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Biochemistry

Seed and vascular expression of a high-affinity transporter for cationic amino acids in *Arabidopsis*

(promoter/retrovirus receptor/amino acid permease/histidine, arginine, and lysine transport)

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ABSTRACT **In most plants amino acids represent the major transport form for organic nitrogen. A sensitive selection system in yeast mutants has allowed identification of a previously unidentified amino acid transporter in *Arabidopsis*. *AAT1* encodes a hydrophobic membrane protein with 14 membrane-spanning regions and shares homologies with the ecotropic murine leukemia virus receptor, a bifunctional protein serving also as a cationic amino acid transporter in mammals. When expressed in yeast, AAT1 mediates high-affinity transport of basic amino acids, but to a lower extent also recognizes acidic and neutral amino acids. AAT1-mediated histidine transport is sensitive to protonophores and occurs against a concentration gradient, indicating that AAT1 may function as a proton symporter. *AAT1* is specifically expressed in major veins of leaves and roots and in various floral tissues—i.e., peduncle and developing seeds.**

Molecular studies in bacterial, fungal, and mammalian organisms have shown that amino acid transport is mediated by multiple families of permeases with partially overlapping specificities. It has, however, been a matter of debate for many years whether plants are simpler and possibly need fewer transporters—e.g., a single general system. The complexity of plant amino acid translocation in time and space made this assumption questionable. Amino acids are distributed through xylem and phloem to supply importing organs—i.e., seeds (1). Transport measurements in several plant species provided evidence for low- and high-affinity transport systems. Uptake studies in plasma membrane vesicles from *Ricinus* and sugar beet leaves have demonstrated the presence of multiple amino acid transporters with overlapping specificities (2–4).

Molecular approaches have allowed cloning of some of the genes involved, thus enabling a dissection and analysis of contributing carriers. High-affinity amino acid permeases (AAPs) with broad substrate specificity were identified by complementation of yeast amino acid transport mutants with *Arabidopsis* cDNA libraries (5–7). The *AAP* genes constitute a gene family of at least six members with differing but overlapping substrate specificities and expression patterns in the plant (7, 8). However, the question remains whether the broad specific AAP family covers all amino acid transport activities present in the plant. In search for carriers able to mediate basic amino acid transport, a sensitive selection system for histidine uptake was established that enabled the identification of further amino acid transporter genes. A cDNA encoding a high-affinity transporter that is unrelated to the previously isolated plant AAPs was identified. Biochemical properties and expression pattern of AAT1 were analyzed.

METHODS

Materials. The cDNA library derived from seedlings of *Arabidopsis thaliana* ecotype Landsberg was a generous gift from M. Minet (9). The cosmid library from *A. thaliana* ecotype C24 was obtained from U. Halfter (10). Plant materials were *A. thaliana* ecotype C24 and *Nicotiana tabacum* SNN. Yeast strains were JT16 [*Mata, hip1-614, his4-401, ura3-52, ino1, can1* (11)] and 22574d [*MATα, ura 3-1, gap 1-1, put 4-1, uga 4-1* (12)].

Yeast Growth, Transformation, and Selection. JT16 was transformed with the *Arabidopsis* cDNA library (13). Transformants were selected on solid SC medium supplemented with 6 mM histidine. Colonies able to grow were tested for growth in liquid medium. Plasmid DNA was isolated and reintroduced into JT16. *AAT1* restored growth of JT16 under selective conditions. To further test the substrate specificity, 22574d was transformed with *AAT1*. Selection was carried out on nitrogen-free medium supplemented with proline, citrulline, or γ-aminobutyric acid as sole nitrogen sources (5). pFL61 (9) or pDR195 (14) served as negative controls.

DNA Work, Transformation, and Analysis of Tobacco. A genomic clone was isolated by using the *AAT1* cDNA as a probe. The promoter of *AAT1* was fused as a 4-kb *Asp* I fragment to *uidA* in pGPTV-Hyg (15). Tobacco was transformed by *Agrobacterium*-mediated transformation (10). Histochemical studies of transgenic plants with 5-bromo-4-chloro-3-indolyl β-D-glucuronide were performed in the presence of methanol to suppress endogenous β-glucuronidase background (16). Comparable expression patterns were found in at least five independent transformants. Both strands of promoter and cDNA were sequenced by nonradioactive fluorescent on-line sequencing (17). Sequences were analyzed by using the University of Wisconsin Genetics Computer Group package (18). To increase the expression of *AAT1*, the cDNA was cloned into *Not* I of pDR195 (14). Southern and Northern blot analyses were performed as described (7).

Transport Measurements. Yeast cells grown to logarithmic or stationary phase were washed in water and resuspended in 50 mM potassium phosphate (pH 4.5). Standard assays contained 100 μl of cells (OD_{600} = 6), 50 μM L-histidine, and the respective competitors at 10-fold molar excess. Reaction mixtures were brought to 200 μl final volume with potassium phosphate buffer and were started by addition of 18.5 kBq of L-[^{14}C]histidine (Amersham). For energization, glucose was added to 10 mM 5 min prior to reaction start. Samples (50 μl) were removed after 10, 60, 150, and 300 sec, transferred to 8 ml of ice-cold water, filtered on glass fiber filters, and washed with 8 ml of water. Uptake of ^{14}C was determined by liquid scintillation spectrometry. Inhibitor studies were carried out as described (7). The capacity to accumulate histidine against concentration gradients was analyzed after centrifugation of cells and radioactivity was determined in sediment and supernatant. Internalized compounds were analyzed by thin-layer

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Abbreviation: AAP, amino acid permease.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X71787 and X92657).

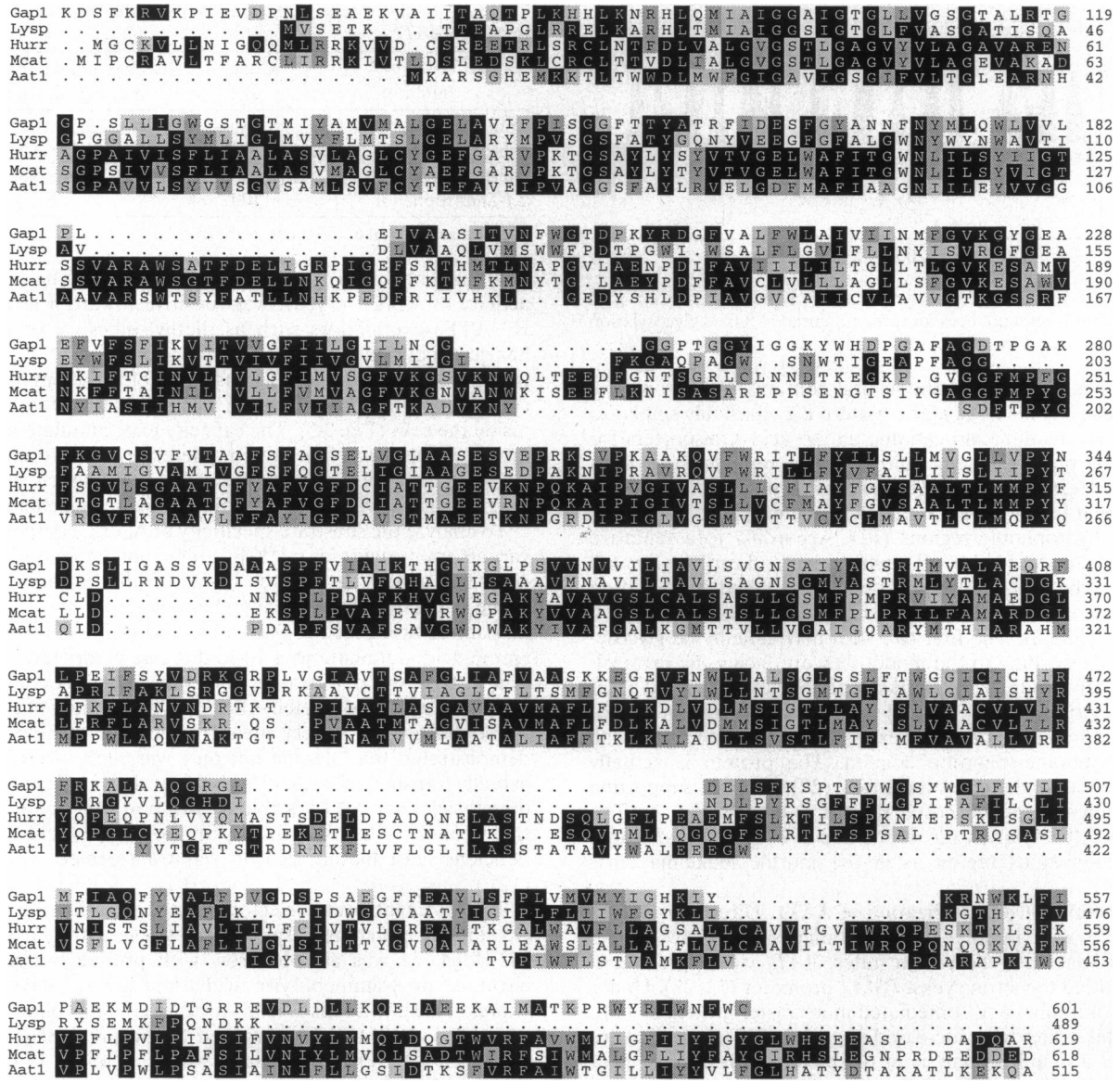

FIG. 1. Comparison between deduced amino acid sequences of AAT1 from *Arabidopsis*, cationic amino acid transporters from mouse (MCAT; ref. 20) and human (HURR; ref. 23), general AAP from yeast (GAP1; ref. 12), and bacterial lysine permease (LYSP; ref. 24).

chromatography on cellulose plates in 1-butanol/acetic acid/water (12:3:5).

RESULTS

Complementation of a Histidine Uptake Deficiency. The yeast strain JT16 carrying mutations in histidine and arginine permease genes and in *His4* requires high concentrations of histidine (30 mM) for efficient growth (11). To identify plant amino acid transporters, JT16 was transformed with a yeast expression cDNA library derived from *Arabidopsis* seedlings (9). After transformation, cells were selected directly on SC medium supplemented with 6 mM histidine. Plasmid DNA was isolated and several classes of clones could be identified. Regarding the restriction map, clones different from previously isolated *AAP* and *NTR* genes were analyzed further (7, 19). To confirm that no reversions or second site mutations had occurred in the transformants, the recombinant plasmids were reintroduced into the mutant. *AAT1* with a size of about 2.1 kb enabled growth on histidine.

Sequence Analysis of *AAT1*. *AAT1* contains an open reading frame of 533 amino acids (58 kDa). AAT1 shares homologies with cationic amino acid transporters from mammals, including the originally identified murine leukemia virus receptor (20–23). Low, but significant, homologies were also found to bacterial (e.g., LYSP; ref. 24) and yeast AAPs (refs. 11 and 12; Fig. 1; Table 1). In contrast, no significant homologies to the AAP family of plant AAPs were detectable (8). This demonstrates the presence of at least two evolutionary distinct protein families involved in plant amino acid transport.

Table 1. Percentage of similarity (upper triangle) and identity (lower triangle) between deduced amino acid sequences of AAT1 from *Arabidopsis*, cationic amino acid transporters from mouse (MCAT) and human (HURR), general AAP from yeast (GAP1), and bacterial lysine permease (LYSP)

	AAT1	MCAT	HURR	GAP1	LYSP
AAT1		59.5	59.5	49.6	48.9
MCAT	32.6		76.2	49.3	49.8
HURR	34.2	59.4		48.3	52.6
GAP1	22.2	19.8	21.7		64.5
LYSP	20.6	22.7	23.2	36.5	

MCAT, ref. 23; HURR, ref. 24; GAP1, ref. 26; LYSP, ref. 25.

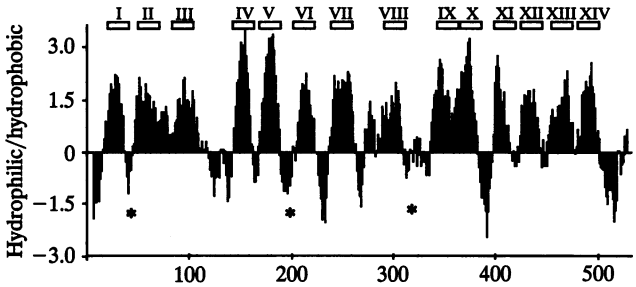

FIG. 2. Hydrophobicity analysis of AAT1 with a window size of 11 (25). Recognition sequences for three potential N-linked glycosylation sites are indicated by asterisks.

AAT1 is highly hydrophobic and contains 14 hydrophobic segments (Fig. 2; ref. 25). The pattern is remarkably similar to that of the mammalian cationic amino acid transporters for which 14 putative membrane-spanning regions have been predicted (26). In contrast, the bacterial LysP and fungal GAP1 that belong to the same protein family contain only 12 distinct hydrophobic regions (12). According to a tentative structural model for MCAT, both amino and carboxy termini are cytosolic (26). In AAT1, potential N-linked glycosylation sites are located in the hydrophilic regions at positions 41, 194, and 337 (Fig. 2). The first two sites may actually be glycosylated, as according to the model they are facing the extracellular matrix, whereas the third one at position 337 would point to the cytosol. In the murine leukemia virus receptor, two N-linked glycosylation sites were found in the loop behind the fifth membrane-spanning domain. The protein is actually glycosylated at these two positions (27), strongly supporting the validity of the proposed structural model (Fig. 2). The N-linked glycosylation site at position 194 in AAT1 is located in a synonymous region as in the murine leukemia virus receptor.

Biochemical Characterization of AAT1. Direct uptake experiments with radiolabeled L-histidine showed low uptake rates. To increase the uptake rates, *AAT1* was expressed in JT16 behind the strong yeast *PMA1* promoter (14, 28). Under these conditions, AAT1 mediated much faster growth of JT16 and uptake rates were increased up to 5-fold. These cells were used for a detailed analysis of the biochemical properties of AAT1. Uptake was pH dependent with an optimum at about pH 4.5–5 (Fig. 3*A*) and saturable with a K_m of 35 μM when determined at pH 4.5. In plants, amino acid transport is active and coupled to a symport of protons (1, 4). The transport activity of AAT1 in yeast was sensitive toward a number of H^+-ATPase inhibitors such as diethylstilbestrol or dicyclohexylcarbodiimide (Table 2). Furthermore, AAT1 was able to transport histidine at least against a 64-fold gradient into yeast cells (Fig. 3*B*). L-[^{14}C]Histidine remained intact for >30 min inside the cells (Fig. 3*C*). The capacity to accumulate histidine inside the cells was blocked completely by the protonophore 2,4-dinitrophenol. Together the data may be taken as indications for an active, proton-coupled transport mechanism.

Table 2. Inhibitor sensitivity of L-[^{14}C]histidine uptake into JT16-*AAT1*

Inhibitor	Conc., μM	Histidine uptake, pmol/min per mg of protein
Control		23.2 ± 1.7
Dicyclohexylcarbodiimide	20	16.2 ± 3.2
Diethylstilbestrol	100	7.0 ± 3.8
2,4-Dinitrophenol	100	<1

Data are expressed as mean ± SD.

To analyze the substrate specificity of AAT1, competition of various amino acids for L-[^{14}C]histidine uptake was performed (Fig. 3*D*). AAT1 has an even higher affinity for arginine and lysine as compared to histidine. However, also neutral amides and amino acids such as glutamine and valine and the acidic amino acid glutamate at a 10-fold excess competed for histidine uptake, whereas proline acted only as a weak competitor. Since D-histidine competed efficiently, AAT1 does not seem to be stereospecific. Direct measurements of alanine uptake demonstrated that alanine not only was recognized but was actually transported by AAT1 (data not shown). To determine directly whether AAT1 is able to transport other amino acids, the proline, citrulline and γ-aminobutyric acid uptake-deficient yeast mutant 22574d was transformed with *AAT1* (12).

Transformants were selected on media containing proline, citrulline, or γ-aminobutyric acid as sole nitrogen sources. 22574d-*AAT1* was able to grow with proline but not with citrulline or γ-aminobutyric acid (data not shown). AAT1-expressing 22574d cells actually took up L-[^{14}C]proline, though to much lower rates as compared to the previously isolated AAPs (5, 7, 8). Proline transport saturated above 15 mM and the K_m was calculated to be ≈3 mM. These data show that AAT1 represents a high-affinity transporter for basic amino acids but to some extent can also transport a spectrum of other

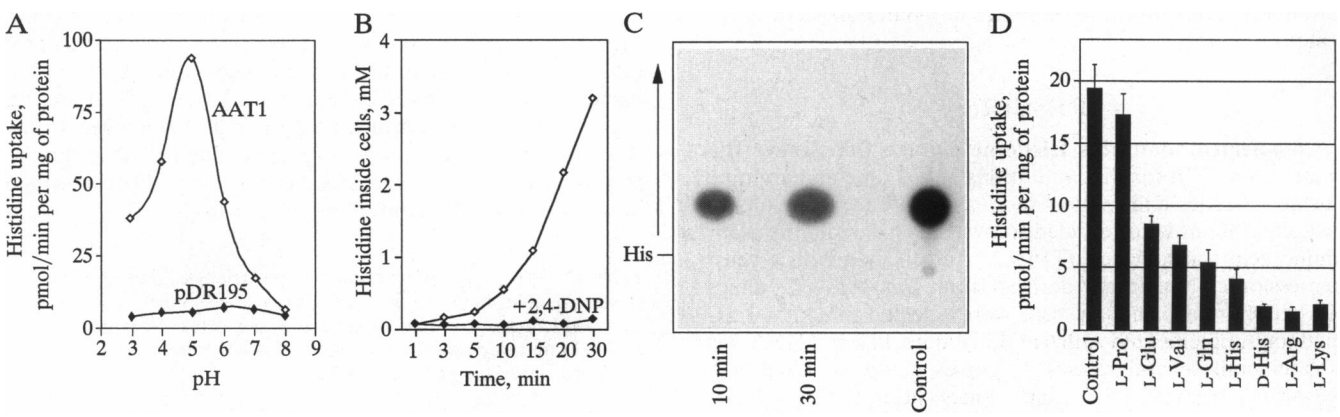

FIG. 3. Biochemical properties of AAT1. (*A*) pH dependence of AAT1-mediated histidine transport. Histidine uptake rates into JT16 transformed with *AAT1* in pDR195 (◇) or pDR195 (◆) were determined in media adjusted to different pH values in the presence of 25 μM histidine. (*B*) Accumulation of L-[^{14}C]histidine from a 50 μM solution by JT16-*AAT1* at pH 4.5 in the presence (◆) or absence (◇) of 100 μM 2,4-dinitrophenol. (*C*) Thin-layer chromatography and autoradiography of radioactive compounds present in JT16-*AAT1* after 10 and 30 min of incubation in 50 μM L-[^{14}C]histidine. (*D*) Amino acid side chain specificity as determined by competition of 50 μM L-[^{14}C]histidine uptake into JT16-*AAT1* at pH 4.5 in the presence of a 10-fold excess of unlabeled amino acids. The mean of three independent competition experiments is shown and standard deviations are given as error bars.

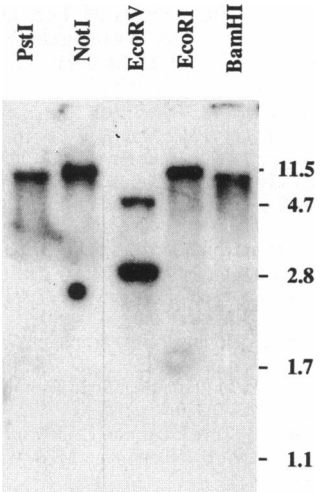

FIG. 4. Southern blot analysis of *AAT1*. Genomic *Arabidopsis* DNA (25 μg) was digested with restriction enzymes and analyzed by Southern hybridization using *AAT1* as probe. The size (kb) is given to the right.

amino acids. To what extent the transport activity—e.g., of proline and alanine—is relevant under physiological conditions remains to be shown.

Expression Profile of *AAT1*. Southern blot hybridizations of genomic *Arabidopsis* DNA with *AAT1* identifies single hybridization signals with several enzymes, indicating that no closely related gene is cross-hybridizing (Fig. 4). Northern analysis shows that *AAT1* hybridized with a transcript of ≈2200 nucleotides. In leaves, roots, flowers, and developing siliques about similar levels of *AAT1* transcripts were found (Fig. 5), whereas stems showed a slightly lower expression. To get an insight into potential functions of AAT1 in long-distance transport, the promoter of *AAT1* was isolated and fused transcriptionally to the β-glucuronidase gene. Histochemical analysis of tobacco transformants showed that *AAT1* is specifically expressed in various floral tissues—i.e., peduncle, sepal, corolla, pistil, and ovaries (Fig. 6 *A* and *B*). Furthermore, *AAT1* expression was found in the major veins of leaves and roots (Fig. 6 *C* and *D*).

DISCUSSION

Amino acids are transported throughout the plant in xylem and phloem. Transport is complex since a mixture of amino acids

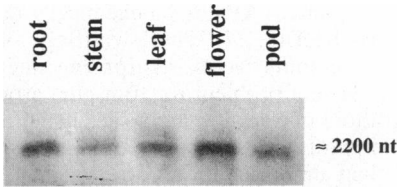

FIG. 5. Organ-specific expression of *AAT1* in *Arabidopsis*. Total RNA (20 μg) from developing leaves, mature leaves, cauline leaves, flowers, green siliques, and roots was analyzed by Northern blot hybridization using *AAT1* as probe.

is translocated (1, 29, 30). Major components of xylem and phloem are acidic amino acids and amides. In contrast, the concentration of basic amino acids in the vascular saps is low, raising the question of how sink tissues are supplied with histidine, arginine, and lysine. In agreement with the low amounts being translocated, sink organs such as seeds generally contain only trace amounts of the essential basic amino acid lysine. Lysine content is also low in many seed proteins and it remains unclear what is the rate-limiting step in lysine accumulation (31). Despite their importance—e.g., for the nutritional quality of seeds—synthesis and accumulation of lysine in seeds and import are poorly understood.

Biochemical and genetic studies have provided evidence for carrier proteins mediating basic amino acid transport (32–37). Complementation of a histidine uptake deficiency of yeast enabled the isolation and characterization of AAT1 that is homologous to amino acid transporters from bacterial, fungal, and mammalian but not plant origin.

The expression pattern of *AAT1* is consistent with three potential functions. (*i*) Vascular expression indicates a role in long-distance translocation. (*ii*) Expression in major veins of leaves and peduncle may indicate a role in xylem/phloem interchange of amino acids. Such exchange is well documented. Transporters must be involved in this process since, despite the presence of amino acids in the xylem, seeds import amino acids almost exclusively from the phloem (38). (*iii*) Expression in ovaries indicates a role in supplying developing embryos with nitrogen.

Characterization of biochemical properties of AAT1 in two yeast mutants deficient in a number of AAPs shows that AAT1 has a high affinity for basic amino acids but, to a lower extent, can also transport neutral and acidic amino acids. With a K_m of 35 μM, AAT1 has a much higher affinity toward histidine

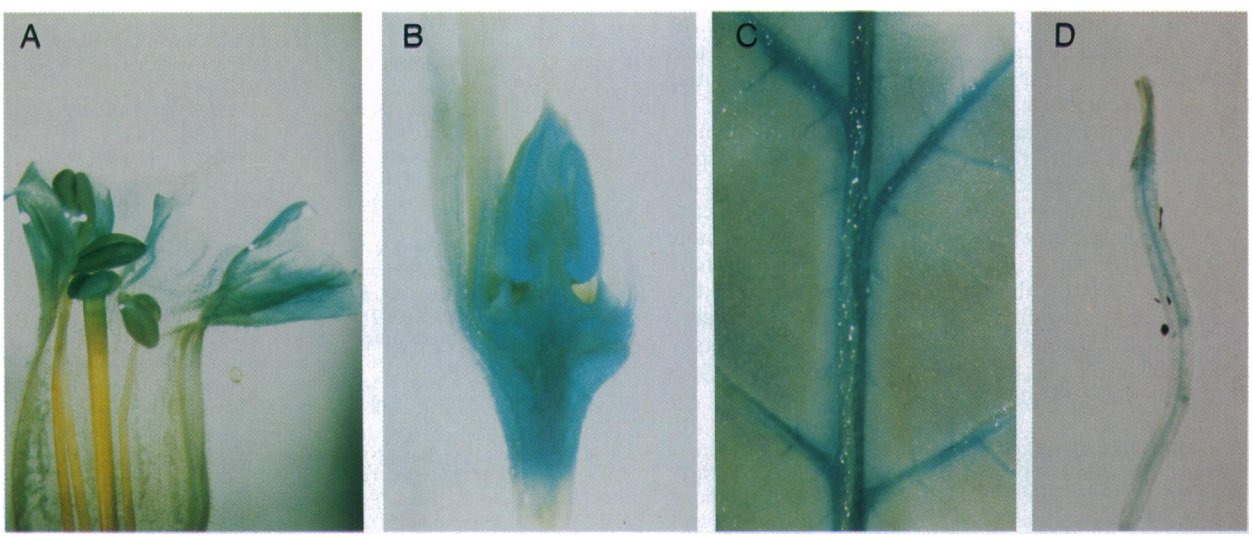

FIG. 6. Histochemical analysis of the *AAT1*–promoter GUS fusion in transgenic tobacco plants. Sections of flowers (top part, *A*; lower part, *B*), leaves (*C*), and roots (*D*) from transgenic plants were stained for 24 hr in 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide and cleared in ethanol.

as compared to plant AAPs or biochemically defined basic systems of sugar beet (8, 37). The low affinity of AAT1 for neutral and acidic amino acids is surprising since the broad specificity may pose a problem for transport efficiency since high concentrations of transport amino acids such as glutamate can act as competitive inhibitors for basic amino acid translocation. All plant amino acid transporters identified so far are broad specific systems. Provided that no other more specific amino acid transporters are present in the plant, the amino acid biosynthesis in source tissues will determine the composition of the vascular saps which in turn will then determine amount and spectrum of amino acids imported into seeds. The analysis of mutants and the similarity in composition and concentration of amino acids between mesophyll cytosol and phloem sap support this hypothesis (29, 39). The broad specificity of amino acid transport systems is unique to plants and may represent a special adaptation to the multiple conditions where the whole spectrum of proteolytic products has to be mobilized—e.g., during germination or senescence. However, this would limit the mobility of amino acids present in low concentrations under normal conditions and might therefore explain the low lysine content in seeds. The hypothesis that the low specificity of AAT1 limits lysine import could be tested by expressing transporters with a higher specificity for lysine (such as MCAT2) ectopically in plants.

With regard to affinity, AAT1 resembles its mammalian homologue, MCAT-2B, a high-affinity form of the mammalian cationic amino acid transporters that has a K_m for arginine of 70 μM (20). The only difference between low- and high-affinity MCAT2 transporters lies in the eighth hydrophilic loop. Interchange of domains between low- and high-affinity MCAT2s allowed delimitation of a region responsible for substrate affinity to this loop. Some residues in this region are conserved also in AAT1. Interchange of domains between different members of the family should allow determination of whether the same region is involved in the affinity for amino acids in AAT1. Active uptake and sensitivity to ATPase inhibitors and protonophores may indicate that AAT1 functions as a proton symporter. In contrast, MCAT2 mediates influx and efflux and seems to function as a facilitator (21). Electrophysiological studies in *Xenopus* oocytes should help to solve this question (21).

Complementation of yeast mutants has been used as an effective tool for expression cloning of >20 different plant transporter genes (40), among them at least 10 different amino acid transporters (this study; refs. 5–8 and 14; D. Rentsch and W.B.F., unpublished results). Surprisingly, no animal transporters have been isolated by this approach (41). The similarity of AAT1 to mammalian amino acid transporters suggests that yeast complementation should be applicable for isolating mammalian transporters in yeast.

Conclusion. Plants contain at least two families of amino acid transporters with overlapping specificities. AAT1 represents a high-affinity transporter for basic amino acids and has similar properties as transport system II described in sugarcane suspension culture cells, whereas the AAPs resemble more system I (36). All amino acid transporters isolated so far have a broad specificity, but with different affinities for individual amino acids, and show differential expression (8). Such a multiplicity of carriers may be required to provide a variety of regulatory mechanisms for adaptation of individual cell types or tissues to varying conditions.

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