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NTR1 encodes a high affinity oligopeptide transporter in *Arabidopsis*

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Abstract Heterologous complementation of yeast mutants has enabled the isolation of genes encoding several families of amino acid transporters. Among them, *NTR1* codes for a membrane protein with weak histidine transport activity. However at the sequence level, NTR1 is related to rather non-specific oligopeptide transporters from a variety of species including *Arabidopsis* and to the *Arabidopsis* nitrate transporter CHL1. A yeast mutant deficient in oligopeptide transport was constructed allowing to show that NTR1 functions as a high affinity, low specificity peptide transporter. In siliques *NTR1*-expression is restricted to the embryo, implicating a role in the nourishment of the developing seed.

Key words: Amino acid transport; Nitrate transport; Peptide transport; Plant; Plasma membrane; Yeast

1. Introduction

Organic nitrogen is mainly transported within the vascular system in the form of amino acids to supply organs that are net importers, e.g. developing seeds. Physiological and biochemical analyses have implicated the involvement of multiple amino acid transport systems (for review cf. [11]). However, during periods of rapid proteolysis, i.e. senescence or germination, the translocation efficiency may be enhanced by direct export of oligopeptides [22]. Transport studies have indicated that during germination of barley grains peptide transport from the endosperm to the embryo is at least as important as amino acid transport [19,21]. In addition, peptide transport activities have been measured in various other tissues [22,24]. The simple kinetics indicate the presence of transporters with a rather low specificity toward the amino acid side chains. Such systems may function in mobilization of the numerous different di- and tripeptides and even peptides up to a chain length of five amino acids seem to be transported [19]. Besides their putative function in nitrogen supply, oligopeptides may be important in wound response [30], in plant pathogen interactions or as compounds affecting the taste and aroma of various foods [22]. The tripeptide glutathione is involved in storage and translocation of reduced sulphur, in detoxification and in redox regulation [32].

Several putative plant peptide transport proteins were identified by sulphydryl group modifying agents and radiolabeled photoreactive peptides as specific inhibitors [16,29]. During the last years, transporter genes from plants for nitrate, ammonium and amino acids were identified and characterized in heterologous hosts (for review cf. [11,13]). Complementation of a yeast mutant defective in peptide transport has allowed to identify the first peptide transporter gene, *AtPTR2*, from plants [39]. AtPTR2 is homologous to peptide transporters from yeast and rabbit, but also to the nitrate transporter CHL1 from *Arabidopsis* [6,31,41]. In parallel, the use of a sensitive complementation system for amino acid transporters led to the identification of NTR1, which functions as a low affinity histidine transporter [10]. Interestingly, NTR1 is also homologous to the peptide/nitrate transporter superfamily. However, due to the low transport activity it remained unclear whether histidine transport represented the major function of NTR1 or only a side activity of a putative peptide or nitrate transporter. To analyze this question, a peptide transport deficient yeast mutant was constructed and the biochemical properties of NTR1 were characterized. Furthermore the expression of *NTR1* in siliques was analyzed in more detail.

2. Materials and methods

Saccharomyces cerevisiae: JT16 (*Mat*-a *hip1-614 his4-401 can1 ino1 ura3-52*; [40]). LR1 (*Mat*-a *hip1-614 his4-401 can1 ino1 ura3-52 ptr2Δ::hisG-URA3-neo-hisG*, this work). LR2 (*Mat*-a *hip1-614 his4-401 can1 ino1 ura3-52 ptr2Δ*::hisG, this work). *Arabidopsis thaliana* L. Heynh. ecotype C24.

2.1. Construction of ptr2 mutants

A 1.6 kb fragment of the yeast *PTR2* gene was amplified by PCR using oligonucleotides ACGCTACAACTTGTCCCC (position 141 to 158 in *PTR2*; [31]) and CGTTAGCTTTAGGTGCGG (position 1753 to 1736 in *PTR2*) and genomic DNA of *S. cerevisiae* as template. The PCR product was subcloned into pBlueskript SK$^-$, the insert was cleaved with *Bcl*I, thus deleting 441 bp of the coding region, and the *Bam*HI/*Bgl*II fragment of pSE1076 containing the *hisG-URA3-neo-HisG* cassette was inserted [1]. The yeast strain JT16 was transformed with the construct [5] and transformants were selected for growth in the abscence of uracil (LR1). Subsequent selection on 5-fluoro-orotic acid selected for excision of the *URA3* cassette by recombination between the *hisG* repeats, thus leading to a ura3$^-$ phenotype (LR2). The correct integration of the disruption cassette into *PTR2* and its deletion upon selection on 5-fluoro-orotic acid was confirmed by PCR and Southern blot analysis (data not shown).

2.2. Yeast growth, transformation and selection

To construct the episomal yeast expression vector YEP 112A1XE, YEP112A1 [33] was cleaved with *Pst*I/*Bam*HI and the annealed syn-

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DEPC, diethylpyrocarbonate; 2,4-DNP, 2,4-dinitrophenol; pCMBS, *p*-chloromercuribezenesulfonic acid; NEM, *N*-ethylmaleimide.

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thetic oligonucleotides GAATTCTAGATCTCGAGCGGCCGCG/GATCCGCGGCCGCTCGAGATCTAGAATTCTGCA were ligated into it. The new multiple cloning site contains the unique restriction sites *Pvu*II,*Pst*I, *Eco*RI, *Xba*I, *Bgl*II, *Xho*I, *Not*I and *Bam*HI downstream of the *ADH1* promoter. YEplac195 [14] was cleaved with *Pst*I and *Eco*RI, made blunt-ended with Klenow fragment and religated. The ADH1 expression cassette was isolated from YEP112A1XE as an 0.7 kb *Sph*I fragment and inserted into the respective site of the modified YEplac195, yielding pMK195. To increase the expression levels, the yeast plasma membrane ATPase promoter *PMA1* was isolated from pRS1024 [36] as a *Hin*dIII/*Xho*I fragment and cloned into the respective sites of pMK195. The resulting plasmid pDR195 allows to clone heterologous genes into the polylinker consisting of *Xho*I, *Not*I and *Bam*HI behind the ATPase promoter. *NTR1* and *AAP2* [25] were cloned into the *Not*I site of pDR195 and expressed in LR2. Transformants were selected on SC medium supplemented with 800 μM L-histidyl-L-leucine or histidine. For nonselective conditions the medium was supplemented with 20 mM histidine.

2.3. Transport measurements

Yeast cells were grown to the logarithmic phase, harvested at an OD_{600} of 0.6, washed and resuspended in 50 mM potassium phosphate (pH 4) to a final OD_{600} of 6. To start the reaction, 100 μl of the cell suspension were added to 100 μl of the same buffer containing 11.6 kBq ^{3}H-labeled L-leucyl-L-leucine (55 Ci/mmol; Biotrend, Köln) and 15 μM of unlabeled dileucine. Samples were removed after 30, 60, 120 and 180 s, transferred to 4 ml ice-cold water, filtered on glass fiber filters and washed with 8 ml water. Competition for dileucine uptake was performed by adding a tenfold molar excess of the respective competitors to 45 μM dileucine. Transport measurements were repeated independently and represent the mean of at least three experiments.

2.4. RNA in situ hybridization

Arabidopsis siliques were fixed in 3.7% formaldehyde, 5% acetic acid and 50% ethanol [2], dehydrated in ethanol and embedded in Fibrowax (Plano, Marburg). Sections (10 μm) were mounted on precoated slides (Star Frost, Wiesbaden). Prior to hybridization sections were soaked in xylene to remove paraffin, rehydrated and deproteinized for 10 min using predigested pronase 500 μg/ml (Calbiochem) at RT. ^{35}S-labeled sense and antisense transcripts of *NTR1* were hydrolyzed to an average length of 0.2 kb and used for hybridization as described [35]. Slides were coated with nuclear track emulsion (NTB2), exposed for seven days and viewed by dark field light microscopy.

3. Results

3.1. Complementation of an oligopeptide uptake deficiency

The yeast strain JT16, carrying mutations in the histidine and arginine permease genes and the *His4* gene requires high concentrations of histidine for efficient growth [40]. A deletion/insertion mutant in the peptide transporter gene *PTR2* of JT16 (strain LR2) was constructed by gene replacement. This peptide transport deficient and histidine auxotrophic strain LR2 was unable to grow on SC medium containing histidine exclusively in the form of a dipeptide. LR2 was transformed with the yeast expression vector pDR195 and the *NTR1*-cDNA in pDR195 under control of the yeast ATPase promoter. Only LR2 cells expressing *NTR1* were able to grow on selective medium containing 800 μM His-Leu (Fig. 1A). Neither the amino acid permease gene *AAP2* in pDR195 [25] nor the vector pDR195 alone mediated growth under these conditions when expressed in LR2. Comparable results were obtained when *NTR1* was expressed under control of the phosphoglycerate kinase promoter (data not shown; [28]). Growth could not be due to the uptake of histidine as a degradation product since the histidine transport capacity was not sufficient to sustain growth of LR2-*NTR1* when comparable amounts of histidine were supplied (Fig. 1B). The amino acid transporter AAP2 thus does not seem to be able to transport dipeptides efficiently.

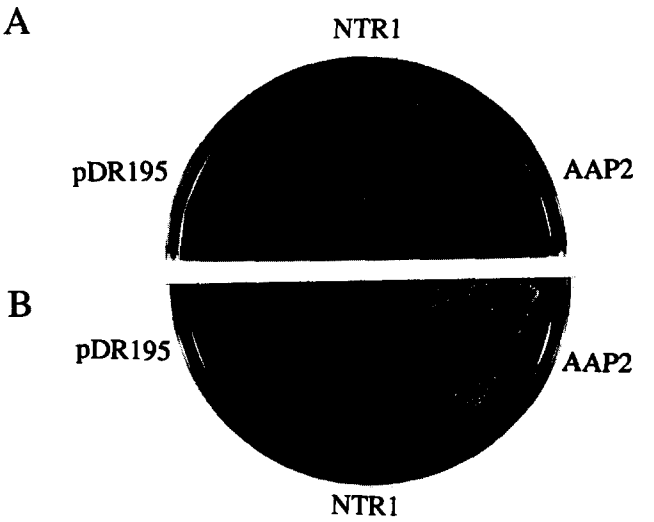

Fig. 1. Complementation of a peptide uptake deficient yeast strain by *NTR1*. Growth of LR2-*NTR1*, LR2-*AAP2* and LR2-pDR195 on SC medium supplemented with 800 μM of His-Leu (A) or 800 μM histidine (B).

Table 1
Effect of inhibitors on dileucine uptake

Inhibitor	Relative uptake of dileucine (%)
None	100
50 μM pCMBS	4.5 ± 1.8
1 mM NEM	22.5 ± 4.9
1 mM DEPC	5.1 ± 1.5
10 μM CCCP	13.5 ± 3.1
100 μM 2,4-DNP	3.2 ± 0.6

Dileucine was present at a final concentration of 15 μM. Inhibitors were added 5 min prior to the reaction start.

3.2. Biochemical characterization of NTR1 expressed in yeast

The peptide transporters isolated so far are rather non-specific, mediating uptake of various oligopeptides [6,31,39]. Assuming that NTR1 also represents a broad specific oligopeptide transporter, the capacity to transport dipeptides was determined directly by measuring the uptake of [^{3}H]dileucine into yeast cells. The transport activity mediated by NTR1 was saturable with a Michaelis–Menten constant of 18 ± 3 μM (data not shown). Similar to data from in planta measurements [18,24,29], the dipeptide uptake activity mediated by NTR1 was sensitive to a number of different inhibitors such as protonophores (CCCP and 2,4-DNP), the histidine-modifying agent DEPC and sulphydryl-group modifying agents as pCMBS and NEM (Table 1) and was pH dependent with an optimum around pH 4.5 (data not shown).

In the absence of PTR function, little peptide transport activity can be detected [23]. The expression system is therefore excellent to study the substrate specificity of heterologous carriers. Since however no other radiolabeled oligopeptides were available, the substrate specificity of NTR1 had to be determined by competition experiments. For this, [^{3}H]dileucine uptake activity was measured in the presence of a tenfold excess of different amino acids or peptides as competitors (Fig. 2). The data show that NTR1 is rather nonselective regarding side

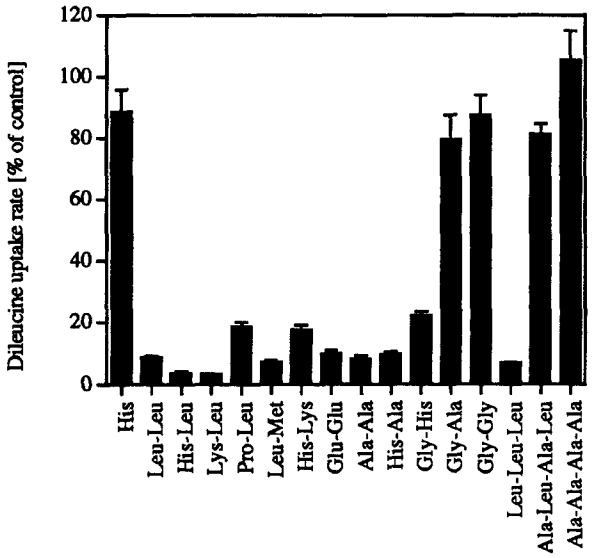

Fig. 2. Specificity of the oligopeptide transporter NTR1. Competition of [^{3}H]dileucine uptake into yeast cells (LR2-*NTR1*) in the presence of a tenfold excess of respective competitors. Uncompeted uptake rates were taken as 100% and correspond to 35 pmol dileucine $min^{-1} \cdot mg^{-1}$ cells.

chains of amino acids, recognizing a number of different dipeptides such as Leu-Leu, His-Leu, Lys-Leu, Pro-Leu, Leu-Met, His-Lys, Glu-Glu, His-Ala and Ala-Ala with high efficiency. Diglycine did not seem to be a good substrate, as was Gly-Ala, whereas Gly-His competed efficiently. The poor competitive ability of glycyl peptides has previously been described for peptide transport in barley [20,37]. Also histidine did not compete efficiently for dileucine transport as could be predicted from the low histidine transport activity of NTR1 in JT16 [10]. Trileucine was an efficient competitor for dileucine uptake, but tetrapeptides such as Leu-Ala-Leu-Ala and tetraalanine did not compete significantly. These data are also in agreement with the substrate specificity of transport activities determined in several plant species [19,34,38].

3.3. *Sequence comparison between peptide and nitrate transporters*

Recently, the peptide transporter gene *AtPTR2* has been isolated from *Arabidopsis*. Sequence comparisons show that *NTR1* is not identical to *AtPTR2*, however, both *Arabidopsis* peptide transporters NTR1 and AtPTR2, the yeast peptide transporter ScPTR2 and the *Arabidopsis* nitrate transporter CHL1 share significant sequence homologies (Fig. 3). Surprisingly, the proteins fall into two groups in which the *Arabidopsis* and yeast *PTR2* genes are more closely related to each other than to *NTR1* and *CHL1* and vice versa. This is obvious both from the presence of typical insertions/deletions in the two groups of proteins and from a computer-aided comparison of members of this protein family (Fig. 4). Significant homologies were also found to the di/tripeptide transporter from *Lactococcus lactis* (DtpT, [15]) and the human and rabbit H^+/peptide cotransporters (hPepT1, [27]; PepT1, [6]) but not to other bacterial peptide transporters (e.g. [42]) and to the putative human peptide transporter Hpt1 [3].

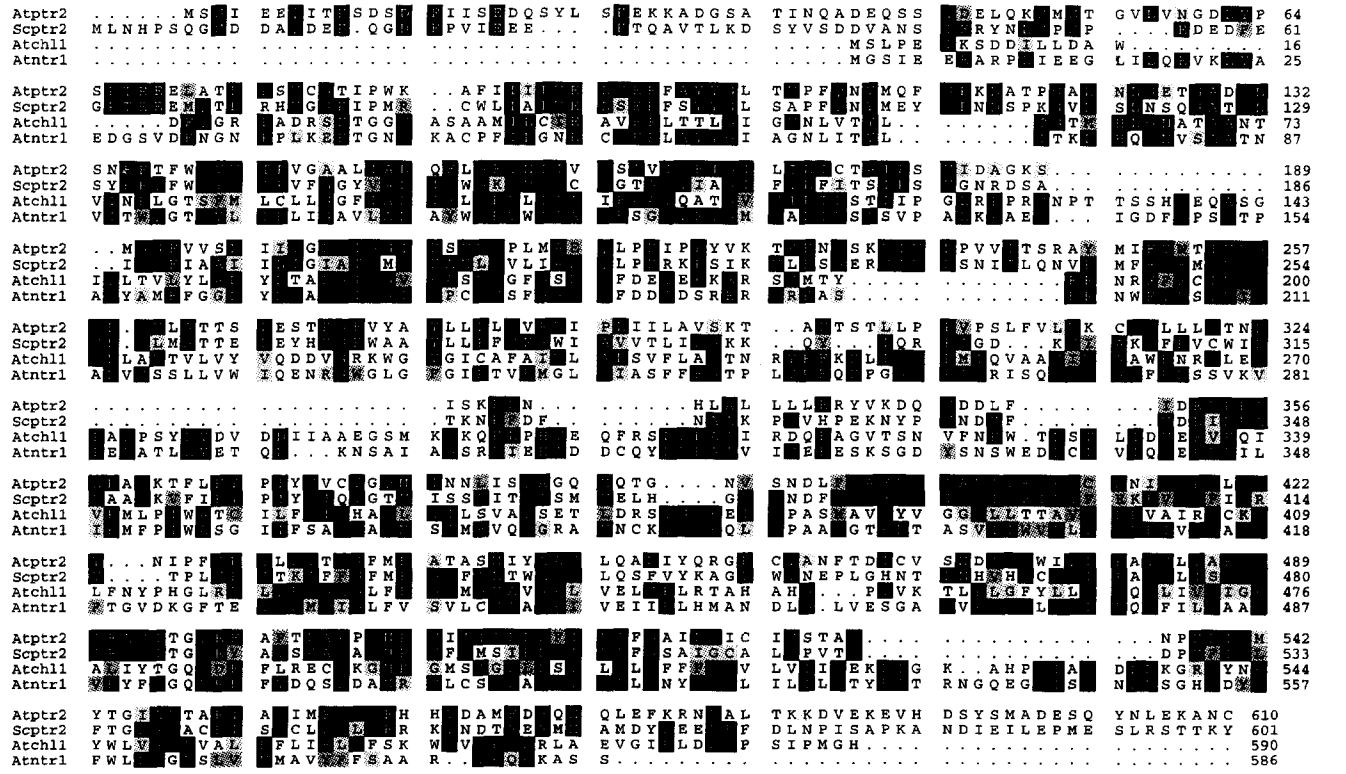

Fig. 3. Amino acid sequence alignment of peptide transporters from *Arabidopsis* (AtPTR2, [39]; AtNTR1, [10]) and yeast (ScPTR2, [31]) and the nitrate transporter AtCHL1 from *Arabidopsis* [41].

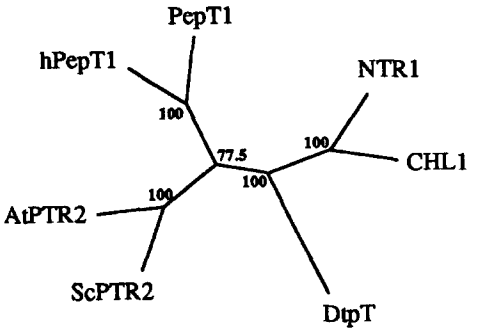

Fig. 4. Computer-aided analysis of the homologies between NTR1 and related proteins. The analysis was performed using the PHYLIP program package [7] with the aligned sequences of NTR1 [10], CHL1 [41], AtPTR2 [39], ScPTR2 [31], PepT1 [6], hPepT1 [27] and DtpT [15]. The comparison was restricted to the region in NTR1 from amino acid position 65 to 452. The numbers indicate the occurrence of a given branch in 100 bootstrap replicates of the given data set.

3.4. *Expression of* NTR1 *in siliques*

To obtain more information on *NTR1* expression in siliques [10], RNA in situ hybridization experiments were performed. At the heart stage of embryo development, expression was detectable in the embryo and to a lesser degree in the seed coat (Fig. 5A). Controls with sense probes showed low unspecific background in the embryo (Fig. 5B), however, in some controls significant background was found in the integuments (data not shown). Therefore we assume, that the signals detected in the seed coat originate from unspecific binding of sense and antisense ^{35}S-labeled RNA and *NTR1*-expression is confined to the embryo.

4. Discussion

4.1. *Analysis of the function of NTR1*

A new class of plant membrane transporters has been identified that seems to comprise proteins covering a wide substrate specificity reaching from nitrate and chlorate (CHL1, [41]) to histidine (NTR1, [10]) and peptides (AtPTR2, [39]). A detailed sequence comparison showed that NTR1 is more related to CHL1 than to the peptide transporters. Nevertheless we wanted to see whether NTR1 is able to mediate peptide transport. For this purpose a yeast mutant was constructed that is deficient in peptide uptake. *NTR1* was able to complement the peptide transport mutation. Furthermore, direct uptake measurements with radiolabeled dipeptide show that NTR1 constitutes a high affinity oligopeptide transporter with low specificity regarding the amino acid side chains. The histidine transport activity described previously thus represents only a side activity of a peptide transporter. On the other hand the amino acid permease AAP2 which mediates strong histidine uptake is not able to complement the peptide transport deficiency. The question remains whether CHL1 is also able to transport peptides and whether chl1 mutants are affected not only in chlorate and nitrate transport but also in peptide transport [4]. This will have to be studied either in the yeast peptide transport mutant described here or in *Xenopus* oocytes [41].

4.2. *Role of NTR1 in oligopeptide transport*

Amino acids represent the major nitrogenous compounds in the vascular system of *Arabidopsis* [26]. However under several environmental, physiological or developmental conditions rapid proteolysis occurs in association with rapid export of organic nitrogen. This includes responses to wounding, senescence, degradation of vegetative storage proteins in leaves dur-

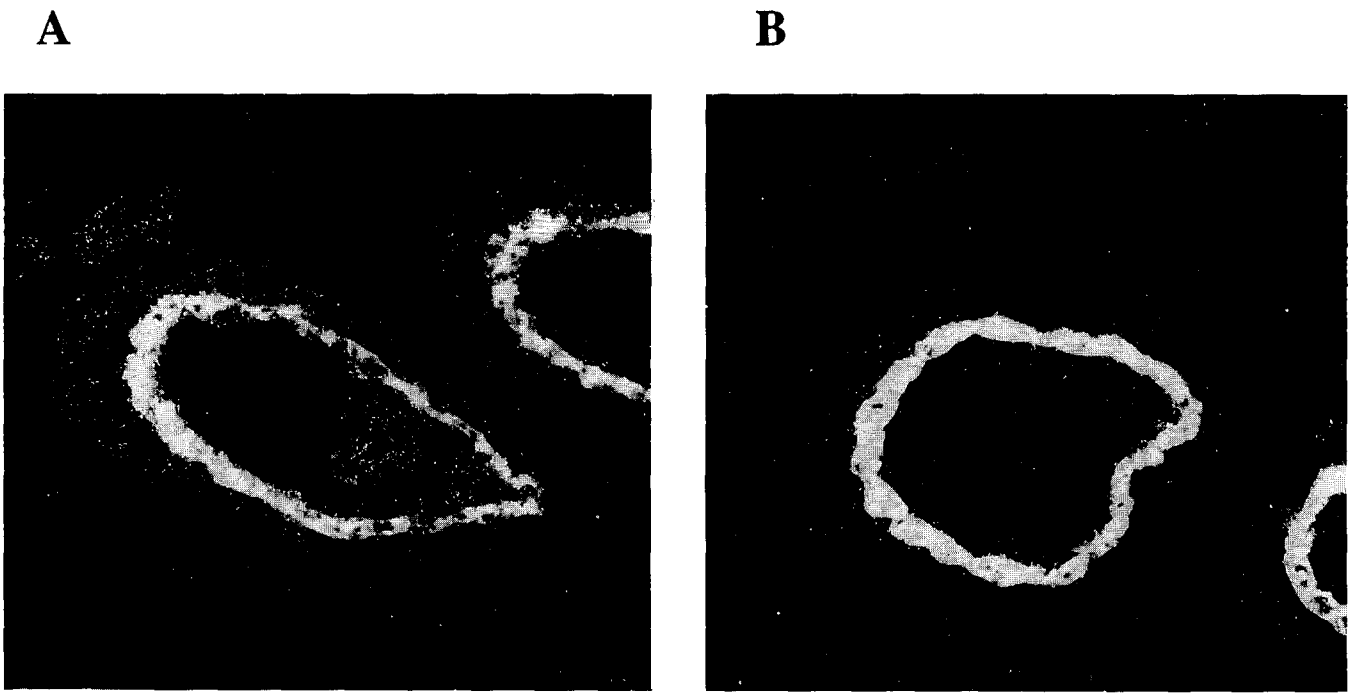

Fig. 5. In situ localization of the peptide transporter mRNA (*NTR1*) in siliques of *Arabidopsis*. Antisense (A) and sense (B) transcripts of *NTR1* were used. The endothelium is reflecting brightly (150× magnification)

ing seed filling, feeding of the embryo by maternal tissues or endosperm and germination. Evidence has been presented that oligopeptide transport is a property of many plant organs [22]. It could be shown that in barley embryos oligopeptide transport activities are higher than those for amino acids [19,21]. Thus, if the degradation of proteins to amino acids or the export capacity is limiting, the transport of proteolytic intermediates, i.e. oligopeptides, may represent an alternative route for translocation of nitrogen. Degradation of oligopeptides could then occur during translocation or after import into the respective sink organs. Since the resulting mixture of oligopeptides is highly complex, the determination of individual oligopeptides would be difficult. This may explain why little is known about peptide transport. An excellent tool to study the role of peptide translocation is to study oligopeptide transport directly.

Regarding the specificity of oligopeptide carriers involved in nutritive peptide transport one might expect a low selectivity towards the amino acid side chains in order to allow proteolysis product translocation. It is unprobable that a cell contains individual transporters for each of the 400 di- or 8000 tripeptides that could possibly be encountered during proteolysis. In agreement with this hypothesis, the two systems identified so far, i.e. NTR1 and AtPTR2, are rather nonspecific and recognize a broad spectrum of di- and tripeptides [39]. Furthermore NTR1 has a high affinity for oligopeptides in agreement with the low concentrations present in plants. *NTR1* expression was found in the embryo, suggesting that it may be involved in supplying the developing embryo with peptides.

As in case of the plant amino acid transporters identified so far [8,9,25,12], the oligopeptide transporters exhibit a very broad specificity. Due to their low specificity towards size and structure of the transported compounds, oligopeptide transporters represent biotechnologically attractive proteins, e.g. to construct designer drugs [17], an effect that could also be exploited to mobilize xenobiotics in the plant.

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