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Functional Characterization and Expression Analysis of the Amino Acid Permease RcAAP3 from Castor Bean[1]

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A polymerase chain reaction-based library screening procedure was used to isolate *RcAAP3*, an amino acid permease cDNA from castor bean (*Ricinus communis*). *RcAAP3* is 1.7 kb in length, with an open reading frame that encodes a protein with a calculated molecular mass of 51 kD. Hydropathy analysis indicates that the RcAAP3 protein is highly hydrophobic in nature with nine to 11 putative transmembrane domains. RcAAP3-mediated uptake of citrulline in a yeast transport mutant showed saturable kinetics with a K_m of 0.4 mM. Transport was higher at acidic pH and was inhibited by the protonophore carbonylcyanide-*m*-chlorophenylhydrazone, suggesting a proton-coupled transport mechanism. Citrulline uptake was strongly inhibited (72%) by the permeable sulfydryl reagent *N*-ethylmaleimide, but showed lower sensitivity (30% inhibition) to the nonpermeable reagent *p*-chloromercuribenzenesulfonic acid. Diethylpyrocarbonate, a histidine modifier, inhibited citrulline uptake by 80%. A range of amino acids inhibited citrulline uptake, suggesting that RcAAP3 may be a broad substrate permease that can transport neutral and basic amino acids with a lower affinity for acidic amino acids. Northern analysis indicated that *RcAAP3* is widely expressed in source and sink tissues of castor bean, and that the pattern of expression is distinct from *RcAAP1* and *RcAAP2*.

In higher plants, inorganic nitrogen is assimilated in the roots or leaves, and the reduced nitrogen (mainly in the form of amino acids, amides, and ureides) is transported around the plant in the vascular system to various metabolically active organs, where it is utilized for growth and development. Transport of solutes across cell membranes is fundamental to this process of partitioning. Studies in castor bean (*Ricinus communis*) have suggested that transport of amino acids across the plasma membrane is mediated by specific transporters driven by the proton electrochemical gradient (Williams et al., 1992, 1996; Weston et al., 1994, 1995). Our previous studies in roots of castor bean using isolated membrane vesicles indicated that several different amino acid transporters exist (Weston et al., 1995). However, these studies were limited by the presence of multiple transporter systems with overlapping specificities.

During the past few years, significant progress has been made toward the understanding of amino acid transport in higher plants using Arabidopsis as a model. This results largely from the isolation of the genes encoding amino acid permeases using complementation of yeast transport mutants (Fischer et al., 1998). The biochemical properties of the Arabidopsis amino acid permeases have been described in a number of cases (Fischer et al., 1995, 1998). However, almost nothing is known about amino acid permeases in other plant species. For example, do related transporters in different species have comparable biochemical properties? Do they show similarities in their tissue distribution? This information is important in providing an insight into their physiological role.

The transport of amino acids is very important during seed germination and seedling development, and castor bean has been used as a model system to investigate this (Williams et al., 1996). The germinating seedling relies exclusively on amino acids derived from the endosperm as a source of nitrogen. Since these are released into the apoplast, specific carriers must exist for transport of amino acids into cotyledon cells and also for loading into the phloem for delivery to the rest of the seedling. We recently isolated two partial cDNA clones (*RcAAP1* and *RcAAP2*) from germinating seedlings of castor bean (Bick et al., 1998) with homology to transporters in the AAP (amino acid permease) gene family from Arabidopsis. Using yeast complementation, we were able to isolate a full-length cDNA for *RcAAP1*. Transport studies showed that this transporter had the highest affinity for basic amino acids (Marvier et al., 1998).

The present paper describes the use of a different strategy for the isolation of amino acid transporter genes. This is the first report, to our knowledge, of the isolation of a plant transporter cDNA based on a screening technique using the PCR that was originally developed by Israel (1993). This method resulted in the isolation of the amino acid permease *RcAAP3* from developing seedlings of castor bean. The biochemical characteristics were investigated following expression of *RcAAP3* in a yeast mutant. Northern analysis indicated that *RcAAP3* has a pattern of expression distinct from the other castor bean amino acid permeases, *RcAAP1* and *RcAAP2*.

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DEPC, diethylpyrocarbonate; NEM, *N*-ethylmaleimide; pfu, plaque forming unit.

MATERIALS AND METHODS

Plant Material

Seeds of castor bean (*Ricinus communis* L. var Sanguineous) were imbibed in cold running water for 24 h and then grown in vermiculite in the dark at 28°C for 6 d. To obtain mature plants, 4- to 5-d-old seedlings were potted in Levington's F2 compost, and grown in a temperate greenhouse for at least 3 months.

Construction of a cDNA Library from Castor Bean Seedlings

Total RNA was extracted from 7-d-old castor bean seedlings (including cotyledons, endosperm, hypocotyl, and roots) using the method described by Logemann et al. (1987). mRNA was isolated from total RNA with an mRNA isolation system (PolyATract, Promega) using biotinylated oligo(dT) in conjunction with streptavadin paramagnetic particles according to the manufacturer's protocol (Promega). Double-stranded cDNA was synthesized from the mRNA (SuperScript Choice system, Life Technologies) according to the manufacturer's instructions, and was cloned into *Eco*RI-digested arms of λgt10. Recombinant phages were packaged in vitro with packaging extract (Gigapack Gold, Stratagene). A primary cDNA library of more than 10^6 pfu in size was constructed and later amplified.

PCR Screening of the cDNA Library

Screening of the castor bean library for amino acid permease cDNAs was carried out at high stringency using a PCR-based technique, largely as described by Israel (1993). The library was divided into 64 wells of a microtiter plate in a grid of eight rows and eight columns, each well containing 3,000 pfu and propagated in bacteria. After amplification of phage in the wells, aliquots of the phage from eight wells across the rows and eight wells down the columns were pooled, giving eight rows and eight columns of samples. PCR was performed on the 16 pooled phage aliquots with nondegenerate oligonucleotide primers designed from the nucleotide sequence of *RcAAP1* (Bick et al., 1998). The *RcAAP1* forward primer (*RcAAP1* F) was 5′GGTGATCCTGTCAATGGCAAGAGG3′ and the *RcAAP1* reverse primer was (*RcAAP1* R) 5′AGGAGACATGTCTCCAAAAGCAGC3′. Positives from the 16 PCR reactions were identified by the amplification of a PCR product of the expected size as visualized by agarose gel electrophoresis.

For the secondary screen the phage from the positive well was subdivided into 64 wells of the microtiter plate (each well containing 100 pfu) repropagated in bacteria and screened using the same PCR protocol. The positives were taken to the tertiary screen with 2 to 3 pfu per well and the procedure was repeated. Individual clones from the final positive wells were plated out as single plaques and screened by plaque PCR. The majority of the randomly picked plaques were found to contain inserts of the desired size. Since the PCR amplification with the primers that we used resulted in the amplification of a single strong PCR product of the expected size, hybridization with an internal oligonucleotide probe as described by Israel (1993) was omitted in the screening procedure.

Subcloning and Sequence Analysis

Phage DNA was isolated from positive plaques using the standard protocols (Sambrook et al., 1989). The cDNAs were excised from phage with *Not*I and subcloned into pBluescript SK− vectors. Subclones were generated for the cDNA and both strands were sequenced using an automated sequencer (LI-COR) with IRD-labeled M13 forward and reverse primers and T7 and T3 promoter primers using a cycle sequencing kit (Thermosequenase, Amersham). Sequence analyses were performed using the University of Wisconsin Genetics Computer Group software offered by the Seqnet computing facility (Daresbury, UK). The cDNA sequence of *RcAAP3* appears in the database under the accession no. AJ132228.

Functional Complementation in a Yeast Transport Mutant

Saccharomyces cerevisiae strain 2512c (*Mat-a*, *gap*1) was modified by the introduction of the *ura3-52* mutation as previously described (Marvier et al., 1998). The resulting strain (referred to as the *gap1* mutant) was maintained on yeast/peptone/dextrose medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose and 2% (w/v) agar. *RcAAP3* cDNA was excised with *Not*I (using the internal restriction sites incorporated into adapters during cDNA library construction) and cloned into the *E. coli*/yeast expression vector NEV-N (Sauer and Stolz, 1994). NEV-N/*RcAAP3* clones containing cDNAs in sense and antisense orientations in relation to the constitutive *PMA1* promoter in the vector were identified by restriction analysis. These constructs, together with the NEV-N control vector, were transformed into the *gap1* mutant essentially as described by Dohmen et al. (1991). Transformants were first selected on synthetic dextrose medium containing yeast nitrogen base (DIFCO Laboratories, Detroit, MI) without ammonium sulfate and amino acids, 2% (w/v) dextrose, 0.5% (w/v) ammonium sulfate, and 2% (w/v) agar. Colonies were washed from the plates with sterile distilled water and plated onto low-citrulline, nitrogen-free medium (DIFCO) supplemented with 2% dextrose and 0.2 mg mL^{-1} citrulline as the sole source of nitrogen and solidified with 2% (w/v) agar. Growth of the yeast amino acid transport mutant containing NEV-N/*RcAAP3* (sense and antisense constructs) and NEV-N controls were analyzed on different liquid media such as yeast/peptone/dextrose, synthetic dextrose, and on a range of citrulline concentrations (0.1–1.0 mg mL^{-1}). A_{600} readings were taken twice a day for 5 d.

Transport Measurements

For transport studies, yeast transformants (NEV-N/*RcAAP3*) were grown to the logarithmic phase, washed in 1% (w/v) Glc in 50 mM potassium phosphate buffer (pH

4.5), and resuspended in 50 mM potassium phosphate buffer (pH 4.5). The cells were preincubated for 5 min in 5 mM Glc (in potassium phosphate buffer, pH 4.5) prior to performing uptake assays. The standard assay contained [^{14}C]citrulline (0.5 mM) and potassium phosphate buffer (pH 4.5). The reaction was started by the addition of 9 to 15 mg fresh weight of cells to give a final volume of 200 μL. For time course experiments, 45-μL samples were taken at 15, 75, 150, and 300 s, transferred to 5 mL of ice-cold water, filtered on glass fiber filters, and washed with 3 $\times$ 5 mL of ice-cold water. Filters were dried and radioactivity determined with liquid scintillation spectroscopy (model 1209 Rackbeta, Pharmacia LKB, Uppsala, Sweden). The inhibitory effect of a range of amino acids on citrulline uptake was determined by including them at a 10-fold molar excess. For inhibition, kinetic, and pH dependence studies, transport was determined from a 150-μL sample after 5 min, during which time uptake was linear.

Southern Analysis

Young leaves obtained from mature castor bean plants were frozen in liquid nitrogen and pulverized using a mortar and pestle. Genomic DNA was isolated according to the method of Murray and Thompson (1980). Samples of genomic DNA (10 μg) were digested with several different restriction enzymes, electrophoresed in 0.8% (w/v) agarose gels, and then transferred onto Hybond N$^+$ (Amersham) membranes using a vacuum-blotting apparatus (Pharmacia Biotech) according to manufacturers' protocol. The blots were hybridized in a buffer containing 5$\times$ SSC, 2% (w/v) Boehringer and Mannheim blocker, 0.02% (w/v) SDS, and 0.1% (w/v) laurylsarcosine at 65°C for 16 h with a ^{32}P-labeled *RcAAP3*-cDNA probe prepared using a labeling kit (Ready-To-Go, Pharmacia Biotech). The blots were washed twice in 2$\times$ SSC and 0.1% (w/v) SDS at room temperature for 15 min and the final washes were done at high stringency (60°C in 0.2$\times$ SSC and 0.1% [w/v] SDS). The blots were autoradiographed at $-$80°C with intensifying screens.

Northern Analysis

Total RNA was extracted from various tissues of castor bean using the method described by Logemann et al. (1987). RNA samples (10 μg) were electrophoresed in 1% (w/v) agarose/formaldehyde gel and blotted onto Hybond N$^+$ membranes (Amersham). The blots were hybridized with ^{32}P-labeled-*RcAAP3* probe at 68°C in a buffer containing 5$\times$ SSC, 1% (w/v) Boehringer blocking agent, 0.1% (w/v) *N*-laurosarcosine, and 0.02% (w/v) SDS. After hybridization the blots were washed under high-stringency conditions (2$\times$ SSC, 0.1% [w/v] SDS, room temperature; 0.5$\times$ SSC, 0.1% [w/v] SDS, 55°C; 0.1$\times$ SSC, 0.1% [w/v] SDS, 68°C). Equal loading of RNA was confirmed by staining with ethidium bromide, stripping the blots, and reprobing with a ^{32}P-labeled 25S ribosomal cDNA probe from *Linum usitatissium* (Goldsbrough and Cullis, 1981) using the same hybridization and washing conditions described above.

RESULTS

Isolation of Castor Bean Amino Acid Permease cDNA (*RcAAP3*) by High-Stringency Library Screening Using PCR

To isolate cDNA clones encoding AAPs, a cDNA library in λgt10 was constructed (using mRNA isolated from 7-d-old castor bean seedlings) and screened using a PCR-based procedure (Israel, 1993). This method relies on the identification of particular cDNA clones in a library using PCR. Nondegenerate oligonucleotide primers (*RcAAP1* forward and reverse) designed from the nucleotide sequence of the *RcAAP1* partial-length cDNA clone were used (Bick et al., 1998). The screening resulted in the isolation of four positive phage pools after primary screening. One of these PCR positives was carried through secondary and tertiary screens, and a clone containing a cDNA 1.7 kb in size was selected for sequencing and further analysis.

Sequence analysis revealed similarities and differences at the nucleotide sequence level with *RcAAP1* and *RcAAP2* (Bick et al., 1998). This indicated that we had isolated a third amino acid permease from castor bean, which we designated as *RcAAP3* (Fig. 1). Thus, this permease is related to but not identical to *RcAAP1*, even though the primers were designed originally from the nucleotide sequence of *RcAAP1*. Comparison of the *RcAAP3* nucleotide sequence with that of *RcAAP1* showed that the forward primer sequence was identical and the reverse primer region differed only in two bases.

RcAAP3 Has the Characteristics of a Hydrophobic Membrane Protein

The 1.7-kb *RcAAP3* cDNA has an open reading frame of 467 amino acids encoding a predicted 51-kD protein (Fig.

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GGTGAGAATACAGCCACAAAGAACCACTTTCCTCCTCAGGTTTTCAGTGTTAATGTTGAC 60
   M  P  P  Q  A  G  S  K  W  Y  D  D  D  G  K  L  K  R  T  G
ATGCCTCCTCAAGCTGGCTCCAAGTGGTATGATGACGATGGCAAACTCAAGCGGACTGGA 120
T  V  W  T  A  S  A  H  I  I  T  A  V  I  G  S  G  V  L  S
ACTGTTTGGACTGCTAGTGCTCACATAATTACAGCTGTGATTGGGTCTGGAGTTCTGTCT 180
L  A  W  A  V  A  Q  L  G  W  V  A  G  P  A  V  M  F  L  F
TTGGCTGGGCGTTGGCTCAGCTTGGATGGGTTGCTGGTCCTGCTGTTATGTTCTTGTTC 240
S  L  V  T  Y  T  S  T  L  L  S  A  C  Y  R  T  G  D  P
TCTTTAGTCACTTACTATACTTCTACTCTGCTCTCCGCTTGCTACCGAACTGGTGATCCT 300
V  N  G  K  R  N  Y  T  Y  M  D  A  V  R  S  N  L  G  G  A
GTCAATGGCAAGAGGAACTATACTTATATGGACGCTGTTCGGTCCAATCTTGGTGGTGCT 360
K  F  K  I  C  G  Y  V  Q  Y  V  N  L  I  G  V  A  I  G  Y
AAGTTTAAGATATGTGGATATGTTCAATACGTGAATCTTATTGGCGTTGCCATTGGTTAC 420
T  I  A  S  S  I  S  M  M  A  V  K  R  S  N  C  F  H  K  S
ACAATAGCATCATCTATCAGTATGATGGCTGTCAAGAGGTCTAATTGTTTCCATAAGAGT 480
E  A  K  N  P  C  H  M  K  C  Q  S  L  H  D  C  I  L  E  V
GAGGCAAAAAATCCATGCCATATGAAATGCCAATCCTTACATGATTGCATTTGGAGGTT 540
V  E  S  S  S  S  Q  I  P  D  F  D  Q  T  M  G  G  L  S  I
GTTGAATCATCTTCTTCTCAGATTCCTGACTTTGATCAAACTATGGGTGGCCTATCCATT 600
V  A  A  I  M  S  F  T  Y  S  T  I  G  L  G  L  G  I  A  E
GTTGCTGCAATCATGTCCTTTACTTACTCAACAATTGGTCTTGGACTAGGAATTGCTGAA 660
V  T  K  N  G  K  A  M  G  S  M  T  G  I  S  I  G  T  V  T
GTTACAAAAAATGGGAAAGCCATGGGAAGTATGACCGGTATTAGCATTGGAACTGTGACT 720
E  T  Q  K  I  W  R  S  F  Q  A  L  G  D  I  A  F  A  Y  S
GAAACACAAAAGATATGGAGGAGCTTCCAAGCTCTTGGTGACATTGCTTTTGCCTACTCT 780
Y  S  L  I  L  I  E  I  Q  D  T  I  R  S  P  P  A  E  S  K
TATTCCCTTATCCTCATTGAAATTCAGGACACGATCAGATCTCCACCAGCAGAGTCCAAA 840
T  M  R  K  A  T  L  I  S  V  S  V  T  T  L  F  Y  M  L  C
ACAATGAGGAAAGCAACTCTAATAAGTGTATCAGTAACAACCCTTTTCTACATGCTATGT 900
G  C  F  G  Y  A  A  F  G  D  M  S  P  G  N  L  L  T  G  F
GGTTGCTTTGGCTATGCTGCCTTTGGAGACATGTCTCCGGGAAATCTCTTAACTGGTTTC 960
G  F  Y  N  P  Y  W  L  L  D  I  A  N  V  A  I  V  V  H  L
GGTTTTTACAACCCATATTGGCTACTAGACATTGCTAACGTTGCAATAGTAGTCCACCTT 1020
V  G  A  Y  Q  Y  Y  C  Q  P  L  F  A  F  V  E  K  A  A  V
GTTGGTGCATACCAAGTCTACTGCCAACCCTTGTTCGCATTCGTCGAAAAAGCAGCAGTA 1080
Q  R  F  P  D  S  E  F  I  L  K  D  I  K  I  P  I  P  G  C
CAAAGATTCCCGGACAGTGAATTCATACTTAAAGATATTAAAATCCCGATTCCTGGATGC 1140
K  P  Y  N  L  N  L  F  R  M  V  W  R  T  V  F  V  I  F  T
AAACCCTACAATCTTAACCTCTTCAGAATGGTATGGAGGACTGTTTTTGTGATCTTCACA 1200
T  V  I  S  M  L  L  P  F  F  N  D  I  V  G  L  L  G  A  L
ACCGTAATTTCGATGCTCCTTCCTTTCTTCAATGACATAGTTGGTCTACTTGGAGCTCTA 1260
G  F  W  P  L  T  V  Y  F  P  V  E  M  Y  I  A  Q  K  K  I
GGATTTGGCCATTAACCGTTTATTTCCCAGTTGAGATGTACATAGCTCAAAGAAGATA 1320
P  K  W  S  T  R  W  L  C  L  Q  I  L  S  A  A  C  L  I  I
CCAAAGTGGAGCACAAGATGGCTTTGCCTCCAAATACTAAGTGCTGCTTGTCTTATTATC 1380
T  I  A  A  A  A  G  S  I  A  G  V  I  V  D  L  K  T  V  K
ACTATAGCTGCAGCTGCTGGCTCAATTGCAGGAGTTATTGTTGATCTCAAGACAGTCAAA 1440
P  F  Q  T  T  Y  *
CCATTCCAGACTACTTACTAGATCAGTTAGAAGTTGAACTGGTATCACCATTGATGAACA 1500
TTTCAAAAACAAGGGAATCCCAACACTAGATTTTAGTTGTTGTTCATGTTCATATGATGC 1560
TCTTTATGTGGTCCTGTTAATTCGCTATGGTAGTTTTTCCATCTCTGTTATTCCTCT 1620
CTCAAAAAAGAAAAATGGGACCATGCCTGTAACATAAGTTAAAAGAAAAGAAAAGG
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Figure 1. Deduced amino acid sequence and nucleotide sequence of *RcAAP3* encoding an amino acid permease from castor bean. The underlined regions indicate the primer annealing sites.

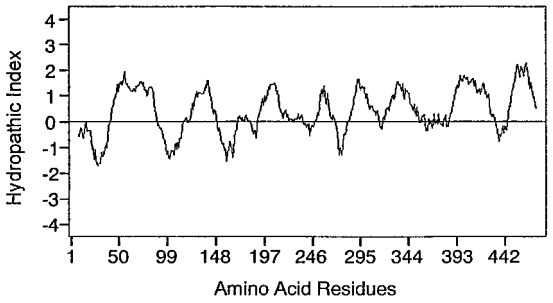

Figure 2. Hydropathy plot for the amino acid sequence of RcAAP3 calculated according to the method of Kyte and Doolittle with a window size of 19 amino acids.

1). It has 60 bases of 5′ UTR and 213 bases of 3′ noncoding region. *RcAAP3* has neither a poly(A^+) tail nor any distinct polyadenylation signals. Hydropathy profile analysis of this transporter, as analyzed by the Kyte-Doolittle algorithm, indicated its highly hydrophobic nature and predicted about nine to 11 putative transmembrane domains (Fig. 2).

RcAAP3 showed significant sequence identities with the castor bean amino acid permeases *RcAAP1* and *RcAAP2*, as well as with all the members of *AAP* gene family of Arabidopsis. RcAAP3 showed highest identity with *RcAAP1* (86%) and 59% with *RcAAP2*. However, *RcAAP2* is only a partial-length sequence and thus the level of identity may change when information for the full-length sequence is

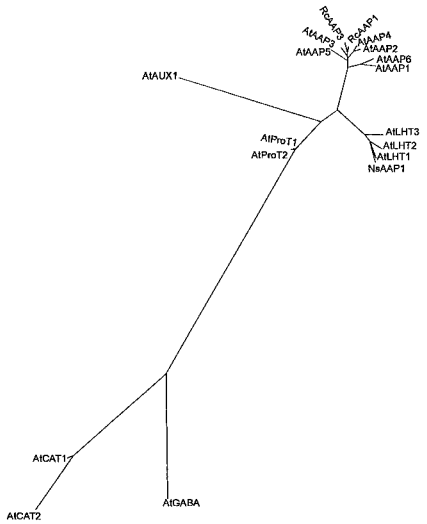

Figure 3. Phylogenetic tree for a range of plant amino acid permeases. The tree was constructed from alignments of full-length amino acid sequences for each gene. This analysis was performed using the phylogeny interference package (Fitch-Margoliash method, version 3.5; J. Felsenstein, unpublished data). Accession numbers: RcAAP1, AJ007574; RcAAP3, AJ132228; AtAAP1, x67124; AtAAP2, X71787; AtAAP3, X77499; AtAAP4, X77500; AtAAP5, X77501; AtAAP6, X95736; AtProt1, X95737; AtProt2, X95738; AtAUX1, X98772; NsAAP1, U31932; AtLHT1, U39782; AtLHT2, AC000103; AtLHT3, AC002294; AtCAT1, X77502; AtCAT2, AC004238; AtGABA, AF019637.

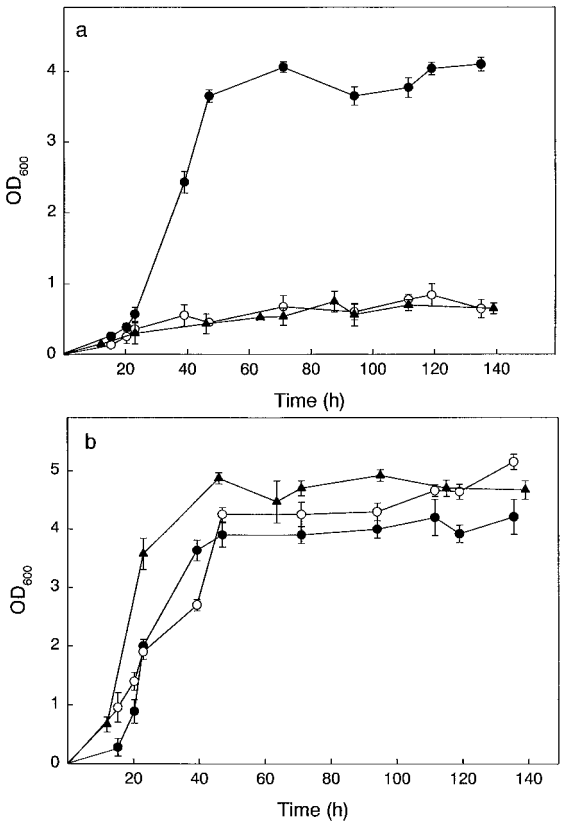

Figure 4. Growth analysis of *gap1* mutants transformed with *RcAAP3*-sense (●), *RcAAP3*-antisense (○), or control NEV-N (▲) vector. a, Growth on low-citrulline medium (0.2 mg mL^{-1}). b, Growth on synthetic dextrose medium. Cultures were grown over a 5-d period at 30°C with shaking. Results shown are the means of three replicates from a representative experiment. The growth curves were repeated twice with similar results.

available. The amino acid identities shared with the members of the Arabidopsis AAP family ranged from 57% to 77%. A phylogenetic analysis of amino acid permease genes is shown in Figure 3.

Heterologous Expression of *RcAAP3* in the Yeast Transport Mutant

The yeast *gap1* mutant is unable to grow on a medium containing citrulline (0.2 mg mL^{-1}) as the sole source of nitrogen due to a mutation in the general amino acid permease (Marvier et al., 1998). Transformation of this mutant with *RcAAP3* in the yeast/*E. coli* shuttle vector NEV-N (Sauer and Stolz, 1994) in sense orientation under the control of a constitutive yeast plasma membrane H^+-ATPase (PMA1) promoter, restored the growth of this mutant on a low-citrulline medium (Fig. 4). Antisense constructs of *RcAAP3* and vector controls grew extremely slowly on the low-citrulline medium (Fig. 4) but were able to grow at high citrulline concentrations, probably due to passive uptake (results not shown). All three types of transformants had similar growth patterns on the ammonium-sulfate-based medium (synthetic dextrose medium) (Fig.

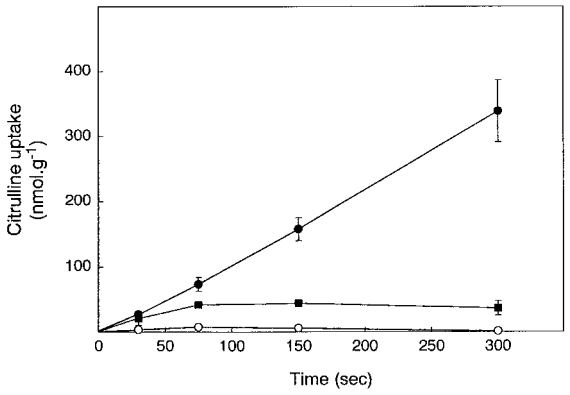

Figure 5. Time course of [^{14}C]citrulline uptake (0.5 mM, 37 kBq) at pH 4.5 by *RcAAP3* sense (●) and antisense (○) transformants. Uptake by *RcAAP3* (sense) transformants in the presence of 5 μM CCCP is also shown (■). Results shown are the means of three replicate experiments.

4). The transport properties of RcAAP3 were investigated further by determining the uptake of [^{14}C]citrulline. The sense construct of RcAAP3 mediated the uptake of [^{14}C]citrulline (Fig. 5), which was linear for at least 30 min (results not shown), whereas the antisense construct showed no significant uptake (Fig. 5).

RcAAP3 Has Properties of a Proton-Coupled Amino Acid Symporter

RcAAP3-mediated citrulline uptake showed saturable kinetics, with a K_m of 0.4 mM and a V_{max} of 384 nmol g^{-1} min^{-1} (Fig. 6). Citrulline uptake was inhibited by the protonophore CCCP (Fig. 5; Table I) and the uptake was higher at acidic pH (Fig. 7), suggesting a proton-coupled transport mechanism. Citrulline uptake was strongly inhibited by the permeable sulfydryl reagent NEM (72% inhibition), but showed lower sensitivity to the nonpermeable reagent *p*-chloromercuribenzenesulfonic acid (30% inhibition) (Table I). DEPC (a His modifier) inhibited citrulline uptake by 80% (Table I). Phenolglyoxal (an Arg modifier) and 2,4,6-trinitrobenzenesulfonic acid (a sulfydryl reagent) showed no appreciable inhibition of RcAAP3-mediated citrulline uptake (Table I).

Table I. *Effect of various reagents on RcAAP3-mediated citrulline transport*

The citrulline concentration was 0.5 mM and the control uptake rate was 187 nmol g^{-1} min^{-1}.

Inhibitor	Inhibition
	%
NEM (1 mM)	72
PCMBS[a] (1 mM)	30
DEPC (1 mM)	80
TNBS[b] (1 mM)	12
Phenolglyoxal (1 mM)	17
CCCP (5 μM)	86

[a] *p*-Chloromercuribenzenesulfonic acid. [b] 2,4,6-Trinitrobenzenesulfonic acid.

RcAAP3-Mediated Citrulline Uptake Is Inhibited by a Wide Range of Amino Acids

A range of amino acids inhibited citrulline uptake mediated by the *RcAAP3* transformants (Fig. 8), suggesting that this permease could have a fairly broad substrate specificity for neutral and basic amino acids and a lower affinity for acidic amino acids. The strongest inhibitors of citrulline uptake were Ala (90% inhibition) and Met (91%); the weakest were Asp (42%), Glu (52%), and Asn (46%) (Fig. 8).

RcAAP3 Is Encoded by a Single Gene and Is Expressed in Both Source and Sink Tissues of Castor Bean

Genomic DNA isolated from castor bean was restricted with five different enzymes and the Southern blots were probed with radiolabeled *RcAAP3* cDNA probes. The probe hybridized strongly to one main restriction band on the blots, indicating that RcAAP3 is encoded by a single

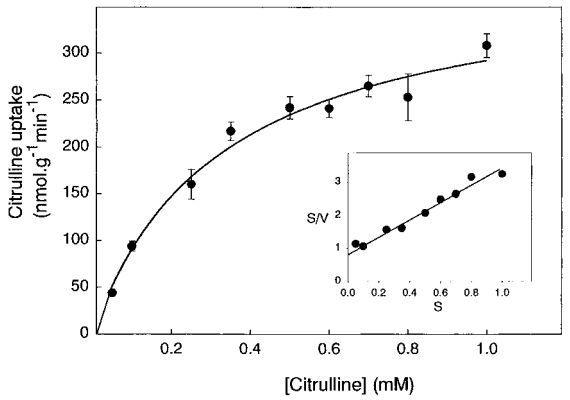

Figure 6. Concentration dependence of [^{14}C]citrulline (9 kBq) uptake by RcAAP3 at pH 4.5. Values for antisense controls have been subtracted. Hanes-Wolf plot of RcAAP3 concentration curve data, K_m = 0.4 mM and V_{max} = 384 nmol g^{-1} min^{-1}. Results are from a representative experiment repeated twice with similar results.

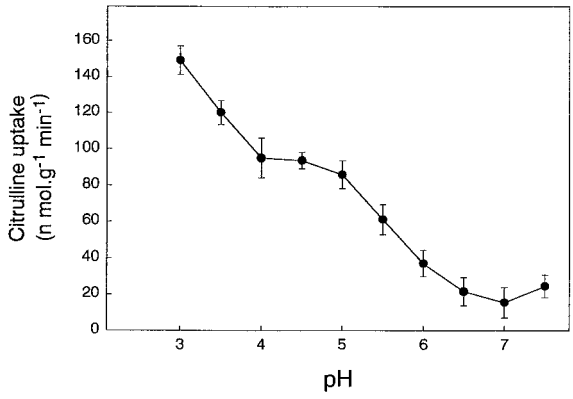

Figure 7. pH dependence of [^{14}C]citrulline uptake (0.5 mM, 37kBq) by *RcAAP3* sense transformants. Values for antisense controls have been deducted. Results shown are the means of two replicate experiments.

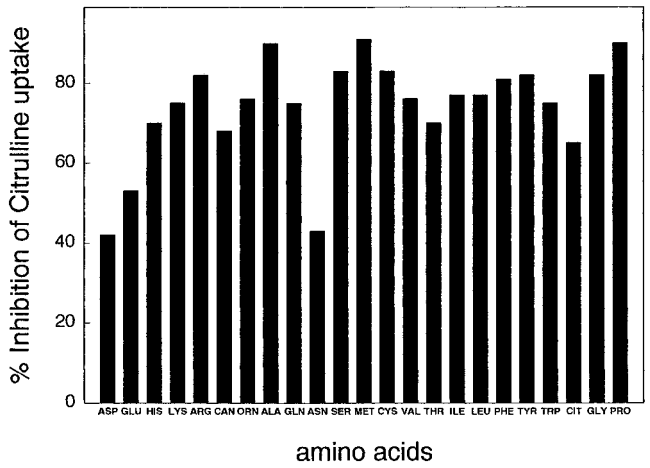

Figure 8. Effect of a range of amino acids on RcAAP3-mediated citrulline uptake. Results show the inhibition of [^{14}C]citrulline uptake (0.1 mM, 9 kBq) by a 10-fold excess (1 mM) of unlabeled amino acids at pH 4.5. Values for antisense controls have not been deducted. Results shown are the means of two replicate experiments.

gene (Fig. 9). The expression of *RcAAP3* was investigated by RNA gel-blot analysis of total RNA isolated from various organs of castor bean (Fig. 10). Transcripts of 1.85 kb were observed in all organs tested, indicating the wide distribution of RcAAP3 in castor bean.

DISCUSSION

Castor bean has been used extensively as a model plant for studying assimilate transport (Komor et al., 1991; Williams et al., 1996). Studies on amino acid transport with

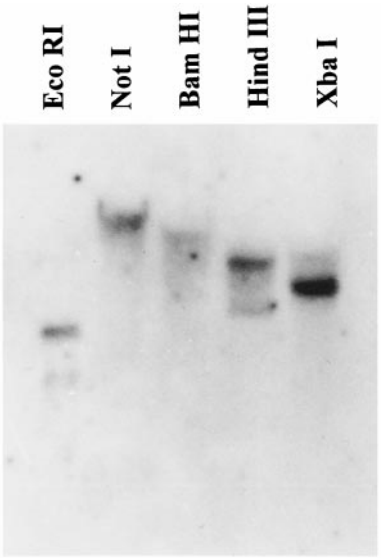

Figure 9. Southern analysis of castor bean genomic DNA using *RcAAP3* as a probe. Genomic DNA (10 μg) was digested to completion with restriction enzymes as indicated and hybridized with ^{32}P-labeled *RcAAP3* cDNA probe and washed under high-stringency conditions. Lane 1, *Eco*RI; lane 2, *Not*I; lane 3, *Bam*HI; lane 4, *Hin*dIII; lane 5, *Xba*I.

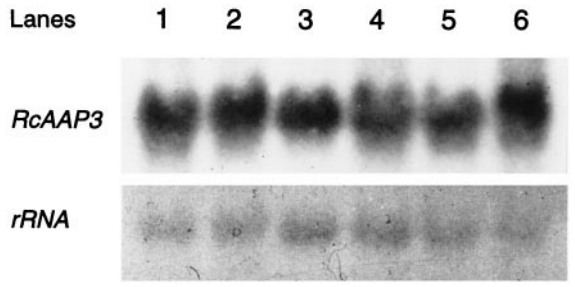

Figure 10. Expression of the castor bean gene, *RcAAP3*, as determined by northern hybridization. Total RNA (10 μg) from various tissues of the seedling (lanes 1–4) and mature plant (lanes 5–6) was hybridized with a ^{32}P-labeled *RcAAP3* cDNA probe. Lane 1, Cotyledons; lane 2, root; lane 3, hypocotyl; lane 4, endosperm; lane 5, sink leaf; and lane 6, source leaf. RNA loading was determined by probing with a ^{32}P-labeled 25S ribosomal cDNA probe.

isolated plasma membrane vesicles from castor bean seedlings suggested the presence of a number of amino acid transporter systems, with evidence in certain cases of a proton symport mechanism (Weston et al., 1994, 1995; Williams et al., 1996). Further studies in castor bean were hampered by lack of data on the biochemical and molecular properties of individual transporters, mainly due to difficulties encountered in the isolation and purification of these proteins. In addition, because of the presence of more than one transporter system in the vesicle preparations, it was difficult to determine the kinetic properties of individual transporters (Williams et al., 1996).

During the past few years, the use of yeast transport mutants and their complementation with plant cDNA libraries has resulted in the isolation and characterization of different families of amino acid permeases from Arabidopsis (Frommer et al., 1993; Kwart et al., 1993; Fischer et al., 1995; Rentsch et al., 1996). The use of sequence homology also enabled the identification of transporters from ESTs and genomic clones (Chen and Bush, 1997). Based on sequence similarities, the plant transporters can be classified into two major superfamilies (Fischer et al., 1998). ATF (amino acid transport family), the largest of these families, includes the AAP gene family (Fischer et al., 1995), transporters that prefer Pro (ProT) (Rentsch et al., 1996), Lys/His transporters (LHT) (Chen and Bush, 1997), and AUX1-related proteins (Bennett et al., 1996). The second superfamily, APC (amino acid-polyamine-choline facilitator), consists of two families: CAT (cationic amino acid transporter), which contains AtCAT1, a high-affinity uptake system for basic amino acids (Frommer et al., 1995), and the GABA permease-related transporter family for which a homolog has recently been identified (Fischer et al., 1998). A high-affinity oligopeptide transporter gene (*NTR1*) and a nitrate transporter (BnNRT1;2) have also been shown to have affinity for His (Rentsch et al., 1995; Zhou et al., 1998).

Studies to date describing the biochemical characteristics of cloned amino acid permeases have been carried out exclusively on those derived from Arabidopsis. Thus, it is important to ascertain if similar properties are displayed by related transporters from other plant species. We have

pursued an alternative methodology for the isolation of castor bean amino acid permeases in addition to the proven yeast complementation strategies. We have recently isolated two partial-length cDNA sequences for amino acid permeases (*RcAAP1* and *RcAAP2*) by RT-PCR from RNA extracted from castor bean seedlings (Bick et al., 1998). Because conventional plaque hybridization screening of libraries for the isolation of cDNA clones is often time consuming, we used an alternative screening approach that involved screening at high stringency using a technique based on PCR (Israel, 1993) with specific primers for the isolation of castor bean amino acid permease cDNA clones. This has resulted in the identification of a third amino acid permease cDNA, *RcAAP3*. This is the first report, to our knowledge, of the use of this PCR-library screening method to isolate a plant transporter gene from a cDNA library. RcAAP3 was isolated entirely by using PCR as a technique without additional hybridization techniques, as reported earlier (Israel, 1993). To isolate related cDNAs from a library (e.g. cDNAs from the same gene family) the level of primer degeneracy and the stringency of the PCR conditions can be altered.

Southern analysis with *RcAAP3* indicated that it was derived from a single gene. On the basis of sequence homology, *RcAAP3* falls into the ATF superfamily and is most closely related to the AAP subfamily of Arabidopsis. Hydropathy analysis indicates that RcAAP3 is a highly hydrophobic protein with nine to 11 transmembrane domains. Chang and Bush (1997) recently provided experimental evidence to suggest the presence of 11 hydrophobic segments in AtAAP1/NAT2, with the N terminus in the cytoplasm and the C terminus facing outside the cell.

Heterologous expression of *RcAAP3* in the yeast transport mutant lacking a functional yeast general amino acid permease (*gap1* mutant) has allowed us to investigate its transport characteristics. Several features indicate that RcAAP3 is a proton-coupled permease: sensitivity to the protonophore CCCP and to uncouplers, increased uptake at acidic pH, and saturable kinetics. However, the pH dependence could be explained by the intrinsic pH optimum of the transporter rather than by a dependence on the proton-motive force; the inhibitors may have a direct effect on the permease as opposed to the driving force. Detailed electrophysiological studies following expression in oocytes could be used to help resolve whether transport is indeed proton coupled.

Protein-modifying reagents such as NEM and DEPC significantly inhibited citrulline uptake. It is not clear at present whether these inhibitors directly affect the permease or if they act on the driving force. Interestingly, RcAAP1 showed only low inhibition by these inhibitors (Marvier et al., 1998); however, this could have been due to differences in the yeast strains that were used for expression. It has previously been found that while NEM inhibits solute transport in intact tissue, it has no effect on uptake into castor bean plasma membrane vesicles. It has been suggested that in castor bean there is little direct effect on the permease but, rather, the response is due to an indirect effect on the driving force (Williams et al., 1992; Weston et al., 1994). The His modifier DEPC has been shown to inhibit amino acid uptake into plasma membrane vesicles (Li and Bush, 1990; Weston et al., 1994). Based on substrate protection assays, Bush et al. (1996) suggested that DEPC binds at, or binding is conformationally linked to, the substrate-binding site. DEPC also inhibited amino acid uptake by the Arabidopsis AAP2 expressed in the yeast transport mutant 22574d (Kwart et al., 1993). Site-directed mutagenesis studies on AAP1 have shown that a single amino acid change at His-337 results in a loss of amino acid transport activity in transformed yeast (Bush et al., 1996). Both RcAAP1 (Marvier et al., 1998) and RcAAP3 contain a conserved His residue at this site and so this may not explain the differences in the response of the transformants to DEPC inhibition.

RcAAP3-mediated citrulline uptake was inhibited by a number of amino acids (neutral, basic, and acidic), indicating that RcAAP3 recognized a broad range of amino acids differing markedly in structure. However, inhibition was higher with neutral and basic amino acids compared with acidic amino acids. Direct transport measurements would be required with individual radiolabeled amino acids in order to differentiate between inhibition due to competition for transport and that for binding alone. Fischer et al. (1995) have grouped the AAPs into two subfamilies based on their recognition of basic amino acids: AAP1, AAP2, and AAP4 recognize acidic and neutral amino acids and ureides, whereas AAP3 and AAP5 are general transporters that also recognize basic amino acids. The inhibition studies indicate that RcAAP3 is similar to the latter subfamily, and in the phylogenetic analysis *RcAAP3* appears to be the most closely related to *AtAAP3* in the Arabidopsis AAP family. Interestingly, however, *RcAAP1* is also closely related to *RcAAP3* and *AtAAP3*, but this permease was shown to have a higher specificity for basic amino acids (Marvier et al., 1998). Further studies are in progress using heterologous expression in oocytes to determine more accurately the specificity of RcAAP1 and RcAAP3.

Northern analysis indicated that *RcAAP3* is expressed in a wide range of tissues in both the developing seedling and mature castor bean plant. This is in contrast to the expression patterns seen for *RcAAP1* and *RcAAP2*, which are predominantly expressed in the cotyledons, to a lesser extent in the roots, and at low levels in endosperm, hypocotyl, and the source and sink tissues of mature plants (Bick et al., 1998). In addition, *AtAAP3*, the most closely related gene in Arabidopsis, is almost exclusively found in roots, where it has been suggested to function in uptake and retrieval of amino acids from the soil (Fischer et al., 1995). This suggests a different biological role for *RcAAP3*, possibly functioning more generally in the accumulation of amino acids for protein synthesis. Further expression analysis at the cellular level would help to clarify this point. Thus, it is possible that highly related transporters (in terms of structure) may serve quite different physiological roles. This may depend on the specific tissue and cell in which they are expressed and also on the availability of particular amino acids in the local environment.

In conclusion, there is a family of amino acid permeases in castor bean (RcAAPs) that are related to the AtAAP family of Arabidopsis. Although the RcAAPs show high

homology to AtAAPs in terms of their protein identity, there are differences both in terms of their tissue-specific expression and their functional characteristics. Even members of the AtAAPs show differences in these properties despite their relatedness. Identification of structural domains involved in the amino acid transport process would help to elucidate these functional differences. It is clear that although Arabidopsis is a useful model, it is also important to study amino acid transporters in other plant species for a more comprehensive understanding of their contribution to nitrogen nutrition and distribution.

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