Functional Characterization of a Putative Nitrate Transporter Gene Promoter from Rice

Ting-Zhang HU1,3, Kai-Ming CAO1, Mian XIA2, and Xi-Ping WANG1,2*

1 Department of Biochemistry, School of Life Science, Fudan University, Shanghai 200433, China;
2 National Center for Molecular Crop Design, Weiming Kaituo Agriculture Biotech Co., Ltd., Beijing 100085, China;
3 Department of Biology, Chongqing Three Gorges University, Chongqing 404000, China

Abstract  Drought is one of the most significant abiotic stresses that influence plant growth and development. Expression analysis revealed that OsNRT1.3, a putative nitrate transporter gene in rice, was induced by drought. To confirm if the OsNRT1.3 promoter can respond to drought stress, a 2019 bp upstream sequence of OsNRT1.3 was cloned. Three OsNRT1.3 promoter fragments were generated by 5'-deletion, and fused to the β-glucuronidase (GUS) gene. The chimeric genes were introduced into rice plants. NRT2019::GUS, NRT1196::GUS and NRT719::GUS showed similar expression patterns in seeds, roots, leaves and flowers in all transgenic rice, and GUS activity conferred by different OsNRT1.3 promoter fragments was significantly upregulated by drought stress, indicating that OsNRT1.3 promoter responds to drought stress and the 719 bp upstream sequence of OsNRT1.3 contains the drought response elements.

Key words: deletion analysis; drought; nitrate transporter; OsNRT1.3 promoter

Plants grow in a dynamic environment that frequently imposes constraints on growth and development. Among the adverse environmental factors commonly encountered by land plants, drought is one of the most significant abiotic stresses that influence plant growth and development and is a major limit on plant productivity in cultivated areas worldwide. To adapt to drought conditions, plants evolve various mechanisms. In plants, at least four independent regulatory pathways exist in response to drought stress: two are abscisic acid (ABA)-dependent and the other two are ABA-independent [1,2].

To overcome limitations of environmental factors and increase crop yield under stress conditions, it is important to improve stress tolerance in crops. The responses of plants to various abiotic stresses have been important subjects of physiological, molecular and transgenic studies [3]. The identification of novel genes, the determination of their expression patterns in response to the stresses, and an improved understanding of their functions in stress adaptation will provide us with the basis of effective engineering strategies to improve stress tolerance [4].

Rice (Oryza sativa), one of the most important crops, has now emerged as an ideal model species for the study of crop genomics due to its commercial value, relative small genome size (approximately 430 Mb), diploid origin and close relationship to other important cereal crops [5]. Rice research has entered a new era after the completion of the entire genomic sequence of rice. It is vital to identify the specific functions of the predicted rice genes and their expression profiles. Sequencing projects increase not only numbers of genomic sequences but also large numbers of expressed sequence tags (ESTs) and full-length cDNA sequences for rice. There are many opportunities to use this sequence information to accelerate the progress toward a comprehensive understanding of genetic mechanisms that control the rice growth and development and their response to the environments [5].

Nitrate transporters (NRTs) have been isolated and characterized in various plant species, including Arabidopsis, tomato, barley, soybean, rice, and Nicotiana plumbaginifolia [6–11]. In higher plants, the molecular basis of root nitrate uptake has been the subject of intensive

©Institute of Biochemistry and Cell Biology, SIBS, CAS
studies during the last decade [12]. Two families of nitrate transporter genes, NRT1 and NRT2, have been identified to contribute to these uptake systems. The NRT1 and NRT2 families are involved in the low-affinity nitrate transport system and the high-affinity nitrate transport system, respectively. Many physiological investigations have led to the conclusion that plants have developed at least four different uptake systems to cope with large variations in nitrate concentrations: constitutive high affinity, inducible high affinity, constitutive low affinity and inducible low affinity [6,13–15]. The complexity of nitrate/nitrite transport is enhanced by the fine regulation that occurs at the transcriptional level. Some genes expressed primarily in root are induced by NO₃⁻ and down regulated by reduced forms of nitrogen such as NH₄⁺ and glutamine [11].

Nitrate transporters belong to the proton-dependent oligopeptide transporter (POT) or peptide transporter (PTR) family and are encoded by a multi-gene family in rice. These POT family proteins are predicted to comprise twelve transmembrane regions. These integral membrane proteins have a PTR2 domain with accession No. PF00854 in the Pfam database of protein families and hidden Markov models (HMMs), http://pfam.wustl.edu/cgi-bin/getdesc?name=PTR2. Ninety-five rice gene models contained the PTR2 domain in the rice annotation database [release 4.0 of The Institute of Genome Research (TIGR) Rice Pseudomolecules and Genome Annotation, http://www.tigr.org/tigr-scripts/e2k1/osa1/putative_function_search.pl]. Some of the putative POT family proteins may have the function of nitrate transporters, and six of them were named as putative nitrate transporters. Investigations into nitrate transporter gene from rice have been minimal. The first low-affinity nitrate transporter gene, OsNRT1, from rice was cloned and characterized, which encodes a constitutively expressed transport system for low-affinity nitrate uptake. OsNRT1 exhibits more functional properties in common with AtNRT1.2 than with AtNRT1.1 [10]. A putative nitrate transporter (LOC_0s10g40600, GenBank accession No. NM_197689) in rice, which contained a PTR2 domain, is one of the six putative nitrate transporters, and here we named it as OsNRT1.3. Amino acid sequence alignment showed that OsNRT1.3 shares a lower degree sequence identity with OsNRT1 (approximately 33.72%), but has higher similarity to their corresponding Arabidopsis and Nicotiana plumbaginifolia homologs, AtNRT1.1 (CHL1), NpNRT1.1 and NpNRT1.2 (amino acid sequence identities are 60.23%, 63.33% and 62.46%, respectively). Also, OsNRT1.3 has a homolog in rice, another putative nitrate transporter (LOC_0s05g05910, GenBank accession No. XM_480163). They showed high homology at the amino acid level (identity is 67.21%).

In this work, as shown by the semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis, OsNRT1.3 was induced by drought. To investigate whether OsNRT1.3 expression is regulated transcriptionally at the cis-acting elements in the promoter region, the 5'-upstream sequence of OsNRT1.3 was isolated and fused to GUS gene. The expression patterns responding to drought, ABA, NaCl, (NH₄)₂SO₄, KNO₃ and NH₄NO₃ were investigated in transgenic rice plants.

### Materials and Methods

#### Plant materials and culture conditions

Oryza sativa cv. Zhonghua 11 was used in all experiments, for investigating OsNRT1.3 gene expression patterns. Rice seeds were soaked in water at 28 °C for 2 d, and then hydroponically grown at 28 °C under a 16/8 h light/dark photoperiod at an intensity of approximately 250 μE/m²/s. Ten-day-old seedlings were divided into four portions for different treatments: water (control), 250 mM NaCl, 100 μM ABA and emergency drought (air-drying on filter paper). The seedlings from each treatment were collected after 0, 1, 2, 6, 10, 24, and 48 h treatment, respectively, frozen in liquid nitrogen and then stored at −80 °C.

Transgenic T2 seedlings cultured for 10 d were treated with emergency drought, simulative drought (15% PEG6000), 100 μM ABA, 250 mM NaCl, 5 mM (NH₄)₂SO₄, 10 mM KNO₃, 100 μM KNO₃ and 1 mM glucose (Gln) for 6 h, respectively. The roots and leaves of seedlings from each treatment were collected, frozen in liquid nitrogen and then stored at −80 °C.

#### RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from the seedlings using Trizol reagent (Gibco-BRL, Grand Island, USA). One microgram of total RNA from each treatment was used for reverse transcriptions using oligo(dT)₁₅ as 3’ primer.

Based on the OsNRT1.3 cDNA sequence, a pair of primers, OsNRT1 sf (5’-AGCAGAGGATGCCACACAG-3’) and OsNRT1 sr (5’-CGATGAGGAAGACGGTGAG-3’), were designed, which produced a 294 bp PCR product. The housekeeping gene ACTIN1 was used as a control [16]. The primers ACTIN1 f (5’-TCCGACATCAAGAAGAAAG-3’) and ACTIN1 r (5’-GATATCACATGACTGACTATG-3’) were designed to get a 242 bp amplification product. The gene fragments of OsNRT1.3 and ACTIN1 were amplified using the two pairs of primers (OsNRT sf/OsNRT sr/OsNRT1 sf/OsNRT1 sr) and the reverse-PCR system of the Thermo Fisher GeneAmp PCR System 2700 instrument.
OsNRT1.3 were generated from 5′ nylon membrane (Amersham, Piscataway, USA). Probes total RNA. Sample was separated with electrophoresis in Northern blot analysis chimeric genes consisting of the as Chen and Ronald described [18]. To construct the fragments were generated. A 2019 bp promoter upstream by

against the expression level of inner control gene

rated in a 2.5% (W/V) agarose gel. The relative expression level of OsNRT1.3 in different conditions was calibrated against the expression level of inner control gene ACTIN1.

**Northern blot analysis**

Northern blot analysis was performed using 20 µg of total RNA. Sample was separated with electrophoresis in 1.2% denaturing agarose gel and then transferred to Hybond nylon membrane (Amersham, Piscataway, USA). Probes were generated from 5′ upstream specific sequence of OsNRT1.3 gene and radiolabeled with [α-32P]dCTP using the random primer system. Standard procedures were used for RNA blot analysis [17].

**Cloning of OsNRT1.3 promoter and generation of OsNRT1.3 promoter::GUS fusions**

Rice genomic DNA was extracted from the seedlings as Chen and Ronald described [18]. To construct the chimeric genes consisting of the GUS gene sequence driven by OsNRT1.3 promoter, three 5′-deletion promoter fragments were generated. A 2019 bp promoter upstream of OsNRT1.3 was amplified from rice genomic DNA with forward primer pNRTf1 (5′-CACCCTGCAATGACTACTTTGATGTCAGCACCC-3′) and the reverse primer pNRTr (5′-CTAGGCGCCTATCACAACACACAG-3′), which was introduced to a PstI site at the 5′-end and an NcoI site at the 3′-end. The amplification product was cloned into pENTR/D-TOPO vector (Invitrogen, Carlsbad, USA), thereafter sequenced, and then subcloned into PstI and NcoI sites of binary vector pCAMBIA1301. It was named as NRT2019::GUS. The 1196 bp fragment of 5′-upstream region of OsNRT1.3 was subcloned into vector pCAMBIA1301 by HindIII and NcoI. The construct was designated as NRT1196::GUS. The 719 bp fragment of the 5′-upstream region was amplified by PCR from NRT2019::GUS with the forward primer pNRTf2 (5′-CACCCTGCAATGACTACTTTGATGTCAGCACCC-3′) and the reverse primer pNRTr. The PCR product was then subcloned into PstI and NcoI sites of pCAMBIA1301 and sequenced; the construct was named as NRT719::GUS.

**Generation and Southern blot analysis of transgenic rice plants**

NRT2019::GUS, NRT1196::GUS and NRT719::GUS were introduced into Agrobacterium tumefaciens AGL0 by the freeze-thaw method [19]. Transformation was carried out using Agrobacterium-mediated cocultivation method [20]. Rice plants were grown in the medium containing 25 mg/L hygromycin B inside the greenhouse. The positive transgenic plants were selected by analyzing hygromycin B resistance, GUS expression and PCR.

About 10 µg of genomic DNA of transgenic plants was digested by EcoRI, separated on 0.8% (W/V) agarose gel, and then transferred to Hybond nylon membrane. Probes were generated from the HPT gene and radiolabeled with [α-32P]dCTP using the random primer system. Standard procedures were used for RNA blot analysis [17].

The seeds from individual lines were harvested. We selected the homozygous transgenic plants with single copy insertion for the following experiments.

**Histochemical GUS assay and GUS activity assay**

Expression profile of GUS gene driven by OsNRT1.3 promoter was identified by histochemical GUS assay [21]. The transgenic rice tissues were stained with GUS staining solution consisting of 50 mM sodium phosphate, pH 7.0, 0.05 mM K3Fe(CN)6, 10 mM Na2EDTA, 0.1% (V/V) Triton X-100, 20% (V/V) methanol, 1 mg/L X-Gluc. After a brief period of vacuum infiltration, the samples were incubated at 37 °C for up to 24 h. The empty vector transgenic rice plants, which carried GUS gene without the promoter, were used as controls.

Quantitative GUS enzymatic assay was performed as described by Jefferson et al. [22]. The roots and leaves of seedlings were ground in liquid nitrogen, GUS extraction buffer (50 mM sodium phosphate, pH 7.0, 1 mM Na2EDTA, 0.1% Triton X-100, 0.1% N-sodium lauryl sarcosyl, 10 mM mercaptoethanol) was added, and finally centrifugation was carried out. Protein concentrations were estimated with the colorimetric method of Bradford [23]. Five microliters of extracted proteins were incubated with 45 µl of GUS enzymatic assay buffer containing 2 mM 4-methylumbelliferyl glucuronide for 20 min at 37 °C. Five microliters of the reaction mixture were added into 195 µl of 0.2 M sodium carbonate to stop the reaction, then its fluorescence was measured with a fluorometer (Tecan, Zurich, Switzerland).

**Results**

OsNRT1.3 expression is induced by drought

Semi-quantitative RT-PCR analysis demonstrated that OsNRT1.3 transcription was upregulated after emergency
drought treatment for 1 h and reached its peak around 6 h, and the OsNRT1.3 expression treated by water had no distinct change in rice seedling (Fig. 1). Treatment with ABA and NaCl did not significantly affect the OsNRT1.3 expression either (data not shown).

Northern blot analysis also suggested that the expression of OsNRT1.3 was induced obviously by drought in rice (Fig. 2).

Cloning of the OsNRT1.3 promoter and generation of OsNRT1.3 promoter::GUS fusion

A 2019 bp promoter upstream of the translation start codon ATG of the OsNRT1.3 was isolated from Oryza sativa cv. Zhonghua 11 by PCR using specific primers pNRTf1 and pNRTr (the OsNRT1.3 promoter sequence has been deposited at GenBank under accession No. DQ323736). A putative TATA-box, CAAT-box and transcription start site (CAC) was detected at position −189 bp, −127 bp, and −93 bp upstream of the translation start codon ATG, respectively (Fig. 3).
To investigate the function of the OsNRT1.3 promoter, the 2019 bp 5' flanking sequence of the OsNRT1.3 gene was isolated and a series of 5' deletion promoter fragments was created. The pCAMBIA1301 was used as a binary vector and the 35S promoter of GUS gene was replaced by the 5' upstream regions of the OsNRT1.3, 2019 bp, 1196 bp and 719 bp in length, respectively (Fig. 4).

**Generation and Southern blot analysis of transgenic rice plants**

NRT2019::GUS, NRT1196::GUS and NRT719::GUS were introduced to A. tumefaciens AGL0, and transformed into rice. More than 20 transgenic lines of each construct were obtained. These transformants were verified by PCR analysis and histochemical GUS assay (data not shown).

Southern blot was used to verify the integration of GUS gene and the copy number of integration. The genomic DNA of wild type and transgenic lines were digested with EcoRI, and hybridized with the probe of radiolabeled HPT gene fragment. The transgenic plants showed specific bands and the wild type had no such hybridization signal. Some transgenic lines that showed a specific band were single copy insertion (Fig. 5).

Independent transgenic lines of homozygous T2 seedlings with single copy insertion for each construct were selected for further analysis.

**Tissue localization of GUS expression directed by the OsNRT1.3 promoter fragments**

Histochemical analyses were performed on seeds and plants during the rapid vegetative growth phase. The representative examples were shown in Fig. 6. The GUS expression was found to be similar in all the NRT promoter::GUS transformants. The GUS expressed mainly in embryo and aleurone layer of seed, root, leaf and flower, which suggested the OsNRT1.3 promoter can direct GUS expression in different tissues of transgenic plants. The NRT2019::GUS, NRT1196::GUS and NRT719::GUS showed similar expression patterns, and the tissue specificity did not change in NRT2019::GUS, NRT1196::GUS and NRT719::GUS transgenic plants after drought treatment (data not shown).

GUS assay showed the inducible activity of OsNRT1.3 promoter

Transgenic rice plants harboring the chimeric NRT2019::GUS, NRT1196::GUS and NRT719::GUS gene
Discussion

In the present study, semi-quantitative RT-PCR and Northern blot analysis revealed that the OsNRT1.3 gene is expressed in low levels in rice seedlings before drought stress. OsNRT1.3 transcript was upregulated after emergency drought treatment for 1 h and reached peak value around 6 h. The finding showed that the OsNRT1.3 gene is related to drought stress in the growth of rice plants. OsNRT1.3 perhaps plays an important role in drought susceptibility in rice.

In order to further investigate the expression profile of the OsNRT1.3 gene, the upstream region of OsNRT1.3 was cloned. The transgenic lines of NRT2019::GUS...
NRT1196::GUS and NRT719::GUS displayed the similar expression pattern and GUS activity (Fig. 7). These findings suggested that the 719 bp fragment of 5′-flanking sequence upstream of OsNRT1.3 was a functional promoter and included the drought response elements and the other main cis-elements.

All the transgenic rice plants exhibited similar induced expression of GUS activity after emergency drought and simulative drought treatment. However, the GUS expression was not affected by ABA, NaCl, (NH4)2SO4, KNO3, and Gln, suggested that the induction of OsNRT1.3 promoter is specific for drought. The increased expression of OsNRT1.3 is specifically linked to water loss from the tissue.

By using PLACE (http://www.dna.aaffrc.go.jp) and Plant CARE (http://intra.psb.ugent.be:8080/PlantCARE/) databases, the 2019 bp upstream region of OsNRT1.3 gene was searched for cis-acting regulatory elements. A TATA-box and a CAAT-box were detected at position −189 bp and −127 bp upstream of the ATG. The putative transcription start site was located at −93 bp upstream of the ATG (Fig. 3). The two MYC-like sequences CATGTG were detected in −1938 bp and −424 bp in the OsNRT1.3 promoter region (Fig. 3). It has been shown that the MYC-like sequence CATGTG plays an important role in the dehydration-inducible expression of the Arabidopsis ERD1 gene, and the expression of GUS gene driven by a 63 bp region containing the CATGTG motif was induced by drought, high salinity, and ABA [24]. However, the GUS activity directed by OsNRT1.3 promoters with different lengths could not be upregulated by ABA and NaCl, which suggests that OsNRT1.3 expression induced by drought in this study might have been controlled by an ABA-independent pathway. The existence of the MYC-like sequence CATGTG in the OsNRT1.3 promoter may have a different role from that in Arabidopsis ERD1 gene.

The motif of DRE/CRT (dehydration-responsive element/C-repeat) cis-acting element is the drought-responsive element in ABA-independent gene expression. The core sequence of DRE/CRT is C/GCGACA [25]. But the typical core sequence of DRE/CRT element was not detected in OsNRT1.3 promoter, suggesting that some novel dehydration-responsive elements or pathways were involved in the drought inducible expression of OsNRT1.3 gene.

In Arabidopsis it has been shown that the nitrate transporter AtNRT1.1 contributes to drought susceptibility [26]. The OsNRT1.3 shows high homology with AtNRT1.1. It is reasonable to deduce that OsNRT1.3 in rice is also involved in drought susceptibility. The finding that OsNRT1.3 expression is specifically induced by drought provides insight into the relationship between drought and nitrate.

Acknowledgements

We thank Dr. Cheng-Cai CHU (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) and his colleagues for help in GUS activity assay.

References

18 Chen DH, Roland PC. A rapid DNA minipreparation method suitable for AFLP and other PCR applications. Plant Mol Biol Rep 1999, 17: 53–57
22 Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 1987, 6: 3901–3907

Edited by Ming-Hua XU