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RESEARCH ARTICLE

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Characterization of promoter expression patterns of *OsNrt2.1*, a nitrate transporter gene of rice (*Oryza sativa* L.)

Abstract Nitrate is one form of available nitrogen nutrient, and its uptake and transport in plants was mediated by nitrate transporters. It is important to elucidate the transcription mechanism of nitrate genes at the molecular level. In this study, the promoter of *OsNrt2.1*, a rice nitrate transporter gene previously cloned in our group, has been characterized. Based on PLACE online analysis, some important cis-regulatory elements were identified. Under the control of the full length fragment of OsNrt2.1 promoter (P-2047), the expression of the reporter gene *Gus* was up-regulated when exposed to low nitrogen. Promoter deletion analysis indicated that the low nitrate responding elements located at position −524 to −1 and −980 to −525. Several cis-regulatory elements such as CIACADIANLELHC and −10 promoter element, located at positions of −524 to −1, −980 to −525, −1487 to −981, and −2047 to −1488 in P-2047, were possibly involved in the circadian regulation of the *OsNrt2.1* gene. Sugar signaling and sugar-responsive motif WBOXHVISO1 and TATCCAOSAMY were also identified in P-2047, suggesting that the exogenous sugar variations resulted from the photosynthesis changes were related to the up-regulated expression of *Gus* during the day time. It is guessed that the expression pattern of *Gus* with low-nitrate inducible and diurnal rhythm was partly, at least, via the mediation of Ca^{2+} signal transduction pathway. Owing to its regulation pattern with low nitrate inducible and typical circadian pattern, the OsNrt2.1 promoter maybe has a potential role in the generation of transgenic crop varieties with high-N use efficiency in the future.

Keywords rice (*Oryza sativa* L.), nitrate transporter gene, OsNrt2.1 promoter, cis-regulatory elements

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1 Introduction

As one form of available nitrogen nutrient to plants, nitrate (NO_3^-) is taken up through active transport processes in the roots. Besides serving as a nutrient, nitrate also plays an important signaling role for plants (Koch, 1997; Forde and Clarkson, 1999; Stitt, 1999; Zhang and Forde, 2000; Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001; Crawford and Forde, 2001). Therefore, in crop production, nitrate can be a significant factor impacting the plant growth and yield formation in that the soil nitrate concentrations may vary by several orders of magnitude (Forde and Clarkson, 1999; Tischner, 2000; Campbell, 2001; Crawford and Forde, 2001).

For the past decade, the transport systems in plants responsible for nitrate uptake have been well characterized physiologically (Glass and Siddiqi, 1995; Crawford and Glass, 1998; Forde and Clarkson, 1999; Crawford and Forde, 2001). In barley, maize and *Lemna*, studies indicate that there exist three primary nitrate transport systems: a nitrate-inducible high-affinity system (iHATS), a nitrate-constitutive high-affinity system (cHATS), and a low-affinity system (LATS) (Glass and Siddiqi, 1995; Crawford and Glass, 1998; Forde et al., 1999). The high-affinity systems show saturable kinetics with K_m values in the range of 10–100 $\mu mol \cdot L^{-1}$, while the low-affinity system shows linear nonsaturable kinetics.

At the molecular level, the updated two gene families, *NRT1* and *NRT2*, have been identified in plants that encode nitrate transporters (Crawford and Glass, 1998;

Daniel-Vedele et al., 1998; Forde, 2000; Crawford and Forde, 2001; Galvan and Fernandez, 2001; Glass et al., 2001; Williams et al., 2001). It is found that several members of the *NRT2* family encode iHATS transporters (Forde, 2000; Cerezo et al., 2001; Filleur et al., 2001; Glass et al., 2001). The *NRT1* family encodes an eclectic mix of nitrate transporters, such as the low-affinity transporter *AtNRT1.2* (Huang et al., 1999), the nitrate/histidine bispecific transporter *BnNRT1.2* (Zhou et al., 1998), and the dual affinity transporter *AtNRT1.1* (*CHL1*) (Tsay et al., 1993; Huang et al., 1996; Touraine and Glass, 1997; Wang et al., 1998; Liu et al., 1999). Expression analysis shows that *NRT2* is strongly influenced by a range of different environmental factors, including being induced by nitrate and repressed by high N status through a negative feedback regulation involving reduced N metabolites such as NH_4^+ or amino acids (Zhuo et al., 1999; Nazoa et al., 2003).

Previously, a rice nitrate transporter gene *OsNrt2.1*, phylogenetically clustered into *NRT2* group and showing a dual-affinity expression pattern, was cloned in our group (Xu et al., 2007). For further investigation of the transcription regulation of *OsNrt2.1*, an approximately 2 kb promoter region of *OsNrt2.1* was cloned, and a series of binary constructs fused different lengths of OsNrt2.1 promoter fragments were constructed. The location regions of putative cis-regulatory elements regulating the downstream gene to be low-nitrate inducible, and circadian pattern were explored, which is based on online analysis and reporter gene *Gus* expression detection under the control of different Nrt2.1 promoter fragments. It is suggested that the OsNrt2.1 promoter maybe has a potential role in the generation of transgenic crop varieties with high-N use efficiency, owing to its regulation with low nitrate inducible and circadian pattern.

2 Methods

2.1 Cloning of OsNrt2.1 promoter

A DNA clone containing the *OsNrt2.1* gene (GenBank accession number AP008208) was identified based on Blast search in the National Center for Biotechnology Information (NCBI) in which the OsNrt2.1 cDNA sequence (AB008519) to be the query. The primer pairs for amplification of the approximately 2-kb flanking region upstream of the OsNrt2.1 translation start codon (ATG) were as follows: 5′-CCCTTGCTGTCAGTGTCAG (forward) and 5′-CCATGGCAACTACTAGCTG (reverse). The PCR reaction was performed in a 20 μL volume containing 1 U *Taq* polymerase (Takara, Dalian, China), 50 ng genome DNA (cv. Nipponbare), 0.25 $\mu mol \cdot L^{-1}$ each forward and reverse primer, and 0.3 $mmol \cdot L^{-1}$ each dNTP, with following thermal reaction parameters: at 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 2.25 min , and a final extension at 72°C for 10 min was added for the improvement of PCR quality. The PCR products (2051 bp) were ligated with pUCm-T vector (Sangon, Shanghai, China) after agarose electrophoreses detection and transformed into *E. coli* strain DH5α. Sequencing was performed (Sangon, Shanghai, China) for the positive transformants for verification of the promoter sequence.

2.2 Identification of regulatory elements in OsNrt2.1 promoter

A search in PLACE (http://www.dna.affrc.go.jp/PLACE/fasta.html), a database of motifs found in plant cis-acting regulatory DNA elements based on previously published reports, was carried out for identification of the regulatory elements in OsNrt2.1 promoter. The sequence of the cis-elements, positions in the promoter, and the putative function of the important cis-regulatory elements were obtained, based on the results of PLACE analysis.

2.3 Construction of binary cassettes of the reporter gene *Gus* driven by different OsNrt2.1 promoter fragments

The OsNrt2.1 promoter was characterized by constructing the binary cassettes fused different lengths of OsNrt2.1 promoter to separately control the β-glucuronidase (Gus) gene expression. The deleted fragments of OsNrt2.1 promoter were all located at the flanking region upstream the translation start codon (ATG) at length of 173 bp, 524 bp, 980 bp, 1487 bp, and 2047 bp, respectively. The above various promoter fragments were amplified using the forward primers 5′-GGAATTCCCGCGCACCTGAAG (173 bp), 5′-GGAATTCCATGGGATAAAGGC (524 bp), 5′-GGAATTCACACGTTAACACTTC (980 bp), 5′-GGAATTCCTCCAGCCTTAGGCT (1487 bp), and 5′-GGAATTCCCTTGCTGTCAGTGTCAG (2047 bp) combined with the reverse primer: 5′- GCCATGGCAACTACTAGCTG, respectively. The deleted promoter fragments were integrated into the binary expression vector pCAMBIA3301 by introducing the restriction digestion sites of *Eco*RI (GAATTC) and *Nco*I (CCATGG) in the forward and reverse primers, respectively. The PCR reaction was the same as that for cloning the promoter mentioned above, except that the template was plasmid isolated from OsNrt2.1 promoter transformant to replace the rice genome DNA. The binary cassettes fused the deleted OsNrt2.1 promoter fragments were constructed based on the ligation reactions of the double digested (*Eco*RI and *Nco*I) promoter fragment and pCAMBIA3301, which was also double digested by the same enzymes.

2.4 Generation of transgenic tobacco plants fusing the binary constructs

The tobacco cultivar *Nicotiana. tabacum* cv. Wisconsin 38 was used for genetic transformation of the binary

constructs, which were fused with different fragments of OsNrt2.1 promoter. The fully expanded leaves from the 30-day-old seedlings growing in the growth chamber were used as the explants. The genetic transformation was performed following the *Agrobacterium tumefaciens* mediated approach, as described by Zhang et al. (2008).

2.5 Identification of the transgenic plants with single-copy insertion

The generated transgenic plants were grown in the growth room to maturity for harvest of the seeds. The T1 seeds were grown in the nutrient soil to the third leaf fully expanded stage in the growth room under a regime of 16 h of light (25°C) and 8 h of dark (20°C). The transformants with single insertion event for each construct were identified based on the plant segregation rates of phosphinothrycin (PPT)-resistant and PPT-sensitive. The plants with an approximately ratio of 3∶1 in PPT-resistant and PPT-sensitive were suggested to occur one transformation event and grown to maturity. The seeds were then used for further analysis.

2.6 Gus activity assays and histochemical staining of the transgenic plants exposed to low nitrogen

The T2-generated transgenic tobacco plants with single-copy insertion for each construct, selected based on PPT solution (5 $mg \cdot L^{-1}$) smearing, were used for assay of Gus activities and histochemical staining analysis. Two to four plants derived from the transformation of various binary constructs were adopted. The seeds were germinated in a growth chamber and then grew hydroponically to the third leaf fully expanded stage by supplement with Murashige and Skoog (MS) nutrient solution and regularly aeration by a pump. For assay of the Gus activities and histochemical staining analysis of transgenic plants responding to the low nitrogen, the nitrate concentration in the MS nutrient solution was adjusted to 20 $\mu mol \cdot L^{-1}$, from the former 5 $mmol \cdot L^{-1}$. After 6 h, 24 h, and 48 h of low-nitrogen treatment, leaves were harvested for the use of Gus activities and histochemical staining. The leaves were harvested before the low-nitrogen treatment was used as the control in the analysis.

The assay of beta-glucuronidase (Gus) activities for the samples followed the descriptions of Xiao et al. (2005). For the histochemical staining, the third leaf was cut out by a scissor and put into a staining solution with the following components: 250 $mg \cdot L^{-1}$ 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexyl ammonium salt (X-gluc), 100 $mmol \cdot L^{-1}$ phosphate buffer (pH 7.0), 0.1% Triton X-100, and 1.0 $mol \cdot L^{-1}$ EDTA. The staining biochemical reaction was performed at 37°C for 16 h. The stained leaves were then decolorized by transferring in 70% ethanol for about 7 days, and the histochemical staining results were recorded by a digital camera.

2.7 Diurnal variation pattern analysis of Gus activities and histochemical staining results of the transgenic plants

The third leaf fully expanded plants prepared for Gus assay and histochemical staining mentioned above were also used for elucidation of the diurnal variation patterns of Gus gene expression under the control of various fragments of OsNrt2.1 promoter. The leaves of plants growing in the normal MS nutrient solution were harvested at 6:00, 9:00, 13:00, 18:00, 20:00, and 22:00. The methods of Gus activity assay and histochemical staining analysis were all the same as those of the low-nitrogen responding treatments mentioned previously.

3 Results

3.1 Identification of the putative cis-regulatory elements in rice OsNrt2.1 promoter

The sequence of flanking region upstream of the translation start codon (ATG) of OsNrt2.1 was shown in Fig. 1, based on the sequencing results of the positive transformants. No base errors were found in this region compared to that in the corresponding DNA clone (GenBank accession number AP008208). The conserved motif of TATA box (TATAAAT) was located at −151 upstream of ATG, whereas two conserved motif of CAAT box were separately located at −174 and −311, suggesting that the 5'-flanking region of OsNrt2.1 had a potential role involved in transcription regulation on the downstream gene.

The important cis-regulatory elements in OsNrt2.1 promoter were identified based on PLACE online analysis. The motif identity, motif sequence, frequency of motif occurrence, motif positions, and the motif putative function is listed in Table 1. In total, 1 motif (EMHVCHORD, TGTAAAGT) responding to nitrogen, 2 motifs (SURECOREATSULTR11, GAGAC) responding to another inorganic nutrient sulfur, 4 motifs (CIACADIANLELHC, CAANNNNATC) involved in circadian transcription regulation, and 1 motif (–10 promoter element, TATTCT) involved in circadian rhythms and light regulation were identified, suggesting that they possibly control the transcription characterization of the downstream genes to some extent. Other cis-regulatory elements probably involved in light-regulated transcription, such as GATABOX, GT1CONSENSUS, IBOX, IBOXCORE, IBOXCORENT, INRNTPSADB, SORLIP1AT, SORLIP2AT, and ASF1MOTIFCAMV, with variable occurrence frequencies, were also identified. In addition, the cis-regulatory elements responding to Ca^{2+} (ABRERATCAL), calmodulin-binding/CGCG box involved in multiple signaling pathways in plants (CGCGBOXAT), early responsive to dehydration (ACGTATERD1), mediation of sugar and hormone regulation (TATCCAOSAMY),

Fig. 1 The OsNrt2.1 promoter sequence

Note: The positions of forward primers and reverse primers for amplification of various length fragments of promoter 2047 bp (P-2047), 1487 bp (P-1487), 980 bp (P-980), 524 bp (P-524), and 173 bp (P-173) were arrow labeled with blue color and red color, respectively. The translation start codon ATG in the reverse primer was highlighted with a black box. The conserved motif TATA (TATAAAT) box and CAAT (CAAT) box, involved in transcription regulation, were highlighted with green color and yellow color, respectively. The putative cis-regulatory element EMHVCHORD (TGTAAAGT) possibly involved in nitrogen response was shown in grey color. The SURECOREATSULTR11 (GAGAC) putatively involved in sulfur-responsive was shown in pink color. The CIACADIANLELHC (CAANNNNATC) putatively involved in circadian transcription regulation was shown in red color. The −10 promoter element (TATTCT) putatively involved in circadian rhythms and light regulation was shown in orange color.

Table 1 Characterization of the putative cis-regulatory elements in OsNrt2.1 promoter

motif identity	motif sequence	frequency of motif occurrence (either orientation)	motif position	putative function
EMHVCHORD	TGTAAAGT	1	1339	nitrogen response
SURECOREATSULTR11	GAGAC	2	1854, −1247	sulfur-responsive element (SURE)
CIACADIANLELHC	CAANNNNATC	4	396, 989, 1032, 2006	circadian expression
−10 promoter element	TATTCT	1	1561	circadian rhythms and light regulation
GATABOX	GATA	17	344, 400, 716, 982, 1588, 1704, 1832, −299, −402, −863, −1009, −1198, −1450, −1627, −1662, −1730, −1837	light regulated, and tissue specific expression; conserved in the promoter of all LHCII type I Cab genes
GT1CONSENSUS	GRWAAW	17	308, 379, 419, 437, 504, 561, 699, 992, 1151, 1588, −359, −861, −1685, −1835, −1180, −1328, −1461	light-regulated transcription and SA-inducible
IBOX	GATAAG	1	−1728	light regulation
IBOXCORE	GATAA	7	1588, −862, −1008, −1197, −1626, −1729, −1836	light regulation
IBOXCORENT	GATAAGR	1	−1727	light regulation
INRNTPSADB	YTCANTYY	2	−700, −1159	light-responsive transcription
SORLIP1AT	GCCAC	6	792, 1086, 1595, 1989, −596, −1483,	light-regulated transcription
SORLIP2AT	GGGCC	1	1320	involved in the network of phytochrome A-regulated
ASF1MOTIFCAMV	TGACG	1	754	light-regulated transcription and transcriptional activation by auxin and/or salicylic acid
ABRERATCAL	MACGYGB	2	1814, −1126	Ca^{2+}-responsive up-regulated
CGCGBOXAT	VCGCGB	2	1935, −1935	calmodulin-binding/CGCG box involved in multiple signaling pathways in plants
ACGTATERD1	ACGT	14	196, 685, 756, 1128, 1421, 1741, 1815, −196, −685, −756, −1128, −1421, −1741, −1815	early responsive to dehydration
TATCCAOSAMY	TATCCA	1	863	mediation of sugar and hormone regulation
WBOXHVISO1	TGACT	3	1649, −102, −322	sugar signaling and sugar-responsive
WBOXNTERF3	TGACY	8	1649, −102, −322, −46, −647, −1029, −1044, −1142	rapid and transient activation of transcription by wounding

sugar signaling and sugar-responsive (WBOXHVISO1), rapid and transient activation of transcription by wounding (WBOXNTERF3), putatively involved in the regulation of various biological processes, and abiotic stresses were also located in the OsNrt2.1 promoter. These results indicated that there existed an intricate and distinct mechanism under the control of the native promoter.

3.2 Construction of the binary cassettes of the reporter gene *Gus* controlled by various fragments of OsNrt2.1 promoter

Based on the plasmid isolated from the positive transformants harboring the OsNrt2.1 promoter, series of flanking region upstream of the ATG with different lengths, including 2047 bp, 1487 bp, 980 bp, 524 bp, and 173 bp, were amplified with the specific forward primers and the reverse primer, respectively. The binary cassette fused with the different lengths of promoter fragments to control the expression of the reporter gene *Gus* is shown in Fig. 2, in which the cassettes were simplified as P-2047, P-1487, P-980, P-524, and P-173, respectively. The PCR products with expected lengths after agarose electrophoresis are shown in Fig. 3.

3.3 Generation of transgenic tobacco plants with the reporter gene *Gus* under the control of various fragments of OsNrt2.1 promoter

The generation process of the transgenic tobacco plants in which the reporter gene *Gus* was under the control of OsNrt2.1 promoter fragments are shown in Fig. 4. Figure 4(a) shows the explants after three days of

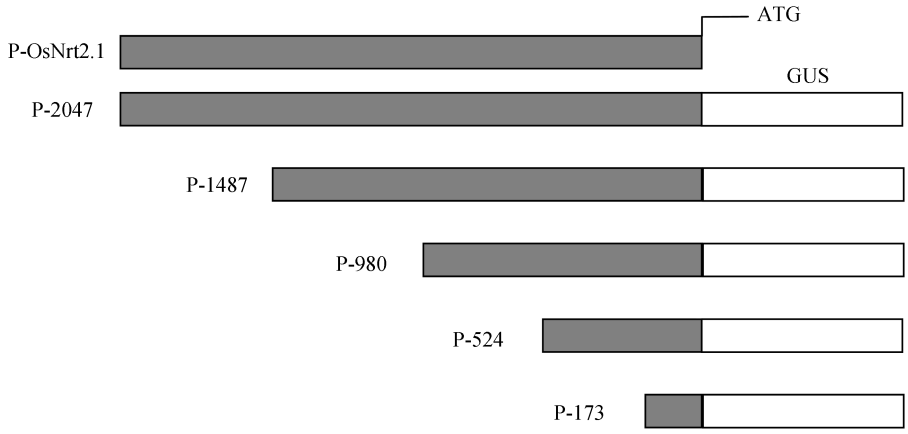

Fig. 2 The diagram of binary cassettes fused the OsNrt2.1 promoter fragments
Note: P-OsNrt2.1 represents OsNrt2.1 promoter. P-2047, P-1487, P-980, P-524, and P-173 represent the binary cassette with the reporter gene *Gus* under the control of the flanking region upstream of the translation codon (ATG) of 2047 bp, 1487 bp, 980 bp, 524 bp, and 173 bp, respectively.

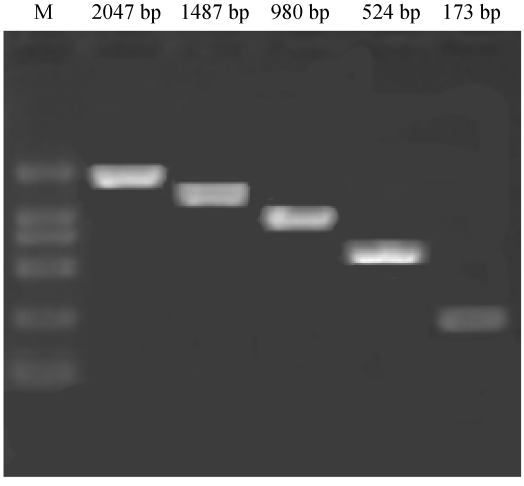

Fig. 3 Agarose electrophoresis results of the PCR products with the expected promoter fragments

Fig. 4 Generation process of the transgenic tobacco plants via *Agrobacterium tumefaciens*-mediated transformation approach
Note: (a) shows the leaf explants after three days of co-cultivation; (b) shows the generated buds with PPT-resistant growing in the bud differentiation medium; (c) shows the generated plant derived from the PPT-resistant bud growing in the rooting culture medium; (d) shows the generated plant derived from the PPT-resistant bud growing in the soil.

co-cultivation before transferring to the selection medium for bud generation. Figure 4(b) shows the buds with PPT-resistant growing in the bud differentiation medium. Whereas, Figs. 4(c) and 4(d) show the generated plants being derived from the PPT-resistant buds growing in the rooting culture medium and soil, respectively.

Identification of the transgenic plants with the exogenous DNA with one insertion event, and T1 plants genetic-transformed with various binary cassettes was performed using PPT-resistant assay by smearing PPT solution ($5\ mg \cdot L^{-1}$) on the leaf surface. Two to four independent transformants with a single copy of the exogenous DNA for various binary constructs were identified, based on the segregation ratio between the plants from PPT-resistance to PPT-sensitivity. Therefore, the above plants with single copy of exogenous DNA were used for further analysis.

3.4 Expression analysis of the reporter gene *Gus* under the control of various OsNrt2.1 promoter fragments when exposed to low nitrogen

Under the control of the full length of OsNrt2.1 promoter (P-2047), the expression level of the *Gus* gene was up-regulated when exposed to low-nitrogen, compared with that to normal nitrogen (CK, $5\ mmol \cdot L^{-1}$ N), based on the histochemical staining results of leaves (Fig. 5) and the *Gus* activity assays (Fig. 6). Meanwhile, there was an

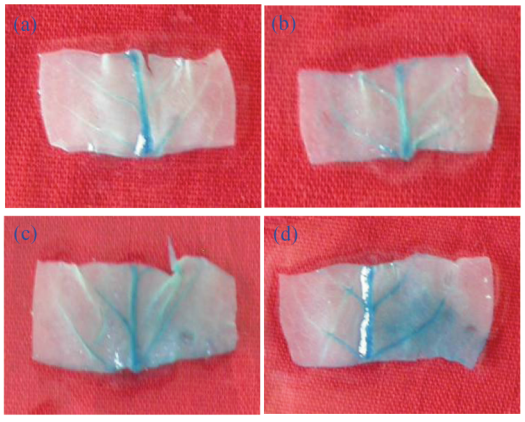

Fig. 5 Gus histochemical staining results of the leaves under the control of OsNrt2.1 promoter (P-2047)
Note: (a) shows the leaf from control plants (0 h, 5 mmol·L^{-1} NO_3^-); (b) shows the leaf from 6 h exposure to low nitrogen (20 μmol·L^{-1} NO_3^-); (c) shows the leaf from 24 h exposure to low-nitrogen (20 μmol·L^{-1} NO_3^-); and (d) shows the leaf from 48 h exposure to low-nitrogen (20 μmol·L^{-1} NO_3^-).

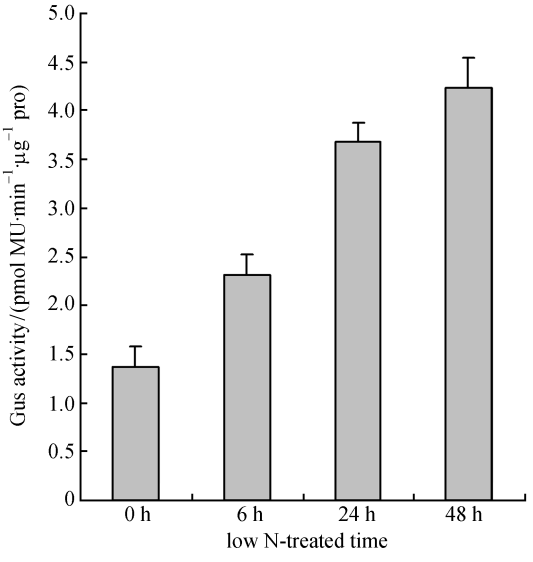

Fig. 6 *Gus* activities of the leaves under the control of OsNrt2.1 promoter (P-2047)
Note: The NO_3^- concentration is 5 mmol·L^{-1} in 0 h and 20 μmol·L^{-1} in 6 h, 24 h and 48 h, respectively.

induction effect on *Gus* gene under low-nitrogen condition, with a trend of gradual intensified staining (Fig. 5) and increased *Gus* activity (Fig. 6), with a prolongation of being exposed to the low-nitrogen in a photoperiodic regime of 6 h (light) to 48 h (light), suggesting that the cis-regulatory elements responding to the nitrogen-starvation stress cue were located in the OsNrt2.1 promoter.

For further identification of the cis-regulatory elements in OsNrt2.1 promoter responding to low-nitrogen stress cue, the transgenic plants fused with binary constructs were generated in which *Gus* was driven by deleted OsNrt2.1 promoter fragments, respectively. Under the control of 173 bp flanking region upstream the translation start codon (ATG) (P-173), negligible *Gus* activities were detected in the leaves of control (5 mmol·L^{-1} NO_3^-) and various exposed duration of low-nitrogen treatments (20 μmol·L^{-1} NO_3^-), such as 6 h, 24 h, and 48 h, showing the lack of indispensible transcription motifs in this fragments (Fig. 7). The *Gus* activities in leaves of P524 were detected in a moderate range, much higher than those in leaves of P-173, indicating that this fragment contained the conserved transcription regulation motifs. Based on PLACE search, one TATA box and two CAAT boxes were identified in this region, which may be related to this regulation effect. In the meantime, it was found that the low-nitrogen responding effects were shown in this fragment, implicating that the cis-regulatory elements responding to low-nitrogen stress cue existed in this region.

The *Gus* activities in leaves of P-980 showed a similar pattern in control and the low-nitrogen treatments as that of P-524, but the values were all increased. This result indicated that putative enhancers or cis-regulatory elements responding to low-nitrogen signal were possibly located in the region from −980 to −525. No changes were found in the leaf *Gus* activities in P-1487 and P-2487, compared to P-980, under the low-nitrogen time points of 0 h, 6 h, 24 h, and 48 h (Fig. 7).

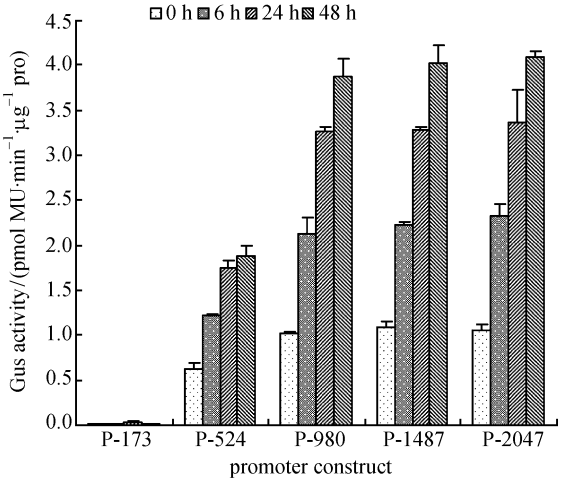

Fig. 7 Gus activities in leaves driven by various fragments of OsNrt2.1 promoter

3.5 Diurnal expression analysis of the reporter gene *Gus* under the control of various fragments of OsNrt2.1 promoter

Typical leaf histochemical staining results at different time points in a whole day are shown in Fig. 8. The leaf samples

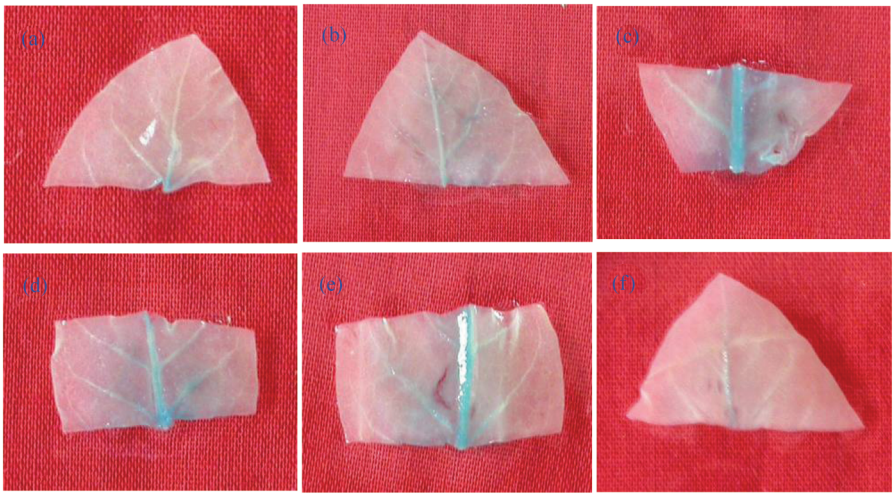

Fig. 8 Diurnal histochemical staining results of the leaves under the control of OsNrt2.1 promoter (P-2047)
Note: Diurnal variation of histochemical staining pattern was separately shown in (a) to (d). (a), (b), (c), (d), (e), and (f) represent 6:00, 9:00, 13:00, 18:00, 20:00, and 22:00, respectively.

for staining were collected from the transgenic plants in which the reporter gene *Gus* was under the control of full length of OsNrt2.1 (P-2047). It was found that the expression pattern of *Gus* to be diurnally varied, with a trend of initial up-regulation from 6:00 to 13:00, then gradual down-regulation from 13:00 to 20:00. At 22:00, the expression level of *Gus* dropped to a similar expression level of 6:00. The *Gus* activity assay results were consistent with those of histochemical staining (Fig. 9). Therefore, the cis-regulatory elements controlling the downstream gene to be circadian expression pattern were located in the OsNrt2.1 promoter.

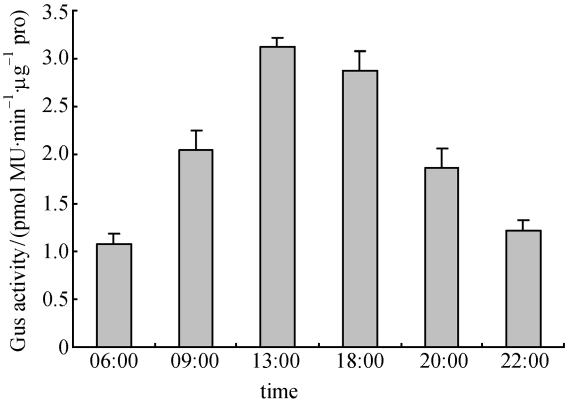

Fig. 9 Diurnal Gus activities in leaves under the control of OsNrt2.1 promoter (P-2047)

For further identification of the cis-regulatory elements in OsNrt2.1 promoter involved in controlling the downstream gene with a diurnally regulated pattern, the leaf samples of transgenic plants in which *Gus* was driven by deleted OsNrt2.1 promoter fragments were collected at various time points around a whole day. Similar to the *Gus* activities evaluated for responding to low-nitrogen stress cue, *Gus* activities at all the checked time points were less detected in the leaves in which *Gus* was under the control of 173 bp promoter fragment (P-173). A diurnal expression pattern could be found in other various transformants separately transformed from binary constructs, in which *Gus* was under the control of promoter fragments of 524 bp (P524), 980 bp (P-980), 1487 bp (P-1487), and 2047 bp (P-2047). However, an increased range both in P-980 when compared to P-524 and in P-1487 and P-2047 when compared to P-980 and P-1487 could be found, respectively. These results indicated that the cis-regulatory elements involved in diurnal control were located at −980 to −525, −1487 to −981, and −2047 to −1488, respectively (Fig. 10).

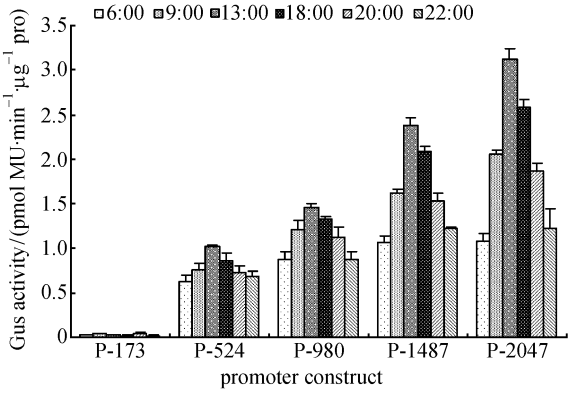

Fig. 10 Diurnal Gus activities in leaves driven by various fragments of OsNrt2.1 promoter

4 Discussion

Nitrogen can be available to plant roots in several different forms, including nitrate (NO_3^-), ammonium (NH_4^+), and organic forms chiefly in amino acids. Nitrate is usually the most abundant source of nitrogen, but in this, the anionic form is readily dissolved in soil water and so is very mobile in the soil profile (Anthony et al., 2007). Physiologically, NO_3^- uptake by the plant roots through three types of transport system to cope with the variations in NO_3^- concentrations in cultivated soils (Crawford and Glass, 1998). Among them, two kinds of transporters comprising saturable high-affinity transport systems (HATS) of mediating NO_3^- uptake from external sources and the uptake at low NO_3^- concentrations (1 $\mu mol \cdot L^{-1}$ to 1 $mmol \cdot L^{-1}$) were encoded by two types of genes known as the *NRT1* and *NRT2* families. Both families transport NO_3^- together with a proton (H^+) in a symport mechanism that is driven by the pH gradients across membranes.

The *NRT2* expression mainly occurs in epidermis and cortex of the mature root regions (Nazoa et al., 2003) and is strongly regulated by a range of different environmental factors, such as the expression of *NRT2.1* induced by low NO_3^-, and repressed by high N status through a negative feedback regulation involving reduced N metabolites such as NH_4^+ or amino acids (Zhuo et al., 1999; Nazoa et al., 2003). However, the regulation mechanisms of plant *NRT2* genes are largely unknown. It is noted that a 431 bp C-hordein promoter of barley (*Hordeum vulgare* L.) lambda-1-17 exhibits a specific response to amino acids and NH_4NO_3 in developing endosperms, while the GCN4 motif ATGA(C/G)TCAT is the dominating cis-acting element in this response. On the other hand, synergistic interaction with the neighboring endosperm motif TGTAAAGT within the bifactorial prolamin element and cooperation with upstream sequences including a second prolamin-like element is an absolute requirement for a strong positive regulation by an optimal nitrogen regime (Müller and Knudsen, 1993). In our present study, we also identified a motif at position 1339 (−764 bp upstream of ATG), suggesting that this motif in OsNrt2.1 promoter was possibly involved in the regulation of low-nitrate response. We also found that another putative cis-regulatory elements responding to low nitrate was located at the region −524 to −173, but the exactly motif needs to be explored further.

Accompanying the onset of light in the morning or dark in the evening, many responses in biological processes express a circadian rhythm with approximately 24-h periods (Dunlap, 1999; Salomé and McClung, 2005). Recent studies have underscored the extent of circadian control on the Arabidopsis (*Arabidopsis thaliana*) transcriptome, with estimates of 10% to 15% (Harmer et al., 2000; Edwards et al., 2006; Covington and Harmer, 2007) of genes affecting all aspects of seedling and plant development, exhibiting clock control. In tomato (*Lycopersicon esculentum* Mill.) plants, net hourly nitrate uptake analysis around a diurnal cycle indicated that the nitrate uptake rate was increased continuously during the light and decreased during the dark periods, implying that some genes for the nitrate transporting system were expressed with a circadian rhythm (Cárdenas-Navarro et al., 1998). Up to date, two important cis-regulatory elements, involving circadian regulation of CIACADIAN-LELHC (CAANNNNATC) and −10 promoter element (TATTCT), have been experimentally confirmed and involved in the circadian regulation of the downstream genes (Piechulla et al., 1998; Thum et al., 2001). In our study, the expression pattern of reporter gene *Gus* under the control of OsNrt2.1 promoter showed a circadian rhythm (Fig. 10). The diurnal changes of *Gus* transcripts under the control of OsNrt2.1 (P-2047) suggested that the circadian-regulated motif was located in the OsNrt2.1 promoter. Based on the PLACE online search, four CIACADIANLELHC motifs located at position −1707, −1114, −1071, and −97 were identified. Similarly, one −10 promoter element located at position −542 was also figured out. Therefore, these motifs could play important roles in regulation *Gus* expression with a diurnal rhythm. In the meantime, under the control of elongated Nrt2.1 promoter fragments, the diurnal oscillation range of *Gus* transcripts was also enlarged in the tested fragment regime. Thus, the above motifs were all possibly involved in the regulation of the diurnal expression rhythm pattern, showing seemly a dose effects on the *Gus* regulations. We also identified several other cis-regulatory elements involved in light regulation in Nrt2.1 promoter with high-occurrence frequencies, such as GATABOX, GT1CONSENSUS, IBOXCORE, and SORLIP1AT, suggesting that the OsNrt2.1 was intricately regulated by light, though the molecular mechanism to be explored.

It is reported that some nitrate transporter genes can be stimulated by light and sugars (Lejay et al., 2003). Exogenous supply of sucrose at the beginning of the night prevents the decline in Arabidopsis nitrate transporter gene *AtNrt1.1* and *AtNrt2.1* expression, indicating that these genes are sugar inducible and that sucrose can mimic the effect of light on their expression. On the other hand, the variations in *AtNrt1.1* and *AtNrt2.1* transcript accumulation parallel those of NO_3^- influx, suggesting that the coordination of root NO_3^- uptake with photosynthesis may rely on the sugar regulation of NO_3^- transporters (Lejay et al., 1999). Thus, the enhancement of *OsNrt2.1* transcripts in the light may also be related to the activation of the more photosynthate resulting from increased photosynthesis in the day regime. The motif WBOXHVISO1 involved in sugar signaling and sugar responsive, as well as the motif TATCCAOSAMY involved in the mediation of sugar and hormone regulation, which are located at Nrt2.1 promoter with three times, indicate these elements play possibly a role in *Gus* responses to the exogenous sugar variations around the whole day.

Most biotic and abiotic stresses elicit an increase in cytosolic free calcium concentrations (Trewavas and Malhó, 1998; Pandey et al., 2000; Sanders et al., 2002). Specific responses to different stimuli can be achieved through variations in the amplitude, duration, location, and frequency of these Ca^{2+}-spikes (McAinsh and Hetherington, 1998). As Ca^{2+} is ubiquitous in stress signaling, it may be an important node at which cross-talk between pathways can occur. In our study, cis-regulatory elements ABRERATCAL and CGCGBOXAT, a Ca^{2+}-responsive up-regulated and calmodulin-binding/CGCG box involved in multiple signaling pathways in plants, respectively, have been identified in OsNrt2.1 promoter, suggesting that the expression pattern of *Gus* with low-nitrate inducible and diurnal rhythm is partly via the mediation through Ca^{2+} signal transduction pathway.

In conclusion, PLACE online search and the reporter gene assay and analysis indicated that there were some important cis-regulatory elements located in the OsNrt2.1 promoter. The expression pattern of reporter gene *Gus* and the native gene with low-nitrate inducible and diurnal rhythm were possibly related to some extent with the nitrogen responding motif EMHVCHORD, the circadian motif CIACADIANLELHC, and the −10 promoter element. Sugar signaling and sugar-responsive motif WBOXHVISO1, and TATCCAOSAMY in the OsNrt2.1 promoter, possibly play roles in responses to the exogenous sugar variations around the whole day. It is guessed that the expression pattern of *Gus* with strong low-nitrate inducible and typical diurnal rhythm is partly, at least, via the mediation through Ca^{2+} signal transduction pathway.

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