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Original Paper

Two-component high-affinity nitrate transport system in barley: Membrane localization, protein expression in roots and a direct protein-protein interaction

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Abstract A high affinity transport system (HATS) for nitrate in plants is operated by a two-component NRT2/NAR2 transport system. However, the regulation and localization of NRT2 and NAR2 at protein level are largely unknown and especially so in crop plant species. In this study with barley (*Hordeum vulgare*), membrane localization, protein expression in the roots, and a direct protein-protein interaction of HvNRT2 and HvNAR2 proteins were investigated. Immunochemical analysis showed that both HvNRT2 and HvNAR2 proteins were co-localized in the plasma membrane of the roots. Expression of HvNRT2 and HvNAR2 proteins was more strongly induced by treatment with higher concentrations of external nitrate, while HATS activity and transcripts for *HvNRT2* and *HvNAR2* were markedly repressed. An affinity column binding analysis using recombinant proteins suggests that the C-terminus of HvNRT2.1 is possibly involved in its binding to the HvNAR2.3 central region and that the Ser463 present in the HvNRT2.1 C-terminus plays a role in the binding ability.

Key words: Barley, membrane localization, NAR2, nitrate, NRT2.

Nitrate is one of the major sources of nitrogen for higher plants and is taken up from the soil by active transporters coupled with H^+ across the plasma membrane (PM) of root cells. Nitrate uptake systems have been classified into two groups: low-affinity transport systems (LATS) and high-affinity transport systems (HATS). The LATS contribute to nitrate uptake at high nitrate concentrations above 1 mM whereas the HATS operate at micromolar concentrations of external nitrate and display Michaelis–Menten kinetics saturating at 0.2–0.5 mM nitrate. The HATS are further divided into two categories: constitutive HATS (cHATS) and inducible HATS (iHATS), which are significantly affected by the supply of external nitrate. Many studies of the molecular basis of nitrate uptake reveal the existence of two gene families, namely the *NRT1* and *NRT2* families, which potentially encode for LATS and HATS respectively. *NRT2* genes are identified in a variety of organisms including fungi, certain yeasts, green algae, and higher plants (Unkles et al. 1991; Quesada et al. 1994; Trueman et al. 1996; Pérez et al. 1997; Quesada et al. 1997; Amarasinghe et al. 1998; Zhuo et al., 1999; Araki and Hasegawa 2006; Tsujimoto et al. 2007). In most species, NRT2 genes are members of a multigene family: for example, seven Arabidopsis genes (*AtNRT2.1-AtNRT2.7*) and four rice genes (*OsNRT2.1-OsNRT2.4*) have been found in their genomes (Orsel et al. 2002; Araki and Hasegawa 2007), and at least four NRT2 genes (*HvNRT2.1-HvNRT2.4*) exist in barley (Vidmar 2000a). Amino acid sequences deduced from these genes indicate that the NRT2 proteins are typically 480–510 amino acids in length and predicted to be integral to membranes with 12 transmembrane helices (Forde 2000).

It has been well documented that iHATS activity is strongly induced by nitrate supply, and is down-regulated by the accumulation of nitrate assimilation products, especially ammonium and glutamine (Crawford and Glass 1998). In several plant species, it has been shown that a particular member of the *NRT2* gene family (e.g., *NpNRT2.1* for *Nicotiana plumbaginifolia*, *AtNRT2.1* for *Arabidopsis*, *HvNRT2.1* for barley) contribute to iHATS, because those transcript levels are highly correlated with changes in iHATS activity in such species (Krapp et al. 1998; Lejay et al. 1999; Zhuo et al. 1999; Vidmar et al. 2000a). Furthermore, *Arabidopsis* T-DNA-insertion

Abbreviations: HATS; high-affinity transport system, PCR; polymerase chain reaction, PM; plasma membrane

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mutants disrupting the *AtNRT2.1* gene or both *AtNRT2.1* and *AtNRT2.2* genes showed significant reduction in iHATS activity by 70 to 80% (Cerezo et al. 2001; Filleur et al. 2001; Li et al. 2007).

However, functional analysis using *Xenopus* oocytes reveal that *NRT2* genes from *Chlamydomonas*, barley and *Arabidopsis* required co-expression with a second type of gene, *NAR2*, to exhibit nitrate transport activity (Zou et al. 2000; Tong et al. 2005; Orsel et al. 2006). At present, two *Arabidopsis NAR2* genes (*AtNAR2.1* and *AtNAR2.2*) and three very similar barley *NAR2* genes (*HvNAR2.1-HvNAR2.3*) have been thoroughly analyzed functionally (Zou et al. 2000; Tong et al. 2005). Among the *NAR2* members from barley and *Arabidopsis*, only HvNAR2.3 and AtNAR2.1 could give nitrate transport activity in the oocyte system when they co-expressed with their putative partner protein, HvNRT2.1 and AtNRT2.1, respectively. Furthermore, it was confirmed that *Arabidopsis* mutants (*atnar2.1-1*) disrupted in *AtNAR2.1* by T-DNA insertion show a greater reduction in HATS activity by more than 90%, even though *AtNRT2.1* is normally expressed (Okamoto et al. 2006; Orsel et al. 2006). It is now generally accepted that HATS is operated by a two-component NRT2/NAR2 transport system.

A strong correlation between HATS activity and transcript levels of *NRT2* gene suggest that the regulation of HATS takes place preferentially at the transcriptional level. In *N. plumbaginifolia*, *Arabidopsis* and barley, *NRT2.1* gene and HATS activity are induced by nitrate and repressed by glutamine (Krapp et al. 1998; Lejey et al. 1999; Vidmar 2000b). However, several reports suggest that the regulation of HATS activity involves posttranscriptional regulation of *NRT2.1*. In *N. plumbaginifolia*, transgenic plants over-expressing the *NpNRT2.1* gene showed a constitutive high level of *NpNRT2.1* transcript, but HATS activity was markedly decreased by addition of ammonium, similar to the wild type (Fraisier et al; 2000). Likewise, in barley, ammonium accumulation in roots of plants treated with the glutamine synthetase inhibitor methionine sulfoximine, decreased root HATS activity but did not change the *HvNRT2.1* transcript level (Vidmar 2000b). Despite these evidences, the detailed mechanisms involved in the post-transcriptional regulation of NRT2 have long been uncertain due to the lack of an antibody for NRT2 protein.

Recently, in *Arabidopsis* localization and regulation of AtNRT2.1 has been studied at protein level using a green fluorescent protein (GFP) fusion strategy and an immunological approach (Chopin et al. 2007; Wirth 2007). In transgenic plants expressing AtNRT2.1-GFP fusion protein, a strong fluorescence was detected mainly in the PM of root cortical and epidermal cells. Interestingly, immunoblot analysis revealed the existence of the major band at 45 kDa (it is probably a monomeric form of AtNRT2.1) and higher molecular mass complexes in both total microsomes and in PM from the roots. Surprisingly, a band at 45 kDa disappeared totally in microsomes from the roots of the mutant *atnar2.1-1*. This result strongly suggests that AtNAR2.1 protein is involved in the expression of AtNRT2.1 protein in the PM. Furthermore, it is revealed that levels of the major form of AtNRT2.1 have not changed in response to the light-dark transition of plants that quickly reduces both *AtNRT2.1* transcript level and HATS activity. Thus, the occurrence of posttranslational regulatory mechanisms of AtNRT2.1 was also revealed.

However, it is still unclear whether the higher molecular mass complexes of NRT2 protein exist commonly in other plant species, whether these complexes play a role in nitrate uptake, and how NRT2 protein interacts with NAR2 protein. Here, membrane localization, protein expression in roots and a direct protein-protein interaction of barley HvNRT2 and HvNAR2 proteins were investigated using immunochemical techniques and an affinity-column binding assay. Our data show that both HvNRT2 and HvNAR2 proteins are co-localized in the PM of barley roots, and the accumulation of HvNRT2 and HvNAR2 proteins is enhanced by the supplement of higher nitrate concentration while HATS activity is strongly repressed. The affinity-column binding assay revealed that the C-terminus of HvNRT2.1 binds to a central loop of HvNAR2.3. Furthermore, the substitution of a serine residue in the C-terminus of HvNRT2.1 results in loss of binding to HvNAR2.3 protein.

Materials and methods

Plant materials and growth conditions

Seven-day-old seedlings of barley (*Hordeum vulgare* L. cv Kawahonami) were used in all physiological experiments. Seeds were surface-sterilized with 1% (v/v) aqueous hypochlorite solution and rinsed with de-ionized water. The seeds were covered with water-moistened paper towel and placed at 23°C in the dark. After 3 d, the seedlings were transferred to 2-L plastic tanks, and grown with N-free solution for 4 d in a growth cabinet (MLR350, SANYO, Osaka, Japan) at 25°C under continuous illumination (250 μEm^{-2} s^{-1}), as previously described (Abdel-Latif et al. 2004). The composition of N-free nutrient solution was as follows: $CaCl_2$ 0.5 mM, $MgSO_4$ 0.2 mM, KH_2PO_4 2.0 mM, Fe(III) EDTA 25 μM, H_3BO_4 50 μM, $MnCl_2$ 9.0 μM, $CuSO_4$ 0.3 μM, $ZnSO_4$ 0.7 μM, $NaMoO_4$ 0.1 μM. The pH of the nutrient solution was adjusted to 5.5 with 1 M KOH. For nitrate treatment of 7d-old seedlings, either 1 mM or 10 mM KNO_3 was added to N-free nutrient solution. The roots were harvested at an appropriate time after the nitrate addition, immediately frozen in liquid nitrogen and stored at −80°C until use.

Purification of total RNA

Total RNA was isolated from frozen root tissue with slight modification of the procedure described by Chemczynski and Sacchi (1987). In brief, each sample (0.5 g) was ground to a fine powder in liquid nitrogen and homogenized with 1 mL of denaturing solution (4 M guanidine isothiocyanate, 50 mM sodium citrate, 1% sarkosyl, 100 mM 2-mercaptoethanol). The homogenate was mixed with 1 mL of water-saturated phenol, 1 mL of chloroform and 0.2 mL of 2 M Na-acetate (pH 4.0), vigorously shaken and centrifuged. Then, an equal volume of iso-propanol was added to the aqueous phase to precipitate RNA. The pellet was washed twice with 3 M Na-acetate (pH 5.2) and once with 70% (v/v) aqueous ethanol. After dissolving the final pellet in distilled water, the RNA was re-precipitated with 1/10 vol of 3 M Na-acetate (pH 5.2). The pellet was collected by centrifugation, washed with 70% (v/v) aqueous ethanol and dried. The dried pellet was dissolved in distilled water and stored −80°C until use.

Cloning of HvNRT2.1 and HvNAR2.3 cDNAs

Full-length cDNAs of *HvNRT2.1* and *HvNAR2.3* were obtained by the PCR-based cloning method. Total RNA isolated from the barley roots which had been treated with 1 mM KNO_3 for 4 h, was transcribed into first-strand cDNA with SuperScript II (Invitrogen, Carlsbad, CA, USA) and oligo$(dT)_{18}$ primer following the manufacturer's instructions. The regions containing the open reading frame of *HvNRT2.1* and *HvNAR2.3* were amplified from the first-strand cDNA by PCR with primers 5′-ATGGAGGTCGAGGCGGGCGC-3′ and 5′-CTCAAAGCTGTTGTAAATTC-3′ for *HvNRT2.1* (accession No. U34198); and 5′-CCCAGCTCCTCTCCTCTCTT-3′ and 5′-GTCCCGACTTGCGAGTTTAG-3′ for *HvNAR2.3* (accession No. AY253450), respectively. The amplified DNA fragments of *HvNRT2.1* (1733 bp) and *HvNAR2.3* (683 bp) were subcloned into a pCR2.1 vector (Invitrogen) and fully sequenced.

Northern blot analysis

For northern blot analysis, equal amounts of total RNA were electrophoresed in agarose/formaldehyde gels and blotted onto nylon membrane (Gene Screen Plus, NEN, Boston, MA. USA). RNA blots were hybridized with ^{32}P-labeled probes for *HvNRT2* and *HvNAR2* mRNAs and washed according to Church and Gilbert (1984), and then exposed to X-ray film. For preparation of hybridization probes, the 1333 bp fragment from +400 to +1733 of *HvNRT2.1* was amplified from a full-length cDNA clone with primers 5′-GTCCGCTTTCTCATTGGC-3' and 5′-CTCAAAGCTGTTGTAAATTC-3′, and a full-length *HvNAR2.3* cDNA was excised from the plasmid with EcoR I. These fragments were separated in 1% agarose gels and purified by a MonoFas DNA purification kit (GL Sciences, Tokyo, Japan). The probes were labeled with [α-^{32}P]dCTP by Prime-a-Gene Labelling system (Promega, Madison, WI, USA).

Expression of recombinant polypeptides and production of antibodies

The recombinant polypeptides for the C-terminus of HvNRT2.1 (HvNRT2.1 C-term) and the central loop of HvNAR2.3 (HvNAR2.3 cent) were individually expressed in *E. coli* BL21-SI using the Gatewey system (Invitrogen) according to the manufacture's instructions. The DNAs encoding the HvNRT2.1 C-term (corresponding to residues 431-507) and the HvNAR2.3 cent (corresponding to residues 76-138) were amplified from the plasmids containing full-length HvNRT2.1 and HvNAR2.3. The primer sequences used were as follows: HvNRT2.1 C-term, 5′-GGGGACAAGTTTGTACAAAAAA-GCAGGCTTGCCCGTCGCTCTTGT-3′ and 5′-GGGGACC-ACTTTGTACAAGAAAGCTGGGTAGTCTCATACGTGCT-GGG-3′; HvNAR2.3 cent, 5′-GGGGACAAGTTTGTACAAA-AAAGCAGGCTTGAAGGTGAGCCTCTGCTAC-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCGTAGGCG CGCACGTAGTAA-3′. Each DNA fragment was cloned into donor vector pDONR221, and subsequently transferred to expression vector pDEST17, which generated a N-terminal polyhistidine (6xHis)-tagged fusion polypeptide. The polypeptides, the HvNRT2.1 C-term (77 amino acid residues) and the HvNAR2.3 cent (63 amino acid residues) were over-expressed in *E. coli* BL21-SI, the bacterial extracts purified using a TALON Metal Affinity Resin (CLONTEC, Mountain View, CA, USA), and used to immunize rabbits. Each antibody was purified from antisera using an antigen-immobilized affinity column.

Preparation of membrane fractions

The frozen barley roots were homogenized in a blender with extraction buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, 5 mM dithiothretol, and 1 μM Leupeptin. Homogenates were filtered through four layers of gauze and centrifuged at 15,000×g for 20 min. Supernatants were centrifuged at 100,000×g for 30 min to precipitate the microsomal fractions. The pellet was resuspended in washing buffer (50 mM HEPES-KOH-pH7.0, 39 mM KCl, 330 mM sorbitol, 0.1 mM EDTA, 1 μM DTT) and gently homogenized in a Potter homogenizer. The plasma membranes were separated from the other membranes by aqueous two-phase partitioning according to the method of Lasson et al. (1987). A microsomal pellet was added to 36-g phase partitioning system (6.5% dextran T-500, 6.5% polyethylene glycol 3350, 5 mM potassium-phosphate (pH7.8), 4 mM KCl, and 250 mM Sucrose). After centrifugation at 2,000×g for 20 min, each phase was mixed and partitioned twice with fresh lower buffer. Then each final phase was diluted with washing buffer and centrifuged at 100,000×g for 30 min to precipitate the membranes. Pellets from the upper phase (plasma membrane enriched fraction) and the lower phase (endomembrane fraction) were resuspended in pH 7 K buffer (50 mM HEPES-KOH-pH 7.0, 39 mM KCl, 330 mM Sorbitol, 0.1 mM EDTA, 1 μM Leupeptin), gently homogenized in a Potter homogenizer and stored at −80°C until use.

Immunoblot analysis

The proteins were solublized from the membrane fraction suspended in pH 7 K buffer by mixing with n-Octyl-β-D-glucoside at a final concentrations of 50 mM. Protein concentrations were determined using a DC protein Assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. Equal amounts of proteins (15 μg) were subjected to 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to PVDF membranes

(Immobilon-P, Millipore, Bedford, MA, USA). The membranes were blocked with 1×TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dry milk, incubated with primary antibodies for 3 h and secondary antibody for 3 h, and washed three times with 1×TBST. The membrane proteins were reacted with primary rabbit antibodies against HvNRT2.1 C-term (anti-NRT2), HvNAR2.3 cent (anti-NAR2), plasma membrane aquaporin (anti-PIP1), ER lumen protein (anti-Bip) and vacuolar H^+-ATPase subunit a (anti-V-ATPase). The dilutions of antibodies were as follows: 1 : 100 dilution for Anti-NRT2 and anti-NAR2, 1 : 3000 dilution for anti-PIP1, anti-Bip and anti-V-ATPase. Reactive proteins were detected with secondary antibody, peroxidase-conjugated goat anti-rabbit IgG (1 : 2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, U.S.A) using ECL Western blotting Analysis System (GE Healthcare, Buckinghamshire, UK) and imaging analyzer (LAS-3000, FUJIFILM, Tokyo, Japan).

Affinity binding assay of HvNRT2.1 C-term and HvNAR2.3 cent

The interaction of native and mutated HvNRT2.1 C-term with HvNAR2.3 cent, which immobilized to the column was investigated. To introduce mutation into the HvNRT2.1 C-term, four serine residues (Ser449, Ser463, Ser482, Ser484) and two threonine residues (Thr453, Thr503) present in HvNRT2.1 C-term were replaced individually with alanine residues using the QuickChange II site-directed mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacture's instructions. For introducing mutation, PCR was performed with plasmid pDEST17 containing the coding region for HvNRT2.1 C-term and site-directed mutagenesis primers. The sets of primers used here are as follows: S449A (5′-TCTTCCCTGCCATCG-CCGACGCCAC-3′ and 5′-GTGGCGTCGGCGATGGCAGG-GAAGA-3′); S463A (5′-CCTCGGAGTGGGCCGAAGAG-GAGAA-3′ and 5′-TTCTCCTCTTCGGCCCACTCCGAGG-3′); S482A (5′-TTGCTGAGAATGCCCGCTCGGAGCG-3′ and 5′-CGCTCCGAGCGGGCATTCTCAGCAA-3′); S484A (5′-AGAATTCCCGCGCGGAGCGCGGTAG-3′ and 5′-CTA-CCGCGCTCCGCGCGGGAATTCT-3′); T453A (5′-GCGCC-GACGCCGCGGAGGAGGAGTA-3′ and 5′-TACTCCTC-CTCCGCGGCGTCGGCGC-3′); T503A (5′-CACCCAACA-ATGCGCCCCAGCACGT-3′ and 5′-ACGTGCTGGGGCGC-ATTGTTGGGTG-3′). The mutated polypeptides were individually expressed in *E. coli* BL21-SI and purified using a TALON Metal Affinity Resin as described above. Native and mutated polypeptides of HvNRT2.1 C-term (0.1 mg each) were individually applied to the HiTrap HP column (1 ml) (GE Healthcare), in which 0.5 mg of HvNAR2.3 cent was immobilized. After washing the column with a 10-fold volume of 1×TBS, polypeptides were eluted by 0.1 M glycine-HCl (pH 2.5) and immediately neutralized with 1/10 vol of 1 M Tris–HCl (pH 8.5). The eluted protein was subjected to SDS-PAGE and detected by immunoblotting using an anti-HvNRT2 antibody.

NO_3^- uptake activity

Seven-day-old seedlings were transferred to 0.2 L of an unlabeled pretreatment solution containing either 1 mM or 10 mM NO_3^- and incubated for 2, 4, 8, 24 or 30 hours. After pretreatment, the seedlings were transferred to an unlabeled uptake solution containing 100 μM NO_3^- for 5 min to equilibrate roots to the conditions to be employed for uptake determination. They were then transferred to 0.2 L of uptake solution containing 100 μM $^{15}NO_3^-$ (atom% ^{15}N: 70.6%). After a 20-min uptake period, plants were transferred back to a 0.2-L vessel of unlabeled solution for 3 min to remove unabsorbed tracer residing in the cell wall space. Roots and shoots were harvested separately, dried overnight at 80°C and ground to a fine powder. The ^{15}N abundance was measured using both a stable isotope mass spectrometer (Delta C; Thermo-Finnigan, Bremen, Germany) and an elemental analyzer (EA1108, Fisons Instruments, Milan, Italy). The rates of $^{15}NO_3^-$ uptake were calculated from the ^{15}N content of both the roots and shoots.

Protein assay

Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Hercules, USA) using BSA as a standard.

Results

Immunological detection of HvNRT2 and HvNAR2 proteins in membrane fractions

A search of the ARAMEMNON database indicated that HvNRT2.1 has 12 transmembrane helices and hydrophilic N- and C-termini, and that HvNAR2.3 has a long central loop between two transmembrane helices (Figure 1A). Therefore, the C-terminus of the HvNRT2.1 (HvNRT2.1 C-term) and the central hydrophilic region of the HvNAR2.3 (HvNAR2.3 cent) were expressed in *E. coli* to obtain the recombinant polypeptides in the soluble form. As shown in Figure 1B, both polypeptides were successfully expressed in *E. coli* and homogeneously purified. The size of the bands at 11 kDa and 10 kDa is exactly matched to the predicted molecular weight of His-tagged HvNRT2.1 C-term and HvNAR2.3 cent. Then, the purified polypeptides were used to immunize a rabbit for antibody production.

The affinity-purified anti-NRT2 and anti-NAR2 were tested by immunoblotting with total microsomal membranes isolated from nitrate-induced barley roots. As shown in Figure 2A, a single band at the position of 50 kDa and 21 kDa was clearly detected with anti-NRT2 and anti-NAR2, respectively. The size of the 50 kDa protein band is not consistent with the molecular mass of 54.7 kDa for HvNRT2.1 calculated from the deduced amino acid sequence. This could be explained by the hydrophobic nature of the protein. Indeed, a discrepancy between apparent and theoretical molecular mass has been observed with other membrane-associated proteins, such as ammonium (Loque et al. 2006) and nitrate transporters (Liu and Tsay 2003; Chopin et al. 2007, Wirth et al. 2007), which all showed immunoblot bands with lower molecular mass than those calculated from their deduced amino acid sequences. In contrast, the band at 21 kDa is consistent with the predicted molecular

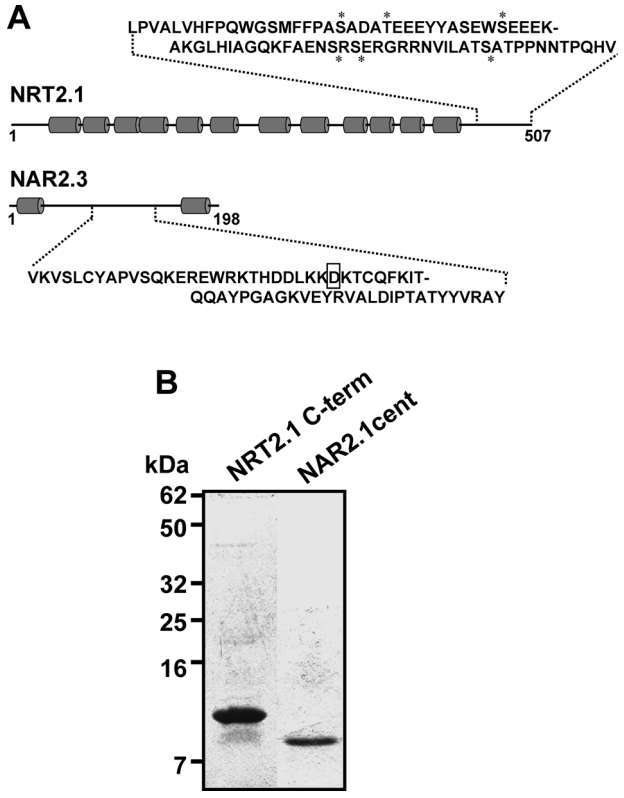

Figure 1. Production of recombinant polypeptides for C-terminus of HvNRT2.1 and the central loop of HvNAR2.3. (A) Transmembrane (TM) helices prediction of HvNRT2.1 and HvNAR2.3 by searching the ARAMEMNON database. Gray cylinders indicate the location of TM helices. The amino acid sequences indicated were the regions expressed in *E. coli* BL21-SI. Alanine-substituted serine and threonine residues in C-terminus of NRT2.1 are marked by asterisks and the functionally important aspartate residue in the central loop of HvNAR2.3 is boxed. (B) Recombinant polypeptides for C-teminus of HvNRT2.1 (HvNRT2.1 C-term) and the central loop of HvNAR2.3 (HvNAR2.3 cent) were homogenously purified from *E. coli* extracts using a metal affinity resin and subjected to a SDS-PAGE (15% gel).

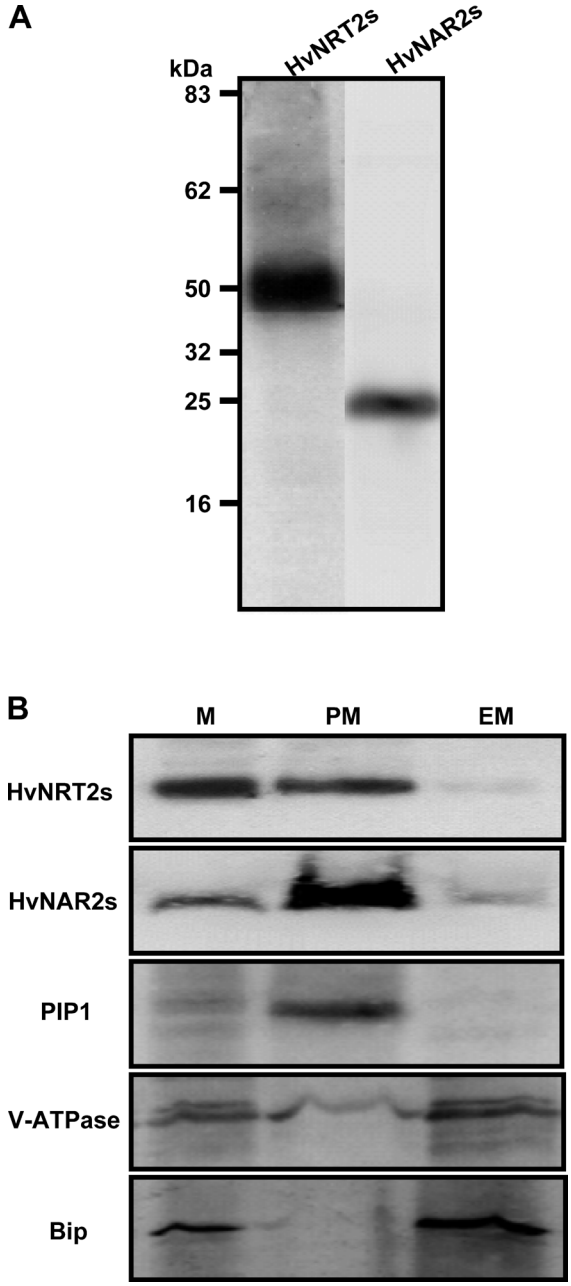

Figure 2. Immunological analysis of HvNRT2 and HvNAR2 in root cell membranes. (A) Immunoblot for HvNRT2 and HvNAR2 using total microsomes extracted from roots of 7-day-old barley seedlings. (B) Immunoblot for HvNRT2, HvNAR2, PIP1 (PM marker), V-ATPase (vacuolar marker), Bip (ER marker) using PM purified from roots of 7-day-old barley seedlings. Proteins were solubilized from microsomes (M), PM and endomembranes (EM), and separated on 12% SDS-PAGE gels (15 μg of protein/lane).

weight for HvNAR2.3. This is due to the low hydrophobic nature of the HvNAR2, which is assumed to have only two membrane spanning regions in the whole amino acid sequence (Figure 1A). However, the cross-reactivity of each antibody to other HvNRT2 or HvNAR2 family members could not be excluded, due to the significant similarity in the molecular mass and amino acid sequences among family members. Therefore, the band detected with our antibodies was indicated as HvNRT2s and HvNAR2s in the Figures.

To verify the membrane localization of HvNRT2 and HvNAR2 proteins, membrane fractions from the barley root were separated by the aqueous two-phase partitioning method and each fraction was subjected to immunoblot analysis. It was shown that HvNRT2s and HvNAR2s were predominantly detected in the PM-enriched upper fraction as was plasma membrane aquaporin (PIP1), whereas vacuolar H^+-ATPase subunit A (V-ATPase) and ER lumen protein (Bip) were detected in the lower fraction enriched in endosomal membranes (Figure 2B). These results indicate that both HvNRT2s and HvNAR2s are localized mainly at the root PM. Similar to the results with the microsomal membrane, only the single band at 50 kDa and 21 kDa was detected by anti-NRT2 and anti-NAR2 in the purified PM fraction.

Time profile of $^{15}NO_3^-$ uptake activity and transcript and protein accumulation for HvNRT2 and HvNAR2 in response to nitrate

The effect of treatment of different nitrate concentrations (1 and 10 mM) on the $^{15}NO_3^-$ uptake rate (HATS activity) and the accumulation of transcripts and proteins for HvNRT2 and HvNAR2, was investigated during various induction periods (0–30 h). The induction pattern of HATS activity in the barley plants treated with 1 mM or 10 mM nitrate were essentially similar; the uptake activity was nearly zero at 0 h, it increased rapidly during the first 8 h and then increased gradually until 48 h. However, maximum HATS activity in plants pretreated with 10 mM nitrate was about 50% lower than in the plants pretreated with 1 mM nitrate (Figure 3A). Northern blot analysis showed that the rapid accumulation of HvNRT2 and HvNAR2 transcripts in barley roots occurred within 2 to 4 h after treatment with 1 mM or 10 mM nitrate. Then, the levels of HvNRT2 transcripts decreased to undetectable levels 30 h after 1 mM nitrate treatment and 24 h after 10 mM nitrate treatment (Figure 3B). These results were consistent with the results previously reported by Vidmar et al. (2000a). On the other hand, the levels of HvNAR2 transcripts slowly increased with treatment with 1 mM nitrate and reached the highest levels at 30 h. When plants were treated with 10 mM nitrate, HvNAR2s transcripts reached maximum levels within 4 h and then decreased steadily (Figure 3B).

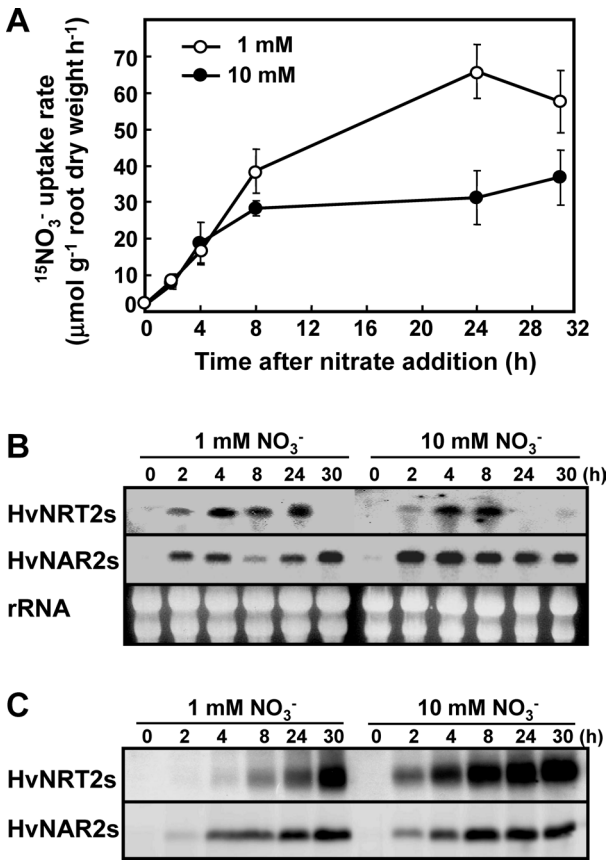

Figure 3. Time profile of nitrate uptake rate and transcript and protein accumulation for HvNRT2 and HvNAR2 in response to provision of nitrate. (A) Effect of pretreatment with different nitrate concentrations (1 and 10 mM) on the $^{15}NO_3^-$ uptake rate. Seven-day-old seedlings were pretreated with 1 mM or 10 mM nitrate for 2, 4, 8, 24, and 30 h, and then root nitrate uptake rate were measured at 100 μM $^{15}NO_3^-$. The values are means of 6 replicates (±SE). Effect of treatment with 1 and 10 mM nitrate on the accumulation of transcripts (B) and proteins (C) for *HvNRT2* and *HvNAR2*. After treatment of barley seedlings with 1 or 10 mM nitrate, total RNA and microsomes were prepared from the roots and subjected to northernblot and immunoblot for HvNRT2 and HvNAR2 as described in 'Materials and Methods'.

Immunoblot analysis showed that HvNRT2 and HvNAR2 proteins in the microsomal fraction of barley roots were detected within 2 h after 10 mM nitrate supply and 4–8 h after 1 mM nitrate supply, they then increased almost linearly until 30 h. The expression level of HvNRT2 protein in roots supplied with 10 mM nitrate was significantly higher than that of plants supplied with 1 mM nitrate (Figure 3C), while no significant difference in the peak levels of HvNAR2 protein between the two nitrate treatments was observed.

Protein-protein interaction between HvNRT2.1 C-terminal and HvNAR2.3 central region

It has long been suggested that the phoshorylation of NRT2 protein is somehow involved in the regulation of the HATS function as was shown to be the case for AtNRT1.1 (Liu and Tsay 2003). Indeed, there are several predicted phosphorylation sites (serine and threonine residues) in the C-terminus of HvNRT2.1 (Figure 1). On the other hand, in the *rnc1* mutant of *Arabidopsis*, it has been shown that a conserved aspartate residue located in the central region of NAR2 protein is important for exhibiting HATS activity (Figure 1, Kawachi et al. 2006). Therefore, the possibility of protein-protein interaction between HvNRT2 C-term and HvNAR2 cent was studied by affinity-column binding analysis. In this experiment, the effect of alanine-substitution of six predicted phosphorylation sites (Ser449, Thr453, Ser463, Ser482, Ser484, Thr503) in HvNRT2 C-term was also investigated. Native, and each of the substituted HvNRT2.1 C-terms were separately applied to the column immobilized with HvNAR2.3 cent. After washing the column, bound polypeptide were eluted by acidic solution and detected by immunoblotting using an anti-HvNRT2. It was clearly shown that the native HvNRT2.1 C-term can interact with HvNRT2.3 cent and substitution of Ser463 to alanine (S463A) in HvNRT2.1 C-term significantly reduced its binding ability to the HvNAR2.3 cent (Figure 4). However, the substitution of other residues in HvNRT2.1 C-term did not affect the interaction between HvNRT2.1 C-term and the HvNAR2.3 cent (Figure 4). It was also confirmed that immunoreactivities of six alanine-substituted proteins

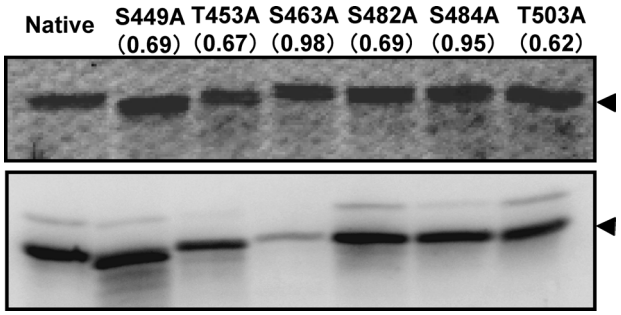

Figure 4. Protein-protein interaction between HvNRT2.1 and HvNAR2.3. Native and mutated HvNRT2.1 C-term, where four serine residues (Ser449, Ser463, Ser482, Ser484) and two threonine residues (Thr453, Thr503) were individually substituted with alanine. These were expressed in *E. coli* and purified by affinity resin. Purified proteins were subjected to 15% SDS-PAGE gels and stained and confirmed with Coomassie blue (upper panel). Native and mutated polypeptides of HvNRT2.1 C-term (0.1 mg each) were individually applied to the HiTrap HP column (1 ml), in which 0.5 mg of HvNAR2.3 cent was immobilized. After washing the column with the buffer, bound polypeptides were eluted by 0.1 M glycine-HCl (pH 2.5), subjected to SDS-PAGE and detected by immunoblotting using an anti-HvNRT2 antibody (lower panel). The values in parentheses are the phoshorylation score for each residue predicted by NetPhos 2.0 sever.

were similar to that of native HvNRT2.1 C-term, and that non-specific interaction between the proteins and resin matrix did not occur (data not shown).

Discussion

It is now accepted that HATS activity is operated by a two-component NRT2/NAR2 transport system. However, the regulation and localization of NRT2 and NAR2 at protein level is largely unknown, especially in crop plant species. This is due to the lack of antibodies against target proteins involved in HATS function in such plants. Here, we report for the first time the generation of two antibodies against HvNRT2 and HvNAR2 proteins that are able to detect both proteins in the same species. With our antibodies, only a single band corresponding to each HvNRT2 and HvNAR2 protein was detected in microsomes and PM fractions from barley roots. In *Arabidopsis*, however, several forms of the AtNRT2.1 proteins (the monomeric form and also one or two higher molecular-mass complexes) were detected in cell membranes (Wirth et al. 2007). The discrepancy in the number of bands detected for NRT2 protein between the two species is not yet clear. The confirmation of the NRT2 form in root PM from other plant species will be important for the future. Immunochemical analysis showed clearly that both the HvNRT2 and HvNAR2 proteins are localized mainly in the PM of barley roots. Previously, it has been shown that co-expression of HvNRT2.1 with HvNAR2.3 in oocytes could only allow nitrate transport activity (Tong et al. 2005). Our results suggest that these two proteins may need to co-exist in the PM to exhibit nitrate transport activity in barley roots. In *Arabidopsis*, it has been reported that AtNRT2.1 protein is absent in PM from the *nar2.1-1* roots suggesting that AtNAR2.1 protein is involved in the targeting of AtNRT2.1 to the PM (Wirth et al. 2007). Furthermore, a "esecretory pathway signal" at the N-terminal of NAR2s is commonly predicted from the amino acid sequence (Orsel et al. 2006). Taken together, it is assumed that NAR2 protein plays a role, not only in establishing HATS activity but also in the targeting of NRT2 to the PM. In a future study, it will be necessary to clarify whether NRT2 and NAR2 proteins are present as a complex in the PM.

The time profile of HATS activity and the transcript levels for *HvNRT2* and *HvNAR2* during nitrate induction period, clearly indicate that iHATS is the main component of HATS activity in this barley cultivar, cv 'Kawahonami'. In contrast, HATS activity was very high in the barley cv 'Steptoe' before nitrate addition, that suggests the existence of cHATS in this cultivar (King et al. 1993). Thus, the presence of cHATS activity seems to depend on cultivar. In the present experiments, it is shown that both HATS activity and HvNRT2 transcript levels increase with the same time dependence during the initial induction period and then, after peaking, the HvNRT2 transcript level rapidly declines to undetectable levels while HATS activity remains constant. This inference is supported by the fact that the HvNRT2 proteins remain abundant for several hours, even after the complete disappearance of NRT2 transcripts (Figure 3). In *Arabidopsis*, it has been shown that the protein synthesis inhibitor cycloheximide has only a slow effect on AtNRT2.1 abundance in PM indicating that the AtNRT2.1 protein is relatively stable (Wirth et al. 2007).

In the present study, it was shown that the HATS activity and HvNRT2 transcript levels in barley plants treated with 10 mM nitrate were significantly repressed when compared with those in plants treated with 1 mM nitrate. This probably reflects a greater down-regulation by larger internal nitrogen pools under such conditions. In contrast, the expression of HvNRT2 proteins was enhanced by the supply of 10 mM nitrate. Thus, the changes in the levels of HvNRT2 proteins in response to excess nitrate supply were totally opposite to those of HATS activity. These results suggest that HATS activity is regulated at post-translational level, perhaps by the modification of HvNRT2.1 which is a main component of barley HATS (Vidmar et al. 2000a). As one of the possibilities for post-translational control of NRT2, a phosphorylation/dephosphorylation mechanism has been proposed because several predicted phosphorylation sites are found in the hydrophilic C-terminus of all NRT2 sequences available so far (Forde 2000). However, the existence of post-translational regulation of barley HATS is still uncertain because the cross-reactivity of antibody

for the HvNRT2.1 C-term to other HvNRT2 family members could not be excluded. For accurate quantification of HvNRT2.1 protein, the generation of mono-specific antibody for HvNRT2.1 will be required.

Previous studies using the yeast split-ubiquitin system suggest that the C-terminus of AtNRT2.1 is somehow involved in its interaction with AtNAR2.1 because the AtNRT2-NubG constructs (where AtNRT2 is fused to the N-terminus domain of the ubiqutin at their C-terminus) never show interaction in combination with AtNAR2.1 in this system (Orsel et al. 2006). However, the region of NAR2 involving in interaction with NRT2 has not been clarified although the functional importance of aspartate residue in the central loop of NAR2 for HATS activity was suggested (Kawachi et al. 2006). In this study, affinity-column binding analysis clearly showed that a direct protein-protein interaction occurred between the HvNRT2.1 C-terminal and the HvNAR2.3 central region. So far, the membrane topologies of both NRT2 and NAR2 have not been defined. To understand how HvNRT2.1 and HvNRT2.3 form complexes in plasma membranes, the membrane topologies of both proteins should be determined experimentally. The alanine-substituted mutant of HvNRT2.1 C-term, S463A, showed a marked reduction in its binding ability to HvNAR2.3 cent indicating that the hydroxyl group of Ser463 of HvNRT2.1 is particularly important for interaction between two proteins. Here, we propose that the phosphorylation/dephosphorylation of Ser463 in HvNRT2.1 may be involved in the regulation of HATS activity or PM targeting of NRT2.1 itself through the association or dissociation of the HvNRT2.1/HvNRT2.3 complex. At present, this idea is difficult to apply to *Arabidopsis* because the serine residue similar to Ser463 of HvNRT2.1 is not conserved in AtNRT2.1.

Here, for the first time, we present evidence that both HvNRT2 and HvNAR2 proteins are co-localized in the plasma membrane of barley roots. This suggests that the two proteins form a complex in the PM. Our results also show a direct protein-protein interaction between the C-terminus of HvNRT2.1 and the central loop of HvNAR2.3. To better understand the regulation of nitrate uptake, an answer will be necessary to the question of how NRT2 and HvNAR2 interact in living cells.

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