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Biochimica et Biophysica Acta 1373 (1998) 321–331

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Cloning of a cDNA coding for an amino acid carrier from *Ricinus communis* (RcAAP1) by functional complementation in yeast: kinetic analysis, inhibitor sensitivity and substrate specificity

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Received 18 May 1998; accepted 22 June 1998

Abstract

A cDNA for the amino acid permease gene RcAAP1 has been isolated from *Ricinus communis* by yeast complementation and subjected to a detailed kinetic analysis. RcAAP1 cDNA is 1.5 kb with an open reading frame that codes for a protein with 486 amino acids and a calculated molecular mass of 53.1 kDa. RcAAP1-mediated histidine uptake was pH dependent with highest transport rates at acidic pH; it was sensitive to protonophores and uncouplers and the K_m for histidine uptake was 96 μM. The substrate specificity was investigated by measuring the levels of inhibition of histidine uptake by a range of amino acids. The basic amino acids (histidine, lysine and arginine) showed strongest inhibition of uptake whereas acidic amino acids competed less effectively. Alanine was the most efficient competitor of the neutral amino acids. Glutamine, serine, asparagine, methionine and cysteine showed moderate inhibition whereas threonine, isoleucine, leucine, phenylalanine, tyrosine and tryptophan showed only low levels of inhibition. Glycine, proline and citrulline caused slight stimulation. More detailed competition kinetics indicated that both lysine and arginine showed simple competitive inhibition of histidine uptake. When direct uptake measurements were carried out, both lysine and arginine were found to be effective substrates for RcAAP1.

Keywords: Amino acid permease; Membrane transport; Yeast complementation; (*Ricinus communis*)

1. Introduction

Nitrogen is an essential element in plants and is crucial for their growth and development. Plants cannot assimilate elemental nitrogen directly and therefore several methods have developed to ensure that all tissues receive an adequate supply. Nitrate and ammonia from the soil are the primary sources of nitrogen although direct uptake of amino acids may also be significant in some plants contributing to their overall nitrogen nutrition [1–3]. However, more importantly, amino acids are vital for nitrogen reallocation in plants, and the cycling of amino acids

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DEPC, diethylpyrocarbonate; DNP, 2,4-dinitrophenol; NEM, *N*-ethylmaleimide; PCMBS, *p*-chloromercuribenzenesulphonic acid; TNBS, trinitrobenzenesulphonic acid; YNB, yeast nitrogen base

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0005-2736/98/$ – see front matter
PII: S0005-2736(98)00117-5

in the xylem and phloem supplies nitrogen to all tissues. Essential to such processes is the transport of amino acids across cell membranes.

Ricinus communis has been used in a number of studies investigating sugar and amino acid transport relating to phloem and xylem loading and has been described as a model system for such studies (see [4]). There is evidence indicating that the transport of these solutes across the plasma membrane is mediated by specific carriers, often driven by proton gradients [5–8]. Studies in *Ricinus* using isolated membrane vesicles have provided evidence for amino acid carriers in the cotyledons [5] and in the roots [7,8]. However, little is known about the biochemical characteristics of the individual carriers in *Ricinus* or their molecular properties. Their physiological role is also unclear but several possibilities exist. In the germinating *Ricinus* seed they are important in the cotyledons for the absorption of amino acids released following protein hydrolysis in the endosperm. Amino acids are then used by cells in the rapidly developing seedling. Carriers also play an important role in the root and it has been demonstrated that *Ricinus* roots accumulate 15–25% of their nitrogen in the form of amino acids from the soil [1]. Carriers may also be involved in the loading of the xylem for transport to the shoot. Other possible functions include retrieval of amino acids lost from the phloem or in allowing high rates of transport into particular cells actively involved in protein synthesis (e.g. those giving rise to lateral roots [9]). Previously, we were able to partially resolve different carriers in the root by carrying out inter-amino acid competition studies with membrane vesicles [8,6]. However, our results suggested that a number of the transporters had overlapping substrate specificity and thus it was difficult to accurately demonstrate the mechanism of transport for individual carriers. Therefore we embarked on studies to isolate cDNAs for individual amino acid carriers from *Ricinus* which would enable us to study the particular transport properties of each transporter separately. This paper describes how this has been achieved using functional complementation of yeast mutants deficient in particular amino acid transport systems. Yeast complementation is a powerful technique which has allowed the isolation of a variety of eukaryotic genes (for a review see [10]). These include both metabolic enzymes such as alcohol phosphotransferase [11] and membrane proteins such as peptide transporters [12], sucrose carriers [13] and amino acid carriers [14,15]. To date, extensive studies on plasma membrane amino acid transporter genes have been carried out with *Arabidopsis*, and several different gene families have been described [16–18]; these include the general amino acid permeases (AAP family), proline transporters (ProT) and basic amino acid transporters (AAT). However, almost nothing is known about amino acid transporter genes in other plant species [6]. This paper describes the isolation of a member of a small gene family of *Ricinus* amino acid carriers, RcAAP1, using yeast complementation and a detailed study characterising the substrate specificity, pH dependence, kinetic parameters and inhibitor sensitivity of the encoded protein.

2. Materials and methods

2.1. Yeast complementation

The *Saccharomyces cerevisiae* strain 2512c (*MAT-a, gap1*) was obtained from the National Collection of Yeast Cultures (AFRC Institute of Food Research, Norwich). Ty mutagenesis of the *URA3* gene in this strain was carried out using the Ty insertion plasmid pMRFW2 [19] kindly provided by Prof. F. Winston (Massachusetts Institute of Technology, MA, USA). A 7-day-old *Ricinus* seedling library was produced in the yeast/*Escherichia coli* shuttle vector NEV-N. Transformation was performed as described previously [20]. Transformants were first selected on SD medium (Difco) which contained yeast nitrogen base (without amino acids and ammonium sulphate), 2% dextrose, 0.5% ammonium sulphate and 2% agar. Transformants were washed from the plates with sterile distilled water and plated onto a low citrulline medium (Difco nitrogen free medium supplemented with 2% dextrose and 0.2 mg/ml citrulline as the nitrogen source and 2% agar). Colonies which were able to grow were cultured further on selective liquid media, and plasmid DNA was isolated for transformation into *E. coli*. Plasmid DNA from one of the isolates was used to transform the yeast mutant JT16 (*Mat-a, hip1-614, his4-401, can1, ino1, ura3-52*) [21]. Transformants

were selected initially on a high histidine-containing medium (YNB without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 2% dextrose, 2% agar and 0.002% inosine, 5.8 mM arginine and 2.9 mM histidine). Colonies that were able to grow were reselected on the same medium but containing low histidine (0.095 mM).

2.2. DNA manipulations

DNA manipulations were carried out essentially as described in [22]. cDNAs were excised with *Not*I and subcloned into pBluescript SK$^-$. Both strands of the recombinant clones were sequenced with a Li-Cor automated sequencer with IRD-labelled T3 and SP6 promoter primers using the Thermosequenase cycle sequencing kit (Amersham, Little Chalfont).

2.3. Transport measurements

For transport studies, yeast cells were grown to logarithmic phase and washed in a solution containing 1% glucose in 50 mM phosphate buffer (pH 4.5) and resuspended in 50 mM phosphate buffer (pH 4.5) to 0.02 g/ml. The standard assay contained 50 μM ^{3}H-labelled histidine (25 mCi/nmol) in potassium phosphate buffer (pH 4.5) and the reaction was started by the addition of 1 mg of cells to give a final volume of 200 μl. Assays were conducted at 22°C. Prior to uptake, cells had a 5 min incubation in 5 mM glucose in 50 mM phosphate buffer (pH 4.5). For time course experiments 45 μl aliquots were removed at appropriate times and transferred to 5 ml of ice cold water, filtered on glass fibre filters and washed with 3×5 ml of ice cold water. Filters were dried, placed in scintillation vials with 5 ml Optiphase Safe liquid scintillation fluid and counted on a Wallac 1209 Rackbeta liquid scintillation counter. For competition studies a range of amino acids were tested in the uptake medium at 10-fold molar excess. In certain cases, competition experiments were conducted with a limited number of amino acids by varying the concentration of the competing amino acid and histidine in the medium to that specified in the results. For kinetic analysis, pH dependence studies and inhibitor sensitivity measurements, transport was determined after 1 min from 150 μl aliquots during which time uptake was linear. The results are presented on a gram fresh weight basis.

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                                                  M  V  E  N  T  A  A  K  N  H  P
AACAAAGGGTGTGTGAAGTTAAGAAAAAGATGGTTGAGAACACAGCAGCCAAGAACCATC                      60
 H  Q  V  F  D  V  S  I  N  M  Q  T  Q  V  V  G  S  K  W  L
CGCACCAGGTTTTTGATGTATCCATTAACATGCAAACTCAAGTTGTTGGCTCTAAATGGC                     120
 D  D  D  G  R  T  K  R  T  G  T  V  W  T  A  S  A  H  I  I
TTGATGATGATGGCCGTACCAAGAGAACTGGGACCGTGTGGACTGCAAGTGCCCACATCA                     180
 T  A  V  I  G  S  G  V  L  S  L  A  W  A  I  A  Q  L  G  W
TAACAGCAGTGATAGGGTCTGGAGTTCTGTCTTTAGCTTGGGCAATCGCTCAGCTTGGGT                     240
 I  A  G  P  A  V  M  F  L  F  S  L  V  T  Y  Y  T  S  T  L
GGATTGCTGGCCCTGCTGTTATGTTCTTGTTCTCTTTAGTCACTTACTATACTTCTACTC                     300
 L  S  A  C  Y  R  S  G  D  P  V  N  G  K  R  N  Y  T  Y  M
TTCTATCTGCCTGCTACCGTTCTGGTGATCCTGTCAATGGCAAGAGGAACTATACTTATA                     360
 D  A  V  R  T  N  L  G  G  A  K  V  K  L  C  G  F  V  Q  Y
TGGACGCTGTTCGCACCAATCTTGGTGGAGCTAAGGTCAAGTTATGTGGATTTGTTCAGT                     420
 L  N  L  F  G  V  A  I  G  Y  T  I  A  S  S  I  S  M  M  A
ATCTGAATCTTTTCGGAGTTGCCATTGGATACACTATCGCGTCGTCTATTAGTATGATGG                     480
 I  K  R  S  N  C  F  H  K  S  G  G  K  N  P  C  H  I  N  A
CTATAAAGAGGTCTAATTGTTTTCACAAGAGCGGAGGCAAAAACCCATGCCATATAAATG                     540
 N  P  Y  M  I  A  F  G  I  A  E  I  I  F  S  Q  I  P  D  F
CCAATCCTTACATGATTGCATTCGGAATTGCTGAAATCATTTTCTCTCAAATTCCTGATT                     600
 D  Q  L  W  W  L  S  I  L  A  A  V  M  S  F  T  Y  S  T  I
TTGATCAGCTATGGTGGCTCTCCATTCTTGCTGCAGTCATGTCCTTTACTTACTCAACCA                     660
 G  L  G  L  G  I  A  Q  V  V  E  N  G  K  A  M  G  S  V  T
TTGGTCTTGGACTTGGAATTGCTCAAGTTGTAGAAAATGGAAAAGCCATGGGAAGTGTGA                     720
 G  I  S  I  G  A  N  V  T  P  T  Q  K  I  W  R  S  F  Q  A
CCGGAATTAGCATTGGAGCGAATGTGACTCCAACCCAGAAGATATGGAGGAGCTTCCAAG                     780
 L  G  D  I  A  F  A  Y  S  Y  S  I  I  L  I  E  I  Q  D  T
CTCTTGGTGACATTGCTTTCGCCTATTCTTATTCCATAATCCTTATTGAAATTCAGGACA                     840
 V  R  S  P  P  S  E  S  K  T  M  K  K  A  T  L  I  S  V  A
CAGTCAGATCCCCACCTTCAGAGTCCAAGACAATGAAGAAAGCAACTCTGATAAGTGTGG                     900
 V  T  T  L  F  Y  M  L  C  G  C  F  G  Y  A  A  F  G  D  M
CAGTAACAACCCTTTTCTACATGCTATGTGGTTGCTTTGGCTATGCTGCTTTTGGAGACA                     960
 S  P  G  N  L  L  T  G  F  G  F  Y  N  P  Y  W  L  L  D  I
TGTCTCCTGGAAATCTACTAACAGGGTTTGGCTTCTACAACCCATATTGGCTACTTGACA                    1020
 A  N  V  A  I  V  V  H  L  V  G  A  Y  Q  V  Y  C  Q  P  L
TTGCTAATGTTGCAATCGTAGTCCATCTTGTTGGCGCGTACCAAGTCTATTGCCAACCCT                    1080
 F  A  F  V  E  K  A  A  A  Q  R  Y  P  D  S  G  F  I  T  K
TGTTTGCCTTCGTTGAAAAAGCAGCTGCACAAAGATATCCAGACAGTGGATTCATTACTA                    1140
 D  I  K  I  P  V  P  G  F  R  P  F  N  L  N  L  F  R  S  V
AAGATATCAAAATCCCAGTTCCTGGTTTCCGCCCCTTTAATCTTAACCTCTTTAGATCGG                    1200
 W  R  T  L  F  V  V  F  T  T  V  I  S  M  L  L  P  F  F  N
TATGGAGGACACTTTTTGTGGTCTTCACAACTGTGATTTCCATGCTCCTTCCGTTCTTTA                    1260
 D  I  V  G  L  L  G  A  L  G  F  W  P  L  T  V  Y  F  P  V
ACGACATAGTTGGTTTGCTTGGAGCTTTGGGATTTTGGCCACTCACGGTTTACTTCCCAG                    1320
 E  M  Y  I  A  Q  K  K  I  P  K  W  S  T  R  W  L  C  L  Q
TAGAAATGTATATAGCACAAAAGAAGATCCCAAAATGGAGCACAAGATGGCTCTGCCTGC                    1380
 I  L  S  A  A  C  L  V  I  T  I  A  A  A  A  G  S  I  A  G
AAATACTAAGCGCTGCTTGCCTTGTTATTACAATAGCTGCTGCTGCTGGATCCATTGCTG                    1440
 V  V  G  D  L  K  S  V  K  P  F  Q  T  S  Y  *
GGGTTGTTGGTGATCTCAAGTCTGTCAAGCCATTCCAGACCTCTTACTGATGAACCCCAG                    1500
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the *Not*I insert in the cDNA clone encoding the *Ricinus* amino acid carrier RcAAP1.

2.4. Kinetic analysis

For competition studies, Dixon plots were carried out to determine whether simple competitive inhibition was occurring. Non-linear regression (performed with the SigmaPlot curve fitter) was used to fit the data to the equation for simple competitive inhibition.

3. Results

3.1. Complementation of a yeast mutant strain (2512c ura3-52) with the Ricinus cDNA library

In order to isolate amino acid transporter genes

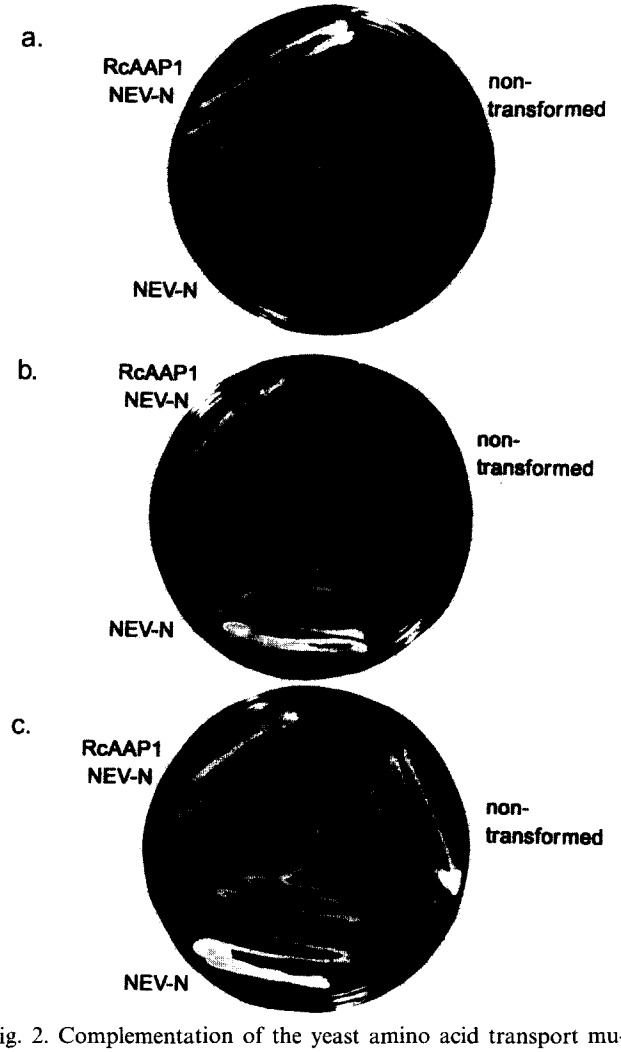

Fig. 2. Complementation of the yeast amino acid transport mutant (JT16) with RcAAP1. The growth of JT16 (untransformed) is shown together with cells transformed with NEV-N alone or the NEV-N/RcAAP1 sense construct on a low histidine (a), high histidine (b) and high histidine plus uracil (c) medium. Results are from a representative experiment repeated at least twice.

from *Ricinus*, the strategy of yeast complementation was used (for a review see [10]). A cDNA library produced from *Ricinus* seedlings was constructed in a yeast-*E. coli* expression shuttle vector, NEV-N [23] using an existing seedling cDNA library which we had previously constructed in λgt10. The yeast mutant strain 2512c which was mutated in the gene for the general amino acid permease (GAP) was used initially in this study. Yeast cells defective in the GAP gene are unable to transport citrulline efficiently and hence grow very slowly on low concentrations of this amino acid [24]; therefore citrulline was used in the selection procedure. It was necessary to modify this particular yeast mutant so that it was suitable for transformation with the *Ricinus* library which contained the *URA3* gene as the selectable marker. The mutant 2512c which was wild type for the *URA3* gene was mutagenised using several different techniques including exposure to UV light, ethidium bromide and NTG. Although several *ura3* isolates were identified, the mutations were found to be unstable and the *ura3* alleles reverted to the wild-type *URA3* form, typically within a 2 week growth period. Therefore, Ty disruption mutagenesis of the *URA3* gene was carried out using *Ty* insertion mutagenesis [19]. The resulting transformants were grown on uracil minimal medium which was supplemented with 5-fluoroorotic acid (5-FOA). This compound is converted to the toxin 5-fluorouracil by the enzyme orotidine-5-phosphate decarboxylase which is encoded by the *URA3* gene. Therefore, yeast capable of growth on 5-FOA media have the *ura3* phenotype. The *ura3-52* genotype of the Ty transformants was confirmed by Southern hybridisation (results not shown). The 2512c *ura3-52* mutant strain (referred to as $gap^{-}ura3^{-}$) was used for complementation.

Complementation of the $gap^{-}ura3^{-}$ yeast mutant was carried out with the *Ricinus* library and transformed cells were first selected on a uracil-free medium containing ammonium as the nitrogen source. Transformants were then plated onto a similar me-

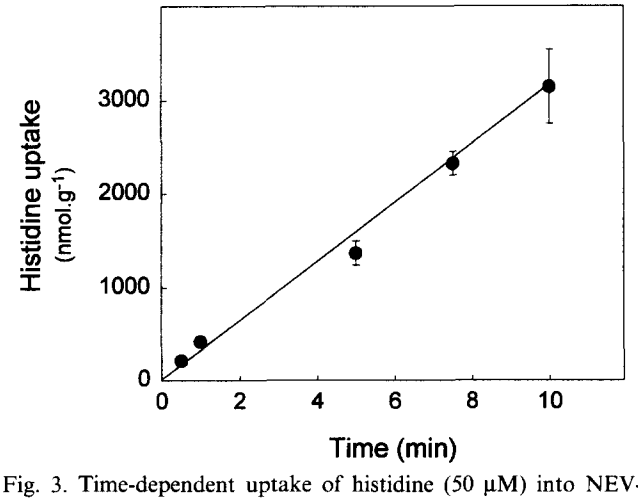

Fig. 3. Time-dependent uptake of histidine (50 μM) into NEV-N/RcAAP1 transformed JT16. Results shown are the mean ± S.E. from three experiments.

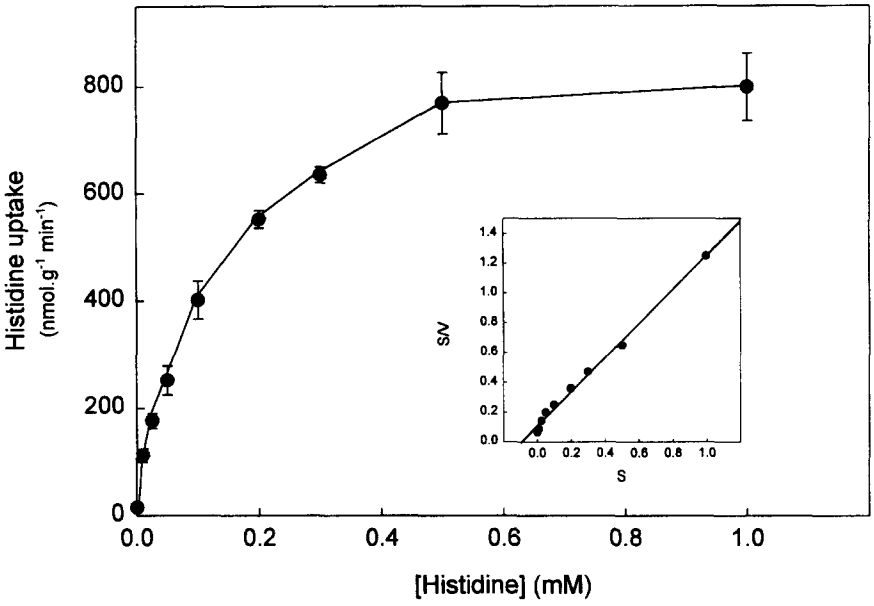

Fig. 4. Concentration dependence of histidine uptake into NEV-N/RcAAP1 transformed JT16. The results shown are the mean ± S.E. of four replicates from two experiments. The insert shows a Hanes-Wolf plot of the data. The K_m determined from linear regression analysis ($R^2 = 0.994$) was 96 μM.

dium in which a low concentration of citrulline (0.2 mg/ml) replaced the ammonium as the nitrogen source. Reselection was carried out on liquid media and this resulted in the isolation of two clones containing inserts each of 1.5 and 1.6 kb. These were subsequently sequenced; one of the inserts (1.5 kb) was identified as the full-length clone, RcAAP1, since it was identical to the partial-length clone (860 bp) previously isolated by RT-PCR [6,9]. The other cDNA (1.6 kb) encoded a protein with high homology to NADPH cytochrome *P*-450 (results not shown) and this was not characterised further.

3.2. Characteristics of RcAAP1 cDNA

The full-length sequence of RcAAP1 is shown in Fig. 1. RcAAP1 has an open reading frame of 486 amino acids. There are 29 bases of 5′ untranslated region while the 3′ untranslated region is truncated with only 10 bases and no distinctive poly(A) tail. The hydropathy profile indicates that this is a hydrophobic protein with 10 to 11 transmembrane domains. RcAAP1 and RcAAP2 (partial-cDNA isolated by RT-PCR [9]) share 62% identity at the amino acid level and RcAAP1 shows varying levels of homology to the AAP family of carriers which have been isolated from *Arabidopsis*, ranging from 59% to 80% [16,17]. Highest homology was observed to AAP3 (80%).

3.3. Functional complementation of the histidine permease yeast mutant (JT16) with RcAAP1

Initial experiments were carried out to measure the uptake of radiolabelled L-citrulline in RcAAP1-trans-

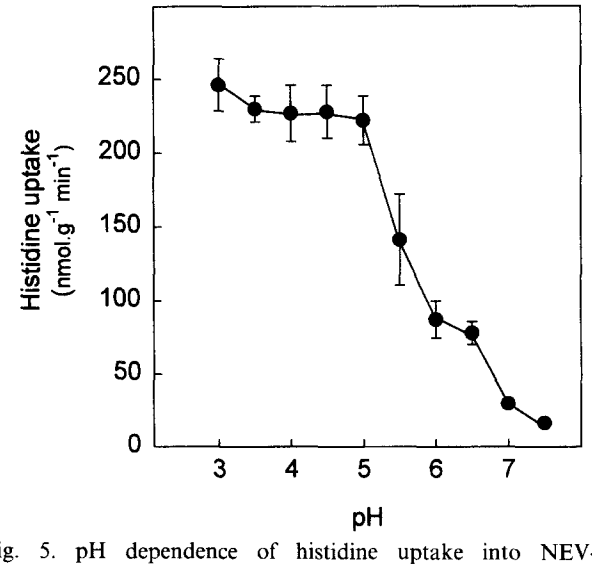

Fig. 5. pH dependence of histidine uptake into NEV-N/RcAAP1 transformed JT16. The results shown are the mean ± S.E. of six replicates from three experiments.

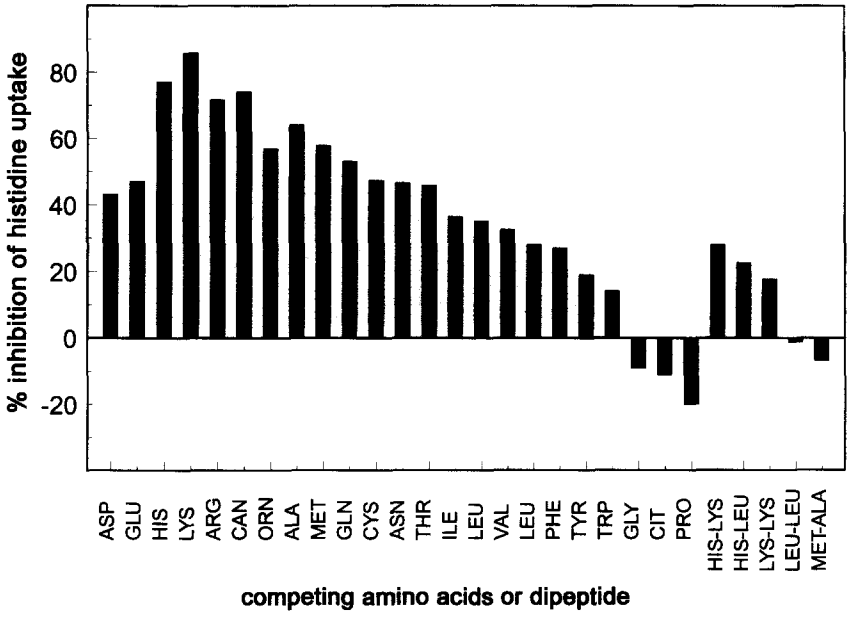

Fig. 6. Competition for histidine (50 μM) uptake into NEV-N/RcAAP1-transformed JT16. Competing amino acids or peptides were present at 10-fold excess (500 μM). Uptake was determined after 1 min and results are the mean % inhibition calculated from three replicated experiments. The control value was 318 nmol g^{-1} min^{-1}.

formed *gap*$^{-}$*ura3*$^{-}$ cells; however, there was no appreciable uptake observed above that seen for vector-transformed control cells. A possible reason for this is that RcAAP1 may have a low affinity for citrulline. Therefore, an alternative yeast strain (JT16) defective in histidine transport was used [21]. JT16 was transformed with the RcAAP1/NEV-N construct and also NEV-N alone. Transformants were initially selected on a high histidine-containing medium (2.9 mM) lacking uracil and then plated on medium containing low histidine. On this latter medium, colonies were only observed on those plates in which the RcAAP1/NEV-N construct was used for transformation. Three of these colonies were reselected on liquid medium and all contained inserts of the correct size. Fig. 2 shows the growth analysis for cells containing the RcAAP1/NEV-N construct, the vector alone and the untransformed JT16 cells. As can be seen, only cells that contain RcAAP1 can grow on low histidine medium whereas all cells can grow on the high histidine medium containing uracil. On high histidine medium, it was consistently observed that RcAAP1 transformants attained the same level of growth as the NEV-N control cells but the rate of growth was always lower; the reasons for this are unclear.

3.4. *Biochemical characterisation of RcAAP1 following heterologous expression in JT16*

Yeast cells transformed with RcAAP1 were tested for transport activity using radiolabelled histidine. RcAAP1-transformed cells showed time-dependent uptake (Fig. 3) and kinetic studies showed that uptake was saturable (Fig. 4) with a K_m of 96 μM. Transport was inhibited by the protonophore CCCP (Table 1) and was pH dependent with highest rates at low pH (Fig. 5), suggesting that RcAAP1 is a proton-coupled symporter. A range of inhibitors

Table 1
Effect of various reagents on RcAAP1-mediated histidine transport

Inhibitor	% inhibition
DNP (100 μM)	92
Azide (1 mM)	69
CCCP (10 μM)	53
PCMBS (1 mM)	45
NEM (1 mM)	21
DEPC (1 mM)	20
Phenylglyoxal (1 mM)	18
TNBS (1 mM)	11

The histidine concentration was 50 μM and the control uptake rate was 310 nmol g^{-1} min^{-1}.

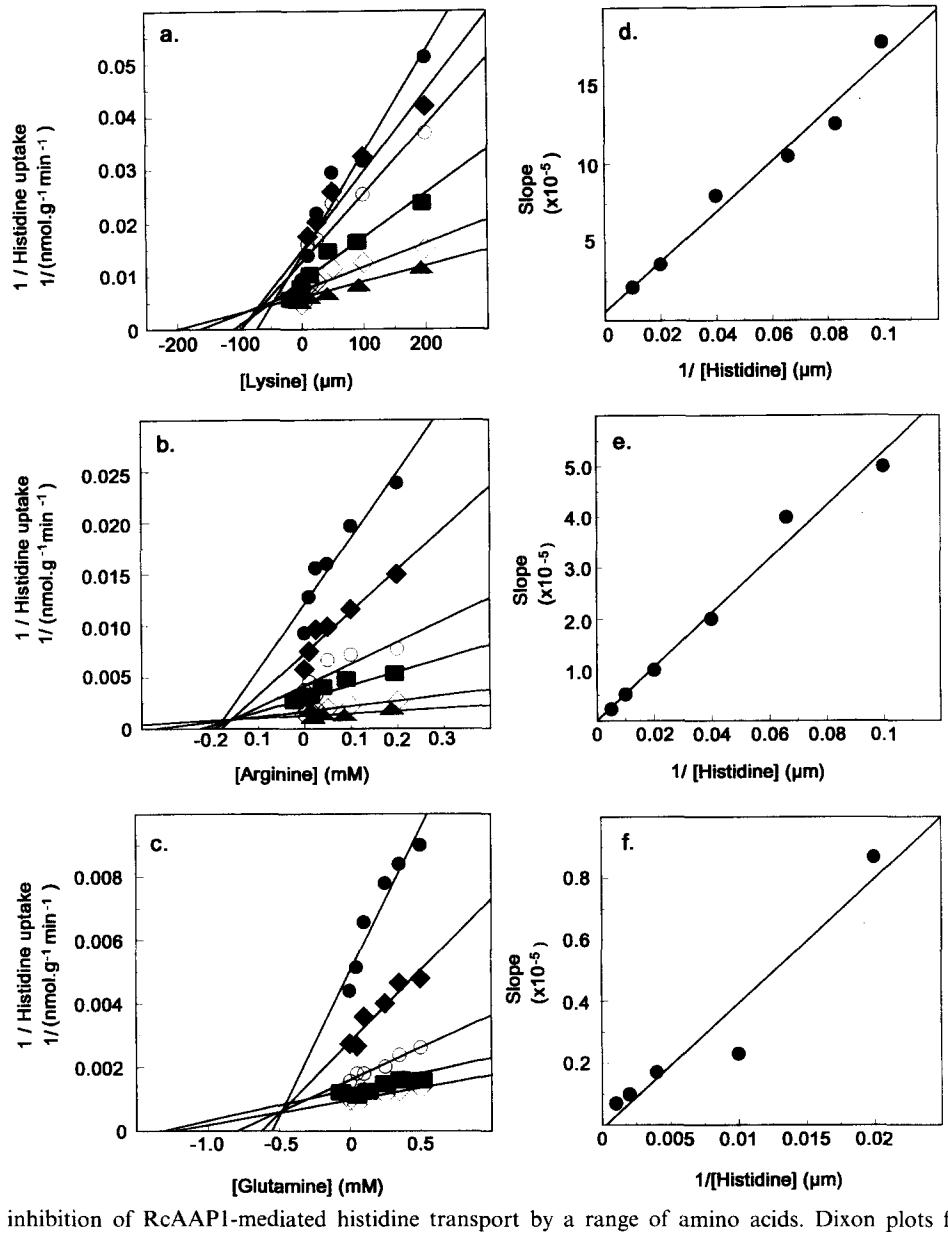

Fig. 7. Competitive inhibition of RcAAP1-mediated histidine transport by a range of amino acids. Dixon plots for competition with lysine (a), arginine (b), and glutamine (c) are shown together with replots of the slopes of the Dixon plots versus (1/histidine) for experiments with lysine (d; $R^2 = 0.94$), arginine (e; $R^2 = 0.99$) and glutamine (f; $R^2 = 0.93$). The results are from a representative experiment repeated at least twice.

were used to determine the inhibitor sensitivity of the transport process (Table 1). Histidine transport was strongly inhibited by the uncouplers DNP and azide. The sulphydryl modifier PCMBS also caused moderate inhibition of transport. DEPC, NEM, phenylglyoxal and TNBS were less inhibitory.

The substrate specificity of RcAAP1 for a range of amino acids and peptides was tested by determining the uptake of radiolabelled histidine in the presence of a 10-fold higher concentration of competing amino acids. As can be seen in Fig. 6, histidine uptake was inhibited by a wide range of amino acids but it was notable that the basic amino acids (histidine, arginine, lysine, and canavanine) appeared to compete most effectively for histidine uptake (71–86% inhibition) with a slightly lower inhibition (57%) by

ornithine. RcAAP1 shows some stereospecificity in that D-histidine only inhibited uptake by 46% (results not shown). The acidic amino acids competed less effectively, with inhibition values of 35–40%. Of the neutral amino acids, only alanine was an efficient competitor (64%) whereas others (glutamine, serine, asparagine, methionine, cysteine) showed only moderate inhibition (46–58%). Certain neutral amino acids (threonine, isoleucine, leucine, phenylalanine, tyrosine and tryptophan) showed only low levels of inhibition (14–37%) whereas glycine, proline and citrulline showed slight stimulation (9–20%). The dipeptides leucine-leucine and methionine-alanine had little effect whereas histidine-leucine resulted in about 25% inhibition and histidine-lysine gave 28% inhibition. Urea and ammonium had little effect.

Further kinetic studies of histidine transport were carried out to analyse the nature of the inhibition observed in the presence of the basic amino acids, lysine and arginine; the neutral amino acid glutamine was also used for comparison. This was achieved by determining their effect at varying concentrations on the level of histidine uptake when the levels of histidine were also varied. Dixon plots of these data are shown in Fig. 7 together with replots of the resulting slopes. This analysis suggested that arginine, lysine and glutamine are inhibiting histidine uptake by simple competitive inhibition and the K_i values (determined from regression analysis of these data fitted to the equation for competitive inhibition) indicate that RcAAP1 has a much higher affinity for arginine and lysine than glutamine (Table 2). In order to investigate whether RcAAP1 could actually transport the other basic amino acids in addition to histidine (i.e. distinguish between competitive binding and competition for transport), direct uptake experiments were conducted for lysine and arginine. Fig. 8

Table 2
K_i values determined for a range of amino acids as transport antagonists of RcAAP1-mediated histidine uptake

Amino acid	K_i (μM)
Lysine	83
Arginine	129
Glutamine	388

The values were obtained from non-linear regression analysis of the data fitted to the Michaelis-Menten equation for competitive inhibition.

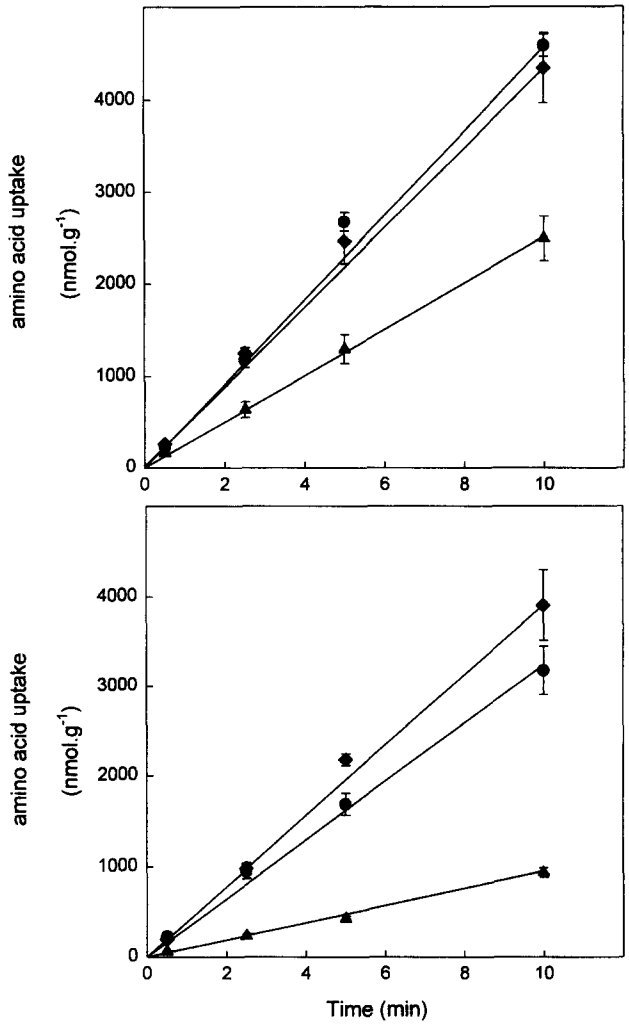

Fig. 8. Time-dependent uptake of histidine (●), lysine (◆), and arginine (▲), in NEV-N-RcAAP1-transformed JT16 cells grown at 0.095 mM histidine (upper) and 0.5 mM histidine (lower). Results in upper panel are the mean ± S.E. of six replicates from three experiments. Results shown in lower panel are the mean ± S.E. of three experiments following subtraction of uptake values for NEV-N-transformed JT 16.

shows the results of experiments in which radiolabelled amino acid uptake was determined in cells grown on low (0.095 mM) and high (0.5 mM) histidine. Under the former conditions, the NEV-N-transformed cells cannot grow and therefore no uptake measurements can be made for these cells. Under the high histidine conditions, RcAAP1-transformed and NEV-N transformed cells can grow and therefore uptake measurements were carried out for each set of cells. The results in Fig. 8 show transport mediated by RcAAP1 since NEV-N control uptake

values have been subtracted. As can be seen, in both sets of histidine conditions, RcAAP1 transformants showed time-dependent uptake of lysine which was comparable to that seen for histidine uptake; arginine uptake was also observed but this was lower than for histidine and lysine transport. Similar results were also observed for cells grown at 1 mM histidine (results not shown).

4. Discussion

Amino acid transporters are crucial in the redistribution of nitrogen around the plant. This work describes the cloning of a cDNA for an amino acid transporter from *Ricinus* (RcAAP1) and its functional characterisation in yeast. Yeast complementation studies have identified a number of gene families encoding proteins responsible for amino acid transport in *Arabidopsis*. The first to be identified, the amino acid permeases (AAPs), contains six members (AAP1–6) [15,17,18]. These have been grouped into two sub-families: the first contains broad specificity transporters that recognise neutral and acidic amino acids and include AAP1, 2 and 4; the second includes general amino acid transporters that, besides acidic and neutral amino acids, also recognise basic amino acids like AAP3 and 5 [25]. The proline transporters are a separate family, which at present has two members (ProT 1,2) [18]. The third represents a high affinity system for the uptake of basic amino acids and so far has only one member (AAT1) [26]. The fourth is a peptide transporter (NTR1) which has a low affinity for histidine and is related to rather non-specific oligopeptide transporters from a variety of species including *Arabidopsis* [27]. Recently, LHT1 was identified and is proposed to represent a new class of amino acid transporter; this is specific for lysine and histidine [28]. Many of these carriers show tissue-specific expression [17,26] and dependence on the nutrient status of the plant [18]. Our studies suggest that *Ricinus* also has a small gene family of amino acid carriers, the members of which are most closely related to the AAP family of *Arabidopsis*. The amino acid carrier genes from *Ricinus* include RcAAP1 (this study), RcAAP2 partial clone [6,9], and RcAAP3 (Neelam et al., unpublished). Preliminary evidence indicates that these may differ in their substrate specificity and also their tissue-specific expression pattern (Marvier and Williams, unpublished). In this paper we report on the isolation of RcAAP1 and provide a detailed kinetic analysis of the transport characteristics following heterologous expression in yeast.

The yeast general amino acid permease mutant 2512c was initially used to screen the *Ricinus* library and RcAAP1 was isolated by selection for growth on a low citrulline medium. Although the cells could grow on this medium there was no appreciable uptake of radiolabelled citrulline above vector-transformed cells. This may be due to a low expression level of the protein in yeast due to incorrect targeting, as is the case with the H^+-ATPase [29]. Another and more likely possibility is that RcAAP1 is not an efficient citrulline transporter, and, while it may support some growth, is unable to show measurable levels of citrulline uptake under the conditions used in these experiments. The expression of RcAAP1 in the histidine transport mutant, JT16, would seem to confirm this view. RcAAP1 transformants showed high levels of histidine uptake which was inhibited by the presence of a number of other amino acids, especially lysine and arginine. Citrulline, however, was not an effective competitor at the concentration used.

The substrate specificity of RcAAP1, as determined by competition studies, suggests that it is a high affinity carrier for basic amino acids with a lower affinity for some neutral and acidic amino acids. The results observed in the competition experiments with basic amino acids suggest that histidine, lysine and arginine are all substrates for RcAAP1 and that they compete for binding to the same active site. This was confirmed by direct uptake measurements where it was seen that the uptake levels of histidine and lysine were similar while arginine uptake was slightly lower. RcAAP1 has highest homology to the *Arabidopsis* amino acid permease AAP3 (80% identity) and shows some similarities to this transporter in substrate specificity, being able to transport basic amino acids; however, there were also some differences, mainly with respect to affinity for proline and citrulline [17]. RcAAP1 has low identity to AAT1 although there do appear to be strong similarities in their transport properties; they are both high affinity carriers for basic amino acids and, as with AAT1, proline appears to be a weak inhibitor of

RcAAP1-mediated uptake. However, unlike AAT1, RcAAP1 does show evidence of stereospecificity in that D-histidine did not compete effectively for uptake. RcAAP1 also shows low identity to LHT1 [28] although both transport lysine and histidine; however, unlike RcAAP1, LT1 does not appear to transport arginine.

RcAAP1-mediated histidine uptake was sensitive to protonophores and showed a marked decrease when measured under alkaline conditions. These results suggest that RcAAP1 may be a proton-coupled carrier, although further studies would be required to confirm this.

Previous work has indicated that RcAAP1 shows highest expression in the cotyledons of germinating seeds [6,9]. Developing seeds represent one of the strongest importers of assimilates and, in *Ricinus*, these are stored in the endosperm mainly in the form of lipids and proteins. During germination, the endosperm contents are ultimately transported to the growing embryo. Certain amino acids resulting from the hydrolysis of storage proteins are used in gluconeogenesis and transferred to the embryo in the form of sucrose; others are transferred directly into the cotyledons and then into the translocation stream to supply the newly developing plant [30]. Several studies have provided evidence for the existence of amino acid carriers in cotyledon cells to allow uptake of amino acids released from the endosperm [30,5,6]. Northern analysis has shown that RcAAP1 is highly expressed in the cotyledons relative to other tissues of the seedling, although it is not known at present in which cells this carrier is expressed [6,9]. Schobert and Komor [31] postulated that intact *Ricinus* cotyledons may possess a basic amino acid transporter at the plasma membrane of both parenchyma and phloem cells; thus RcAAP1 could conceivably serve this function. In situ hybridisation studies to map the expression of this gene should help resolve this question. A lower but significant level of RcAAP1 expression was observed in the root [6,9]. Schobert and Komor [1] found a differential distribution of amino acids within the root tissue and xylem sap of *Ricinus* and this could reflect the differential specificity of the transporters responsible for amino acid uptake from the soil and the xylem-loading systems, as well as compartmentation and metabolism of particular amino acids. The in situ studies indicate that RcAAP1 is predominantly expressed in the cells adjacent to the xylem poles and one possible function could be the loading of amino acids into the xylem [9]. However, Schobert and Komor [2] observed that the basic amino acids were fairly poorly represented in the xylem sap and that arginine showed a relatively low transfer to the xylem compared to glutamine. Thus RcAAP1 may have an alternative function in these cells (see [9] for a discussion). A clearer picture will undoubtedly emerge when we are able to map the location of the other amino acid carriers in *Ricinus* and determine their particular biochemical properties.

Acknowledgements

We are grateful to Prof. Norbert Sauer for the kind gift of the NEV-N vector and Dr. Dan Bush for the JT16 yeast mutant and advice with culture conditions. We would like to thank the BBSRC for financial support.

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