Production and characterization of transgenic rice plants carrying a high-affinity nitrate transporter gene (OsNRT2.1)

Hisato Katayama*1), Mari Mori2), Yoko Kawamura3), Toshinori Tanaka3), Masashi Mori†) and Hiroshi Hasegawa5)

1) Shiga Prefecture Agricultural Technology Promotion Center, Azuchi, Shiga 521-1301, Japan
2) Shiga Prefecture Government, 4-1-1 Kyo-machi, Otsu, Shiga 520-8577, Japan
3) Okinawa Institute of Science and Technology Promotion Corporation, Onna-son, Kunigami, Okinawa 904-0411, Japan
4) Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi-machi, Ishikawa 921-8836, Japan
5) School of Environmental Science, University of Shiga Prefecture, 2500 Hassaka, Hikone, Shiga 522-8533, Japan

Enhancing nitrogen utilization is important in maintaining crop yield under low nitrogen conditions. Therefore, rice plants (Oryza sativa L.) were transformed with pMLH7133-RNRT2, which bears OsNRT2.1, a major gene involved in nitrate uptake under low nitrate concentrations. Two transgenic plants harboring an OsNRT2.1 transgene were identified and used for further study. In transgenic plants, OsNRT2.1 was expressed constitutively in roots, while in wild-type plants, the transcript was detected 30 min after starting to supply nitrate. No co-suppression between transgene and host gene was observed, as was observed on introduction of a nitrate reductase gene from the same species. One of the transformants grew faster than the wild-type cultivars when KNO₃ or NH₄NO₃ was supplied as a nitrogen source. However, no increase in nitrate uptake by young plants was observed. These results suggest that constitutive expression of OsNRT2.1 might enhance vegetative growth and that introduction of genes involved in nitrate uptake from the same species may be useful for genetic improvement of plant growth under low nitrogen conditions. Further studies are necessary to understand the molecular and physiological bases of expression of NRT2.1 in relation to plant growth.

Key Words: transgenic rice, high-affinity nitrate transporter, NRT2, nitrate uptake, plant growth.

Introduction

Nitrogen fertilization is essential in increasing grain yield, in particular for crops that have been principally selected for their adaptation to high fertilizer input (Castelberry et al. 1984). In recent years, however, leaching of fertilized nitrogen from the soil has become a serious environmental problem and efficient management of nitrogen manure is needed for today’s agriculture. As nitrate is the major source of nitrogen, genetic improvement of the nitrate assimilation pathway is thought to be essential for solving the problem.

Recently, it has been recognized that nitrate uptake by root epidermal cells is the first step of the nitrate assimilation pathway in higher plants. Nitrate uptake consists of a low-affinity transport system (LATS) at higher nitrate concentrations (>1 mM) and a high-affinity transport system (HATS) at lower nitrate concentrations (<1 mM) (Glass and Siddiqi 1995, Crawford and Glass 1998, Forde 2000); uptake by the two systems is mediated by NRT1 and NRT2, respectively (Forde 2000). HATS are further subdivided into constitutive (cHATS) and inducible (iHATS) systems (Wang and Crawford 1996, Crawford and Glass 1998, Forde 2000).

Paddy rice (Oryza sativa L.), a major crop in East and Southeast Asia, prefers ammonium to nitrate as a nitrogen source, but researchers have recently shown that rice efficiently uses the nitrate formed by nitrification in the rhizosphere (Wang et al. 1993, Kirk and Kronzucker 2005, Duan et al. 2006). It is of practical importance to improve the efficiency of nitrate uptake in paddy rice. As the nitrate concentration in the rhizosphere of paddy fields is estimated to be 1–10 μM (Kirk and Kronzucker 2005), in rice, NRT2 appears to play a major role in nitrate uptake (Araki and Hasegawa 2006). As in higher plants such as Arabidopsis thaliana (Orsel et al. 2002) and barley (Vidmar et al. 2000), rice has NRT2 family genes that are differentially regulated. In rice, four NRT2s have been identified and OsNRT2.1 and OsNRT2.2 are thought to play a major role in nitrate assimilation (Araki and Hasegawa 2006).

In this study, for improving nitrate utilization of paddy rice under low-nitrogen input conditions, OsNRT2.1, one of the candidate genes for breeding rice for more efficient use
of nitrate, was constitutively expressed by introducing more than one copy of OsNRT2.1 fused to a constitutive 35S promoter using Agrobacterium tumefaciens. Nitrate uptake ability and vegetative growth of the transgenic plants were examined.

Materials and Methods

Cloning of OsNRT2 and plasmid used for transformation

The clone RNRT1 was obtained from a rice root cDNA library using a barley cDNA homologue of high-affinity nitrate transporter genes of Chlamydomonas reinhardtii and Aspergillus nidulans. RNRT1 was subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA) and named pRNRT1. The RNRT1 sequence has been registered in DDBJ under the accession number AB008519 (OsNRT2).

Plasmid pMLH7133-RNRT2 was constructed as shown in Fig. 1. A cDNA fragment of pRNRT1, obtained by digesting with EcoRV and BamHI, was ligated with the plasmid pMLH7133-GUS cut with EcolI and BamHI. Plasmid pMLH7133-GUS (Mochizuki et al. 1999) was a gift from Dr. Ohashi, National Institute of Agrobiological Science, Japan.

Plant materials and callus induction

Rice (Oryza sativa L. cv. Yumemouri) calli were prepared as described by Urushibara et al. (2001). Briefly, seeds were sterilized with 1% (v/v) sodium hypochlorite for 20 min, rinsed three times with sterile distilled water, and then plated on N6 medium (pH 5.8, Chu et al. 1975) supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.38 mg/L casamino acids, 2.88 mg/L proline, 30 g/L sucrose and 4 g/L GelRite at 25°C in the dark for callus induction.

Transformation and reproduction for examination

Rice transformation using Agrobacterium tumefaciens was carried out as proposed by Hiei et al. (1994). Calli were pre-cultured for 2 days were incubated for 2 min with A. tumefaciens strain EHA105 harboring pMLH7133-RNRT2 for infection. The A. tumefaciens strain was a gift from Dr. Hood, ProdiGene Inc., USA. Infected calli were cultured on N6 medium (pH 5.8) supplemented with 10 mg/L acetosyringone, 2 mg/L 2,4-D, 30 g/L sucrose, 10 g/L glucose and 2 g/L GelRite at 25°C in the dark. After 3 days, calli were thoroughly rinsed with 500 mg/L carbenicillin solution, and then cultured on N6 medium (pH5.8) supplemented with 500 mg/L carbenicillin, 50 mg/L hygromycin B, 2 mg/L 2,4-D, 30 g/L sucrose and 4 g/L GelRite at 25°C under fluorescent lighting (70 μmol·s⁻¹·m⁻²). About 2 weeks later, surviving calli were transferred to regeneration medium (N6 medium containing 50 mg/L hygromycin, 60 g/L sucrose and 10 g/L agarose [Type I, Sigma], pH5.8) at 25°C under fluorescent lighting (70 μmol·s⁻¹·m⁻²).

Regenerated plants were grown to maturity in 6-inch pots (Fujiwara Sci. Co.) in a greenhouse under natural daylight and daylength (30°C for 16 h during the day and 25°C for 8 h during the night) and the progeny were harvested. In the second generation, seeds of plants confirmed to be transgenic by PCR were cultured under the same conditions as the originally generated plants (first generation) and the progeny were harvested. Seeds of the third generation (T3), which were surface-sterilized with 1% (v/v) sodium hypochlorite for 20 min and rinsed with sterile distilled water, were sown and cultured on MS medium (pH5.8) supplemented with 50 mg/L hygromycin B, 30 g/L sucrose and 4 g/L GelRite in a growth chamber at 25°C under fluorescent lighting (16 h daylength, 70 μmol·s⁻¹·m⁻²). Germinated plants that were selected as possible transgenics were used for further experiments.

DNA analyses

Genomic DNA was isolated from the leaves of the selected plants identified as possible transformants using a Plant Genomic DNA Mini Kit (Viogene). The transgene was detected in genomic DNA using two primer sets: 5′-AGCTGC TCTTCTTCACGTGC-3′ and 5′-CAAATGTTTGAACGA TCAGG-3′, which contain sequences corresponding to the coding region of OsNRT2.1 cDNA and the nopaline synthetase terminator, respectively, and 5′-GAGAGTTCATG GCAACTACT-3′ and 5′-TTCGAAGACCTTCTCAGTA-3′, which contain sequences corresponding to the coding region of OsNRT2.1 cDNA and the 35S promoter, respectively. PCR conditions consisted of annealing for 10 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C.

Southern blot analyses were carried out as described by Sambrook et al. (1989). Genomic DNA was isolated from leaves using a CTAB method (Murray and Thompson

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**Fig. 1.** Binary vector used for rice transformation (pMLH7133-RNRT2). Expression of the NRT2.1 gene is regulated by 7 copies of an enhancer of the cauliflower virus 35S promoter (E7) with the tobacco mosaic virus omega sequence insertion (O) and the first intron of the gene for phaseolin (I). The hygromycin-resistance gene (hpt) serves as a selectable marker in rice transformation. RB: right border sequence, LB: left border sequence. Restriction sites are indicated under the vector.
1980). Following digestion with HindIII, 5 μg of DNA was electrophoresed through a 0.7% (w/v) agarose gel and transferred to a Hybond N+ nylon membrane (Amersham). A DIG-labeled probe corresponding to the hygromycin-resistance gene was transcribed from pRNRT1 using T7 polymerase with a PCR DIG Probe Synthesis Kit (Roche). Detection was carried out using a DIG Luminescent Detection Kit (Roche).

**RT-PCR analyses**

Seedlings at the three-leaf stage were treated with 200 μM KNO₃ containing 400 μM CaSO₄ solution after pretreatment with 400 μM CaSO₄ for 24h. Total RNA was extracted from rice roots and leaves using an RNeasy Plant Mini Kit (Qiagen) and treated with an RNase-Free DNase Set (Qiagen). RT-PCR was performed following the protocol of a One-Step RT-PCR Kit (Qiagen). Specific primers were used for this experiment: 5'-GCTAGTGTGGCCATGGA CTCG-3' and 5'-ACGACAGCAACCTGATCGT-3' for OsNRT2.1, 5'-TGGAGCGAACAGGAAGAAG-3' and 5'-GCTCCTATATTAGCTGTGGCT-3' for OsNRT2.2, 5'-GCCATCCAAAGATCGGTAG-3' and 5'-TGTGGAGCT TCCCGTAGTTG-3' for OsNRT2.3, and 5'-ATCGTTCCT CTTGCTCTGCAA-3' and 5'-TACCTGGACCGCTGAA GAA-3' for OsNRT2.4. The specific primers 5'-CTTCAT AGGAATGGAAGCTGCGGGTA-3' and 5'-GCTCATC GACGGAGAAGAG-3' were used for actin as the constitutive internal standard. Conditions for RT-PCR consisted of reverse transcription for 30 min at 50°C, followed by 30 cycles of 15 s at 94°C, 30 s at 58°C, and 1 min at 72°C.

**Nitrate uptake measurement**

Seedlings at the three-leaf stage that had been cultivated for 2 weeks in distilled water were transferred into a solution containing 200 μM KNO₃ and 400 μM CaSO₄ for 24h to induce nitrate uptake. After induction, plants were transferred individually into a 50 ml solution containing 20 μM or 200 μM KNO₃ with 400 μM CaSO₄ in a controlled chamber at 25°C under fluorescent lighting (70 μmol s⁻¹ m⁻²). After 2h, the nitrate concentration of the solution was measured with an ion chromatograph (LC solution, Shimadzu) equipped with an anion specific column (IC-A3, Shimadzu). The fresh weight of each plant was also measured after nitrate treatment. Nitrate uptake was evaluated by the disappearance of nitrate from the solution by kinetic analysis using the method of Hasagawa and Ichii (1994). Each experiment, which used three to five plants, was replicated three times.

**NR activity**

Plants were cultivated for 4 weeks in modified Kasugai’s nutrient solution (pH 5.8, 0.2 mM nitrate as nitrogen source) at 25°C under fluorescent lighting (16h daylength, 70 μmol s⁻¹ m⁻²). Preparation of the crude extract and measurement of NADH-NR and NADPH-NR activities were performed according to the procedure of Kleinhofs et al. (1986).

**Growth of transgenic plants in different nitrogen sources**

Seedlings that had been cultured with distilled water to the three-leaf stage for 2 weeks were cultured with KNO₃ or NH₄NO₃ as nitrogen source at a N concentration of 200 μM. Other major and minor elements were supplied with Kasugai’s nutrient solution (Kasugai 1939). Plants were grown in a controlled chamber at 25°C under fluorescent lighting (16h daylength, 70 μmol s⁻¹ m⁻²). The culture solution was exchanged every week. The pH of the culture solution was adjusted to 5.8 with 0.1 N HCl or 0.1 N NaOH every 2 days. The plant height, root length and total fresh weight were measured every week. Each experiment was replicated three times using fifteen plants.

**Results**

**Production of transgenic rice plants overexpressing OsNRT2.1**

In this experiment, 100 hygromycin-resistant calli were obtained from about 200 calli infected with *A. tumefaciens* harboring OsNRT2.1. From the surviving calli, about 100 regenerated plants grew to maturity. In the second generation (T2), from 25 transformants confirmed by PCR and RT-PCR analysis, the progeny of two transformants (Nrt201 and Nrt204), which showed better growth without any morphological changes, were selected for further studies. Transformation was confirmed in the T3 generation. Genomic Southern blotting analysis using all T3 lines detected bands of the 35S transgene in each line, indicating that one copy of 35S transgene had been introduced (Fig. 2). Two lines, Nrt201-2 and Nrt204-5, from Nrt201 and two lines, Nrt204-1 and Nrt204-2, from Nrt204 were used for further study.

**Expression of NRT2s in roots and shoots**

In the wild-type plants, transcripts of OsNRT2.1, OsNRT2.2 and OsNRT2.4 in roots were detected starting 30 min after the beginning of nitrate treatment, suggesting that a short induction period exists in rice as reported by Araki and Hasegawa (2006). Transcripts of OsNRT2.3 were constitutively expressed. Semi-quantitative RT-PCR analysis showed that the amount of OsNRT2.1, OsNRT2.2 and OsNRT2.4 transcripts increased with nitrate treatment time and peaked 3 h after the beginning of treatment. On the other hand, in all of the transgenic plant lines, OsNRT2.1 and OsNRT2.2 were constitutively expressed in roots (Fig. 3A). No difference in the expression pattern of NRT2 family genes was found among all transgenic lines. Fig. 3 shows the expression pattern of NRT2 family genes in Nrt204-1 as a representative. There was no difference in the expression of other genes involved in nitrate uptake (OsNARs and OsNRT1) in roots between wild-type and transgenic plants; in both wild-type and all transgenic plant lines, OsNAR2.1 transcript was induced within 15 min after transferring plants to nitrate from a N-free solution, while OsNAR2.2 and OsNRT1 were constitutively expressed (data not shown).

In wild-type plants, the transcript level of OsNRT2s in
plants was 1.13 μmol g⁻¹ f.w. h⁻¹. However, the nitrate uptake of the Nr204-derived lines, Nr204-1 and Nr204-2, was lower than that of wild-type at both nitrate concentrations. Nitrate uptake was 0.13 μmol g⁻¹ f.w. h⁻¹ in Nr204-1 and 0.25 μmol g⁻¹ f.w. h⁻¹ in Nr204-2, at 20 μM and 0.72 μmol g⁻¹ f.w. h⁻¹ and 1.10 μmol g⁻¹ f.w. h⁻¹, respectively, at 200 μM.

There was no significant difference in NR activity of the leaves between transgenic and wild-type plants cultivated hydroponically for 4 weeks. NR activity of Nr201s, Nr204s and wild-type plants was 24.88 ± 4.94, 21.22 ± 2.55 and 21.25 ± 5.21 nmol NO₃⁻ g⁻¹ f.w. min⁻¹, respectively.

**Growth and morphological characters**

When KNO₃ or NH₄NO₃ was used as nitrogen source at 200 μM of N, the plant height and total fresh weight of both Nr204-1 and Nr204-2 increased compared to that of wild-type plants for both nitrogen sources throughout the cultivation period (Fig. 4 and Fig. 5). There was no difference in root length between transgenic and wild-type plants. The plant height of Nr204-1 was higher than that of Nr204-2. On the other hand, there was no significant difference in plant height between Nr201s and wild-type plants when KNO₃ or NH₄NO₃ was applied as nitrogen source (Fig. 4). No difference in the morphological characters at maturity was observed between transgenic and wild-type plants.

**Discussion**

In this study, an additional copy of *OsNRT2.1*, a candidate gene for nitrate use efficiency in rice (Araki and Hasegawa 2006), was transformed into a *japonica* cultivar, Yumeoumi. Of the transformant lines analyzed, the growth of two lines derived from one of the transformants, Nr204, was accelerated under low nitrate conditions, suggesting that constitutive overexpression of *OsNRT2.1* stimulates vegetative growth in rice. However, the nitrate uptake ability of Nr204-derived lines was less than that in wild type and no stimulation of NR activity was detected. Although constitutive expression of *OsNRT2.1* in roots did not affect nitrate uptake or NR activity, constitutive overexpression of *OsNRT2.1* in shoots may provide better vegetative growth (Fig. 3). Crawford (1995) described the regulation of nitrate and/or nitrogen metabolic pathway as complex and diverse. Recent research showed that the effects by the alternation of a single gene involved in nitrate assimilation are often different from the predicted one on nitrate metabolism. For example, it has been reported that NR-deficient mutants or transformants in barley (Abdel-Latif *et al.* 2004), *Nicotiana plumbaginifolia* (Dorbe *et al.* 1992) and rice (Omura and Hasegawa 2001) grow vigorously when nitrate is applied as the sole source of nitrogen. The present results were also unexpected. The transformant lines, Nr204-1 and Nr204-2, grew well without increased levels in nitrate uptake despite constitutive expression of both *OsNRT2.1* and *OsNRT2.2*.

The most interesting finding of this experiment was that a gene transferred from the same species was well expressed.
As observed for the genes involved in nitrate assimilation in higher plants, introduction of a target gene from the same species allowed co-suppression of both the transgene and host gene and did not result in acceleration of nitrate utilization or enhanced growth. In the case of NR, introduction of *Nicotiana plumbaginifolia* (*NpNia*) cDNA resulted in co-suppression of the host gene and the 35S transgene encoding NR and lethality (Vincentz and Caboche 1991, Dorlhac et al. 1994). When *NpNRT2* was introduced into *N. plumbaginifolia*, the gene was expressed but neither accelerated influx by roots nor accumulation of nitrate in tissues was observed, suggesting that regulation of HATS is controlled at the post-transcriptional level for NRT2 (Fraisier et al. 2000). However, in the present experiment using rice, increasing the gene dosage by introducing *OsNRT2.1* resulted in constitutive expression and enhancement of vegetative growth without co-suppression of the host gene and transgene. The cooperative effect between host *OsNRT2.1* and transformed *OsNRT2.1* might be related to the correlation between *OsNRT2.1* and *OsNRT2.2* because *OsNRT2.1* and *OsNRT2.2*, which have the same ORF with different 5′-UTR and 3′-UTR, are expressed coordinately (Araki and Hasegawa 2006, Miller et al. 2007). In transgenic plants, the 35S transgene encoding *OsNRT2.1* was expressed constitutively, whereas the host gene encoding *OsNRT2.2* was also expressed constitutively, unlike in wild-type plants. These observations suggest that the constitutive expression of *OsNRT2.1* led to altered regulation of the metabolic pathway, and was followed by the constitutive expression of *OsNRT2.2*. Although the constitutive expression of *OsNRT2.1* and *OsNRT2.2* remains to be clarified, the transgenic plants used in this experiment are useful for the research of nitrate metabolic pathways.

In today’s agriculture, it is necessary to breed crop cultivars that efficiently use nitrogen, because such cultivars are likely to grow better under low nitrogen input conditions. The present results that when transferring an additional copy of NRT2 into the same species, the transformants showed better vegetative growth under very low nitrate conditions, imply the possibility of breeding rice cultivars with efficient utilization of nitrate by use of the transgenic plants. However, the relationship between expression of this gene involved in nitrate assimilation and vegetative growth is still unclear. Further investigations are necessary.

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**Table 1.** Nitrate uptake in 20 and 200 μM KNO₃ of transgenic and wild-type plants cultured for 2 weeks

<table>
<thead>
<tr>
<th>Lines</th>
<th>Nitrate uptake (μmol g⁻¹ f.w. h⁻¹)</th>
<th>20 μM</th>
<th>200 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td>0.33 ± 0.030</td>
<td>1.13 ± 0.179</td>
</tr>
<tr>
<td>Nrt201-2</td>
<td></td>
<td>0.41 ± 0.012</td>
<td>1.42 ± 0.105</td>
</tr>
<tr>
<td>Nrt201-5</td>
<td></td>
<td>0.41 ± 0.018</td>
<td>1.17 ± 0.047</td>
</tr>
<tr>
<td>Nrt204-1</td>
<td></td>
<td>0.13 ± 0.012**</td>
<td>0.72 ± 0.062</td>
</tr>
<tr>
<td>Nrt204-2</td>
<td></td>
<td>0.25 ± 0.018</td>
<td>1.10 ± 0.047</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of three replications.

**Significant difference at the 1% level when compared with the wild-type plant (Student’s t-test)**
Acknowledgments

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Literature Cited


