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Cloning a plant amino acid transporter by functional complementation of a yeast amino acid transport mutant

(symport/membrane protein/plasma membrane/heterologous expression/nucleotide sequence)

LI-CHU HSU*, TZYY-JEN CHIOU*, LISHAN CHEN*, AND DANIEL R. BUSH*†‡

*Department of Plant Biology, 196 Plant and Animal Biotechnology Laboratory, 1201 West Gregory, University of Illinois, Urbana, IL 61801; and †Photosynthesis Research Unit, U.S. Department of Agriculture–Agricultural Research Service, Urbana, IL 61801

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ABSTRACT **Amino acids are transported across the plasma membrane of plant cells by proton–amino acid symports. We report here the successful cloning of a neutral amino acid carrier by functional complementation. A histidine transport deletion mutant of *Saccharomyces cerevisiae* was transformed with an *Arabidopsis thaliana* cDNA library constructed in a yeast expression vector. Forty transformants, out of 10^5, allowed growth on a histidine-limiting medium. The acquired ability to grow on low histidine was shown to be strictly dependent on the protein encoded by the expression plasmid. Histidine and alanine transport activity were 10- to 20-fold greater in the transformants. The transport kinetics, inhibitor sensitivity, and substrate specificity match those of neutral system II, a neutral amino acid carrier we previously described in plasma membrane vesicles isolated from leaf tissue. The cDNA insert is 1.7 kb with an open reading frame that codes for a protein containing 486 amino acids with a calculated molecular mass of 52.9 kDa and three sites of potential N-linked glycosylation. Hydropathy analysis of the deduced amino acid sequence suggests this is an integral membrane protein with 10–12 membrane-spanning α-helices. Overall, the sequence of this amino acid carrier is not closely related to any other protein sequences in the GenBank data base. Interestingly, however, there are small regions of sequence that exhibit significant levels of similarity with at least seven other integral membrane proteins.**

Although plants are photoautotrophic organisms, they are composed of many heterotrophic tissue systems that are dependent upon sugar and amino acid import for normal growth and development (1, 2). These heterotrophic tissues include many systems of unqualified biological significance, including the roots, young expanding leaves, and the reproductive organs and developing seed. While many plant cells possess the ability to incorporate inorganic nitrogen into amino acids, most heterotrophic tissues do not have access to adequate quantities of inorganic substrate and are therefore dependent upon amino acid import from the primary sites of nitrogen assimilation. In spite of the obvious significance of nitrogen redistribution in the plant, however, little is known about the transport proteins mediating this essential process. We are interested in amino acid transporters because of their central role in this important resource allocation system.

Amino acids are actively transported into plant cells by proton-coupled symporters (3). These systems link translocation across the plasma membrane to the proton-motive force generated by a P-type, H^+-pumping ATPase (4, 5). Recent experiments with purified plasma membrane vesicles provided evidence for at least four amino acid carriers, including an acidic amino acid symporter, a basic amino acid symporter, and two symporters for the neutral amino acids (6, 7). Traditional biochemical strategies used to identify these porters have been hampered by the low abundance of membrane-bound transport proteins. In an alternative approach to learning about these amino acid symporters, we have turned to functional complementation as a powerful strategy for identifying cDNAs that code for these critical transport systems. In this approach a plant cDNA library, constructed in a yeast expression vector, is screened for sequences that encode peptides that complement a yeast amino acid transport mutant. We report here the molecular cloning and transport characterization of a plant amino acid carrier[§] functionally expressed in a yeast histidine transport mutant.

MATERIALS AND METHODS

Strains and Media. The strain of *Saccharomyces cerevisiae* used in this study [JT16 (8); *MATa hip1-614 his4-401 ura3-52 ino1 can1*] was kindly provided by G. Fink (Whitehead Institute, Cambridge, MA). JT16 was maintained on complete yeast extract/peptone/dextrose medium supplemented with 650 μM histidine (8). Ura^+ transformants were selected on S1 medium, which contains 2% glucose, 0.17% yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate, 0.002% inosine, 0.1% arginine, 1 M sorbitol, and 3.9 mM histidine. Plasmids were propagated in *Escherichia coli* strain XL1-Blue on Luria–Bertani medium supplemented with ampicillin (60 μg/ml).

cDNA Library. An *Arabidopsis thaliana* (Columbia ecotype) cDNA library, constructed in a yeast expression vector (λYES; ref. 9), was obtained from R. Davis (Stanford University, Stanford, CA). This library was made from mRNA prepared from the aboveground parts of plants at all stages of development (9). The cDNAs were inserted into an *Xho* I site flanked by *Eco*RI sites. For this vector, expression is controlled by the inducible *GAL1* promoter.

Transformation. Electroporation was used to efficiently introduce the cDNA library into JT16 cells (10). Yeast cells were harvested during midlogarithmic-phase growth and suspended in 1 M sorbitol at $\approx 1 \times 10^{10}$ cells per ml. Forty-microliter aliquots of this yeast suspension were mixed with 0.5 μg of plasmid DNA. Electroporated cells were collected in 1 ml of 1.0 M sorbitol and spread onto S1 medium in a 15-cm plate.

Screening. Stable transformants were identified by screening on S1 medium for complementation of the nutritional deficiency in uracil biosynthesis. Ura^+ transformants were pooled and then plated on a histidine-limiting medium (HLM)

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Abbreviations: HLM, histidine-limiting medium; FOA, 5-fluoroorotic acid.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L16240).

consisting of 4% galactose, 0.17% yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate, 0.002% inosine, 0.1% arginine, and 130 μM histidine. Transformants that grew on HLM were selected and rechallenged on a histidine-free medium (HLM minus histidine) and a glucose-containing medium (glucose instead of galactose as the carbon source in HLM). Additionally, 5-fluoroorotic acid (5-FOA) was used to cure positive transformants of the newly acquired plasmid (11).

DNA Sequencing. The sequence of the transporter cDNA clone was determined by the dideoxy chain-termination method (12) using Sequenase version 2.0 (United States Biochemical). Three fragments from the cDNA insert (1.7-kb *Eco*RI–*Eco*RI, 0.5-kb *Nsi* I–*Nsi* I, and 0.8-kb *Nsi* I–*Ava* II) were subcloned into pBluescript II KS. Double-stranded template DNA was sequenced by using T3 and T7 primers of pBluescript as well as specific oligonucleotide primers deduced from the partially sequenced insert. Standard molecular techniques, unless otherwise stated, were performed according to Sambrook *et al.* (13). DNA sequence analysis was done with DNA Strider 1.1 (Ch. Marck and C.E.A., Cedex, France). Protein sequence comparisons were performed with the BLAST (basic local alignment search tool) search program (14).

Northern Analysis. RNA was extracted from *A. thaliana* (Columbia ecotype) using a hot borate extraction method (15). Twenty micrograms of total RNA was electrophoresed in 1.5% agarose and transferred to a nylon membrane (13). A NAT2 probe was prepared with random hexamer-primed [α-^{32}P]dATP labeling of the insert from p4-28.

Transport Measurements. Cells were grown to midlogarithmic phase, washed, and suspended in HLM minus histidine and arginine (pH adjusted to 5.0 with NaOH) at 200–300 mg of cells per ml. Transport was initiated by diluting 5–10 μl of cells into 500 μl of suspension solution containing 0.2–1.0 μCi (1 Ci = 37 GBq) of ^{14}C-labeled amino acid and unlabeled amino acid to the desired final concentration. At predetermined time points, an aliquot of suspended cells was delivered onto a micropore filter. Transport solution was aspirated into a vacuum flask, and the cells were rinsed with 2 ml of cold, unlabeled suspension solution. Accumulated radioactivity was measured by scintillation spectroscopy. All transport experiments were repeated at least three times with duplicate samples included for each treatment.

RESULTS AND DISCUSSION

Positive Transformants Complement the His$^-$ Phenotype. The strategy used here to clone a plant amino acid carrier was to screen an *Arabidopsis* expression library for sequences that encode proteins that functionally complement the yeast histidine transport mutant JT16. This yeast strain was a good recipient for the expression library because it is both a histidine permease deletion mutant (*hip1*) and a histidine auxotroph (*his4*) (8). Although JT16 grows well in the presence of high histidine (3.9 mM), growth is severely limited on low histidine (130 μM) medium containing ammonia (data not shown). Ammonia is a required addition for the His$^-$ phenotype because it suppresses the expression of the yeast general amino acid permease (16), which provides an alternative pathway for histidine uptake. We exploited the His$^-$ phenotype by selecting for plasmids that enabled JT16 to grow on low, nonpermissive concentrations of histidine.

An *A. thaliana* cDNA library, constructed in the yeast expression vector λYES, was transformed into the JT16 cell line. Approximately 10^5 Ura$^+$ transformants were identified on the uracil-free S1 medium. These cells were collected and spread on 100 plates, at about 10^4 cells per plate, containing a second selective medium in which galactose was used to induce the expression of the plant cDNAs and the histidine concentration was lowered to 130 μM. Five days later, 400 colonies were identified that acquired the ability to grow on HLM. Because we introduced a 10-fold amplification of those cells collected in the initial screen, the selected colonies represent ≈40 positive clones from the original 10^5 Ura$^+$ transformants.

Since JT16 is both a histidine auxotroph and uptake mutant (8), it is possible that positive transformants complemented the biosynthetic pathway rather than histidine transport. To test this, transformants were plated on histidine-free HLM. None of the positive colonies were able to grow in the absence of histidine, suggesting the positive transformants acquired histidine transport activity.

To make sure that the suppression of the transport-deficient phenotype is due to a peptide encoded by the expression plasmid, His$^+$ transformants were transferred to a modified HLM containing glucose as the sole source of carbon. Glucose represses the *GAL1* promotor engineered into the vector, thus eliminating the expression of any encoded plant peptides (8). Approximately 98% of the transformants lost the ability to grow on low histidine. In control experiments, JT16 also failed to grow. These observations suggest transformant growth on limiting histidine is dependent upon the expression of the encoded plant protein.

To provide unequivocal evidence that the encoded plant protein is responsible for suppression of the histidine-limiting phenotype, we used 5-FOA to cure these cells of the plasmid (11). 5-FOA is a pyrimidine analog that reacts with orotidine-5′-phosphate decarboxylase (the *URA3* gene product) to produce a toxic product, 5-fluorouracil. Ura3$^-$ mutant strains containing a plasmid-borne Ura3$^+$ function are unable to grow on medium containing 5-FOA because of the toxic byproduct. Thus, 5-FOA provides a strong selection pressure to lose the plasmid-borne orotidine-5′-phosphate decarboxylase activity in our positive transformants. We randomly picked 30 His$^+$ transformants from the original 400 and spread ≈10^6 cells of each on medium containing 0.1% 5-FOA. We recovered plasmid-free colonies for 23 of the 30 transformants challenged with 5-FOA; of these, 16 lost the ability to grow on low histidine. This is consistent with growth on low histidine being dependent upon functional expression of the plant cDNA insert in the expression plasmid.

Plasmid DNAs were transferred into *E. coli* for eight of the original transformants that lost the His$^+$ phenotype in the 5-FOA experiment (3-34, 4-5, 4-28, 6-3, 7-23, 7-51, 8-30, and 10-31). The cDNA inserts isolated from these plasmids were ≈1.7 kb, with the exception of 10-31, and restriction analysis of the inserts yielded identical patterns (data not shown), suggesting they were derived from the same mRNA. Eventually a single transformant, 4-28, and plasmid, pNAT2, were chosen for further analysis.

Transport Analysis. If pNAT2 encodes a plant amino acid carrier, then 4-28 should exhibit greater histidine transport activity than JT16. Indeed, 4-28 histidine transport was 10-fold higher than that observed for JT16 (Fig. 1*A*). Previous research from the Bush laboratory identified two carriers for the neutral amino acids in plasma membrane vesicles isolated from plant tissue (6, 7). In those experiments, isoleucine and alanine were shown to be prototypical substrates for neutral systems I and II, respectively. We examined isoleucine and alanine transport in 4-28 and found only alanine transport to be significantly greater than that observed in JT16 (Fig. 1*B*). Since alanine transport activity was substantially larger than that measured for histidine, we focused on alanine for subsequent analysis.

Alanine transport into 4-28 exhibited saturable, concentration-dependent influx that is consistent with carrier-mediated uptake (Fig. 2). Significantly, the apparent K_m for alanine transport into 4-28, 292 ± 42 μM, is virtually identical to that determined for alanine uptake into isolated plasma membrane

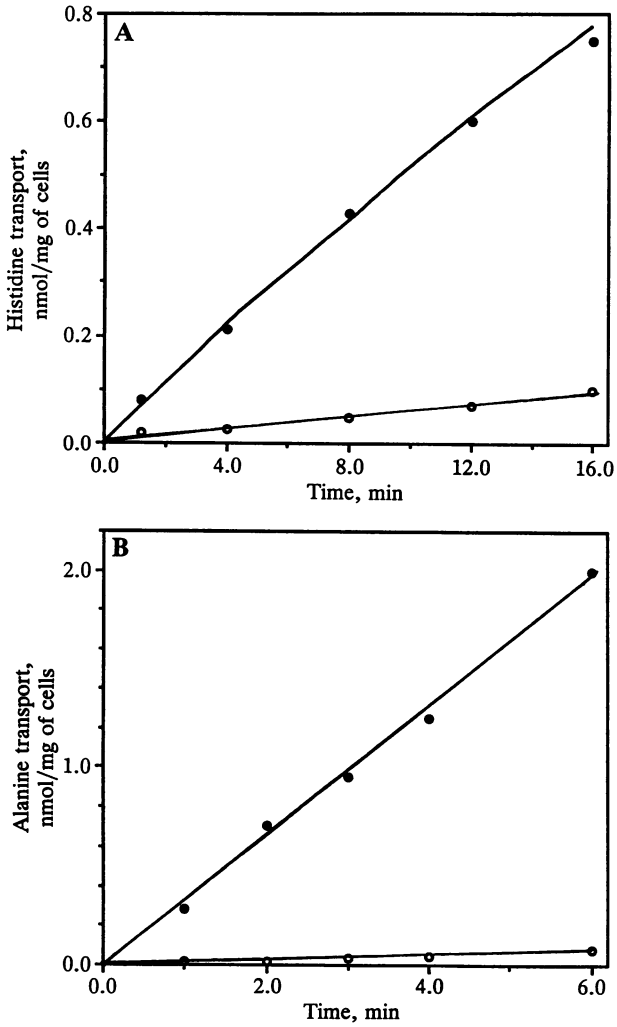

FIG. 1. Time course of histidine (*A*) and alanine (*B*) transport into the histidine deletion mutant JT16 (○) and the transport-complemented transformant 4-28 (●). The transport solutions included 250 μM histidine or 200 μM alanine.

vesicles (6). Although a low-affinity transport system with linear kinetics was present in both JT16 and 4-28 at high alanine concentrations (5–50 mM; data not shown), it contributed <5% of the total transport activity in solutions below 1 mM alanine. The transport kinetics of the high-affinity uptake system in 4-28 suggest that pNAT2 encodes the amino acid carrier previously described as neutral system II (6).

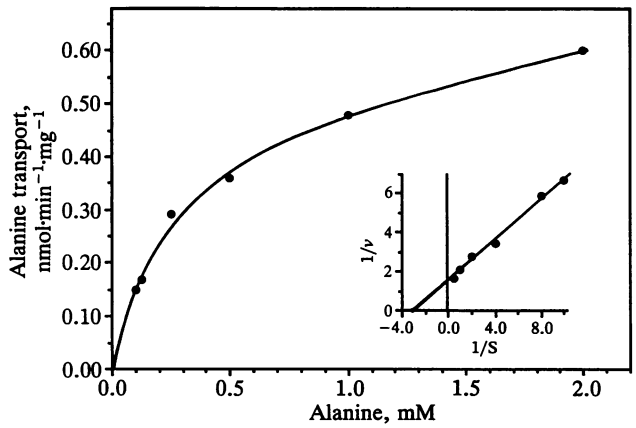

FIG. 2. Concentration dependence of alanine transport into transformant 4-28. (*Inset*) Lineweaver–Burk plot of the same data.

Neutral system II is a neutral amino acid symport that exhibits broad specificity for the aliphatic neutral amino acids (6). Surprisingly, however, it does not appear to be an effective carrier for isoleucine, valine, threonine, and proline (6, 7). We have investigated the structural determinants involved in substrate recognition by this porter and concluded that branching at the β-carbon of these amino acids produces steric interactions that block successful binding and translocation by this carrier (17). To investigate further the hypothesis that pNAT2 encodes neutral system II, we screened several amino acids as potential competitive inhibitors of alanine transport (Table 1). Leucine and methionine were strong antagonists of alanine uptake, whereas isoleucine, glutamate, and lysine were much less active. This pattern of transport competition is very similar to that previously observed for neutral system II in isolated plasma membrane vesicles (6).

The neutral amino acid carriers we described in isolated plasma membrane vesicles were proton-coupled symporters (3, 6, 18). Alanine transport into 4-28 was significantly inhibited in the presence of carbonylcyanide *m*-chlorophenylhydrazone (Table 1), a protonophore that dissipates the proton-motive force that exists across the yeast cell's plasma membrane. Although interpretation of protonophore sensitivity is compromised when applied to living cells, this observation is consistent with direct coupling to the proton electrochemical potential. In addition, alanine transport exhibited an acidic pH optimum, and it was very sensitive to chemical modification by diethyl pyrocarbonate (Table 1). Significantly, each of these transport characteristics parallel those previously documented for neutral system II in isolated plasma membrane vesicles (6).

Taken together, the transport kinetics, substrate specificity, and inhibitor sensitivity of alanine uptake into 4-28 provide compelling evidence that the cDNA insert in pNAT2 codes for neutral system II.

DNA Sequence and Expression of NAT2. Sequence analysis of the insert isolated from pNAT2 identified a 1458-bp open reading frame within the 1665-bp cDNA (Fig. 3). The open reading frame codes for a protein containing 486 amino acids with a calculated molecular mass of 52,861 Da and three sites of potential N-linked glycosylation. We have designated this gene as *NAT2* for neutral amino acid transporter II. Northern blot analysis using *NAT2* sequence as the probe detected an ≈1.8-kb message in leaf RNA (data not shown), demonstrating that this insert was derived from *Arabidopsis* cDNA. Hydropathy analysis (19) of the deduced amino acid sequence suggests that NAT2 is a typical integral membrane

Table 1. Antagonists, inhibitors, and pH optimum of alanine transport into transformant 4-28

Condition	% control activity
200 μM alanine, pH 5.0	100
+ 1 mM methionine	38
+ 1 mM leucine	43
+ 1 mM isoleucine	79
+ 1 mM glutamate	83
+ 1 mM lysine	95
+ 10 μM CCCP	12
+ 1 mM DEPC	3
200 μM alanine, pH 4.5	112
200 μM alanine, pH 6.0	58
200 μM alanine, pH 7.0	21

The transport solution included 200 μM alanine in the presence or absence of the indicated compound at the pH given. Cells were pretreated for 5 min in carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and diethyl pyrocarbonate (DEPC) before adding alanine. One hundred percent activity = 0.24 nmol of alanine per min per mg of cells.

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                             30                            60                                90
AATTCCTCGAGCTACGTCAGGGCTTAAAACATTTATTTTATCTTCTTCTTGTTCTCTCTTTCTCTTTCTCTCATCACT ATG AAG AGT TTC AAC ACA GAA GGA CAC AAC
                                                                                Met Lys Ser Phe Asn Thr Glu Gly His Asn

            120                                     150                                     180
CAC TCC ACG GCG GAA TCC GGC GAT GCC TAC ACC GTG TCG GAC CCG ACA AAG AAC GTC GAT GAA GAT GGT CGA GAG AAG CGT ACC GGG ACG
His Ser Thr Ala Glu Ser Gly Asp Ala Tyr Thr Val Ser Asp Pro Thr Lys Asn Val Asp Glu Asp Gly Arg Glu Lys Arg Thr Gly Thr

            210                                     240                                     270
TGG CTT ACG GCG AGT GCG CAT ATT ATC ACG GCG GTG ATA GGC TCC GGA GTG TTG TCT TTA GCA TGG GCT ATA GCT CAG CTT GGT TGG ATC
Trp Leu Thr Ala Ser Ala His Ile Ile Thr Ala Val Ile Gly Ser Gly Val Leu Ser Leu Ala Trp Ala Ile Ala Gln Leu Gly Trp Ile
            300                                     330                                     360
GCA GGG ACA TCG ATC TTA CTC ATT TTC TCG TTC ATT ACT TAC TTC ACC TCC ACC ATG CTT GCC GAT TGC TAC CGT GCG CCG GAT CCC GTC
Ala Gly Thr Ser Ile Leu Leu Ile Phe Ser Phe Ile Thr Tyr Phe Thr Ser Thr Met Leu Ala Asp Cys Tyr Arg Ala Pro Asp Pro Val
            390                                     420                                     450
ACC GGA AAA CGG AAT TAC ACT TAC ATG GAC GTT GTT CGA TCT TAC CTC GGT GGT AGG AAA GTG CAG CTC TGT GGA GTG GCA CAA TAT GGG
Thr Gly Lys Arg Asn Tyr Thr Tyr Met Asp Val Val Arg Ser Tyr Leu Gly Gly Arg Lys Val Gln Leu Cys Gly Val Ala Gln Tyr Gly

            480                                     510                                     540
AAT CTG ATT GGG GTC ACT GTT GGT TAC ACC ATC ACT GCT TCT ATT AGT TTG GTA GCG GTA GGG AAA TCG AAC TGC TTC CAC GAT AAA GGG
Asn Leu Ile Gly Val Thr Val Gly Tyr Thr Ile Thr Ala Ser Ile Ser Leu Val Ala Val Gly Lys Ser Asn Cys Phe His Asp Lys Gly
            570                                     600                                     630
CAC ACT GCG GAT TGT ACT ATA TCG AAT TAT CCG TAT ATG GCG GTT TTT GGC ATT ATT CAG GTT ATT CTT AGC CAG ATC CCA AAT TTC CAC
His Thr Ala Asp Cys Thr Ile Ser Asn Tyr Pro Tyr Met Ala Val Phe Gly Ile Ile Gln Val Ile Leu Ser Gln Ile Pro Asn Phe His
            660                                     690                                     720
AAG CTC TCT TTT CTT TCC ATT ATG GCC GCG GTC ATG TCC TTT ACT TAT GCA ACT ATT GGA ATC GGT CTA GCC ATC GCA ACC GTC GCA GGT
Lys Leu Ser Phe Leu Ser Ile Met Ala Ala Val Met Ser Phe Thr Tyr Ala Thr Ile Gly Ile Gly Leu Ala Ile Ala Thr Val Ala Gly
            750                                     780                                     810
GGG AAA GTG GGT AAG ACG AGT ATG ACG GGC ACA GCG GTT GGA GTA GAT GTA ACC GCA GCT CAA AAG ATA TGG AGA TCG TTT CAA GCG GTT
Gly Lys Val Gly Lys Thr Ser Met Thr Gly Thr Ala Val Gly Val Asp Val Thr Ala Ala Gln Lys Ile Trp Arg Ser Phe Gln Ala Val
            840                                     870                                     900
GGG GAC ATA GCG TTC GCC TAT GCT TAT GCC ACG GTT CTC ATC GAG ATT CAG GAT ACA CTA AGA TCT AGC CCA GCT GAG AAC AAA GCC ATG
Gly Asp Ile Ala Phe Ala Tyr Ala Tyr Ala Thr Val Leu Ile Glu Ile Gln Asp Thr Leu Arg Ser Ser Pro Ala Glu Asn Lys Ala Met
            930                                     960                                     990
AAA AGA GCA AGT CTT GTG GGA GTA TCA ACC ACC ACT TTT TTC TAC ATC TTA TGT GGA TGC ATC GGC TAT GCT GCA TTT GGA AAC AAT GCC
Lys Arg Ala Ser Leu Val Gly Val Ser Thr Thr Thr Phe Phe Tyr Ile Leu Cys Gly Cys Ile Gly Tyr Ala Ala Phe Gly Asn Asn Ala
           1020                                    1050                                    1080
CCT GGA GAT TTC CTC ACA GAT TTC GGG TTT TTC GAG CCC TTT TGG CTC ATT GAC TTT GCA AAC GCT TGC ATC GCT GTC CAC CTT ATT GGT
Pro Gly Asp Phe Leu Thr Asp Phe Gly Phe Phe Glu Pro Phe Trp Leu Ile Asp Phe Ala Asn Ala Cys Ile Ala Val His Leu Ile Gly
           1110                                    1140                                    1170
GCC TAT CAG GTG TTC GCG CAG CCG ATA TTC CAG TTT GTT GAG AAA AAA TGC AAC AGA AAC TAT CCA GAC AAC AAG TTC ATC ACT TCT GAA
Ala Tyr Gln Val Phe Ala Gln Pro Ile Phe Gln Phe Val Glu Lys Lys Cys Asn Arg Asn Tyr Pro Asp Asn Lys Phe Ile Thr Ser Glu
           1200                                    1230                                    1260
TAT TCA GTA AAC GTA CCT TTC CTT GGA AAA TTC AAC ATT AGC CTC TTC AGA TTG GTG TGG AGG ACA GCT TAT GTG GTT ATA ACC ACT GTT
Tyr Ser Val Asn Val Pro Phe Leu Gly Lys Phe Asn Ile Ser Leu Phe Arg Leu Val Trp Arg Thr Ala Tyr Val Val Ile Thr Thr Val

           1290                                    1320                                    1350
GTA GCT ATG ATA TTC CCT TTC TTC AAC GCG ATC TTA GGT CTT ATC GGA GCA GCT TCC TTC TGG CCT TTA ACG GTT TAT TTC CCT GTG GAG
Val Ala Met Ile Phe Pro Phe Phe Asn Ala Ile Leu Gly Leu Ile Gly Ala Ala Ser Phe Trp Pro Leu Thr Val Tyr Phe Pro Val Glu
           1380                                    1410                                    1440
ATG CAC ATT GCA CAA ACC AAG ATT AAG AAG TAC TCT GCT AGA TGG ATT GCG CTG AAA ACG ATG TGC TAT GTT TGC TTG ATC GTC TCG CTC
Met His Ile Ala Gln Thr Lys Ile Lys Lys Tyr Ser Ala Arg Trp Ile Ala Leu Lys Thr Met Cys Tyr Val Cys Leu Ile Val Ser Leu
           1470                                    1500                                    1530
TTA GCT GCA GCC GGA TCC ATC GCA GGA CTT ATA AGT AGT GTC AAA ACC TAC AAG CCC TTC CGG ACT ATG CAT GAG TGAGTTTGAGATCCTCAAG
Leu Ala Ala Ala Gly Ser Ile Ala Gly Leu Ile Ser Ser Val Lys Thr Tyr Lys Pro Phe Arg Thr Met His Glu
   1560                          1590                          1620                          1650
AGAGTCAAAAATATATGTAGTAGTTTGGTCTTTCTGTTAAACTATCTGGTGTCTAAATCCAATGAGAATGCTTTATTGCTAAAACTTCATGAATCTCTCTGTATCTACATCTTTCAATC
   1680
TAAGCTACGTCAGGGCCCTGACGTAGCTCGAGGAATT
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FIG. 3. Nucleotide and deduced amino acid sequence of NAT2. Sites of potential N-linked glycosylation are double underlined. Sequences representing the *Xho* I–*Eco*RI linker of the vector are underlined.

protein with 10–12 membrane-spanning domains (Fig. 4). Although the location of potential glycosylation sites can indicate which hydrophilic domains are on the extracellular face of the plasma membrane (Fig. 4), it should be noted that these same regions in NAT2 are also rich in basic amino acid residues, which can be important topogenic determinants in specifying intracellular domains (27, 28). Consequently, definitive statements regarding membrane topology must await supporting biochemical evidence.

A search of the GenBank data base (February 1993) did not identify any strong sequence homologies, which suggests that NAT2 belongs to a class of membrane transport proteins that

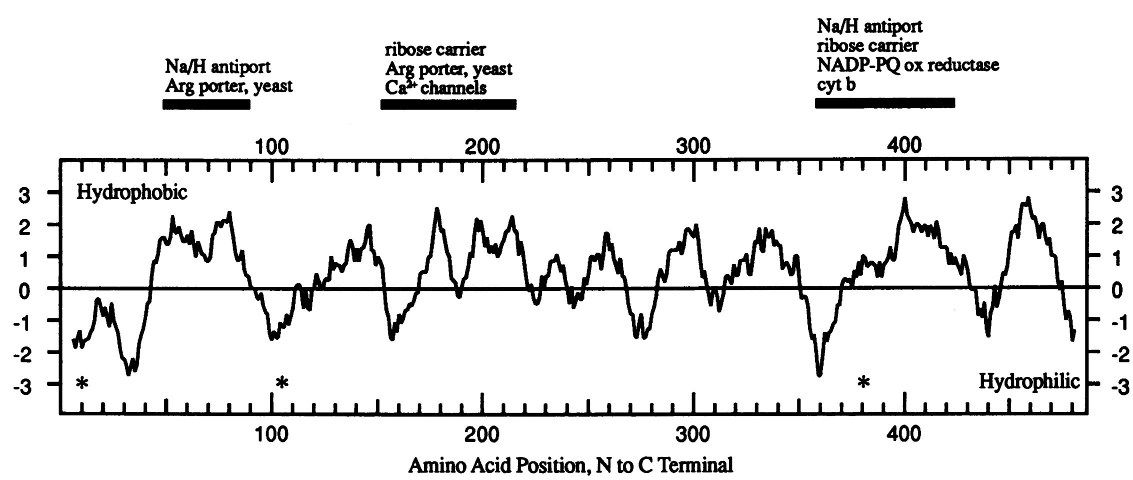

FIG. 4. Hydropathy analysis of the predicted amino acid sequence of NAT2. Positive values indicate hydrophobic domains. Sites of potential N-linked glycosylation are indicated with an asterisk above the *x* axis. Thick horizontal bars are used to designate regions in the primary sequence that exhibit short runs of sequence similarity with other integral membrane proteins. Specific membrane proteins containing similar sequences to a given region are listed above the bars. NAT2 sequences that are similar to these proteins are (*i*) 69–84 and 172–204 to the arginine permease (20), (*ii*) 49–91 and 377–412 to the Na^+/H^+ exchanger (21), (*iii*) 360–403 to cytochrome *b* (22), (*iv*) 177–211 to a calcium channel (23), (*v*) 76–91 and 366–386 to plastoquinone oxidoreductase (24), (*vi*) 156–218 and 395–425 to the ribose high-affinity carrier (25), and (*vii*) 64–85 to brain glucose carrier (26).

have not been previously identified. Significantly, however, three regions of the NAT2 protein sequence exhibited short runs of sequence similarity (from 25% to 42% identity) to several integral membrane proteins, including the yeast arginine carrier, a sodium/proton exchanger, cytochrome *b*, a glucose carrier, a ribose carrier, and several calcium channels (Fig. 4; refs. 20–26). At this time, it is difficult to ascertain whether these regions of similarity are the remnants of sequence divergence from a common ancestral gene or the result of convergent evolution in solving the unique chemical requirements imposed on functional domains of membrane-spanning sequences.

Conclusions. Amino acid transporters play an important role in satisfying the nutritional requirements of the heterotrophic tissues of the plant. As part of our interest in understanding the function of specific porters in assimilate partitioning, we report here the successful cloning of a plant amino acid carrier by functional complementation of a yeast amino acid transport mutant. The transport properties of the heterologously expressed carrier are very similar to those defined for a neutral amino acid symporter using plasma membrane vesicles isolated from sugar beet leaf tissue (6, 7). We conclude, therefore, that NAT2 codes for the amino acid carrier we previously identified as neutral system II.

Molecular cloning by functional complementation has contributed to the recent identification of a plant potassium channel (29, 30) and sucrose carrier (31). We anticipate the successful application of this approach to additional transport systems and, therefore, consider it a powerful new tool in plant transport biology. In addition, this system of heterologous expression provides an opportunity to use site-specific mutagenesis to explore more easily the difficult questions concerning carrier structure and function.

Note Added in Proof. Since acceptance of this work, we have learned that another group has cloned this carrier by complementing a proline transport mutant in *S. cerevisiae* (32). The authors suggest this porter may be neutral system I based on proline transport activity. In contrast, the transport analysis and comparison to earlier biochemical descriptions reported here strongly implicate neutral system II. That neutral system II possesses a limited ability to move proline is consistent with our previous conclusion (6,7) that the plant amino acid carriers are relatively specific for a given group of amino acids, but that each porter exhibits some level of crossover specificity for amino acids outside its primary transport group. Thus, we continue to support the conclusion that NAT2 codes for neutral system II.

We gratefully acknowledge Peter Jennetten for his assistance in measuring amino acid transport activity.

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