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Functional Characterization of a Putative Nitrate Transporter Gene Promoter from Rice

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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

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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 NO_3^- and down regulated by reduced forms of nitrogen such as NH_4^+ 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 Genomic 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_Os10g40600, 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_Os08g05910, 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, $(NH_4)_2SO_4$, KNO_3 and NH_4NO_3 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 investing *OsNRT1.3* gene expression patterns. Rice seeds were soaked in water at 28 ºC for 2 d, and then hydroponically grown at 28 ºC under a 16/8 h light/dark photoperiod at an intensity of approximately 250 μE/m^2/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 ºC.

Transgenic T2 seedlings cultured for 10 d were treated with emergency drought, simulative drought (15% PEG6000), 100 μM ABA, 250 mM NaCl, 5 mM $(NH_4)_2SO_4$, 10 mM KNO_3, 100 μM KNO_3 and 1 mM glutamine (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 ºC.

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 olig$(dT)_{18}$ as 3′ primer.

Based on the *OsNRT1.3* cDNA sequence, a pair of primers, OsNRT sf (5′-AGCAGAGGATGCCACACAG-3′) and OsNRT 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′-TCCGTGACATCAAGGAAA-AG-3′) and ACTIN1 r (5′-GATATCAACATCGCACTTCA-TG-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 and ACTIN1 f/ACTIN1 r) in PCR reaction. Amplification was carried out by initial denaturation at 94 ºC for 2 min followed by 28 cycles of 94 ºC denaturation for 30 s, 58 ºC annealing for 30 s, and 72 ºC elongation for 30 s. PCR products from each amplification reaction were separated 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-^{32}P]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′-CACCCTGCAGTACAAGTTGGATGTACGCACC-3′) and the reverse primer pNRTr (5′-CTAGCCATGGCGCCATATCAACAACAACAAG-3′), which was introduced to a *Pst*I site at the 5′-end and an *Nco*I site at the 3′-end. The amplification product was cloned into pENTR/D-TOPO vector (Invitrogen, Carlsbad, USA), thereafter sequenced, and then subcloned into *Pst*I and *Nco*I 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 *Hin*dIII and *Nco*I. 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′-CACCCTGCAGATCACAGCCTCAGAGAATGGG-3′)and the reverse primer pNRTr. The PCR product was then subcloned into *Pst*I and *Nco*I 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 *Eco*RI, 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-^{32}P]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 $K_3Fe(CN)_6$, 10 mM Na_2EDTA, 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 ºC 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 Na_2EDTA, 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 ºC. 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**).

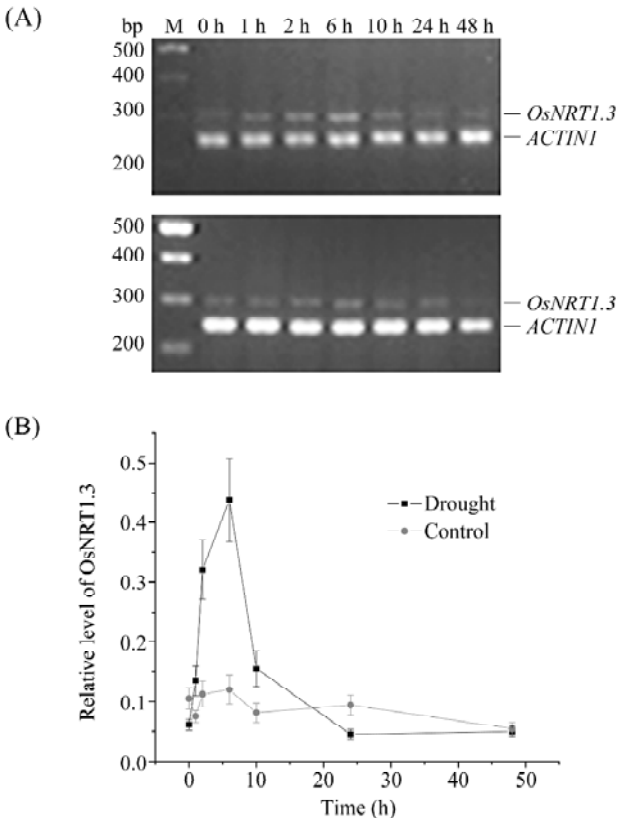

Fig. 1 The relative expression level of *OsNRT1.3* gene in response to emergency drought

(A) Rice seedlings treated by emergency drought (upper) and treated with water as control (bottom). (B) The quantity of the fractions of the PCR products was determined by light density scanning gel using gel image analysis system, the relative expression level of *OsNRT1.3* in different conditions was calibrated against the expression of *ACTIN1*. The averages and standard deviations were obtained from three independent experiments.

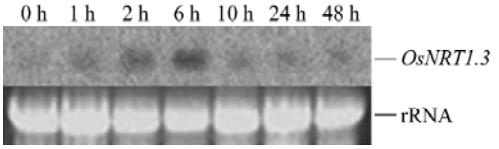

Fig. 2 Northern blot analysis of *OsNRT1.3* gene expression

Rice seedling was treated by emergency drought for different time.

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**).

pNRT f1 Myc-like sequence
–2019 tacaagttggatgtacgcaccacactcgtacgtgtacatgtgggtgtgttctagcacacg
–1959 tttatttttatcaaatttgtctcatagaactttcatatcacaccgtatgagatttggttc
1899 atacacacattgaacttccgagtcatgaaacatatgaggtgttaggagaaaaatcctctg
–1839 acaaaactttccaaatgacatggtcactgatatgtggatccgtcgatggtgggatccata
–1779 tgtcagcgatcatgttcctctaactttacgtcggaggatcctaatccaaagtgttattaa
–1719 ttagatatagaaaggtattttgattagcttagctggtttcctcattaattagctatatat
–1659 ttacgtggagccaaaacttacctcttaatcaattgcttaaatttaatcatgccccttaa
–1599 ctctatattagtaagacaattaaaggagagagctgtaaaacagctcaagagccaaacagc
–1539 tagtactactgtactagctaggtcggcacatcacaggcagggagcaggaaagagataagg
–1479 agaggcaggttcagagtcatccacggtggagtcgattgagctgatcatacacagcttcac
–1359 aacaaagctgctaacgtcaacgcatgtcgctgtatgagagagctatagcttgctgcatgc
–1419 ctagctgatgctgtccttcagagaaatcgacattgatgggtgtgtataattcgtgttaaa
–1299 attagaagtttggttgaaattggaattatatgatagaaaagttagaagtttgtgtgcgta
*Hin*dIII
–1239 gaaaagttttgatgtgatgaaaaaactgtaagtttaaagaaaaagcttggaactaaacaa
–1179 gggcatcgtctgagtctgaatttctattcgtgtcttcatctcaagaaaatacttctcaat
–1119 atacgccggccgtacactcgtcgtagtaccactatacgttcaatgcgaggtagaaagatg
–1059 caggttcaaattgttgttgctaccgactaattaatctgttcatctgaatctgaatttttg
–999 tcatattttgggcccttccagtttcaaatatatatcgatcgggcagtggttttaactgaa
–939 ctaatgtacttacgaggatgatcaaaacaaaattaatgatctaaaagctgaaatgcatga
879 gaacctaattgtgaggctcctagttacacgacaaatcagaatctgatgtagtgaatattt
–819 ttctccaagcgggttagtagtatctttcagattcacacgcatctctttttcccctacaaaca
pNRT f2
–759 cttcacctttttgcaagatgctactactaattactaaacatcacagcctcagagantgg
–699 gcagcacacattgatcataccacacactgttccctgcacataaaaagtttcagtcaaaaa
–639 attatatatcttgatacacagtagtcaagatcacttgtgttccatcaaaattgtatctgc
–579 tcctagtttcagaacgtctcttgactgcaaaatccagtttgtccacaagtatttctccaaa
–519 ttttccaagtgtgtaaactgtggtagttgagattaacgttgtaaccgtccattgtgggaa
Myc-like sequence
–459 actgccatcatctcaacttttctgaccaaacacacacatgtgttgtaattagtcaagaaaa
–399 tcctggagaagtagtaaatcctaatccagcatttgatcctccactaatcctaccaattaa
–339 cgagtcgtactcaataattgctctgagagcgattaaattaacgagtgccagagtcatggga
–279 gaaagaattagaaaattggaggagatactgattcccccacataacaccacttgatactat
CAAT-box
–219 ttctcttaattaattactccctttaattaatcaatcacacctataattacccccccagtgca
TATA-box
–159 cgaatcccgatgcaccacccccctctccgcctatataaggccaccactcggacacggtgg
Transcription start site
99 cttcatcacagcatccatcgccgccggccaccaccaccaccgccgccgccgtcttactct
pNRT r
–39 ctctgtctcgagctcgagcttgttgttgttgatatggccgATG

Fig. 3 Nucleotide sequence of the *OsNRT1.3* promoter

The 2019 bp 5′-flanking sequence upstream of the translation start codon ATG was isolated from *Oryza sativa* cv. Zhonghua 11 by polymerase chain reaction. The primers and *Hin*dIII site used for 5′-deletion are underlined. The putative TATA-box, CAAT-box and Myc-like sequences are shaded. The putative transcription start site (cac) and the translation start codon ATG are in bold.

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 *Eco*RI, 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

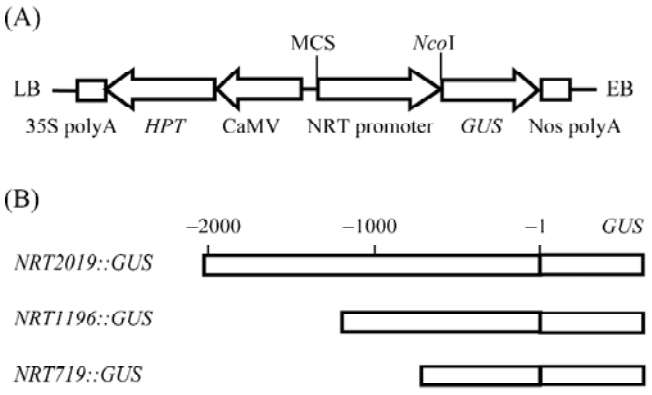

Fig. 4 Schematic diagram of plant expression cassettes

(A) The T-DNA region of plant expression vector. (B) Schematic diagram of *OsNRT1.3* promoters::*GUS* expression cassettes. CaMV, cauliflower mosaic virus; LB, left border; MCS, multiple cloning site; NRT, nitrate transporter; RB, right border.

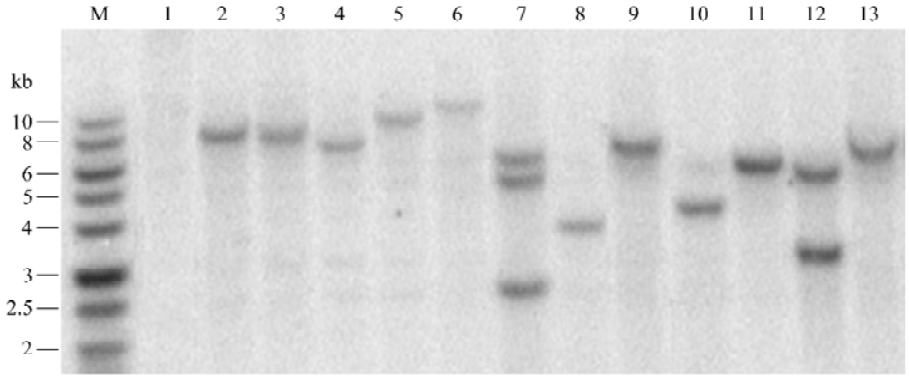

Fig. 5 Southern blot analysis of transgenic rice

M, marker; 1, wild type; 2–5, independent transgenic lines of *NRT2019::GUS*; 6–9, independent transgenic lines of *NRT1196::GUS*; 10–13, independent transgenic lines of *NRT719::GUS*.

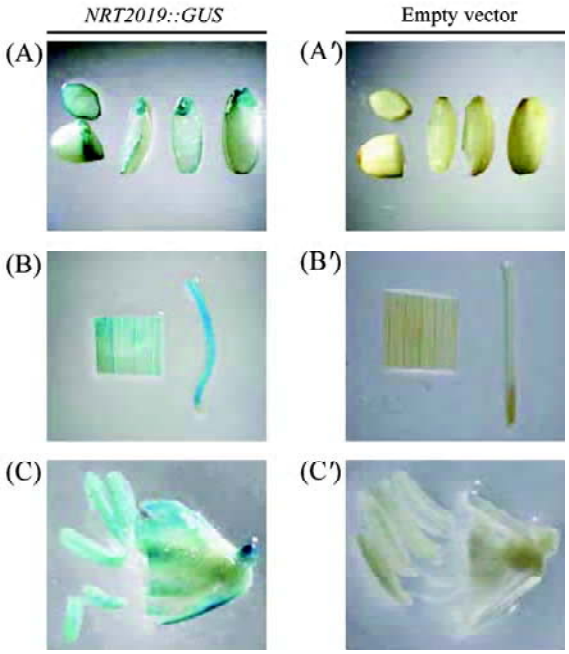

Fig. 6 Histochemical assay of β-glucuronidase (GUS) gene activity in transgenic rice plants

(A,A′) Seed. (B,B′) Leaf and root of 20-day-old seedlings. (C,C′) Flower.

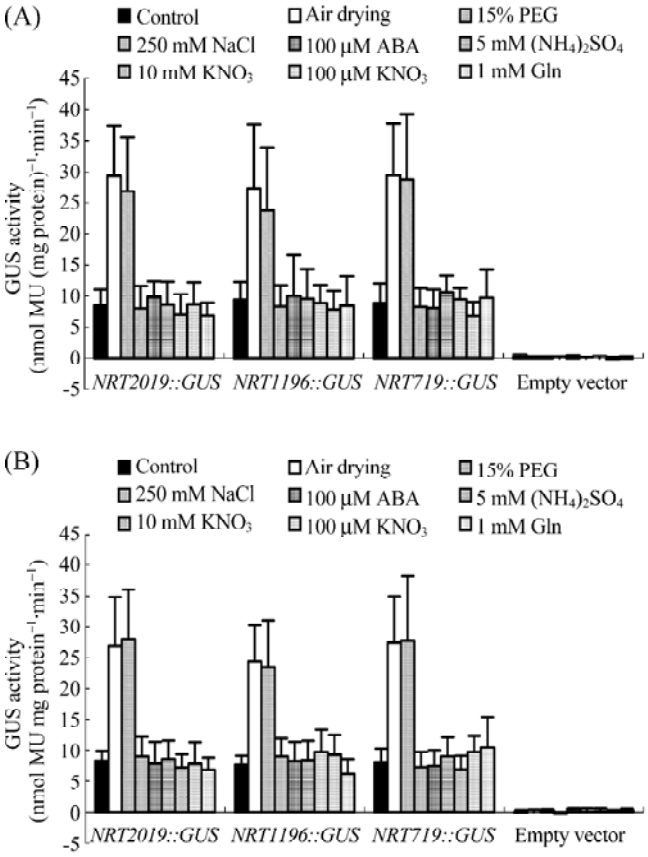

Fig.7 β-glucuronidase (GUS) gene expression directed by the *OsNRT1.3* promoter fragments in response to different treatments

(A) Roots. (B) Leaves. Transgenic rice seedlings were grown for 10 d and were treated by air-drying, 15% PEG6000, 100 μM abscisic acid (ABA), 250 mM NaCl, 5 mM $(NH_4)_2SO_4$, 10 mM KNO_3, 100 μM KNO_3 and 1 mM Gln for 6 h. Untreated transgenic rice seedlings were used as controls. The average GUS activity and standard deviations were obtained from three independent transgenic lines.

were hydroponically grown for 10 d. The roots and leaves of seedlings treated by emergency drought and simulative drought were harvested for GUS activity assay. For every chimeric gene, we selected at least three homozygous T2 seedlings from independent lines to assay the GUS activities. Despite the variability of GUS activity measured in the seedling extracts from different lines, the roots and leaves of all plants exhibited similar increase in GUS activity after drought treatment. The GUS activity conferred by different *OsNRT1.3* promoter fragments was upregulated up to about 3 times that of the untreated transgenic plants. The induction patterns of transgenic rice plants harboring the chimeric *NRT2019::GUS*, *NRT1196::GUS* and *NRT719::GUS* gene were similar. The induction expression pattern of the *GUS* gene of the seedlings treated by simulative drought was consistent with that of drought stress (**Fig. 7**).

To further investigate the expression pattern of *OsNRT1.3*, transgenic seedlings were treated with 100 μM ABA, 250 mM NaCl, 5 mM $(NH_4)_2SO_4$, 10 mM KNO_3, 100 μM KNO_3 and 1 mM Gln, respectively, and then the roots and leaves of seedlings were harvested for GUS activity assay. The results showed that the *OsNRT1.3* promoter can not respond to ABA, NaCl, $(NH_4)_2SO_4$, KNO_3, and Gln (**Fig. 7**).

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, $(NH4)_2SO_4$, KNO_3 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.affrc.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 −1983 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 CATGT 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.

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