Characterization of cytokinin and adenine transport in Arabidopsis cell cultures

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ABSTRACT

Cytokinins are distributed through the vascular system and trigger responses of target cells via receptor-mediated signal transduction. Perception and transduction of the signal can occur at the plasma membrane or in the cytosol. The signal is terminated by the action of extra- or intracellular cytokinin oxidases. While radiotracer studies have been used to study transport and metabolism of cytokinins in plants, little is known about the kinetic properties of cytokinin transport. To provide a reference data set, radiolabeled trans-zeatin was used for uptake studies in Arabidopsis cell culture. Uptake kinetics of trans-zeatin are multiphasic, indicating the presence of both low- and high-affinity transport systems. The protonophore CCCP is an effective inhibitor of cytokinin uptake, consistent with H⁺-mediated uptake. Other physiological cytokinins such as isopentenyladenine and benzylaminopurine are effective competitors of trans-zeatin uptake, whereas allantoin has no inhibitory effect. Adenine competes for zeatin uptake indicating that the degradation product of cytokinin oxidases is transported by the same systems. Comparison of adenine and trans-zeatin uptake in Arabidopsis seedlings reveals similar uptake kinetics. Kinetic properties as well as substrate specificity determined in cell cultures is compatible with the hypothesis that members of the plant-specific PUP family play a role in adenine transport for scavenging extracellular adenine, and may in addition be involved in low affinity cytokinin uptake.
INTRODUCTION

Due to their immobile nature, plants require highly efficient mechanisms for acclimation to rapidly changing environmental conditions and for communication between the distal organs of the plant. Besides classical hormones, such as steroids, oligopeptides and eicosanoid-like compounds, plants have developed a specific set of phytohormones. Most phytohormones are synthesized by few conversions from common metabolic intermediates. The phytohormone cytokinin is mainly generated by isopentylation of AMP, ADP or ATP by adenosine-phosphate –isopentenyltransferase (IPT), resulting in the production of corresponding isopentenyladenosine-5′-phosphates. These isopentenyladenine (iP-) nucleotides are converted to trans-zeatin (tZ) derivatives by trans-hydroxylases CYP735A1 and CYP735A2 (Takei et al., 2004b). Further dephosphorylation and deribosylation of iP- and tZ-nucleotides result in their biological activity. In addition, cytokinin nucleotides can be activated by phosphoribohydrolase LOG (Kurakawa et al., 2007). In Arabidopsis seven IPT genes have been identified (AtIPT1, AtIPT3-AtIPt8, Kakimoto, 2001; Takei et al., 2001), which are specifically expressed in a wide range of organs and cell types (Miyawaki et al., 2004; Takei et al., 2004a). Their products, preferentially isopentylate ATP and ADP (Kakimoto, 2001; Sakakibara, 2006) are responsible for the bulk of isopentenyladenine- and trans-zeatin-type cytokinins (Miyawaki et al., 2006). In addition, degradation of isopentylated tRNAs has been suggested as a possible source of cytokinins. Two Arabidopsis tRNA- isopentenyltransferases AtIPT2 and AtIPT9 catalyze isopentylation of tRNA and are required for cis-zeatin type cytokinins synthesis (Golovko et al., 2002; Miyawaki et al., 2006).

The level of cytokinins in plants is controlled by de novo biosynthesis, conversion between free bases, nucleosides and nucleotides, inactivation, degradation and translocation. The irreversible degradation of cytokinins is catalyzed by cytokinin oxidase/dehydrogenase (CKX). Analysis of the entire Arabidopsis genome identified seven CKX genes (AtCKX1 to
AtCKX7, Schmülling et al., 2003; Werner et al., 2006); the rice genome contains at least eleven CKX homologues (OsCKX1 to OsCKX11, Werner et al., 2006). Interestingly a rice CKX was identified as a major QTL controlling grain number (Ashikari et al., 2005). Ectopic overexpression of cytokinin oxidase genes has dramatic effects on the root/shoot ratio and leaf cell number (Werner et al., 2001). The presence of signal sequences together with heterologous expression data indicate that some of the enzymes are secreted, while others may be localized in the vacuole or in the cytoplasm (Werner et al., 2003). Besides a role in cytokinin degradation, secreted cytokinin oxidases may thus also control cytokinin concentrations available to the plasma membrane localized cytokinin receptors. In Arabidopsis three plasma membrane cytokinin receptors have been identified (AHK2, AHK3 and AHK4/CRE1/WOL), belonging to the class of two component histidine kinase systems (Higuchi et al., 2004; Müller and Sheen, 2007a). Downstream elements of the cytokinin-mediated signal transduction cascade are histidine phosphotransfer proteins (AHP’s) and nuclear response regulators (ARR’s), serving as transcriptional regulators (Müller and Sheen, 2007b). In addition to plasma membrane associated receptors the presence of potential intracellular receptors has been shown, suggesting that besides extracellular signal perception, the uptake of cytokinins may also be important (Kulaeva et al., 1995; Brault et al., 1997; Kulaeva et al., 1998).

The distinct sites of expression of genes for cytokinin biosynthesis (Miyawaki et al., 2004; Takei et al., 2004a), degradation (Werner et al., 2003, 2006) and signaling (D’Agostino et al., 2000; Kiba et al., 2002; Mason et al., 2004; Tajima et al., 2004; Ferreira and Kieber, 2005; Mason et al., 2005; Yokoyama et al., 2007; Ishida et al., 2008) lead to the hypothesis for local function of cytokinins. On the other hand the translocation of cytokinins from roots to shoots via the xylem and their reflux occurring in the phloem, support the hypothesis that they serve as long-distance signaling molecules (Weiler and Ziegler, 1981; Beveridge et al., 1997; Emery et al., 2000). Thus, multiple cellular importers and exporters are required to
allow efficient mobilization and targeted translocation. However, in contrast to the mechanisms of polar transport of the hormone auxin, little is known about the mechanisms of cytokinin transport (Swarup et al., 2000). It has been shown that cell cultures rapidly take up and inactivate free cytokinin bases by glycosylation and storage in the vacuole, indicating the presence of cellular uptake systems (Fußeder et al., 1989). Furthermore, if a significant portion of cytokinin is catabolized extracellularly by CKX, transporters are required for the recycling of the released adenine. Efficient salvage of adenine to AMP and ATP is important for the synthesis of nucleotide cofactors and DNA, and for the supply of the cell with energy carriers (Lee and Moffat, 1994; Moffat and Ashihara, 2002).

Cellular uptake and turnover of cytokinin appears to be highly controlled since a local supply of cytokinins by microorganisms on senescing leaves leads to delay of senescence. Furthermore, grafts of wild type shoots onto root stocks of transgenic tobacco, overproducing cytokinins due to ectopic expression of the Agrobacterium tumefaciens ipt gene, did not lead to release of lateral shoot buds from suppression or to accelerated senescence (Faiss et al., 1997). Localized induction of ipt gene expression also had only local effects, leading to a paracrine hypothesis of cytokinin action. Thus the major step, which is least understood and molecularly still not defined, is the role of cytokinin and cytokinin catabolite transport.

Recently, low affinity transporters for cytokinin nucleobases belonging to the PUP (purine permease) family in Arabidopsis (Gillissen et al., 2000; Bürkle et al., 2003) und for cytokinin nucleosides belonging to the ENT (equilibrative nucleoside transporter) family in rice and Arabidopsis (Hirose et al., 2005; Hirose et al., 2008) have been identified. The cytokinin transporters of the PUP family have been isolated by an indirect approach based on the structural similarity of cytokinins and purine bases. If metabolism occurs at least in part in the apoplasm, one may expect the coexistence of adenine and cytokinin transport systems in the plasma membrane. A purine transport-deficient yeast mutant fcy2 was used for suppression cloning of the plant transporter gene PUP1 (for Arabidopsis thaliana purine
permease 1), which belongs to a plant-specific gene family encoding a new class of small, integral membrane proteins. PUP1 mediates high affinity transport of adenine and other nucleobases (Gillissen et al., 2000). Competition studies showed that PUP1 was also able to recognize cytokinins. The ability of PUP1 to transport cytokinins was demonstrated by direct uptake of radiolabeled trans-zeatin into yeast expressing this transporter (Bürkle et al., 2003). Analysis of expression using promoter reporter fusions indicates that PUP1 may play a role in the retrieval of nucleobase derivatives, potentially including xylem-delivered cytokinins, in the epithem of hydathodes and at the stigma surface of siliques (Bürkle et al., 2003). Evidence for this hypothesis is provided by a recent study demonstrating an accumulation of free cytokinins in shoot tissues known for high transpiration such as hydathodes, guard cells, trichomes and stigmas in ARR5-GUS plants (Aloni et al., 2005). Two homologues of PUP1 were cloned and expressed in yeast. PUP2 is able to mediate adenine uptake and to recognize trans- and cis-zeatin, isopentenyladenine, kinetin and benzylaminopurine as substrates, in contrast PUP3 did not show detectable activity in yeast. The PUP2 promoter drives expression of the GUS reporter gene in the phloem of Arabidopsis leaves. This result, together with recent findings demonstrating the expression of IPT3 (Miyawaki et al., 2004; Takei et al., 2004a) in companion cells of sieve elements and the accumulation of cytokinins in phloem of ARR5-GUS plants (Aloni et al., 2005) may implicate a role of PUP2 in phloem loading and transport of adenine and possibly cytokinins. The present study describes the properties of adenine and trans-zeatin transport in cultured Arabidopsis cells. The uptake of trans-zeatin is inhibited by other cytokinins as well as adenine.
RESULTS

Stability of radiolabeled trans-zeatin in Arabidopsis cell culture supernatant

Cytokinins are irreversibly degraded by cytokinin oxidases to produce the structurally related adenine. The presence of signal sequences and the accumulation of extracellular cytokinin oxidase activity in yeast indicate that some of the enzymes may be secreted (Werner et al., 2001). The tritium label of radiolabeled trans-zeatin is located on the purine ring (Fig. 1). To verify uptake of intact trans-zeatin into the cells, rather than the degradation product $^{14}$C-adenine, the stability of the radiolabeled trans-zeatin in Arabidopsis cell culture supernatant was tested. HPLC analysis showed that almost all the detected radioactivity had the same retention time as trans-zeatin; whereas no radioactivity peak with the retention time corresponding to adenine was detected (Fig. 1). These results indicate that at least within the first 5 minutes no significant degradation of trans-zeatin occurs in the cell supernatant and support the notion that the measured uptake corresponds to import of trans-zeatin into Arabidopsis cultured cells.

The low affinity transport system for trans-zeatin in Arabidopsis cell culture

The presence of a low affinity transport system for trans-zeatin in Arabidopsis cell culture was analyzed by direct uptake measurements with radiolabeled $^3$H-trans-zeatin. The uptake rate at a substrate concentration of 20 µM was linear for at least three minutes and sensitive to the protonophore CCCP, potentially suggesting that energization is necessary for the transport (Fig. 2A, B). At 20 µM substrate concentration the uptake rates were in the range of 0.4 pmol trans-zeatin mg$^{-1}$ fresh weight min$^{-1}$ (Fig. 2A), or an estimated 0.09 fmol trans-zeatin cell$^{-1}$ min$^{-1}$ corresponding to 741 fmol trans-zeatin cm$^{-2}$ membrane surface of an average culture cell (diameter 31.05±7.33 µm; n=36). Uptake rates were concentration-dependent, however they did not show clear Michaelis-Menten saturation (Fig. 2C). Using an Eadie-Hofstee plot of the data two kinetic components with $K_m$ values of 4 µM and ~100
µM was determined (inset in Fig. 2C), suggesting the presence of at least two transport systems with different affinity.

Competition experiments with a 10-fold excess of unlabeled trans-zeatin, adenine, isopentenyladenine and benzylaminopurine reduced the uptake rate, indicating that adenine and cytokinins may be taken up by a common transport system (Fig. 2D). The uptake was significantly inhibited by trans-zeatin riboside. Allantoin had no inhibitory effect, properties similar to that of the Arabidopsis transporter PUP1 when expressed heterologously in yeast (Gillissen et al., 2000).

**High affinity transport of trans-zeatin in Arabidopsis cell culture**

The presence of nanomolar concentrations of cytokinin in phloem and xylem sap (Beck and Wagner, 1994; Takei et al., 2001) may suggest that physiologically relevant high affinity transport systems may exist in plant cells. Therefore uptake measurements with nanomolar trans-3H-zeatin concentrations in Arabidopsis cultured cells were performed to test for the presence of such a system. As shown in Fig. 3A and B, Arabidopsis cells take up trans-zeatin at a substrate concentration of 90 nM. The uptake rates remain constant for at least three minutes and are completely abolished by the protonophore CCCP, compatible with the involvement of a secondary active transport system. At 90 nM substrate concentration the uptake rates were in the range of 0.092 pmol trans-zeatin mg⁻¹ fresh weight min⁻¹ (Fig. 3A) or an estimated 0.021 fmol trans-zeatin cell⁻¹ min⁻¹ corresponding to 173 fmol trans-zeatin cm⁻² membrane surface of an average cell culture cell (diameter 31.05 ± 7.33 µm; n=36). The uptake rates were concentration dependent and displayed saturation kinetics (Fig. 3C) with a $K_m$ value of 211 nM (inset in Fig. 3C).

Surprisingly also the high affinity import of trans-3H-zeatin into Arabidopsis cells was strongly inhibited by an excess of unlabeled adenine. Also other cytokinins such as isopentenyladenine, benzylaminopurine and trans-zeatin riboside significantly reduced the
uptake rates, whereas allantoin did not compete (Fig. 3D). Together the results indicate that also the high affinity transport system recognizes both cytokinins and adenine as substrates.

**Relative uptake rates for adenine and trans-zeatin in Arabidopsis cell culture**

Since transport studies in cultured cells and yeast transformed with PUP1 or PUP2 indicate that adenine and cytokinins use the same low affinity transport system (this study, Gillissen *et al.*, 2000; Bürkle *et al.*, 2003), the uptake rates for cytokinin and adenine were compared. At a substrate concentration of 50 µM the uptake of adenine into Arabidopsis cells was about 3-fold higher compared to that of trans-zeatin (Fig. 4A). The difference was even greater at a substrate concentration of 200 nM (Fig. 4B). The transport system thus appears to have a higher capacity for adenine, alternatively additional adenine transporters may contribute to the observed rates.

**Uptake of adenine and trans-zeatin by Arabidopsis seedlings**

The presence of a transport system for adenine and cytokinins was also demonstrated in 8d-old Arabidopsis seedlings by direct uptake studies with radiolabeled trans-zeatin and adenine. Adenine and trans-zeatin are both taken up by Arabidopsis seedlings (Fig. 5A). The uptake is sensitive to the protonophore CCCP, suggesting that transport is energy-dependent. Similar as in the case of the cell culture the uptakes rates are higher for ³H-adenine compared to ¹⁴C-trans-zeatin.

The Arabidopsis purine permeases PUP1 and PUP2 may contribute to the observed accumulation of adenine and cytokinins, since RT-PCR analysis with gene-specific primers demonstrated high expression in Arabidopsis seedling tissue (Fig. 5B). As shown in Fig. 5C, reporter gene activity was detectable at the tip of cotyledons in PUP1-GUS seedlings at a similar developmental stage as used for the uptake studies, and in the vascular tissue of PUP2-GUS cotyledons. For PUP3 no RT-PCR signal and no GUS activity was obtained in Arabidopsis seedlings, consistent with its pollen-restricted expression pattern described
previously (Bürkle et al., 2003). The PUP family of Arabidopsis comprises 21 members, of which 12 are present on the ATH1 chip (22k full genome Arabidopsis Affymetrix GeneChip). Fig 5D summarizes the organ-specific expression data for the PUP gene family from the Genevestigator database (Zimmermann et al., 2005). Only a subset of 12 PUPs is represented on the chip. Among the ones present on the chip, PUP11,14 and 18 show highest expression levels across all tissues. In roots, PUP 4 is expressed to high levels in addition to PUP11,14 and 18. PUP4 is relatively closely related to PUP1 and 2, while the other three PUPs share only a low level of homology (Bürkle et al., 2003). Overall, the majority of PUPs was found to be expressed in callus and cell suspensions (Fig. 5 D). For none of these members, function as a purine transporter has been demonstrated yet.

**Sensitivity of germination to adenine**

Nucleobases deriving from deteriorating biological material are present at low levels in soil. While Arabidopsis germination is unaffected on media containing up to 2 mM cytosine, thymine or uracil, seedling development was severely affected on media supplemented with 2 mM adenine (Fig. 6). Guanine was not tested due to its low solubility in water. Lower adenine concentrations affected seedling growth as well and lead to chlorosis. Growth arrest in roots was detectable at concentrations above 0.5 mM adenine (Fig. 5N). As expected based on the hypothesis that adenine competes for cytokinin, the observed phenotype is apparently different from growth inhibition by isopentenyladenine (Fig. 6N-P). Lateral root formation was inhibited even at the lowest cytokinin concentrations, while it was less affected by adenine.
DISCUSSION

Previous studies had shown that cell suspension cultures of *Chenobium rubrum* and tobacco are able to take up and metabolize cytokinins (Laloue *et al.*, 1981; Fußeder *et al.*, 1989). However, to the best of our knowledge, no detailed analysis of the characteristics of cytokinin uptake systems has been published to date. Here a detailed analysis of the kinetics of $^3$H-labelled *trans*-zeatin uptake in Arabidopsis cell cultures is provided. Cytokinin uptakes rates were concentration-dependent and showed multiphasic characteristics, indicating the presence of multiple low- and a high-affinity transport systems. In the low-affinity range two activities with apparent $K_m$ values for *trans*-zeatin of 4 µM and ~100 µM could be identified, respectively. In addition, a high-affinity transport system was identified in the Arabidopsis cell cultures, which is characterized by a $K_m$ of 200 nM for *trans*-zeatin. The affinity of the high and medium affinity systems is compatible with the observed range for cytokinins in plants (Komor, 1993 #6903; Weiler, 1981 #2349; Beck, 1994 #2299; Takei, 2001 #2344). In contrast, it is at present not clear whether the observed systems with an affinity of 100 µM will be relevant in planta. It should however be kept in mind, that comparable low affinities have previously been described for enzymes involved in cytokinin biosynthesis and metabolism (Turner *et al.*, 1987; Dixon *et al.*, 1989; Galuszka *et al.*, 1999; Bilyeu *et al.*, 2001), leaving the possibility open that low affinity cytokinin transport could play a physiological role. The systems appear to be able to transport a variety of cytokinins since *trans*-zeatin uptake was inhibited by other cytokinins such as isopentenyladenine and benzylaminopurine.

Moreover, cytokinin uptake into cell cultures was competitively inhibited by adenine, lending support to the hypothesis of the existence of a common transport system for adenine and cytokinins. Such systems may either contribute to retrieval of cytokinin-oxidase derived adenine from the apoplasm, or they may serve functions in retrieval of adenine derived from other biological processes. As a consequence, one would expect that transport rates of
cytokinins will be influenced whenever adenine is present due to the higher capacity for adenine transport. However, in general, adenine levels appear to be very low (Ashihara et al., 1990). Interestingly, adenine uptake rates were significantly higher compared to uptake rates for trans-zeatin when analyzed in Arabidopsis cells. Similar as in cell cultures, also the uptake of trans-zeatin into seedlings was sensitive to competition by adenine. To test for the physiological relevance of the competition of cytokinin and adenine for uptake, the effect of both set of compounds on root growth was tested. Surprisingly, adenine, but not other nucleobases inhibited root development. Despite the fact that both adenine and cytokinin affect root growth and development, the phenotypes were clearly different, a finding compatible either with a cytokinin-independent mechanism or which could be due to reduced cytokinin flux in the root.

Previous studies had suggested a diffusion mechanism for cytokinin uptake based on the high permeability of cell membranes to free cytokinin bases and ribosides (Laloue et al., 1981). The sensitivity of the cytokinin uptake into Arabidopsis cells and seedlings to CCCP suggests that high, medium and low affinity systems are mediated by secondary active transport systems. Similarly uptake into seedlings was protonophore-sensitive. Taken together, the data show that Arabidopsis cell cultures have at least three kinetically distinct uptake systems for cytokinins that share similar properties such as protonophore-sensitivity and competition by adenine.

**Candidates for adenine and cytokinin transport function**

The molecular nature of the cytokinin transport systems described here is still unknown. Using suppression cloning in yeast, a new family of plant-specific transporters had been identified, members of which mediate protonophore-sensitive uptake of both adenine and cytokinins (Gillissen et al., 2000; Bürkle et al., 2003). When expressed in yeast, the $K_m$ values for the Arabidopsis transporters PUP1 and PUP2 for trans-zeatin were in the range of 35-40 µM. The affinity of PUP1 for adenine was similar to that for trans-zeatin in low
micromolar range (Bürkle et al., 2003). The affinities of the PUPs for adenine were in a similar range as the value of 370µM obtained for uptake in Ricinus cotyledons (Kombrink and Beevers, 1983). A variety of PUP members are expressed in Arabidopsis callus and cell cultures; it is thus possible that well-characterized PUPs, or other members of the family that have not been functionally characterized yet, could contribute to the observed cytokinin uptake in the cell cultures. In principle, three hypotheses can be formulated: either the affinity of PUPs expressed in yeast does not correspond to their in planta activity due to the lack of additional factors or posttranslational modifications; other members of the PUP family, which contains 21 members in the Arabidopsis genome are responsible for the high affinity component (Bürkle et al., 2003); or other transporters unrelated to purine permeases may be involved in the cytokinin transport in the nanomolar range. Direct proof for a role of PUPs in cytokinin transport in planta is still outstanding. The large number of paralogs will potentially make analysis of insertional mutants difficult due to overlapping activities and compensation phenomena.

Interestingly the adenine uptake into Arabidopsis cells was also not competed by trans-zeatin riboside possibly indicating that the transport of this compound occurs via transporters with selectivity for other cytokinins but not to adenine. Potential candidates for such function could be the newly identified and characterized plant ENTs (Möhlmann et al., 2001; Li et al., 2003; Wormit et al., 2004), which transport cytokinin ribosides (Hirose et al., 2005; Sun et al., 2005; Hirose et al., 2008). Isopentenyladenine riboside inhibited adenosine transport mediated by one of the four rice homologues of the ENT family, OsENT2, indicating that OsENT2 can recognize cytokinin nucleosides. Furthermore, direct measurements with radiolabeled cytokinins showed that OsENT2 transports isopentenyladenine riboside and trans-zeatin riboside with K_m values in the low affinity range of 32 and 660 µM, respectively (Hirose et al., 2005). The Arabidopsis genome contains eight ENT-genes (AtENT1-AtENT8, Li et al., 2003). In competition experiments, the uptake of adenosine by AtENT6-expressing
yeast cells was significantly inhibited by trans-zeatin riboside and isopentenyladenine riboside. Direct transport measurements revealed $K_m$ values of 17 µM for isopentenyladenine riboside and 630 µM for trans-zeatin riboside (Hirose et al., 2008). Interestingly the adenosine uptake mediated by OsENT2 was also significantly inhibited by trans-zeatin, whereas adenine had no inhibitory effect (Hirose et al., 2005). This finding indicates that the substrate specificity OsENT2 is not only restricted to cytokinin nucleosides and that at least OsENT2 can also recognize other cytokinins. Although competition studies with some AtENTs revealed no indication for transport of kinetin or zeatin (Wormit et al., 2004), it can not be excluded that other members of the Arabidopsis ENT family may be involved in the trans-zeatin riboside uptake system described in cultured cell in this study. Since AtENTs function as nucleoside transporters (Möhlmann et al., 2001; Li et al., 2003; Wormit et al., 2004) they may be also responsible for the transport of cytokinin ribosides.

In summary, the present study provides evidence for the presence of proton-coupled high, medium and low affinity cytokinin transport systems in Arabidopsis culture cells. The transport systems share some properties with PUP1 and 2, the so far only functionally characterized members of this large gene family with 21 members. The characterization of other PUP family members, the characterization of cell cultures from insertional mutants, RNAi-repressed or overexpressor Arabidopsis plants and cloning of new transporters different from purine permeases may help to obtain further information regarding the cytokinin and purine transport and its function in planta.
MATERIALS AND METHODS

Transport measurements in Arabidopsis suspension cells

The suspension cell culture from Arabidopsis thaliana ecotype Landsberg erecta (May et al., 1998) was a gift from Mike Bevan (John Innes Institute, Norwich, UK). Cells were maintained as described by Fuerst et al. (1996). For the uptakes experiments cells were harvested five days after transfer into new medium by centrifugation at 300 rpm for 3 min and resuspended in fresh cytokinin-free medium containing 10 mM potassium phosphate buffer (pH 5.7) at a concentration of 0.25 ml packed cells per 1 ml of suspension. To start the reaction 850 µl cells were mixed with 50 µl medium containing trans-2-3H-zeatin at a final concentration of 43.2 Bq/µl (specific activity: 1.2 TBq/mmol; OlchemIm, Olomouc, Czech Republic). Adenine was added as 14C-adenine of a final concentration of 12.3 Bq/µl (specific activity: 10.6 GBq/mmol); Amersham Pharmacia, Buckinghamshire, UK). The unlabeled analogs (Sigma) were added to the final concentration as indicated. Samples of 170 µl were removed after 1, 2, 3, and 4 minutes, filtered on glass fiber filters and washed twice with 10 ml medium. Radioactivity on the filters was determined by liquid scintillation spectrometry (Beckman). For the uptake measurements in the nanomolar range, cells were additionally washed with medium and diluted 1:12. The final concentration for trans-2-3H-zeatin was 14.4 Bq/µl. The protonophore CCCP was added two minutes before and one and half minutes after starting the assay. Competition experiments were performed with a 10-fold excess (2 µM or 200 µM) of the respective unlabeled competitor. Cell diameter was determined as described by Richard et al. (2001).

Analysis of trans-3H-zeatin stability in Arabidopsis cell culture supernatant

The Arabidopsis suspension cells were harvested by centrifugation, washed and diluted as described above. After 6 h incubation in fresh medium, an aliquot of 850 µl cells was mixed with 50 µl trans-3H-zeatin containing medium to the final concentration of 500 nM and
incubated for further 5 minutes. The sample was than centrifuged for 3 min at 500 rpm and filtered. 20 µl of the obtained supernatant was analyzed by HPLC using the LB 507B radiodetector (Berthold, Wildbad, Germany). Adenine (Sigma) and trans-zeatin (Sigma) were used as standards for estimation of the retention time.

**Transport measurements into Arabidopsis seedlings**

_Arabidopsis thaliana_ seeds (ecotype Columbia-0) were plated on Murashige Skoog medium supplemented with 2% sucrose (2MS). Uptake measurements were performed with ten 8d-old seedlings in 2MS-medium containing trans-2-^3^H-zeatin (final concentration 43.2 Bq/µl) or ^13^C-adenine (final concentration 12.3 Bq/µl) and the unlabeled analog to the final concentration of 5µM. Seedlings were floated in the medium containing the radiotracer. After 45 min the seedlings were washed twice with 5 mM adenine solution and incubated over night in p-diisobutyl-cresoxyethoxyethyl dimethylbenzylammonium hydroxide to solubilize the tissue. Radioactivity was quantified by scintillation counting.

**RT-PCR analysis**

RNA for RT-PCR analysis was extracted from 8d-old Arabidopsis seedlings grown on 2MS-medium according to the SDS/Phenol method. Aliquots of 2 µg RNA were used as template for first strand synthesis using RETROscript Kit (Ambion, Austin, USA). Two µl of the first strand cDNA or 150 ng RNA (as negative control) were used for PCR with gene specific primers. To avoid amplification of genomic DNA, reverse primers were positioned on intron/exon borders. A 524 bp PUP1 fragment was amplified by 32 PCR cycles using the oligonucleotides: 5’-CTAACAACGCGGAAAACAAGC-3’ and 5’-CTCTTGCTATCACCTTAAAATCTC-3’. A 619-bp PUP2 transcript was obtained by 32 PCR cycles with specific primers: 5’-TATCTTGGTACCAAAGGATCTGGTTTCCAAGC-3’ and 5’-TCCTGCTATCACCTTGAAATCG-3’. The primers (5’-
ACAATGTGGGTGATAGTACAAG-3’ and 5’-CTTTGGTAAGGCCTTGAAAATC-3’.

used to analyze the expression of PUP3 amplifying a 515 bp region.

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LEGENDS TO FIGURES

Figure 1. Radiochemical purity and stability of trans-3H-zeatin. Radiolabeled trans-2-3H-zeatin was mixed with 2MS-medium (blue line) or with cultured cells (red line) to a final concentration of 500 nM in the total volume of 900 µl and incubated for 5 minutes. Samples were centrifuged, filtrated and the resulting supernatant was analyzed by HPLC using a radioactivity detector. Retention times of adenine (4.63 min) and trans-zeatin (13.91 min) are marked by arrows.

Figure 2. Kinetics of trans-zeatin uptake into Arabidopsis suspension cells in the “low-affinity” range. A, B. Time and energy dependence. Arabidopsis cells were assayed for trans-2-3H-zeatin uptake at 20 µM substrate concentration, pH 5.7, in the presence (○) or absence (□) of 100 µM carbonyl cyanide m-chlorphenyl-hydrazone (CCCP). CCCP was added two minutes before (A) and one and half minutes after starting the assay (B). C. Trans-zeatin uptake at different substrate concentrations. Inset displays an Eadie-Hofstee plot of the data; the two fitted curves indicate the presence of at least two transporter activities with $K_m$ values of 3.9 and 98.8 µM, respectively. D. Inhibition of trans-zeatin uptake by adenine and other cytokinins. Uptake of trans-3H-zeatin was determined at 20 µM trans-zeatin in presence of 10-fold excess (200 µM) of unlabeled competitors. BA, benzylaminopurine; iPA, isopenetyladenine; ZR, trans-zeatin riboside. Values represent the mean of three independent measurements ± SD; WW, wet weight.

Figure 3. Kinetics of trans-zeatin uptake into Arabidopsis suspension cells in the “high-affinity” range. A, B. Time and energy dependence. Arabidopsis cells were assayed for trans-2-3H-zeatin uptake at 90 nM substrate concentration, pH 5.7, in the presence (○) or absence (□) of 100 µM carbonyl cyanide m-chlorphenyl-hydrazone (CCCP). CCCP was
added two minutes before (A) and one and half minutes after starting the assay (B). C. 

*Trans*-zeatin uptake at different substrate concentrations. The inset is an Eadie-Hofstee plot of the data, showing a transport activity with the $K_m$ value of 211 nM. D. Inhibition of *trans*-zeatin uptake by adenine and by cytokinins. Uptake of *trans*-H-zeatin was determined at 200 nM *trans*-zeatin in the presence of 10-fold excess (2 µM) of unlabeled competitors. BA, benzylaminopurine; iPA, isopenetyladenine; ZR, *trans*-zeatin riboside. Values represent the mean of three independent measurements ± SD; WW, wet weight.

**Figure 4.** Comparison of uptake rates for adenine and *trans*-zeatin in Arabidopsis suspension cells. The uptake of adenine (○) and *trans*-zeatin (□) was determined at 50 µM (A) and 200 nM (B) substrate concentrations. Results represent the mean of three measurements ± SD; DW, dry weight; WW, wet weight.

**Figure 5.** Adenine and cytokinin accumulation in 8d-old Arabidopsis seedlings. A. Uptake of adenine and *trans*-zeatin into Arabidopsis seedlings in presence and absence of CCCP. B. Analysis of PUP1, PUP 2, and PUP 3 expression in Arabidopsis seedlings by RT-PCR. RNA from 8d-old Arabidopsis seedlings was converted to cDNA by reverse transcription. A 524 bp fragment of *PUP1*, and a 619 bp fragment of *PUP2* were amplified by 32 cycles (+: with template, -: without template, M: molecular mass marker). C. Analysis of PUP1 and 2 expression in Arabidopsis seedlings by promoter-GUS fusions. Arabidopsis plants expressing either PUP1- or PUP2-GUS (line 10 and 50, respectively) were stained with 1 mM X-gluc, cleared in ethanol and documented (PUP3-GUS did not show staining). D. Tissue-specificity of PUP family members derived from Genevestigator (Zimmermann et al., 2005). Members not present on the ATH1 22k Arabidopsis Affymetrix GeneChip are PUP2
Figure 6. Effect of adenine on germination and growth of Arabidopsis seedlings. A-M.

Growth of Arabidopsis seedlings on media containing nucleobases. Arabidopsis seeds were plated on 2MS-medium supplemented with adenine (0.5mM A; 1 mM B; 2 mM C) cytosine (0.5mM D; 1 mM E; 2 mM F), uracil (0.5mM G; 1 mM H; 2 mM I) and thymine (0.5mM K; 1 mM L; 2 mM M) and grown for 18 days in the light. N,O. Sterilized seeds of Arabidopsis were plated on 2MS medium supplemented with adenine (N) or isopentenyladenine (O) at the concentrations indicated. P. Root length at different adenine and isopentenyladenine concentrations.
Figure 1. Radiochemical purity and stability of trans-\(^3\)H-zeatin. Radiolabeled trans-2-\(^3\)H-zeatin was mixed with 2MS-medium (blue line) or with cultured cells (red line) to a final concentration of 500 nM in the total volume of 900 \(\mu\)L and incubated for 5 minutes. Samples were centrifuged, filtrated and the resulting supernatant was analyzed by HPLC using a radioactivity detector. Retention times of adenine (4.63 min) and trans-zeatin (13.91 min) are marked by arrows.
Figure 2. Kinetics of trans-zeatin uptake into Arabidopsis suspension cells in the “low-affinity” range. A, B. Time and energy dependence. Arabidopsis cells were assayed for trans-2-^3^H-zeatin uptake at 20 μM substrate concentration, pH 5.7, in the presence (○) or absence (□) of 100 μM carbonyl cyanide m-chlorophenyl-hydrazone (CCCP). CCCP was added two minutes before (A) and one and half minutes after starting the assay (B). C. Trans-zeatin uptake at different substrate concentrations. Inset displays an Eadie-Hofstee plot of the data; the two fitted curves indicate the presence of at least two transporter activities with $K_m$ values of 3.9 and 98.8 μM, respectively. D. Inhibition of trans-zeatin uptake by adenine and other cytokinins. Uptake of trans-^3^H-zeatin was determined at 20 μM trans-zeatin in presence of 10-fold excess (200 μM) of unlabeled competitors. BA, benzylaminopurine; iPA, isopenethyladenine; ZR, trans-zeatin riboside. Values represent the mean of three independent measurements ± SD; WW, wet weight.
Figure 3. Kinetics of trans-zeatin uptake into Arabidopsis suspension cells in the “high-affinity” range. A, B. Time and energy dependence. Arabidopsis cells were assayed for trans-2-^3^H-zeatin uptake at 90 nM substrate concentration, pH 5.7, in the presence (○) or absence (□) of 100 μM carbonyl cyanide m-chlorophenyl-hydrazone (CCCP). CCCP was added two minutes before (A) and one and half minutes after starting the assay (B). C. Trans-zeatin uptake at different substrate concentrations. The inset is an Eadie-Hofstee plot of the data, showing a transport activity with the \( K_m \) value of 211 nM. D. Inhibition of trans-zeatin uptake by adenine and by cytokinins. Uptake of trans-^3^H-zeatin was determined at 200 nM trans-zeatin in the presence of 10-fold excess (2 μM) of unlabeled competitors. BA, benzylaminopurine; iP, isopenetyl adenine; ZR, trans-zeatin. Means represent the mean of three independent measurements ± SD; WW, wet weight.
Figure 4. Comparison of uptake rates for adenine and trans-zeatin in Arabidopsis suspension cells. The uptake of adenine (○) and trans-zeatin (□) was determined at 50 μM (A) and 200 nM (B) substrate concentrations. Results represent the mean of three measurements ± SD; DW, dry weight; WW, wet weight.
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Figure 6. Effect of adenine on germination and growth of Arabidopsis seedlings. A-M. Growth of Arabidopsis seedlings on media containing nucleobases. Arabidopsis seeds were plated on 2MS-medium supplemented with adenine (0.5mM A; 1 mM B; 2 mM C) cytosine (0.5mM D; 1 mM E; 2 mM F), uracil (0.5mM G; 1 mM H; 2 mM I), and isopentenyladenine (0.5mM J; 1 mM L; 2 mM M) and grown for 18 days in the light. N, O. Seedling growth in Arabidopsis were plated on 2MS medium supplemented with adenine (N) or isopentenyladenine (O) at the concentrations indicated. P. Root length at different adenine and isopentenyladenine concentrations.