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Molecular Cloning and Functional Analysis of Rice (*Oryza sativa* L.) *OsNDR1* on Defense Signaling Pathway

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A novel rice (*Oryza sativa* L.) gene, homologous to Arabidopsis pathogenesis-related *NDR1* gene, was cloned from cDNA library prepared from 30 min *Magnaporthe grisea* -treated rice seedling leaves, and named as *OsNDR1*. *OsNDR1* encoded a 220-amino-acid polypeptide and was highly similar to the Arabidopsis *AtNDR1* protein. *OsNDR1* is a plasma membrane (PM)-localized protein, and presumes through sequence analysis and protein localization experiment. Overexpression of *OsNDR1* promotes the expression of *PBZ1* that is essential for the activation of defense/stress-related gene. The *OsNDR1* promoter did not respond significantly to treatments with either SA, PBZ, or ETP. Exogenously applied BTH induces the same set of SAR genes as biological induction, providing further evidence for BTH as a signal. Presumably, BTH is bound by a receptor and the binding triggers a signal transduction cascade that has an ultimate effect on transcription factors that regulate SAR gene expression. Thus *OsNDR1* may act as a transducer of pathogen signals and/or interact with the pathogen and is indeed another important step in clarifying the component participating in the defense response pathways in rice.

Keywords : Benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), *Magnaporthe grisea*, *Oryza sativa* L., *OsNDR1*, system acquired resistance (SAR)

Disease resistance mechanism of plant has been a major research area for many researchers in order to solve the yield loss problems by pathogens. Breeders have been trying to generate resistant cultivars through introgression of resistance genes which confer complete resistance into the customer most wanted, but susceptible cultivars. There have been many reports on cloning and functional analysis of R-genes for the purpose of using them for molecular breeding sources of various plants (Dilbirligi et al., 2004). However, as proved by the breeding programs so far, a simple introgression of R-genes into the susceptible cultivars for generating resistant one result in resistance breakdown through the appearance of new races infesting R-gene-mediated new resistant cultivars (Kiyosawa, 1982). This unexpected disease occurrence brings the necessity of developing durable resistant cultivars.

Previous studies have established that mutations in the *AtNDR1* gene in *Arabidopsis thaliana* suppress the resistance response of three resistance protein, *RPS2* (Bent et al., 1994; Mindrinos et al., 1994), *RPM1* (Grant et al., 1995), and *RPS5* (Simonich and Innes 1995; Warren et al., 1998), to *Pseudomonas syringae* pv. tomato (pst) strain DC3000 containing the cognate effector genes, *AvrRpt2* (Innes et al., 1993; Whalen et al., 1991), *AvrRpm1* (Ritter and Dangl et al., 1995), and *AvrPhB* (Puri et al., 1997), respectively. Many of the major components in Arabidopsis, as many of the major components in Arabidopsis disease resistance signaling, including *RPM1*, *RPS2*, and the Pst effector molecules *AvrRpt2*, *AvrB*, and *AvrRpm1* are localize at the plasma membrane (PM) (Coppinger et al., 2004). *AtNDR1* is also plasma membrain (PM)-localize protein, and undergoes several post-translational modifications including carboxy-treminal processing and N-linked glycosylation (Coppinger et al., 2004). It is suggested that *NDR1* may exist in a protein complex with other R proteins at the PM. *AtNDR1* appears to possess an uncleaved N-terminal signal anchor in addition to a C-terminal GPI anchor (Coppinger et al., 2004). Sequence analysis also predicts a hydrophobic core between amino acids 20 and 33 of the putative signal anchor, suggestive of a transmembrain domain. That localization of *AtNDR1* at the PM via a GPI anchor is a particularly interesting discovery. GPI modification would potentially place *AtNDR1* on the outer surface of the PM, perhaps allowing *AtNDR1* to act as an intercellular transducer of pathogen signals. *AtNDR1* may also function at the site of pathogen-plant cell contact, perhaps through direct interaction with the pathogen (Coppinger et al., 2004).

Systemic acquired resistance (SAR) is a pathogen-

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induced disease resistance response in plants that is characterized by broad spectrum disease control and an associated coordinate expression of a set of SAR genes (Delaney et al., 1994; Ross 1961). Higher plants can be induced to become resistant against a variety of pathogens either by infection with a pathogen that forms necrotic lesions or by treatment with resistance-inducing compounds such as salicylic acid (SA) or 2,6-dichloroisonicotinic acid (Kuc, 1982; Métraux et al., 1991; Ross, 1961). Benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) is a novel synthetic chemical capable of inducing disease resistance in a number of dicotyledenous and monocotyledonous plant species (Friedrich et al., 1996). BTH does not cause an accumulation of salicylic acid (SA), an intermediate in the SAR signal transduction pathway in tobacco (Friedrich et al., 1996). As BTH also induces disease resistance and gene expression in transgenic plants expressing the *nahG* gene, it appears to activate the SAR signal transduction pathway at the site of or downstream of SA accumulation. BTH and SA induce the tobacco *PR-1a* promoter using similar *cis*-acting elements and gene expression is blocked by cycloheximide treatment. Thus, BTH induces SAR based on all of the physiological and biochemical criteria that define SAR in tobacco (Friedrich et al., 1996).

OsNDR1 encodes a protein of unknown function. Here we report that *OsNDR1* is plasma membrain (PM)-localize protein that would potentially place *OsNDR*1 on the outer surface of the PM, perhaps allowing *OsNDR1* to act as a transducer of pathogen signals and/or interact directly with the pathogen, and demonstrated that 2Kb of 5'flanking DNA was sufficient to impart BTH inducibility to a reporter gene.

Materials and Methods

Plant materials. Rice (*Oryza sativa* L. japonica-type cv. Nipponbare) was grown under white fluorescent light (wavelength 390-500 nm, 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 12 h photoperiod) at 25°C and 70% relative humidity as described previously (Agrawal et al., 2001, 2002a, 2002b).

Generation of Transgenic Plants. Transgenic Arabidopsis plants were generated with the *OsNDR* promoter::*GUS* in pCAMLA vector using the floral dip method (Clough and Bent, 1998).

cDNA library construction and screening. The poly $(A)^+$ RNA (5 μg) was prepared from total RNA isolated from rice seedling leaves after treatment with 1×10^5 spore suspension of *Magnaporthe grisea* for 30 min using a kit (Stratagene, USA), which served as the template for cDNA synthesis and construction of the Uni-ZAP XR vector. (Stratagene, USA). The amplified library contained 4×10^9 pfu/ml. The *OsNDR1* cDNA clone was screened and cloned by plaque hybridization method using PCR product of a part of the *OsNDR1* amplified by primer set. (*NDR*-S: 5'-TCGACTTGTTAATGTACTCTTGCACTG-ATC-3', *NDR*-AS: 5'-CGGCAGCCGG TACGGCGGC-ATTATTG-3'). ORF (Open reading frame) region. Hybridization was performed as described (Agrawal et al., 2001, 2002a, 2002b). Autoradiography was carried out overnight with X-ray films (Kodak, Japan) using two intensifying screens at –80°C. Positive λ phage clones were selected and phagemids (pBluescript SK-) were rescued through in vivo excision protocol (Stratagene, USA).

Chemicals. Salicilic acid (SA) and ethephone (ETP) were purchased from Sigma (St. Louis, USA). Probenazole (PBZ) were obtained from Wako Pure Chemical Industries, Ltd. and Benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) got from Hayashi Pure Cemical Ind., Co., Ltd. All other chemicals used in this study were of analytical grade. Stock solutions were prepared as reported previously (Agrawal et al., 2001, 2002a, 2002b).

DNA sequencing and analysis. Both strands of the recombinant phagemids of positive clones were sequenced using a dye-terminator cycle sequencing kit, and an automated capillary DNA sequencer (Genetic Analyzer ABI 310, PE Applied Biosystems). All sequencing data were analyzed using GenetyX software (SDC Software Development, Japan). Homology of nucleotide and amino acid sequence was analyzed using GenetyX and CLUSTAL-W programs against sequences in the GenBank and EMBL DNA database. The phylogenetic tree was constructed by the Neighbour-joining method using the GenetyX program (Software Development Co., Japan).

Reverse Transcription (RT)–PCR. Transcript levels were measured by RT-PCR. Total RNA was isolated from whole plants or plant materials using the Trizol reagent (Invitrogen, USA). Total RNA samples were treated extensively with RNase-free DNase I to remove any contaminating genomic DNA. The first-strand cDNA was synthesized using *Pfu* Turbo polymerase (Stratagene, USA) from 1 μg of total RNA in a 20-μL reaction volume, and 1 μL of the reaction mixture was subject to subsequent PCR in a 50-μL reaction volume. The RT-PCR runs were 15 to 30 cycles, depending on the linear range of PCR amplification for each gene, with each cycle at 94°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1 min, with a final cycle at 72°C for 5 min to allow the completion of the polymerizations. The primers were as follows: 5'-

ATGTCGGTGAT CAAGGATTGT-3' (*OsNDR1*-F) and 5'-GACATCGACGGCGCATGCAGC-3' (*OsNDR1*-R) for OsNDR1; 5'-TCCATCTTGGCATCTCTCAG-3' (*Actin*-F) and 5'-GTACCCGCATCAGGCATCTG-3' (*Actin*-R) for *Actin* as a control.

Generation of transgenic rice with *OsNDR1*. Cultivar of Japonica rice, Nipponbare was used in transformation. The 1.1 Kb OsNDR1 cDNA was delivered to rice callus under the control of CaMV 35S promoter. Agrobacterium tumefaciens strain LBA4404 (Hoekema et al., 1983) was used for transformation. All the transformation procedure of *OsNDR1* was carried out by (Hiei et al., 1994) doing some modification. Transformed calli were selected on the gradually increased level of hygromycin (30 mg and 60 mg hygromycin/l) media during in vitro culture. After shooting and rooting from the transformed and selected callus, seedlings were acclimated for 3-4 days in water and they were grown in soil in a greenhouse. Seeds of T1 lines were analyzed for hygromycin resistance and hygromycin-resistant seedlings were used for functional analysis and T2 homozygote selections.

Localization and visualization analysis. GFP fusion protein was constructed with a full length *OsNDR1* clone was with the GFP N-terminus in a CaMV35S promoter. For transient localization assay, the *OsNDR1::GFP* construct DNA was purified as plasmids using Qiagen plasmid miniprep kit (Qiagen, USA). Plasmid DNA was mixed with tungsten particles and particles coated with *OsNDR1::GFP* construct plasmids were transformed into onion cells by particle bambardment (Bilang and Bogorad 1996). Leaf samples were placed on MS media in the petri plate. The epidermal cells expressing *GFP* images were identified using a Olympus microscope with epifluorescence using *GFP*-optimized ND filter sets (Olympus, Japan). Digital images were collected using Olympus IX70 fluorescence microscope (Olympus, Japan) with I.CAMSCOPE digital camera (Sometech, Korea) and software (MicroFire, USA). Images were further processed with Photoshop 5.0 software (Adobe).

GUS staining. Seeds of *Arabidopsis thaliana* (L.) Heynh., Col-2, were sterilized and germinated under the same conditions as previously reported (Dubrovsky et al. 2000). To detect GUS activity the material was stained as described by Hemerly et al. (1993), and cleared by using the method of Malamy and Benfey (1997).

Results

Identification and characterization of the *OsNDR1* gene. The *OsNDR1* genes were isolated using gene specific PCR primers that were derived from conserved sequences of previously cloned *AtNDR1* gene from Arabidopsis. Polymerase chain reaction (PCR) amplification was performed on genomic DNA from rice. DNA fragments of the expected sizes were recovered from an agarose gel and TA-cloned into the plasmid vector. To isolate *OsNDR1* gene from rice, a cDNA library was constructed from poly(A)$^+$ RNA from pathogen induced seedling rice leaf that were cultured under 1×10^5 spore suspension of *M. grisea*, and screened using the PCR fragments as probes. DNA sequencing of the obtained full-length cDNA clones revealed that the *OsNDR1* cDNA was 660bp in length and had an open-reading frame (ORF) that putatively encoded a protein of 220 amino acids (GenBank accession no. AK103610) (Fig. 1). Among the *NDR* proteins in the NCBI database, *OsNDR1* showed highest similarity to rice putative *NDR* (62.07%/Gene accession number XP_507419) and Arabidopsis *AtNDR1* (41.44%/Gene accession number AAO41900), respectively.

Comparisons of the amino acid sequences of *OsNDR1* with that of Arabidopsis *AtNDR* revealed that the amino acid residues were conserved throughout the entire region of the protein (Fig. 2).

Prediction of the hydrophobicity of the deduced amino acid sequences indicated that OsNDR1 proteins most likely contain 1 specific transmembrane-spanning domains (Fig. 3), similar to the other *NDR* polypeptides from Arabidopsis. We used the TMHMM program (Sonnhammer et al., 1998) to

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        10        20        30        40        50        60        70        80
atgtcggtgatcaaggattgtgggggcacaaggggtgcgagtgcgagaaggagaaaggtgcaccggcggctgtgctgggc
M  S  V  I  K  D  C  G  G  H  K  G  C  E  C  E  K  E  K  V  H  R  R  L  C  W  A

        90       100       110       120       130       140       150       160
gctggtggcgctggtgctcctgacgctcttcatcgtgctggtggtgtggctggcgctgcggccgcacaagccgcggttct
 L  V  A  L  V  L  L  T  L  F  I  V  L  V  V  W  L  A  L  R  P  H  K  P  R  F  Y

       170       180       190       200       210       220       230       240
acctgcaggacctgtccgtgctgtgcctcaacgtgacgccgccggcgtccgcctacctcttcaccaccatgcaggcgacg
  L  Q  D  L  S  V  L  C  L  N  V  T  P  P  A  S  A  Y  L  F  T  T  M  Q  A  T

       250       260       270       280       290       300       310       320
gtggcggcgcgcaacgacaacggccgcgtgggcgtctactacgacaaggtggacgtgtacgcgcagtacaaggacgtggc
V  A  A  R  N  D  N  G  R  V  G  V  Y  Y  D  K  V  D  V  Y  A  Q  Y  K  D  V  A

       330       340       350       360       370       380       390       400
catcacggtgccgacgaggctcccccgtggagtaccagggccactacgaccagtccgtctggtccccccttcctccaatcgc
 T  T  V  P  T  R  L  P  V  E  Y  Q  G  H  Y  D  Q  S  V  W  S  P  F  L  Q  S  L

       410       420       430       440       450       460       470       480
tcgaccacgtcgtcctcccccccaacctcgccgtcgcccctcgcccaggacgagaccgccggctacgtcctcgtcgacatc
  D  H  V  V  L  P  P  N  L  A  V  A  L  A  Q  D  E  T  A  G  Y  V  L  V  D  I

       490       500       510       520       530       540       550       560
cgcctcgacggctgggtccgatggaaggtcggcacctggatctccggccactaccacctccgcgtcaactgccccgccct
R  L  D  G  W  V  R  W  K  V  G  T  W  I  S  G  H  Y  H  L  R  V  N  C  P  A  L

       570       580       590       600       610       620       630       640
cctcaccgtcaacgacggcaagggcagctacggcgtcaactacggcggcggcgatggctacttccgcttccagcaagcag
 L  T  V  N  D  G  K  G  S  Y  G  V  N  Y  G  G  G  D  G  Y  F  R  F  Q  Q  A  A

       650       660       670
ctgcatgcgccgtcgatgtctag
  A  C  A  V  D  V  *
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Fig. 1. Analysis of rice cDNA Sequence Encoding *OsNDR1* Protein. Nucleotide and deduced amino acid sequences of the *OsNDR1* gene. Horizontal arrows indicate the position and orientations of primers, *OsNDR1*-F and *OsNDR1*-R, used for RT-PCR amplification.

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NDR1 protein            1: MSVIKDCGGHKGCECEKEKVHR-RLCWALVALVLLTLF--IVLVVWLALR-PHKPRFYLQDLSVLCLNVTPPAS------ 70
arabidopsis-AAO41900    1: M--KD-CENHGH-SRRKLIRR---IFWSIIFVLFIIFL--TILLIWAILQ-PSKPRFILQDATVYAFNVSGNPPNILTS- 69
arabidopsis-BAB08955    1: MTSKD-CGSHDSHSSCN--RKI-VI-WTISIILLLILV--VILLVWAILQ-PSKPRFVLQDATVFNFNVSGNPPNLLTS- 71
rice- BAD28833          1: MSKE--KH-HKREH-H--LRRCCGG-MAACILALVLVVGFIAL-VVYLALRPSKPSFYLQDLQLRSVDLGDPSLSATA-- 70
rice-CAE01528           1: MGKD--KH-HR-DW-I--LRRCCGS-IAACILTLAVLVGFIVL-VIYLAIHPSKPSFYLQDVQLRNIDLSDPAISLNL-- 69
rice-XP_507419          1: MSIK-HCEQHKDC--ERQRLYR-RCCAAIFGILLLLLL--IVLIVWLILR-PTKPRFYLNDLTVVCLNVTTGGSYAGATA 73

NDR1 protein           71: --AYL-FTT--MQATVAARNDNGRVGVYYDKVDVYAQYKDVAITVPTRLPVEYQGHYDQSVWSPFLQSLDHVVLPPNLAV 145
arabidopsis-AAO41900   70: -N----F-----QITLSSRNPNNKIGIYYDRLDVYATYRSQQITFPTSIPPTYQGHKDVDIWSPFVYGTSVPIAPFNGVS 139
arabidopsis-BAB08955   72: -N----F-----QFTLSSRNPNDKIGIYYDRLDVYASYRSQQITLPSPMLTTYQGHKEVNVWSPFVGGYSVPVAPYNAFY 141
rice- BAD28833         71: ----------QVTLASRNPNDHVGVHYRRLDVFVTYRDEPVTVPVSLPPTYQGHRDVTIWSPVLSGESVPVAGFVADA 138
rice-CAE01528          70: ----------QVTIASRNPNDRVGVYYKTLHVFTTYREEPITVPVELPAIYQGHKDVSVWSPVMSGESVPVGQYVADA 137
rice-XP_507419         74: SSGYFSFLTVTMQTTLAARNGNERVGIYYDRADVYAEYKGLRITVPTSLPPVYQGHPDLTVWSPFLSGNN-VQLPPYLAV 152

NDR1 protein          146: ALAQDETAGYV-LVD-IRLDGWVRWKVGTWISGHYHLRVNCPALLTVND----GKGSYGVN-----YGGGDGYFRFQQAA 214
arabidopsis-AAO41900  140: LDTDKDN-GVVLLII-RA-DGRVRWKVGTFITGKYHLHVKCPAYINF------GNKANGVI-----VGDNAVK--YTFTT 203
arabidopsis-BAB08955  142: LDQDHSS-GAIMLML-HL-DGRVRWKVGSFITGKYHLHVRCHALINF------GSSAAGVI-----VG----K--YMLTE 201
rice- BAD28833         139: LRQDVAA-GYVALQVKV-D-GRVKWKVGSWVSGSYHLFVSCPAMLASAGPGGVG-PMPLGGASAAVVNGTGAGA-VASLR 213
rice-CAE01528          138: MRQDIAA-GYVLLHVKV-D-GRVKWKVGSWVSGGYHLFVTCPALLAASGGN-VGGAFAMSATAGG---GAGGNATV-SLK 209
rice-XP_507419         153: SITQDETAGYL-LVT-IRVDGWIRYKAGAFITGHYHLRVRCPALLIVND----GRGSYGSN-----SGGGNGYFRFQRAA 221

NDR1 protein          215: --A---CAVDV                                                                  220
arabidopsis-AAO41900  204: --S---CSVSV                                                                  209
arabidopsis-BAB08955  202: --T---CSVSV                                                                  207
rice- BAD28833        214: FTQPTGCSVEV                                                                  224
rice-CAE01528         210: FAQAADCTVDV                                                                  220
rice-XP_507419        222: --A---CVVDV                                                                  227
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Fig. 2. Alignment of amino acid sequence of *OsNDR1* and related protein from rice and dicot species. The predicted *OsNDR1* seuences is compared with the primary structure of the Arabidopsis (Gene accession number AAO41900 and BAB08955) and rice (Gene accession number BAD28833, CAE01528 and XP_507419). Conserved residues among the rice and Arabidopsis are black boxed.

estimate the length of the transmembrane domain and accordingly named the constructs with the corresponding length in amino acids for the transmembrane domain. *OsNDR*1 clearly harbors 23 hydrophobic amino acids, all of them having the highest chance to be integral in a membrane. We chose this sequence that reason: the limits between the membrane part and the flanking regions are clear. As shown in Fig. 3-A, the amino acids chosen for deletions is fully inside the expected transmembrane domain and therefore should affect only its length.

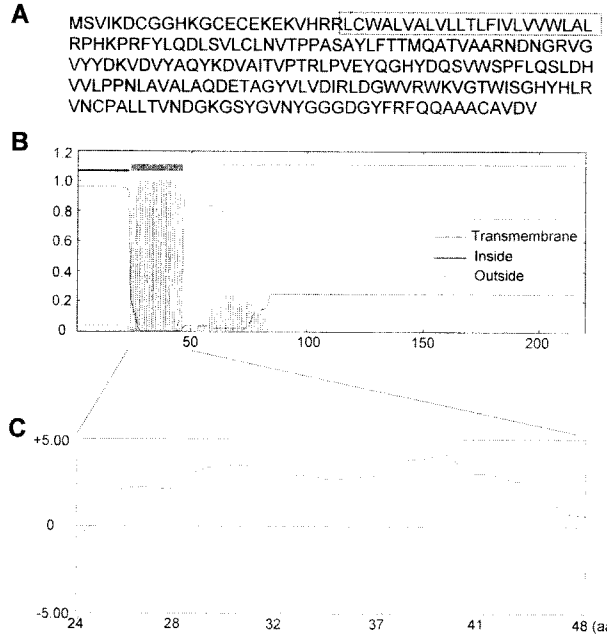

Fig. 3. Structure of *OsNDR1*. A) Hydropathy profile of the OsNDR1 protein was calculated by the algorithm of Sonnhammer et al. (1998) and Krogh et al. (2001) (http://www.cbs.dtu.dk/servieces/TMHMM/). B) Hydropathy plots of OsNDR1 using the program of Kyte and Doolittle (1982). The hydropathy index of each residue is represented on the y axes. The position of the transmembrane domain estimated by the TMHMM program is underlined. C) Amino acid sequence of *OsNDR1*. The putative transmembrane region of OsNDR1 is square box.

We examined to search additional homologs to *OsNDR1* in the rice cDNA database of DDBJ (http://www.ddbj.nig.ac.jp/E-mail/homology-j.html) and the rice genome database of Tiger rice genome project (http://www.tigr.org/tdb/e2k1/osa1/) and NCBI BLAST search (http://www.ncbi.nlm.nih.gov/blast/). Interestingly, Additional three *OsNDR1* homologs were existed. A phylogenetic tree was constructed, based on the homologous full-length sequences, which revealed a closeness of *OsNDR1* with the other rice putative *NDR* (Fig. 4). This result clearly indicated that monocot *OsNDR1* protein is grouped separately from dicot *NDR*.

Determination of the transcript level by RT-PCR. RT-PCR analysis of the spatial expression of *OsNDR1* indicated that its transcripts were present in all organs tested (Fig. 5). The expression level was higher in young shoot (10d ays after germination) and leaf sheaths than in mature leaf and roots (young root – 10 days after germination and mature root). *OsNDR1* transcript was strongly present from panicle before heading, and steady expression of the gene was observed to stage of maturity of pollen (Fig. 5).

***GFP::OsNDR1* fusions localize to the Plasma membrane.** To test whether *OsNDR1* could direct membrane targeting, we established a method for assaying protein localization using transient expression of green fluorescent protein (*GFP*) fusion in onion epidermal cells. We cloned full length *OsNDR1* cDNAs in frame with the *GFP* C-terminus in a CaMV35S promoter construct. For our localization assay, cells in onion epidermis cell were transiently transformed by particle bombardment (Bilang

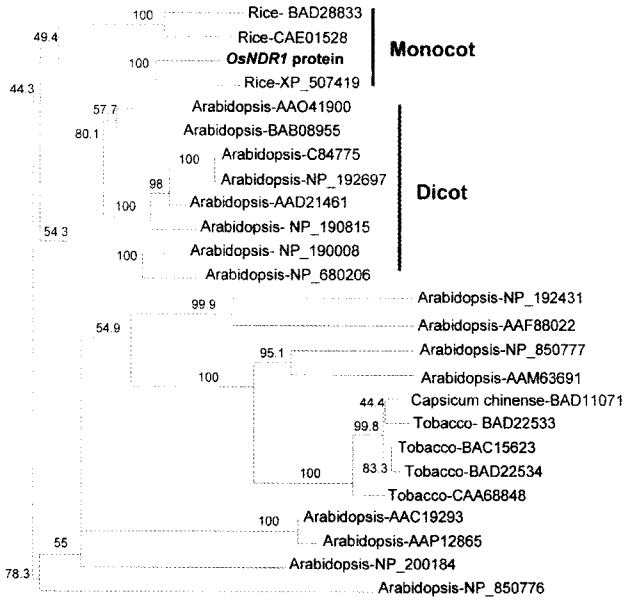

Fig. 4. Phylogenetic tree of *NDR* protein from rice and different dicot species. The Neighbour-joining method was used to generate a dendrogram of *NDR* proteins. Bootstrap values expressed as percentage (over 1000 replicates) are shown at the corresponding nodes. Sources of the genes are indicated in parentheses follows.

Fig. 5. Developmental stage and tissue specific expression pattern of *OsNDR1* in the rice organs. *OsNDR1* was expressed at all the rice organs. *OsNDR1* accumulation in the shoots or panicles is higher than that of the roots or leaves. Young shoot (1), root (2); 14-day-old seedling, Mature leaf (3) and leaf sheath (4); 50-day-old after seedling, Mature leaf (5), leaf sheath (6); 70-day-old after seedling, Mature root (7), panicle before heading (8), after heading (9), and at maturity of pollen (10); 90-day-old after seedling.

and Bogorad 1996), enabling us to observe tens to hundreds of transformed cells per explants (Fig. 6).

We found that the entire periphery of *GFP::OsNDR1* transformed cells fluoresced distinctively, whereas the *GFP*-only control cells fluoresced primarily in the cytoplasm and nucleus (Fig. 6). Microscopy imaging under UV light confirmed that the *GFP::OsNDR1* fluorescence was almost exclusively at the cell periphery, and thus the fusion was associated with either cell wall or plasma membrane. These observations, together with the prediction analysis of the hydrophobicity, indicate that *OsNDR1* may have role of transmembrane protein in rice.

Expression of *OsNDR1* in rice Up-regulates *PBZ1* (*PR10*). To determine whether *OsNDR1* play a role in

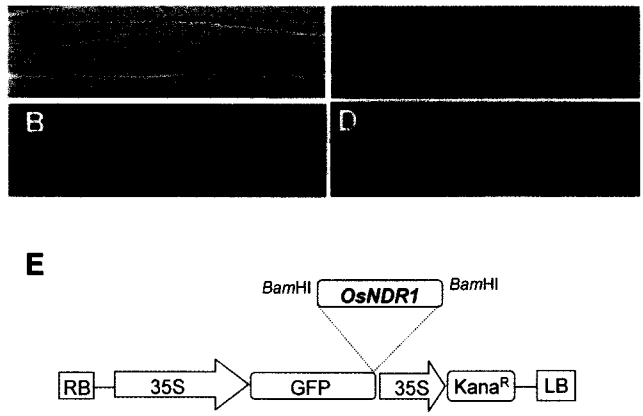

Fig. 6. Plasma membrane Localization of the *GFP::OsNDR1* protein. (A) and (B) The control GFP protein. (C) and (D) The *GFP::OsNDR1* fusion protein. The proteins are transiently expressed in onion epidermal cells. Individual cells are seen in a differential interference contrast image ([A] and [C]) and a corresponding epifluorescence image ([B] and [D]), respectively. E) Structure of *GFP::OsNDR1*.

activating *PBZ1* (*PR10*) genes in rice, the expression of different classes of *PBZ1* (*PR10*) genes was examined in transgenic plants having CaMV*35S::OsNDR1*. Up-regulation of *PBZ1* (*PR10*) is essential for the activation of defense/stress-related gene (Jwa et al., 2001). As shown in Fig. 7, expression of *OsNDR1* in rice caused an increase in the steady state abundance of *PBZ1* (*PR10*) transcripts. This finding is suggested *OsNDR1* may have a definite involvement in defense response in rice and that the transcriptional activation of the *OsNDR1* gene may involve upstream of *PBZ1* (*PR10*). Therefore, cloning of *OsNDR1* is indeed another important step in clarifying the components participating in the defense response pathway in rice. Finally, the genetic manipulation of this gene may help, not only in understanding the function(s) of this gene, but also a

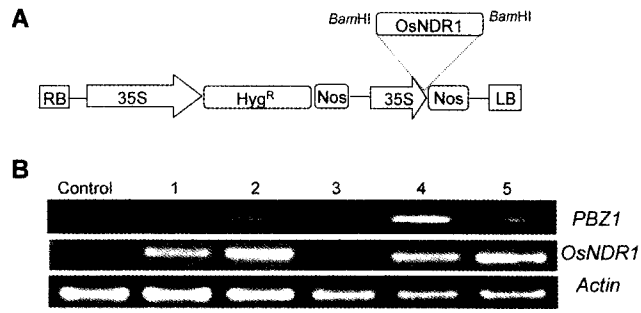

Fig. 7. Over-expression of *OsNDR1* in rice causes constitutive up-regulation of *PBZ1* (*PR10*) Genes. A. Structure of CaMV35S promoter::*OsNDR1*. Five individual transgenic lines (homozygous, from the T3 generation) were chosen for analysis for each construct expressing *OsNDR1*. RT-PCR control showing that actin transcripts are detected consistently in all of the samples.

step toward unraveling the complexity in the signal pathway(s) that mediate the defense response(s).

Expression of *OsNDR1* promoter in Arabidopsis. We also analyzed the regulation of the *OsNDR1* promoter in transgenic Arabidopsis. The main promoter::reporter construct used in this work was ~2 Kb promoter portion fused to *β-glucuronidase* (*GUS*) to create *OsNDR1::GUS*. The *OsNDR1::GUS* construct was introduced into Arabidopsis. Approximately 10 independent transformants were obtained by selection for hygromycin resistance. The organ specificity of *OsNDR1::GUS* expression was determined by in situ histochemical staining for *GUS* activity.

In leaf primordia, cotyledon, *OsNDR1::GUS* is weakly expressed and *GUS* expression is somewhat stronger in vascular bundles (Fig. 8). In hypocotyls and stems, *OsNDR1::GUS* expression is primarily restricted to vascular bundles, little expression is found in parenchyma and epidermal tissues (Fig. 8). In the primary root of 10-d plants, *OsNDR1::GUS* expression was detected in the elongation zone within the central cylinder including pericycle (Fig. 8). It was maintained in vascular cylinder up to young differentiation zone and then the expression was associated preferentially with protophloem cells. Our detailed analysis of *OsNDR1::GUS* line demonstrated the same expression pattern in mature differentiation zone (Fig. 8). During lateral root formation, *OsNDR1::GUS* expression was not detected in primordial and started to weakly appear in lateral root only after emergence, also in its central cylinder (Fig. 8). The overall expression pattern in the lateral root was similar to that in the primary root. In young lateral root expression was maintained in the vascular cylinder (Fig. 8).

The expression of *OsNDR1::GUS* in transgenic Arabidopsis is consistent with the results of RNA gel blot analysis in rice (Fig. 8) and indicates that the ~2 kb of 5' flanking region of *OsNDR1* confers expression pattern upon a distantly related dicot plant, Arabidopsis.

Effects of Benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) on GUS Expression. To determine the amount of 5' flanking sequence from the *OsNDR1* gene sufficient to confer SA, PBZ, BTH, ETP inducibility, promoter region (~2 Kb) was constructed and fused to the GUS reporter gene. Expression of the GUS reporter gene following BTH treatment was assayed in 11-20 independent transformants. The results shown in Fig. 9 indicate that GUS activity was maximally induced by BTH in plants. The tissue specificity of GUS induction following BTH treatment of a number of independent lines was also analyzed.

The promoter activity was increased in the elongation zone, in the central cylinder, cotyledon and leaf primordia when plants were treated with BTH (Fig. 8), indicating importance of BTH signal in the *OsNDR1* expression. Interestingly, Compared to untreated plants *OsNDR1::GUS* expression in other-treated seedlings was the same (Fig. 8). This data confirmed interaction of *OsNDR1* with BTH signaling. Therefore, BTH has the same effect on *OsNDR1* gene expression BTH based on both the promoter elements involved and the tissue specificity of *OsNDR1* expression.

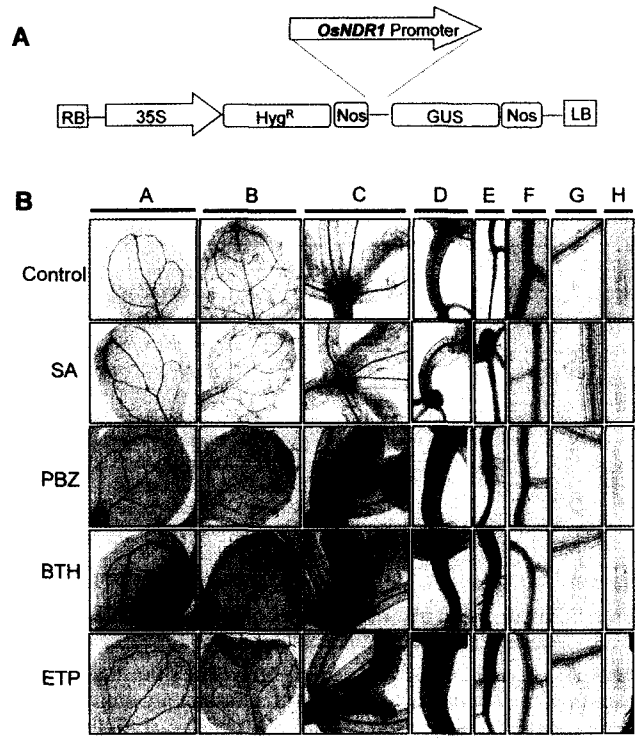

Fig. 8. *OsNDR1* regulation pattern using *GUS* protein and change of the *GUS* accumulation under various chemical treatments. *GUS* protein was expressed in the stem more than other organs. BTH significantly induces expression of the *GUS* protein. A. Structure of *OsNDR1::GUS*. SA; Salicilic acid, PBZ; Probenazole, BTH; Benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, ETP; Ethephone, (A) Cotyledon, (B) First leaf, (C) Shoot-hypocotyl junction, (D) Hypocotyl, (E) and (F) Primary root, (G) Lateral root, (H) Primary root tip. Seedling were grown for 7 days in hormone-free medium and then transferred to agar plates containing various concentrations of SA (25 μM), PBZ (125 μM), BTH (200 μM) and ETP (1 mM) for 24 hours. Plants were grown on 1% sucrose and 0.2x MS medium during 8 days under long day condition (16 h light/8 h dark).

Dicussion

In our research for novel gene up-regulated in rice (*Oryza sativa* L. cv. Nipponbare) and responding to *Magnaporthe grisea* treatment, a cDNA clone coding for a transcript with a predicted gene product showing high similarity to the

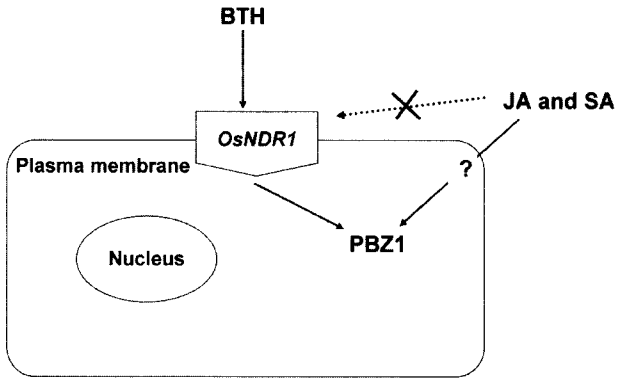

Fig. 9. Integration of BTH signals pathway is mediated by *OsNDR1*. *OsNDR1* acts as downstream of BTH and upstream of *PBZ1*. Arrows represent promotion and effects.

Arabidopsis *AtNDR* gene was isolated and designated as *OsNDR1*.

Identification of this new *NDR* gene in rice confirmed our initial assertion that this gene is indeed a rich source of transcripts associated with the defense response. Identification of *OsNDR1* is an important step toward first isolating rice *NDR* gene, and second, their detail characterization to understand their precise function/role in rice. Although many *NDR* genes have been isolated from Arabidopsis, rice and tobacco, the precise molecular function of *NDR* remains unclear. A direct comparison with the expression profiles of the available homologous *OsNDR1* genes is not possible due to the lack of detailed studies on the effect of these signaling molecules in other plants. A study on the cloning of Arabidopsis *NDR* gene only reported avirulent pathogens induces an increase in its mRNA level (Century et al., 1997).

The *OsNDR1*, which has transmembrane domain as detected by a homology search and similarity with other *NDR* proteins, may have related genes in the rice genome. Base on our results, it is believed that there are other putative *NDR* genes still undiscovered in rice, and which is the focus of our continuing work of *NDR* genes in rice.

Phylogenetic tree analysis revealed that the *OsNDR1* protein involved in putative rice *NDR* subfamily group, and was separated from Arabidopsis and other dicot *NDR* proteins. The observed differences in the sequences of *OsNDR1* and that of the dicot *NDR* may reflect the differences between monocots and dicots.

We have shown that *OsNDR1* is a PM (Plasma membrane)-localized protein. Localization of *OsNDR1* at the PM is a particularly interesting discovery. *OsNDR1* may function at the site of microbe-plant cell-to-cell contact, perhaps through direct interaction with the pathogen. Many of the major components in Arabidopsis disease resistance signaling, including *RIN4*, *RPM1*, *RPS2*, and the Pst effector molecules *AvrRpt2*, *AvrB*, and *AvrRpm1*, are also localized at the PM (Coppinger et al., 2004). This result has led us to speculate that *OsNDR1* may exist in a protein complex with other R proteins at the PM. To this end, we will investigate whether OsNDR1 interacts with *RPS2*, *RIN4*, and/or *RPM1*, and *OsNDR1* topology in PM in order to reveal further clues as to the molecular function of *OsNDR1* in disease resistance.

JA and SA are important inducers of defense-related genes in plants (Agrawal et al., 2001, 2002a, 2002b; Jwa et al., 2001). Several *PR* proteins, such as *PR1*, *PR2*, and *PBZ1* (*PR10*) are expressed in an SA- and/or JA-dependent manner (Agrawal et al., 2001; Agrawal et al., 2002a; Agrawal et al., 2002b). In this study, The *OsNDR1* promoter did not respond significantly to treatments with either SA or JA. BTH was identified in a biological screen as a compound that caused disease resistance in tobacco and cucumber (Friedirich et al., 1996). It is unclear how to determine whether it is capable of inducing a systemic signal. However, BTH does not induce SA accumulation and BTH could induce the responses following an independent signaling pathway (Friedirich et al., 1996). These results indicate *OsNDR1* has a different signaling pathway compared to *PBZ1* (*PR10*). These differences in expression are most probably due to the fundamental differences on defense mechanism. These data strongly suggests that *OsNDR1* may be playing unknown important role(s) in regulating the expression of *PBZ1* (*PR10*). Transgenic rice plants carrying the *CaMV35S::OsNDR1* fusion gene showed also over-expression of *PBZ1* (*PR10*). This result suggests that the *OsNDR1* protein might have a role, as a positive regulator of pathogen signal pathways. Further analyses of *OsNDR1* over-expressed rice will help to elucidate the role of OsNDR1 or its gene product in PR gene regulation and in response to pathogen.

Exogenously applied BTH induces the same set of System acquired resistance (SAR) genes as biological induction, providing further evidence for BTH as a signal (Friedrich et al., 1996). However, little is known about the mechanism of SAR gene induction. Presumably, BTH is bound by a receptor and the binding triggers a signal transduction cascade that has an ultimate effect on transcription factors that regulate SAR gene expression. However, the extent of 5' flanking DNA sufficient for pathogen and BTH induction or regulation by wounding is still unclear. To address the questions raised by these studies, we have performed deletion analysis on the *OsNDR1* promoter linked to a reporter gene encoding β-glucuronidase (*GUS*). Analysis of the 2 kb sequence upstream of the *OsNDR1* revealed two W-box elements in the promoter region of *OsNDR1* (TTGAC at −710 bp and AGTCA at −701 bp). W boxes are *cis*-acting elements often found in

promoters of many SA- and pathogen-responsive genes, such as *NPR1* and *PR1* (Lebel et al., 1998; Yu et al., 2001). They are binding sites for the WRKY family of transcription factors for the transcriptional regulation of defense-related genes (Eulgem et al., 2000; Rushton and Somssich 1998). However, this promoter is not activated by SA. Therefore, we anticipated that W-box of *OsNDR1* may have the different function or SA independent signaling pathway. A future challenge is to understand the basis of this specificity.

Based on data presented in this study, it is believed that the *OsNDR1* protein may have a definite involvement in defense response in rice, and that transcriptional activation of the *OsNDR1* gene may involve downstream of BTH and upstream of *PBZ1* (*PR10*) (Fig. 9). Therefore, cloning of *OsNDR1* is indeed another important step in clarifying the components participating in the defense response pathways in rice. Finally the genetic manipulation of this gene may help, not only in understanding the exact functions of this gene, but also a step toward unraveling the complexity in the signaling pathway that mediate the defense response.

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