Two short sequences in OsNAR2.1 promoter are necessary for fully activating the nitrate induced gene expression in rice roots

Xiaoqin Liu1,2*, Huimin Feng1,2*, Daimin Huang1,2, Miaoquan Song1,2, Xiaorong Fan1,2 & Guohua Xu1,2

Nitrate is an essential nitrogen source and serves as a signal to control growth and gene expression in plants. In rice, OsNAR2.1 is an essential partner of multiple OsNRT2 nitrate transporters for nitrate uptake over low and high concentration range. Previously, we have reported that −311 bp upstream fragment from the translational start site in the promoter of OsNAR2.1 gene is the nitrate responsive region. To identify the cis-acting DNA elements necessary for nitrate induced gene expression, we detected the expression of beta-glucuronidase (GUS) reporter in the transgenic rice driven by the OsNAR2.1 promoter with different lengths and site mutations of the 311 bp region. We found that −129 to −1 bp region is necessary for the nitrate-induced full activation of OsNAR2.1. Besides, the site mutations showed that the 20 bp fragment between −191 and −172 bp contains an enhancer binding site necessary to fully drive the OsNAR2.1 expression. Part of the 20 bp fragment is commonly presented in the sequences of different promoters of both the nitrate induced NAR2 genes and nitrite reductase NIR1 genes from various higher plants. These findings thus reveal the presence of conserved cis-acting element for mediating nitrate responses in plants.

Nitrate in plants serves as a nutrient as well as a signal which induces changes in growth and gene expression1–7. Large number of the genes in plants are involved in nitrate responses and regulation. When plants were exposed to nitrate, expression of nitrate transporters genes (NRT/NAR) and nitrate assimilation related genes (NIA, Nir) were immediately induced or enhanced8–14. Genome-wide gene expression analyses have showed that expression of a wide spectrum of the genes involved in nutrient uptake, metabolism, growth and development are rapidly altered by nitrate15–21.

The cis-regulatory modules in responses to nitrate supply are emerging in plants. To date, several putative transcription factors linking to nitrate regulation have been reported, including NPL22,23, SPL24, TGA1 and TGA425. In addition, a handful of cis-acting element(s) in the promoters of nitrate regulated genes has been identified. In Arabidopsis, a 150 bp fragment from the promoter of a nitrate transporter gene AtNRT2.1 was shown to be a nitrate specific regulation region26. A 43 bp sequence containing the fragment 5′-GACcCTTN10AAG-3′ in the promoter of a nitrite reductase gene AtNIR1 has emerged as nitrate-responsive cis-regulatory elements27. A 180 bp fragment from the promoter of nitrate reductase gene AtNIA1 contains three elements corresponding to the predicted binding motifs of nitrate enhancer28. However, motif analysis showed that these reported fragments and the putative nitrate responsive cis-acting elements [5′-GATA-3′, 5′-A(C/G)TCA-3′, 5′-GACcCTTN10AAG-3′] are not commonly presented in the promoters of nitrate responsive genes from different plant species29.
There might be multiple cis-elements involved in different response of the genes to nitrate and nitrogen (N) supplies in plants.

During the past two decades, the nitrate transport and signaling in plants have been well characterized. A nitrate inducible gene, NAR2.1 (nitrate assimilation related gene), has been defined as the gene encoding nitrate accessory protein. NAR2 is mainly expressed in roots and induced by nitrate and suppressed by ammonium. In Arabidopsis, expression of NAR2.1 is required for the activities of multiple NRT2 nitrate transporters for both constitutive and inducible high affinity nitrate uptake. The expression of AtNAR2.1 and AtNRT2.1, AtNRT2.2 was coordinately induced by low external nitrate concentration and sudden N deprivation, and suppressed by high nitrate supply. In rice, OsNAR2.1 is a partner protein interacting with OsNRT2.1, OsNRT2.2, and OsNRT2.3a, affecting nitrate uptake over low and high concentration ranges. The expression of OsNAR2.1, OsNRT2.1, OsNRT2.2 and OsNRT2.3a genes was coordinately induced by both low and high nitrate concentrations in roots, and knockdown of OsNAR2.1 in turn synchronously suppressed expression of OsNRT2.1, OsNRT2.2 and OsNRT2.3a.

The cis-acting regulatory components for sensing nitrate in rice were scarcely reported. Previously, we have shown that a region from the position –311 to –1 bp, relative to the translation start site in the promoter of OsNAR2.1, was found to contain the nitrate responsive cis-element(s), while no similar cis-element(s) is presented in the promoters of OsNAR2.1 and OsNRT2.3. In this study, we revealed that the 20 bp sequence between –191 and –172 bp in addition to –129–1 bp region contains the nitrate responsive cis-element(s) which are required for enhancing expression.

Results

A 192 bp region at the upstream from translational start codon of OsNAR2.1 gene is sufficient for fully mediating the nitrate induced expression. Previously, we identified that the –311/–1 bp region of OsNAR2.1 promoter contains the nitrate regulated element(s). To further dissect the nitrate response cis-element(s), we first made different deletions from the upstream of TATA-box region (–129/–123 bp) and generated three fragments of –284/–1 bp, –192/–1 bp and –129/–1 bp of OsNAR2.1 promoter (Fig. 1a,b). These truncated promoter regions were respectively fused with beta-glucuronidase (GUS) reporter gene and transformed into rice (cv. Nipponbare). We generated twenty independent transgenic lines for each of the constructs harboring the different lengths of OsNAR2.1 promoter (Fig. S1), and nearly all of these lines had the responses of the GUS reporter to nitrate in their roots (Fig. 1c). The histochemical staining of GUS reporter in the transgenic lines showed that these promoters were not activated by exogenously supplied ammonium (Fig. 1d–f). In contrast, the nitrate induced GUS expression controlled by all these promoters and the expression pattern in both the root and root-shoot junction were similar for the lines transformed with –311p:GUS, –284p:GUS, and –192p:GUS (Fig. 1d,e). However, the 129p:GUS transgenic lines showed a remarkably suppression of GUS activity compared with other transgenic lines (Fig. 1d,e). Quantitative analysis of GUS reporter enzyme activity in the transgenic rice roots confirmed the visible difference (Fig. 1f). Furthermore, qRT-PCR analysis using the roots of WT and the transgenic lines revealed that supply of nitrate in comparison to ammonium strongly elevated the levels of endogenous OsNAR2.1 mRNAs (Fig. S2). No significant difference of the abundance of OsNAR2.1 transcripts was observed between WT and the transgenic lines (Fig. S2), indicating that transforming the GUS report construct into rice did not affect the expression of endogenous OsNAR2.1 gene. Although the –129/–1 bp promoter drove the GUS activity only about 40% of that by –192/–1 bp promoter, their expression patterns in both roots and root-shoot junction were similar to that obtained with the –311/–1 bp promoter (Fig. 1d,e; Fig. S1). The GUS expression patterns indicate that the cis-regulatory elements involved in nitrate induced gene expression locate in the –192/–1 bp region of the promoter, and the –192/–129 bp region might contain the nitrate cis-element(s) which are required for enhancing OsNAR2.1 expression.

A 129 bp region at the upstream from translational start codon of OsNAR2.1 gene is essential for mediating basic nitrate response. To further characterize if the –129/–1 bp region is critical for OsNAR2.1 to sensing nitrate supply, we generated three –129 bp deleted promoters with different lengths (–311/–129 bp, –284/–129 bp, –192/–129 bp) and the –129 bp promoter in which both CaMV 35S minimal promoter (min) and GUS reporter gene were fused in the constructs (Fig. 2a). Twenty independent transgenic lines for harboring each of the constructs were tested for detecting the GUS expression under either nitrate or ammonium supply condition (Fig. S1). It showed that all the three –129 bp deleted promoters of OsNAR2.1 gene spanning –311/–129 bp, –284/–129 bp and –192/–129 bp region, respectively, lost the function in driving the nitrate induced GUS activity in both the roots and root-shoot junction (Fig. 2b–d). The quantitative GUS activity measurement confirmed the visible results (Fig. 2e). The insertion of the GUS construct with the different promoters did not affect the response of endogenous OsNAR2.1 expression to nitrate (Fig. S2). In contrast, the transgenic lines expressing –129/–1::min::GUS containing the TATA-box (–129/–123 bp) showed nitrate induced GUS activity, even
though the activity was much less stronger than that driven by the –311::GUS (Fig. 2c–e). These results suggest the sequence of –129/–1 bp indeed be required for the nitrate regulated expression of OsNAR2.1.

The 20 bp sequence between –191 bp and –172 bp of the OsNAR2.1 promoter contains the transcriptional enhancer element(s) for sensing nitrate supply. Since the nitrate induced GUS activity driven by the –129/–1 bp promoter was much less strong than that by the –192/–1 bp promoter of OsNAR2.1 gene (Figs 1 and 2), the –192/–129 bp region may have the nitrate enhancer binding site for activating transcriptional expression. Interestingly, we found the 20 bp fragment of

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**Figure 1. Deletion analysis of the OsNAR2.1 311 bp promoter fragment in rice transgenic lines for nitrate enhancer elements.** (a) TATABOX position in OsNAR2.1 promoter. (b) Schematic representation of the diagram of binary cassettes fused the OsNAR2.1 promoter fragments with GUS reporter gene. –311::GUS, –284::GUS, –192::GUS, and –129::GUS, represent the binary cassette with GUS under the control of the flanking region upstream of the translation start codon (ATG) of 311 bp, 284 bp, 192 bp, and 129 bp, respectively. (c) Analysis was performed on 10 and 20 independent lines transformed with each construct. (d,e) Histochemical analysis of GUS activity in the roots (d) and root-shoot junction (e) of the representative transgenic line grown in the nutrient solution containing 0.2 mM NH₄⁺ or 0.2 mM NO₃⁻ for seven days. Bars: 1 mm (d) and 0.5 mm (e). (f) Quantification of the root GUS activity. Analysis was performed on six independent transgenic lines grown in either ammonium or nitrate solution. Each GUS activity assay was performed for each line as described in "Materials and Methods". a, b and c indicate the significant difference at p < 0.05 between the four lengths of OsNAR2.1 promoter treated with different forms of N. Values are mean ± SE of six biological replicates.
5′-GCCTCTT(GAATCCAACG)AAG-3′ at the region between –191 bp and –172 bp of the OsNAR2.1 promoter showed a high similarity with the motif 5′-GACTCTTN10AAG-3′ in the AtNIR1 promoter which is critical for nitrate inducibility27. In addition, we found that the 20 bp sequence in OsNAR2.1 promoter is relatively conserved in the putative promoters of NAR2 genes from different plant species including Arabidopsis, bean, birch and tobacco (Fig. 3).

To test if this 20bp-sequence functions as the putative nitrate enhancer element in the –192/–129bp region, the effects of mutating this fragment on the promoter activity were examined. We generated four –192/–1bp promoters with the 20bp-sequence mutations of M1 (6 bp), M2 (8 bp), M3 (17 bp), M4 (19 bp) and one synthetic promoter with the fusion of four copies of the 20 bp sequence (4 × 20 bp) to the 35S minimal promoter (Fig. 4a,b). These point or site mutated or synthetic promoters were further

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**Figure 2. Identification of the region required for the nitrate response using the CaMV 35S minimal promoter.** (a) Schematic representation of cis-activation of the CaMV 35S minimal promoter by sequences located upstream of the TATA box of OsNAR2.1 in the transgenic lines. Constructs of –311/–129::min::GUS, –284/–129::min::GUS, –192/–129::min::GUS and –129/–1::min::GUS are the different promoter fragments fused with 35S minimal promoter and GUS reporter gene. (b) Analysis was performed on 20 independent transgenic lines for each construct. (c,d) GUS expression pattern by histochemical staining in the roots (c, Bars: 1 mm) and root-shoot junction (d, Bars: 0.5 mm). (e) Quantification of the root GUS activity. Analysis was performed on six independent transgenic lines grown in either ammonium or nitrate solution. Each GUS activity assay was performed for each line as described in “Materials and Methods”. a, b and c indicate the significant difference at p < 0.05 between the four lengths of OsNAR2.1 promoter treated with different forms of N. Values are mean ± SE of six biological replicates.
effects of many nitrate induced gene expression (Fig. 4). In general, promoter activity is determined by the combined conserved sequence is necessary for the full activation of the native promoter by nitrate. Konishi and Yanagisawa (2010) have defined that the 43 bp sequence containing the 20 bp cis-element for responding to nitrate supply and transferring this signal to other nitrate responsive genes 32,37. In this study, we detected that a conserved sequence of 20 bp::mini::GUS transgenic lines had no GUS activities under the same nitrate treatment (Fig. 4d–f), which implied that the 20 bp sequence itself is not enough for conferring the nitrate signal to induce the transcriptional expression.

**Discussion**

Nitrate supply can trigger the rapid change of expression of the genes involved in nitrate uptake and assimilation, as well as their associated carbon and energy metabolism20,28,46. Although there are common responses of the genes encoding two components of high affinity nitrate transporters (NAR2.1 and its associated NRT2s) as well as a number of the genes encoding nitrate and nitrite reductase to different N forms, the identity of the nitrate regulatory factor(s) and conserved cis-acting element(s) were uncertain in plants36. In our current study, we analyzed the OsNAR2.1 promoter and identified that both –129/–1 bp and native –311/–193 bp fragment of OsNAR2.1 promoters (M3 and M4) drastically decreased the promoter activity to drive GUS reporter under the same nitrate treatment (Fig. 4d–f). Interestingly, the M3 and M4 mutations resulted in the same activity of –192/–1 bp and native –129/–1 bp promoter of OsNAR2.1 in responses to nitrate (Fig. 4d–f), indicating that the 20 bp cis-sequence contains essential cis-element for enhancing the nitrate response of OsNAR2.1 in rice. However, 4 × 20 bp::mini::GUS transgenic lines had no GUS activities under the same nitrate treatment (Fig. 4d–f), which implied that the 20 bp sequence itself is not enough for conferring the nitrate signal to induce the transcriptional expression.

**Figure 3. Analysis of the 20 bp conserved sequence in the promoters of NAR2s from different plant species.** Alignment of conserved sequences in the NAR2 gene promoters from *Brachypodium distachyon* (*Western Poplar*), *Pomus trichocarpa* (*Western Poplar*), *Solanum lycopersicum* (*Tomato*), *Arabidopsis thaliana*, *Vitis vinifera* (*Grape*), *Oryza sativa* (*rice*). M2, mutation of non-fully conserved 8 nucleotides in the sequence between –191bp and –172bp of OsNAR2.1 promoter. The highly conserved nucleotides are highlighted with yellow color.
Figure 4. Analysis of the –191 bp to –172 bp sequence as a conserved transcription enhancer element in OsNAR2.1 promoter. (a) Comparison of the essential nitrate enhancer element in AtNIR1 gene and the 20 bp sequence with different mutations between –191 bp and –172 bp of OsNAR2.1 promoter. M1, 6 bp mutation; M2, 8 bp mutation; M3, 17 bp mutation; M4, 19 bp mutation. The highly conserved nucleotides and mutated nucleotides are labeled with red and blue color, respectively. (b) The diagram of binary cassettes of the 4×20 bp::min::GUS, representation of a reporter construct with a synthetic promoter in which four copies of the 20-bp sequence are placed upstream of the 35S minimal promoter. (c) Analysis was performed on 20 independent transgenic lines for each construct. (d,e) GUS expression pattern by histochemical staining in the roots (c, Bars: 1 mm) and root-shoot junction (d, Bars: 0.5 mm) of M1::GUS, M2::GUS, M3::GUS, M4::GUS and 4×20 bp::min::GUS transgenic lines. (e) Quantification of the root GUS activity. Analysis was performed on six independent transgenic lines grown in either ammonium or nitrate solution. Each GUS activity assay was performed for each line as described in “Materials and Methods”. a, b and c indicate the significant difference at p < 0.05 between the site mutations and 4×20 bp::min::GUS of OsNAR2.1 promoter treated with different forms of N. Values are mean ± SE of six biological replicates.
the 20bp fragment itself is not enough for conferring the nitrate signal to induce the transcriptional expression. Interestingly, point mutations in not completely conserved positions (M1 and M2) did not affect the promoter activity in driving GUS expression (Fig. 4d–f). The expression pattern implies that the discontinuous 12 bp region in the motif (Figs 3 and 4) maybe the key biding site of transcription enhancer for mediating the nitrate regulation. Since the 12 bp are highly conserved among the promoters of known plant NAR2 members (Fig. 3), it will be interesting to explore whether they perform a similar function for other NAR2s genes in different plant species.

Within the 20bp of OsNAR2.1 promoter (Fig. 3), the highly conserved sequence 5′-AATCCAAC-3′ has been reported to be specifically binding site with a GBF factor isolated from nuclear extracts of tomato and Arabidopsis46. In addition, the sequence 5′-CTCTT-3′ in the 20bp region is putative nodulin consensus sequences as a cis-acting elements controlling expression of the root nodule-specific soybean leghemoglobin gene47,48. To date, no trans-acting factor that directly regulates the nitrate-responsive transcription have been identified in rice. Our identification of the relative conserved sequence will facilitate a search for a novel-type of transcription factor in sensing nitrate signaling.

The –129/–1bp fragment of OsNAR2.1 promoter could drive the nitrate induced gene expression (Fig. 1), while the fragment between –311/–129bp was not able to cis-activate the transcription (Fig. 2), implicating that the 129bp region also have the cis-regulatory elements in controlling the promoter activity. The GATA transcription factors have been predicted to be involved in regulating nitrate acquisition pathways49–51, while R2R3-MYB is involved in nitrate signaling52. Interestingly, the 129bp cis-acting sequence contains the motif potentially being able to interact with GATA transcription factors and a binding site of the transcription factor R2R3-MYB (Table S1).

Some transcription factors bind multiple recognition sequences53–55. Multiple transcription factors function as a hub to perceive phosphate and mycorrhiza signals in plants have been well characterized56,57. For example, two conserved cis-acting elements, MYCS and P1BS, are involved in the regulation of mycorrhiza-activated phosphate transporters in eudicot species57. A single pair of the core motif in a large number of nitrate responsive genes is neither specific to nitrate responsive genes, nor common to all nitrate responsive genes and is randomly distributed throughout the genomes in both Arabidopsis and rice55. So, we deduced that the relative conserved 20bp sequence in –129/–129bp region is required to allow the enhancement of the GUS expression via the –129bp fragment as combining sites of nitrate signal factor(s). However, whether these motifs are sufficient to confer the transcriptional regulation or need to interact with other elements remain unknown.

Materials and Methods

Construction of reporter vectors. The –311::GUS, –284::GUS, –192::GUS, and –129::GUS were obtained by fusing 311bp, 284bp, 192bp, 129bp fragments corresponding to the sequence located upstream of the initiating codon of OsNAR2.1 to the b-glucuronidase (GUS) coding sequence using the primers showing in Table S2. The obtained DNA fragment for the OsNAR2.1 promoter was digested with Ncol and HindIII. These cloned fragments were used to replace the 35S-promoter which was inserted at upstream of the 5′ end of the GUS reporter gene in the pCB302-35S-GUS vector27.

The 35S minimal promoter (min) is a 62 bp fragment with HindIII and Xho I sites located respectively at the 5′ and 3′ ends. Four copies of 20bp (4 × 20bp) were commercially synthesized by GenScript company. Chimaeric promoter constructs (–311/–129::min, –284/–129::min, –192/–129::min, –129/–1::min, 4 × 20bp::min) were obtained by inserting PCR-amplified fragments corresponding to the –311/–129, –284/–129, –192/–129, –129/–1 and 4 × 20bp sequences of OsNAR2.1 promoter in sense orientation into the HindIII and XhoI site of min in 35min-LUC vector27. We replaced the 35S promoter sequence in the pCB302-35S-GUS vector with the –311/–129::min, –284/–129::min, –192/–129::min, –129/–1::min and the 4 × 20bp::min, respectively. Primers are showed in Table S3.

Reporter constructs with mutated OsNAR2.1 promoters were generated by PCR using 192::GUS plasmid of OsNAR2.1. For the M1, M2, M3 and M4 mutation, we got the 6bp, 8bp, 17bp and 19bp nucleotides mutation on the basis of 20bp sequence using primers in Table S4. The constructs were obtained and transformed into callus initiated from N. splendida seeds were germinated on a plastic support netting (mesh 1 mm2) mounted in plastic containers for one week. Uniform seedlings were selected and then transferred to a tank containing 8L of IRRI nutrient solution for 4 weeks at pH 5.5. After N starved for 4 days, seedlings were grown for seven days in the culture solution for nitrate or ammonium treatment with solution refreshed every 2 days.

Rice Transformation. The constructs were obtained and transformed into callus initiated from the seeds of rice (Nipponbare) by Agrobacterium tumefaciens (strain EHA105)-mediated transformation54. Rice embryonic calli were induced on N6 media and transformation was performed by Agrobacterium-mediated co-cultivation54. Transgenic plants were selected on a medium containing 50 mg/L glyphosate (Roche, Indianapolis, IN, USA).

Plant material growth conditions. Both WT and the transgenic seeds of rice (cv. Nipponbare) were surface-sterilized with 10% (v/v) H2O2 for 30 min and rinsed thoroughly with deionized water. The sterilized seeds were germinated on a plastic support netting (mesh 1 mm2) mounted in plastic containers for one week. Uniform seedlings were selected and then transferred to a tank containing 8L of IRRI nutrient solution for 4 weeks at pH 5.5. After N starved for 4 days, seedlings were grown for seven days in the culture solution for nitrate or ammonium treatment with solution refreshed every 2 days.
Seedlings were then collected for the analysis of the nitrate induction of GUS activity assay. IRRI nutrient solution (1.25 mM NH₄NO₃, 0.3 mM KH₂PO₄, 0.35 mM K₂SO₄, 1 mM CaCl₂·2H₂O, 0.15 mM Na₂EDTA, 20 μM NaFeEDTA, 20 μM H₂BO₃, 9 μM MnCl₂·4H₂O, 0.32 μM CuSO₄·5H₂O, 0.77 μM ZnSO₄·7H₂O and 0.39 μM Na₃MoO₄·2H₂O, pH 5.5) were supplied as described previously, and replaced every two days. To inhibit nitrification, 7 μM dicyandiamide (DCD-C₂H₄N₄) was mixed into all the solutions. Plants were grown in a growth chamber (Thermoline Scientific Equipment Pty. Ltd., Smithfield, Australia) at 30 °C during the day and 22 °C during the night with a 16-h light/8-h dark regime. The relative humidity was controlled at approximately 70%.

qRT-PCR analysis of OsNAR2.1 expression. Total RNA was isolated from the roots of rice seedlings. RNA extraction, reverse transcription, and quantitative reverse-transcription PCR (qRT-PCR) were performed as described previously. Primer sets for OsNAR2.1 (AP004023.2) as follows: Forward: 5′-CAGTCGGTTTGGTTGTCAG-3′; Reverse: 5′-TGGAGGAGCCGTGGATGC-3′.

Quantitative measurement of GUS activity. Histochemical GUS staining was performed as described previously, and quantification of the extractable GUS enzymatic activity using fluorescent substrate was carried out according to the method described by Jefferson et al. Samples (1–10 mg of root tissues) frozen in liquid N₂ were disrupted for 1 min, then suspended into 1 mL GUS extraction buffer (50 mM Na₃PO₄, pH 7.4, 10 mM EDTA, 0.1% Triton X-100 (Sigma, St-Louis, MD, USA), 0.1% sodium lauryl sarcosine, 10 mM β-mercaptoethanol). Reactions were initiated by mixing 50 mL of protein extract with 120 mL of 1 mM p-nitrophenyl-β-D-glucuronide (Sigma-Aldrich) at 37 °C for 1 to 4 h (GUS activity stayed linear for up to 16 h), and were stopped by adding 800 mL 125 mM Na₂CO₃, then measured with a Wallac Victor 2 spectrofluorimeter (Perkin Elmer, Waltham, MA, USA) at 355 nm excitation and 460 nm emission. Protein concentration was quantified using the Protein Assay reagent (Bio-Rad Laboratories, http://www.bio-rad.com).

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Author Contributions
G.X., X.L. and H.F. conceived the study and designed the experiments. X.L., D.H., H.F., X.F. and M.S. performed the experiments. G.X. and X.L. analyzed the data. G.X., X.L. and H.F. wrote the manuscript. All authors read and approved the final manuscript.

Additional Information
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