Genetic Differences in Nitrate Uptake in Two Clones of the Common Reed, *Phragmites australis*

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Phragmites australis, common reed, could be useful in removing eutrophic substances from river and lake water. In this study, the genetic differences in nitrate uptake ability of the reed were investigated with a view to breeding a reed plant useful for phytoremediation. Two reed clones (W-6 and W-8) isolated from a reed community in the lakeside wetland along Lake Biwa, Japan, were used for a study on the physiological and molecular basis of nitrate uptake. K_m s for nitrate uptake were 80.8 and 45.2 μM and $V_{\max}$ s for nitrate uptake were 10.62 and 2.37 $\mu\text{mol g}^{-1}$ root f.w. h^{-1} in W-6 and W-8, respectively, suggesting that there were critical differences in kinetic parameters for nitrate uptake. To investigate these differences at the molecular level, we isolated a high-affinity nitrate transporter gene (*NRT2*) from the two reed clones and analyzed the reed *NRT2* structure and expression. The deduced amino acid sequence of the reed *NRT2* consisted of 523 residues and had a high similarity to *NRT2* from other monocots. Reed *NRT2* was strongly expressed in roots treated with 200 μM nitrate. There were three amino acid substitutions of the reed *NRT2* between W-6 and W-8. Differences in *NRT2* transcription were also observed between the two clones. It was not clear whether the difference in kinetic parameters for nitrate was due to the reed *NRT2* structure or expression. These results indicate the possibility of selecting genotypes more useful for the removal of nitrate.

Key Words: *Phragmites australis*, high-affinity nitrate transporter, nitrate uptake, Michaelis-Menten kinetics, gene isolation.

Introduction

Phragmites australis, a common reed, is a well-known marsh plant that is distributed from tropical to cool-temperate zones (Koppitz 1999) and can grow under both freshwater and saline conditions (Asaeda *et al.* 2003), suggesting a very wide adaptability. In recent years, the reed has attracted attention as a useful plant for removing eutrophic substances to improve river and lake water quality. Furthermore, genetic diversity among reed populations has been reported worldwide (Koppitz 1999), and polyploidy and isozyme polymorphisms have been demonstrated in the reed communities along Lake Biwa (Ishii and Kadono 2001, Nomiya *et al.* unpublished).

At present, a serious environmental problem in lakes such as Lake Biwa (Shiga Prefecture, Japan) is the phenomenon of algal bloom, which is caused by an excess of nitrogen and phosphorus in the lake water. Eutrophic substances such as nitrate, ammonium and phosphate must be removed

from the lake and river water to prevent this phenomenon. Not only prevention of nitrate leakage from paddy and upland fields into the lake, but also removal of nitrate is important, because nitrate is a major source of the nitrogen excess. For this purpose, the reed is thought to be useful for taking up nitrates without using fossil and atomic energy, and projects on restoration of reed communities have been started. To evaluate how efficiently nitrate can be removed by the reed, we need to understand the nitrate uptake mechanism of the reed.

In higher plants, nitrate is taken up by nitrate transporters located in the plasma membrane of root surface cells (Tsai *et al.* 1993, Forde 2000, Orsel *et al.* 2002a). There are two nitrate transport systems, the high-affinity transport system (HATS) at a low nitrate concentration (< 1 mM) and the low-affinity transport system (LATS) at a high nitrate concentration (> 1 mM) (Forde 2000). HATS is induced by nitrate (iHATS) or is constitutively expressed (cHATS) (Wang and Crawford 1996). HATS and LATS are encoded by a high-affinity nitrate transporter gene (*NRT2*) and a low-affinity nitrate transporter gene (*NRT1*), respectively, and both genes are identified and characterized at a molecular level in several higher plant species (Tsai *et al.* 1993,

Trueman *et al.* 1996, Quesada *et al.* 1997, Krapp *et al.* 1998). *NRT2* is found in several families (Forde 2000, Orsel *et al.* 2002b).

The nitrate concentration of river water flowing into Lake Biwa is estimated to be less than 100 μM (Lake Biwa Research Institute 2002). Therefore, *NRT2* probably plays a major role in removing nitrate from the river water. In this paper, we report on the structure and function of the reed *NRT2* and discuss the possibility of manipulating the reed *NRT2* as the first step of breeding of reed with higher nitrate removal potential.

Materials and Methods

Plant materials and cultivation

Two reed clones, named W-6 and W-8, were isolated from seeds obtained from plants growing naturally on the lakeside of Lake Biwa in Moriyama-City, Shiga Prefecture, Japan and maintained at the Shiga Prefecture Agricultural Research Center. Two reed clones randomly chosen from these clones were used in this experiment. They were cultivated hydroponically in 60 L plastic containers with a water culture solution containing nitrate as a sole nitrogen source in a greenhouse kept at over 15°C. The composition of the culture medium was KNO_3 0.2022 g/L, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 0.2362 g/L, KH_2PO_4 0.068 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1232 g/L, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.0288 g/L, citric acid 0.0228 g/L, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.0036 g/L, and H_3BO_3 0.0058 g/L. Other minor elements were present in the tap-water. Throughout the cultivation, the water level was kept about 10 cm above the root.

To propagate small plants from each clone, we cut culms at the water surface. After removal of leaves, culms were laid on a plastic tray filled with distilled water in a growth chamber, controlled at 27°C and 60% relative humidity. After two weeks, small plants that had developed to more than 2 cm from each node were excised from each side of the node. These plants were wrapped with urethane at the basal part of the shoot and were inserted into holes made in polystyrene foam covering the top of a 15 L container filled with a culture solution as described above. Plants were cultivated in the growth chamber under the same conditions as described above. One or two weeks later, small plants with two or three expanding leaves were used for measuring nitrate uptake.

Nitrate uptake measurement

Small plants were cultured using a 400 μM CaSO_4 solution for nitrate starvation for 24 hours and then were transferred into the 50 ml solution containing 200 μM KNO_3 and 400 μM CaSO_4 to induce nitrate uptake for 16 hours. After the induction, each small plant was soaked in a 50 ml solution containing 10–400 μM KNO_3 with 400 μM CaSO_4 in a biotron (Nippon Medical and Chemical Instruments, Japan) controlled at 25°C under fluorescent lighting (70.9 $\mu\text{mol m}^{-2} \text{ s}^{-1}$, 16 h photoperiod). After 2 hours, the nitrate concentration of the solution was measured by ionchromatography

(DX-3000, Dionex, USA) using an anion-specific column (IonPacR AS12A, Dionex, USA). The fresh weight of each plant was also measured. The nitrate uptake was evaluated by the disappearance of nitrate from the solution. Analysis of nitrate uptake with Michaelis-Menten kinetics was carried out using the same method as Hasegawa and Ichii (1994). Each experiment with five to ten plants was replicated three times.

Isolation of the reed *NRT2*

The mRNA of the root treated with 400 μM KNO_3 and 400 μM CaSO_4 solution for 16 hours was purified with a QuickPrep micro mRNA purification kit (Amersham Bioscience, USA). Complimentary DNA was constructed from the RNA. The 642 bp fragment of the reed *NRT2* ORF was amplified by RT-PCR using two primers (P-RT-1: 5'-ATGA GCACCATGTTCAACAG-3', P-RT-2: 5'-CAGAAGGAGA AGAGGACCAT-3') designed based on the *Oryza sativa* high-affinity nitrate transporter gene sequence (*OsNRT2*: GenBank accession no. AB008519). The program of the PCR amplification step was 94°C for 5 min for 1 cycle followed by 94°C for 30 sec, 55°C for 30 sec, and 68°C for 50 sec for 30 cycles. As a result, a putative reed *NRT2* fragment was obtained.

The reed *NRT2* termini were cloned by 5'- and 3'-RACE-PCR using a 5'-Full RACE Core set and 3'-Full RACE Core set (TaKaRa, Japan), respectively, following the manufacturer's protocol. An oligonucleotide P-3'-1: 5'-CCATTACCGCCATGGTCCTCTTCTCCTTCTG-3' was used for 3'-RACE-PCR with the 3-site adaptor primer (3'-Full RACE Core set: TaKaRa, Japan). Five specific primers were prepared for 5'-RACE as indicated in the 5'-RACE kit manufacturer's protocol, P-5'-5: 5'-P-CGCACTTGCGGAT GA-3', P-5'-4: 5'-GCTGTTGAACATGGTGCTCA-3', P-5'-2: 5'-GTTACCGTCCCGATGATCT-3', P-5'-3: 5'-CTC CTCATGCCGCTCGTCTA-3' and P-5'-1: 5'-CCACGCAG CTCCTCATG-3'. The full-length of the reed *NRT2* was amplified using P-RT5: 5'-GCAGCAAGTCCAAGAAAGAAG CTAG-3' containing the revealed sequence in RACE and the 3-site adaptor primer (3'-Full RACE Core set, TaKaRa, Japan). The amplification was carried out as follows: 94°C for 2 min, then 35 cycles of 94°C for 25 sec, 59°C for 30 sec and 72°C for 2 min.

Sequence analysis

The PCR products were integrated with pCR®4-TOPO® vector (Invitrogen, USA) for sequencing. Sequencing was performed using a BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, USA) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer's instructions or ordered from Hokkaido System Science Co., LTD., Japan.

Southern blot analysis

Southern blot analysis was performed according to the Roche Molecular Biochemicals DIG Application Manual

for Filter Hybridization (Roche Japan, Japan). A DIG-labeled RNA probe containing 642 nt of the reed *NRT2* sequence was prepared using the cDNA integrated with pCR[®]4-TOPO[®] vector (Invitrogen, USA) and T3 and T7 RNA polymerase (Stratagene, USA; DIG RNA Labeling Kit (SP6/T7), Roche, Germany).

Genomic DNAs were isolated by the CTAB-based method (Tel-Zur *et al.* 1999) with a minor change. Approximately 20 µg genome DNA was digested with restriction enzymes (*XhoI*, *SacI* or *EcoRI*), which did not have restriction sites in the probe used. Hybridization was performed at 50°C for 16 h after addition of the DIG-labeled probe into the new DIG Easy Hyb Granules solution (Roche, Germany), which was the same solution as that used for prehybridization. Detections were carried out as indicated by the manufacturer's protocol.

Northern blot analysis

Total RNAs were isolated by phenol-chloroform extraction (Liu *et al.* 1994) with a minor change. Hybridization was performed at 68°C with 5 µg of total RNA as a template and the RNA probe described in Southern blot analysis, according to the manufacturer's protocol. Ethidium bromide-stained RNA is shown as a loading control.

Result

Nitrate uptake

When nitrate was applied at concentrations of 10–400 µM KNO₃, nitrate uptake by small reed plants rose with the increase in initial nitrate concentration. Net nitrate uptake of W-6 was higher than that of W-8 in the range of nitrate concentrations applied in this experiment (Fig. 1). At the lowest concentration, the difference between W-6 and W-8 was about 3-fold. The difference in nitrate uptake seemed to become greater with the increase in nitrate concentration and, in particular, the highest difference was found at the concentration of 200 µM. The difference between the clones was due to the response to nitrate concentration; that is, nitrate uptake was saturated at an initial concentration of 50 µM in W-8, while the uptake was saturated at an initial concentration of 200 µM in W-6. The relationship between initial nitrate concentration and nitrate uptake ratio could be ana-

lyzed with Michaelis-Menten kinetics. There were also critical differences in kinetic parameters for nitrate uptake between the two clones (Fig. 1). K_m s were 80.8 and 45.2 µM and V_{max} s were 10.62 and 2.37 µmol g⁻¹ root f.w. h⁻¹ in W-6 and W-8, respectively. No significant differences in morphological characters were found between W-6 and W-8.

Isolation of the reed *NRT2*

In this experiment, an 1858 bp cDNA fragment was isolated by PCR and RACE methods and was sequenced. In the isolated cDNA fragment, a start codon was found at 108 bp and a stop codon at 1676 bp, indicating that the length of the open reading frame (ORF) was 1569 bp. The cDNA had 88% similarity to *Zea mays NRT2* (*ZmNRT2*: GenBank accession no. AY129953) and 87% similarity to *OsNRT2*. The deduced amino acid sequence from the isolated cDNA had 523 amino acid residues and had 12 spanning regions across the plasma membrane as predicted with TMpred (Hofmann and Stoffel 1993), which was applied with a minimum of 20 and maximum of 32. At an amino acid sequence level, the deduced sequence had 81% similarity to *ZmNRT2* and 80% similarity to *OsNRT2*. The reed *NRT2* also had a specific nitrate/nitrite transporter 'signature motif', A-G-W/L-G-N-M-G (Trueman *et al.* 1996) (Fig. 2), which is located between amino acids residues 189 and 195. The phylogenetic relation of *NRT2* in higher plants determined using a ClustalW program available at DDBJ (<http://hypenig.nig.ac.jp>) and Tree view (Page 1996) is shown in Fig. 3, in which the reed *NRT2* is shown to be included in a monocot group consisting of rice and maize but is different from the group of the tribe Triticeae. There were three substitutions at amino acid residues 202, 380 and 464 from a comparison with amino acid sequences of the reed *NRT2* between W-6 and W-8 (Fig. 2).

Southern blot analysis was performed using genomic DNAs of two reed clones, which were digested with *XhoI*, *SacI* or *EcoRI*, and the reed *NRT2*-specific probe. The probe hybridized strongly to five major bands and weakly to four other bands, suggesting that both clones had more than five copies of the reed *NRT2*. There were no differences in the copy number between W-6 and W-8.

Expression of the reed *NRT2*

Northern blot analysis showed that the expression of the reed *NRT2* gene was induced 3 hours after nitrate treatment and was strongly expressed in the root (Fig. 4) as demonstrated in other *NRT2* (Forde 2000). However, expression of *NRT2* was not detected in leaves (Fig. 4). There were differences in *NRT2* transcript abundance and expression between W-6 and W-8 (Fig. 4). In W-8, the expression was highest after 3 to 6 hours since nitrate was added and slightly declined until 16 h after nitrate addition, while in W-6, *NRT2* expression was highest 11 h after nitrate treatment.

Discussion

In this experiment, the two kinetic parameters, K_m and

Fig. 1. Difference in nitrate uptake by roots between W-6 (triangle) and W-8 (square).

Fig. 2. Comparison of NRT2 amino acid sequences among reed (W-6 and W-8), monocotyledons (OsNRT2 and *Hordeum vulgare* NRT2 (HvNRT2)) and *Arabidopsis thaliana* NRT2 (AtNRT2). GenBank protein ID numbers are OsNRT2, BAA33382; HvNRT2, AAC49531; AtNRT2.1, AAC64170. Solid bar shows the specific nitrate/nitrite transporter 'signature motif'. "◇" indicates amino acid substitution between W-6 and W-8.

$V_{\max}$, for HATS for nitrate uptake of two reed clones were 80.8 and 45.2 $\mu\text{mol l}^{-1}$ for K_m and 10.62 and 2.37 $\mu\text{mol g}^{-1}$ root f.w. h^{-1} for $V_{\max}$ in W-6 and W-8, respectively. These values were roughly coincident with those obtained in other higher plants (Rao and Rains 1976, Doddema and Telkamp 1979, Goyal and Huffaker 1986, Barro *et al.* 1994, Hasegawa and Ichii 1994). Intraspecific differences in nitrate uptake ability have been demonstrated in some plant species such as *O. sativa*, *Lolium perenne* and *Trifolium repens* (Hasegawa and Ichii 1994, Høgh-Jensen *et al.* 1997). A very interesting result was the clear differences in nitrate uptake kinetic pa-

Fig. 3. Phylogenetic analysis of NRT2 proteins. Amino acid sequences of NRT2 were aligned with ClustalW. GenBank protein ID numbers are *Phragmites australis* reed NRT2, BAC76606; *Zea mays* ZmNRT2, AAN05088; *Oryza sativa* OsNRT2, BAA33382; *Hordeum vulgare* HvNRT2.1, AAC49531; HvNRT2, AAC49532; *Triticum aestivum* TaNRT2.1, AAK19519; *Arabidopsis thaliana* AtNRT2.1, CAB09794; *Brassica napus* BnNRT2, CAC05338; *Glycine max* GmNRT2, AAC09320; *Lotus japonicus* LjNRT2, CAC35729; *Nicotiana tabacum* NtNRT2.1, CAD89798; NtNRT2.2, CAD89799; *Lycopersicon esculentum* LeNRT2.1, AAF00053; LeNRT2.2, AAF00054; LeNRT2.3, AAK72402; *Chlorella sorokiniana* CsNRT2, AAK02066.

Fig. 4. Northern analysis of the reed NRT2 expression in roots and leaves. Total RNA (5 μg) was extracted from roots, which were cultured with 400 μM nitrate. Each number means time (h) after nitrate treatment. Loading controls stained with ethidium are shown separately for roots and leaves.

rameters between the two clones; about a 2-fold difference in K_m and more than a 4-fold difference in $V_{\max}$. As both clones were progenies of the reed plant collected in the same community, the differences in nitrate kinetic parameters

indicated the presence of genetic variability in the reed community. This suggests that useful genotypes for efficient removal of nitrate from river and lake water can be found in the reed community, because diverse chromosome numbers have been reported in the reed community (Ishii and Kadono 2002) and isozyme polymorphism has also been reported in reeds distributed in the Lake Biwa region (Nomiya *et al.* unpublished). From these results, it should be possible to breed a unique reed with increased nitrate uptake ability. Further screening and selection for nitrate uptake in reeds are necessary.

As the first step in the molecular analysis for differences in nitrate uptake ability in the two clones, we attempted to isolate the reed *NRT2*. A putative reed *NRT2* cDNA fragment (1858 bp) was isolated using PCR and RACE methods and its deduced amino acid sequence of putative *NRT2* had a high similarity to *NRT2* identified in other higher plants, notably being very similar to that in the monocot group (Fig. 3). Furthermore, there was a 'signature motif' only for the nitrate/nitrite transporter family in the major facilitator superfamily (Fig. 2; Trueman *et al.* 1996, Amarasinghe *et al.* 1998). The putative reed *NRT2* had 12 spanning regions estimated by the deduced amino acid sequence, and they were the same as those of in other plant *NRT2* (Forde 2000). Northern blot analysis revealed that the cDNA isolated in this experiment was transcribed specifically in the roots treated with nitrate. These results indicated that the isolated cDNA is *NRT2* of reed. (GenBank accession no. AB096061.)

In higher plants, *NRT2* comprises several families (Orsel *et al.* 2002b). In reed, Southern blot analysis demonstrated more than five bands thought to be *NRT2* in both reed clones, indicating that there are also several families of reed *NRT2*. However, to determine the number of copies, the polyploidy level must be taken into account, because diversity in polyploidy among the reed populations around Lake Biwa has been reported (Ishii and Kadono 2001). As the polyploidy level of the two clones was still unclear, further analysis of the banding pattern of Southern blot in relation to polyploidy is necessary. Three amino acid substitutions were found between W-6 and W-8. At present, it is not clear whether the difference in nitrate uptake ability is due to the amino acid substitutions or not.

The reed *NRT2* was induced by nitrate and the transcription started within 3 hours after nitrate treatment (Fig. 4). The expression pattern of reed *NRT2* revealed a difference between the two clones: in W-8 the reed *NRT2* maintained a high expression level from the start of transcription, while the amount of *NRT2* mRNA in W-6 was gradually increased from the beginning of transcription. It is not clear whether the difference in the nitrate uptake ratio is due to the difference in reed *NRT2* expression between W-6 and W-8. Further investigation is necessary on the relationship time-dependent *NRT2* expression and nitrate uptake.

When reed plants grow under flooded conditions, ammonium is thought to be a major source of nitrogen as rice.

Under such conditions, the expression of *NRT2* is repressed (Orsel *et al.* 2002a). However, reed roots from the nodes can develop into the water, where the most available nitrogen form is nitrate. Improvement of ability of nitrate uptake from roots that have developed from the nodes into water is an excellent strategy for removing nitrate. Furthermore, the present results imply the possibility of selecting reed genotypes with high removing other eutrophic substances and pollutants.

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