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Cloning and Functional Characterization of a *Brassica napus* Transporter That Is Able to Transport Nitrate and Histidine*

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A full-length cDNA for a membrane transporter was isolated from *Brassica napus* by its sequence homology to a previously cloned *Arabidopsis* low affinity nitrate transporter. The cDNA encodes a predicted protein of 589 amino acid residues with 12 putative transmembrane domains. The transporter belongs to a multigene family with members that have been identified in bacteria, fungi, plants, and animals and that are able to transport a range of different nitrogen-containing substrates, including amino acids, peptides, and nitrate. To identify the substrates of this plant gene, we have expressed the protein in *Xenopus* oocytes. The properties of the transporter are consistent with a proton cotransport mechanism for nitrate, and the voltage dependence of the K_m for nitrate was determined. The K_m for nitrate was shown to increase from 4 to 14 mM as the membrane voltage became more negative from −40 to −180 mV. Oocytes expressing the gene could accumulate internal nitrate to concentrations higher than those measured in water-injected controls. A range of different substrate molecules for the transporter was tested, but of these, histidine gave the largest currents, although the affinity was in the millimolar range. The pH dependence of the activity of the transporter was different for the substrates, with histidine transport favored at alkaline and nitrate at acid external pH. Kinetic analysis of the mechanism of histidine transport suggests a cotransport of protons and the neutral form of the amino acid, with the K_m for histidine decreasing at more negative membrane voltages. This gene is the first member of this family of transporters for which the transport of two very different types of substrate, nitrate and histidine, has been demonstrated.

A family of mammalian peptide transporters (1, 2) has been identified, and sequence comparisons have shown that it includes plant peptide transporters (3–5). The mammalian peptide transporters have been shown to transport a broad range of substrates, including di- and tripeptides (2) and free amino acids (6). The family members are characterized by all having a consensus motif, and they have been named the proton-dependent oligopeptide transporter (POT) family (7), or as most members are peptide transporters, they have also been called the PTR family (8). However, the family also includes plant members that have been identified as nitrate transporters (9, 10). In this paper, we show that another member of this family, isolated from the plant *Brassica napus*, can transport both the amino acids and nitrate when expressed in *Xenopus* oocytes.

In soil, the nitrate concentrations can vary from >1 μM to >10 mM depending on factors such as rainfall and fertilizer supply (11). Soil also contains other forms of nitrogen, including ammonium and amino acids, and these may also be nitrogen sources available to plants (*e.g.* Ref. 12). Nitrate uptake by plants has been shown to have biphasic kinetics, with different affinities for external nitrate; one uptake system has K_m values for nitrate in the micromolar range, and the other in the millimolar range (13). Furthermore, these transporters show differing patterns of induction by nitrate, with two different high affinity systems, only one of which is nitrate-inducible; the other is constitutively expressed (14). Examples of each type of gene have been cloned: a low affinity transport system (9) and a high affinity transport system (15).

After isolating a transporter gene, the best way to characterize the electrophysiological properties of the protein is to heterologously express the transporter in *Xenopus* oocytes. The advantage of this approach is that the activity of the transporter in the oocyte plasma membrane can then be assayed by measuring the nitrate-elicited current. In addition, in these experiments, the membrane voltage, a parameter that is usually variable in most uptake experiments, can be controlled. The control of membrane voltage is achieved by using the two-electrode voltage-clamp technique on an oocyte that is expressing the transporter. A low affinity nitrate transporter from *Arabidopsis* has been cloned, and its K_m for nitrate was determined when expressed in *Xenopus* oocytes. The K_m for nitrate of the *AtNRT1* (formerly called *CHL1*) gene expressed in oocytes was measured as 8.5 mM at −60 mV, but the voltage dependence of this property was not determined (16). This characterization was performed using oocytes in a mannitol-based external solution, in place of the more usual NaCl-based oocyte saline. This solution was chosen because *AtNRT1* may also be able to transport chloride (17), and a nitrate-elicited current may be hidden in the background chloride current that would be present in a more typical saline. However, we have used a typical saline to demonstrate nitrate transporter activity in oocytes injected with *BnNRT1;2* (alternative name *BnNRT1B*) cRNA. Furthermore, we employed the activity of endogenous anion channels to demonstrate that oocytes expressing this related transporter from *Brassica napus*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U17987.

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(*BnNRT1;2*) can accumulate nitrate, but not chloride. These results show that this low affinity nitrate transporter is able to transport several different nitrogen-containing molecules; however, the transport requires high external concentration of the substrate, suggesting that if present in root cells, it is most likely to function in uptake of nitrate from the soil when it is available at high concentrations. However, the production of mRNA for the transporter is induced in roots by treatment with only low external concentrations of nitrate.

EXPERIMENTAL PROCEDURES

Plant Material—Oilseed rape seedlings (*Brassica napus* L. cv. Kentan nova) were grown hydroponically for 1 week and supplied with a nitrogen-free but otherwise complete nutrient solution at pH 6.5 (18). The plants were maintained in a growth chamber under a photon flux density of 200 μmol m^{-2} s^{-1} (400–700 nm) with a diurnal light cycle of 16 h of light followed by 8 h of darkness. The temperature and humidity were kept at 25 °C and 80%, respectively.

Isolation of RNA and Synthesis and Cloning of cDNA—Total RNA from roots supplied with 25 mM KNO_3 for 3 h was phenol/chloroform-extracted and purified according to Öhlen *et al*. (18) and subsequently used for preparing poly(A)$^+$ RNA with oligo(dT)$_{25}$-Dynabeads (Dynal, Oslo, Norway). The mRNA was converted to cDNA using a TimeSaver cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden), ligated to dephosphorylated λgt10 arms, and *in vitro* packaged (Stratagene). Approximately 2.0×10^5 plaques from the unamplified λgt10 cDNA library were screened at medium hybridization stringency. The [^{32}P]dCTP-labeled probe was prepared from *CHL1* cDNA (kindly provided by Dr. N. Crawford) using a DECAprime DNA labeling kit (Ambion Inc.). Positive clones of interest were subcloned into pBluescript II KS$^+$ (Stratagene), amplified in XL1-Blue, and sequenced using the Sequenase II kit (Amersham International, Buckinghamshire, United Kingdom) and synthetic oligonucleotide primers.

Subcloning—For expression of the *BnNRT1;2* transporter in the *Xenopus* oocytes, the cDNA insert was excised from pBluescript, blunted, and inserted into the *Bgl*II site of the dephosphorylated *Xenopus* expression vector pSP64T. This vector provides 5′- and 3′-flanking sequences from the *Xenopus* β-globin gene to any cDNA that provides its own initiation codon (19, 20).

Ribonuclease Protection Assay (RPA)[1]—The expression of *BnNRT1;2* mRNA in roots was deduced by a ribonuclease protection assay (HybSpeed™ RPA, Ambion Inc.). A probe for the 3′-untranslated region was used to minimize cross-hybridization to related transcripts since cDNA cloning and Southern analyses indicated that there are several homologous genes in the rape genome. The RPA analysis was performed according to the manufacturer's recommendations using 25 μg of root total RNA and 3×10^4 cpm of the gel-purified riboprobe. All samples were normalized to 50 μg of RNA with yeast total RNA. The 388-base pair RNA probe was transcribed and labeled with [^{32}P]UTP to high specific activity (MAXIscript, Ambion Inc.) after digestion of the plasmid with *Bpu*A1. Digestion of the probe target mRNA was performed with RNase T only, as the 3′-untranslated region is rather AU-rich. RNA was extracted as described above, before as well as 1 and 6 h after the addition of 100 μM KNO_3.

cRNA Preparation—A full-length cDNA for the *B. napus* gene *BnNRT1;2* was constructed in a *Xenopus* expression vector (pSP64T). The construct was linearized by digestion with *Bam*HI, and cRNA was transcribed and capped using an SP6 mRNA mMachine™ *in vitro* transcription kit (Ambion Inc.) according to the manufacturer's instructions.

Oocyte Preparation and Injection—Oocytes were removed and treated as described previously (21). Stage V or VI oocytes (22) were chosen for injection with 50 nl of *BnNRT1;2* cRNA (1 μg μl^{-1}) or 50 nl of diethyl pyrocarbonate-treated water and were assayed for transporter activity 4–5 days after injection.

Electrophysiology—Oocyte currents were measured using the two-microelectrode voltage-clamp method as described previously (21). A mannitol bath saline containing 0.15 mM $CaCl_2$, 230 mM D-mannitol, and 10 mM HEPES was used to demonstrate the function of the nitrate transporter *CHL1* (*AtNRT1;1*) from *Arabidopsis* in *Xenopus* oocytes (9); this solution was chosen because the transporter may have been able to transport chloride ions (17). We attempted to use this saline, but in control water-injected oocytes, we found that an inward current was elicited by the application of a range of different external salt solutions, the size of which depended on the concentration applied (data not shown). To avoid these background problems for all subsequent measurements, we decided to use a more usual frog saline containing 116 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.2). Other details of the electrophysiology methods have been described previously (21).

Each *I-V* curve was performed at the maximum steady-state current before, during, and after any treatments, and only in cases where identical before and after results were obtained were data used for further analysis. Substrate-elicited currents were obtained by subtracting the currents measured before addition from those obtained during the addition of substrate. Exposure to nitrate was for <1 min to minimize the accumulation of nitrate within the cell. At any given membrane potential, steady-state currents measured as a function of external substrate concentration were fitted to single Michaelis-Menten functions by a nonlinear least-squares method using SigmaPlot software (Jandel Scientific, Erkrath, Germany). These fits yielded the maximal currents (i_{max}) and the half-maximal nitrate concentrations (K_m) (23). In all experiments, membrane potentials were allowed to adjust for at least 5 min after changing the external pH before any treatments were applied.

To demonstrate the activity of BnNRT1;2 in driving the accumulation of nitrate inside oocytes, we took advantage of the presence of an already characterized endogenous anion channel activity in the oocyte plasma membrane. These anion channels are activated by transient clamping of the oocyte membrane voltage to values more negative than −150 mV and so are described as being hyperpolarization-activated with a selectivity sequence of $I^- > NO_3^- > Br^- > Cl^-$ (24). By deliberately stimulating the opening of these channels in oocytes immersed in solutions containing either 120 mM nitrate or chloride and then measuring the associated tail currents, we could determine the internal concentrations of nitrate and chloride. For measurement of the reversal potentials, the membrane channel currents were activated by a 3-s prepulse voltage step to a range of different test voltages, from −150 to −200 mV, and then the voltage was clamped to values between −80 and +10 mV in 10-mV steps. The decay phases of the tail currents were fitted to an exponential function, and the extrapolated initial amplitudes were plotted as a function of tail potential. The interpolated x intercept of this plot was taken as the reversal potential (see Ref. 24). The internal nitrate concentrations were then estimated from the reversal potential (E) using the Nernst equation with 120 mM external nitrate. These measurements of internal nitrate were then checked by measuring the internal nitrate concentration of the oocytes with nitrate-selective microelectrodes. These electrodes were made and used as described previously (25).

Uptake of ^{14}C-Labeled Histidine—Five cRNA-injected oocytes and 12 water-injected oocytes were incubated in 1 ml of saline at pH 5.5 for 30 min at 20 °C, after which they were all transferred to 1 ml of saline containing 5 mM ^{14}C-labeled L-histidine (Amersham International) for a further 1 h at the same temperature. Uptake was then terminated by transferring the oocytes five times into 2-ml washes of ice-cold unlabeled saline. Finally, individual oocytes were transferred to plastic vials, each containing 0.2 ml of 5% (w/v) SDS, and then 4 ml of scintillation mixture (Packard Ultima Gold) was added. The radioactivity accumulated by each oocyte was measured on a Packard liquid scintillation analyzer (Model 2500TR).

RESULTS

Isolation of Clones and Sequence Analysis—The screening of the λgt10 library demonstrated that cDNA homologous to the *AtNRT1* cDNA (*CHL1*) was rather abundant; ~10 positive plaques of 100,000 were obtained. Sequence analysis revealed that one of the cDNA clones (*BnNRT1;2*) (26) practically represented a full-length clone, 2.0 kilobase pairs in length. It contains an open reading frame corresponding to a 65-kDa protein consisting of 589 amino acids. By sequence comparisons, *BnNRT1;2* was shown to be highly homologous to *AtNRT1* both at the nucleotide level (85%) and at the level of deduced amino acid sequence. The protein corresponding to the 2.0-kilobase pair clone contains 12 hydrophobic amino acid segments. These putative membrane-spanning regions are separated into two groups, six in each, with a long putative cytoplasmic loop in the middle containing charged amino acids. The proposed topology of the protein is almost identical to the one

[1] The abbreviations used are: ribonuclease protection assay; MES, 2-(*N*-morpholino)ethanesulfonic acid.

TABLE I
Comparison of the amino acid sequence identity of BnNRT1;2 to various mammalian and plant members of the PTR family

Name	Identity in the amino acid sequence to *BnNRT1;2*	Substrate and source reference
	%	
AtNRT1 (*CHL1*)	96.9	Nitrate transport (9)
LeNRT	82.4	Nitrate transport (10)
AtPTR2B	65.9	Peptide transport (3, 26)
HPEPT1	56.0	Peptide transport (2)
PepT1	51.7	Peptide/histidine transport (6)
PHT1	51.2	Peptide transport (1)
AtPTR2A	50.9	Peptide transport (4)

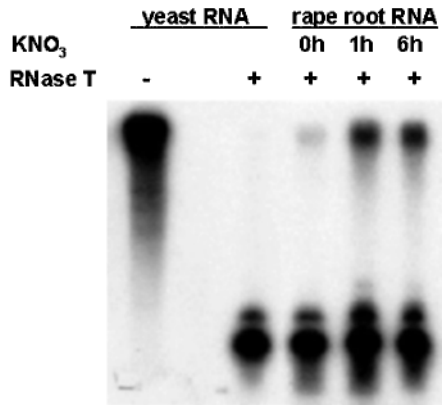

FIG. 1. **Northern blot analysis showing root-specific expression and nitrate induction of *BnNRT1;2*.** Shown are the results from RPA analysis of total RNA extracted from *B. napus* roots before (0 h) as well as 1 and 6 h after the addition of 100 μM KNO_3. The *left* part of the autoradiogram shows the probe yeast RNA control, undigested and after digestion. All digestions were carried out with RNase T, and the same amount of probe was added to each lane.

predicted for *AtNRT1* (*CHL1*) (see Ref. 9), and it retains the consensus motif (FY*XX*IN*X*GSL) described for the protein family (7, 8). A comparison of the amino acid identity between *BnNRT1;2* and some other members of the family is shown in Table I. The comparison shows the most similarity to the *Arabidopsis* (9) and tomato (10) homologues, which are 97 and 82% homologous to *BnNRT1;2*, suggesting that both of these are nitrate transporters. The identity comparison with other members of the family suggests that they are more distantly related, and they have all been shown to be peptide transporters (Table I).

Root mRNA Expression—Significant hybridization of the 3′-untranslated region of the *BnNRT1;2* clone to total RNA from roots was only obtained if the roots had been pretreated with nitrate, as deduced from RPA analysis (Fig. 1). This result clearly demonstrates that the gene coding for this specific protein is highly nitrate-regulated. The same pattern, as seen here at 100 μM nitrate, can also be seen after the addition of 10 μM nitrate (data not shown), indicating that the sensitivity of the system for nitrate perception is very high.

Steady-state Nitrate-dependent Currents in Xenopus Oocytes Injected with BnNRT1;2 cRNA—The steady-state currents of the transporter activity were measured as a function of membrane voltage and nitrate concentration in oocytes that had been previously injected with *BnNRT1;2* cRNA. No nitrate-elicited currents for water-injected oocytes could be measured (data not shown). Fig. 2*A* shows the *I-V* difference curves obtained from a cRNA-injected oocyte on treatment with sodium nitrate ranging from 1 to 20 mM at pH_o 5.5. The maximal currents varied between oocytes and were typically in the range 150–200 nA, although currents of up to 300 nA were recorded in some oocytes. Inward currents became larger at more negative membrane potentials and also increased as a function of nitrate concentration, saturating at 15 mM nitrate. A maximum current of −180 nA was measured in this experiment. These *I-V* difference curves could all be fitted to a Michaelis-Menten function; the fits for −100, −140, and −180 mV are shown in Fig. 2*B*. The voltage dependence of the kinetic parameters obtained from the full set of fitted data is shown in Fig. 3 (*A* and *B*). Both K_m and i_{max} are voltage-dependent; K_m increased from 4 mM at −40 mV to 9 mM at −140 mV (Fig. 3*A*), and i_{max} increased from −20 nA at −20 mV to −270 nA at −160 mV (Fig. 3*B*).

Reversal Potential and Nitrate Permeability of Hyperpolarization-activated Currents—Reversal potentials were determined from the tail currents for both water- and cRNA-injected oocytes (see "Experimental Procedures"), and values are shown in Table II. In saline containing 128 mM Cl^-, the reversal potentials were −15.7 and −20.3 mV for *BnNRT1;2*- and water-injected oocytes, respectively (Table II). These values are not significantly different and are close to the oocyte chloride ion equilibrium potential (−18 mV), assuming an internal concentration of 62 mM (27). This result suggests that the internal chloride concentrations are not significantly different between cRNA- and water-injected oocytes. However, when external chloride was replaced by a similar concentration of nitrate, the reversal potentials were significantly different between *BnNRT1;2*- and water-injected oocytes: −18.8 mV for the former and −50.5 mV for the later (Table II). Using the Nernst equation, the estimated internal nitrate concentrations from the reversal potentials are 17 mM in water-injected oocytes and 57 mM nitrate in *BnNRT1;2*-injected oocytes (Table II). The oocytes were voltage-clamped at −50 mV when exposed to 120 mM nitrate before the tail current measurements. At this membrane potential, the oocytes could passively accumulate 17 mM nitrate, which is much lower than that estimated from the reversal potential in *BnNRT1;2*-injected oocytes. However, the resting potentials of oocytes prior to voltage clamping are also shown in Table II; from these, the equilibrium concentrations could also be calculated, and these are shown Table II. The measurements of internal nitrate were confirmed using nitrate-selective microelectrodes (Table II). The results in Table II show that cRNA-injected oocytes accumulate more nitrate than similar water-injected oocytes. However, in no case were the accumulations of nitrate large enough to demonstrate that active transport of nitrate is occurring (*i.e.* the measured accumulation was significantly larger than that predicted from the Nernst equation or equilibrium potential).

Other Substrates for the Transporter—Although the gene was isolated by its sequence homology to a low affinity nitrate transporter, it belongs to a family of transporters that includes peptide and amino acid proton cotransporters, we tested other substrates in oocytes that had been injected with *BnNRT1;2* cRNA. These experiments showed that the oocytes showed larger currents when supplied with histidine than when treated with nitrate. However, several different substrates were tested, including other basic amino acids and anions. The results of these currents relative to the histidine currents for a range of different amino acids are shown in Table III. Only the basic amino acids lysine and arginine appeared to be transported; the other amino acids tested did not elicit significant currents in oocytes injected with *BnNRT1;2* cRNA when compared with water-injected controls (Table III). Micromolar concentrations of ammonium did not elicit currents, and concentrations above 0.5 mM gave large currents in both cRNA- and water-injected oocytes. Also, D-histidine, free imidazole, and

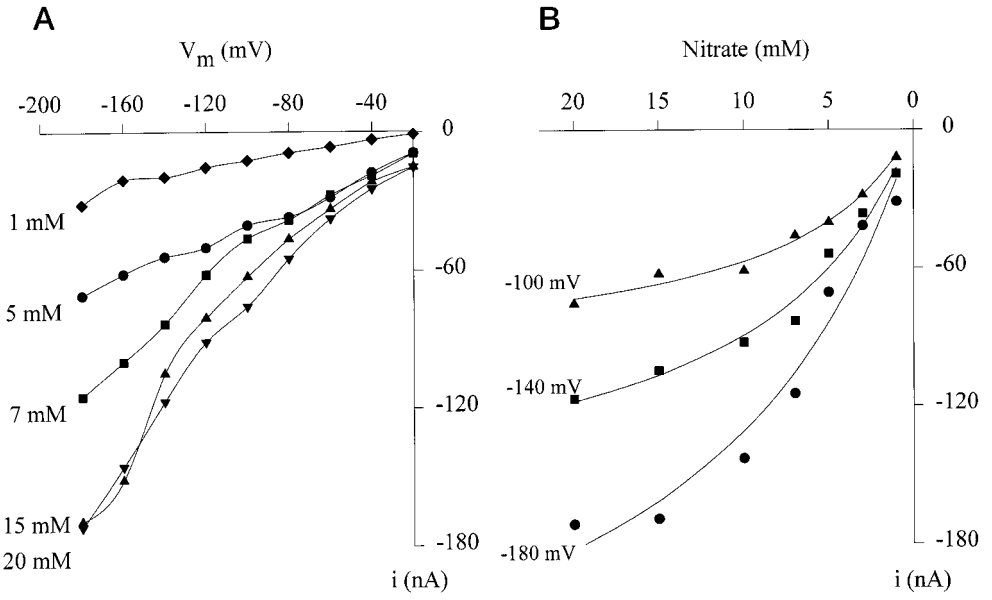

FIG. 2. **Nitrate-elicited currents of *BnNRT1;2* expressed in *Xenopus* oocytes in saline.** The *BnNRT1;2* cRNA-injected oocytes were exposed to sodium nitrate (1, 5, 7, 15, and 20 mM) in saline at pH 5.5. The nitrate-elicited currents were measured while the oocyte plasma membrane was clamped from −50 mV to between −20 and −180 mV for 120 ms with −20-mV increments for each nitrate concentration. The current-voltage difference relationship (*A*) was obtained by subtracting the *I-V* relations determined before nitrate addition from that obtained after nitrate addition. The voltage-response curves (*B*) were obtained by plotting nitrate-elicited currents in *A* against external nitrate concentrations and were fitted to the Michaelis-Menten equation (see "Experimental Procedures") at three different membrane voltages (−100, −140, and −180 mV).

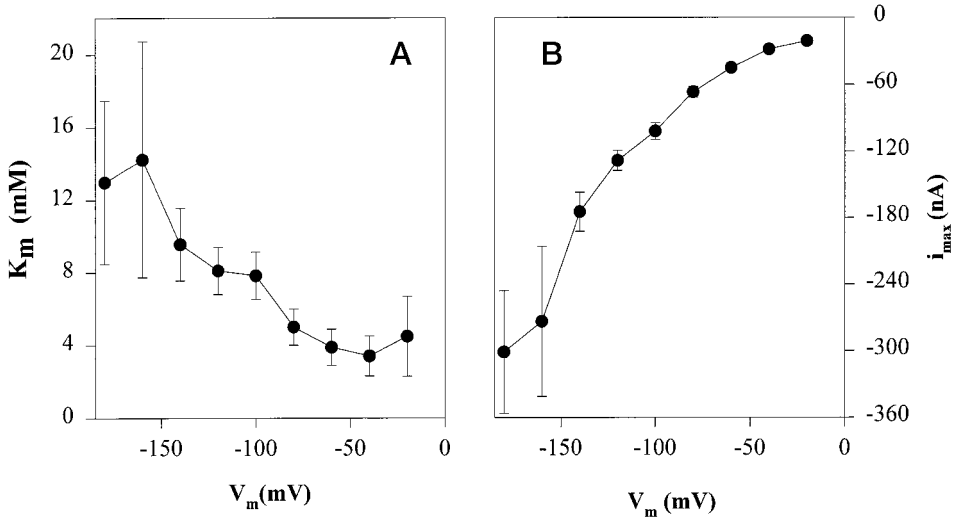

FIG. 3. **Voltage dependence of K_m and i_{max} for nitrate determined in saline at pH 5.5.** The parameters were determined by fitting *I-V* response curves in Fig. 3 to the Michaelis-Menten equation (see "Experimental Procedures") at each voltage.

the dipeptide histidine-leucine failed to elicit significant current in cRNA-injected oocytes (data not shown). Other anions tested included nitrite, cyanate, and chlorate, all of which elicited small negative currents, which were not significantly different from those obtained in control water-injected oocytes. Fig. 4*A* shows the result of experiments in which cRNA-injected oocytes were incubated in radiolabeled L-histidine at pH_o 5.5; these oocytes accumulated significantly more histidine. Fig. 4*B* shows the *I-V* difference curve for the same cRNA-injected oocytes treated with L-histidine, nitrate, and L-histidine plus nitrate at pH_o 5.5. The *I-V* curve shows that 10 mM L-histidine elicited a larger current than 10 mM nitrate, but when nitrate and L-histidine were applied at the same concentration in the same solution, the current obtained was the same size as that obtained with histidine alone.

pH Dependence of Nitrate- and Histidine-elicited Currents—Fig. 5 shows nitrate-elicited currents at two different pH values in a *BnNRT1;2*-injected oocyte. Nitrate applied externally at 10 mM elicited larger currents (negative currents, cation-inward) at more acid pH_o (pH 6.0) than at pH_o 7.2 (Fig. 5*A*). In contrast, L-histidine transport had a very different pH_o optimum compared with nitrate; the largest currents were obtained at more alkaline pH_o (Fig. 5*B*). Similar treatments elicited no more than 1-nA currents in water-injected oocytes.

Kinetics of Histidine Transport—The concentration dependence of the histidine-elicited currents at pH_o 8.5 could be fitted to a Michaelis-Menten function at membrane voltages more negative than −100 mV. However, in these oocytes, the expression of *BnNRT1;2* was lower than that in previous experiments; for example, the i_{max} value for nitrate-elicited currents at pH_o 5.5 was only 10–20 nA (data not shown). The fitted lines and values at −100, −120, −160, and −180 mV are shown in Fig. 6*A*. The voltage dependence of the kinetic parameters obtained from the fitted data is shown in Fig. 6*B*. Both K_m and

i_{max} are voltage-dependent; K_m decreased from 25 mM at −100 mV to 1.4 mM at −180 mV, and i_{max} increased from −170 nA at −100 mV to −260 nA at −180 mV (Fig. 6*B*).

DISCUSSION

In this paper, we have shown that oocytes injected with cRNA encoding *BnNRT1;2* exhibited nitrate- and histidine-elicited currents. These results, together with the increased accumulation of nitrate and radiolabeled histidine in cRNA-injected oocytes, suggest that both substrates can be transported by *BnNRT1;2*; however, we will begin by discussing whether there are other interpretations of these observations.

One explanation for the apparent transport of two such different substrates is that the expression of *BnNRT1;2* stimulates the expression or activity of an endogenous transporter present in the oocyte plasma membrane. There are already a few examples of this phenomenon; the injection of cRNA for *minK* (28) and *IsK* (29) has been shown to activate endogenous oocyte channels. However, if the two different substrates were each transported by different membrane proteins, then the currents obtained when both substrates are supplied simultaneously should be additive, and this was not the case (see Fig. 4*B*). Furthermore, water-injected control oocytes did not show any significant histidine- or nitrate-elicited currents and so do not provide any evidence for either endogenous transport system in oocytes. Another possibility is that the positively charged form of histidine can donate or substitute for protons in the cotransport mechanism. This would seem unlikely because histidine transport can occur without any nitrate in the external solution, but uncoupled proton transport has been described for a H^+/sucrose cotransporter (30). If *BnNRT1;2* also showed uncoupled proton transport, then perhaps histidine could be transported by the same mechanism, but we could find no evidence for uncoupled proton cotransport by *BnNRT1;2* (data not shown). We conclude that *BnNRT1;2* codes for a transporter that, when expressed in oocytes, can transport both nitrate and amino acids.

The gene *BnNRT1;2* belongs to the emerging PTR family of transporters, which have been shown to transport various substrates ranging from peptides and amino acids to nitrate (8). Although, mammalian and plant members of the family have been shown to transport peptides and histidine (3, 6), this is first example that has been shown to transport both nitrate

TABLE II

Reversal potentials (E_v and E) of hyperpolarization-activated currents, resting potentials, and calculated and measured internal nitrate concentrations for water- and BnNRT1;2-injected oocytes

Reversal potentials were determined from the tail currents (24) measured in either 120 mM sodium chloride or nitrate in the external solution, which also contained 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM MES (pH 5.5). The electrical potential values are shown as the mean ± S.E. (number of oocytes). The Nernst equation was used to calculate the internal concentrations of nitrate using the measured membrane potentials and the external concentration. The internal nitrate concentrations of oocytes are expressed as means and confidence limits because they were calculated using an antilog conversion, the values are not normally distributed, and S.E. cannot be determined. Oocytes were all incubated in the nitrate or chloride solution for between 20 and 30 min before the determination of the tail currents, and the oocytes had been previously stored in a modified Barth's saline (21).

Parameter	Water-injected oocyte	*BnNRT1;2* RNA-injected oocyte
E_v (mV)	−20.3 ± 0.9 (4)	−15.7 ± 2.1 (6)
E (mV)	−50.5 ± 6.4 (4)	−18.8 ± 4.2 (6)
Measured mean resting potential (mV)	−42.2 ± 5.4 (4)	−31.9 ± 5.6 (6)
Calculated internal [NO_3] (mM) from E	16.7 (13, 22)	57 (49, 68)
Theoretical nernstian internal [NO_3] (mM)	23 (19, 29)	34.5 (28, 43)
Measured internal [NO_3] (mM)	8 (3, 15) (4)	41 (29, 55) (4)

TABLE III

Currents elicited by a range of different amino acid substrates in oocytes injected with BnNRT1;2 cRNA

Each amino acid was applied at a concentration of 5 mM. The elicited currents are shown as a percentage of that measured by treatment with 10 mM histidine. This was the maximum current, and all were applied at an external pH of 5.5. Substrate-elicited currents from water-injected oocytes were typically 5 nA (data not shown), equivalent to 2% of the histidine-elicited current.

Substrate	Histidine current at −160 mV
	%
Arginine	50
Lysine	47
aa mixture 1[a]	14
aa mixture 2[b]	15

[a] aa mixture 1 contained alanine, leucine, glycine, glutamine, and threonine.

[b] aa mixture 2 contained asparagine, proline, valine, aspartic acid, isoleucine, and tyrosine.

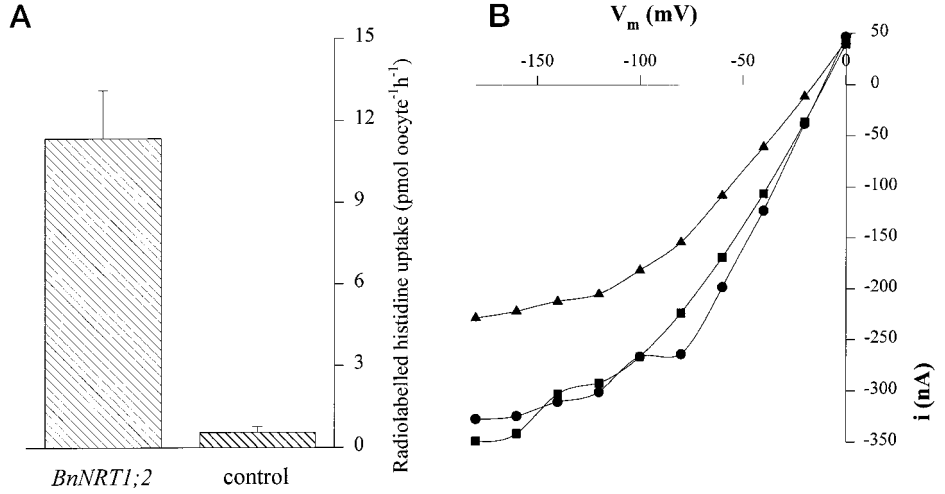

FIG. 4. **Histidine and nitrate uptake by oocytes.** *A*, radiolabeled histidine uptake by *BnNRT1;2* cRNA- and water-injected oocytes in saline at pH_o 5.5. *B*, *I-V* difference curve showing histidine-elicited current compared with nitrate-elicited current from the same oocyte. The *I-V* difference relationships for the same oocyte that had been previously injected with cRNA for *BnNRT1;2* were determined at pH_o 5.5 with 10 mM nitrate (▲), 10 mM histidine (●), or 10 mM histidine + 10 mM nitrate (■).

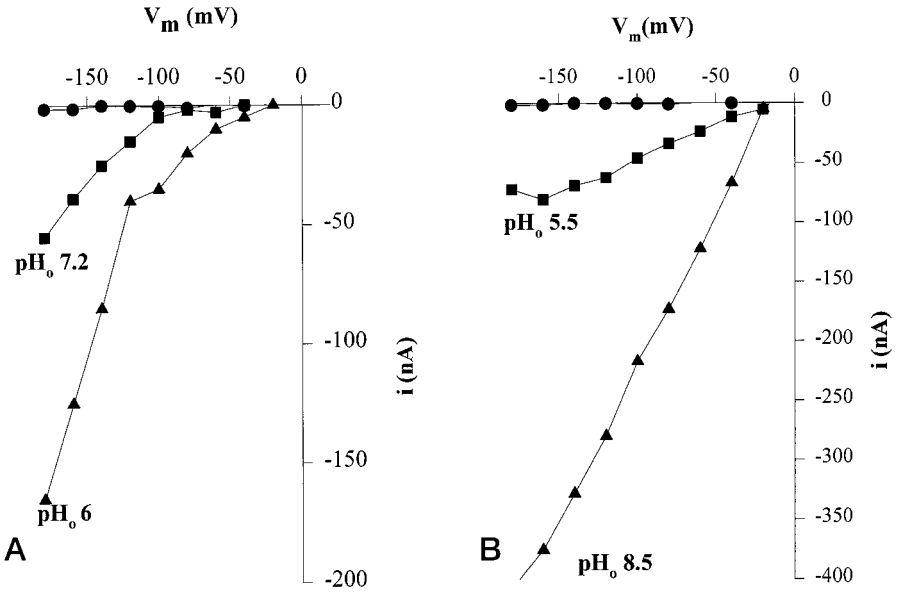

FIG. 5. **pH dependence of nitrate- and histidine-elicited currents in *BnNRT1;2* cRNA- and water-injected oocytes.** *A*, the *I-V* difference relationships were determined at pH_o 7.2 (■) and pH_o 6.0 (▲) when the oocyte was exposed to 10 mM nitrate after previously allowing a 5-min adjustment period to each different proton concentration. The *I-V* difference relationship of a water-injected oocyte is also shown (●). *B*, *I-V* difference relationships of histidine-elicited currents in *BnNRT1;2* cRNA-injected oocytes at pH_o 5.5 (■) and pH_o 8.5 (▲). The *I-V* difference relationship of a water-injected oocyte is also shown (●).

and amino acids. Furthermore, the *I-V* difference curves show that both types of substrate generate inward cation currents, which would be consistent with the idea that transport is proton-coupled, as is found for the members of this family that have been characterized. Comparative analysis of the amino acid sequence has indicated that the family is distinct from other families of secondary transporters and can be divided into two subfamilies (8). The genes *BnNRT1;2*, *AtNRT1*, and *AtPTR2-B* are all classified in one subfamily, suggesting that the nitrate transport activity we have observed may also be found for *AtPTR2-B* (3). However, nitrate did not compete with radiolabeled dileucine uptake in yeast cells expressing *AtPTR2-B* (5). The results shown in Fig. 4*B* suggest that supplying both substrates together may not necessarily decrease the uptake of one of them because the histidine-elicited current was not altered by the addition of nitrate to the bathing solution.

A membrane protein that is able to transport such very different types of substrate is unusual in biology. These substrates have very different sizes, and so presumably they have two different binding sites on the protein. As each substrate-elicited current was not additive when supplied together, some models for the transport can be discounted. For example, there cannot be two different forms of the protein, each transporting the different substrates, because this would also result in additive currents when both substrates were supplied. However, both substrates were required in millimolar concentrations, and other plant transporters have been identified that have higher affinities for both substrates (*e.g.* Refs. 15 and 31). The *in vivo* biphasic uptake observed for nitrate (13) and the multiphasic amino acid uptake kinetics (32) are explained by the activity of transporters with differing substrate affinities. The related plant peptide transporter *AtPTR2-B* also required millimolar concentrations of histidine to complement the yeast amino acid uptake mutant (33), but it is not known whether the amino acid transport activity is important *in vivo* (3).

The rat peptide/histidine transporter was shown to have a K_m for histidine of 17 μM (6), but in contrast, much higher (millimolar) concentrations of histidine were required for the currents obtained in this work and for yeast complementation

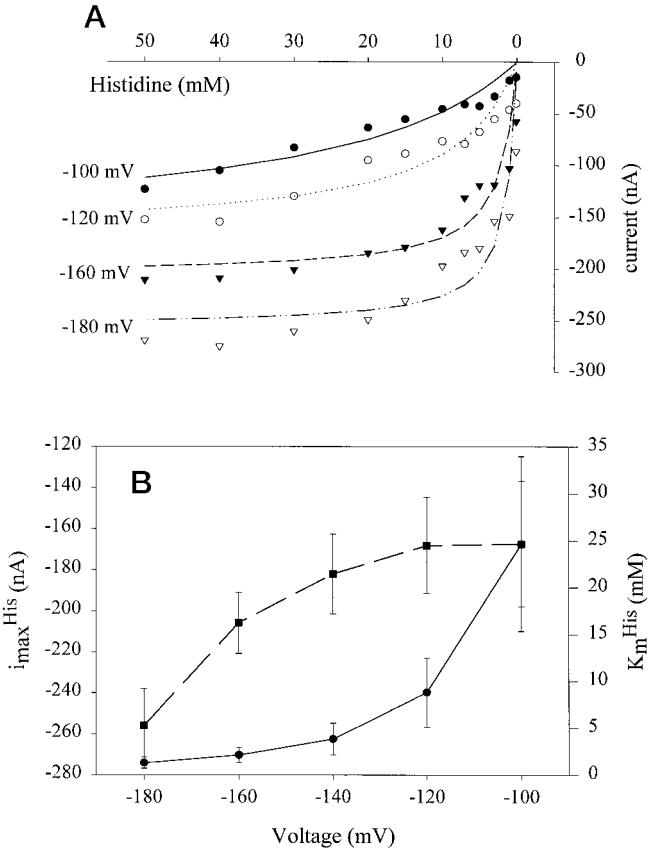

FIG. 6. **Kinetics of histidine-elicited currents at pH_o 8.5 in *BnNRT1;2* cRNA-injected oocytes.** *A*, the *I-V* difference relationships were determined for oocytes treated with differing concentrations of histidine. The voltage-response curves were obtained by plotting histidine-elicited currents against external histidine concentrations, and the data were fitted to the Michaelis-Menten equation (see "Experimental Procedures") at four different membrane voltages (−100, −120, −160, and −180 mV). *B*, voltage dependence of the K_m (●) and i_{max} (■) for histidine. These parameters were determined from the fitted curves like those shown in *A*.

by *AtPTR2-B* (33). Furthermore, the uptake of radiolabeled histidine was greatest at pH_o 5.5 (6), which again contrasts with the pH_o optimum for the activity of *BnNRT1;2* (Fig. 5*B*). At pH_o 5.5, the 1+ form of histidine is the chief ionic species present in the solution, although a smaller amount of the zwitterion will also be present (0.28 times less). The other ionic forms of histidine will be far less abundant, and in a millimolar solution of histidine, only micromolar concentrations of the 2+ form and even less of the 1− form will be present. More alkaline pH_o will increase the amounts of the zwitterion and the 1− form as the histidine-elicited currents increase, so it seems likely that one or both of these species are transported, but it is difficult to identify which ionic species of histidine is transported. The alkaline pH_o optimum does not suggest a histidine channel mechanism for uptake because the positively charged forms are less abundant. However, at these pH_o values, there will also be a smaller proton gradient to drive the cotransport, but the uptake can be driven by a more negative membrane potential. This idea is supported by the actual shape of the *I-V* relationship shown in Fig. 5*B*; at the more alkaline pH, the slope of the line increased, indicating that the histidine current has become more voltage-dependent. Another unrelated plant amino acid transporter (*AAP5*) has been shown to transport the neutral form of histidine in cotransport with protons (34). Therefore, it is possible that *BnNRT1;2* also transports histidine as the zwitterion in cotransport with protons, and the kinetic analysis in Fig. 6 supports this hypothesis. The transport of the neutral form of histidine could explain the requirement for relatively high concentrations of histidine at pH_o 5.5. It is unclear whether the low affinity amino acid transport activity of *BnNRT1;2* is a major function of the nitrate transporter *in planta*.

For more information on the function of *BnNRT1;2 in vivo*, it will be important to identify in which root cell type this transporter is expressed because the external concentrations of substrate will determine its role in the plant. Genes homologous to *BnNRT1;2* are expressed in the root hair cells of tomato, suggesting that these genes are involved in uptake from the soil as they are present at the soil/root interface (10). This location is consistent with the nitrate uptake role of the transporter, as nitrate can be present in soil at these concentrations, particularly in an agricultural soil (11), but histidine is unlikely to be present in the soil solution at millimolar concentrations. The role of *BnNRT1;2* in transporting amino acids suggests that it could be located in supplying sink tissues such as developing seeds. The peptide/histidine transporter isolated from *Arabidopsis* was found to be expressed in the leaves and developing pods (3, 33). However, *BnNRT1;2* is expressed in the root and so could be involved in supplying the growing roots with histidine or the uptake of nitrate. The diverse pH optima for each of the substrates may indicate distinct roles in different parts of the plant according to the external pH, perhaps as a nitrate transporter at the soil interface or a histidine transporter into developing cells of the root tip. There may be other substrates for the transporter, such as particular peptides, that have yet to be determined. The low affinity nitrate transporter is induced by very low external concentrations of nitrate outside the roots, so the production of low affinity nitrate transporters does not require the presence of high external concentrations of nitrate. This result may indicate that the induction of nitrate transport in plants is dependent on the presence of nitrate in the environment and not the actual concentration of available nitrate.

The transport of two such different substrates by a single protein suggests that there may be other carriers with this type of multipurpose function. The design of the oocyte experiments is limited by the substrates that are offered by the experimenter, and usually the choice of these depends on the assignment of a gene to a family and therefore a particular function, and appropriate substrates are applied to assay activity. Perhaps some already characterized peptide and amino acid transporters of this family have other anion substrates yet to be identified. The affinity of the transporter may be very important in defining the *in vivo* function; for example millimolar concentrations of some substrates, particularly peptides, may not exist *in vivo*. Finally, the naming of transporter genes according to their substrate becomes very difficult, and the most likely *in vivo* substrate could depend on where the gene is expressed.

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