Salvage pathways play an important role in providing nucleobases to cells, which are unable to synthesize sufficient amounts for their needs. Cellular uptake systems for pyrimidines have been described, but in higher eukaryotes, transporters for thymine and uracil have not been identified. Two plant transporters, AtUPS1 and PvUPS1, were recently identified as transporters for allantoin in Arabidopsis and French bean, respectively. However, Arabidopsis, in contrast to tropical legumes, uses mainly amino acids for long distance transport. Allantoin transport has not been described in the Brassicaceae. Thus, the physiological substrates of ureide permease (UPS) transporters in Arabidopsis may be compounds structurally related to allantoin. A detailed analysis of the substrate specificities of two members of the AtUPS family shows that AtUPS1 and AtUPS2 mediate high affinity uracil and 5-fluorouracil (a toxic uracil analogue) transport when expressed in yeast and Xenopus oocytes. Consistent with a function during germination and early seedling development, AtUPS1 expression is transiently induced during the early stages of seedling development followed by up-regulation of AtUPS2 expression. Arabidopsis ups2 insertion mutants and ups1 lines, in which transcript levels were reduced by post-transcriptional gene silencing, are more tolerant to 5-fluourauracil as compared with wild type plants. The results suggest that in Arabidopsis UPS transporters are the main transporters for uracil and potentially other nucleobases, whereas during evolution legumes may have taken advantage of the low selectivity of UPS proteins for long distance transport of allantoin.

Purine and pyrimidine nucleotides are building blocks for DNA and RNA synthesis and essential precursors for enzyme cofactors (NAD, FAD, 5-adenosylmethionin, thiamin), signal molecules (e.g. cyclic nucleotides or the thyohormone cytokinin), and plant secondary metabolites (e.g. caffeine). They can be synthesized *de novo* or obtained by recycling of nucleosides and nucleobases (salvage pathways) (1, 2). The enzymes involved in salvage pathways frequently act with cellular import systems for their substrates in a concerted manner. Thus, most unicellular organisms including prokaryotes, fungi, and cellular parasites possess specialized transporters for the capture of nucleosides and nucleobases from their environment, which are used for nucleotide synthesis with lower net energy cost compared with *de novo* synthesis. In some extreme cases obligate parasites have lost the capacity to produce nucleotides via *de novo* synthesis, and import of nucleobases or nucleosides represents the only route to nucleotide formation. Uptake systems for nucleosides and nucleobases are also present in multicellular organisms. Moreover, the expression of uptake and release systems in distinct animal cell types seems to play a crucial role in the recovery and the distribution of nucleobases and nucleosides passing tissue barriers, e.g. in the kidney, intestine, and placenta (3). These transport systems, therefore, play not only an important role in organism physiology but also in the pharmaco-dynamics of anti-tumor compounds, such as 5-FU (4).

In plant cells adenylates comprise the largest nucleotide pool followed in size by uridylates. UDP-sugars serve as activated intermediates in the synthesis of sucrose and cell wall polymers (5). Of the free pyrimidine bases, plants apparently can only salvage uracil, whereas they are unable to salvage cytosine due to the lack of cytokine deaminase (5). Cells often rely on salvage pathways when the activities of enzymes for *de novo* synthesis are low or absent (e.g. during early stages of seed germination) or when demand for nucleobases is high (e.g. when cells divide rapidly). Although salvaging of nucleobases and nucleosides predominates at the inception of germination, its importance declines when germination proceeds and *de novo* synthesis takes over (6). Salvaging is often accompanied by transport of nucleobases and nucleosides from storage tissues, as was demonstrated for germinating castor bean (7). A high affinity transporter system for uracil with a $K_m$ of ~40 μM was described (7).

Despite the apparent physiological importance of high affinity transport of free pyrimidines, in particular of uracil, no transporters for these substrates have been identified in plants or other higher eukaryotes until now. A number of membrane protein families mediating transport of nucleobases or nucleosides into the cell have been described to date; they comprise (i) the nucleobase ascorbate transporters (NAT; Archaea, eubac-
High Affinity Pyrimidine Transporters from Arabidopsis

Recently, members of the UPF family mediating allantoin transport were identified in Arabidopsis and French bean (13, 14). Allantoin is an important transport form of organic nitrogen in legumes, whereas Arabidopsis is considered to preferentially transport amino acids. Therefore, the substrate specificities of AtUPS1 and AtUPS2, related members of the UPF family from Arabidopsis, were analyzed in detail. Using heterologous expression in yeast and in Xenopus oocytes, it was demonstrated that pyrimidines are excellent substrates for both AtUPS1 and AtUPS2. The affinity for uracil was several-fold higher than for allantoin. In plants, expression of both AtUPS1 and AtUPS2. The affinity for uracil was several-fold higher than for allantoin. In plants, expression of AtUPS1 and AtUPS2 was high during periods of increased demand of nucleotides, i.e. early seedling development, indicating a function in salvage pathways, e.g. by the utilization of pyrimidines from seed storage tissue. In planta, AtUPS function as major transporters for uracil analogs and potentially uracil itself, as demonstrated by the increased tolerance to 5-FU of plants in which AtUPS1 was inhibited by PTGS or in plants that carry T-DNA insertions in AtUPS1.

EXPERIMENTAL PROCEDURES

**UPF1 and UPF2 Expression Constructs**—AtUPS1 was amplified by PCR from a cDNA library from 5-day-old seedlings from Arabidopsis thaliana (ecotype Col-0) with the primers 5'-ATAGGATCCATCATCAT-TAGAGCCCGAAGA-3' and 5'-ATATCTAGATACCTTTCTATGGCAGAAGA-3' and cloned into pCRTopoBlunt (Invitrogen). Sequencing revealed mutations of A to T (at position 276 of the open reading frame) and T to C (at position 771) starting 2 days after injection. The standard perfusion solution (wash solution) contained 100 mM choline chloride, 2 mM CaCl2, 2 mM MgCl2 in 5 mM Mes-Tris, pH 5.5. Substances were dissolved in this solution to final concentrations of 200 μM, and the pH values were adjusted to 5.5 with Mes or Tris. For voltage-current analysis, currents were recorded from oocytes at -100 mV by applying an IV protocol. The IV protocol consisted of 200-ms pulses of voltages ranging from -140 to 0 mV in steps of 20 mV. Substrate evoked currents were constant during these short pulses. Net currents induced by substrates were obtained by subtracting the background currents (measured with “wash solution” alone) before and after the addition of substrates. To avoid endogenous currents, which were slowly activated at more negative voltages, current elicited by 120 mM NA2 was not analyzed (in some cases only voltages in the range of 0 to −100 mV were analyzed). Differences between non-injected and water-injected oocytes were used as controls.

**T-DNA Insertion Lines**—tjuatlanica ecotype Col-0 was crossed throughout the experiment (13). Seeds of the T-DNA insertion lines Salk_143013 (designated up2-1) and Salk_044551 (designated up2-2) were obtained from the Arabidopsis Biological Resource Center (Columbus, Ohio) (25). Homozygous plants were isolated from T3 plants and confirmed in the T4 generation. Genomic DNA was prepared as described (www.dartmouth.edu/~jjack/TAILDNPrep.html). Homozygous lines were screened by two sets of PCR. The first PCR was performed using gene-specific primers 5'-GAATTAAGTTTGCCTGAC-AGC-3' and 5'-GAACCTAGCAAAGGTAGTGTC-3' (Salk_044551) or 5'-CTAGTGAACACATACAGGACGAG-3' and 5'-GTATCTTCTACCTTCTGAC-A-3' (Salk_143013). The second PCR was carried out with the T-DNA left border primer 5'-CTTTGACTGTTAGGTCCAC-3' and the primer 5'-GAACCTAGCAAAGGTAGTGTC-3' (Salk_044551) or 5'-GTATCTTTACCTTCTGAC-A-3' (Salk_143013). Plants were classified homozygous if no PCR product was obtained by the first PCR reaction, and fragments of expected size confirming the T-DNA insertion were amplified by the second PCR reaction. Localization of T-DNA insertions was analyzed by sequencing. Homozygous lines were taken for further investigations.

**PTGS Construct**—A 521-bp AtUPS1 fragment (sense) was amplified by PCR from pFL61UPS1 using gene-specific primers 5'-GTATCTCAGAACACATACAGGACGAG-3' and 5'-GAACCTAGCAAAGGTAGTGTC-3', and a 521-bp fragment (antisense) was amplified using primers 5'-CTCGAGTTTTGGAATTTGTTGATTGTTG-3' and 5'-TGATCTTCTACCTTCTGAC-A-3', and a 521-bp fragment (antisense) was amplified using primers 5'-CTCGAGTTTTGGAATTTGTTGATTGTTG-3' and 5'-TGATCTTCTACCTTCTGAC-A-3'. An 882-bp fragment of the second intron of AAtAAP6 (At5g94630) was amplified from BAC K6M13 using primers 5'-TAATGCTTATCCTCATGAGTCGAC-3' and 5'-TCTGAGTACGGAGACTGTTCCTTCCTCCTATGTT-3'. All fragments were

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cloned into pCR4TopoBlunt and sequenced. The UPS1 antisense fragment was excised with XbaI/XhoI and ligated into the pCR4TopoBluntAAP6intron construct. The resulting construct was subsequently cleaved with HindIII/BamHI (XbaI/BamHI) and introduced into pCB302.3 (MBI) was used as template for PCR reactions. As an internal control a

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\text{RevertAid™ H minus M murine leukemia virus reverse transcriptase}
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was extracted (30). cDNA synthesized with

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\text{mutant strain transformed with either pMD200UPS1 or pMD200UPS2 or empty vector (pMD200) was tested for growth on 0.2 mM uracil as sole source of pyrimidines (B).}
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planted in the greenhouse were transformed with (T1 to T3 generation) were recovered by BASTA™ (phosphinothricin). Agrobacterium using the floral dip method (28) and transgenic plants

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\text{AtUPS1 and AtUPS2} \text{ promoters with restriction}
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excised by SpeI/BamHI (XbaI/BamHI) and introduced into pCB302

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\text{UTS1} \text{ gene was amplified by PCR (26 cycles)}
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with primers 5’-CTGATACACCGGTATTTGCTGG-3’ and 5’-GGACC- TGCCTCTACATACTCG-3’. For AtUPS1 (AtUPS2) primers 5’-CAATT- TCTGCAAGCAAGGTTAAC-3’ and 5’-ACAAGTGAAGGTGCTGTA- ACGG-3’ (5’-CATCAGAAGCAGGAGTACTATG-3’ and 5’-CTCA- AAGGAGAGCCCTGAAGC-3’) were used to amplify a 481-bp (484 bp) fragment by PCR in 30 or 32 cycles. Total RNA was isolated from 9-day-old seedlings grown in liquid cultures using RNeasy (Qiagen), treated with DNase I, and reverse-transcribed. PCR (29 cycles each) of AtUPS1 (AtUPS2) templates with primers 5’-GCACAATAACGGAT- TGTTG-3’ and 5’-ATGTTAAATATCAGAAMACTTAAAGG-3’ (5’- GTACTGTTGTAAGCCTCG-3’ and 5’-TTGCTCATATTGTTAGAT- GACC-3’) produced a 187-bp (289 bp) fragment. For AtACT2 a 192-bp fragment was amplified by PCR (26 cycles) using primers 5’-TCTTCA- CTTCTCATAGGCG-3’ and 5’-ATTGTTGAATATATCATCAGCC-3’.

**RESULTS**

AtUPS1 and -2 Mediate Allantoin Uptake—Out of the five members of the UPS family in Arabidopsis, AtUPS1 and AtUPS2 seem to have arisen from a recent gene duplication as well as AtUPS 2 and AtUPS4. To be able to compare the properties of these two groups, the AtUPS2 cDNA (At2g03530) was isolated from a seedling CDNA library. AtUPS2 showed 74% sequence identity to AtUPS1 on the amino acid level. The yeast mutant dal4 dal5, deficient in the uptake of allantoin and allantoic acid, previously used to isolate AtUPS1 (13), was used to demonstrate that AtUPS2 was able to suppress the mutant phenotype in a growth assay using 5 mM allantoin as the sole source of nitrogen (Fig. 1A). Colonies of cells expressing AtUPS2 or AtUPS1 were several times bigger as those formed by control cells transformed with the empty vector. AtUPS1 colonies grew slightly faster than AtUPS2 colonies. In addition, uptake of [14C]allantoin, due to AtUPS2 expression, was directly shown. The uptake rate was ~75% of the rate observed for AtUPS1-expressing yeast, whereas the uptake rates of dal4 dal5 yeast mutant cells transformed with the empty vector was ~3% of the rate observed upon AtUPS1 or AtUPS2 expression (Supplemental Fig. 1). Thus, both proteins mediate allantoin transport.

AtUPS1 and AtUPS2 Also Transport Uric acid—Because there is no evidence that Arabidopsis uses allantoin as a long distance transport form of nitrogen, it was conceivable that in Arabidopsis UPS has a different physiological substrate. Previous studies had shown that AtUPS1 recognizes ozo derivatives of N-heterocycles (13). Because the structure of uracil fits the criteria established previously, and since uracil transport in plants has been described but transporters have not been identified, we tested whether AtUPS1 or AtUPS2 would be able to mediate uracil uptake. The yeast mutant fur4, deficient in
uracil uptake, grew on 0.2 mM uracil as the sole source of pyrimidines when expressing AtUPS1 or AtUPS2, demonstrating that both transporters mediate uracil uptake (Fig. 1B).

Functional Expression of AtUPS1 and AtUPS2 in Xenopus Oocytes—To test the substrate specificities of AtUPS1 and AtUPS2 more directly, both transporters were functionally expressed in *X. laevis* oocytes. Expression of AtUPS1 induced inward currents in oocytes bathed in buffer with allantoin in a flow system. When clamped at $-100$ mV, significant inward currents were also recorded from AtUPS2-expressing oocytes upon the addition of allantoin (Fig. 2A), whereas no significant currents were detected in non-injected control oocytes (Fig. 2, B and C). To define the substrate specificity in detail, a large number of compounds were tested. Criteria for the selection of potential substrates were (i) molecules belonging to the group of nucleobases and derivatives, (ii) compounds transported by other nucleobase transporters, and (iii) compounds with similar structural features. Currents were induced by cyclic purine degradation products and pyrimidines. In case of allantoin, hydantoin-, cytosine-, thymine- or uracil-induced currents remained stable or slowly decreased until washout of the substrate. However, when flushed with xanthine, the evoked current decreased within 10 s to a lower steady state level (Fig. 2A). A similar effect was found for uric acid (not shown). Despite lower current levels, AtUPS1 behaved in a manner similar to AtUPS2. Clamping oocytes to $-100$ mV for longer periods resulted in small shifts of the base line (Fig. 2A). To minimize errors conferred by this process, further investigations were done using brief substrate flushing periods and the IV protocol described under “Experimental Procedures.” The allantoin-induced currents recorded from AtUPS1- or AtUPS2-expressing oocytes increased in response to further hyperpolarization of the membrane potential (Fig. 2C) and displayed clear voltage dependence.

Steady state currents induced by different substances were plotted as a percentage of those caused by allantoin set to 100% (Fig. 3, A and B). The cyclic purine degradation products uric acid and xanthine induced currents in AtUPS1- and AtUPS2-expressing oocytes ($-60$ and $50\%$ for AtUPS1, 75 and 40% for AtUPS2, respectively). All pyrimidines and their tested derivatives (cytosine, uracil, thymine, dihydrouracil, and 5-FU) induced currents of different magnitude (Fig. 3A). Slight differences were found between AtUPS1 and AtUPS2 in response to thymine and cytosine ($-92$ and $105\%$ for thymine, 50 and 75% for cytosine, respectively), whereas uracil, dihydrouracil, and 5-FU were transported with comparable rates ($-91, 110,$ and $140\%$, respectively). Transporters for some of the tested compounds have already been identified in plants, e.g. for nucleosides (AtENT) (31), for the secondary metabolite caffeine (AtPUP), and for the purine bases adenine, guanine, and hypoxanthine (AtPUP) (11, 12). None of these substrates induced currents in AtUPS1 or AtUPS2-expressing oocytes (Fig. 3, A and B). Moreover, ascorbic acid failed to produce currents.

![Figure 2](image1.png)

**Fig. 2. Functional expression of AtUPS2 in Xenopus oocytes.** AtUPS2 expressing oocytes (A) or non-injected oocytes (B) were flushed with solutions containing 200 $\mu$M of the indicated substances, pH 5.5, and inward currents were recorded at $-100$ mV. C, allantoin-induced currents were recorded with an IV protocol for control oocytes (filled circles), AtUPS1 expressing oocytes (open circles), and AtUPS2 expressing oocytes (open squares) (C). Data points represent the means ± S.D. from at least three oocytes of different batches.

![Figure 3](image2.png)

**Fig. 3. Substrate specificity of AtUPS1 (filled bars) and AtUPS2 (open bars)-expressing oocytes** were flushed with solutions containing 200 $\mu$M concentrations of the indicated substances, pH 5.5 (A). Competition experiments were performed with equal concentrations (200 $\mu$M) of allantoin and tested compounds in a mixture, pH 5.5. Currents determined at $-100$ mV with an IV protocol are presented as the percentages to the currents induced by 200 $\mu$M allantoin alone (B). Data points represent the means ± S.D. from at least three oocytes of different batches. Structural formulas are given accordingly.
are also not transported, confirming that the ureido residue is not a structural determinant for substrates. Hydantoin, a synthetic compound lacking the ureido substitution at position 5 of allantoin, induced currents in AtUPS1- and AtUPS2-expressing oocytes (−108 and 127%, respectively) (Fig. 3A). No inward currents could be recorded for barbituric acid and orotic acid even though they seemed to resemble the structure of pyrimidines, except in having additional substitutions in the meta position to the oxo residues (Fig. 3B). A mechanism of co-transport of one proton with the neutral substrate had been suggested for AtUPS1 (13). The percentage of neutral and charged molecules varies between substrates at pH 5.5 used in the assays. In the case of allantoin, hydantoin, and uracil more than 99% of all molecules are uncharged, whereas in the case of cytosine 89% of all molecules are negatively charged (−80 for uric acid and 20% for xanthine). At least 99.9% of orotic and barbituric acids carry one negative net charge. To elucidate whether these almost exclusively negatively charged compounds are transported without being electrically detected due to proton co-transport, competition experiments were performed. Allantoin and other compounds were mixed at equal concentrations (200 μM), resulting in almost the same current levels as induced by allantoin alone. These results indicate that these compounds are not transported or bound by AtUPS1 or AtUPS2 (Fig. 3B). In summary, all transported substrates were heterocyclic compounds with similar electron density distribution established by the N-hetero-atoms and the substituents. AtUPS1 and AtUPS2 transport a similar range of substrates covering cyclic purine degradation products and pyrimidines.

AtUPS1 and AtUPS2 Have a High Affinity to Uracil—Because the affinities of transporters for their substrates, in conjunction with the local metabolite concentration in the plant, can be informative about the physiological function of the transporters, the kinetic parameters for allantoin and uracil transport were determined for AtUPS1 and AtUPS2. Currents mediated by allantoin or uracil depended on the concentrations of the compounds and displayed saturation kinetics (Fig. 4). For AtUPS1, an apparent $K_m$ for allantoin of 75 ± 2.7 μM was determined. This value is significantly higher as compared with that obtained by measuring $^{14}$C-allantoin uptake in yeast (52 ± 2 μM) (13). The discrepancy might be due to the use of different expression systems (yeast versus X. laevis oocytes) and/or different measurement techniques (detection of radioactivity versus electrophysiological detection of co-transported protons). AtUPS2 had a 3-fold lower $K_m$ of 26 ± 5.4 μM. Both transporters showed higher affinities to uracil with $K_m$ values in the range of 5.9 ± 0.7 μM (AtUPS1) and 6.2 ± 1.6 μM (AtUPS2; Fig. 4). Thus, AtUPS1 and AtUPS2 have 11- and 5-fold higher affinity to uracil than to allantoin.

AtUPS1 and AtUPS2 Are Expressed during Seedling Development—AtUPS1 expression was found to be induced under conditions of nitrogen starvation (13). Nitrogen starvation causes a multitude of effects including nucleobase deficiency (31). To investigate whether AtUPS transcript levels might be increased under conditions of increased demand for nucleobases, mRNA levels of AtUPS1 and AtUPS2 were studied in seedlings during the first 11 days of development. AtUPS1 levels were high at day 1, declining continuously to steady levels after day 5 (Fig. 5). In contrast, expression of AtUPS2 was hardly detectable during the first 2 days of seedling development, whereupon expression levels increased until day 9. To study tissue-specific expression, promoter regions of AtUPS1 and AtUPS2 were isolated and fused translationally to the uidA gene. GUS expression, driven by the AtUPS1 promoter, was observed in 2-day-old seedlings (Fig. 6A). GUS staining was detected in the whole seedling with exception of the radicle. At day 3 GUS activity was present in the hypocotyl and in cotyledons but limited to hydropylot and hydathodes (staining of hydathodes was not always observed) from day 4, further decreasing with age.

GUS activity driven by the AtUPS2 promoter was in agreement with the reverse transcription-PCR data. Starting at day 4, blue staining was observed in cotyledons, increasing in intensity and distribution during subsequent days (Fig. 6B). At later stages (11 days) GUS activity was high in the cotyledons and primary leaves. In addition, staining was observed in the stelle of roots beginning at about day 5 (Figs. 6B and 7). To localize AtUPS2 expression in the root stelle at the cellular level, transverse sections of plant roots were analyzed. Highest GUS activity was found in the pericycle, and weaker activity was detectable in all cells of the stelle, except in mature xylem tracheary elements (Fig. 7).

To investigate if AtUPS expression is directly regulated by the availability of pyrimidines, the effects of uracil or uridine supplementation and of N-(phosphonomethyl)-L-aspartate, a potent transition-state analog inhibitor of aspartate transcarbamoylase causing pyrimidine starvation (32), were studied in Arabidopsis seedlings. Pyrimidine supplementation or starvation caused no more than slight variations of AtUPS expression levels (Supplemental Fig. 2). These data do not provide evidence for a principal regulation of AtUPS expression by pyrimidine availability and suggest that other regulatory mechanisms may predominate in the context of plant development.

AtUPS1 PTGS Lines and AtUPS2 Knock-out Mutants Are Less Sensitive to 5-Fluorouracil—To test whether AtUPS1 and AtUPS2 serve as uracil transporters in planta, AtUPS2 T-DNA insertion lines ups2-1 and ups2-2 and several AtUPS1 PTGS

with that obtained by measuring $^{14}$C-allantoin uptake in yeast (52 ± 2 μM) (13). The discrepancy might be due to the use of different expression systems (yeast versus X. laevis oocytes) and/or different measurement techniques (detection of radioactivity versus electrophysiological detection of co-transported protons). AtUPS2 had a 3-fold lower $K_m$ of 26 ± 5.4 μM. Both transporters showed higher affinities to uracil with $K_m$ values in the range of 5.9 ± 0.7 μM (AtUPS1) and 6.2 ± 1.6 μM (AtUPS2; Fig. 4). Thus, AtUPS1 and AtUPS2 have 11- and 5-fold higher affinity to uracil than to allantoin.

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AtUPS1 PTGS Lines and AtUPS2 Knock-out Mutants Are Less Sensitive to 5-Fluorouracil—To test whether AtUPS1 and AtUPS2 serve as uracil transporters in planta, AtUPS2 T-DNA insertion lines ups2-1 and ups2-2 and several AtUPS1 PTGS
lines were analyzed (Supplemental Fig. 3). The insertion in
ups2-1 is localized in the first exon 16-bp downstream of
the ATG, causing a deletion of 25 bp of the coding sequence. Line
ups2-2 carries a T-DNA insertion in the third intron of
AtUPS2. In either line no AtUPS2 mRNA was detectable by
reverse transcription-PCR, indicating that both T-DNA inser-
tions prevent the generation of stable full-length transcripts
(Fig. 8). AtUPS1 mRNA levels were severely reduced in five
independent PTGS lines, as shown by reverse transcription-
PCR (Fig. 8). Due to the high sequence similarity of AtUPS1
and AtUPS2 in the region used for PTGS, mRNA levels of
AtUPS2 were also reduced in 2 of the 5 lines. No phenotypic
alterations were found for any of the lines when plants were
grown in soil or on agar. However, when germinated on the toxic
uracil analog 5-FU (200 μM), wild type seedlings showed severe
developmental retardation (33), whereas AtUPS1 PTGS plants as
well as ups2-1 and ups2-2 mutants grew significantly better (Fig.
9). The reduced sensitivity to 5-FU suggests that the UPS trans-
porters serve as the major uptake systems for 5-FU.
High Affinity Pyrimidine Transporters from Arabidopsis

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Given that germination and seedling development is not affected in the mutants with decreased expression of AtUPS1 and AtUPS2, other transporters for uracil or uridine may be able to compensate the reduced UPS transporter activity. The Arabidopsis genome encodes five members of the UPS family. Despite intensive screens, we have been unable to amplify transcripts for AtUPS3 and AtUPS4, although Expressed Service Tags have been identified for AtUPS4. AtUPS5 is transcribed and processed in two different mRNA splicing variants, but their functions remain unknown. In addition, a putative membrane protein (At5g03555) shares 30% sequence identity with the uracil transporter FUR4 of Schizosaccharomyces pombe on the protein level. To date efforts to demonstrate pyrimidine transport for this protein have been unsuccessful in our group. However, it is important to note that the protein encoded by At5g03555 shares also similarity with other PRTs that are transporters for adenine and cytosine (FCY2) or the vitamin thiamine (THI10). Eight proteins of the ENT family are encoded in the Arabidopsis genome. Although substrate specificities and expression of several AtENTs have been recently reported (31, 38), their functions, e.g., during germination, remain unclear.

AtUPS1 and AtUPS2 Serve Distinct Functions—AtUPS1 and AtUPS2 share a high degree of homology at the amino acid level (74% identity) and have similar predicted transmembrane structures (aramemnon.botanik.uni-koeln.de). Phylogenetically related proteins may either transport distinct substrates, have different affinities for a given substrate, or they may be expressed at different times or in different locations on cellular or subcellular level. For instance, the S. cerevisiae DAL4 is 70% identical to FUR4, but it serves different functions as a transporter for allantoin or uracil, respectively (34). In contrast, AtUPS1 and AtUPS2 seem to transport the same range of substrates. A notable difference is the 3-fold higher relative affinity of AtUPS2 for allantoin. All prediction programs of subcellular targeting in the data base Aramemnon consistently predict AtUPS1 to localize to the secretory pathway, whereas three programs suggest that AtUPS2 is in the secretory pathway; one predicts plastidic and one predicts mitochondrial localization (aramemnon.botanik.uni-koeln.de). The uptake efficiency observed in yeast and oocytes expressing AtUPS indicates that both proteins can be functionally targeted to the plasma membrane of these cells. Because mistargeting may occur in heterologous expression systems, subcellular localizations have to be experimentally validated in future studies. The differential expression of AtUPS1 and AtUPS2 mRNA indicates that, despite the similarity in biochemical function, the genes have distinct functions in the plant.

AtUPS1 is strongly expressed in seedlings during the early stages of germination and seedling development. Previous observations in germinating castor bean suggested that RNA from endosperm is degraded and released to the apoplasm in the form of nucleosides and free nucleobases (7). Castor bean cotyledons were shown to be able to take up nucleosides and nucleobases. The high expression of AtUPS1 at day 1 after imbibition may, thus, be taken as an indication that AtUPS1

2 A Schmidt, Y.-H. Su, R. Kunze, S. Warner, M. Hewitt, R. D. Slocum, U. Ludewig, W. B. Frommer, and M. Desimone, unpublished results.
represents a transporter involved in salvaging of free pyrimidines originating from storage tissue mobilization during germination of Arabidopsis plants. The previously observed induction of AtUPS1 expression under N-starvation may be explained by a decreased availability of organic nitrogen (e.g., for nucleotide biosynthesis), necessitating an up-regulation of salvage pathways (13, 31). It has been recently reported that members of the Arabidopsis ENT family were strongly induced by 5-FU-mediated thymidylate starvation (31). We also observed up-regulation of AtENT1 in response to N-(phosphonacetyl)-L-aspartate-mediated general pyrimidine starvation and down-regulation in response to exogenous uracil,2 suggesting that transporter induction might be a general mechanism in response to changes in nucleotide availability. In contrast, only minor changes in AtUPS1 and AtUPS2 transcript levels were observed in response to changes in pyrimidine availability, suggesting that other factors may be more important in regulating expression of these transporters.

AtUPS2 presents a spatially and temporally distinct expression pattern as AtUPS1, indicating a different function. In tropical legumes high amounts of allantoin are produced in root nodules, transferred to the xylem vessels, and exported to the shoot via the transpiration stream. It has been postulated that PvUPS1 serves in the transfer of allantoin through the endodermis and for the loading into the xylem (14). Arabidopsis plants can use allantoin as the sole nitrogen source, which suggest that an allantoin transporter is localized in the roots. AtUPS2, but not AtUPS1, localizes in cells of the root stеле. Thus, AtUPS2 may serve for utilization of a range of substrates present in the rhizosphere including pyrimidines but also allantoin, xanthine, and uric acid. Alternatively, AtUPS2 may be involved in utilization of pyrimidines synthesized in leaves and transported to the roots via the phloem.

Inhibition of either AtUPS1 or AtUPS2 expression led to similar seedling phenotypes when grown on plates containing 5-FU. However, AtUPS1 and AtUPS2 do not serve redundant functions. Thus, it is likely that both proteins are necessary for effective 5-FU transport from the external medium to the location where the toxic effect occurs, presumably in a sequential manner. According to the functions hypothesized above, AtUPS2 may allow 5-FU allocation in the plant, whereas AtUPS1 may transport it in cotyledon cells with photosynthetic potential other functions of this family of N-heterocycle proton cotransporters.

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REFERENCES
1. Moffatt, B. A., and Ashihara, H. (2002) in The Arabidopsis Book (Somerville, C. R., Meyerowitz, E. M., ed) pp. 1–20, American Society of Plant Biologists, Rockville, MD
2. van der Graaff, E., Hooykaas, P., Len, W., Lerchi, J., Kunze, R., Sonnewald, U., and Böltz, R. (2004) Front Biosci. 9, 1803–1816
3. de Koning, H., and Diallinas, G. (2000) Mol. Memb. Biol. 17, 75–94
4. Damaraju, V. L., Damaraju, S., Young, J. D., Baldwin, S. A., Mackey, J., Sawyer, M. B., and Cass, C. E. (2003) Oncogene 22, 7524–7536
5. Luo, M., Orsi, R., Patrucco, E., Pancaldi, S., and Cella, R. (1997) Biochim. Biophys. Acta 1345, 1–8
6. Xiang, C., Han, P., Lutziger, I., Wang, K., and Oliver, D. J. (1999) Nature 398, 70–75
7. Lepisiker, H., de Freger, A., Desimine, M., Schumacher, K., and Tegeder, M. (2002) Plant Cell 14, 87–90
8. Hokry, A., Horky, P., and Abell, A. (2001) Plant Physiol. 126, 1–25
9. Combrink, E., and Bevers, H. (1983) Plant Physiol. 73, 370–376
10. Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U. V., Chen, X. Z., Wang, Y., Brubaker, R. F., and Hediger, M. A. (1999) Nature 398, 70–75
11. Gillissen, B., Bürkle, L., André, B., Kühn, C., and Frommer, W. B. (2000) Plant Cell 12, 291–300
12. Burke, L., Cederh, A., Döpé, C., Stransky, H., Okumoto, S., Gillissen, B., Kühn, C., and Frommer, W. B. (2003) Plant J. 34, 13–26
13. Desimine, M., Catoni, E., Ludewig, U., Hülpert, M., Schneider, A. K., Kunze, R., Tegeder, M., Frommer, W. B., and Schumacher, K. (2002) Plant Cell 14, 87–90
14. Pöllä, H. C., Freich, A., Desimine, M., Schumacher, K., and Tegeder, M. (2004) Plant Physiol. 134, 664–675
15. Cass, C. E., Young, J. D., and Baldwin, S. A. (1998) Biochem. Cell Biol. 76, 761–770
16. Hyde, R. J., Cass C. E., Young, J. D., and Baldwin, S. A. (2001) Mol. Memb. Biol. 18, 53–63
17. Möhlmann, T., Mezher, Z., Schwerdtfeger, H., and Neuhaus, E. (2001) FEBS Lett. 509, 370–374
18. Andersen, P. S., Frees, D., Fast, R., and Mygind, B. (1995) J. Bacteriol. 177, 2008–2013
19. Jund, E., Weber, E., and Chevallier, M. R. (1988) Eur. J. Biochem. 171, 417–424
20. Ludewig, U., van Wieren, N., and Frommer, W. B. (2002) J. Biol. Chem. 277, 13548–13555
21. Ihlenbr, R. S., Strasser, A. W., Honer, C. B., and Hollenberg, C. P. (1991) Yeast 7, 691–692
22. Rentse, D., Lalo, M., Rouha, I., Schmelzer, E., Delrot, S., and Frommer, W. B. (1995) FEBS Lett. 370, 264–268
23. Gietz, R. D., and Schiestl, R. H. (1995) Methods Mol. Cell Biol. 5, 255–289
24. Gueldner, U., Heck, S., Fiedler, T., Reinhauer, J., and Heumann, E. (1994) Nucleic Acids Res. 32, 2519–2524
25. Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., et al. (2003) Science 301, 653–657
26. Luo, M., Orsi, R., Patrucco, E., Panzaldi, S., and Cella, R. (1997) Plant Mol. Biol. 33, 709–723
27. Xiang, C., Han, P., Lutziger, I., Wang, K., and Oliver, D. J. (1999) Plant Mol. Biol. 40, 711–717
28. Clough, S. J., and Bent, A. F. (1998) Plant J. 16, 735–745
29. Martin, T., Wöhrer, E. V., Hummel, S., Willnitzer, L., and Frommer, W. B. (1992) in GUS Protocols (Gallagher, S. R., ed) pp. 23–43, Academic Press, Inc., San Diego, CA
30. Chang, S., Puryear, J., and Caimir, J. (1993) Plant Mol. Biol. Rep. 11, 13–16
31. Li, G., Liu, K., Baldwin, S. A., and Wang, D. (2003) J. Biol. Chem. 278, 35732–35742
32. Bassett, E. V., Boucher, E. Y., Carr, J. M., Williamson, C. L., and Slocum, R. D. (2003) Plant Physiol. Biochem. 41, 695–703
33. Boyes, D. C., Zayed, A. M., Ascenzi, R., McCaskill, A. J., Hoffman, N. E., Davis, K. R., and Gorlach, J. (2001) Plant Cell 13, 1499–1510
34. Yao, H. S., Cunningham, T., and Cooper, T. G. (1992) Yeast 8, 997–1006
35. Amilis, S., Koukaki, M., and Diallinas, G. (2001) Mol. Memb. Biol. 18, 370–376
36. Meintanis, C., Karagouna, A. D., and Diallinas, G. (2000) Mol. Memb. Biol. 17, 47–57
37. Pinson, B., Chevallier, J., and Urban-Grimal, D. (1999) Biochem. J. 339, 37–42
38. Wurmir, A., Traub, M., Flörchinger, M., Neuhaus, H. E., and Möhlmann, T. (2004) Biochem. J., in press
UPS1 and UPS2 from *Arabidopsis* Mediate High Affinity Transport of Uracil and 5-Fluorouracil

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