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Functional Characterization of a Maize Purine Transporter by Expression in *Aspergillus nidulans*

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We have characterized the function of Leaf Permease1 (LPE1), a protein that is necessary for proper chloroplast development in maize, by functional expression in the filamentous fungus *Aspergillus nidulans*. The choice of this ascomycete was dictated by the similarity of its endogenous purine transporters to LPE1 and by particular genetic and physiological features of purine transport and metabolism in *A. nidulans*. When *Lpe1* was expressed in a purine transport–deficient *A. nidulans* strain, the capacity for uric acid and xanthine transport was acquired. This capacity was directly dependent on *Lpe1* copy number and expression level. Interestingly, overexpression of LPE1 from >10 gene copies resulted in transformants with pleiotropically reduced growth rates on various nitrogen sources and the absolute inability to transport purines. Kinetic analysis established that LPE1 is a high-affinity (K_m = 30 ± 2.5 μM), high-capacity transporter specific for the oxidized purines xanthine and uric acid. Competition studies showed that high concentrations of ascorbic acid (>30 mM) competitively inhibit LPE1-mediated purine transport. This work defines the biochemical function of LPE1, a plant representative of a large and ubiquitous transporter family. In addition, *A. nidulans* is introduced as a novel model system for the cloning and/or functional characterization of transporter genes.

INTRODUCTION

Pyrimidine and purine biochemistry have central roles in the growth and development of higher plants. DNA and RNA synthesis rely on a steady flow of new nucleotides, and the turnover of nucleic acids results in the catabolism of nucleotides through salvage pathways (Ross, 1981). Nucleobase derivatives play important roles in cell energization, senescence, and defense reactions. In tropical legumes, nitrogen storage and transport in the form of ureides (allantoin and allantoic acid) are dependent on both nucleobase biogenesis and catabolism (Schubert and Boland, 1990). Nucleobase biochemistry yields important secondary compounds such as cytokinins and the alkaloids theobromine and caffeine (Chen, 1997). Purine catabolism is associated with the infection of crop plants by fungi (Montalbini, 1992, 1995). All are dependent on the intracellular and often intercellular exchange of nucleobase metabolites. The extensive compartmentalization of nucleobase metabolism suggests that several nucleobase transporters with distinct specificities function in different membranes.

Although several nucleobase transport activities have been detected in plants, the corresponding genes have not been cloned. Only recently, *AtPUP1*, a gene from Arabidopsis, has been shown to encode a protein that, on introduction into a *Saccharomyces cerevisiae* strain, functions as a transporter for purine-related compounds (Gillissen et al., 2000). The *AtPUP1* gene was isolated by direct complementation of a *S. cerevisiae* mutant deficient in adenine uptake (*fcy2*) and subsequently shown to transport adenine and cytosine with high affinity. Competition studies also showed that purines, phytohormones (zeatin and kinetin), and alkaloids (caffeine) are potent inhibitors of adenine and cytosine uptake, indicating that several purine-related compounds also may be transported by this system. Interestingly, AtPUP1-like sequences are present only in plants and show no similarity to known nucleobase transporters from other systems (De Koning and Diallinas, 2000). The physiological role of AtPUP1 or other similar proteins in plants remains unknown.

Nucleobase transporter genes from bacteria and fungi have been described in recent years. These genes and similar sequences of unknown function present in databases encode proteins that constitute two major families named nucleobase–ascorbate transporters (NATs) and purine-related transporters (PRTs) (De Koning and Diallinas, 2000). PRT sequences are restricted to prokaryotes and fungi. In contrast, NAT sequences are ubiquitous, are well represented in plants, and include transporters specific for L-ascorbic acid (see below). To date, 13 full-length plant genes, the maize *Leaf permease1* (*Lpe1*), a paralog from the

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ice plant, and 11 paralogs from Arabidopsis are known. In addition, partial sequence information is available from ~30 expressed sequence tag clones from maize, Arabidopsis, rice, tomato, soybean, cotton, rapeseed, aspen, and citrus. Comparison of plant NAT reveals substantial amino acid similarities (~50% identity and ~70% similarity) along the entire length of complete protein sequences. Given that 85% of the Arabidopsis genome sequence is available for analysis, this putative nucleobase transporter family might consist of 14 or 15 members. However, the function of these genes is not known.

The physiological importance of the *Lpe1* gene was demonstrated recently (Schultes et al., 1996). A functional *Lpe1* gene is necessary for proper chloroplast development and membrane integrity in maize, because mutant *lpe1-m1* leaf tissue displays a cell autonomous defective chloroplast phenotype most apparent in leaf bundle sheath cells. Although the function of LPE1 remains unknown, related bacterial homologs are uracil or xanthine transporters (Andersen et al., 1989; Ghim and Neuhard 1994; Turner et al., 1994; Christiansen et al., 1997), and two *Aspergillus nidulans* homologs are transporters specific for uric acid, xanthine, and other purines (Gorfinkiel et al., 1993; Diallinas et al., 1995). Interestingly, mammalian homologs specifically transport L-ascorbic acid (Daruwala et al., 1999; Rajan et al., 1999; Tsukaguchi et al., 1999).

In this article, the biochemical function of LPE1 is investigated using *A. nidulans* as a novel model system. The choice of this ascomycete was based on the current state of the art of nucleobase transport and metabolism in this fungus. Our results demonstrate that LPE1 is a highly specific and efficient uric acid–xanthine transporter that also can bind, but is unable to transport, ascorbate at high concentrations. We also show that functional expression of LPE1 is directly dependent on the levels of *Lpe1* expression in *A. nidulans* and discuss the consequences of this observation with regard to the cloning and expression of transport proteins in this novel model system.

RESULTS

Rationale for the Functional Expression of LPE1 in *A. nidulans*

There are serious disadvantages to investigating the putative transport function of LPE1 in planta. First, difficulties in manipulating plant cells and quantifying solute transport render these cells poor candidates for primary transport studies. Second, the high number of NAT genes in plant genomes opens the possibility of solute transport redundancy; thus, transport studies comparing *lpe1-m1* with wild-type cells would be uninformative. Alternatively, the functional characterization of LPE1 is amenable to heterologous complementation studies in simple model microorganisms, and the choice of the most appropriate system is of prime importance. We chose *A. nidulans* as our model system for the study of LPE1 for several reasons.

LPE1 has a structure that is characteristic of hydrophobic transmembrane proteins (Schultes et al., 1996). The functional expression of eukaryotic transmembrane proteins is dependent on proper intracellular targeting, which implicates specific interactions with several cellular factors. Thus, the use of a simple eukaryotic system seems, a priori, more appropriate than a bacterial system for expressing LPE1. *S. cerevisiae* has proved to be a unique model system for cloning or expressing transporters from higher organisms (Frommer et al., 1993; Hsu et al., 1993; Chiou and Bush, 1996; Eide et al., 1996; Hogue et al., 1996; Mäser et al., 1999; Vickers et al., 1999). However, certain transporters cannot be identified in yeast either because their substrates are not transported or metabolized or because of the presence of several transporters with overlapping transport specificities. Thus, heterologous complementation studies are not possible or meaningful. Most wild-type *S. cerevisiae* strains have "lost" the ability to catabolize purines, although they still can break down allantoin or allantoic acid to urea and ammonia. This is reflected in the evolution of their uptake systems, which include transporters specific for salvageable purines and pyrimidines but lack transporters for the oxidized purines uric acid and xanthine.

A. nidulans is a nonpathogenic ascomycete that in recent years has proved to be an excellent system for genetic and molecular studies. Nucleobase metabolism and transport have been characterized extensively (Scazzocchio, 1994). Like plants, *A. nidulans* can use end products or intermediates of purine catabolism as nitrogen sources and possesses a rich repertoire of transporters specific for all purines and uracil. All three genes involved in purine transport, *uapA*, *uapC*, and *azgA*, have been cloned and studied at the molecular level. The UapA protein is a high-affinity, high-capacity transporter specific for uric acid and xanthine (Darlington and Scazzocchio, 1967; Diallinas and Scazzocchio, 1989; Gorfinkiel et al., 1993). The UapC protein is a high-affinity, moderate-capacity carrier for uric acid and xanthine, but it also transports with very low capacity all other purines and purine analogs (Scazzocchio and Gorton, 1977; Diallinas et al., 1995). UapA and UapC belong to the NAT family and are significantly similar to LPE1 and other plant proteins. Structure–function studies using chimeric UapA/UapC proteins have shown that a short region that includes two transmembrane segments (TMSs) is critical for the function and specificity of these transporters (Diallinas et al., 1998). Other studies have shown that substitutions of specific amino acid residues in adjacent hydrophilic loops (Diallinas et al., 1998; Meintanis et al., 2000) or in the last TMS (M. Koukaki, S. Amillis, and G. Diallinas, unpublished data) alter the specificity of UapA for different purines, pyrimidines, or nucleosides. The third gene, *azgA*, which encodes a high-affinity, high-capacity transporter specific for adenine, guanine, and hypoxanthine, was cloned recently (Darlington and

Scazzocchio, 1967; C. Drevet and C. Scazzocchio, unpublished data). Finally, genes involved in uracil transport (*fiul*) also have been identified genetically (Palmer et al., 1975; S. Amillis and G. Diallinas, unpublished data). Loss-of-function mutations in any of these genes can be detected in simple growth tests in which purines are provided as sole nitrogen sources or in the presence of toxic purine or pyrimidine analogs (Palmer et al., 1975; Diallinas and Scazzocchio, 1989; Scazzocchio, 1994). Thus, any foreign gene complementing *uapA*$^-$, *uapC*$^-$, *azgA*$^-$, or *fiul*$^-$ mutations can be detected easily in appropriate media, cloned, and studied in detail by direct transport measurements.

To achieve the expression of LPE1 in *A. nidulans*, we constructed vector pAN-Lpe1, which is shown in Figure 1 (for details, see Methods). This vector carries the *Lpe1* coding region as a translational fusion with *uapA* 5′ and 3′ regulatory sequences and the *A. nidulans argB* gene as a selection marker (see below). *uapA* regulatory sequences were selected to achieve *Lpe1* expression levels similar to those of the homologous endogenous transporter UapA. The *Lpe1* coding region in vector pAN-Lpe1 encodes a protein identical to LPE1 except for the amino acid residue at position 2, in which aspartate is substituted by proline.

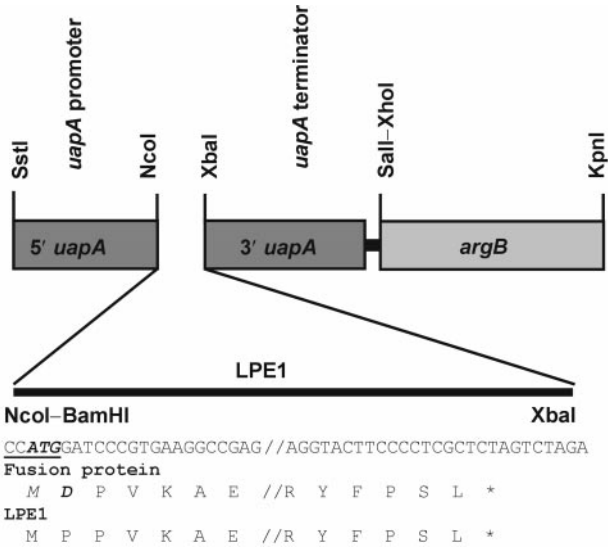

Figure 1. Construction of Plasmid pAN-Lpe1.

The coding region of *Lpe1* inserted as a translational fusion with the *uapA* expression vector. The fused LPE1 protein differs at amino acid position 2, with aspartate substituting for proline. For details of plasmid construction, see Methods. Boldface, italic "D" indicates the only amino-acid difference of the fusion protein compared to the LPE1 protein. Asterisk indicates termination codon; letters at the bottom indicate LPE1 amino-acid sequence. Slashes indicate amino-acid sequence not shown in the figure. Underlining indicates restriction site of NcoI.

Lpe1 Complements a *uapA*$^-$*uapC*$^-$*azgA*$^-$ Strain for Growth on Uric Acid

Plasmid pAN-Lpe1 and control plasmids pAN510 and pIJL16 (Diallinas et al., 1998) were introduced by transformation into strain ACZ, which lacks all major purine transporters (*uapA*$^-$*uapC*$^-$*azgA*$^-$) and thus cannot grow on purines as sole nitrogen sources (for plasmids, full genotypes, and other experimental details, see Methods). In addition, the presence of a loss-of-function allele of *argB* in this strain results in the inability to grow in the absence of arginine from the growth medium. All plasmids used in transformations include a wild-type copy of the *argB* gene and thus allow the selection of *argB*$^+$ transformants in arginine-free minimal medium. Several transformants obtained with each plasmid were selected and purified on selective medium. Genomic DNA was isolated from all purified transformants and amplified by polymerase chain reaction (PCR) using *uapA*-specific primers corresponding to 17 nucleotide sequences present immediately upstream of or downstream from the start or stop codons of the *uapA* gene, respectively. These primers (see Methods) allowed the amplification of the genomic *uapA* loss-of-function allele from all transformants (2.1 kb), the amplification of the plasmid *uapA* wild-type allele in ACZ:pAN510 transformants (2.1 kb), and the amplification of *Lpe1* sequences (1.6 kb) only from transformants carrying the pAN-Lpe1 construct. The identity of the amplified bands was confirmed by DNA gel blot analysis using *uapA*- and *Lpe1*-specific probes (data not shown). These results strongly suggested that isolated strains were true transformants and that intact *Lpe1* sequences had been integrated into the genome of the recipient strain.

Selected transformants were analyzed for their ability to grow on various purines as sole nitrogen sources. All transformants carrying *Lpe1* sequences (LP2, LP4, LP5, and LP6) could grow, albeit at various rates, on uric acid (Figure 2) and xanthine (data not shown). None of the transformants could grow on adenine, hypoxanthine, or guanine (data not shown). Growth on uric acid or xanthine was not affected by the temperature or pH of the medium when these were kept within the physiological range for *A. nidulans* (25 to 37°C and pH 5.5 to 8.0, respectively; data not shown). Transformant LP2 grew very strongly and had a compact morphology, similar to that of wild-type strains or *uapA*$^-$*uapC*$^-$*azgA*$^-$ transformants carrying a wild-type *uapA* gene (ACZ:pAN510). Transformant LP5 also had a compact morphology but grew less well than did LP2. DNA sequencing of the amplified genomic *uapA* gene from both LP2 and LP5 confirmed the presence of the total loss-of-function mutation in the recipient strain. These observations exclude the possibility that uric acid or xanthine transport in these transformants is due to the generation of chimeric transporter genes or reversion to the original *uapA*$^-$ mutation.

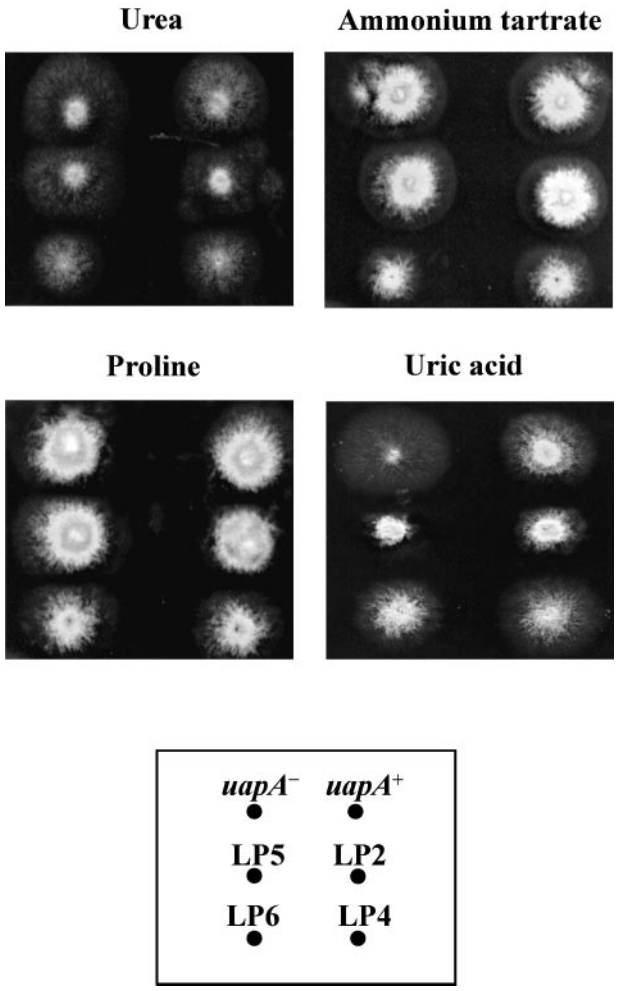

Figure 2. Growth of *A. nidulans* Strains on Various Nitrogen Sources.

Growth of control strains (ACZ:plJL16 [*uapA*$^-$] and ACZ:pAN510 [*uapA*$^+$]) and ACZ:pAN-Lpe1 transformants (LP2, LP4, LP5, and LP6). Growth was on *A. nidulans* minimal medium supplemented with 0.1 mg/mL uric acid or 5 mM proline, urea, or ammonium tartrate as sole nitrogen sources. Growth tests were performed for 48 hr at 37°C. Strain terminology is described in the text.

Transformants LP4 and LP6 showed reduced growth on uric acid or xanthine but also lacked the characteristic compact morphology of LP2, LP5, and control (ACZ:pAN510) transformants. More important, transformants LP4 and LP6 also showed reduced growth on several other nitrogen sources unrelated to purines, such as proline, urea, and ammonium tartrate (Figure 2). In addition, their phenotype proved to be mitotically unstable, because after repeated replica plating and growth their ability to grow on purines was reduced further (data not shown).

LPE1 Expression Levels Determine the Ability of ACZ:pAN-Lpe1 Transformants to Grow on Purines

To investigate the nature of the plasmid integration events that led to transformants being able to grow on uric acid and xanthine, we performed DNA gel blot analysis of genomic sequences extracted from transformants LP2, LP4, LP5, and LP6. DNA gel blot analysis was performed with *Lpe1*-, *uapA*-, and *argB*-specific radiolabeled probes as described in Methods. Selected results are shown in Figure 3. Integration of plasmid sequences on the genome was detected in all cases, confirming the fact that isolated strains were true transformants. Integration events took place by heterologous recombination. This was demonstrated by the presence of intact *uapA*- and *argB*-specific bands corresponding to the genomic copies of these genes (data not shown). As was suggested by PCR analysis (see above), *Lpe1* sequences did not participate in recombination events, as judged by the fact that all transformants showed intact *Lpe1*-specific bands (Figure 3). In all transformants, multiple copies of pAN-Lpe1 were integrated into the genome. Transformant LP2, which showed the best growth on uric acid or xanthine, had three copies. Transformant LP5, which showed reduced growth on these purines, arose from an identical integration event as LP2 and had the most copies (>10). Interestingly, unstable transformants LP4 and LP6, which showed reduced growth on purines and on other nitrogen sources, result from a different heterologous event (data not shown) compared with that of LP2 and LP5, also leading to the integration of multiple pAN-Lpe1 copies (>10; data not shown). These results suggest that although the integration of intact *Lpe1* sequences into the genome of the recipient strain is responsible for growth on uric acid or xanthine, the rate of growth on these purines is directly dependent on the nature of the integration event.

To further investigate the relationship between *Lpe1* expression and growth on uric acid or xanthine, we performed RNA gel blot analysis of total RNA extracted from stable transformants LP2 and LP5. Our results (Figure 4) show a stable *Lpe1*-specific message (1.7 kb). This message is strongly induced in the presence of uric acid, as expected due to the presence of *uapA* promoter and terminator sequences upstream of and downstream from, respectively, the *Lpe1* open reading frame (Diallinas and Scazzocchio, 1989). The steady state levels of *Lpe1* mRNA in LP5 (>10 *Lpe1* copies) were estimated to be 3.4-fold higher than those in LP2 (three *Lpe1* copies). These results show that *Lpe1* gene expression is critical for complementing the genetic deficiency of the recipient strain for uric acid or xanthine uptake and suggest that very high levels of *Lpe1* mRNA have a negative effect on the expression of the LPE1 transporter.

To further investigate the association between LPE1 expression levels and the capacity to transport purines, we took advantage of particular features of the *A. nidulans* system. When *A. nidulans* transformants carrying multiply inte-

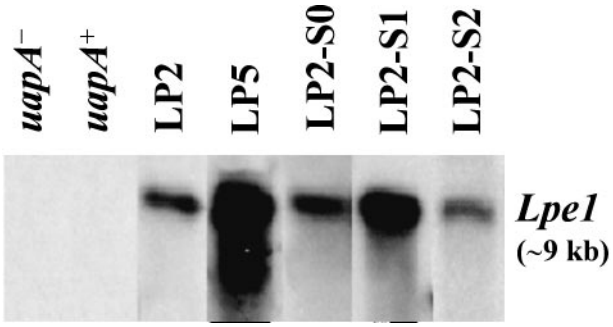

Figure 3. Copy Number of *Lpe1* in *A. nidulans* Original Transformants and Progeny.

DNA gel blot analysis of genomic DNA of control strains ACZ:pIJL16 (*uapA*$^-$) and ACZ:pAN510 (*uapA*$^+$), transformants ACZ:pAN-Lpe1 (LP2 and LP5), and selfed LP2 strains (LP2-S0, LP2-S1, and LP2-S2). Approximately 5 μg of DNA was digested with PstI, an enzyme that does not cut within the plasmid pAN-Lpe1. A randomly labeled full-length *Lpe1* cDNA was used as a ^{32}P-labeled radioactive probe. The length of the hybridized band is indicated at right.

grated copies of plasmids undergo a meiotic cycle (selfing; Tilburn et al., 1983), the number of integrated copies in some progeny is reduced or increased due to unequal genetic crossover. We analyzed 50 colonies arising from selfing of transformant LP2 by growth tests on uric acid or xanthine as sole nitrogen sources, and 48 of them retained the capacity to grow on these purines (data not shown). Two isolates (LP2-S1 and LP2-S2) lost this capacity (Figure 5). We performed both DNA and RNA gel blot analyses of total genomic DNA and RNA, respectively, isolated from LP2-S1 and LP2-S2 and from LP2-S0 (a colony from progeny that conserved the capacity for purine uptake). The results are shown as part of Figures 3 and 4. LP2-S1 had an increased number of *Lpe1* copies (>10) that was compatible with a sixfold increase in *Lpe1* mRNA levels compared with the parental strain LP2, which carried three copies of *Lpe1*. LP2-S2 retained a single copy of *Lpe1* and showed 2.5-fold reduced expression of *Lpe1* mRNA compared with the parental strain. LP2-S0 retained two copies of *Lpe1* and showed 80% of *Lpe1* mRNA levels compared with the parental strain. These results provide compelling evidence that the level of *Lpe1* mRNA expression, which is directly dependent on the number of *Lpe1* copies integrated into the genome, determines the capacity of the recipient strain to grow on uric acid or xanthine.

Biochemical Properties of LPE1

To further characterize the biochemical function of the LPE1 protein, we performed radiolabeled purine uptake assays by using the LP2 transformant. We calculated the K_m of LPE1 for ^{3}H-xanthine and measured the initial uptake rates of ^{3}H-xanthine accumulation in the absence or presence of other "cold" purines and pyrimidines in germinating conidiospores of LP2. The method for uptake measurements has been described (Tazebay et al., 1995; Diallinas et al., 1998). The K_m for xanthine was 30 ± 2.5 μM. The K_m for uric acid showed a similar value (30 to 35 μM), as estimated indirectly by the corresponding competitive inhibition constant value (data not shown).

Figure 6 shows the competition profile of various purines and pyrimidines on the uptake of 10 μM ^{3}H-xanthine. LPE1-mediated ^{3}H-xanthine uptake was equally well inhibited by excess amounts (300 μM) of "cold" xanthine or uric acid but was not inhibited by adenine, guanine, hypoxanthine, uracil, thymine, cytosine, or caffeine. These results showed that LPE1 has specificity and kinetic profiles very similar to those of the *A. nidulans* UapA transporter and that both proteins act as very efficient and highly specific uric acid–xanthine transporters.

Considering the similarity of LPE1 to mammalian ascorbate transporters (Daruwala et al., 1999; Rajan et al., 1999; Tsukaguchi et al., 1999), we also investigated whether LPE1 is capable of recognizing L-ascorbic acid. A similar analysis

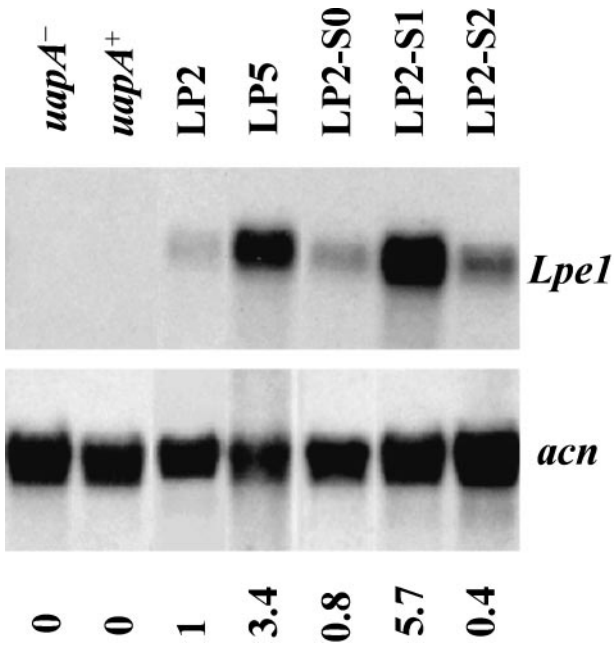

Figure 4. Expression of *Lpe1* in *A. nidulans*.

RNA gel blot analysis of 10 μg of total RNA isolated from control strains ACZ:pIJL16 (*uapA*$^-$) and ACZ:pAN510 (*uapA*$^+$), transformants ACZ:pAN-Lpe1 (LP2 and LP5), and selfed LP2 strains (LP2-S0, LP2-S1, and LP2-S2). A randomly labeled full-length *Lpe1* cDNA was used as a ^{32}P-labeled radioactive probe. Control hybridization with an actin (*acn*) radiolabeled probe is shown below. Numbers below the blot show relative estimations of *Lpe1* expression after normalization by using the levels of the constitutively expressed *acn* gene.

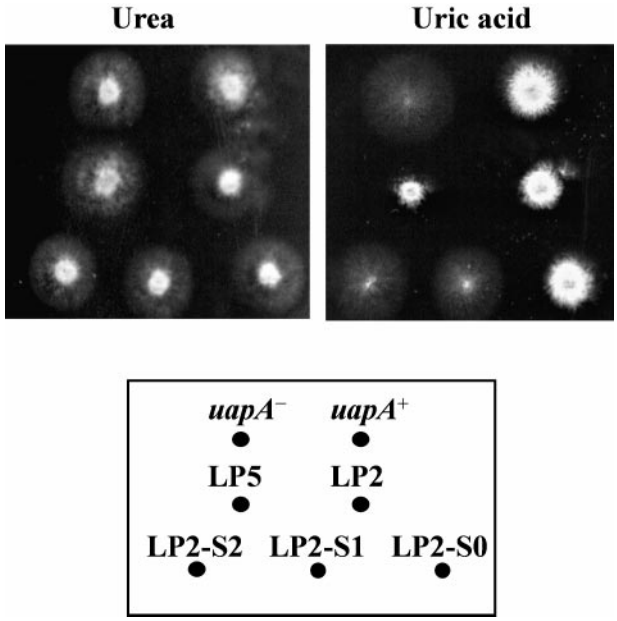

Figure 5. Growth of Selected Selfed LP2 Progeny on Uric Acid.

Growth of control strains (ACZ:pIJL16 [*uapA*$^-$] and ACZ:pAN510 [*uapA*$^+$]), transformants ACZ:pAN-Lpe1 (LP2 and LP5), and selfed LP2 strains (LP2-S0, LP2-S1, and LP2-S2). Growth was on *A. nidulans* minimal medium supplemented with 0.1 mg/mL uric acid or 5 mM urea as sole nitrogen sources. Growth tests were performed for 48 hr at 37°C. Strain terminology is described in the text.

was performed for UapA. We measured initial ^{3}H-xanthine uptake rates in the presence of cold ascorbic acid at a concentration of 35 μM to 100 mM in strains expressing LPE1 (LP2) or UapA (ACZ:pAN510). Figure 7A shows that high levels (>30 mM) of L-ascorbic acid nearly completely inhibited LPE1- or UapA-mediated ^{3}H-xanthine uptake. Inhibition of LPE1-mediated xanthine uptake was stronger than inhibition of UapA-mediated xanthine uptake. The concentration of ascorbic acid needed to achieve 50% inhibition (PC_{50}) of 10 μM ^{3}H-xanthine uptake via LPE1 or UapA was estimated to be 11 or 15 mM, respectively (data not shown). The effect of ascorbic acid was specific to these transporters because it had no effect on carrier-mediated ^{3}H-L-proline uptake (Figure 7B). To further investigate the nature of ascorbic acid inhibition of LPE1, we performed a kinetic analysis of initial uptake rates of ^{3}H-xanthine in the presence of different concentrations of ascorbic acid. Table 1 shows that the K_m for xanthine was progressively shifted to higher values with increasing amounts of inhibitor. These results are compatible with a mechanism of competitive inhibition in which xanthine and ascorbic acid compete for the same binding site. To determine whether ascorbic acid actually is transported within germinating conidiospores of *A. nidulans*, we performed ^{14}C-L-ascorbic acid uptake assays at final substrate concentrations of 1 to 5 mM. Our results show that ascorbic acid was not transported by LPE1 or UapA (data not shown).

Table 2 further summarizes experiments concerning the biochemical properties of LPE1. LPE1-mediated xanthine uptake was found to be sensitive to protonophores and to H^+-ATPase inhibitors but remained fairly stable at a pH range close to the physiological range for *A. nidulans* (pH 5.5 to 8.0; data not shown). Xanthine uptake also was found to be independent of the presence of Na^+ from the uptake medium. Similar data were obtained with UapA-mediated xanthine uptake and indicate a common mechanism of proton-coupled xanthine transport.

DISCUSSION

LPE1 Is a High-Affinity Uric Acid–Xanthine Transporter

The work presented in this article describes the use of the ascomycete *A. nidulans* as a model system in which to study the function of LPE1, a plant representative of the NAT family. LPE1 was shown to be a high-affinity, high-capacity uric acid–xanthine transporter very similar to the *A. nidulans* UapA transporter. Both transporters are highly specific for oxidized purines, their pH range for full activity is very simi-

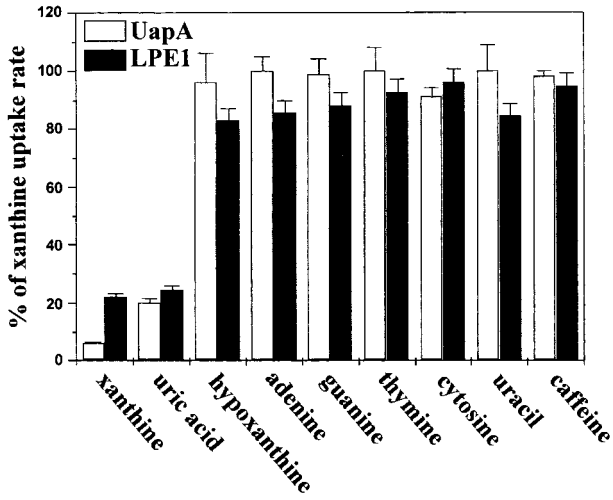

Figure 6. Substrate Specificity of UapA and LPE1.

Specificity was determined by inhibition of ^{3}H-xanthine (10 μM) uptake by excess (300 μM) unlabeled nucleic acid bases and derivatives. The xanthine uptake rate in the absence of inhibitor was taken as 100%. Data represent means of at least three independent experiments. For experimental details, see Methods. Error bars indicate mean standard deviation values from at least three independent experiments.

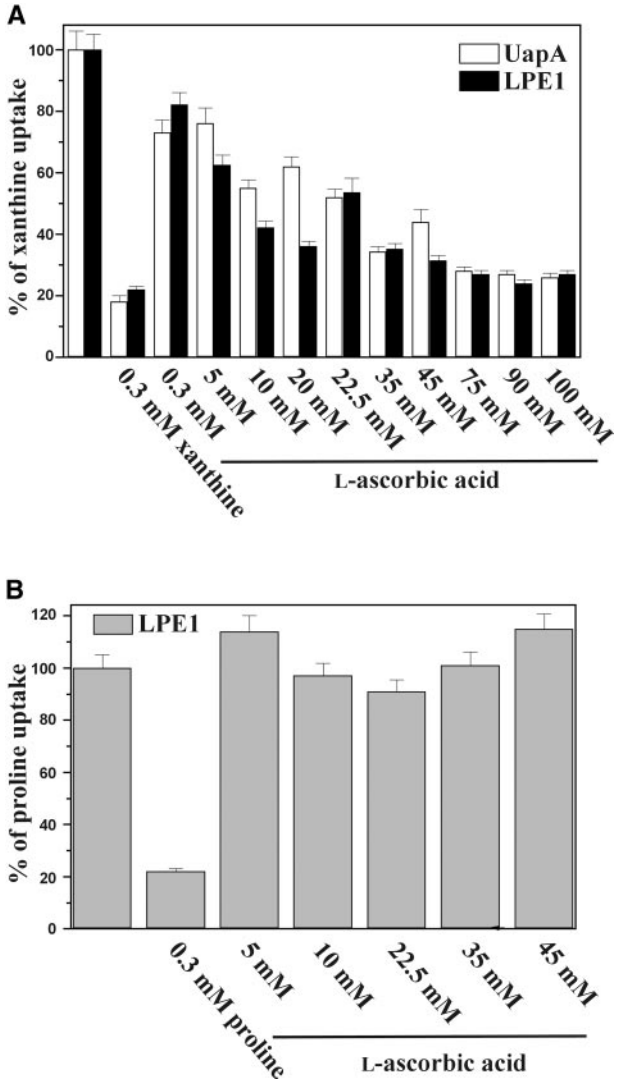

Figure 7. Inhibition of ^{3}H-Xanthine (10 μM) or ^{3}H-L-Proline (10 μM) Uptake by L-Ascorbic Acid in ACZ:pAN510 (UapA) and ACZ:pAN-LPE1 (LPE1) Strains.

Uptake of ^{3}H-xanthine **(A)** and ^{3}H-L-proline **(B)** was determined in the presence of different concentrations of L-ascorbic acid. Data represent means of at least three independent assays. For experimental details, see Methods. Error bars indicate mean standard deviation values from at least three independent experiments.

lar, and both catalyze high-affinity transport of their substrates through a proton-coupled process. Both proteins also are competitively inhibited by high levels of ascorbic acid and present only minor kinetic differences. The K_m for xanthine of LPE1 is fourfold higher than that of UapA (30 versus 7.5 μM), whereas the PC_{50} of L-ascorbic acid is relatively lower in LPE1 compared with that in UapA (11 versus 15 mM). Given that antibodies to LPE1 are not available, we cannot compare the absolute capacities of LPE1 and UapA, because this is directly dependent on the amount of protein present in the plasma membrane. Expression from two or three copies of *Lpe1* is comparable to expression from a single copy of *uapA*, but this might be due simply to more efficient topogenesis of the native UapA compared with that of the foreign LPE1.

NAT Proteins Have Evolved Different Specificities That Cannot Be Deduced by Sequence Similarities

Primary sequence and evolutionary analyses of plant NAT sequences have shown that they are probably more related to metazoan NAT sequences (including the mammalian ascorbate transporters) than to microbial nucleobase transporters (De Koning and Diallinas, 2000). However, the results presented here clearly confirmed that LPE1 is a purine transporter. NAT proteins have evolved different solute specificities that cannot be predicted by overall sequence similarities. Interestingly, an amino acid residue that has been shown to be critical for specificity in UapA (Q449) is better conserved in LPE1 than in mammalian ascorbate transporters (De Koning and Diallinas, 2000; Meintanis et al., 2000). This observation suggests that differences in a limited number of critical amino acid residues in NAT proteins might define specificity. This assumption is supported experimentally by our unpublished results (M. Koukaki, S. Amillis, and G. Diallinas, unpublished results), which showed that UapA can be converted into a uracil, an adenine-hypoxanthine, or an adenosine transporter with one or two amino acid substitutions.

Although uric acid and ascorbic acid are structurally unrelated substances, both are substrates of NAT proteins. LPE1 and UapA can transport uric acid and xanthine but can only bind ascorbic acid, whereas human SVCT-1 transports ascorbic acid but is inhibited by xanthine (Tsukaguchi et al., 1999). This indicates that NAT proteins, unlike most solute symporter families, are characterized by significant structural plasticity that allows for the evolution of different solute specificities among chemically unrelated compounds. Interestingly, uric acid and ascorbic acid are both outstanding

Table 1. K_m Values for ^{3}H-Xanthine Uptake in Strain ACZ:pAN-Lpe1 (LPE1) in the Presence of Different L-Ascorbic Acid Concentrations

L-Ascorbic Acid Concentration (mM)	K_m (μM)
—[a]	30 ± 2.5
5	47 ± 2.0
10	59 ± 2.5
45	92 ± 3.0

[a] No ascorbic acid.

Table 2. Influence of Inhibitors and Protonophores on Xanthine (10 μM) Uptake Rate in ACZ:pAN510 (UapA) and ACZ:pAN-Lpe1 (LPE1) Strains

	^{3}H-Xanthine Uptake Rate (pmol min^{-1} 10^7 conidiospores^{-1})	
Inhibitor	UapA	LPE1
Without inhibitor	0.209	0.236
N,N′-dicyclohexylcarbodiimide (100 μM)	0.027	0.029
Carbonyl cyanide *m*-chlorophenyl-hydrazone (30 μM)	0.010	0.032

antioxidants because of their action as oxygen free radical scavengers (Ames et al., 1981; Nyyssonen et al., 1997), and uric acid also stabilizes ascorbic acid as a radical scavenger (Sevanian et al., 1991).

In Search of the Physiological Role of LPE1

What is the physiological role of LPE1 in maize? We suggest that LPE1 acts as a xanthine and uric acid transporter in plant cells. It is unclear in which cellular membrane LPE1 resides, because xanthine is involved in both intracellular and intercellular metabolite exchanges. There is evidence to support the possibility that LPE1 resides in plastid membranes. Maize *lpe1-m1* mutant leaves are pale green and deficient in pigment, and aberrant membrane structures are evident in both bundle sheath and mesophyll chloroplasts (Schultes et al., 1996). Although the plastid is the main site for purine biochemistry, the catabolism of xanthine to uric acid is believed to occur in peroxisomes, necessitating the transport of xanthine across plastid membranes (Schubert and Boland, 1990). A disruption in xanthine transport could lead to the accumulation of xanthine or purine intermediates in the plastid. Such metabolite accumulation may affect other plastid biochemical pathways and lead to reduced pigment levels, photosensitivity, and altered plastid morphology in high light.

Alternately, LPE1 may localize to the plasma membrane. In this case, LPE1 may serve to shuttle uric acid or xanthine into the apoplast. This work shows that LPE1 is functionally targeted to the plasma membrane of *A. nidulans*. Computer analysis revealed that LPE1 has 80 to 90% probability to be a plasma membrane protein and 50% probability to be plastidic and contains no discernible chloroplast signal sequence. Intercellular exchange of xanthine or xanthine derivatives is known to play an important role in ureide synthesis in the root nodules of tropical N_2-fixing legumes such as soybean (Schubert and Boland, 1990). Xanthine catabolism is involved in plant–fungus interactions. In incompatible fungus–host interactions in bean and wheat, xanthine catabolism via xanthine oxidase generated activated oxygen radicals, most likely as part of the hypersensitive response (Montalbini, 1992, 1995). This burst of oxygen radical formation occurs on the apoplastic portion of the plasmalemma (Baker and Orlandi, 1995), suggesting that xanthine may be actively transported out of the cell. As mentioned above, uric acid functions together with ascorbate as a free radical scavenger. Ascorbate is known to play a role in protection from ozone (Conklin et al., 2000) and in regulating cell wall expansion in the plant apoplast (Noctor and Foyer, 1998). Perhaps uric acid is transported out of the cell via LPE1 to serve similar functions. Future research will seek to determine the subcellular location of LPE1. This information will be useful for devising experiments to address the compartmentalization of nucleobase biochemistry in plant cells.

Several observations suggest that LPE1 and the recently identified AtPUP1 protein, which also transports purine-related solutes, have distinct roles in plants. The two proteins are structurally unrelated. LPE1 and similar transporters (>450 amino acid residues) are predicted to have secondary structures with 10 to 12 TMSs, whereas AtPUP proteins are shorter (345 to 419 amino acid residues) and have predicted secondary structures of 9 to 10 TMSs. Although LPE1 homologs are abundant in archeae, bacteria, fungi, plants, and metazoans, AtPUP1 homologs are restricted to plants. The lack of AtPUP transporters from complete genome sequences of prokaryotes, fungi, and metazoans suggests that these transporters serve plant-specific biochemical processes. In contrast, LPE1 should serve cellular functions common to all domains of life. LPE1 and AtPUP1 specificities also are very different. LPE1 transports uric acid and xanthine and binds ascorbic acid, whereas AtPUP transports adenine, hypoxanthine, and cytosine and binds cytokinins, phytohormones, and alkaloids. *Lpe1* is expressed abundantly in roots and at lower levels in etiolated and green leaves (Schultes et al., 1996). Interestingly, the AtPUP1 transporter, which has a mirror image expression profile, is expressed in all plant tissues except for roots (Gillissen et al., 2000).

A. nidulans as a Novel System for Cloning and Studying Foreign Transporters

Cloning of eukaryotic transporter genes by functional complementation in yeast has proved to be a very efficient methodology (Frommer et al., 1993; Hsu et al., 1993; Chiou and Bush, 1996; Eide et al., 1996; Hogue et al., 1996; Mäser

et al., 1999; Vickers et al., 1999; Gillissen et al., 2000). In the present study, we showed that *A. nidulans* also can be used for transporter functional complementation. This is an important consideration for the investigation of higher organism transporters, which are difficult to clone or express in other systems. In particular, the *A. nidulans* system will serve as a valuable tool not only for discovering the function of NAT proteins but also for elucidating the molecular determinants that define their solute specificities. This work and the recent improvements of transformation efficiencies in *A. nidulans* (Dawe et al., 2000; G. Diallinas, unpublished results) indicate that *A. nidulans* can be used to directly clone novel transporter genes by complementation with cDNA libraries.

An interesting aspect also arising from this work is that the introduced transporter gene expression level is critical for the successful expression of the corresponding protein. We observed previously that eight- to 10-fold overexpression of the *A. nidulans* endogenous *uapA* or *uapC* transporter genes from multiply integrated copies or from up-promoter mutations led to a generalized reduction of growth rates on various substances related to their substrates (G. Diallinas and C. Scazzocchio, unpublished data). Studies with the UapA, UapC, and PrnB (the major proline transporter; Sophianopoulou and Scazzocchio, 1989) proteins fused to the green fluorescent protein have shown that overexpression of these transporters leads to increased intracellular accumulation (Valdez-Taubas et al., 2000; S. Tavoularis, C. Scazzocchio, and V. Sophianopoulou, submitted manuscript). Other studies in yeast also have suggested that "toxic" effects from overexpression of transporter genes might be due to "crowding" during transporter topogenesis from the endoplasmic reticulum and via the Golgi to the plasma membrane (Loayza et al., 1998). In the case of LPE1 overexpression, we observed that sixfold overexpression completely blocked purine uptake and in some cases reduced growth on other nitrogen sources. The negative effects of LPE1 overexpression may be due to the lack of sequences necessary for the recycling and turnover of endogenous overexpressed transporters. These findings should be considered in experiments designed to clone foreign transporters by direct complementation of microbial transporter mutants. In such cases, finely regulated promoters should be used for the controlled expression of transporter sequences.

METHODS

Media, Growth Conditions, and Strains for *Aspergillus nidulans*

Minimal medium and complete medium for *A. nidulans* have been described previously (Cove, 1966; Scazzocchio et al., 1982). Supplements were added when necessary. In growth tests, all purines were used at a final concentration of 0.1 mg/mL. Other nitrogen sources (proline, urea, and ammonium tartrate) were used at 5 mM final concentration. The principal recipient strain used was *yA2argB2-uapC201uapC401uapA24azgA4* (ACZ). *biA1*, *pabaA1*, and *argB2* indicate auxotrophy for biotin, *p*-aminobenzoic acid, and arginine, respectively. *yA2* results in yellow conidia. These markers do not affect the regulation of gene products involved in purine catabolism. *uapA24*, *azgA4*, and *uapC401* are genetically obtained loss-of-function mutations in the corresponding genes. *uapC201* is a promoter mutation (Diallinas et al., 1995).

DNA Manipulations and Plasmid Construction

Plasmid isolation from *Escherichia coli* strains and DNA manipulations were performed as described previously (Sambrook et al., 1989). Total genomic DNA or RNA isolation from *A. nidulans* strains has been described (Lockington et al., 1985; Chomczynski and Sacchi, 1987). DNA and RNA gel blot analyses were performed as described by Tazebay et al. (1997) and Meintanis et al. (2000). The *A. nidulans* actin gene was used as an internal control to quantitate the amount of RNA loaded in different lanes (Tazebay et al., 1997). Polymerase chain reaction (PCR) was performed using AmpliTaq DNA polymerase (Perkin-Elmer) and the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). Oligonucleotide primers specific for the *uapA* gene were used for sequencing and PCR amplifications (Diallinas et al., 1995). For oligonucleotide synthesis and DNA sequencing of plasmid constructions and PCR products, we used the in-house Yale Medical School facility (Howard Hughes Medical Institute Biopolymer/W.M. Keck Foundation Biotechnology Resource Laboratories, Yale University School of Medicine, New Haven, CT) and the ABI 310 genetic analyzer at the Institute of Biology (National Center for Scientific Research "Demokritos"). Control plasmids pIJL16 and pAN510, including the *argB* and the *argB* and *uapA* genes, respectively, have been described (Diallinas et al., 1998). Plasmids were introduced into *E. coli* by standard transformation procedures (Sambrook et al., 1989). QuantiScan version 1.25 (BIOSOFT, Ferguson, MO) was used to estimate the number of plasmid copies integrated into the genome or the level of LPE1 expression in various transformants.

Construction of Plasmid pAN-Lpe1

Plasmid pAN503 (Gorfinkiel et al., 1993) was cleaved with XbaI and relegated to isolate the ~800 bp *uapA* 3′ untranslated region as plasmid pNS321. Primers 503-4 (5′-GAGCTCGATCTGCGAGATCCTGGGGTCT-3′) and 503-3 (5′-TCTAGAATCTAGGATCCCATGGTGGAAGATGGGTCAA-3′) were used to amplify the *uapA* 5′ untranslated region and the ATG codon from pAN503. The resulting PCR product was cleaved with SstI and XbaI and cloned into pNS321 to generate the *uapA* translational cassette plasmid pNS335. The *Lpe1* coding region was amplified from pNS175 (Schultes et al., 1996) by using primers Lpe1-22 (5′-GCTCTAGACTAGAGCGAGGGGAAGTACCTGC-3′) and Lpe1-23 (5′-CGGGATCCCGTGAAGGCCGAGGACCTGGTTG-3′), cleaved with BamHI and XbaI, and cloned into vector pOK12 (Vieria and Messing, 1991) to form pNS336. The chimeric *uapA/Lpe1* gene was generated by cloning the BamHI-XbaI *Lpe1* fragment from pNS336 into pNS335 to form pNS345. The *A. nidulans argB* locus was amplified from pIJL16 (Diallinas et al., 1998) with primers 5′-GGGGTACCGTCGACCTACAGCCATTG-3′ and 5′-CCGCTCGAGGGGGTAGTCATCTAATG-3′, cleaved with XhoI and KpnI, and cloned into pNS345 (SalI and KpnI) to form pAN-Lpe1. DNA sequence analysis verified the integrity of the *uapA/Lpe1* translational fusion.

Purine Transport Assays

8-^{3}H-xanthine, 1-^{14}C-L-ascorbic acid, or 2,3,4,5-^{3}H-L-proline uptake was assayed in germinating conidiospores at 37°C as described previously (Diallinas et al., 1995; Tazebay et al., 1995). Standard uptake assays for the determination of initial uptake rates were performed in *A. nidulans* minimal medium (pH 6.5) by using 10 μM 8-^{3}H-xanthine or 2,3,4,5-^{3}H-L-proline or 1 to 5 mM 1-^{14}C-L-ascorbic acid. Radioactivity was determined in sediment and supernatant by liquid scintillation (Beckman Instruments). Transport measurements were repeated independently, and the reported results represent the mean of at least three experiments. Initial velocities were corrected by subtracting background uptake values, evident in the triple *uapA*$^{-}$*uapC*$^{-}$*azgA*$^{-}$ mutant, which are due to genetically undefined minor purine transporters present in all strains used in uptake assays. The apparent K_m and maximal velocity values for 8-^{3}H-xanthine were determined from double reciprocal plots of the initial uptake rates against substrate concentration. The optimized substrate concentration range used 1, 2, 5, 10, 20, 50, 70, 80, and 100 μM. Competition assays were performed in the presence of both 10 μM 8-^{3}H-xanthine and 300 μM "cold" purines or pyrimidines. Competition assays with fresh L-ascorbic acid were performed at a final pH of 5.5 in a concentration range of 35 μM to 100 mM. At this pH, at which *A. nidulans* transporters are not affected, fresh solutions of L-ascorbic acid remain stable.

To calculate the concentration of L-ascorbic acid resulting in 50% inhibition (PC_{50}) of 8-^{3}H-xanthine uptake, we used a constant 8-^{3}H-xanthine concentration (10 μM) and a concentration range of unlabeled L-ascorbic acid from 35 μM to 100 mM. Assays to establish the competitive nature of ascorbic acid inhibition were performed as described for the calculation of the K_m for xanthine but in the presence of three different concentrations of L-ascorbic acid (5, 10, and 45 mM). All transport assays were performed at least three times. The errors listed in Table 1 and in Figures 6 and 7 are standard errors of the mean value. Radiolabeled 8-^{3}H-xanthine (specific activity, 9.8 Ci/mmol) and 2,3,4,5-^{3}H-L-proline (specific activity, 120 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). Radiolabeled 1-^{14}C-L-ascorbic acid (specific activity, 6 mCi/mmol) was obtained from DuPont–New England Nuclear Life Science Products (Boston, MA).

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