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Cloning of an *Arabidopsis* histidine transporting protein related to nitrate and peptide transporters

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Abstract

In plants, many of the proteins involved in transport of nitrogenous compounds have not been identified so far. The use of heterologous complementation in yeast mutants has enabled the isolation of a gene family encoding amino acid permease (AAP). A highly sensitive selection procedure was used to identify other proteins capable of transporting amino acids. In addition to members of the AAP gene family, an integral membrane protein (NTR1) that shows significant similarities to the low affinity nitrate transporter from *Arabidopsis* and to peptide transporters from yeast and rabbit was identified. *NTR1* seems to be involved in the supply of reproductive organs with nitrogen as it is expressed at low levels in leaves and highly in developing pods.

Key words: Plasma membrane; *Arabidopsis thaliana*; Multigene family; Amino acid transport

1. Introduction

In higher plants, inorganic nitrogen is usually absorbed from the soil as ammonium or nitrate. Depending on the species, fixation occurs either directly in the roots or nitrate is transported to the leaves, where it is reductively assimilated to produce amino acids. Reduced nitrogen is transported mainly in the form of amino acids within the vascular system. Amino acids are distributed within the plant through both xylem and phloem to supply organs that are net importers, e.g. reproductive organs such as seeds, with reduced nitrogen. The existence of multiple amino acid transport systems has been postulated (for review see [6]). During periods of rapid proteolysis, e.g. senescence or germination, the translocation efficiency may be enhanced by direct export of peptides. The best studied system is peptide transport in barley embryos during germination [8]. In addition, peptide transport has been demonstrated for other tissues [10].

Due to the high complexity of the transport of nitrogenous compounds in plants, a molecular dissection is required. To circumvent the problems associated with biochemical identification of transport proteins, expression cloning in oocytes has been used as an effective tool to isolate amino acid and peptide transporter genes from mammalian organisms [4,7,13,14]. In plants, ammonium and amino acid transporter genes were isolated by complementation of yeast mutants defective in the respective uptake systems [5,11,15,19]. A low affinity nitrate transporter has recently been isolated from a T-DNA tagged *Arabidopsis* mutant that shows homologies to the peptide transporters from fungal and animal sources [4,21,24]. Here we describe the use of a sensitive complementation system to identify new amino acid transporter genes. A gene that is related to both the nitrate and the peptide transporters was further characterized regarding DNA sequence and expression pattern in the plant.

2. Materials and methods

2.1. Materials and strains

The cDNA library from *Arabidopsis* seedlings was a generous gift from Mich le Minet [18]. The following *Saccharomyces cerevisiae* strains were used: JT16 (*Mat-a, hip1-614, his-401, ura3-52, ino1, can1* [23]) and 22574d (*MAT-α*, ura 3-1, gap 1-1, put 4-1, uga 4-1 [12]). The plant material was *Arabidopsis thaliana* L. Heynh. ecotype C24.

2.2. Yeast growth, transformation and selection

Yeast strain JT16 was transformed with a cDNA expression library derived from *Arabidopsis* seedlings [3]. Transformants were selected directly on solid SC medium supplemented with 6 mM histidine. Colonies able to grow were tested for growth in liquid medium containing different amounts of histidine. Plasmid DNA was isolated and reintroduced into the mutant strain JT16. The cDNA clone NTR1 was able to restore the growth of the mutant on medium supplemented with 6 mM histidine. For non-selective conditions, the medium was supplemented with 30 mM histidine. To test the substrate specificity, yeast strain 22574d was transformed with the cDNA clone. Selection was carried out on nitrogen-free medium supplemented with proline, citrulline or γ-aminobutyric acid as the sole nitrogen sources. The empty vector pFL61 served as a negative control whilst the amino acid transporters AAP1 or AAP2 were used as positive controls [15,18].

2.3. DNA sequence analysis

The cDNA was excised with *Not*I, subcloned into pBluescript SK– (Stratagene, Heidelberg) and both strands were sequenced with T7 polymerase (Pharmacia, Freiburg) from a set of subclones and by using synthetic oligonucleotides. The DNA sequence was analyzed using the UWGCG package [2] and has been submitted to the EMBL DataBank under the accession number X77503.

2.4. Southern and Northern blot analysis

Genomic DNA from *Arabidopsis* was isolated according to [22] and

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RNA was isolated as described by [17]. Standard procedures were used for electrophoretic separation and transfer to nylon membranes (Amersham, Braunschweig). The cDNA was radiolabelled using the Random Primed DNA Labelling Kit (Boehringer, Mannheim). DNA and RNA filters were hybridized for 16 h at 42°C in a buffer containing 6× SSC, 5× Denhardt's, 0.5% SDS, 100 μg/ml tRNA and 50% formamide; subsequently filters were washed 3 times for 20 min in 2× SSC, 0.1% SDS.

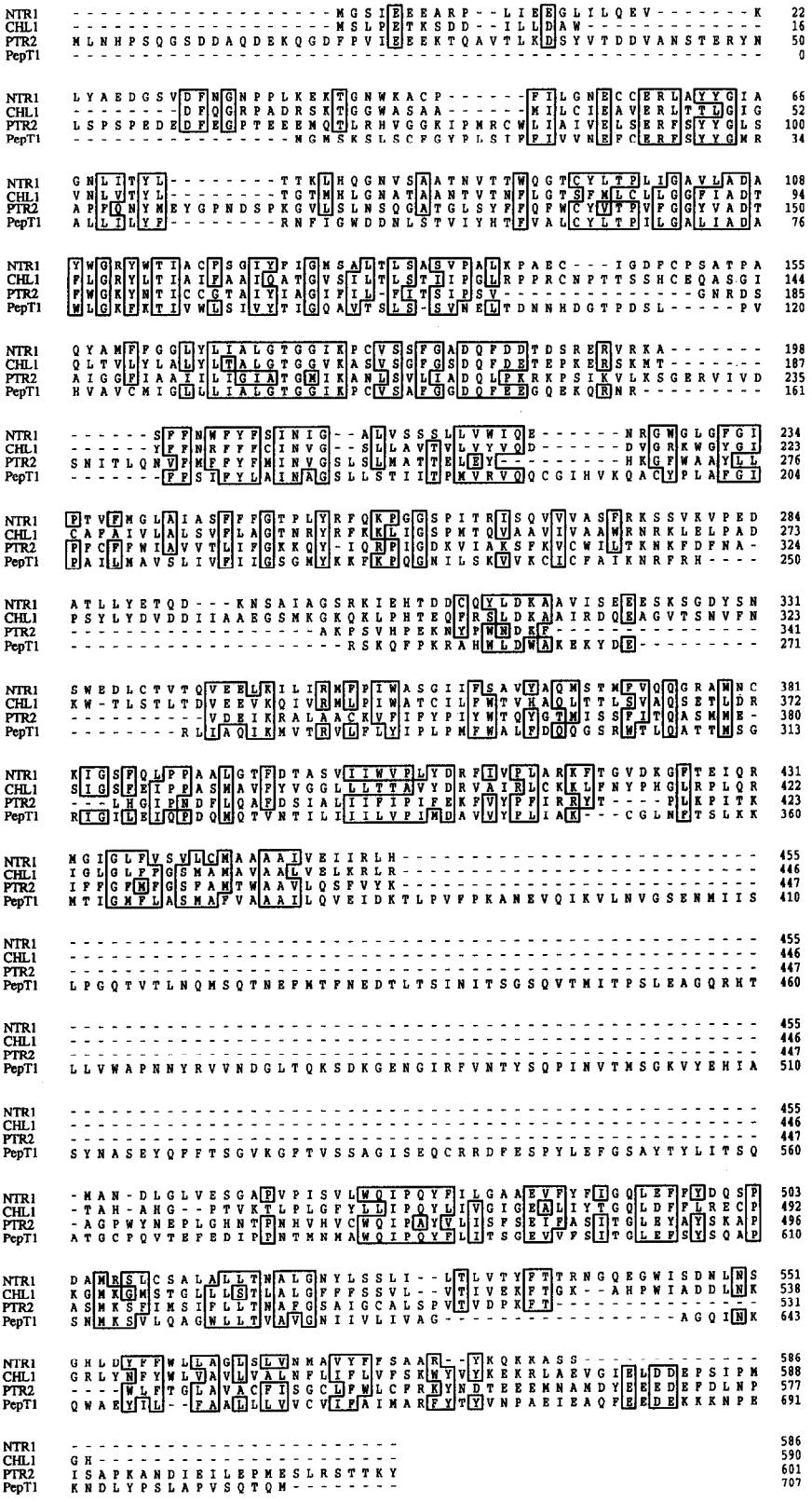

Fig. 1. Alignment of the deduced amino acid sequence of the *NTR1* gene (this work), the nitrate transporter *CHL1* [24] (both from *Arabidopsis thaliana*), the yeast peptide transporter gene *PTR2* [21], and the rabbit oligopeptide transporter *PepT1* [4].

3. Results

3.1. Isolation of NTR1 by complementation of a yeast amino acid transport mutant

The *Saccharomyces cerevisiae* strain JT16, which carries mutations in the histidine and arginine permease genes and in histidine biosynthesis, requires high concentrations of histidine (30 mM) for efficient growth [23]. To identify genes encoding plant amino acid transporters, JT16 was transformed with an episomal plasmid containing a cDNA library derived from *Arabidopsis* seedlings under the control of the phosphoglycerate kinase promoter [18]. After transformation, the cells were plated directly on selective SC medium supplemented with 6 mM histidine. Despite the high concentration of histidine, this condition does not allow background growth of JT16 transformed with the vector pFL61 alone, the selection thus being very sensitive. Plamid DNA was isolated and several classes of clones could be identified. To confirm that no reversion, e.g. of the *HIS4* gene or second site mutation, had occurred in the transformants, the recombinant plasmids were re-introduced into the mutant. Some were able to grow in the absence of histidine, indicating either supression or reversion of the *his4* mutation. The majority of clones belonged to the AAP gene family (data not shown). Among the colonies that showed slow growth on histidine, one clone with a size of about 2.1 kb was isolated and named *NTR1*.

3.2. Sequence analysis of NTR1

The clone contains a large open reading frame of 587 amino acids with a 21 bp untranslated leader that is not preceeded by a stop codon in-frame to the first ATG, and encodes a protein with a predicted molecular mass of 64.5 kDa. A search through the databases shows 50–60% similarity to the *Arabidopsis* nitrate transporter (CHL1) and to the yeast (PTR2) and rabbit (PepT1) peptide transporters, but no homologies to the other *Arabidopsis* amino acid permeases [4,15,21,24]. A number of additional *Arabidopsis* genes related to this family of proteins were identified in the database for expressed sequence tags (accession numbers dbest 31639/21313/34272/35050/14262/32819/14089/14055 and 35079; [1]). Alignment of the derived amino acid sequences of NTR1, CHL1, PTR2, PepT1 shows that all four are related (Fig. 1). The large insertion in PepT1 may not be relevant for function as it is absent from PTR2 and the other proteins. Hydropathy analysis shows that the predicted protein is highly hydrophobic and contains 12 putative membrane spanning regions (Fig. 2; [16]). The structure is highly similar to that of the related transporter proteins. Two potential N-linked glycosylation sites of NTR1 are located in the hydrophilic region at position 81 and 86, that according to a model based on the hydropathy analysis faces the extracellular matrix in the loop between the first and second membrane spanning domain. A potential N-linked glycosylation site is found in a synonymous position in CHL1 and PepT1. A new family of nitrogen transporters seems to emerge that is different from the sugar, ammonium and amino acid transporters [5,9,12,19].

3.3. Organ-specific expression of NTR1 in the plant

To prove that *NTR1* is actually a plant gene, Southern blot hybridizations of *Arabidopsis* DNA were performed. Under stringent conditions, single hybridization signals were found in genomic DNA restricted with *Eco*RI, *Eco*RV and *Not*I. For *Pst*I, which cleaves once inside the cDNA, two bands were found in the hybridization, and for *Hin*dIII, which contains multiple sites in the cDNA, two bands were detected when probed with the *NTR1* cDNA (Fig. 3). This result is in agreement with the assumption that no other closely related gene is cross-hybridizing under these conditions.

At a comparable stringency, *NTR1* hybridizes to a transcript with an approximate length of 2,400 nucleotides on Northern blots of total RNA from *Arabidopsis*. To analyze the expression profile in mature plants, RNA

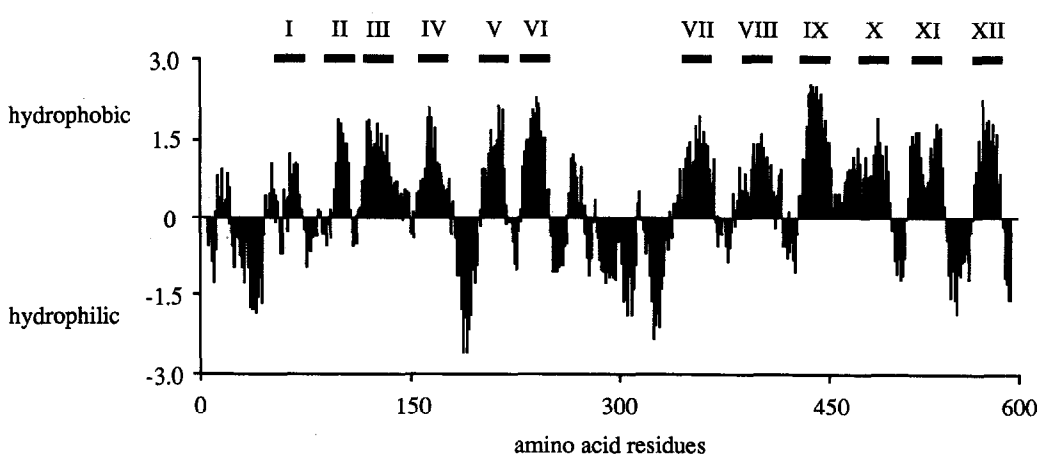

Fig. 2. Hydropathy plot calculated from the amino acid sequence of NTR1. The analysis was performed according to [16] with a window of 11 amino acids. Hydrophobic regions were given a positive hydropathy index. Tentative mebrane spanning α-helices are indicated by bars (I–XII).

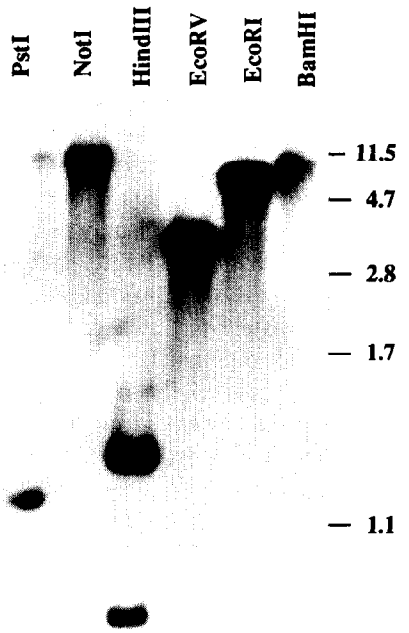

Fig. 3. Southern bloth analysis of *NTR1*. Equal amounts of genomic *Arabidopsis* DNA (50 μg) were analyzed by Southern blot hybridization using ^{32}P-labelled full-length cDNA of *NTR1* as probe.

was isolated from different organs. A high level of expression was found only in developing pods, an intermediate level in leaves, and low levels in other tissues and in seedlings (Fig. 4). The expression pattern is thus similar to that of the amino acid permease *AAP1* [15].

3.4. *Functional analysis of* NTR1

Functional expression of NTR1 in the histidine mutant enables the histidine transport-deficient yeast mutant JT16 to grow on media containing 6 mM histidine, a condition under which the wild-type is unable to grow (Fig. 5). The growth, however, is slow compared to both AAP1 and AAP2 (data not shown). Direct uptake measurements with [^{14}C]histidine were not sensitive enough to detect significant uptake above background. To enhance the level of expression, NTR1 was expressed in the mutant under control of the yeast plasma membrane ATPase promoter (*PMA1*). Transformants of JT16 were able to grow on media containing 3 mM histidine (data not shown). However, also in this case the uptake rates were not sufficient to be distinguishable from the background. To see whether NTR1 is able to transport other amino acids with higher efficiency, the proline, citrulline and γ-aminobutyric acid uptake-deficient mutant 22574d was transformed with *NTR1* [12]. Transformants were first selected for growth on minimal medium in the presence of ammonium and subsequently tested for growth on media containing proline, citrulline or γ-aminobutyric acid as sole nitrogen sources. However, 22574d-NT1 was unable to grow with 4.3 mM proline, citrulline or γ-aminobutyric acid, demonstrating that none of the three serves as substrate (data not shown).

4. Discussion

The use of yeast mutants deficient in amino acid transport has allowed the isolation of different cDNAs from plants, which encode broad specificity amino acid transporters [15]. The proteins encoded by the amino acid permease genes isolated so far do not cover all postulated activities and tissues. Therefore we established a very sensitive selection system in yeast to isolate other transporters for amino acids. JT16 that is deficient in both histidine and arginine uptake has been chosen, as the selection is tight also at high concentrations of histidine and thus should be more sensitive than JT48 [23]. On the basis of the success in expressing two permease cDNA clones in this strain, JT16 was transformed with an *Arabidopsis* cDNA library and selected for growth on medium containing 6 mM histidine [15]. The majority of the clones isolated was identical to *AAP1* and *AAP2* or were other members of this gene family (Frommer, unpublished results). Furthermore new genes could be identified that mediate growth of JT16 on media containing 6 mM histidine and that also encode integral membrane proteins. The gene described in this manuscript is a typical membrane protein with 12 putative membrane spanning regions and is homologous to two other types of transporters, namely the low affinity nitrate transporter from *Arabidopsis* and peptide transporters from yeast and mammalian origin [4,21,24].

The transport rates of NTR1 were too low to analyse the uptake of radiolabelled histidine into the yeast cells. In contrast to the AAP amino acid permeases, NTR1 does not mediate uptake of a number of other amino acids as shown by expression in a yeast mutant deficient in the uptake of citrulline, proline and γ-amino butyric acid [15]. Several reasons may be responsible for the low uptake rates, including low levels of expression in yeast, e.g due to incomplete targeting as has been observed in case of the H^+-ATPase [20]. Another possibility is that

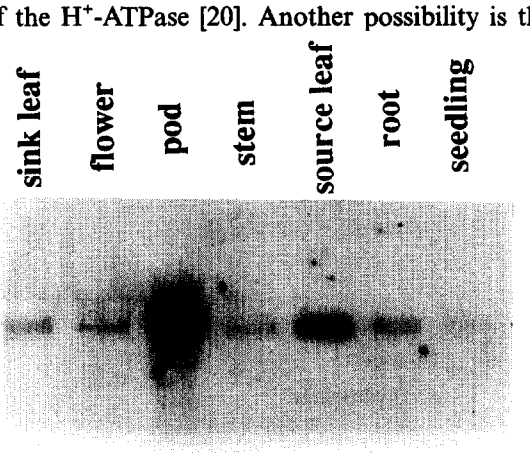

Fig. 4. Organ specific RNA expression of the amino acid permease gene *NTR1* in *Arabidopsis*. Total RNA (20 μg) from developing leaves, mature leaves, cauline leaves, flowers, green pods and roots was analyzed by Northern blot hybridization using a ^{32}P-labelled full-length cDNA from *NTR1* as probe.

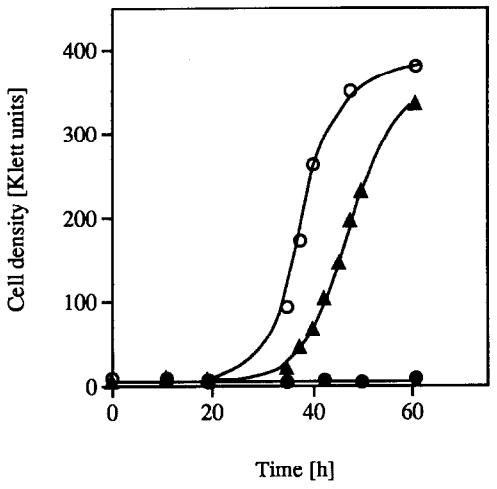

Fig. 5. Ability of NTR1 to complement histidine uptake in JT16. Growth rates of JT16 transformed either with the plasmid pFL61 or the *NTR1* cDNA in pFL61 in media containing 6 mM and 30 mM histidine (JT16-pFL61/30 mM histidine ○, JT16-pFL61/6 mM histidine ●, JT16-NTR1/6 mM histidine ▲).

in vivo NTR1 is not a transporter for histidine, but that histidine transport is a side activity of a nitrate or peptide transporter with low affinity for histidine. The low uptake rates did not allow for competition of histidine uptake by nitrate or by peptides to be tested. So far no data are available as to whether CHL1 or the peptide transporters also transport histidine. Expression of these proteins in JT16 and expression of NTR1 and CHL1 in the yeast peptide transport mutant or expression in oocytes, as shown for the rabbit oligopeptide transporter, may be tools to study the inter-relationship of the transport activities of the three types of transporters [4,21,24]. Due to the comparable sequence similarities of the different transporters, the comparison did not help in defining the actual function.

At the RNA level, *NTR1* is expressed in seedlings and in green pods and thus shows a similar expression pattern to the two AAP genes described so far [15]. This may be taken as an indication that NTR1 is also involved in the transport of nitrogenous compounds from the vascular system to the developing embryo and subsequently plays a role in the mobilization of stored nitrogen during germination.

All four proteins (NTR1, CHL1, PTR2 and PepT1) seem to be involved in the transport of nitrogenous compounds. According to the data from the expressed sequence tags, *Arabidopsis* contains a number of related proteins which might serve diverse functions in nitrogen transport. A similar diversity in substrate recognition as in case of the nitrogen transporters was found for another transporter family, i.e. the Na^+-dependent transporters in mammalian systems, where related proteins transport glucose, amino acids, choline, nucleosides, or *myo*-inositol [14]. The diverse functions of the related proteins represent an excellent system for the study of structure–function relationships in yeast by mutagenesis.

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Note added in proof

After acceptance of this manuscript, H.Y. Steiner informed us about the identification of a peptide transporter from *Arabidopsis* that is not identical to NTR1 but also belongs to the same family of related proteins (Steiner, H.Y. et al., Plant Cell, in press).