A Nitrate-Inducible GARP Family Gene Encodes an Auto-Repressible Transcriptional Repressor in Rice

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(Received September 26, 2012; Accepted January 7, 2013)

Nitrogen is the most important macronutrient in plants and its supply induces responses in gene expression, metabolism and developmental processes. However, the molecular mechanisms underlying the nitrogen responses remain poorly understood. Here we show that the supply of nitrate but not ammonium immediately induces the expression of a transcriptional repressor gene in rice, designated NIGT1 (Nitrate-Inducible, GARP-type Transcriptional Repressor 1). The results of DNA-binding site selection experiments and electrophoretic mobility shift assays indicated that NIGT1 binds to DNA containing either of two consensus sequences, GAATTC or GAATAATTC. In transient reporter assays, NIGT1 was found to repress transcription from the promoters containing the identified NIGT1-binding sequences in vivo. Furthermore, NIGT1 repressed the activity of its own promoter, suggesting an autorepression mechanism. Consistently, nitrate-induced NIGT1 expression was found to be down-regulated after a transient peak during nitrate treatment, and the nitrate-induced expression of NIGT1 decreased in transgenic rice plants in which this gene was constitutively overexpressed. Furthermore, the chlorophyll content that could be a marker of nitrogen utilization was found to be decreased in NIGT1 overexpressors of rice grown with nitrate medium but not with ammonium medium. Thus, we propose NIGT1 as a nitrate-inducible and autorepressible transcriptional repressor that may play a role in the nitrogen response in rice. Taken together with the fact that the NIGT1-binding sites are conserved in promoter sequences of Arabidopsis NIGT1 homologs, our findings imply the presence of a time-dependent complex system for nitrate-responsive transcriptional regulation that is conserved in both monocots and dicots.

Keywords: Nitrate response • Rice • Transcriptional repressor.

Abbreviations: Chl, chlorophyll; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; GST, glutathione S-transferase; GUS, β-glucuronidase; LUC, luciferase; NR, nitrate reductase; NIR, nitrite reductase; NRT, nitrate transporter; qRT-PCR, quantitative reverse transcription–PCR.

Introduction


Nitrate, the major nitrogen source for most land plants, is reduced to ammonium by two enzymes, nitrate reductase (NR) and nitrite reductase (NIR), and is then incorporated into glutamine in plant cells. On the other hand, it is also well known that nitrate induces the expression of NR and NIR genes as well as nitrate transporter (NRT) genes. Furthermore, previous comprehensive analyses of transcripts in Arabidopsis have revealed that nitrate induces or represses the expression of a wide variety of genes, including not only metabolic enzyme genes but also genes encoding putative regulatory proteins such as transcription factors, protein kinases and phosphatases (Wang et al. 2001, Wang et al. 2003, Scheible et al. 2004, Wang et al. 2007). Because the nitrate-inducible modulation of gene expression occurs even in mutant plants without NR activity, nitrate itself
is believed to function as a signaling molecule (Scheible et al. 1997, Wang et al. 2004). Furthermore, the nitrate-inducible expression of NR and NIR genes and the activity of the nitrate-responsive cis-element found in the NIR gene promoters do not require de novo protein synthesis (Gowri et al. 1992, Sakakibara et al. 1996, Price et al. 2004, Konishi and Yanagisawa 2010, Konishi and Yanagisawa 2011). Thus, it is also believed that nitrate post-translationally activates a pre-existing but as yet unidentified transcription factor, which induces or represses regulatory genes to actuate response mechanisms.

Regulation of gene expression by nitrate is fundamental to plant growth. However, the mechanism underlying such regulation is still largely unknown, as only some components of the mechanisms, including putative transcription factors and a sensor protein, have been characterized (Castaings et al. 2009, Ho et al. 2009, Hu et al. 2009, Wang et al. 2009). It has been shown that overexpression of nitrate-inducible Arabidopsis genes for LBD (LATERAL ORGAN BOUNDARY DOMAIN) proteins (LBD37, LBD38 and LBD39) reduced the expression of NRT and NR genes in Arabidopsis, suggesting that these putative transcription factors are involved in regulation caused by the nitrate signal (Rubin et al. 2009). In another study, an Arabidopsis nitrate-inducible gene, GNC (GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED), was proposed to encode a putative transcription factor from the GATA family that is involved in nitrate response (Bi et al. 2005). Because the product of GNC was later identified as a repressor of the gibberellin signaling pathway (Richter et al. 2010), GNC may be a factor that connects nitrate regulation with hormonal regulation. However, other nitrate-inducible transcription factor genes in higher plants remain to be characterized.

In our current study, we focus on a gene encoding a putative GARP-type transcription factor (Os02g0325600) from rice (Oryza sativa L.), which we designated NIGT1 (Nitrate-Inducible, GARP-type Transcriptional Repressor 1). Our previous transcriptome analysis of nitrogen response pathways in rice has indicated that several genes encoding putative transcription factors are induced by the supply of ammonium nitrate and also that NIGT1 was one of the earliest and most strongly induced genes (M. Shigyo et al. unpublished data). We therefore characterized the NIGT1 gene and here reveal that it encodes a novel transcriptional repressor of the regulatory network underlying the nitrate response in rice. We show that the NIGT1 expression is rapidly and specifically induced by nitrate and that NIGT1 negatively regulates its own expression. These findings suggest that nitrate is also an important nitrogen signal in rice, a plant which prefers to utilize ammonium as a nitrogen source because the major form of nitrogen in paddies where rice grows is ammonium and nitrate is a minor component (Sasakawa and Yamamoto 1978, Britto et al. 2001, Kronzucker et al. 2001). More significantly, the identification of such a transcription factor suggests the presence of a complex mechanism of transcriptional regulation for an appropriate nitrogen response in plants.

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**Results**

**Nitrates induces the expression of the NIGT1 gene in rice**

To verify the temporal induction pattern of NIGT1 expression through the supply of a nitrogen source, we performed quantitative reverse transcription–PCR (qRT–PCR) analysis using RNA from rice shoots treated with various inorganic or organic nitrogen compounds. As shown in Fig. 1A, NIGT1 expression was found to be induced by nitrate but not ammonium, glutamine or trans-zeatin, a plant hormone involved in the nitrate response (Sakakibara et al. 1998). Time course analysis revealed that NIGT1 expression was induced within 1 h of nitrate treatment, peaking approximately 3 h later and then decreasing during further nitrate treatment (Fig. 1B). We also show that such nitrate-inducible expression of NIGT1 occurred not only in leaf blades and sheaths but also in roots (Fig. 1C) and that low concentrations of nitrate (<1 mM) were sufficient to induce the nitrate-dependent expression of NIGT1 (Fig. 1D). On the other hand, the analysis of the subcellular localization of NIGT1 using green fluorescent protein (GFP) revealed that the fluorescence image of NIGT1–GFP perfectly overlapped with that of nuclear staining, indicating that NIGT1–GFP fusion protein predominantly accumulates in the nucleus (Fig. 1E). These results suggest that nitrate is a signaling molecule even in rice, and that it transiently induces the expression of a nuclear protein, NIGT1.

**NIGT1 binds to DNA in a sequence-specific manner**

The GARP domain is a single Myb-related DNA-binding domain, which was named using the initials of the GARP domain-containing proteins, GOLDEN2, ARR-B and Psr1 (Riechmann et al. 2000). GARP family proteins can be classified into several subgroups (Eshed et al. 2001), and several studies have reported the binding sequences of a few GARP family proteins (Rubio et al. 2001, Hosoda et al. 2002, Waters et al. 2009). The reported binding sequences differ from one another, and NIGT1 is a member of a subgroup for which the DNA binding activity has not been previously reported for any member. We therefore investigated the DNA binding activity of NIGT1 in a DNA-binding site selection experiment using a library of randomly synthesized DNA and recombinant NIGT1 protein (Fig. 2A, B). The DNA sequences selected in this experiment contained either of two sequences, GAATC or GAATATTG, which are referred to as core-1 sequence and core-2 sequence, respectively, hereafter (Fig. 2A, B). The core-2 sequence is a palindrome composed of two copies of the GAAT sequence that matches part of the core-1 sequence. Thus, GAAT sequences in a palindrome appear to be sufficient for NIGT1 binding, but additional C may be required if the sequence is present alone.

To confirm the binding of NIGT1 to these sequences, we performed electrophoretic mobility shift assay (EMSA). When we used synthetic DNA containing the core-1 sequence as the
biotin-labeled probe, a complex of NIGT1 and this DNA probe was detected as a shifted band. Furthermore, this complex formation was inhibited by the presence of a 10-fold molar excess of a non-labeled probe DNA but not a mutant core-1 competitor DNA, indicating a sequence-specific binding of NIGT1 to the core-1 sequence in vitro (Fig. 2D). The specific binding of NIGT1 to the core-2 sequence was similarly confirmed (Fig. 2E).

Furthermore, the core-1 and core-2 sequences functioned similarly as competitor DNA in binding of NIGT1 to the core-1 sequence (Fig. 2F), indicating that NIGT1 binds similarly to these sequences at least in vitro using the same binding sites in the protein.

**NIGT1 is a transcriptional repressor**

The activity of NIGT1 as a transcription factor was examined by co-transfection of various reporter and effector constructs into maize mesophyll protoplasts and measurement of reporter enzyme activity (Fig. 3). The expression of NIGT1 could not activate transcription from the promoter containing four copies of the core-1 sequence or two copies of the core-2 sequence upstream of the 35S minimal promoter, whereas NIGT1 fused to the transcriptional activation domain of VP16 (NIGT1–VP16) was found to promote transcription from these promoters (Fig. 3B). Mutations within the core-1 and core-2 sequences diminished any positive effects of NIGT1–VP16 on transcription. Similar but more prominent effects were observed using NIGT1 fused to two copies of the VP16 transcriptional activation domain (NIGT1–VP32). Hence, we concluded that NIGT1 binds to the core-1 and core-2 sequence in vivo but did not activate transcription.

Because we could not detect any function of NIGT1 itself as a transcriptional activator, we speculated that this protein might be a transcriptional repressor. To evaluate this hypothesis, we employed an engineered plant promoter that harbored eight copies of the bacterial LexA DNA-binding protein site between the 35S minimal promoter and a 35S enhancer (NIGT1–VP32). Hence, we concluded that NIGT1 binds to the core-1 and core-2 sequence in vivo but did not activate transcription.
would enable us to detect transcriptional repressor activity. The expression of LexA alone did not cause significant effects on the activity of this promoter. LexA fused to the VP16 transcriptional activation domain (LexA–VP16) strongly activated this promoter, whereas LexA fused to the transcriptional repressor domain of SUPERMAN (LexA–SUPRD) (Hiratsu et al. 2003) repressed the promoter activity. These results indicated that our system using LexA fusions is a viable tool to monitor both transcriptional activator and repressor activity. Using this system, we evaluated the activity of NIGT1 as a transcription factor. Similar to LexA–SUPRD, the expression of LexA–NIGT1 fusion protein down-regulated reporter enzyme activity, indicating that NIGT1 is a transcriptional repressor (Fig. 4B).

**NIGT1 represses its own promoter activity**

The accumulation of NIGT1 transcript peaked at about 3 h after nitrate treatment and decreased thereafter (Fig. 1B). Furthermore, we identified six putative sites for NIGT1 binding in the NIGT1 promoter (Fig. 5A). We therefore hypothesized that NIGT1 might function as a repressor of its own expression. This hypothesis was also supported by the results of a comparison of promoter sequences of NIGT1 and its Arabidopsis homologs, which were identified by a survey using the SALAD database (http://salad.dna.affrc.go.jp/salad/). The result of the survey indicated that NIGT1 forms a subgroup of the GARP family together with four rice genes (Os01g0176700, Os03g0764600, Os07g0119300 and Os12g0586300) and seven Arabidopsis genes (At1g25550, At1g68670, At1g13300, At3g25790, At2g03500, At4g37180 and At1g49560). Among them, four Arabidopsis genes (At1g25550, At1g68670, At1g13300 and At3g25790), which were previously shown to be nitrate-inducible genes (Scheible et al. 2004), showed the closest relationship with NIGT1. In contrast, the other genes appeared not to be nitrate inducible based on publicly available expression data in Arabidopsis and the unpublished result of our own nitrogen-responsive transcriptome analysis using rice seedlings. The exception is Os12g0586300, the expression of which could not be confirmed. Hence, NIGT1 and its
Arabidopsis homologs appear to be specifically associated with the nitrate response. When we aligned the promoter sequences of the \textit{NIGT1} gene and its Arabidopsis homologs, we found four sequence motifs that are highly conserved (Fig. 5A). Interestingly, three out of these four conserved motifs were putative \textit{NIGT1}-binding sites (two core-1 sequences and one core-2 sequence), implying that these sites might be functional cis-elements in both rice and Arabidopsis.

To test the possibility of negative autoregulation of the \textit{NIGT1} gene, we performed transient reporter assays using the wild-type and mutated \textit{NIGT1} promoters (Fig. 5B). The results indicated that \textit{NIGT1} represses the activity of the \textit{NIGT1} promoter, whereas \textit{NIGT1} fused to four copies of the VP16 transcriptional activation domain (\textit{NIGT1}–VP64) activates this promoter (Fig. 5C). Furthermore, mutations in the putative \textit{NIGT1}-binding sites that are conserved in both rice and Arabidopsis promoters (M1-\textit{NIGT1pro}-LUC) resulted in remarkably decreased effects of \textit{NIGT1} and \textit{NIGT1}–VP64. On the other hand, \textit{NIGT1} barely repressed the mutated promoter in which all six putative \textit{NIGT1} binding-sites were disrupted (M2-\textit{NIGT1pro}-LUC). Although \textit{NIGT1}–VP64 unexpectedly repressed the M2 promoter, this might be an artifact because this protein also repressed the 35S minimal promoter to a similar extent. These results suggested that \textit{NIGT1} autoregulates its expression negatively through conserved consensus sites in its own promoter.

Reduced expression of endogenous \textit{NIGT1} in transgenic rice plants overexpressing \textit{NIGT1}

To elucidate further the \textit{NIGT1} autoregulatory loop, we generated transgenic rice plants overexpressing \textit{NIGT1} under the control of the maize ubiquitin gene promoter. The \textit{NIGT1} transcripts in these transformed plants, which were from both endogenous and introduced \textit{NIGT1} genes, were far more abundant than in the vector control lines, independently of nitrate
treatment (Fig. 6A). This indicated that the expression of the introduced NIGT1 was much stronger than that of the endogenous gene. As expected from the strong expression of the introduced NIGT1 gene, qRT–PCR analysis revealed that the nitrate-induced expression level of endogenous NIGT1 was lower in the NIGT1 overexpressors, although the effects of this overexpression was limited under conditions of nitrate absence.

![Fig. 5](image)

**Fig. 5** Autoregulation of the NIGT1 gene. (A) Alignment of nucleotide sequences for the promoters of NIGT1 and Arabidopsis homologs. Nucleotides that are completely conserved among the five promoters and nucleotides that are well conserved are highlighted in black and gray, respectively. The consensus sequences for NIGT1 binding that are conserved in both rice and Arabidopsis promoters and present only in the rice promoter are indicated by red and blue boxes, respectively. The transcription start site of each gene is indicated by an arrow. Nucleotide numbers are relative to the translational start site of each gene. (B) Effector and reporter constructs used. Reporter constructs were generated with the wild-type or mutated NIGT1 promoter. Putative NIGT1-binding sites that are conserved in both rice and Arabidopsis promoters were disrupted in the M1-NIGT1pro-LUC construct, whereas all putative NIGT1-binding sites in the proximal region of the NIGT1 promoter were disrupted in the M2-NIGT1pro-LUC construct. Nucleotides in the putative NIGT1-binding sites were mutated in the same way as in Figs. 2C and 3A. (C) Relative LUC activity obtained with various effector and reporter constructs and normalized to GUS activity from an internal control plasmid. Values are the means ± SD (n = 3), and the value obtained with the empty vector (none) was set to 1.
Effects of overexpressed NIGT1 on the chlorophyll content

We did not find significant effects of NIGT1 overexpression on the fresh weight, height or root length of rice plants. However, we observed a reduction in the chlorophyll (Chl) content in the independently generated NIGT1 overexpressors grown with medium that contained nitrate alone as the nitrogen source, although such a reduction was not detected in the NIGT1 overexpressors grown with ammonium medium (Fig. 6B). As Chl is a compound involving nitrogen and its content could be a marker of nitrogen status in plants (Wood et al. 1992), the overexpression of NIGT1 might have its repressive effects upon the expression of some genes related to nitrate utilization and then decrease the Chl content. Similar to NIGT1 expression, expression of several nitrate-inducible genes, such as NR and NIR genes, is generally down-regulated after prolonged treatment (Kramer et al. 1989, Cheng et al. 1991, Zhuo et al. 1999, Nazoa et al. 2003, Hu et al. 2009, Rubin et al. 2009). We therefore examined the expression levels of these genes in the NIGT1 overexpressors but did not find any significant modulations in their expression levels (data not shown, see Discussion).

Discussion

In our current study, we identified a nitrate-inducible GARP-type transcriptional repressor in rice, NIGT1. The results of our expression analyses revealed that NIGT1 is rapidly and specifically induced by nitrate, suggesting that nitrate is recognized as a nitrogen signal in rice, even though this plant prefers...
to use ammonium as its nitrogen sources (Sasakawa and Yamamoto 1978, Britto et al. 2001, Kronzucker et al. 2001). Functional analyses of NIGT1 revealed that it functions as a transcriptional repressor, which negatively regulates its own expression through direct interactions with the binding sites in its own promoter. Furthermore, we also revealed that the modified expression of NIGT1 impacts on the Chl content. These results suggest that NIGT1 is a nitrate-inducible and auto-repressible transcriptional repressor which might be involved in control of nitrate utilization. Because NIGT1-binding sites are conserved in the rice NIGT1 promoter and the promoters of Arabidopsis homologs, the NIGT1-mediated regulation may also be conserved in monocots and dicots.

**Negative feedback regulation in nitrogen response**

Nitrate-induced expression is generally down-regulated after prolonged treatment (Kramer et al. 1989, Cheng et al. 1991, Zhuo et al. 1999, Nazoa et al. 2003, Hu et al. 2009, Rubin et al. 2009). It has been suggested previously that downstream metabolites of the nitrate assimilation pathway trigger such negative feedback events (Vincentz et al. 1993, Loqué et al. 2003, Nazoa et al. 2003). However, because the nitrate-inducible gene NIGT1 was found to be auto-repressed in this study, nitrate itself may be an additional signaling molecule that gives rise to negative feedback regulation in nitrate response. The mechanisms underlying nitrate-regulated gene expression may be more complex than previously envisaged and our data are indicative of a new mechanism for regulation by nitrate. Such a negative effect of nitrate on nitrate-inducible gene expression has been observed in the analysis of an Arabidopsis high-affinity NRT gene, AtNRT2.1, the expression of which is repressed by a high concentration of nitrate in the presence of ammonium (Krouk et al. 2006).

Arabidopsis genes, LBD37–LBD39, are other nitrate-inducible genes encoding putative transcription factors. Recently, these genes have been shown to be involved in the repression of nitrogen-responsive gene expression and nitrogen metabolism (Husbands et al. 2007, Rubin et al. 2009). On the other hand, another study using the yeast two-hybrid system revealed that LBD37 is one of the proteins to interact with transcriptional co-repressors TOPLESS/TPLs (Causier et al. 2012). Therefore, LBD37–LBD39 proteins may also function as transcriptional repressors of nitrogen utilization. Although both NIGT1-mediated and LBD37–LBD39-mediated regulations appear to be nitrate-inducible negative regulations for proper nitrate response, these regulatory processes should be distinguished from one another. The expression of the LBD genes was induced by various nitrogen sources such as ammonium and glutamine (Rubin et al. 2009), whilst the NIGT1 expression was specifically induced by nitrate. Thus, the LBD proteins probably function as mediators of nitrogen sufficiency (Rubin et al. 2009), whereas NIGT1 probably plays a role in nitrate-specific regulation in nitrogen response.

**Physiological roles of the NIGT1-mediated regulation**

We showed that the overexpression of NIGT1 results in a reduced Chl content in rice. This phenotype was not observed when the NIGT1 overexpressors were grown with ammonium media, suggesting that NIGT1 hampers nitrate-induced positive regulation and then produces this effect. As the Chl content could be a marker of the nitrogen utilization efficiency (Wood et al. 1992), NIGT1 overexpression might negatively influence the nitrogen utilization efficiency and then reduce the Chl content. However, the expression of NR and NIT genes, which is strongly induced by nitrate and then down-regulated, was not significantly reduced in the NIGT1 overexpressors (data not shown). The Chl content is not solely dependent on the efficiency of nitrogen utilization but also dependent on other environmental factors. Thus, at this stage, we could not rule out the possibility that the NIGT1 overexpression exerted its effects on other physiological processes rather than the nitrogen utilization. Furthermore, a research group has recently reported that the overexpression of an Arabidopsis homolog of NIGT1, HRS1 (At1g13300), resulted in a shorter primary root under low phosphate conditions, although it did not affect the phosphate response (Liu et al. 2009). The same group also reported that the HRS1 knockout mutant (hrs1-1) exhibited significantly delayed germination (Liu et al. 2009, Wu et al. 2012). These phenotypes, caused directly or indirectly by modification of the expression levels of NIGT1 or its Arabidopsis homolog, are very divergent. Further analysis of effects of the NIGT1 overexpression on growth and various markers for nitrogen utilization efficiency, namely nitrate, total nitrogen, amino acid and Rubisco protein contents, under various growth conditions would be necessary to clarify the physiological relevance of NIGT1 regulation to the nitrogen utilization efficiency.

The identification of direct target genes of NIGT1 and its Arabidopsis homologs would be likely to provide essential clues to addressing the physiological roles of NIGT1 and its homologs. The result of our recent transcriptome analysis suggested that the expression levels of a number of genes were modified in the NIGT1 overexpressors (Supplementary Table S1). The list of genes up-regulated and down-regulated in the NIGT1 overexpressors did not include the genes that are directly involved in nitrogen assimilation or Chl biosynthesis, but NIGT1 itself and several genes encoding regulatory proteins, such as transcription factor, protein kinase and phosphatase, and ubiquitin ligase. The modified expression of these genes might be associated with the phenotype of the NIGT1 overexpressors. However, further intensive analyses would be necessary to identify direct target genes of NIGT1 and genes whose functions are directly associated with the phenotype of the NIGT1 overexpressors.
**DNA recognition by GARP domains**

The results of our present analyses additionally provide a new insight into DNA recognition by the GARP family proteins. Several earlier studies have reported the binding sequences of some members of the GARP family. In reported binding experiments in vitro, Arabidopsis PHR1 involved in the phosphate starvation response has been shown to bind to the GNATATNC sequence (Rubio et al. 2001), whereas Arabidopsis ARR10, which plays an essential role in cytokinin signal transduction, binds the AGATT sequence (Hosoda et al. 2002). Other Arabidopsis members, GLK1 and GLK2, that are involved in the synchronized expression of a suite of nuclear photosynthetic genes have been suggested through comparisons of the DNA sequences of their target promoters to bind the CCAATC sequence (Waters et al. 2009). In our current study, we show that the GAATC sequence is one of the recognition sequences of NIGT1 (the core-1 sequence). This sequence is identical in part to the ARR10-binding sequence in the complementary strand, AATCT. Structural analysis of ARR10 previously indicated that 11 amino acid residues in the GARP domain of the protein interact with DNA (Hosoda et al. 2002). Among these, six residues (W188, S229, L231, Q232, K233 and K239) interact with the AATC sequence, which is present in both ARR10- and NIGT1-binding sequences. Significantly, these amino acid residues, except for K239, are conserved in NIGT1. Conversely, E225 and A228 of ARR10 that interact with the last nucleotide ‘T’ in the ARR10-binding sequence are not conserved in NIGT1 (D278 and K281, respectively). Therefore, the GARP domain probably provides a backbone for similar DNA binding, but precise DNA recognition of each GARP domain is likely to be determined by specific amino acid residue differences.

On the other hand, NIGT1 also binds to the core-2 sequence, GAATTTTC. Because this sequence is palindromic and contains four out of five nucleotides found in the core-1 sequence (GAATC), the same amino acid residues of NIGT1 probably recognize the GAAT sequence in the core-1 and core-2 sequences. Interestingly, the core-2 sequence is one variant of another palindromic sequence, GNATATNC, which is the consensus binding sequence of another Arabidopsis GARP family protein, PHR1 (Rubio et al. 2001). It is difficult to find any similarity between the binding sequences of PHR1 (GNATATNC) and ARR10 (AGATT) by direct comparison of these sequences. However, the fact that core-1 and core-2 sequences are similar to the binding sequences of ARR10 and PHR1, respectively, suggests that GARP domains in PHR1 and ARR1 also interact with DNA in a similar manner. Consistent with the fact that the PHR1 recognition sequence is palindromic, it has been shown that PHR1 binds to DNA as a dimer. Hence, differences in complex formation in addition to substituted amino acid residues might contribute to diversity in the recognition sequences of respective GARP family proteins. Thus, even if recognition sequences of respective GARP family proteins are seemingly very different, the GARP domain provides the molecular basis for their sequence-specific interaction with DNA.

**Materials and Methods**

**qRT–PCR analysis**

Rice seedlings (Oryza sativa L. cultivar Nipponbare) grown for 2 weeks with distilled water at 25°C under a day/night cycle of 16/8 h with approximately 200 µE m⁻² s⁻¹ light were treated with various compounds 3 h after the onset of the photoperiod. Preparation of RNA from rice tissues and qRT–PCR were performed as described previously (Aki and Yanagisawa 2009, Konishi and Yanagisawa 2010). The PCR primers used were specific for the 3’-non-coding region and the coding region of NIGT1 transcripts and rice U8Q5 transcripts. Primers used are listed in Supplementary Table S2.

**Plasmid construction**

A vector for the expression of NIGT1 fused to GFP in plant cells, p35SC4PPDK-NIGT1-GFP, was constructed by replacement of EIN3 cDNA in p35SC4PPDK-EIN3(1–449)-GFP (Yanagisawa et al. 2003) with a cDNA encoding full-length NIGT1, which was obtained by RT–PCR using the primers shown in Supplementary Table S2. The NIGT1 cDNA, together with a DNA fragment encoding a MYC epitope tag, was also inserted between the BamHI and XhoI sites of pGEX-6P-1 (GE Healthcare) to generate pGEX-NIGT1-MYC, a plasmid enabling the expression of glutathione S-transferase (GST) fused to NIGT1 in Escherichia coli.

The NIGT1 cDNA fragment was also used to generate effector plasmids for transient reporter assays. One such plasmid, p35SS-C4PPDK-NIGT1-MYC, was generated by replacing EIN3 cDNA in p35SS-C4PPDK-EIN3-MYC (Yanagisawa et al. 2003) with NIGT1 cDNA. Another effector plasmid for expression of NIGT1 fused to the VP16 transcriptional activation domain in plant cells was produced by replacing EIN3 cDNA in the p35SS-C4PPDK-EIN3(1–449)-VP16 plasmid (Konishi and Yanagisawa 2008) with NIGT1 cDNA. The effector plasmids p35SS-C4PPDK-NIGT1-VP32 and p35SS-C4PPDK-NIGT1-VP64 were produced by replacing the sequences for MYC-tags in p35SS-C4PPDK-NIGT1-MYC with a DNA fragment encoding two or four copies of the VP16 domain, respectively. Construction of effector plasmids p35SS-C4PPDK-LexA-MYC, p35SS-C4PPDK-LexA-VP16 and p35SS-C4PPDK-LexA-SUPRD was described previously (Sugiyama et al. 2012). To generate p35SS-C4PPDK-LexA-NIGT1, the sequence for the VP16 transcriptional activation domain in p35SS-C4PPDK-LexA-VP16 was replaced with NIGT1 cDNA.

For four reporter plasmids for use in transient reporter assays, pCore-1-LUC, pCore-2-LUC, pMut-1-LUC and pMut-2-LUC, the EIN3-binding sequence upstream of the 35S minimal promoter in pEBS-LUC (Yanagisawa et al. 2003) was removed and replaced with inserts containing the NIGT1-binding sequences identified in this study (core-1 and core-2...
sequences) and corresponding mutated sequences (mut-1 and mut-2 sequences), which were generated using ligation of synthetic oligonucleotides (Supplementary Table S2). A reporter plasmid containing eight copies of the LexA-binding sites between a 35S enhancer (position −45 to −284) and the 35S minimal promoter truncated at −72 was generated by replacing the 35S enhancer region (−45 to −421) in a previously constructed reporter plasmid (Sugiyama et al. 2012) with a truncated 35S enhancer sequence (−45 to −284), because NIGT1-binding sequences were found in the region from −421 to −284. Another reporter plasmid, pNIGT1pro-LUC, was constructed by replacement of the 35S promoter and the omega enhancer sequence upstream of the LUC gene in pJ301 (Luehrs et al. 1992) with the NIGT1 promoter (−2,256 to −1, relative to the translational start), which was obtained by PCR amplification using specific primers (Supplementary Table S2). Two reporter plasmids, pM1-NIGT1pro-LUC and pM2-NIGT1pro-LUC, in which NIGT1-binding sites in pNIGT1pro-LUC were differently mutated were generated by PCR. Because we used PCR techniques for plasmid construction, all of the resulting constructs were verified by DNA sequencing.

Analysis of the subcellular localization of NIGT1

The plasmid p35S-C4PPDK-NIGT1-GFP was transfected into maize protoplasts which were then incubated for 16 h at 23°C in the dark. Nuclear staining was performed with Hoechst 33258 at the final concentration of 15 μg l⁻¹. The protoplasts were observed using a fluorescence microscope (Olympus) equipped with a digital camera (Olympus) as described previously (Yanagisawa 2001, Aki and Yanagisawa 2009).

DNA-binding site selection experiment

The GST–NIGT1 fusion protein was produced using pGEX-NIGT1-MYC in E. coli and used for DNA-binding site selection experiments, as described previously (Yanagisawa 1997, Yanagisawa and Schmidt 1999) with slight modifications. GST–NIGT1 anchored on glutathione–Sepharose 4B resin (GE Healthcare) was incubated with a library of randomly synthesized DNAs, which were produced by the annealing of two oligonucleotides (5′-GCAAGCTTCTGCAGTTGGATCAGN3′ and 5′-GCTAGCTTCGCTGCGTATT-3′) followed by treatment with the Klenow fragment of E. coli DNA polymerase I. DNA bound to GST–NIGT1 was recovered and amplified by PCR using the primers shown in Supplementary Table S2. After four rounds of selection, DNA sequences were determined.

EMSA

The oligonucleotide pairs (Supplementary Table S2) were annealed to generate probe and competitor DNAs including the core-1 sequence, mut-1 sequence, core-2 sequence and mut-2 sequence. EMSA was performed using biotin-labeled DNA probes as described previously (Konishi and Yanagisawa 2008).

Transient reporter assays

An effector plasmid and a reporter plasmid were co-transfected into mesophyll protoplasts prepared from the second leaves of maize seedlings (strain NS120, Kaneko Seeds Co., LTD.), together with an internal control plasmid harboring the GUS gene under the control of the maize ubiquitin gene promoter (Yanagisawa and Sheen 1998), as described previously (Yanagisawa 2000). Transfected protoplasts were incubated for 16 h, and LUC and GUS activity was measured as described previously (Yanagisawa et al. 2003).

Generation and analysis of transgenic rice plants

The 35S promoter and GUS gene in pCB302-HYG-3SS-GUS (Kato et al. 2010), which harbored a hygromycin resistance gene as a selection marker, were replaced with the maize ubiquitin gene promoter and NIGT1 cDNA fused to a DNA sequence encoding two copies of MYC tags, respectively, to construct a binary vector pCB-HYG-Zmubi-NIGT1-MYC. Using the resultant plasmid, transformation of rice (O. sativa L. cultivar Nipponbare) was performed by Inplanta Innovations Inc. The vector control plants were also generated using pCB-HYG-Zmubi-GUS, which harbored the GUS gene instead of NIGT1 cDNA. Seeds of the T1 generation were germinated on agar plates containing 50 mg l⁻¹ hygromycin B, and the resulting seedlings were grown on the plates for 5 d to select transformants. For RNA preparation, the selected seedlings were further grown hydroponically for 9 d using distilled water at 25°C under a day/night cycle of 16/8 h with approximately 200 μE m⁻² s⁻¹ light and then treated with 10 mM KNO₃ or KCl for 2 h. For the measurement of Chl contents, the selected seedlings were further grown hydroponically for 5 weeks using a nutrient solution [0.37 mM CaCl₂, 0.17 mM NaH₂PO₄, 0.47 mM MgSO₄, 0.27 mM K₂SO₄, 45 μM Fe(III)EDTA, 15 μM H₃BO₃, 4.6 μM MnSO₄, 0.1 μM NaMoO₄, 0.15 μM ZnSO₄, 0.16 μM CuSO₄, 2 mM MES-KOH (pH 5.7)] supplemented with 1 mM NH₄Cl or KNO₃ at 25°C under a day/night cycle of 16/8 h with approximately 200 μE m⁻² s⁻¹ light. The nutrient solution was changed every 2 d. Measurements of Chl contents were performed according to the method of Moran (1982).

Supplementary data

Supplementary data are available at PCP online.

Funding

This research was supported in part by the Japan Science and Technology Agency (JST) [CREST program]; the Ministry of Education, Culture, Sports, Science and
Technologies of Japan (MEXT) and Japan Society for the Promotion of Science (JSPS) [KAKENHI (Nos. 21114004 and 22380043)].

Acknowledgments

We thank Professor Peter Quail (University of California, Berkeley) and the Rice Genome Resource Center (Ibaraki, Japan) for kindly providing the maize ubiquitin gene promoter and a full-length rice cDNA clone, respectively. We thank Dr. Tomohiro Kurai (our laboratory) for excellent assistance with rice cultivation. We also thank Professor Kazuko Yamaguchi-Shinozaki and Dr. Junya Mizoi (The University of Tokyo, Japan) for helping with analysis of microarray data.

References


A nitrate-inducible transcriptional repressor in rice

