OsNAC111, a Blast Disease–Responsive Transcription Factor in Rice, Positively Regulates the Expression of Defense-Related Genes

Naoki Yokotani,¹ Tomoko Tsuchida-Mayama,² Hiroaki Ichikawa,² Nobutaka Mitsuda,³ Masaru Ohme-Takagi,^{3,4} Hisatoshi Kaku,⁵ Eiichi Minami,¹ and Yoko Nishizawa¹

¹GMO Research Center, and ²Division of Plant Sciences, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan; ³Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Higashi 1-1-1, Tsukuba, Ibaraki 305-8562, Japan; ⁴Institute for Environmental Research and Technology (IEST), Saitama University, Saitama 338-8570 Japan; ⁵Sakata Seed Corporation, Kimitsu Research Station, 358 Uchikoshi, Sodegaura, Chiba 299-0217, Japan

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Plants respond to pathogen attack by transcriptionally regulating defense-related genes via various types of transcription factors. We identified a transcription factor in rice, OsNAC111, belonging to the TERN subgroup of the NAC family that was transcriptionally upregulated after rice blast fungus (*Magnaporthe oryzae*) inoculation. OsNAC111 was localized in the nucleus of rice cells and had transcriptional activation activity in yeast and rice cells. Transgenic rice plants overexpressing *OsNAC111* showed increased resistance to the rice blast fungus. In *OsNAC111*-overexpressing plants, the expression of several defense-related genes, including pathogenesis-related (PR) genes, was constitutively high compared with the control. These genes all showed blast disease-responsive expression in leaves. Among them, two chitinase genes and one β -1,3-glucanase gene showed reduced expression in transgenic rice plants in which OsNAC111 function was suppressed by a chimeric repressor (*OsNAC111-SRDX*). OsNAC111 activated transcription from the promoters of the chitinase and β -1,3-glucanase genes in rice cells. In addition, brown pigmentation at the infection sites, a defense response of rice cells to the blast fungus, was lowered in *OsNAC111-SRDX* plants at the early infection stage. These results indicate that OsNAC111 positively regulates the expression of a specific set of PR genes in the disease response and contributes to disease resistance.

Plants have developed defense mechanisms to protect themselves from pathogens. These defense responses are triggered by receptor-mediated recognition of microbe-associated molecular patterns (MAMPs) or resistance-gene-mediated recognition of effector or avirulence proteins, and the transduction of these signals causes transcriptional alteration of a large number of defense-related genes (Schwessinger and Ronald 2012; Stergiopoulos and de Wit 2009). Pathogenesis-related (PR) genes are major defense-responsive genes and are categorized into 17 groups. The PR genes in each group compose a multi-

gene family and encode proteins predicted to play roles in antifungal activity, mechanical protection, antioxidation, or regulation of the hypersensitive reaction to resist pathogens (van Loon et al. 2006).

The expression of defense-related genes is regulated by various transcription factors, which also exhibit defense-responsive expression, through specific types of *cis* elements in their promoter regions (Singh et al. 2002). WRKYs are well-characterized plant-specific transcription factors that play an important role in the regulation of defense-related gene expression via the W-box (CCGACT/C), a *cis* element found in a wide range of disease-responsive genes in various plant species (Rushton et al. 2010). AP2/ERFs, another group of plant-specific transcription factors, are involved in the signaling of defense-related plant hormones (ethylene and jasmonate [JA]) and regulate the expression of some PR genes via the GCC-box element (AGCCGCC) (Licausi et al. 2013). Members of the TGA family of bZIP transcription factors bind the *as-1* element (TGACG) and play a role in salicylic acid (SA)-mediated systemic acquired resistance (Zhang et al. 1999).

Rice is one of the most important cereal crops, and provides a model system for molecular and genetic studies of monocots. Rice blast fungus (*Magnaporthe oryzae*, *Oryzae* isolate) is a hemibiotrophic pathogen that causes blast disease in rice, which causes major losses in the global rice yield (Talbot 2003). Genome-wide transcriptome analysis using microarray (Li et al. 2006; Mosquera et al. 2009) and RNA-Seq (Kawahara et al. 2012) techniques has demonstrated that the expression of transcription factors of several families is induced by *M. oryzae* infection. They include WRKY, ERF/AP2, bHLH, MYB, and NAC, and are predicted to regulate defense responses to *M. oryzae*; in particular, the function of WRKYs has been intensively analyzed (Chujo et al. 2007; Shimono et al. 2007; Yokotani et al. 2013).

NAC (NAM, ATAF1/2, and CUC2) is a plant-specific family of transcription factors and is characterized by a conserved DNA-binding NAC domain in the N-terminal region (Puranik et al. 2012). NAC transcription factors were originally identified as petunia NAM (Souer et al. 1996) and *Arabidopsis* CUC2 (Aida et al. 1997), both of which play roles in the determination and development of the shoot apical meristem. Thus far, it has been reported that NAC proteins are involved in plant growth, development, hormone signaling, and biotic and

Corresponding author: Y. Nishizawa; E-mail: ynishi@affrc.go.jp

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abiotic stress responses (Puranik et al. 2012). Ooka and associates (2003) classified NAC proteins into two groups and 18 subgroups based on phylogenetic analysis. In rice, more than 150 NAC genes have been identified in the genome (Nuruzzaman et al. 2010), and some of them are involved in the disease response. OsNAC6 participates in responses to drought, high salinity, and blast disease (Nakashima et al. 2007). OsNAC4 is upregulated during nonhost defense responses and regulates hypersensitive cell death (Kaneda et al. 2009). Rice RIM1 is a negative regulator of resistance to *Rice dwarf virus* (Yoshii et al. 2009). Virus-induced silencing of *ONAC122* or *ONAC131*, which are closely related rice NAC proteins, caused increased susceptibility to blast disease, suggesting that both *ONAC122* and -131 positively regulate blast disease resistance (Sun et al. 2013). However, the mode of action of rice NAC proteins besides OsNAC4 (Kaneda et al. 2009) in the disease response has not been revealed.

We previously analyzed the transcriptome in rice leaves 36 h postinoculation (hpi) with *M. oryzae* (Yokotani et al. 2013). *OsNAC111* (Yilmaz et al. 2009) (identical to *ONAC017*), located at Os11g0154500 in Rice Annotation Project (RAP) locus ID, was one of the *M. oryzae*-responsive transcription factor genes. OsNAC111 is classified into the TERN subgroup of group I (Ooka et al. 2003) but no members of this subgroup have been characterized for biological function. Here, we report the functional analysis of *OsNAC111*. Our data demonstrate that OsNAC111 acts as a transcriptional activator, and plays a positive role in disease resistance through regulating the expression of a specific set of defense-related genes.

RESULTS

Blast-responsive expression of *OsNAC111*.

OsNAC111 encodes a 294-amino-acid protein. OsNAC111 contains one NAC domain consisting of five subdomains (A to E) at the N terminus (Supplementary Fig. S1A and B). No other characterized motifs or domains such as nuclear localization signals or transcriptional regulation motifs were found in OsNAC111. A phylogenetic tree based on the amino acid sequences of OsNAC111 and other reported NAC proteins placed OsNAC111 into the same clade as tobacco TERN (Ooka et al. 2003) and *Arabidopsis* ANAC061, which is transcriptionally activated by a fungal MAMP, chitin (Libault et al. 2007). We examined the expression pattern of *OsNAC111* after inoculation with *M. oryzae* by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis (Fig. 1A). The expression of *OsNAC111* was nearly undetectable in untreated or mock-treated rice plants and was induced by both compatible and incompatible strains of *M. oryzae* within 1 day postinoculation (dpi). This result suggests that OsNAC111 plays a role in the basal resistance. The expression of *OsNAC111* was not induced by wounding or application of JA or benzothiadiazole (BTH), a chemical activator of SA signaling (data not shown).

Nuclear localization of the OsNAC111 protein.

To investigate the subcellular localization of OsNAC111, expression plasmids carrying a green fluorescent protein (GFP) gene, GFP-fused OsNAC111 (*OsNAC111-GFP*), and *DiscoSoma* red fluorescent protein (*DsRed*) were co-introduced into rice coleoptile cells by particle bombardment. The fluorescence from nonfused GFP and *DsRed* (controls) was detected in both the nucleus and cytosol (Fig. 1B), whereas that from *OsNAC111-GFP* was detected only in the nucleus, indicating that the OsNAC111 protein is targeted to the nucleus.

Fig. 1. Molecular characterization of OsNAC111. **A**, Expression of *OsNAC111* in response to inoculation with *Magnaporthe oryzae*. The expression of *OsNAC111* in leaf blades was quantified by quantitative reverse transcription polymerase chain reaction analysis. The bottom numbers indicate days postinoculation (dpi). Expression levels are expressed as ratios of the transcript level at 0 day. Data are represented as mean values $\pm$ standard error (SE) value for three replicates. **B**, Subcellular localization of green fluorescent protein (GFP)-fused OsNAC111 protein in etiolated rice coleoptile cells. The upper photograph is a fluorescence image of the GFP control. The lower photographs are fluorescence images of GFP-fused OsNAC111 (NAC111-GFP) coexpressed with the *DiscoSoma* red fluorescent protein (*DsRed*) control. Bar = 100 μ m. **C**, Yeast two-hybrid assay of OsNAC111. Whole and partial deletion constructs of OsNAC111 were fused to the GAL4 DNA-binding domain (DB) and expressed in the yeast strain AH109. HIS3 activity was indicated by viability on a histidine-lacking medium (HIS⁻). LacZ activity was assessed by β -galactosidase (β -gal) filter lift assay. The NAC domain is shaded in gray. **D**, Luciferase (LUC) reporter assay of GAL4DB and GAL4 element-mediated LUC activity by OsNAC111 and OsNAC111-SRDX in cultured rice cells. Data are represented as mean values $\pm$ SE for four replicates. Significantly higher and lower LUC activity compared with the DB control is denoted by single and double asterisks, respectively ($P < 0.05$ by *t* test).

Transcriptional activator activity of OsNAC111 in yeast and rice cells.

First, the transcriptional regulatory activity of OsNAC111 was tested using a yeast reporter assay system. A whole or partial coding sequence of OsNAC111 was fused with the GAL4 DNA-binding domain (DB) and introduced into the yeast strain AH109. The yeast transformed with whole OsNAC111 or the C-terminal region survived on histidine-lacking medium (Fig. 1C). The LacZ activity of yeast transformants was consistent with survivability on histidine-lacking medium (Fig. 1C). These results indicated that OsNAC111 exhibits transactivation activity and that the C-terminal region next to the NAC domain is required for this activity.

Next, the transcriptional activator activity of OsNAC111 was examined in rice cells. The effector plasmid expressed OsNAC111 fused with DB at the N terminus (DB-OsNAC111). An effector plasmid expressing DB alone was used as a control. The reporter plasmid carried the firefly luciferase (*LUC*) gene under the 35S promoter and the GAL4 upstream activation sequence (UAS). Both effector and reporter plasmids were co-introduced into rice cultured cells. The LUC reporter activity was considerably enhanced by coexpression with DB-OsNAC111 compared with the DB control, indicating that OsNAC111 also exhibits transactivation activity in rice cells (Fig. 1D). In contrast, DB-OsNAC111 fused with the EAR-motif repression domain (SRDX) (Hiratsu et al. 2003) repressed LUC reporter activity, confirming that fusion of the repressor domain converted OsNAC111 from an activator to a repressor (Fig. 1D).

Overexpression and functional suppression of OsNAC111 affect responses to *M. oryzae*.

To analyze the biological function of *OsNAC111*, transgenic rice plants overexpressing OsNAC111 (*N111-OX*) or OsNAC111 fused with the repression domain (*N111-SRDX*) were produced using the maize *polyubiquitin-1* promoter (*Ubi-1*) (Supplementary Fig. S2A). The three lines expressed *OsNAC111* at >50 times higher levels than the nontransformed control (NT). The *N111-OX* plants showed dwarfism and the total fresh weight of 28-day-old seedlings was reduced to 70% compared with NT plants. On the other hand, the *N111-SRDX* plants showed normal growth, similar to NT plants.

The function of OsNAC111 in the disease response was examined by testing the resistance of *N111-OX* plants against *M. oryzae*. Two independent *N111-OX* plants showed reduced blast disease symptoms compared with NT plants (Fig. 2A). In quantitative genomic PCR analysis, more than 10-fold lower levels of fungal DNA were detected from *N111-OX* plants compared with NT plants (Fig. 2B). Infection hyphae growing in the inner epidermal cells of leaf sheaths at 36 hpi are shown in Figure 2C. The microscopic observation indicated that

Fig. 2. Effects of overexpression and functional suppression of OsNAC111 on disease responses to a compatible strain (Ina86-137) of *Magnaporthe oryzae*. **A**, Blast disease symptoms of the rice seedlings. Sixth leaf blades of rice seedlings were sprayed with conidia suspended in water. Photographs show leaf blades at 5 days postinoculation (dpi). Enlarged photographs of disease symptoms in a nontransformant (NT) and an overexpressor (OX#08) are also shown. Black and white bars indicate 10 and 2 mm, respectively. **B**, Quantification of fungal growth by measuring the amount of genomic DNA in rice leaf blades. The amount of *M. oryzae* 28S ribosomal DNA relative to rice genomic *eEF1* DNA was determined by quantitative polymerase chain reaction analysis. Values are represented as mean values $\pm$ standard error (SE) for five leaf blades. Significantly higher values compared with the nontransformed control are denoted by asterisks (* and ** indicate $P < 0.05$ and 0.01 , respectively, by Dunnett's test on log-transformed data). **C**, Representative photographs of the leaf sheaths of NT and *N111-OX* plants at 36 h after inoculation with *M. oryzae*. White, gray, and black arrowheads indicate "no invasion", "invasion of one cell", and "invasion of two or more cells", respectively. In *N111-OX* plants, hyphal growth after penetration was suppressed. **D**, Invasion rate of the infection hyphae in rice leaf sheaths. Data are expressed as the ratio of the invasion pattern of infection hyphae per 200 appressoria. Values are represented as mean values $\pm$ SE for four leaf sheaths. Significantly different values compared with the nontransformed control are denoted by asterisks (* and ** indicate $P < 0.05$ and 0.01 , respectively, by Dunnett's test). **E**, Development of brown pigmentation at the early stage of blast disease in NT and *N111-SRDX* plants. Photographs show bleached sixth leaf blades at 3 dpi. **F**, Changes in the size of brown area in leaf blades after inoculation of blast fungus in NT and *N111-SRDX* plants. Values are expressed as mean values $\pm$ standard deviation for four leaf blades.

hyphal growth after penetration was suppressed in *N111-OX* plants (Fig. 2D). These results indicated that overexpression of *OsNAC111* enhances resistance against *M. oryzae*. However, hypersensitive cell death of host cells, which is a typical reaction in the incompatible interaction (Koga et al. 2004), was not observed (data not shown).

On the other hand, the *N111-SRDX* plants were similar to NT plants in disease symptoms at 5 dpi (Fig. 2A), fungal multiplication (Fig. 2B), and growth (Fig. 2D). However, brown pigmentation was suppressed in *N111-SRDX* plants at the early stage of infection compared with NT (Fig. 2E and F). Reduced pigmentation was also observed in the incompatible interaction (Supplementary Fig. S3), indicating that *OsNAC111* is involved in the accumulation of a brown-colored substance near the penetration site.

Overexpression of *OsNAC111* affects the expression of defense-related genes.

To identify candidates for the *OsNAC111* target genes, gene expression profiling was performed by microarray analysis. Total RNA was isolated from the untreated leaf blades of NT, *N111-OX#08*, and *N111-SRDX#05* plants. After statistical processing, 71 genes were selected by applying the following filter: average maximum/minimum difference greater than 5 and false discovery rates (FDR) < 0.01 (Supplementary Fig. S4). Among them, 54 genes were highly expressed in *N111-OX#08* compared with NT and *N111-SRDX#05*. They included 14 *PR*

genes categorized into six groups: *PR1*, *PR2*, *PR3*, *PR4*, *PR5*, and *PR8* (Table 1). *N111-OX#08* also highly expressed other defense-related genes, including *RIR1b* (Schaffrath et al. 2000), *OsERG1* (Kim et al. 2000), *DRR206* (Culley et al. 1995), and *OsCPS4* (Toyomasu et al. 2014). In addition, several genes that are known to be involved in disease-responsive signal transduction such as receptor-like kinases and WRKY transcription factors were also upregulated in *N111-OX#08* plants. The results of the microarray analysis were confirmed by qRT-PCR analysis for eight genes (seven *PR* genes and *RIR1b*). The expression of all genes tested was constitutively upregulated in two independent *N111-OX* lines (Fig. 3A).

Microarray analysis revealed that the expression levels of most genes upregulated in *N111-OX#08* were comparable between NT and *N111-SRDX* plants. This result was probably attributable to the extremely low expression levels of these genes in untreated NT plants. Thus, we compared the expression levels of these genes in detail between NT and *N111-SRDX* plants by qRT-PCR analysis after inoculation with *M. oryzae*. In NT plants, the expression of all eight defense-related genes tested was induced by blast inoculation within 1 dpi (Fig. 3B; Supplementary Fig. S5). Among them, three genes (*PR2*, *PR3*, and *PR8/OsChib3a*) were expressed at very low levels even after inoculation of *M. oryzae* in two independent *N111-SRDX* lines (Fig. 3B), strongly suggesting that the expression of these three genes was under the control of *OsNAC111*. The expression of another five genes (*PR1*, *PR4*, *PR3/OsChia1c*, and *PR5*) was increased to levels similar to those in NT plants.

OsNAC111 enhances the promoter activity of defense-related genes.

To determine whether *OsNAC111* promotes transcription of defense-related genes, a reporter assay was performed with the promoters of *PR2* (Os07g0539900) and *PR8/OsChib3a* (Os01g0660200), which were upregulated in *N111-OX* and not activated by *M. oryzae* infection in *N111-SRDX*. A reporter plasmid harboring the *LUC* reporter gene fused to the 1,500-bp upstream region of *PR2* or *PR8/OsChib3a* was co-introduced with an effector plasmid expressing *OsNAC111* under the 35S promoter in rice cultured cells. *OsNAC111* markedly induced *LUC* activity compared with the vector control (Fig. 4), demonstrating that *OsNAC111* transcriptionally activates the promoters of *PR2* and *PR8/OsChib3a*.

DISCUSSION

In this study, we characterized the molecular and physiological function of a rice NAC family gene, *OsNAC111*, which transcriptionally responded to *M. oryzae* infection. *OsNAC111* contains a conserved NAC DB in its N terminus. *OsNAC111* localized to the nucleus (Fig. 1B) and had transcriptional activator activity in the C-terminal region next to the NAC DB (Fig. 1C and D). These characteristics are typical of NAC family transcription factors (Puranik et al. 2012). Ooka and associates (2003) classified NAC proteins into 18 subgroups based on phylogenetic analysis and proposed a relationship between the structure of NAC domains and their biological functions. However, information on the function of TERN subgroup members was lacking. Recently, it was reported that VpNAC1, an NAC protein of Chinese wild *Vitis pseudoreticulata*, induced the expression of *PR* genes when overexpressed in tobacco (Zhu et al. 2012). The authors described VpNAC1 as a TERN subfamily member but it is classified into a different subgroup from TERN. In the present study, we demonstrated that *OsNAC111* regulates the expression of several defense-related genes. *ANAC061* is a chitin-responsive gene (Libault et

Table 1. Defense-related genes upregulated in *N111-OX#08* identified by microarray analysis^a

RAP locus ID	Description	Fold (OX/NT)
<i>PR</i> gene		
Os07g0129300	<i>PR1</i>	333.2
Os01g0713200	<i>PR2/β-1,3-glucanase</i>	95.5
Os01g0940800	<i>PR2/β-1,3-glucanase</i>	75.2
Os01g0941400	<i>PR2/α-1,3-glucanase</i>	16.3
Os01g0944500	<i>PR2/β-1,3-glucanase</i>	8.4
Os03g0600600	<i>PR2/β-1,3-glucanase</i>	8.7
Os07g0539900	<i>PR2/β-1,3-glucanase</i>	45.5
Os01g0940700	<i>PR2/β-1,3-glucanase/Gns5</i>	7.0
Os03g0132900	<i>PR3/chitinase class I</i>	31.1
Os06g0726100	<i>PR3/chitinase class I/OsChia1c</i>	117.8
Os11g0592100	<i>PR4/barwin homolog</i>	35.3
Os03g0663400	<i>PR5/thaumatin</i>	14.5
Os12g0629700	<i>PR5/thaumatin</i>	312.9
Os01g0660200	<i>PR8/chitinase class III/OsChib3a</i>	52.3
Protein kinase		
Os01g0117200	Receptor-like cytoplasmic kinase/ <i>OsRLCK16</i>	22.0
Os02g0807800	Wall-associated receptor-like kinase/ <i>OsWAK20</i>	19.7
Os10g0180800	Wall-associated receptor-like kinase/ <i>OsWAK112d</i>	81.0
Os01g0132100	Leucine-rich repeat receptor-like kinase	42.7
Os02g0609900	Leucine-rich repeat receptor-like kinase	134.6
Os11g0625900	Leucine-rich repeat receptor-like kinase	29.6
Os07g0538200	Cysteine-rich receptor-like kinase	20.1
Transcription factor		
Os05g0571200	WRKY transcription factor/ <i>OsWRKY19</i>	59.3
Os01g0584900	WRKY transcription factor/ <i>OsWRKY77</i>	20.0
Other defense-related gene		
Os10g0569800	Defense-related gene/ <i>RIR1b</i>	26.1
Os01g0841700	Elicitor-responsive gene/ <i>OsERG1</i>	39.6
Os01g0879200	Disease-resistance response gene/ <i>DRR206</i>	25.8
Os04g0178300	<i>syn-Copalyl diphosphate synthase/OsCPS4</i>	23.7

^a RAP = Rice Annotation Project and PR = pathogenesis-related.

al. 2007); therefore, although the functions of ANAC061 and TERN have not been characterized, NAC proteins of the TERN subgroup might play a role in disease responses.

N111-OX plants showed increased blast disease resistance in their leaf blades and leaf sheaths (Fig. 3A–D) but did not show enhanced hypersensitive cell death (data not shown), suggesting that OsNAC111 has a different function from OsNAC4, which positively regulates hypersensitive cell death (Kaneda et al. 2009). The suppression of OsNAC111 function did not affect the growth of *M. oryzae* (Fig. 2C and D), which is probably because of the limited suppression of the defense-related gene expression in *N111-SRDX* plants, as discussed later. However, the phenotype of *N111-SRDX* plants indicates that OsNAC111 is involved in dark-brown pigmentation. Dark-brown pigmentation is considered to be related to phenylpropanoid metabolism and to protect cells via antioxidation of cellular components and mechanical reinforcement of cell walls (Pourcel et al. 2006; Sridhar and Ou 1974). Some rice lesion mimic mutants that spontaneously form dark-brown spots, such as *spotted leaf* and *blast lesion mimic*, exhibit enhanced disease resistance (Wu et al. 2008; Yin et al. 2000). Therefore, dark-brown pigmentation is a defense response. The dark-brown area in *N111-OX* plants was also reduced (Supplementary Fig. S6) but this result is attributable to lowered fungal multiplication in *N111-OX* plants. Considering the phenotypes of both *N111-OX* and *N111-SRDX* plants, we concluded that OsNAC111 acts as a positive regulator of disease resistance. The expression of *OsNAC111* was not induced by BTH or JA (data not shown). Thus, unlike the BTH-responsive positive regulator of disease

resistance OsWRKY45, which is considered to be an SA-signaling mediator (Shimono et al. 2007), OsNAC111 might play a role in an SA-independent defense signaling pathway.

Microarray analysis revealed that 54 genes were upregulated in *N111-OX* plants under normal growth conditions, 27 genes of which were predicted to be associated with defense mechanisms (Table 1). They included *RIR1b*, which confers blast disease resistance when overexpressed, although its molecular function remains unknown (Schaffrath et al. 2000). *PR2* and *PR3/8* encode β -1,3-glucanase and chitinase, respectively, which are well-known antifungal hydrolytic enzymes. Genes for receptor-like kinases and WRKY transcription factors—for example, OsWRKY77 (Lan et al. 2013), which positively regulates the expression of defense-responsive genes—were also upregulated in *N111-OX* plants. These results strongly suggest that the enhanced blast resistance in *N111-OX* plants results from the constitutively higher expression of these defense-related genes. *N111-OX* plants showed growth reduction under normal growth conditions. This phenotype might be a side effect of constitutive activation of defense responses. Consistent with this, plant activators such as BTH induce disease resistance via priming of defense-related genes but suppress plant growth (Heil and Baldwin 2002).

Among the 54 candidate target genes of OsNAC111 revealed by microarray analysis, all 8 genes tested were dramatically upregulated after inoculation with *M. oryzae* (Fig. 3B). Furthermore, the induction of the expression of three PR genes (*PR2*, *PR3*, and *PR8/OsChib3a*) after *M. oryzae* infection was suppressed in *N111-SRDX* plants (Fig. 3B), suggesting that the

Fig. 3. Effects of overexpression and functional suppression of OsNAC111 on the expression of defense-related genes. **A**, Expression of defense-related genes in nontransformant (NT) and *N111-OX* plants under normal growth conditions. Total RNA was extracted from sixth-leaf blades. **B**, Expression of defense-related genes in NT and *N111-SRDX* plants after inoculation with a compatible strain of *Magnaporthe oryzae* (Ina86-137). Transcript levels are expressed relative to the transcript level in the *N111-OX#08* line, which was assumed to be 100. Data are represented as mean values $\pm$ standard error for three replicates.

expression of these genes depends largely on OsNAC111 function. These data strongly suggest that OsNAC111 is a key transcription factor that upregulates a set of defense-related genes. In fact, a reporter assay demonstrated that OsNAC111 activates transcription from the promoters of two *PR* genes (*PR2* and *PR8/OsChib3a*) in rice cells (Fig. 4), indicating that OsNAC111 transactivates these genes directly. Several studies have been conducted to characterize the direct target genes and DNA-binding specificity of NAC transcription factors. A rice NAC transcription factor, IDEF2, regulates iron homeostasis via recognizing the iron-deficiency-responsive IDE2 element, CA(A/C)G(T/C)(T/C/A)(T/C/A) (Ogo et al. 2008). *Arabidopsis* RD26/ANAC072 binds to the CATGTG motif-containing drought-responsive *cis*-acting element of the *EDR2* gene (Tran et al. 2004). However, these NAC-binding sequences were not found in the promoter regions of the two *PR* genes tested. Thus, OsNAC111 might bind to unidentified *cis* elements. DNA-protein-binding analysis may reveal novel *cis* elements and further elucidate the regulatory mechanism of OsNAC111. The expression of five defense-related genes tested (*PR1*, *PR3/OsChia1c*, *PR4*, *PR5*, and *RIR1b*) was increased after *M. oryzae* infection in *N111-SRDX* plants at levels similar to those in NT plants, which indicates that OsNAC111 contributes to the expression of these genes to a lesser extent. Probably, the expression of these genes is regulated by other transcription factors in addition to OsNAC111. In fact, the majority of OsNAC111 target gene candidates contained pathogenesis-responsive *cis* elements such as W-box, GCC-box, and *as-1* elements in their promoter regions (data not shown).

In the present study, we performed molecular characterization and physiological analysis of a rice disease-responsive transcription factor, OsNAC111. Our data demonstrated that the expression of several defense-related genes depends on this novel NAC member, which provides new insight into the function of NAC transcription factors in disease responses.

Fig. 4. Transactivation of the promoters of defense-related genes by OsNAC111. The 1,500-bp upstream region of pathogenesis-related (*PR*)2 or *PR8/OsChib3a* was used as the promoter. Values are expressed as mean values $\pm$ standard error for three independent experiments.

MATERIALS AND METHODS

Plants and pathogens.

Rice plants (*Oryza sativa* L. 'Nipponbare') carrying the blast-resistance gene *Pia* were used in this study. Rice plants were grown in a chamber under a cycle of 14 h of light (28°C) and 10 h of darkness (24°C) in hydroponic culture, as previously described by Tanabe and associates (2006). *M. oryzae* isolates Ina86-137 (MAFF 101511, race 007.0) and P91-15B (race 001.0) were used as virulent and avirulent strains, respectively.

RNA isolation and qRT-PCR.

Total RNA was extracted using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan). First-strand cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). qRT-PCR was performed using 1 $\times$ SYBR Premix Ex Taq II (Takara Bio). The primer sequences are listed in Supplementary Table S1. The relative levels of transcripts were quantified using an MX3000P system (Stratagene, La Jolla, CA, U.S.A.). The data were normalized to the expression of *eEF-1 α* encoding the eukaryotic elongation factor 1- α (Jain et al. 2006).

Fungal inoculation and microscopic observation.

Rice plants at the six-leaf stage (25 days old) were inoculated with *M. oryzae*. Rice seedlings were placed on moistened filter paper in petri dishes and sprayed with washed conidia suspended at 5×10^5 ml⁻¹. The sprayed seedlings were incubated at 25°C in the dark for 24 h, and then under a cycle of 14 h of light and 10 h of darkness. Brown pigmentation was measured using image analysis software (ImageJ) after chlorophyll was removed by boiled ethanol. Blast disease development was quantified by measuring *M. oryzae* genomic DNA relative to rice genomic DNA by quantitative genomic PCR analysis (Zellerhoff et al. 2006). Total DNA was extracted from leaves in extraction buffer containing 0.5% (wt/vol) sodium dodecyl sulfate, 25 mM EDTA, 250 mM NaCl, and 50 mM Tris-HCl (pH 8.0), and purified with chloroform/isoamyl alcohol (24:1, vol/vol) followed by isopropyl alcohol precipitation. The genomic DNA of *M. oryzae* and rice was measured using specific primers for the *Mo28S rRNA* gene and *OsEF1 α* , respectively, by qPCR analysis, as previously described (Yokotani et al. 2013).

The plant response to the blast fungus at the early stage was observed in the inner epidermal cells of the leaf sheath under a microscope. Sheaths of the fifth leaves of rice plants at the 5.6-leaf stage were detached and inoculated with a suspension of conidia (1×10^5 conidia/ml), then incubated at 25°C in the dark for 36 h. After fixation in a solution of formaldehyde/acetic acid/ethanol/water (5:5:45:45, vol/vol/vol/vol), the level of infection was evaluated by observing intact appressoria under a light microscope. The samples were scored as "no invasion", "invasion of one cell", or "invasion of two or more cells" (Tanabe et al. 2006).

Transient expression of OsNAC111-GFP in rice cells.

The coding region of OsNAC111 was fused with a coding region of synthetic *GFP* gene (Niwa et al. 1999) and inserted into the *XbaI/SacI*-digested pBI221 vector (Clontech, Mountain View, CA, U.S.A.) to replace the β -glucuronidase (*GUS*) gene. The nonfused GFP driven by the *Cauliflower mosaic virus* 35S promoter in pBI221 was used as a control. For the transient expression assay, 1 μ g of plasmid DNA was introduced into etiolated rice coleoptile cells using a PDS-1000/He particle delivery system (Bio-Rad, Hercules, CA, U.S.A.). The DsRed-monomer gene (Clontech) driven by the 35S promoter

in pBI221 was coexpressed as a gene expression marker and subcellular (cytosol and nuclear) localization marker. After overnight incubation in the dark, cells were observed under a fluorescence microscope.

Transactivation assay in yeast.

To express the N-terminal GAL4 DB fusion protein in yeast, whole or truncated coding sequences of OsNAC111 were cloned into the *Bam*HI site of pGBKT7 (Clontech). The yeast strain AH109 harboring the *HIS3* and *LacZ* reporter genes was transformed with the recombinant plasmid. *HIS3* activity was assessed by conducting a viability test on a histidine-lacking medium. *LacZ* activity was tested by performing the β -galactosidase filter assay according to the manufacturer's instructions (Clontech).

LUC reporter assay in cultured rice cells.

To express the N-terminal GAL4DB fusion protein in rice cells, the coding sequence of OsNAC111 or OsNAC111 fused in-frame to the EAR-motif repression domain of a rice SUPERMAN-like protein (RAP locus ID; Os05g0286100) was inserted into the *Bam*HI site of 35S-GAL4DB-NOS (Ohta et al. 2001). The 35S-GAL4-TATA-LUC plasmid was used as a GAL4-responsive reporter. To express the OsNAC111 without tags, the coding sequence of OsNAC111 was inserted into the *Xba*I/*Sac*I-digested pBI221 to replace the *GUS* gene. The plasmid expressing the Renilla LUC (*RLUC*) gene under the 35S promoter was used as an internal control.

For cotransfection assays, 2 μ g of the reporter plasmid, 2 μ g of the effector plasmid, and 0.04 μ g of the internal control plasmid were mixed and introduced into suspension-cultured rice cells (line Oc) (Baba et al. 1986) using the PDS-1000/He particle delivery system (Bio-Rad). Transformed rice cells were incubated for 24 h at 24°C in the dark. The LUC assay was performed using the Dual-Luciferase reporter assay system (Promega Corp., Madison, WI, U.S.A.) according to the manufacturer's instructions. LUC activity was measured using a TD20/20 luminometer (Turner Designs, Sunnyvale, CA, U.S.A.).

Rice transformation.

For overexpression of *OsNAC111*, the coding region of *OsNAC111* was inserted into the binary vector pBI333 (Nishizawa et al. 1999) with the maize *Ubi-1* promoter. For overexpression of *OsNAC111-SRDX*, the coding region of *OsNAC111* was inserted into the binary vector pSMAHdN643-UGWRD (Tanaka et al. 2012), which expresses the EAR-motif repression domain-fused protein between the *Ubi-1* promoter and the *NOS* terminator. The resultant vectors were introduced into rice via *Rhizobium radiobacter* strain EHA105. Rice transformation was performed as described by Toki and associates (2006).

Microarray analysis.

The sixth leaf blades of six-leaf-stage plants were used for the microarray experiment. Total RNA was isolated using the RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.). Cy3-labeled complementary RNA was prepared from 200 ng of total RNA and hybridized to a rice 44K oligo microarray based on RAP according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA, U.S.A.). The slide images were scanned with a DNA microarray scanner (Agilent Technologies) using the manufacturer's Feature Extraction software. The data were analyzed using R/Bioconductor. Four independent plants were used for each sample as biological replicates. After log2 transformation and global normalization, changes in gene expression were considered to be statistically significant when

their FDR was <0.01 using a one-way analysis of variance FDR test (Benjamini and Hochberg 1995). The data were z-score transformed across each gene and presented as a heat map represented by two-dimensional hierarchical clustering.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

GRASSIUS, Grass Regulatory Information server: grassius.org
 Bioconductor open source software: www.bioconductor.org