A Nitrite Transporter Associated with Nitrite Uptake by Higher Plant Chloroplasts

Miwa Sugiura, Mihaela N. Georgescu and Masaaki Takahashi *

Department of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka, 599-8531 Japan

Chloroplasts take up cytosolic nitrite during nitrate assimilation. In this study we identified a nitrite transporter located in the chloroplasts of higher plants. The transporter, CsNitr1-L, a member of the proton-dependent oligopeptide transporter (POT) family, was detected during light-induced chloroplast development in de-etioliating cucumber seedlings. We detected a CsNitr1-L-green fluorescent protein (GFP) fusion protein in the chloroplasts of leaf cells and found that an immunoreactive 51 kDa protein was present in the isolated inner envelope membrane of chloroplasts. CsNitr1-L has an isoform, CsNitr1-S, with an identical 484 amino acid core sequence; however, in CsNitr1-S the 120 amino acid N-terminal extension is missing. Saccharomyces cerevisiae cells expressing CsNitr1-S absorbed nitrite from an acidic medium at a slower rate than mock-transformed control cells, and accumulated nitrite to only one-sixth the concentration found in the wild type. These results show that it is possible that both CsNitr1-L and CsNitr1-S encode efflux-type nitrite transporters, but with different subcellular localizations. CsNitr1-L may possibly load cytosolic nitrite into chloroplast stroma in the chloroplast envelope during nitrate assimilation. The presence of genes homologous to CsNitr1-L in the genomes of Arabidopsis and rice indicates that facilitated nitrite transport is of general physiological importance in plant nutrition.

Keywords: Chloroplast envelope — Cucumis sativus (cucumber) — Nitrate assimilation — Nitrite transporter — Proton-dependent oligopeptide transporter (POT).

Abbreviations: CaMV35S, cauliflower mosaic virus 35S RNA; dNTP, deoxyribonucleoside-5'-triphosphate; GFP, green fluorescent protein; POT, proton-dependent oligopeptide transporter; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription—PCR.

The nucleotide sequence reported in this paper has been registered in the European Molecular Biology Laboratory (EMBL) database under the accession number Z69370.

Introduction

Nitrate assimilation by plants is one of the two primary pathways in the biological conversion of inorganic nitrogen into reduced nitrogenous compounds in the global nitrogen cycle. Nitrate assimilation occurs mostly in leaf mesophyll cells, and the resulting reduced nitrogen species are then used to synthesize all required amino acids and amino acid-derived compounds. Reactions throughout the course of the nitrate assimilation pathway occur in separate cells and cellular compartments; nitrate is first transferred from root epidermal cells to the cytosol of leaf cells via vascular tissues, and the subsequently reduced nitrogen species move, also via vascular tissues, from chloroplasts to sink organs (Frommer et al. 1994b, Forde 2000). The nitrogenous metabolites involved in this pathway are mostly ionic at physiological pH and need specific transporters to allow them to permeate the biological membranes. The CHL1 gene, which encodes a nitrate transporter, was first cloned from Arabidopsis thaliana (Tsay et al. 1993) and functions in roots, probably in the uptake of nitrate from soil solution. By virtue of the proton/nitrate co-transport function of CHL1 (Tsay et al. 1993), CHL1 and subsequently discovered plant homologs are known as the proton-dependent oligopeptide transporter (POT) family (Paulsen and Skurray 1994). Following the discovery of CHL1, NPT1, which is also a POT, was identified as a transporter of amino acids and/or oligopeptides functioning in sink organs (Rentsch et al. 1995). Thus, assimilation of nitrate is a polygenic system involving multiple enzymes catalyzing successive conversions from nitrate to nitrite, ammonia, glutamine and glutamate, and transporters connecting the reactions taking place in different cellular compartments (Crawford and Arst 1993, Hoff et al. 1994).

Nitrite, a metabolite of nitrate assimilation, is generated in the cytosol. It must be transferred immediately to the chloroplast stroma to sustain high nitrogen use efficiency and to prevent the accumulation of toxic nitrite in the cytosol (Hoff et al. 1994). Nitrite is taken up by

*Corresponding author: E-mail, mtakahashi864@ybb.ne.jp; Fax, +81-72-254-9451.
illuminated chloroplasts in vitro (Anderson and Done 1978, Brunswick and Cresswell 1988a). Two different mechanisms have been proposed to explain this phenomenon: free diffusion of nitrous acid through a sink effect due to nitrite consumption in illuminated chloroplasts, or active transport coupled with photochemical energy transduction. Shingles et al. (1996) deduced that the rate of diffusional transfer would be comparable with that of nitrite reduction by chloroplasts if nitrite (plus nitrous acid) was present in the extra-chloroplastic space at a concentration higher than 0.5 mM at pH 4. The chloroplast stroma is separated from the cytosol by envelope membranes. Free diffusion of a weak acid through biological membranes may be possible, especially when the concentration of its acid form is high, for instance at low pH, whereas with decreasing concentrations of the acid form, active transport of the basic form becomes essential for plant growth, as observed for borate transport through root pericycle cell membranes (Takano et al. 2002). Rexach et al. (2000) found a nitrite transporter (NAR1) in *Chlamydomonas* chloroplasts, knockout of which caused nitrogen deficiency in the cell under conditions of limiting nitrate supply. Considering that the physiological concentration of nitrite can be as low as 10 μM (Siddiqi at al. 1992, Vaucheret et al. 1992, Kawamura et al. 1996, Rockel et al. 2002), a nitrite transporter might also be of importance in nitrite uptake by higher plant chloroplasts. Besides NAR1 in *Chlamydomonas* (Rexach et al. 2000), several transporters have been found to be involved in the uptake or efflux of nitrite by unicellular microorganisms: nitrate/nitrite-bispecific transporters have been found in *Synechococcus* sp. (NRTA; Omata 1995), *Escherichia coli* (NARK; Noji et al. 1989) and *Hansenula polymorpha* (YNT1; Machín et al. 2004). Homologs of the *ynt1* gene have been found in plants; however, these homologs are expressed only in roots, presumably acting as a high-affinity nitrate transporter to absorb nitrate from the soil solution (Forde 2000). Despite the importance of nitrite transfer in nitrite assimilation by higher plants, this process remains poorly understood. In our preliminary experiments on the direct measurement of high-affinity nitrite uptake by isolated chloroplasts, driven by an external acidic proton gradient (Takahashi et al. 1998), when the rate of uptake was plotted relative to the increase in nitrite concentration a saturation curve resulted. We therefore believe that an active transport process catalyzed by an as yet unknown nitrite transporter exists. More than a hundred membrane proteins in chloroplast envelopes function in the cross-talk between metabolic processes taking place in the chloroplast and other cellular compartments (Ferro et al. 2002, Schleiff et al. 2003). Here we describe the first confirmed nitrite transporter in the inner envelope of higher plant chloroplasts.

### Results

**Isolation of two POT family cDNAs with matching sequences**

Etiolated plants develop chloroplasts upon illumination via light-induced expression of photosynthesis-related nuclear genes (Thompson and White 1991). When cucumber seedlings that had been grown in the dark for 72 h after imbibition were placed under continuous light at 28°C, photosynthetic oxygen evolution began at 24 h, had risen rapidly by 36 h in parallel with the greening of cotyledons, and reached a rate of 50 μmol (mg Chl)⁻¹ h⁻¹ at 48 h, then activity slowly increased to the level seen in the mature plant (data not shown).

We constructed cDNA libraries from mRNAs of cucumber seedlings illuminated as described above for 6 and 12 h. By screening these libraries for a metal-binding thylakoid membrane protein, we found a 1,575 bp cDNA encoding an entire 484 amino acid hydrophobic protein. We named this cDNA *CsNitr1-S* because of its function and length as explained below. By 5′-RACE (rapid amplification of cDNA ends) primed with an internal sequence of *CsNitr1-S*, and using mRNAs from 2 d de-etiolating cucumber seedlings, we found another mRNA, which we named *CsNitr1-L*. The suffix -*L* was given because the mRNA encoded an isoform of *CsNitr1-S* with a 120 amino acid N-terminal extension (EMBL accession No. Z69370). Full-length *CsNitr1-L* was obtained by 3′-RACE, and its sequence was found to match exactly with that of *CsNitr1-S*, except that the sequence from bases 158 to 375 of *CsNitr1-L* is missing in *CsNitr1-S*. Analysis of the genomic DNA and cDNA sequences of *CsNitr1-L* and *CsNitr1-S* showed that bases 158–375 in *CsNitr1-L* corresponded to the second exon of the *CsNitr1-L* gene (Fig. 1). Thus, it is highly likely that the two mRNAs are transcribed from a single gene, and that a cryptic intron is recognized and excised from transcripts of the *CsNitr1-L* coding sequence, yielding the *CsNitr1-S* transcript. The *CsNitr1-S*
mRNA also appears to be spliced at a splice acceptor site 11 bp upstream from the third exon of the CsNitr1-L sequence. This alternative splicing leaves a 5'-GTTATTTG TAG-3' sequence in the CsNitr1-S mRNA, which we found in the λgt10 libraries. This 11 bp insertion in the 5'-non-coding region does not affect the translation initiation site of CsNitr1-S. The matching sequence in CsNitr1-L and CsNitr1-S is the core sequence encoding the functional transporter domain, and the additional N-terminal extension in the L-isoform might mean that it has a different targeting signal from that of CsNitr1-S.

Fig. 2 shows the amino acid sequence of CsNitr1-L (and CsNitr1-S) aligned with five homologous proteins. CsNitr1-L encodes a membrane protein with 11 transmembrane regions, as shown by the lines on the sequence labeled with Roman numerals. Between transmembrane regions V and VI, there is a 70 amino acid long hydrophilic loop. This structure of a long central hydrophilic loop interconnecting.
two pairs of 5–6 transmembrane regions is characteristic of the facilitative transporter protein superfamilies (Sadée et al. 1995). The arrow on the second transmembrane region of CsNitr1-L indicates the N-terminal methionine of CsNitr1-S, showing that the first transmembrane domain and half of the second transmembrane domain of CsNitr1-L are missing in the CsNitr1-S sequence.

Among the aligned sequences from the protein databases, At1g68570 from *Arabidopsis thaliana* (Arabidopsis Genome Initiative 2000) and AK070558, AK120596 and AK110441 from *Oryza sativa* cv. japonica (Rice Full-Length cDNA Consortium 2002) had the highest identity, 57–58%, with CsNitr1-L (and CsNitr1-S). AK070558, AK120596 and AK11041 are very similar to each other (59% identity), except that an N-terminal 107 amino acid sequence is missing from AK110441, just as observed for CsNitr1-S. It is interesting to note that the N-terminus of AK11041 is in exactly the same position as that of CsNitr1-S.

A nitrate transporter (CHL1) and other POT family members also have sequences that are homologous to that of CsNitr1-L; however, the amino acid sequence identity for CHL1 and CsNitr1-L (38.1%) is lower than the identities for CsNitr1-L and At1g68570, AK070558, AK120696 and AK110441.

**Light-dependent and nutrient-specific expression of CsNitr1-L in leaves**

Northern blot analysis revealed light-induced expression of a transcript in cucumber seedlings (Fig. 3a). The size of this transcript, about 2,100 nucleotides, agrees with the size of cloned *CsNitr1-L* cDNA [1,947 bp including a 20 bp poly(A) tail]. There was no visible signal from this transcript in the etiolated seedlings before illumination was applied (–6 h and 0 h). After 6 h illumination, a positive signal appeared in the greening seedlings. The mRNA accumulated with continued illumination and seemed to reach a plateau after 36 h. At 60 h after illumination was first applied, the cotyledons had almost completed their expansion and greening, and had a Chl content of 100 μg (mg protein)⁻¹.

*CsNitr1-S* was cloned from the cDNA libraries constructed from mRNAs of 6 and 12 h illuminated cucumber seedlings. At 6 h illumination there were no clear separate bands. *CsNitr1-S* is transcribed at too low a level in the seedlings to be detected by standard Northern blotting. Reverse transcription–PCR (RT–PCR) was conducted to determine separately the expression of *CsNitr1* transcripts during the greening of cucumber seedlings. RT–PCR amplifying the 5’-terminal sequence using mRNAs of 6 h light-grown seedlings gave two bands with a difference of about 220 bp in length (Fig. 3b). The bands were identified as 5’ fragments of *CsNitr1*-L and *CsNitr1*-S by sequence analysis. The mRNA for *CsNitr1*-L was present in the leaves (Fig. 4a, lane 2) but was limited in the roots (Fig. 4a, lane 1) of 7 d light-grown cucumber seedlings. We found that the nitrogen source also affects the level of *CsNitr1*-L expression. RNA was isolated from the first leaves of 7 d light-grown plants with different nitrogen sources. Expression of *CsNitr1*-L was seen even with no exogenous nitrogen, and was increased when plants were supplemented with nitrate (Fig. 4b, lanes 1 and 2). We thus assume that endogenous nitrate in cucumber seed cells is able to induce *CsNitr1*-L expression, given that nitrate was detected at a concentration of 17.9 (± 8.7; n = 4) nmol (g FW)⁻¹ in seedlings that had been grown for 7 d even without a nitrate supply. With ammonia as the sole nitrogen source, the level of mRNA was slightly lower than seen in the leaves of water-grown plants when expression was judged on an equal RNA basis. On an equal actin basis, there was no difference in expression irrespective of whether exogenous ammonia was supplied, suggesting that ammonia neither induced nor suppressed the nitrate-induced expression of *CsNitr1*-L (Fig. 4b, compare lane 3 with lanes 1 and 4). The possible requirement of light for transcription of *CsNitr1*-L in leaves suggests that the function of *CsNitr1*-L is coupled to photosynthesis.

![Fig. 3](https://academic.oup.com/pcp/article-abstract/48/7/1022/1855680)

(a) Total RNA (20 μg) of cucumber seedlings sampled after the indicated durations of illumination were subjected to Northern blot analysis as described in the text. Membranes were exposed to imaging plates for 3 d and read by using a Fuji Film FLA3000 imaging analyzer. A gel stained with ethidium bromide is shown in the bottom panel (labeled rRNA). (b) RT–PCR products of *CsNitr1*-L and *CsNitr1*-S in cotyledons after the indicated durations of illumination. The primers used were 5'-GCAAAGAGGT AAATAAGAATGG-3' and 5'-TTTCGGTTCTTGAAGGCCGC-3', corresponding to bases 1–22 and bases 836–817 in the *CsNitr1*-L sequence, respectively. A 10 μl aliquot of the PCR product (35 cycles) was run in a 2% agarose gel and stained with ethidium bromide.
Localization of CsNitr1-L protein in the chloroplast envelope

Determining the subcellular localization of CsNitr1 isoforms is important for determining their function in cellular metabolic processes. As described above, CsNitr1-L has an N-terminal extension. PSORT (Nakai and Horton 1999; http://psort.hgc.jp/) predicts that CsNitr1-L is targeted to the chloroplast. To test this hypothesis, we constructed transgenic tobacco plants expressing a full-length CsNitr1-L–green fluorescent protein (GFP) fusion protein. The location of the GFP fluorescence (Fig. 5b) coincides with the locations of intracellular particles emitting Chl fluorescence, as shown in Fig. 5a.

The localization of CsNitr1-L in chloroplasts was further confirmed by immunodetection of the protein in purified intact spinach chloroplasts. We chose spinach chloroplasts for the isolation of chloroplast envelopes because chloroplasts remain intact in spinach, and yield a large amount of purified envelope membranes (Keegstra and Yousif 1986). Chloroplast envelopes consist of double membranes with different densities (Cline et al. 1981, Douce and Joyard 1990). In the present study, the inner envelopes were separated from the outer ones by sucrose density gradient centrifugation of osmotically ruptured chloroplasts. Fig. 6 (right panel) shows a Western blot of the inner and outer envelope membrane proteins of spinach chloroplasts using antibodies raised against the hydrophilic central loop of CsNitr1-L (and CsNitr1-S); a single immunoreactive band of 51 kDa can be seen. The molecular mass of CsNitr1-L as deduced from the amino acid sequence is 67,580 Da, which may include the mass of an N-terminal targeting sequence that can be cleaved off on incorporation of the functional protein into an envelope membrane. Since a highly hydrophobic protein migrates faster on SDS–PAGE than expected on the basis of its molecular weight (Flügge and Heldt 1991), the immunoreactive band of 51 kDa was deemed to be the mature form of spinach Nitr1-L protein.

Fig. 6 shows that a spinach Nitr1-L protein was localized in the envelope fractions of chloroplasts and was present at higher levels in the inner envelope membranes, where many metabolite translocators for photosynthesis are present (Flügge and Heldt 1991, Ferro et al. 2002). Triose phosphate–phosphate translocator, a marker protein for the inner envelope (Cline et al. 1981, Flügge and Heldt 1991), was found as a 29 kDa band, primarily in our inner envelope preparation. Judging from the immunostaining,
Delayed accumulation of nitrite in CsNitr1-S-transformed yeast cells

Up-regulation of the transcription of CsNitr1-L by light, sequence homology to POT family members and localization in the chloroplast envelope all suggest that CsNitr1-L may be involved in transfer of a nitrogenous substance that is involved in chloroplast nitrogen metabolism. Among the possible transport functions that occur in chloroplast envelopes, nitrite transport seems the most likely candidate function for CsNitr1-L, given the above-mentioned findings. To investigate this possibility, we attempted to show nitrite transport activity by using S. cerevisiae cells transformed with CsNitr1-S.

Loddenkötter et al. (1993) succeeded in expressing a functional mature form of triose phosphate translocator in Schizosaccharomyces pombe cells, but heterologous proteins of chloroplast envelope origin were localized in the mitochondrial membranes of the host cell. With the possible integration of CsNitr1-S into plasma membranes as predicted by PSORT software (certainty = 0.640 for plasma membranes, 0.460 for the Golgi apparatus, 0.370 for endoplasmic reticulum membranes and 0.100 for the endoplasmic reticulum lumen; Nakai and Horton 1999), we introduced CsNitr1-S cDNA into respiration-deficient S. cerevisiae \( \rho^0 \) cells to reduce the undesirable incorporation of the recombinant protein into mitochondria. When CsNitr1-S was expressed in S. cerevisiae \( \rho^0 \) cells under the control of a moderately strong constitutive ADH promoter (Mumberg et al. 1995), a recombinant protein of 51 kDa was successfully detected by Western blotting. The mature form of Arabidopsis Nitr1-S is 3 kDa smaller than the CsNitr1-S expressed in S. cerevisiae, as described above. Differences in post-translational modification between yeast and plants might affect the migration distance of the S-type nitrite transporter on SDS–PAGE.

Hinze and Holzer (1985) reported that nitrite accumulated in S. cerevisiae cells when the cells were incubated with nitrite (and/or nitrous acid) at pH 5.0, although nitrite is not useful for the growth of these cells (Barnett et al. 1983) but rather is toxic to them. The S. cerevisiae \( \rho^0 \) cells transformed with the empty vector (pAAH5) absorbed nitrite at nearly a constant rate for 20 min from the start of incubation at pH 5.0 in the presence of 10 mM sodium nitrite and 20 mM glucose. Nitrite accumulation is represented by the decrease in the amount of nitrite in the supernatant relative to that in the starting solution. The averages of three different incubations are plotted with error bars (± SD).

Fig. 6 Localization of the Nitr1-L protein in the envelope membranes of spinach chloroplasts. The left panel shows a Coomassie brilliant blue-stained gel after SDS–PAGE of inner and outer envelope proteins (50 and 44 \( \mu \)g of protein per lane, respectively), and the right panel is a corresponding immunostained gel. Lane I, inner envelope membranes; lane O, outer envelope membranes. The numbers represent the positions of the indicated molecular mass standards (Amersham Pharmacia Biotechnology). The asterisk indicates triose phosphate–phosphate translocator, an inner envelope marker.

Fig. 7 Delayed accumulation of nitrite in S. cerevisiae cells expressing CsNitr1-S. Saccharomyces cerevisiae cells harboring pAAH5 (a) and two separately cloned cells harboring pAAH5:CsNitr1-S (b and c) were incubated for the indicated times at 30°C in 50 mM MES-NaOH, pH 5.0, containing 100 \( \mu \)M sodium nitrite and 20 mM glucose. Nitrite accumulation is represented by the distance of the S-type nitrite transporter on SDS–PAGE.
A nitrite transporter of higher plant chloroplasts

Table 1: Effect of CsNitr1-S expression on accumulation of nitrite in S. cerevisiae cells

<table>
<thead>
<tr>
<th>Plasmid used to transform S. cerevisiae cells</th>
<th>Intracellular nitrite nmol (10^7 cells)^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAAH5(a)</td>
<td>0.90 ± 0.27</td>
</tr>
<tr>
<td>pAAH5::CsNitr1-S(b)</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>pAAH5::CsNitr1-S(c)</td>
<td>0.13 ± 0.03</td>
</tr>
</tbody>
</table>

After incubation of yeast cells in 50 mM MES-NaOH, pH 5.0, containing 100 μM sodium nitrite and 10 mM glucose for 30 min at 30°C, intracellular nitrite was determined as described in the text. Yeast cells harboring plasmids followed by (a), (b) and (c) correspond to the cells used in Fig. 7a, b and c, respectively. Each value is the mean ± SD of three independent measurements.

Table 2: Effect of CsNitr1-S expression on uptake of aspartate, glutamate and glycine by S. cerevisiae cells

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Plasmid used to transform S. cerevisiae cells</th>
<th>Uptake rate pmol (10^7 cells)^{-1} %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>pAAH5(a)</td>
<td>0.017 ± 0.004 100</td>
</tr>
<tr>
<td></td>
<td>pAAH5::CsNitr1-S(b)</td>
<td>0.016 ± 0.003 94</td>
</tr>
<tr>
<td></td>
<td>pAAH5::CsNitr1-S(c)</td>
<td>0.014 ± 0.006 82</td>
</tr>
<tr>
<td>Glutamate</td>
<td>pAAH5(a)</td>
<td>0.018 ± 0.007 100</td>
</tr>
<tr>
<td></td>
<td>pAAH5::CsNitr1-S(b)</td>
<td>0.016 ± 0.003 89</td>
</tr>
<tr>
<td></td>
<td>pAAH5::CsNitr1-S(c)</td>
<td>0.016 ± 0.004 89</td>
</tr>
<tr>
<td>Glycine</td>
<td>pAAH5(a)</td>
<td>0.200 ± 0.028 100</td>
</tr>
<tr>
<td></td>
<td>pAAH5::CsNitr1-S(b)</td>
<td>0.195 ± 0.035 98</td>
</tr>
<tr>
<td></td>
<td>pAAH5::CsNitr1-S(c)</td>
<td>0.170 ± 0.042 85</td>
</tr>
</tbody>
</table>

Amino acid uptake was determined as described in the text. Yeast cells harboring plasmids followed by (a), (b), and (c) correspond to the cells used in Fig. 7a, b and c, respectively. Each uptake rate is the mean ± SD of two independent measurements and is also expressed as percentage (control = 100).

Nitrite accumulation in Arabidopsis mutants with a T-DNA insertion in the putative nitrite transporter gene

CsNitr1-L has a single homolog, At1g68570, in the Arabidopsis genome (Fig. 2). Three homozygous T-DNA insertion lines (Alonso et al. 2003) of At1g68570 were obtained from the Arabidopsis Biological Resource Center (ABRC) and were cultivated with 1/1000-diluted Hypoxen containing 0.76 mM nitrate and 2 mM ammonia as nitrogen sources. T-DNA is inserted in the third exon of At1g68570 in SALK_076219 and at the same position in the second intron in SALK_076121 and SALK_130095, as shown in Fig. 8a. An RT–PCR product of At1g68570, which could be detected in the leaves of Col-0 grown for 3 weeks, was completely absent in all of the three T-DNA mutant lines at the same growth stage, despite the presence of the transcript for act1 in T-DNA mutants as in Col-0 (Fig. 8b). Western blotting shows two immunoreactive bands in the leaves of Col-0 (Fig. 8c, lane 1). The major band, 51 kDa in size, was AtNitr1-L, the size of which agreed well with the molecular mass of spinach Nitr1-L (Fig. 5) in the chloroplast envelope. The other band, 105 kDa in size (marked by an asterisk to the left), could be a dimeric form, since the molecular mass is approximately twice that of the monomeric unit. Short, cold SDS treatment to avoid degradation of the AtNitr1-L molecule by proteases may be a factor in stabilization of the molecule by proteases may be a factor in stabilization of the...
AtNitr1-L was completely absent in SALK_076219, as expected from the T-DNA insertion in exon 3 (Fig. 8c, lane 2). The dimeric form was also absent in SALK_076219. The absence of both bands in SALK_076219 and the existence of both bands in Col-0 provide confirmation that the 105 kDa band is a dimeric form of AtNitr1-L. In SALK_076121 and SALK_130095, T-DNA is inserted in the same position in intron 2 at -35 bp relative to the translation start codon of At1g68570 in Arabidopsis leaves. Healthy leaves from 3-week-old Col-0 and SALK mutant plants were used for RT–PCR and Western blotting. (a) The At1g68570 locus in Arabidopsis with T-DNA insertion sites in SALK mutants. Closed boxes are exons. Horizontal arrows indicate the positions of PCR primers. (b) Expression of the At1g68570 gene in Col-0 and SALK mutants. The primers shown in (a) were used to detect the expression of At1g68570 using RT–PCR. The expression level of an actin gene was used as an internal control. A 10 μl aliquot of the PCR product (27 cycles for actin1 and 35 cycles for At1g68570) was run in a 2% agarose gel and stained with ethidium bromide. (c) Immunodetection of Arabidopsis Nitr1s in Col-0 and SALK mutants using anti-CsNitr1 antibodies. Whole leaf proteins from Col-0, SALK_076219, SALK_076121 and SALK_130095 were applied to lanes 1, 2, 3 and 4, respectively (5.0 μg of Chl per lane). The left panel shows a Coomassie brilliant blue-stained gel and the right panel shows the corresponding immunostained gel. The numbers on the left refer to the molecular mass standards in lane M (Amersham Pharmacia Biotechnology).

Fig. 8 Expression of the At1g68570 gene in Arabidopsis leaves. Healthy leaves from 27-day-old plants of each SALK mutant and Col-0 were used for nitrite determination. Leaves were frozen under light conditions to avoid an increase in nitrite concentration upon the light–dark transition (Riens and Heldt 1992) and were then homogenized with water. Chl and nitrite were determined as described in the text. Shaded and open bars show nitrite concentration in μmol (mg Chl)$^{-1}$ and in μmol (g FW)$^{-1}$, respectively. The average of three different extractions is shown with error bars (± SD).

Fig. 9 Nitrite accumulation in the leaves of At1g68570 insertion mutants. Healthy leaves from 27-day-old plants of each SALK mutant and Col-0 were used for nitrite determination. There were two immunoreactive bands with different molecular masses for both mutants: 48 and 98 kDa (Fig. 8c, lanes 3 and 4). The molecular masses are smaller than those of AtNitr1-L (Fig. 8c, compare lane 1 with lanes 3 and 4) and probably correspond to AtNitr1-S and its dimer, respectively. Despite the undetectable amount of the S-type variant in Col-0, it could be overexpressed in the mutants, probably under the control of a strong promoter in the T-DNA region, or it could adapt to modified circumstances due to the loss of AtNitr1-L function. AtNitr1-L was not expressed at all in any mutant. Growth of the T-DNA mutants was slower than that of wild-type plants, and bolting was delayed by about 1 week. Enhanced senescence of older leaves and root elongation, which are often associated with nitrogen deficiency (Marschner 1993), were seen in the T-DNA mutants when the mutants were cultivated with nitrate as the sole nitrogen source.
reductase (Vaucheret et al. 1992). Accumulation of nitrite could be a consequence of reduced nitrite uptake by chloroplasts because the L-type nitrite transporter is missing.

These results provide evidence that an efflux-type nitrite transporter exists in higher plants. Given the novel function of the nitrite transporter it encodes, we named the gene CsNitr1. CsNitr1-L mRNA encodes a nitrite transporter of the chloroplast envelope that transports cytosolic nitrite into the chloroplast stroma during nitrate assimilation.

**Discussion**

In de-etiolating cucumber seedlings, we found an mRNA encoding CsNitr1-S, a member of the POT family. Two members of the POT family from Arabidopsis have been shown to have a transport function: At1g12110 is a nitrate transporter (CHL1; Tsay et al. 1993) and At2g02040 is a histidine (and oligopeptide) transporter (NTR1; Rentch et al. 1995). In the present report, we show that CsNitr1-S is a transporter of another nitrogenous compound, nitrite, in higher plants. Three classes of nitrite transporter have so far been identified: the Chlamydomonas nitrite transporter (NAR1), which is homologous to the E. coli nitrite extrusion protein (NARK); the yeast Hansenula nitrate/nitrite-bispecific transporter (YNT1); and the Synechococcus NRTA ABC (ATP-binding cassette)-type nitrate/nitrite transporter. In higher plants, a different class of transporter, comprising members of the POT family, is herein shown to operate in nitrite uptake by chloroplasts.

The nitrite transport function of CsNitr1-S was deduced from the modified nitrite accumulation kinetics of CsNitr1-S-transformed yeast cells. Saccharomyces cerevisiae cells absorb nitrite from the growth medium at acidic pH in the presence of an energy source, as shown in Fig. 7. Nitrite accumulation was delayed when the yeast cells expressed CsNitr1-S (Fig. 7b, c). Takano et al. (2002) reported a similar effect on the borate uptake of yeast cells expressing a boron transporter of Arabidopsis. They concluded that the Arabidopsis boron transporter is involved in efflux. The delay in nitrite accumulation suggests that permeation of the cytosolic nitrite through yeast plasma membranes could be ascribed to CsNitr1-S cDNA. We therefore propose that CsNitr1-S is an efflux-type nitrite transporter.

The CsNitr1-S protein has an isoform, CsNitr1-L, the expression of which coincides with chloroplast development, especially in nitrate-fed plants. Chloroplast targeting of CsNitr1-L is predicted by PSORT (Nakai and Horton 1999). The N-terminal target sequences of envelope membrane proteins were found to be less conserved but longer in length than those for nuclear-encoded precursors of thylakoid and stromal proteins (Kammerer et al. 1998). CsNitr1-L contains characteristic structural features in the N-terminal region that have been seen in some chloroplast targeting signals: for example, the region is rich in basic and hydroxylated residues and contains a short hydrophobic region (Bruce 2000). Ferro et al. (2002) identified 54 envelope membrane proteins in spinach chloroplasts and found four common characters: (i) a predicted chloroplast target sequence at the N-terminus; (ii) fewer than 100 amino acid residues in each transmembrane domain; (iii) at least four transmembrane domains; and (iv) a pI higher than 8.8. CsNitr1-L fulfills the first three conditions, but not (iv); the pI of the putative mature form of CsNitr1-L is estimated to be 8.49 (Arabidopsis At1g68570 and rice chloroplast-type AK070558 have theoretical pI values of 8.92 and 9.70, respectively). Empirical evidence has shown that CsNitr1-L localizes to the chloroplast envelope, as expected. Nitrite uptake by the chloroplast in the course of nitrate assimilation is synonymous with an efflux of nitrite to an extra-cytoplasmic space, the chloroplast stroma. CsNitr1-L may function to load cytosolic nitrite into chloroplasts at the chloroplast inner envelope.

The results presented here may provide some molecular detail for the process of nitrite utilization by higher plants. Since nitrite uptake in higher plant chloroplasts was proposed by Brunswick and Cresswell (1988a, 1988b) to occur via active transfer, the molecular mechanisms by which this might occur have remained poorly understood. The genome of A. thaliana contains 50 POT family genes (http://www.membranetransport.org/), among which At1g68570 is the only CsNitr1-L homolog (Fig. 2). In contrast to the diversity of CHL1 and NTR1, according to phylogenetic analysis by CLUSTAL W (Thompson et al. 1994), At1g68570 has no isoforms in Arabidopsis. The purpose of diversity is to prepare multiple copies of a gene; different genetic loci may be associated with different expression patterns, while a modified sequence may confer different lifespans and catalytic activities on isoforms. Plant cells must have multiple isoforms of nitrate and oligopeptide transporters to support the long transport route of nitrate, amino acids and oligopeptides through multiple cells from the source organ to sink organs. Diversity, however, might not have evolved for At1g68570, because one copy of a nitrite transporter gene may be enough to support efficient nitrate assimilation given that nitrite is generated and metabolized within the same cell, although in separate intracellular spaces. If the function of At1g68570 is lost, it cannot be compensated for by the action of other proteins. Vaucheret et al. (1992) reported a 5-fold increase in nitrite concentration in the leaves of a nitrate reductase-deficient mutant of tobacco, which was most probably a result of the reduced nitrite consumption in chloroplasts.
This must also be the case when Arabidopsis mutants lack facilitated nitrite transfer through the chloroplast envelope. Mutation of At1g68570 resulted in accumulation of nitrite at an unusually high concentration, as expected (Fig. 9). In contrast to Arabidopsis, a strain of japonica rice naturally accumulated a large amount of leaf nitrite (Sustiprijatno et al. 2006), which was similar to the level observed in the nitrite transporter-less mutants of Arabidopsis. Overexpression of CsNitr1-L produced an inverse effect on the concentration of nitrite in rice: the leaf nitrite concentration in CsNitr1-L-transgenic rice was reduced to one-third that in the untransformed rice (Sustiprijatno et al. 2006).

Kawamura et al. (1996) reported that the average nitrite content in leaves for several dicotyledonous plant species was <10 nmol (g FW)$^{-1}$. To permit utilization of nitrite in this concentration range, a high-affinity transporter is likely to be involved. The affinity, $K_m$, of a transporter for a solute molecule can be estimated from an analysis of uptake kinetics with varying solute concentrations. The $K_m$ of CsNitr1-S for nitrite was roughly estimated from the accumulation kinetics of nitrite by yeast cells as follows. Assuming that the volume of a S. cerevisiae vegetative cell is 56 $\mu$m$^3$ on average (Woldringh et al. 1993), the nitrite concentration is estimated to be about 1.6 mM in control yeast cells on the basis of the amount of nitrite that was taken up during the first 10 min of incubation in 100 $\mu$M nitrite. If we assume the same cell volume for CsNitr1-S-transformed cells, a nitrite content of 0.06 nmol (10$^7$ cells)$^{-1}$ would give a concentration of about 0.2 mM, which is only twice the concentration of nitrite present in the incubation medium, as compared with the 16-fold increase relative to the medium in the control cells. For CsNitr1-S-transformed yeast cells, the rate of uptake for the second 10 min period of incubation was significantly faster than that for the first 10 min period (Fig. 7b, c). These upward concave kinetics might occur if the efflux rate of nitrite became saturated when the intracellular concentration of nitrite exceeded 0.2 mM. We estimated that the $K_m$ of CsNitr1-S for nitrite is around 0.1 mM using a half-saturating concentration for nitrite efflux. This value of $K_m$ corresponds to that for a high-affinity transporter. The identical amino acid sequence of the catalytic core parts of CsNitr1-L and CsNitr1-S indicates that both isoforms have the same molecular structure and, hence, the same affinity for the nitrite molecule. It is noteworthy that the $K_m$ for nitrite of CsNitr1-S (and CsNitr1-L) agrees with the half-saturating concentration of nitrite for nitrite uptake by illuminated chloroplasts (Anderson and Done 1978, Brunswick and Cresswell 1988a).

Brunswick and Cresswell (1988b) suggested that cysteine residue(s) are involved in nitrite uptake by illuminated spinach chloroplasts, because uptake was inhibited by treatment of the chloroplasts with thiol-modifying reagent. In the catalytic core sequence shared by CsNitr1-L and CsNitr1-S, four cysteine residues are conserved among all CsNitr1-L and CsNitr1-S homologs in Arabidopsis and rice, as shown in Fig. 2: two in the hydrophilic region near the N-terminus of the core sequence, one in the hydrophilic region between the membrane-spanning regions III and IV, and the last in membrane-spanning region XI. The former two cysteine residues are conserved in >35 of the 50 POT family members in Arabidopsis. Oligopeptide transport by NTRI-transformed yeast cells was inhibited by thiol modifiers (Rentsch et al. 1995). The involvement of cysteine residue(s) in the molecular mechanism of nitrite transport will be tested by further research, for example studies involving site-directed mutagenesis.

The full nucleotide sequence of CsNitr1-S completely matches that of CsNitr1-L, indicative of alternative splicing to yield two isoforms of different lengths. A peroxisomal and several chloroplastic enzymes have different isoforms targeting mitochondria or cytosol, but are encoded by single genes, respectively, using different transcription start sites (Obara 2002), different translation start codons (Glackin and Grula 1990, Watanabe et al. 2001) or alternative splicing (Yoshimura et al. 2002). Splicing a cryptic intron containing one exon results in the use of a different translation start site to encode CsNitr1-S. A short fragment of intron is occasionally retained in CsNitr1-S mRNA, as also occurs in the transcription of a hydroxypyruvate reductase (Mano et al. 2000) and a copper-binding protein (Burkhead et al. 2003), although both result in modification of the C-terminus. CsNitr1-L and CsNitr1-S are believed to have the same transport activity but possibly have different subcellular localizations. At1g68570 also encodes an S-type isoform, as exists in cucumber (Fig. 8c). In the genome of rice, however, there are two genes (AK070558 and AK120596) encoding CsNitr1-L homologs, and one copy of the S-type gene of nitrite transporter (AK110444) is located adjacent to AK070558 in chromosome 5. As described above, the steady-state concentration of nitrite in rice leaves is quite high in comparison with that in dicotyledonous plants. To utilize nitrite over such a wide concentration range, rice might have developed isoforms with different $K_m$ values for nitrite.

Another fate of nitrite in plants is nitrite-dependent nitric oxide generation in the extracellular apoplastic space (Stohr and Ulrich 2002). Nitric oxide was suggested to be a signal for light-induced de-etiolation (Beligni and Lamattina 2000). Expression of CsNitr1-S at the early stage of greening of etiolated seedlings as well as the predicted localization at the plasma membrane might assign another role for the nitrite transporter in the development of plant organs.
Materials and Methods

Plant materials

Cucumber (Cucumis sativus L. cv. Jibai) seeds were imbibed in aerated water for 24 h and grown for 72 h in the dark at 28 °C on vermiculite (GS; Nittai, Aichi, Japan) wetted with distilled water, unless otherwise indicated. Etiolated seedlings were then placed under continuous white light (120 μmol m⁻² s⁻¹).

Seeds of A. thaliana (L) Heynh. ecotype Columbia (Col-0) and corresponding T-DNA-tagged lines were provided by Dr. S. Goto (Miyagi University of Education, Sendai, Japan) and the ABRC at Ohio State University (Columbus, OH, USA), respectively. The seeds were sown on rock wool (Airrich; Taiyo Kogyo, Tokyo, Japan) wetted with water, stratified for 3 d at 4 °C in the dark and cultivated on 1/1,000-diluted Hyponex 6-10-5 medium (Hyponex Japan, Osaka) at 22 °C under continuous light (180 μmol m⁻² s⁻¹).

Cloning of CsNitr1 cDNAs

Poly(A)⁺ RNAs isolated from de-etiolating cotyledons 6 and 12 h after illumination was first applied were used to construct a cDNA library (λgt10) with a cDNA synthesis kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). Fifty thousand plaques in the λgt10 libraries were screened by plaque hybridization using radiolabeled degenerate probes [5'-TC(TG)GTCT(CAGA) (A/G)ATGTGGCA(T/C)-3'] and 5'-AG(T)CTT(TTG)CAAGA(G) ATGTGGCA(T/C)-3'], designed to isolate the cDNAs of metal-binding proteins of chloroplast thylakoids. Five positive clones in λgt10 were subcloned into pBluescript KS⁺ (Stratagene, La Jolla, CA, USA) at the Not I site and sequenced in both directions by the dyeoxy chain termination method (Sanger et al. 1977) using Sequenase Ver. 2 (Toyobo, Osaka, Japan). A 1.6 kb cDNA whose expression was preliminarily shown to be light-inducible was named CsNitr1-L and subjected to further study.

The sequence of the 5' end of CsNitr1-L mRNA was determined using a 5'-RACE kit (Life Technologies, Gaithersburg, MD, USA). Total RNA was isolated from 100 mg of fresh leaves from 7 d light-grown cucumber by using an isolation kit (RNeasy plant mini kit; Qiagen, Hilden, Germany). First-strand cDNA synthesis was primed using 5'-TTCGCCCTGCAATACCGACC-3' and 5'-CTATGTTAATCTTCTCCTTCCAA-3', which contained full-length CsNitr1-L, reverse primer [5'-CTAGGAGATTGACCCCTCT-3'] and the adaptor primer. Amplified DNA was recovered from the agarose gel, cloned into the pBluescript KS⁺ (+) vector and subjected to sequence analysis.

DNA extraction and PCR amplification of genomic DNA fragments

Genomic DNA was isolated from cucumber seedlings 10 d after germination. Leaves pulsedized in liquid N2 were mixed and incubated with a solution containing 0.15 M sorbitol, 23 mM EDTA, 0.83 M NaCl, 0.83% (w/v) cetyl trimethylammonium bromide, 0.83% (w/v) Na-lauroylsarcosine, 0.5% (w/v) sodium bisulfite and 0.125 M Tris–HCl, pH 8.25, at 65 °C for 2 h. DNA was recovered in an aqueous solution after extraction with equal volumes of chloroform/isoamylalcohol (24:1) and precipitated in 70% (v/v) ethanol. PCR (5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 45 °C and 5 min at 72 °C) was conducted using a Gene Amp 2400R PCR System (Perkin Elmer, Boston, MA, USA) in a reaction mixture (25 μl) containing 0.65 μg of cucumber DNA, 0.2 μM forward primer [5'-GGTAATTAAG AATTGAGATTGACCCCTCT-3'] (bases 8–36 of CsNitr1-L), 5'-TGGAAGTAGAGATGAAAGAGAGAAGAC-3' (bases 95–124 of CsNitr1-L) or 5'-TGGAAATAGAATGGCGAT AGATTG-3' (bases 157–182 of CsNitr1-L)], 0.2 μM reverse primer 5'-CTAATTATCTTCCCTTCCCTCCA-3' (bases 1774–1750 of CsNitr1-L), 0.2 mM of each dNTP, 2 μl of DNA polymerase (Taq; Toyobo), 1.5 mM MgCl₂ and 2.5 μl of 10× PCR buffer (Toyobo). PCR products using each primer were separated in a 1% agarose gel, extracted, quantified and sequenced.

RNA extraction, Northern blotting and RT–PCR analyses

Cotyledons of cucumber seedlings grown as above were collected at 6 and 0 h before and at 6, 15, 24, 36 and 60 h after illumination was first applied, frozen in liquid N2, and stored at –85 °C. The tissues were ground in liquid N2, mixed with 100 mM Tris–HCl, pH 8.5, containing 20 mM aurin tricarboxylic acid, 10 mM L-β-ascorbate, 100 mM EDTA, 1 M LiCl, 100 μM dNTP, 25 mM Tris–HCl, pH 8.5, and extracted with phenol–chloroform as described by Wardsworth et al. (1988). RNA was precipitated in 3 M LiCl, dissolved in 2% (v/v) potassium acetate and precipitated in 70% (v/v) ethanol. The concentration of RNA was determined spectrophotometrically assuming that 1 A₂₆₀ corresponds to 38 μg ml⁻¹.

RNA was fractionated by electrophoresis in 1.5% agarose gels containing 2.2 M formaldehyde (Sambrook and Russell 2001) and transferred in the presence of 0.01 N NaOH/3 M NaCl onto a nylon membrane (Hybond-N⁺; Amersham Pharmacia Biotech) for 2 h. The membrane was neutralized with 6× SSC (0.3 M sodium acetate and 3.0 M NaCl) and incubated with a probe in a prehybridization solution containing 0.5 M sodium phosphate, pH 7.2, 7% SDS and 1 M EDTA for 12 h at 68 °C. The probe was synthesized using a DNA labeling kit (Ready-To-Go DNA-labeling beads; Amersham Pharmacia Biotech) with [32P]dCTP (222 TBq mmol⁻¹; Amersham Biosciences, Buckinghamshire, UK) and the NorI-digested fragment of pBlutis-1, which contained full-length CsNitr1-S. The membrane was washed once with 1× SSC containing 0.1% SDS for 10 min at 25 °C and three times with 0.5× SSC containing 0.1% SDS for 10 min at 68 °C. Radioactive images on the membranes were captured using a Fuji FLA3000 imaging analyzer (Fuji Film, Tokyo).

For semi-quantitative determination of mRNA by RT–PCR, the cDNAs for CsNitr1-L and actin were amplified by PCR (1 min at 94 °C, followed by 27 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C) in reaction mixtures (25 μl) containing [32P]dCTP (740 kBq) and 0.2 μM primer solution [5'-GCCAAGAGGTA AATAAGAATG-3'] and 5'-TTTCTGTTCTTGAAGGCGC-3' (CsNitr1-L), or 5'-GGCCGGTGTTGTCCCTTCTA-3' and 5'-AGAGAATGGCTG-3' (cucumber actin; accession No. AB010922). A 5 μl aliquot of the PCR product was separated by 5% PAGE using a Tris-borate-EDTA buffer system (Sambrook...
and Russell 2001) unless otherwise stated. After the gel was fixed in 7% (v/v) acetic acid, radioactive bands were visualized by autoradiography and excised from the gel for 32P counting. PCR was performed using variable cycles to determine the logarithmic amplification for CsNitr1-L and actin. Amplification of all samples was logarithmic from 24 to 27 cycles. Therefore, 27 cycles of PCR were used to detect differences in the expression of CsNitr1-L in various tissues and under various growth conditions.

For expression analysis of At1g68570, total RNA was extracted from leaves of 3-week-old A. thaliana, 2.0 μg of which was used for cDNA synthesis as described above. Primers for At1g68570 were 5'-GGATTAAATTACGATGCCCTTTCATC TTG-C-3' and 5'-CA CCTAAGGGAGGA TATGTC-3'. Primers for actin1 (At2g37620) were 5'-TAA CCAGAAGCTATACGT GAGA-3' and 5'-CACCAC TGACGCAAACTTTACC-3'.

Preparation of antibodies

Anti-CsNitr1 antibodies were raised against a 67 amino acid central hydrophilic region of the protein fused with bacterial maltose-binding protein. A DNA fragment, encoding Lys252–Asp318 of CsNitr1-L (or Lys132–Asp198 of CsNitr1-S) was amplified from pBnitr1-L by PCR using an Ex-Taq DNA polymerase (TAKARA BIO INC., Kusatsu, Japan) with a forward primer with an EcoRI site (5'-ACAACGAGAATCCGCAAGTGGGAGC-3') and a reverse primer with a SalI site (5'-GGTGTCGCTACCTATACGGGATCGTT-3'), digested by EcoRI and SalI, and inserted into the EcoRI/SalI site of the pMal-c2 plasmid (Biolabs, Beverly, MA, USA). The E. coli TB1 cells that harbored the resulting plasmid were harvested after a 3 h culture with 0.3 mM isopropylthio-β-d-galactopyranoside at 30 °C. The fusion protein was isolated from the cell lysate by an affinity chromatography using an amylose resin (Biolabs) according to the manufacturer’s instructions, followed by gel filtration using Sephadex G-100 (Pharmacia LKB Biotechnology). The fusion protein (1 mg) was dialyzed against phosphate-buffered saline, vortexed with RIBI adjuvant (Corixa, Hamilton, MT, USA) and used to immunize rabbits. Immunization was boosted on day 28 by using the same amount of antigen emulsified as above. The antiserum was taken on day 40 and freed of anti-maltose-binding protein antibodies by passing crude antisera through a maltose-binding protein column. The column was made by conjugating BrCN-activated Sepharose 4B (Pharmacia LKB Biotechnology) with the bacterial maltose-binding protein that was obtained as above from E. coli cells transformed with the control pMal-c2 plasmid.

Functional expression of CsNitr1-S in yeast cells

Saccharomyces cerevisiae AH22 (p') cells were transformed using the LiCl method (Oeda et al. 1985) with the pAAH5 expression vector (Ammerer 1983). A NotI–SacI fragment of pBnitr1-L was blunted and inserted into the filled HindIII site of the pAAH5 vector. The direction of CsNitr1-S in pAAH5::CsNitr1-S was confirmed from the size of the NotI–PstI restriction fragment. Saccharomyces cerevisiae cells harboring the pAAH5::CsNitr1-S plasmid were selected on a plate containing 0.67% (w/v) N-base without amino acids (Difco Laboratories, Detroit, MI, USA), 2% (w/v) glucose and 20 μg ml⁻¹ histidine at pH 5.4 (Sherman 1991). Expression of CsNitr1-S in the yeast cells was confirmed by Western blotting. The recombinant yeasts were liquid cultured at 30 °C in the same medium as above to an A600 of 0.6, at which time cell number (colony-forming units) was estimated.

Agrobacterium-mediated transformation of tobacco

Tobacco (Nicotiana tabacum cv. petite Havana SR1) was transformed by leaf disc infection with Agrobacterium tumefaciens LBA4404 (Rogers et al. 1986) harboring a derivative of the pBI21 vector to express a fusion protein, CsNitr1-L–GFP (sGFP(S65T)). The binary vector and the A. tumefaciens strain were obtained from Clontech Laboratories (Palo Alto, CA, USA). A DNA sequence encoding full-length CsNitr1-L was obtained from pBnitr1-L by PCR with Ex-Taq DNA polymerase using a forward primer with an XbaI site (5'-AACCTAGGACCAAAGGGTAATAAGA ATGGG-3') and a reverse primer with a BamHI site (5'-CACCAGCCTATCTTATAGTTGC-3'). The PCR product was digested by XbaI and BamHI and inserted into the XbaI–BamHI sites of a pUC18 derivative into which a 0.4 kb cauliflower mosaic virus 35S RNA (CaMV35S) promoter, a 1 kb sGFP(S65T) and a 0.2 kb terminator of the nopaline synthase gene had previously been inserted in the HindIII–XbaI, BamHI–PstI and PstI–EcoRI sites, respectively, by Niwa et al. (1999). In-frame fusion of the C-terminal alanine of CsNitr1-L to the N-terminal methionine of sGFP(S65T) with a glycine-serine linker was confirmed by DNA sequencing. The constructed vector was digested with XbaI and EcoRI, and the resulting CsNitr1-L::sGFP(S65T)::NOS terminator was inserted between the XbaI and EcoRI sites of pBI21 to make the vector pBI21::CsNitr1-L::sGFP(S65T). This vector was used to transform A. tumefaciens LBA4404 via electroporation as described by Walkerpeach and Velten (1994). Transgenic tobacco plants (T0) grown from calli of infected leaf cells were screened on Murashige-Skoog plates (Murashige and Skoog 1962) containing 100 μg ml⁻¹ kanamycin. Fluorescence images of GFP and Chl in leaf epidermal cells of 40 day T1 transgenic plants were obtained using the GFP(R) and B-2A excitation-emission filter sets, respectively, and a Nikon Eclipse E800 fluorescence microscope (Nikon, Tokyo) equipped with an Olympus DP70 digital camera (Olympus, Tokyo).

Preparation of chloroplast envelope membranes and Western blotting

Inner and outer envelope membranes of chloroplasts were isolated from spinach leaves. Spinach leaves from a local farm were homogenized using an isolation buffer containing 5 mM HEPES-KOH, pH 6.8, 0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂ and 1 mM sodium pyrophosphate. Homogenates were filtered through four sheets of Miracloth (Calbiochem, San Diego, CA, USA), and 4 ml of the filtrate was layered over 10 ml of a Percoll density gradient (Pharmacia LKB Biotechnology) from 20% (v/v) to 80% (v/v) in the isolation buffer. After centrifugation at 17,800 x g for 10 min using a RPS40T rotor and a Hitachi CP-36GFI centrifuge (Hitachi, Tokyo, Japan), intact chloroplasts were isolated. Intact chloroplasts were broken by two cycles of freezing and thawing, and were then centrifuged in a discontinuous sucrose density gradient to obtain inner and outer envelope membranes as described by Cline et al. (1981).

Aliquots of the envelope membrane preparations were lysed with 2% (w/v) SDS, 6 M urea and 1 mM phenylmethylsulfonyl fluoride at 5 °C, and subjected to SDS–(12%) PAGE using the method of Laemmli (1970) within 10 min after SDS treatment. Protein bands were blotted on a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) using the semi-dry blot method for 2 h at 2 mA cm⁻² in the presence of 0.1 M Tris, 0.192 M glycine, 5% (v/v) methanol and 0.05% SDS. The membrane was immuno-decorated with 1/1,000-diluted antiserum for 2 h at 25 °C, then with 1/5,000-diluted alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA), and stained using the

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nitra blue tetrazolium–bromochloroindolyl phosphate method (Sambrook and Russell 2001).

Measurement of nitrite and amino acid uptake by yeast cells

The recombinant S. cerevisiae cells cultured as described above were precipitated by centrifugation at 5°C, then washed twice with and suspended in 5 mM sodium phosphate, pH 7.2. The yeast cells (0.4 × 10⁷ cells) were incubated in 250 µl of 50 mM MES-NaOH, pH 5.0, containing 100 µM sodium nitrite and 20 mM glucose, at 30°C with shaking. After being incubated for 10 or 20 min, the reaction mixtures were centrifuged at 10,000×g for 1 min at 5°C, and an aliquot of the supernatant was subjected to nitrite determination. To collect cellular fluid for the direct determination of intracellular nitrite, the pellets were washed three times with 0.5 ml of 5 mM sodium phosphate, pH 7.2, by 10 s centrifugation, suspended in 100 µl of the same buffer, vortexed with an equal volume of acid-washed glass beads and centrifuged at 10,000×g for 5 min at 5°C. The nitrite concentration in the supernatant was then determined.

For measurement of amino acid uptake, the S. cerevisiae cells (2.1 × 10⁸ cells) were incubated in a reaction mixture (500 µl) containing 10 mM glucose, 5 mM sodium phosphate, pH 7.2, and 8.10 µM [14C]glycine, 16.4 µM [14C]aspartate or 1.12 µM [14C]glutamate for 15 min at 30°C. The reaction was stopped by filtration through a cellulose acetate filter (0.8 µm pore size; Advantec, Tokyo, Japan). The yeast cells were washed three times with 5 ml of 5 mM sodium phosphate, pH 7.2, and lysed in 5 ml of Clearsol (Nacalai Tesque, Kyoto, Japan) to determine the radioactivity entrapped in the cells.

Nitrite, nitrate, Chl and protein assays

Nitrite was determined according to the method of Hageman and Reed (1980). For the measurement of nitrite in leaf homogenates, protein impurities were removed prior to determination by the addition of zinc acetate followed by 2 min centrifugation at 300×g. Nitrate was determined as described by Riens and Heldt (1992). Chl and protein were quantified by using the methods of Arnon (1949) and Lowry et al. (1951), respectively.

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