Novel P-Type ATPases Mediate High-Affinity Potassium or Sodium Uptake in Fungi

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Fungi have an absolute requirement for K⁺, but K⁺ may be partially replaced by Na⁺. Na⁺ uptake in Ustilago maydis and Pichia sorbitophila was found to exhibit a fast rate, low Kₘ, and apparent independence of the membrane potential. Searches of sequences with similarity to P-type ATPases in databases allowed us to identify three genes in these species, Umacu1, Umacu2, and PsACU1, that could encode P-type ATPases of a novel type. Deletion of the acu1 and acu2 genes proved that they encoded the transporters that mediated the high-affinity Na⁺ uptake of U. maydis. Heterologous expressions of the Umacu2 gene in K⁺ transport mutants of Saccharomyces cerevisiae and transport studies in the single and double Δacu1 and Δacu2 mutants of U. maydis revealed that the acu1 and acu2 genes encode transporters that mediated high-affinity K⁺ uptake in addition to Na⁺ uptake. Other fungi also have genes or pseudogenes whose translated sequences show high similarity to the ACU proteins of U. maydis and P. sorbitophila. In the phylogenetic tree of P-type ATPases all the identified ACU ATPases define a new cluster, which shows the lowest divergence with type IIC, animal Na⁺,K⁺-ATPases. The fungal high-affinity Na⁺ uptake mediated by ACU ATPases is functionally identical to the uptake that is mediated by some plant HKT transporters.

Potassium and Na⁺ were present in the sea, where the early evolution of life took place, but only K⁺ was selected to maintain the electrical and osmotic equilibria of the cells. Constricted by the K⁺ environment, further evolution of the cellular physiology added to the original requirements; the K⁺ dependence on many physiologic processes that adapted to the cation was present. This K⁺ dependence of living cells was not a restriction for life in the sea, where K⁺ was abundant, but involved a nutritional problem for the start of terrestrial life on the rocks emerging from the sea, probably in the Precambrian (27). Therefore, before plants and fungi could colonize terrestrial environments (12, 26) they had to sustain a complex process of physiological adaptations to a new medium in which K⁺ and Na⁺ concentrations were very low.

A crucial role in this terrestrial adaptation was played by high-affinity K⁺ transporters, whose existence has been well established for several plant and fungal species (44); but very little is known of other cooperative processes that may alleviate these strict K⁺ requirements. Among these, an emerging notion is that Na⁺ can be used as a substitute for K⁺. It has been known for a long time that Na⁺ enhances the growth of fungi (17) and plants (14, 28, 49) under conditions of K⁺ deficiency, but this effect had been considered accidental or under a weak physiological regulation. In contrast with this view, the characterization of high-affinity Na⁺ uptake in several plants and the fact that it takes place only when K⁺ has been exhausted (21) strongly suggest that Na⁺ plays a physiologically programmed role in plants given an insufficient supply of K⁺. Although this conclusion is simple, the process and regulation of high-affinity Na⁺ uptake may be complex and difficult to dissociate from K⁺ uptake, because the plant transporters that mediate this Na⁺ uptake (21) belong to a large family of K⁺ transporters (44). Notably, at least one of them, HKT1, from wheat, has been shown to function as an Na⁺-K⁺ symporter (47).

The notion of a physiologically programmed use of Na⁺ as a substitute for K⁺ and the mechanistic links between K⁺ and Na⁺ uptake raise the question of whether this may be a process that is not exclusive to plants but rather a general one that exists in nonanimal eukaryotic cells adapted to terrestrial environments. If this were the case, it should exist in fungi because of the parallel life of fungi and plants as soil inhabitants and their concurrent colonization of terrestrial environments. We investigated this possibility and found that high-affinity Na⁺ uptake does occur in fungi and is mediated by transporters whose sequences define a novel phylogenetic cluster of P-type ATPases that is close to that of animal Na⁺,K⁺- and H⁺,K⁺-ATPases. We show that these ATPases mediate high-affinity K⁺ uptake primarily and Na⁺ uptake secondarily, when K⁺ is at very low concentrations.

MATERIALS AND METHODS

Strains and growth conditions. Ustilago maydis strain FB1 (a alb) (7) and Pichia sorbitophila strain CBS7064 (6) were used throughout this study. The Escherichia coli strain DH5α was routinely used for plasmidic DNA propagation. The Saccharomyces cerevisiae strains used were W303.1A (Mat a ade2 ura3 trpl leu2 his3) and its derivatives WΔΔ (Mat a ade2 ura3 trpl trk1::Leu2 trk2::His3), which is deficient in the endogenous K⁺ uptake systems TRK1 and TRK2 (25), and MAS (Mat a ade2 ura3 trpl trk1::Leu2 trk2::His3 ena1-4::His3 nha1Δ::Leu2), a strain obtained for this work by crossing the aforementioned WΔΔ with B51 (Mat a ade2 ura3 trpl ena1-4::His3 nha1Δ::Leu2) (6), which is deficient in the Na⁺ efflux systems ENA1-4 and NHA1. Fungal strains were normally grown either in the complex medium YPD (1% yeast extract, 2% peptone, 2% glucose) or in the minimal SD medium (48), supplemented with 50 mM K⁺ when used for the K⁺ uptake mutants. Growth at

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low K⁺ and Na⁺ concentrations occurred on arginine phosphate (AP) medium (46) supplemented with the indicated K⁺ and Na⁺ concentrations (the basal medium contains 5 μM K⁺ and 20 μM Na⁺).

Recombinant DNA techniques. Manipulation of nucleic acids was performed by standard protocols or, when appropriate, according to the manufacturer's instructions. PCRs were performed with a Perkin-Elmer thermocycler by using the Expand-High-Fidelity PCR system (Roche Molecular Biochemicals). Some of the PCR fragments were first cloned into the PCR2.1-TOPO vector by using the TOPO TA cloning kit (Invitrogen). For yeast expressions, the genes were cloned into vector pYEPG15 (16). DNA sequencing was performed on an automated ABI PRISM 377 DNA sequencer (Perkin-Elmer). DNA and total RNA were prepared by using the DNeasy and RNeasy plant kits (Qiagen), respectively. PCR amplifications of mRNA fragments were carried out with double-stranded cDNA synthesized from total RNA by using the cDNA Synthesis System kit (Roche). The full-length ACU1 cDNA of P. sorbitophila was obtained by using the 5'3' RACE Kit (Roche) as described earlier for other fungal ATPases (9). The full-length Umacu1 and Umacu2 cDNAs were obtained by reverse transcription-PCR from RNA extracted from K⁺-starved cells of *U. maydis*, by using specific primers whose sequences contained the predicted START and STOP codons. The three genes Umacu1, Umacu2, and PeACU1 were amplified from genomic DNA by PCR with the same primers as for the cDNAs. The pseudogene of *Candida albicans* was amplified from genomic DNA of strain SC5314 (37). The substitution of the AAG codon for the stop TAG codon at position 1066 was carried out by PCR in two steps by using primers that included one mutated nucleotide. First, we amplified a 1,110-bp DNA fragment by using a sense-specific primer upstream of the start codon NaK1 (5'-TTCAA GAAATCAAATAAACACAT-3') and the antisense mutator primer NaKMUT3 (5'-GAGAATTTTTTCACTGTTGAAATG3') (the changed nucleotide is underlined). We then amplified a 2,210-bp DNA fragment by using a sense mutator primer NaKMUT3 (5'-CCACCTTAAAAAGGAATTTATAGA3') (the changed nucleotide is underlined) and the antisense specific primer downstream of the stop codon NaK2 (5'-CTAAATACCTGCTCCCAAACTG-3') (A3). In a second step, a PCR was carried out by using the two DNA fragments obtained above as templates and primers NaK1 and NaK2, which amplify the complete gene.

Real-time PCR assays. The results of real-time PCR assays reported in Table 1 were obtained for cells that were grown either in YPD or in AP minimal medium with 3 mM KCl. The latter were also exposed to the same medium for 5 h. These starved cells were spun down and suspended in 10 mM tartaric acid and pH brought to 3.5 with arginine (2 h); addition of 0.5 mM Ca²⁺ and pH brought to 8.0 with arginine (2 h); addition of 100 mM NaCl (2 h); without K⁺ (4 h); and without arginine and pH brought to 6.5 with KOH (6 h).

### Table 1. Effects of growth conditions on *Umacu1* and *Umacu2* transcripts

| Gene | YPD | AP control medium | AP medium with indicated modification* | pH 3.5 | pH 8.0 | K⁺ starvation | N starvation |
|------|-----|-------------------|----------------------------------------|--------|--------|----------------|-------------|
| Umacu1 | <0.01 | 2.3 | 0.08 | 1.5 | 0.07 | 150 | 0.12 |
| Umacu2 | 0.17 | 8.1 | 0.23 | 6.7 | 0.23 | 530 | 0.93 |

* Cells were grown in AP medium with 3 mM K⁺ and transferred to the indicated modified medium for 2 to 6 h as described in the text.

Cation uptake experiments. Cation uptake experiments were performed with K⁺-starved cells. First, cells were grown in AP medium supplemented with 3 mM K⁺, washed in water, and then inoculated in K⁺- and Na⁺-free AP medium for 5 h. These starved cells were spun down and suspended in 10 mM morpholinéthanesulfonic acid (MES) brought to pH 6.0 with ultrapure Ca(OH)₂ and supplemented with 2% glucose (testing buffer). K⁺-starved cells with a high Na⁺ content for measuring K⁺ and Na⁺ exchange were prepared in the same way as regular K⁺-starved cells, except for an Na⁺-loading period of 30 min in 100 mM Na⁺ AP medium before the starvation step. The uptake tests were started by the addition of the selected cation, and then, at intervals, samples of the suspended cells were either centrifuged or filtered to determine either the time course of the depletion of the cation in the testing buffer or the increase of the cation content inside the cells. In both cases, the cation contents were determined by atomic emission spectrophotometry. Both methods, as well as the statistical analyses of the results, have been described previously (3, 24, 46). For each experiment, four or five independent replications were carried out.
Na⁺ influx was influenced by the presence of K⁺ and by the K⁺ content of the cells. In experiments with K⁺-starved cells, the rate of Na⁺ uptake started to decrease when the Na⁺ taken up amounted to approximately 50 nmol mg⁻¹. This effect, the rapid initial rate of uptake, and the very low Kₘ reduced the effective sampling time of test experiments to just a few minutes, making the number of effective samples insufficient for a good statistical fitting of the results. However, from the data obtained in experiments using very low Na⁺ concentrations (<20 μM) we calculated that the Kₛ of Na⁺ influx in both U. maydis and P. sorbitophila were below 5 μM Na⁺.

Although a complete biochemical characterization of Na⁺ influxes was not tackled, we found that in both U. maydis and P. sorbitophila high-affinity Na⁺ uptake was not inhibited by Ba²⁺, which inhibits the corresponding uptake in plants (21). This suggested that a TRK-HKT transporter was not involved. Subsequently, we found that the uncoupler CCCP (carbonyl cyanide m-chlorophenylhydrazone), at 100 μM, inhibited high-affinity Na⁺ uptake completely in U. maydis, which always respires, but only partially in P. sorbitophila, which can ferment. The latter result suggested that Na⁺ influx was not a secondary process energized by the membrane potential. Taking into consideration the effects of CCCP in S. cerevisiae (24), it was extremely unlikely that a 100 μM concentration did not completely uncouple the plasma membrane of P. sorbitophila.

Cloning of the genes encoding the high-affinity Na⁺ transporters. Assuming that the described high-affinity Na⁺ uptake was not a secondary process and was mediated by P-type ATPases, we carried out BLAST searches in the database GENOLEVURES (http://cbi.labri.u-bordeaux1.fr/Genolevures) for P. sorbitophila and in the database of the U. maydis genome sequence (Bayer CropScience). By using as queries the amino acid sequences of the S. cerevisiae and N. crassa ENA ATPases (10), we identified the partial sequence of a gene of P. sorbitophila (accession number, axthha002g12ep1) and two complete genes of U. maydis. All of them encoded a novel kind of P-type ATPases, which showed maximum sequence similarity to animal Na⁺,K⁺-ATPases. These findings fulfilled minimal conditions of functionality and prompted us to further investigate whether they mediated the Na⁺ uptake that we were studying. Therefore, we cloned the corresponding genes and cDNAs, i.e., P. sorbitophila ACU1 and U. maydis acu1 and acu2 (mnemonics for alkali cation uptake ATPase). In all three cases, the genes did not have introns and the ORFs comprised 3,218, 3,236, and 3,389 bp starting at the first ATG triplet of the ORFs and could encode polypeptides of 1,073, 1,079, and 1,130 amino acid residues with calculated molecular masses of 122,98, 119,68, and 123.50 kDa, respectively. Sequence analyses of the translated proteins proved that they met all the characteristics of typical P-type ATPases and that they conserved some of the amino acids involved in cation binding in Na⁺,K⁺-ATPases (Fig. 2).

To investigate the involvement of the discovered transporters in the nutrition of the fungus under different growth conditions, the transcript abundance of the two genes of U. maydis was tested by real-time PCR (in Table 1 we list the ratios of the content of the cDNA corresponding to each gene to the content of the actin cDNA in the same sample). The rich mediumYPD was used as the least stressing growth condition and minimal AP medium with 3 mM K⁺ as the basal condition over
which we imposed high (8.0) or low (3.5) pH, 100 mM Na\textsuperscript{+}/H\textsuperscript{−}, K\textsuperscript{+}/H\textsuperscript{−}, starvation, or nitrogen starvation. The variations of the transcript abundance of the two genes followed similar patterns, although under all conditions the transcripts of acu\textsubscript{2} were more abundant than those of acu\textsubscript{1}. In rich medium, the expression of acu\textsubscript{2} was low and acu\textsubscript{1} was not expressed. In minimal medium the only condition that clearly triggered strong expressions was K\textsuperscript{+}/H\textsuperscript{−} (and Na\textsuperscript{+}/H\textsuperscript{−}) starvation. Under all the other conditions the transcript abundances of the ATPases were 10\textsuperscript{2} to 10\textsuperscript{4} times lower than those of actin, higher at pH 6.5 or 3.5 and lower at 100 mM Na\textsuperscript{+}/H\textsuperscript{−}, pH 8.0, or nitrogen starvation. These results suggest that the UmAcu ATPases have only one function, which is triggered when the cells are suffering K\textsuperscript{+} starvation.

According to their sequence similarities, the ATPases encoded by the cloned genes could mediate K\textsuperscript{+}/H\textsuperscript{−} or Na\textsuperscript{+}/H\textsuperscript{−} uptake.
Therefore, in order to study their functions, we inserted the genes in the yeast expression vector pYPGE15 (16) and transformed the constructs into a K⁺ transport mutant (trk1Δ trk2Δ) (32) of S. cerevisiae. For this mutant the Na⁺ and K⁺ uptake rates at low K⁺ or Na⁺ concentrations (<2 mM) are undetectable; tests aimed at solving the problem of the inhibition of the Na⁺ transport by K⁺ have been described (21).

We obtained the transformants for PsACU1 and Umacu2 but not for Umacu1, probably because it was toxic for bacteria. Although many constructs of fungal cDNAs encoding P-type ATPases that are toxic for E. coli can be obtained directly with yeast (9, 10), in the case of Umacu1 all attempts failed.

Growth tests with the PsACU1 or Umacu2 yeast transformants revealed that both genes suppressed the defective growth of the trk1Δ trk2Δ mutant at low K⁺ (the mutant grows poorly at 1 mM K⁺, and the transformants grew at 0.1 mM K⁺) in AP medium without Na⁺ (Fig. 3). These tests are normally carried out at pH 6.5, and at this pH PsACU1 and acu2 showed similar capacities for suppressing the defect of the trk1Δ trk2Δ mutant. At pH 5.0 the trk1Δ trk2Δ mutant is more defective, and the PsACU1 transformant did not grow at 0.3 mM K⁺, whereas the Umacu2 transformant grew at 0.1 mM K⁺ as rapidly as mutants expressing the N. crassa HAK1 (high-affinity K⁺) transporter (25). This suggested that both PsACU1 and Umacu2 encoded K⁺ uptake transporters and that the transporter encoded by Umacu2 was very active in yeast.

We then carried out uptake experiments in testing buffer. The PsACU1 transformant took up K⁺ and Na⁺ but did so too slowly to be studied with our experimental approach. In contrast, in the Umacu2 transformant the uptake was more rapid and we were able to perform uptake kinetic studies that demonstrated that Umacu2 encoded a transporter that mediated K⁺ or Na⁺ uptake. The high-affinity uptake kinetics exhibited by this transporter (Kₘ = 0.5 to 1.5 μM for both cations) and the decrease in rate when modest amounts of cations had been taken up (compare the response to the external cation in the main panel to the one in the inset in Fig. 4) suggested that the product of Umacu2 was involved in the Na⁺ uptake that we had found in U. maydis. Moreover, Umacu2 encoded a high-affinity uptake system that was similarly effective for Na⁺ and for K⁺. Although the uptake test results were less conclusive, it seems likely that PsACU1 encodes an ATPase similar to that encoded by Umacu2.

**UmAcu2 does not exchange K⁺ and Na⁺.** Sequence similarities between the ACU ATPases of U. maydis and P. sorbitophila with animal Na⁺,K⁺-, and H⁺,K⁺-ATPases (see Fig. 6 below) suggested that the mechanism of K⁺ uptake of ACU ATPases was the exchange of external K⁺ with internal Na⁺ or H⁺. However, K⁺-Na⁺ exchanges could be ruled out in the experiments described above (Fig. 4) because K⁺-starved cells of the trk1Δ trk2Δ yeast mutant transformed with Umacu2 took over 100 nmol of K⁺ mg⁻¹ although the cells had low amounts of Na⁺ (Umacu2 yeast cells grown with K⁺ in the absence of added Na⁺ did not have Na⁺, but during the starvation process they took up all the Na⁺ that contaminated the medium; typical acu2 K⁺-starved cells contained 300 and 30 nmol of K⁺ and Na⁺ mg⁻¹, respectively). This suggested that the mechanisms of UmAcu2 could be similar to those of gastric (38) or nongastric (29) H⁺,K⁺-ATPases. Because the latter can exchange Na⁺ for K⁺ under certain conditions (19, 23, 40), we tested whether Acu2 exchanged Na⁺ and K⁺. For this purpose, we transformed the pYPGE15-Umacu2 plasmid into a yeast strain mutant for both K⁺ uptake and Na⁺ influx (trk1Δ trk2Δ ena1Δ Δ nha1Δ) and tested K⁺ uptake and Na⁺ loss in K⁺-starved cells that had been previously loaded with Na⁺, which contained 150 nmol of K⁺ and Na⁺ mg⁻¹. In these cells, the rate of K⁺ uptake was not enhanced by the internal Na⁺ and Na⁺ was not lost. However, because it is impossible to prepare a buffer without Na⁺, the loss of intracellular Na⁺ had to be measured as the increment of the Na⁺ content in the testing buffer over the content at the beginning of the experiment. Considering the detection limits under these conditions, the
conclusion of several experiments is that in more than 90% of the cycles the enzyme did not exchange Na⁺ for K⁺.

Disruption of the UmAcu1 and UmAcu2 genes. The transport functions of the Acu1 and Acu2 ATPases in U. maydis were then further investigated by undertaking the single and double disruptions of the genes. The hypothesis that the UmAcu1 and UmAcu2 ATPases mediated high-affinity K⁺ or Na⁺ uptake systems in U. maydis was confirmed with the Δacu1 Δacu2 double mutant. Here, high-affinity Na⁺ uptake completely disappeared and K⁺ uptake was slow in the micromolar range of concentrations (Fig. 5). By sequence analysis we further identified the presence of a trk1 gene in the genome of U. maydis, which suggested that K⁺ uptake in the Δacu1 Δacu2 double mutant could be mediated by a Trk1 transporter. In a preliminary study we found that the $K_{m}$ of the rate of K⁺ uptake in the Δacu1 Δacu2 double mutant was approximately 0.3 mM K⁺.

In experiments of K⁺ or Na⁺ depletion the single mutants Δacu1 and Δacu2 took up K⁺ or Na⁺ with high affinity, exhibiting rates that were very similar to those exhibited by the wild-type strain, except for Na⁺ uptake in the Δacu2 mutant, which was slower (Fig. 5). The existence of K⁺ uptake in the double mutant did not inconvenience the study of K⁺ uptake in the single Δacu1 and Δacu2 mutants because the K⁺ uptake rate in the double-mutant strain was much slower than in the single-mutant strains. Therefore, considering that our strains are completely isogenic, it was evident that high-affinity K⁺ and Na⁺ influxes were mediated by the Acu2 ATPase in the Δacu1 strain and by the Acu1 ATPase in the Δacu2 strain. To sum up, K⁺ uptake in K⁺-starved cells of U. maydis was dominated by the concurrent functions of Acu1 and Acu2 ATPases, whereas other transport systems, including Trk1, made a minor contribution. In contrast, high-affinity Na⁺ uptake was dominated only by Acu2 because the double mutant did not take up Na⁺ uptake in the wild-type strain, Δacu1 and Δacu2 single mutants, and Δacu1 Δacu2 double mutant of U. maydis. In the single mutants the experiments were started at 60 and 10 μM (inset) to show the regulation of the enzyme by the internal cations and its very low $K_{m}$. Experiments, as described in the legend to Fig. 4 for measurement of Na⁺ (closed circles) and K⁺ (open triangles), were performed at the following cell densities (dry weights): wild-type strain, 0.49 mg ml⁻¹ and Na⁺/H₁¹₀₀₁, 0.53 mg ml⁻¹ for the K⁺ experiment and 0.94 mg ml⁻¹ for the Na⁺ experiment; Δacu1 main panel, 0.58 mg ml⁻¹ for both the K⁺ and Na⁺ experiments; Δacu1 inset, 0.30 mg ml⁻¹ for both the K⁺ and Na⁺ experiments; Δacu2 main panel, 0.51 mg ml⁻¹ for both the K⁺ and Na⁺ experiments; and Δacu2 inset, 0.60 mg ml⁻¹ for both the K⁺ and Na⁺ experiments.

![FIG. 5. Acu1 and Acu2 transport K⁺ and Na⁺ in U. maydis as shown by results for K⁺ and Na⁺ uptake in the wild-type strain, Δacu1 and Δacu2 single mutants, and Δacu1 Δacu2 double mutant of U. maydis.](image)

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UmAcu2 ATPases for NH$_4^+$ by measuring K$^+$ uptake in the presence of NH$_4^+$. In experiments such as those whose results are shown in the insets of Fig. 5, the presence of 0.1 mM NH$_4^+$ did not produce any noticeable effect, and the K$^+$ left in the external medium was still undetectable (0.05 M) at the end of the experiments. Even at 1 mM ammonium, the only appreciable effect was that K$^+$ uptake ceased at a concentration of 0.1 M. These results indicated that ammonium (at a concentration of up to 1 mM) is not transported or is poorly transported by the UmAcu ATPases.

ACU ATPases possibly exist in many fungi. In the genome of S. cerevisiae there are no genes encoding ATPases that are similar to the ACU ATPases of P. sorbitophila and U. maydis (18), and we also found that they are absent in the genomes of N. crassa and S. pombe. However, we identified one gene in C. albicans and two genes in M. grisea that suggested that these ATPases may exist in many other fungi. Fragments of putative genes were also found in M. graminicola (COGEME accession number, mga0392f), F. sporotrichioides (COGEME accession number, FS-t2a06fs.fl), and A. fumigatus (gnl|TIGR_5085|contig:4901:a_fumigatus and gnl|TIGR_5085|contig:4951:a_fumigatus). Interestingly, the C. albicans sequence was apparently a pseudogene, because there was a stop codon in position 1066 (with the adenine of the first ATG codon in the ORF considered nucleotide +1). The complete pseudogene comprised 3,243 bp and apparently did not have introns. We cloned the putative gene and sequenced it, finding that the sequence in the database was correct. The mutation of the TAG stop codon to AAG, which encodes a lysine residue, as in PsACU1, was sufficient to restore the activity of the pseudogene. The mutated pseudogene suppressed the defective growth of the trk1/trk2 mutant at low K$^+$ in a manner almost identical to that described for PsACU1 (Fig. 3).

A phylogenetic study of the ACU ATPases along with other P-type ATPases revealed that ACU ATPases were more related to animal Na$^+$/K$^+$- or H$^+$/K$^+$-ATPases (type IIC [2]) than to Ca$^{2+}$- or ENA-ATPases (types IIA, IIB, and IID). However, they are rather divergent from type IIC sequences, including those of Heterosigma akashiwo, Blastocladia emer-sonii (20), and Dictyostelium discoideum (Fig. 6), and from the prokaryotic sequences recorded elsewhere (11). The divergence, estimated as the number of amino acid changes per 100 positions, between animal Na$^+$/K$^+$-ATPases and fungal ACU ATPases is similar to that existing between plasma membrane

![Phylogenetic relationships of fungal ACU ATPases with other P-type ATPases.](http://genome.imb-jena.de)
**DISCUSSION**

The existence of active K\(^+\) uptake in fungi and plants was established many years ago because, under certain growth conditions, the membrane potential is positive relative to the equilibrium potential of K\(^+\) across the plasma membrane (44). When this existence was established, the most persistent notion, which arose from reasoning by analogy with the Na\(^+\) pump of animal cells (15), was that a K\(^+\)-ATPase mediated K\(^+\) uptake (31, 39, 51, 52, 55, 56). However, the incomplete biochemical support for the K\(^+\)-ATPase and the findings that active K\(^+\) uptake was mediated by a K\(^+\)-H\(^+\) symporter in *N. crassa* (45) caused the attention given to active K\(^+\) transporters. This conclusion, together with the unmistakability of the translated sequences of all ACU proteins to P-type ATPases (Fig. 2 and 6), allows us to classify the ACU transporters as K\(^+\)-ATPases. The extraordinary capacity exhibited by the UmAcu1 and UmAcu2 ATPases (as observed in the Δacu2 and Δacu1 mutants, respectively) for depleting the K\(^+\) of the external medium and concentrating it in the cells more than 10\(^4\)-fold, their very low K\(_m\) (possibly as low as 0.5 μM K\(^+\)), and their almost exclusive expression under conditions of K\(^+\) starvation suggest that the function of these ATPases is to take up K\(^+\) from media in which the cation is at extremely low concentrations—a function that is similar to that reported for the Kdp-ATPase of *E. coli* (43).

The old discussion about plant K\(^+\)-ATPases raises a question about the existence of ACU ATPases in plants. They do not exist in *Arabidopsis* or rice (8), and we did not find in databases a plant gene sequence that could encode an ACU ATPase. However, these findings cannot be taken as a definitive proof. First, *Arabidopsis* and rice are not representative of all plant species and ACU ATPases do not exist in all fungi. Second, the absence of expressed sequence tags with ACU sequences in databases can be expected if ACU mRNAs are expressed only in K\(^+\)-starved plants and consequently, the corresponding cDNAs do not exist in current libraries.

In *U. maydis*, the Acu2 ATPase mediated a high-rate, high-affinity Na\(^+\) uptake and Acu1 was also an Na\(^+\) transporter, albeit with lower activity. These findings, the sequence similarities, and the phylogenetic relationships (Fig. 6) strongly suggest that, in general, fungal ACU ATPases have the function of K\(^+\) or Na\(^+\) uptake described here for the *U. maydis* ATPases. However, neither the question of whether some ACU ATPases transport K\(^+\) or Na\(^+\) exclusively nor that of whether Na\(^+\) transport may be the primary function in some of them can be answered now. The simplest hypothesis, however, is that both functions take place concurrently, though with different degrees of discrimination between K\(^+\) and Na\(^+\). K\(^+\)-ATPases are also present in fungi and consequently, the corresponding cDNAs do not exist in current libraries.

**TABLE 2.** Number of TRK, HAK, and ACU genes in the genomes of fungi for which the complete sequences are available\(^a\)

| Species            | TRK | HAK | ACU |
|--------------------|-----|-----|-----|
| *S. cerevisiae*    | 2   | 0   | 0   |
| *S. pombe*         | 2   | 0   | 0   |
| *N. crassa*        | 2   | 1   | 0   |
| *U. maydis*        | 1   | 0   | 2   |
| *M. grisea*        | 2   | 1   | 2   |
| *C. albicans*      | 1   | 1   |     |

\(\text{a} \) BLAST searches were carried out with NcTRK1, NcHAK1, and PsACU1 protein sequences against the translated genomic sequences in the databases given in Materials and Methods.

\(\text{b} \) --- this species has a pseudogene, as explained in the text.

and SERCA Ca\(^{2+}\)-ATPases (types IIA and IIB [2]), and for that reason we propose naming fungal ACU ATPases type IIE (Fig. 6).

**Several high-affinity K\(^+\)** transporters coexist in fungi. High-affinity K\(^+\) uptake in fungi is mediated by HAK transporters (4, 25), and this implies a certain degree of functional redundancy if ACU ATPases coexist with HAK transporters. This redundancy is less significant if they coexist with TRK K\(^+\) transporters, because these transporters have a lower concentrative capacity and, in fact, TRK transporters coexist with HAK transporters (5, 25). Therefore, to update the models of K\(^+\) uptake in fungi we investigated the presence of TRK and HAK transporters and ACU ATPases in those fungal species for which the complete genomic sequences are available, i.e., *S. cerevisiae*, *S. pombe*, *C. albicans*, *U. maydis*, and *M. grisea*. Table 2 summarizes the results, which revealed that the models of K\(^+\) uptake in fungi are very diverse. The only type of K\(^+\) transporters that seem to be present in all fungi is TRK, probably because these transporters have a role in controlling the membrane potential (5, 32). The HAK transporters or ACU ATPases may be present or not, either together or independently.
which is mediated by HKT secondary transporters (21). The mechanistic differences between the two transporters demonstrate that high-affinity Na\(^+\) uptake developed independently in fungi and in plants and suggest that Na\(^+\) uptake probably fulfilled a very important biological function in the evolution of plants and fungi. Such an important function does not apply to all contemporary fungi, because high-affinity Na\(^+\) uptake does not exist in some of them (e.g., *S. cerevisiae*). Therefore, a likely hypothesis is that the importance of Na\(^+\) uptake and, hence, the selective pressure that determined the characteristics of the fungal ACU ATPases and plant HKT transporters took place early in the evolution of fungi and plants. This probably occurred when associations of fungi and plants colonized the harsh environment of the emerged land and continental water in the Cambrian era (12) or even earlier (26, 27).

In these environments, the mineral nutrients were at very low levels and Na\(^+\) was probably used as a substitute for K\(^+\). Although K\(^+\) is indispensable in fungi and plants, Na\(^+\) can substitute for a substantial amount of K\(^+\) with very little toxicity. In *S. cerevisiae* most of the Na\(^+\) is confined to the inside of the vacuole (54; K. Venema, personal communication), it can be expected that cells with a big vacuole can sequester in it significant amounts of Na\(^+\), thus avoiding its toxic effects in the cytoplasm (1) and reducing notably the K\(^+\) requirements. This use of Na\(^+\) could have been a selective advantage for millions of years in the oligotrophic soils of Paleozoic ages (41, 42), and it may persist in many contemporary oligotrophic environments in which fungi and plants thrive.

K\(^+\) uptake in fungi is especially complex because they thrive in very diverse environments, which implies a great variability in the K\(^+\) concentrations to which fungi are exposed. For example, K\(^+\) concentrations are very low in many soils but very high in decaying plant materials or in the environment of fungus-plant symbioses (10). Some species meet all these environmental variations, such as plant pathogens that also grow in soil, whereas other species are adapted to more constant environments, such as fermenting yeast. Therefore, the variability of the transport systems among different fungal species (Table 2) is not surprising, although it cannot currently be explained on functional grounds. The absence of HAK transporters and ACU ATPases in *S. cerevisiae* may be explained by the high K\(^+\) content of fruit, an environment in which the organism thrives, but the coexistence of ACU ATPases, which exhibited excellent performances for transporting both K\(^+\) and Na\(^+\), and HAK transporters, which transport only K\(^+\) (44), in *M. grisea* does not have a simple explanation now.

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