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Localization of a new class of transporter for nitrogenous compound in chloroplast envelopes

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Introduction

In the nitrogen cycle, NO_3^-/NO_2^- assimilation by plants is one of the two primary pathways for the conversion of inorganic nitrogen into amino acids and proteins. The reductive assimilation of NO_3^- is a polygenic functional system involving sequential conversions of NO_3^- to NO_2^-, NH_4^+, glutamine, and glutamate (Crawford and Arst Jr. 1993; Hoff et al. 1994). Chloroplasts are separated from the cytosol by envelope membranes. A low steady state concentration of NO_2^- in mesophyll cells (Vaucheret et al. 1992; Siddiqi et al. 1992; Kawamura et al. 1996) suggests that the transfer of NO_2^- through the envelope membranes is fast enough to sustain NO_2^- reduction in the chloroplast stroma. There are two phases in the NO_2^- uptake of chloroplast: a saturable active transport (Brunswick and Cresswell 1988) and a passive influx of NO_2^- along the concentration gradient (Shingles et al. 1996). The facilitated transfer of NO_2^- might account for most of the NO_2^- uptake by chloroplasts from the cytosol, which contains a relatively low concentration of NO_2^-, in order to maintain the catalytic activity of stromal nitrite reductase and to lower the cytosolic concentration of NO_2^-, which might be toxic to cellular metabolism.

NO_3^- assimilation, which includes NO_2^- reduction, uses reductants provided by photosynthetic electron transport. The genes for NO_3^- assimilation, for example, nitrite reductase and glutamine synthetase, are expressed in parallel with the development of the cotyledons (Suzuki and Rothstein 1997). The NO_2^- transporter might be synthesized during the light-dependent development of chloroplasts as are other photosynthesis-related proteins that start to be expressed in etiolated shoots upon illumination (Thompson and White 1991). We cloned a gene for a transporter of chloroplast from the cDNA library of the greening cucumber cotyledons.

Materials and methods

Cucumber (*Cucumis sativus* L.) cotyledons that had been grown for 72 h in the dark at 28^{o}C were then grown for 36 h under continuous light. mRNA was isolated from these cotyledons and was used to prepare a cDNA library. A 1.6-kb cDNA for a greening-plant specific mRNA was cloned from the library. The cDNA from the NotI-digested clone was subcloned into *pBluescript* KS+

(Promega) to construct *pBnitr1.6*. The sequence of the 5' end of the mRNA was captured by 5'RACE. Synthesis of first-strand cDNA was primed using an antisense sequence corresponding to a position from 567 to 548 of the cDNA in *pBnitr1.6*. The first-strand cDNA was purified, tailed with dCTP at the 3' end, and amplified by PCR. The cDNA from 5'RACE was cloned in the *pCRII-TOPO* vector (Invitrogen), digested by *Xba*I and *Bst*EII, and inserted into the same site of *pBnitr1.6* to construct *pBnitr* that harbors full-length cDNA, *Nitr1*.

To analyze the expression of *Nitr1*, cucumber cotyledons were homogenized with liquid N_2, mixed with 4 M guanidine thiocyanate that contained 25 mM Na-citrate, 1.5% lauroylsarcosine, and 0.01% ß-mercaptethanol at room temperature, and extracted with phenol-chloroform. RNA was precipitated with LiCl, denatured at 65°C, and subjected to agarose gel electrophoresis with formaldehyde. RNAs were blotted onto a nylon membrane, baked at 80°C for 2 h, and hybridized at 68°C for 6 h with a radiolabeled probe. The membrane was washed at 68°C with the 1 x SSC containing 0.1% SDS.

To obtain recombinant Nitr1 protein, a fragment of *Nitr1* cDNA, which corresponded to Lys^{281}-Ala^{604}, was ligated after the *Sal*I site of the *pMal*-c2 expression plasmid with a Gly-Ile-Asp spacer. The *E. coli* TB1 cells that contained the resulting plasmid were harvested after a 3-h culture at 30°C after the addition of 0.3 mM IPTG. The fusion protein was purified by amylose affinity chromatography, dialyzed against phosphate-buffered saline, and used to immunize rabbits. Immunization was boosted 4 times by the same amount (0.2 mg) of antigen. Anti-Nitr1 antibodies were obtained by removing the anti-bacterial maltose binding protein antisera. Fab fragments were prepared according to Harlow and Lane (1989).

NO_2^- uptake by chloroplasts was assayed as the light-induced decrease of NO_2^- concentration in the extra-chloroplast space. Spinach leaves were homogenized with an isolation buffer that contained 5 mM HEPES-KOH, pH 6.8, 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM Na-pyrophosphate. Homogenates were filtered through 4 layers of cheesecloth and centrifuged at 4,000 x *g* for 10 s. Pellets were suspended in the isolation buffer at a Chl concentration of 2.5 mg ml^{-1} and 2 ml was layered on 10 ml of a 20% - 80% Percoll (Pharmacia) gradient. After centrifugation at 17,800 x *g* for 10 min, the band of intact chloroplasts was collected. The entire procedure was conducted at 5°C. The chloroplasts (50 µg of Chl) were incubated with the Fab fragments in 1 ml of the isolation medium for 30 min at 5°C, mixed with 1 ml of the isolation buffer that contained 100 µM KNO_2, and illuminated for 5 - 10 min by a red light (440 µE m^{-2} s^{-1}) at 25°C. The reaction mixtures were centrifuged at 10,000 x *g* for 3 min at 5°C and the NO_2^- concentration in the supernatant was colorimetrically determined.

Results

When cucumber seedlings, dark-grown for 3 days after germination, were illuminated, a mRNA that hybridized with *Nitr1* was produced and its accumulation peaked at 24-h illumination (Fig.

1A), which occurred at the same time as the opening and greening of the cotyledons. The mRNA for *Nitr1* was present in the leaves (Fig. 1B, lane L) and in the fruits (Fig. 1B, lane Fr) but was little in the roots, stems, and flowers (Fig. 1B, lanes R, S, and Fl). Light-induced expression of *Nitr1* mRNA in green tissues suggests that the biosynthesis of the Nitr1 protein might be correlated with the development of photosynthetic functions. Supplement of 20 μM Norflurazon to the nutrient caused heavy chlorosis on the cucumber leaves. Chloroplast proteins and Nitr1 protein were missing in the Norflurazon-treated leaves (data not shown).

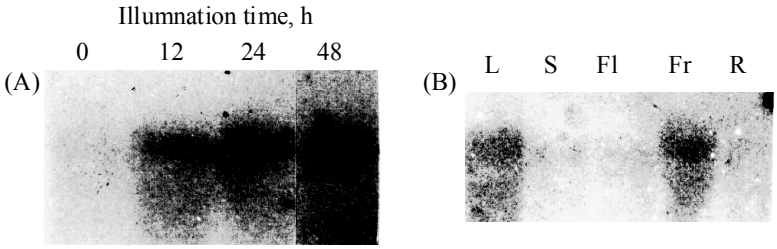

Fig. 1. Light-induced expression of *Nitr1* in the green tissues of the cucumber.

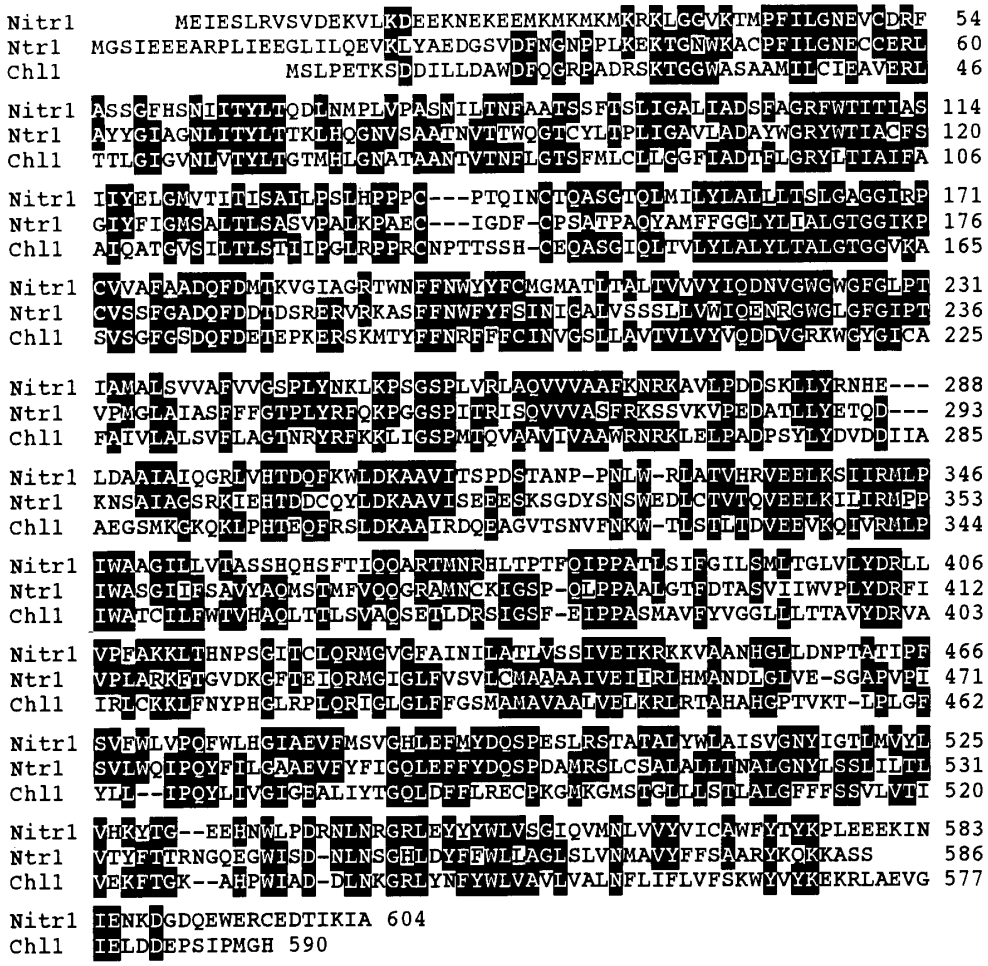

Fig. 2. Alignment of the amino acid sequence of Nitr1 with the sequences of the *Arabidopsis* NO_3^- transporter, CHL1 and the *Arabidopsis* peptide transporter, NTR1. Reversed letters indicate conserved residues among these sequences.

Nitr1 is 1,916 bp long with a 1,815-bp open reading frame preceded by an 18-bp noncoding region [EMBL accession number Z69370]. The molecular weight of the encoded protein of 604 amino acids is 67,579. Figure 2 shows the sequence of Nitr1, which is aligned with its homologs, the *A. thaliana* NO_3^- transporter (CHL1; Tsay et al. 1993) and oligopeptide transporter (NTR1; Rentsch et al. 1995). The homology of Nitr1 is 38.1% for CHL1 and 41.8% for NTR1 in its amino acid sequence, respectively. The hydropathy plot indicates that *Nitr1* encodes a membrane protein with 11 transmembrane regions. Between the 5th and 6th hydrophobic regions, there is a 70-amino acid hydrophilic loop. This structure with 5 plus 6 transmembrane regions and the interconnecting hydrophilic loop is characteristic of the transporter families, although most transporter proteins have a structure with 6 plus 6 transmembrane regions (Sadée et al. 1995). The conserved amino acid residues of the above three proteins are located mostly at the membrane surface.

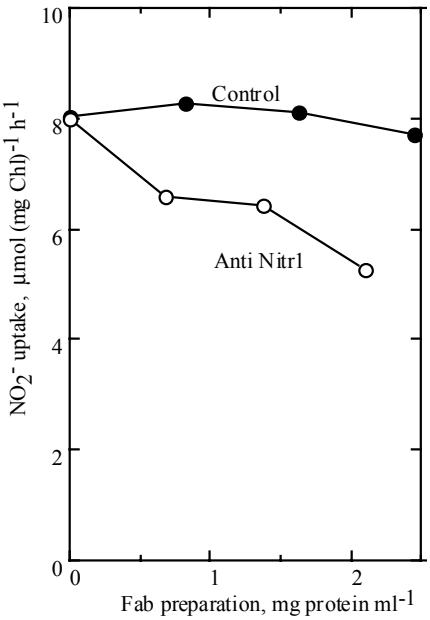

Fig. 3. Inhibition of NO_2^- uptake by intact chloroplasts by the Fab fragments of anti-Nitr1 antibodies.

NO_2^- can be taken up by chloroplasts on illumination even in the isolation medium at neutral pH (Brunswick and Cresswell 1988). Uptake kinetics by spinach intact chloroplasts was linear for first 10 min after the start of illumination. However, the rate of uptake was lowered when chloroplasts were incubated with the Fab fragments of anti-Nitr1 antibodies prior to the start of illumination (Fig. 3, open circles). Fab fragments of control sera from preimmune rabbit showed no effect on the NO_2^- uptake kinetics (closed circles).

Discussion

The cloned cDNA, *Nitr1*, is a homolog of *CHL1* and *NTR1* of *A. thaliana*. Figure 4 shows the inter-relatedness of some of these homologs. It is obvious that Nitr1 is distinct from NO_3^- transporters and peptide transporters. Similarity of amino acid sequences suggests that *Nitr1* is

not a cucumber *CHL1* or *NTR1* but a gene for a transporter for a nitrogenous compound other than NO_3^-, because the amino acid substitution is more than the phylogenic variation: there is only a 9% difference between the CHL1s of *A. thaliana* and *B. napus* (RCH2), and less than a 30% difference between the nuclear-coded chloroplast protein, Cab1, PetE, and RbcS, of *Arabidopsis* and the cucumber.

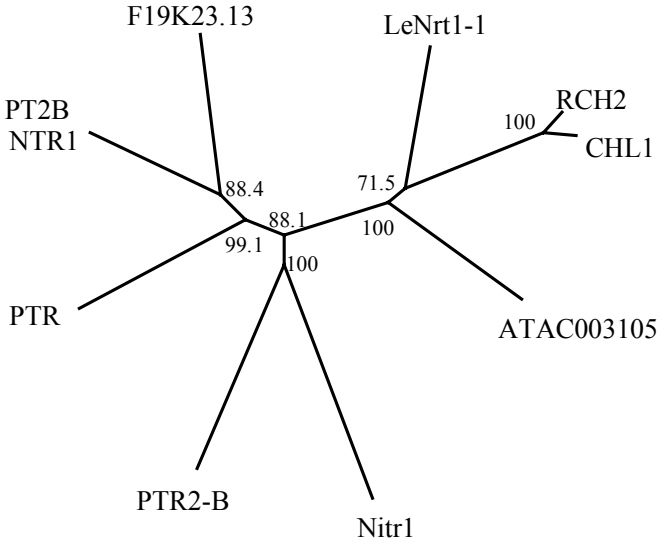

Fig. 4. Relatedness of plant homologs of nitrate and peptide transporters showing the unique position of *Nitr1*. The unrooted tree was drawn by CLUSTAL W (Thompson et al. 1994). CsNitr1, *C. sativus* putative NO_2^- transporter (this study); CHL1, *A. thaliana* NO_3^- transporter ; RCH2, *B. napus* NO_3^- transporter (Muldin and Ingemarsson 1995); LeNrt1-1, *L. esculentum* NO_3^- transporter (Lauter et al. 1996); ATAC003105-6, *A. thaliana* putative NO_3^- transporter; F19K23.13, *A. thaliana* F19K23.13 gene product; PTR2-B, *A. thaliana* peptide transporter (Song et al. 1996); NTR1, *A. thaliana* histidine transport protein; PTR, *H. vulgare* peptide transporter. The region of His^{301} to Gly^{520} of Nitr1 was compared. The numbers represent the probability of the indicated branch in 100 bootstraps of the given data.

CHL1 encodes a low affinity NO_3^- transporter which transfers NO_3^- with H^+ from soil into root cells (Crawford 1994). A low affinity transporter with a K_m for NO_2^- of 0.1 - 0.2 mM could mediate the NO_2^- uptake by chloroplasts (Anderson and Done 1978, Brunswick and Cresswell 1988) and it is a H^+-NO_2^- symporter (Takahashi et al. 1998). It is likely that Nitr1 is involved in NO_2^- uptake across the chloroplast envelopes because of the sensitivity of such function to anti-Nitr1 antibodies. The molecular size of the reduced Fab fragments is about 25 kDa. The outer envelope is considered freely permeable to small molecules like NO_2^- and most transporters are located on the inner membrane of chloroplast envelopes. Additional work will be necessary to prove this function of Nitr1 in the uptake of NO_2^- by chloroplast.

Analysis of *Arabidopsis* genome sequence has revealed that only one homolog of high sequence homology (about 60%) can be found for *Nitr1* of cucumber although over 50 members of NO_3^- and peptide transporter family were identified in the genome (The *Arabidopsis* Genome Initiative 2000). It is interesting to note that the expression of *Arabidopsis* counterpart of cucumber *Nitr1* is limited in the illuminated leaves (Sugiura et al. unpublished). The study of plant with different level of *Nitr1* expression would help understanding its function in the transport of nitrogenous compound when the plants grow with different inorganic N sources.

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