RESEARCH PAPER

Expression of a RING-HC protein from rice improves resistance to *Pseudomonas syringae* pv. *tomato* DC3000 in transgenic *Arabidopsis thaliana*

Ming-Yan Cheung¹, Nai-Yan Zeng¹, Suk-Wah Tong¹, Francisca Wing-Yen Li¹, Kai-Jun Zhao², Qi Zhang², Samuel Sai-Ming Sun¹,³ and Hon-Ming Lam¹,³,*

¹ Department of Biology, The Chinese University of Hong Kong, Shatin, Hong Kong SAR
² Institute of Crop Sciences, The Chinese Academy of Agricultural Sciences, Beijing, PRC
³ Molecular Biotechnology Program, The Chinese University of Hong Kong, Shatin, Hong Kong SAR

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Abstract

A cDNA clone (*OsRHC1*) was obtained, which encodes a novel RING zinc finger protein sharing similar structural features (multiple transmembrane domains at the N-half; a unique RING zinc finger consensus Cys-X₂-Cys-X₁₁-Cys-X-His-X₂-Cys-X₂-Cys-X₂-Cys at the C terminus) to a group of closely related annotated proteins from both monocots and dicots. *OsRHC1* was found to be localized on plasma membrane of rice cells and induced by wounding in rice lines containing *Xa* loci. Ecotopic expression of the *OsRHC1* cDNA from rice (a monocot) in transgenic *Arabidopsis thaliana* (a dicot) enhanced the defence response toward *Pseudomonas syringae* pv. *tomato* DC3000, suggesting that *OsRHC1* may confer broad-spectrum disease resistance. The protective effects were neutralized in the presence of MG132 or in an *npr1-3* mutation background, indicating that the function of *OsRHC1* is dependent on the ubiquitin-mediated protein degradation via the 26S proteasome and the presence of the key defence response regulator NPR1.

Key words: E3 ubiquitin ligase, plant disease resistance, RING zinc finger protein, signal transduction.

Introduction

Preformed and induced defence mechanisms provide a wide spectrum of resistance toward numerous pathogens encountered by the plant host (Seo *et al.*, 1999; van Wees *et al.*, 1999, 2000; Cheong *et al.*, 2002; Collins *et al.*, 2003; Holub and Cooper, 2004; Assmann, 2005). Pathogen-specific defence responses are usually initiated by the recognition of a pathogen avirulent (Avr) protein by the corresponding resistance (R) protein of the host. The specific Avr-R interaction formulated the gene-for-gene hypothesis (Flor, 1956). Ultimately, the plant host will produce a series of defence molecules (including pathogenesis-related proteins) to restrict or kill the pathogens (Veronese *et al.*, 2003). The processes between the two ends involve a complex signal transduction network (van Wees *et al.*, 2000; Anderson *et al.*, 2004; Apel and Hirt, 2004; Durrant and Dong, 2004; Li *et al.*, 2006) which is yet to be fully elucidated.

In *Arabidopsis thaliana*, many important hubs of the defence signalling network have been identified by molecular genetic approaches, including EDS1, NPR1, and NDR1 (Bonas and van den Ackerveken, 1999; Chern *et al.*, 2001; Glazebrook, 2001; Després *et al.*, 2003). Using similar tactics, together with biochemical studies, the involvement of phytohormone signals in defence responses has been corroborated in *A. thaliana*, especially the roles of salicylic acid (SA) (Thomma *et al.*, 1998, 2001). The importance of other phytohormones such as jasmonic acid (JA) and ethylene (ET) has gradually been revealed (Thomma *et al.*, 1998, 2001; van Wees *et al.*, 2000).

Many known signalling strategies are employed in plant defence responses. For instance, some R proteins are receptor kinases (Song *et al.*, 1995; Sun *et al.*, 2004) while other protein kinases also play significant roles (Romeis *et al.*, 2000). Biochemical signals such as
calcium flux (Levine et al., 1996) and oxidative burst (Baker and Orlandi, 1995; Levine et al., 1996) are important. Furthermore, there are several reports on the participation of other signalling components such as G-proteins (Legendre et al., 1992; Assmann, 2005) and RING (Really Interesting New Gene) zinc finger proteins (Takai et al., 2002; Wang et al., 2006).

RING zinc finger proteins are a group of diverse proteins with highly conserved zinc binding domains (Jouzeiro and Weissman, 2000). Based on the type of cysteine and histidine residue combination, the RING zinc finger domain can be classified into canonical and modified RING zinc fingers. The canonical RING zinc finger can be further grouped into two subclasses: HC subclass (consensus: C-X2-C-X9-39-C-X1-3-H-X2-3-C-X2-C-X4-48-C-X2-C) and H2 subclass (consensus: C-X2-C-X9-39-C-X1-3-H-X2-3-C-X2-C-X4-48-C-X2-C) (Stone et al., 2005). Modified RING zinc fingers include RING-C2, RING-v, RING-D, RING-S/T, and RING-G (Stone et al., 2005).

Many members of the RING zinc finger protein family (including both HC and H2 subclasses) are E3 ubiquitin ligases (Stone et al., 2005). Different subclasses of the RING zinc finger domain determine specificity toward different E2 ubiquitin conjugating enzymes (Huibregtse et al., 1995). Other RING zinc finger proteins can bind to nucleic acids or interact with other protein targets (Stone et al., 2005). Besides the ubiquitin-mediated degradation pathway, RING zinc finger proteins also play important roles in organelle transport and transcription/translation regulations (Stone et al., 2005).

In this work, the cloning and characterization of a novel RING zinc finger protein gene (OsRHC1) from rice is reported. OsRHC1 is differentially expressed by wounding in near isogenic lines containing the Xa14 or Xa23 locus, but not in the corresponding susceptible recurrent parents. Ectopic expression of OsRHC1 in transgenic A. thaliana enhances its resistance towards bacterial pathogens and this protective function depended on the action of the 26S proteasome.

Materials and methods

**Plant materials and chemicals**

The bacterial blight resistant near isogenic rice lines (CBB14 and CBB23) and their susceptible recurrent parents (SN1033 and JG30, respectively) were constructed by Professor Q Zhang at the Chinese Academy of Agricultural Sciences (Zhang et al., 1996, 2001). A. thaliana wild-type Col-0 and Oryza sativa cv. Aichi asashi are laboratory stocks. The npr1-3 mutant and *Pseudomonas syringae* pv. *tomato* DC3000 were gifts from Dr C Deprés at Brock University and Dr C Lo at University of Hong Kong, respectively. Enzymes and reagents for molecular studies were from Applied Biosystems (Foster City, CA), Clontech Laboratories, Inc. (Palo Alto, CA), Bio-Rad Laboratories (Hercules, CA), Promega Biosciences (San Luis Obispo, CA), and Roche Diagnostic Ltd (Basel, Switzerland). DNA oligos were from Integrated DNA Technol-ogies, Inc. (Corallville, IA), Invitrogen Corp. (Carlsbad, CA), and Tech Dragon Ltd (Hong Kong). Chemicals for plant growth and tissue cultures were from Sigma-Aldrich Co. (St Louis, MO). Metro-mix200 soil for growth of *A. thaliana* was from Humvert International Supplier (Earth City, MO).

**Plant growth, pathogen inoculation and titre determination, and treatment of the proteasome inhibitor MG132**

Bacterial blight resistant near isogenic rice lines were grown on regular field soil in a greenhouse (temperature 24–28 °C; RH 70–80%; under natural light). Inoculation of the *Xoo* races LN44 and P6 was performed by the clipping method (Zhang et al., 1996). Mock inoculation and wounding treatment followed the same procedure except that the pathogen was replaced by water. For the time-course experiments, samples were collected at 0, 2, 4, and 6 d at around the same time of the day (between 08.00 h and 10.00 h). Day 0 sample was collected before treatment. Aichi asashi rice calli were first induced and cultivated in N6D (Sigma) solid medium for 4 weeks and subcultured in N6D liquid medium for 2 weeks (with shaking) before using in the subcellular localization study. Cells in calli were separated by repeated aspirating up and down using a pipette.

*A. thaliana* was grown in a growth chamber (temperature 22–24 °C; RH 70–80%; light intensity 80–120 μE of a 16/8 h light/dark cycle). Preparation of the *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) culture, inoculation (by a dipping method), and subsequent titre determination were performed as previously described (modified from Uknnes et al., 1992; Kim and Delaney, 2002). The 26S proteasome inhibitor (MG132, Sigma C2211) was applied to transgenic *A. thaliana* using a protocol modified from previous reports (Ohate-Sánchez and Singh, 2002; Guo and Ecker, 2003; Abas et al., 2006; Dong et al., 2006). In brief, 50 mg l−1 MG132 dissolved in 1% (v/v) DMSO supplemented with 0.01% (v/v) Silwet L-77 (behe seeds VIS-01) were poured onto MS square plates to cover the roots but not the aerial tissues of the seedlings. Mock treatment was performed with the same procedures except that no MG132 was added. After 4 h, the seedlings were harvested for RNA extraction.

**Construction of transgenic A. thaliana lines**

OsRHC1 cDNA was inserted into a binary vector (Brears et al., 1993) and the transgene expression was driven by the Cauliflower Mosaic Virus 35S promoter. *Agrobacterium*-mediated transformation was performed using a vacuum infiltration method (Bechtold and Pelletier, 1998). Transgenic plants with a single insertion locus were screened by the kanamycin resistance phenotype (encoded by the selection marker gene in the binary vector) of offspring. A 3:1 (resistant: sensitive) ratio verified by Chi-Square test in the T1 generation suggested a single insertion event.

**DNA sequencing, RNA extraction, northern blot, reverse-transcription, and real-time PCR**

DNA sequencing was performed by a thermal sequencing method according to the manufacturer’s manual (Applied Biosystems 4337455). Total RNA was extracted by a phenol extraction method (Ausbel et al., 1995). Northern blot analysis was performed using OsRHC1 cRNA probe generated by the DIG labelling kit (Roche 11175025910). The cRNA probe covered the full-length coding region of the OsRHC1 cDNA clone. Procedures for blotting and transferring were according to standard procedures (Sambrook et al., 1989).

For gene expression studies via real time PCR, cDNA samples were generated by reverse transcription (18-mer oligo-dT; SUPERSCRIPT® II RNaseH (Invitrogen 18064-071)) of DNase I (Invitrogen 18068-015)-treated RNA samples.
At least two independent batches of plant samples were used to perform the real-time PCR gene expression studies and gene expression patterns were consistently observed. All PCR products were sequenced at least once to verify that the right targets were being quantified.

Real-time PCR amplification of cDNA was conducted using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) in a 96-well PCR plate with a dome cap. The reaction was carried out in a 20 μl reaction volume containing 10 μl SYBR Green PCR Master Mix (Applied Biosystems 4309155) with 0.3 μM each of the forward and reverse primers. Primers for real-time PCR were designed by the program Primer Express (Applied Biosystems). All reactions were set independently for at least four times and at least three sets of consistent data were used for analysis. Expression level of actin (O. sativa OsAct1D; accession number: X15865) (Wasaki et al., 2003), with the primer set 5′-CTTCATAGGAAATGGAAGCTGCAGGTA-3′ and 5′-GACCACCTTGATCTGTAATGTA-3′, and tubulin (A. thaliana β-tubulin 4, accession number: M21415) (Tsun et al., 2002), with the primer set 5′-AAAGGTGTCGAGGATGAGTT-3′ and 5′-GGACTTGGACCTTGGTTTGG-3′, were used for signal normalization of real-time PCR studies in rice and A. thaliana, respectively. Relative gene expression was calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001). To validate the reliability data, amplification efficiencies between the target genes and the housekeeping genes of all the real-time PCR reactions were compared, and dissociation curves of all PCR products were examined to ensure the quality of PCR.

The primer sets for real-time PCR studies include OsRHC1: 5′-AAAGAGAGACAGCCGGTTAT-3′ and 5′-GCTCTCAATTCCCTGT-3′; PR1: 5′-CTCCATGCTGATACGTT-3′ and 5′-GCTCTCAATTCCCTGT-3′; and PR2: 5′-ACCACACATGACTGTCCTC-3′ and 5′-ACACACATGACTGTCCTC-3′. The primer set 5′-TTCCTGGTCTGTAACCTTTCC-3′ and 5′-TAAAGGTTGGAAGCTGCAGGTA-3′ were used in the 5′-RACE reactions to amplify the full-length coding region, PCRs using the primer pair 5′-ACACACATGACTGTCCTC-3′ and 5′-ACACACATGACTGTCCTC-3′ were performed. All clones were stored in the plasmid vector pBlueScript KSII(+) and propagated in the E. coli strain DH5α.

Protein extraction, antibodies, and western blot analysis
Membrane-bound and soluble proteins were separated by a fractionation method (modified from Jiang and Rogers, 1998). Primary antibodies (polyclonal) targeting the OsRHC1 protein was raised by a commercial service (Invitrogen, Custom antibody) via injecting a synthetic peptide (‘N-CGYPPEVVVRKMPKR-D-C’) into rabbits and antibodies were purified using affinity column before use. Anti-rabbit secondary antibody conjugated to an alkaline phosphatase (provided in Western Breeze™ Immunodetection Kit, Invitrogen WB7106) was used to recognize the primary antibodies.

For western blot analysis, the proteins were electrophoretically separated on a polyacrylamide gel (4% stacking; 10% resolving) before transferred to an activated PVDF membrane (pretreated in absolute methanol for 20 min followed by protein transfer buffer for 15 min) using the Trans-Blot™ SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad 170-3949). The blocking and detection steps were performed according to the manufacturer’s manual (Western Breeze™ Immunodetection Kit, Invitrogen WB7106). A parallel SDS–PAGE was performed to illustrate even loading of protein samples (see Supplementary Fig. S1 at JXB online). The specificity of the anti-OsRHC1 antibody was tested (see Supplementary Fig. S2 at JXB online).

Immunolabelling and confocal microscopy
Rice callus cells were fixed and immunolabelled as described previously (Jiang and Rogers, 1998). All confocal images were collected using the confocal laser scanning microscope Olympus Fluoview 300. Laser excitation (543 nm Green HeNe) was followed by signal detection using specific filter (610 nm long pass filter). The OsRHC1 primary antibodies were labelled with Rhodamine-conjugated secondary antibody (pseudocolored in red).

 Molecular cloning of OsRHC1
A partial cDNA clone of OsRHC1 was first obtained via suppression subtractive hybridization techniques with the PCR-select cDNA subtraction kit (Clontech 637401), using total RNA extracted from 6–8-week-old CBB14 (tester) and SN1033 (driver) rice lines collected 4 d after pathogen (Xoo race LN44) inoculation. A partial cDNA clone of OsRHC1 was determined by the 5′ Rapid Amplification of cDNA Ends (5′-RACE) method using a commercial kit (Clontech 634914). Gene specific primers 5′-TTCTCCATGTTCGTAACCTTTCC-3′, 5′-TACAATGTTGGAAGCTGCAGGTA-3′ and 5′-ACACACATGACTGTCCTC-3′ were used in the 5′-RACE reactions. To amplify the full-length coding region, PCRs using the primer pair 5′-ACACACATGACTGTCCTC-3′ and 5′-ACACACATGACTGTCCTC-3′ were performed. All clones were stored in the plasmid vector pBlueScript KSII(+) and propagated in the E. coli strain DH5α.

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In rice, more than 30 resistance loci (Xa loci) against the pathogen Xanthomonas oryza pv. oryza were identified and six Xa genes were cloned mainly by map-based cloning approaches (Song et al., 1995; Yoshimura et al., 1998; Iyer and McCouch, 2004; Sun et al., 2004; Gu et al., 2005; Chu et al., 2006). At the other end, several pathogenesis-related (PR) genes were reported to contribute directly to the resistance mechanism (Li et al., 2006). However, only a few key components of the signal transduction pathway from the onset of R protein–Avr protein interaction to the actual resistance development were studied (Lieberherr et al., 2005; Wang et al., 2006). To obtain new signal transduction components related to Xoo resistance in rice, we searched for cDNA clones differentially expressed in rice lines harbouring Xa loci. One partial cDNA clone obtained (see Materials and methods) was chosen for further studies. Using the DNA sequence information of this partial clone, a 5′-Rapid-Amplification of cDNA Ends (5′-RACE) experiment and subsequent PCR amplifications using specific primers were performed. The resulting cDNA clone (GenBank accession number EF584506) encodes an intact open reading frame of 409 amino acids residues. EF584506 is 99% identical to a directly deposited rice cDNA clone

Results
Cloning of the OsRHC1 cDNA clone from Oryza sativa (rice) encoding a protein with a RING-HC domain

Cloning of the OsRHC1 cDNA clone from Oryza sativa (rice) encoding a protein with a RING-HC domain
The predicted amino acid sequence of the OsRHC1 protein was compared with two RING zinc finger proteins, EL5 (RING-H2 subclass) (Takai et al., 2002) and XB3 (RING-HC subclass) (Wang et al., 2006) from rice that are involved in disease resistance. No significant homology was found except at the RING zinc finger domain (data not shown). The RING zinc finger domain of OsRHC1 is located at the C-terminus (Fig. 1A) while such a domain in EL5 and XB3 is located in the middle portion or close to the C-terminus of the protein, respectively. Prediction by the TopPred program (von Heijne, 1992; Claros and von Heijne, 1994) and the iPSORT programs (Bannai et al., 2002) suggested that OsRHC1 may possess multiple transmembrane domains (Fig. 1B) while EL5 only has one and XB3 does not possess any transmembrane region with high certainty (data not shown).

BlastP analysis revealed that OsRHC1 shares high amino acid sequence homology to seven other annotated proteins deposited in GenBank from various plant species (Fig. 1A). These proteins exhibit greater than 50% identity (spanning full length) to OsRHC1, with multiple transmembrane domains at the N terminal half, and a RING-HC domain at the C-terminus. The consensus of the RING-HC domain for this group of proteins is Cys-X2-Cys-X11-Cys-X-His-X3-Cys-X2-Cys-X6-Cys-X2-Cys. There is apparently no published information on the functions of these homologues.

To verify that the OsRHC1 is membrane bound as depicted by bioinformatics tools, membrane-bound and soluble proteins were separated using a fractionation protocol (modified from Jiang and Rogers, 1998). Western blot analysis confirmed that the OsRHC1 protein was tightly associated to membranes (Fig. 2A). Using confocal microscopy, the subcellular localization of the OsRHC1 in rice callus was tracked further (Fig. 2B). The signals were clearly observed in plasma membrane (Fig. 2B). Autofluorescence in nuclei was also seen in all cells examined, including negative controls without antibody treatments (data not shown).

The OsRHC1 clone is wound-inducible in the rice lines CBB14 and CBB23

To study the expression pattern of OsRHC1, real-time PCR analyses were performed using reverse-transcribed RNA samples (see Materials and methods) from two near isogenic pairs (CBB14 containing Xa14 and its susceptible recurrent parent SN1033; CBB23 containing Xa23 and its susceptible recurrent parent JG30). When an incompatible Xoo strain (LN44 for Xa14 and P6 for Xa23) was inoculated, the rice lines containing Xa14 or Xa23 exhibited an induction of OsRHC1 gene expression while the susceptible recurrent parents were non-responsive (Fig. 3). However, such induction was also observed in mock inoculated samples which had been wounded, suggesting that OsRHC1 could be wound inducible. The amplitude of induction was much stronger in the case of CBB23 which harbours the Xa23 locus that confers broad spectrum resistance (Zhang et al., 2001).

The effect of wounding on OsRHC1 expression in the CBB23 line and its susceptible recurrent parent JG30 was analysed further. Both RNA and protein samples were collected 2, 4, and 6 d after wounding by leaf clipping. The induction peak of OsRHC1 gene expression appeared on day 4 after treatment in CBB23 (Fig. 4A). Western blot analysis of membrane-bound proteins showed that the production of the OsRHC1 protein in CBB23 was greatly enhanced on day 6 (Fig. 4B), after the induction of gene expression on day 4. The response in JG30 was not obvious when compared to CBB23, indicating that the presence of the Xa23 locus may play a role in the wounding induction of OsRHC1.

Expressing the OsRHC1 clone in transgenic Arabidopsis thaliana enhances the resistance to the bacterial pathogen Pseudomonas syringae pv. tomato DC3000 (Pst DC3000)

To test whether OsRHC1 is a component of the defence pathway that can mediate broad spectrum (e.g. cross-species) resistance, a gain-of-function test in a heterologous

Fig. 1. Sequence alignment and membrane topology analysis of the OsRHC1 protein. (A) The full-length amino acid sequence of OsRHC1 was aligned to seven annotated proteins exhibiting high degree of similarity, using the Clustal W program (Thompson et al., 1994). These proteins were deposited in GenBank and originated from different plant species including, Arabidopsis thaliana (NP_564945), Brassica oleracea (AAW17137), Trifolium pratense (BAC71207), and Medicago truncatula (ABJ90658). ‘*’: Conserved amino acid residues; ‘:’: conserved substitutions; and ‘.’: semi-conserved amino acid substitutions. The transmembrane domains of OsRHC1 predicted by the TopPred program (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) were underlined. The eight conserved Cys and His residues co-ordinating two zinc ions in the RING-HC domain were boxed. The spacing between the Cys and His residues were found to be conserved. (B) Schematic drawing of the membrane topology of OsRHC1.
Expression of a RING-HC protein improves resistance
A system using transgenic *A. thaliana* was performed. The *OsRHC1* clone was inserted into a binary vector, placed under the control of the constitutive Cauliflower Mosaic Virus 35S promoter, and transformed into the *A. thaliana* ecotype Col-0 (see Materials and methods). Only positive transformants containing a single insertion locus were propagated to obtain homozygous lines for further experiments. The transgene expression in three independent homozygous transgenic lines was examined by northern blot analysis (Fig. 5A).

Pst DC3000 inoculation caused severe yellowing and necrosis in infected Col-0 (Fig. 5B) and transgenic plants transformed with the empty vector (see Supplementary Fig. S3 at *JXB* online). On the other hand, the disease symptoms were much reduced in all *OsRHC1* transgenic lines. The titres of pathogen inside the rosette leaves were consistent with the observed phenotypes (Fig. 5C). Furthermore, the H-2–9 line that exhibited the highest level of transgene expression also gave the lowest pathogen titre (compare Fig. 5A and C).

**Constitutive expression of OsRHC1 activates the expression of defence marker genes in transgenic Arabidopsis thaliana**

The expression of four defence marker genes was tested, including *PR1*, *PR2*, *PDF1.2*, and *Thi2.1*. In *A. thaliana*, these genes are indicators of defence pathways mediated by different phytohormones including SA, JA, and ET (Thomma et al., 1998; Spoel et al., 2003; Thibaud et al., 2004; Devoto and Turner, 2005).

In 6-week-old seedlings under regular growth conditions, all four defence marker genes exhibited enhanced expression when compared to the wild type Col-0 (Fig. 6A). The fold of induction was particularly high for the *PR1* and the *PDF1.2* genes which are mediated by two different signalling pathways. Among three independent transgenic lines tested, the H-2–9 line that showed highest expression of the transgene and best protection in the pathogen inoculation experiment also gave the highest fold of induction of *PR1* and *PDF1.2* (comparing Figs 5 and 6A).

When the plants were subjected to the challenge of *Pst* DC3000, the levels of *PR1* and *PR2* transcripts in Col-0 increased (see Supplementary Fig. S4 at *JXB* online) but the expression levels of these genes were still higher in transgenic lines (Fig. 6B). While the level of *Thi2.1* in Col-0 did not alter significantly by the pathogen inoculation (see Supplementary Fig. S4 at *JXB* online), its expression was elevated in the transgenic lines (Fig. 6B). The expression of *PDF1.2*, on the other hand, was repressed by *Pst* DC3000 inoculation in both Col-0 (see Supplementary Fig. S4 at *JXB* online) and transgenic lines (Fig. 6B).

As discussed in the Introduction, many members of the RING-HC subclass are E3 ubiquitin ligases (Stone et al., 2005). To show the relationship between the function of
OsRHC1 and ubiquitin-mediated protein degradation, the effects of MG132 (a 26S proteasome inhibitor) on the expression of defence marker genes in the transgenic lines were studied. Four-week-old seedlings were subjected to MG132 treatment. Expression of the transgene was not affected by such treatment (data not shown). In Col-0, no significant effects of MG132 on the expression of defence marker genes were observed. On the other hand, the induction effects of overexpressing OsRHC1 on the four defence marker genes were diminished under MG132 treatment (Fig. 7).

The protective function of the OsRHC1 clone in transgenic Arabidopsis thaliana is dependent on the function of NPR1

The above results indicated that OsRHC1 encodes a new component of the defence signal transduction pathway in rice that is also functional in A. thaliana. Making use of the model plant system, we tried to position the function of OsRHC1 in relation to a known hub in the defence signalling network, NPR1 that mediates both SA and JA/ET signals plays a central role in defence signalling in A. thaliana. OsRHC1 was transformed into the npr1-3 A. thaliana mutant that is depleted of NPR1 (Yu et al., 2001). Independent transformants with a single insertion locus were selected. At the time of inoculation, the expression of transgene (under the control of the Cauliflower Mosaic Virus 35S promoter) in individual lines was examined with real-time PCR. The steady-state level of OsRHC1 in an npr1-3 background was found to be comparable to that in the transgenic lines with a Col-0 background (data not shown).

When the npr1-3 transgenic lines were subjected to the challenge of Pst DC3000, no protection effects could be observed in the transgenic lines. Both the disease symptom development (data not shown) and pathogen titre (Fig. 8A) of these transgenic lines resembled that of the untransformed npr1-3 mutant. Consistent with this observation, no significant increase in the expression of four selected defence marker genes was found in any of these transgenic lines without (Fig. 8B) or with (see Supplementary Fig. S5 at JXB online) pathogen inoculation.
Discussion

A cDNA clone (OsRHC1) was obtained from rice encoding a novel type of RING zinc finger protein that is related to plant disease resistance. Homologues exhibiting high amino acid sequence identities to the OsRHC1 protein can be found in various plant species of both dicots and monocots (Fig. 1A). The OsRHC1 protein and its homologues exhibit several features distinguishing them from other RING zinc finger proteins, including the presence of multiple transmembrane domains in the N-terminal half and a zinc finger consensus Cys-X2-Cys-X11-Cys-X-His-X3-Cys-X2-Cys at the...
C-terminus (Fig. 1). The presence of transmembrane domains and the absence of other protein targeting signals suggest a plasma membrane localization, as demonstrated in the case of OsRHC1 (Fig. 2). To the best of our literature survey, the functional aspect of this group of proteins in relation to the plant defence response is still unclear. Therefore, the possible functions of OsRHC1 as a prototype were studied.

Under pathogen inoculation or mock inoculation treatments, both CBB14 (containing Xa14) and CBB23 (containing Xa23) rice lines exhibited higher level of OsRHC1 transcripts, comparing to their susceptible recurrent parents SN1033 and JG30, respectively (Fig. 3). Further gene expression analysis showed that OsRHC1 is induced by wounding and this induction is dependent on the presence of the Xa genes (Fig. 4). The observation that the induction fold of OsRHC1 in CBB23 was higher than that in CBB14 (Fig. 3) is of particular interest. While Xa14 only confers resistance to a narrow spectrum of Xoo races, Xa23 leads to broad-spectrum Xoo resistance (Zhang et al., 2001). Therefore, OsRHC1 may be involved in the signal transduction process initiated from more than one Xa gene.

RING zinc finger proteins often involved in plant defence responses (Takai et al., 2002; Devoto et al., 2003; Wang et al., 2006; Goritsching et al., 2007). In rice, EL5 and XB3 belong to the RING-H2 and RING-HC subclasses, respectively (Takai et al., 2002; Wang et al., 2006). The EL5 protein is structurally related to the ATL family which consists of a large number of members in both A. thaliana and rice (Serrano et al., 2006). The gene expression of several ATL genes in A. thaliana is induced by fungal elicitors (Serrano et al., 2006). Similarly, the EL5 protein in rice cells is rapidly induced by fungal elicitors (Takai et al., 2002), suggesting a role in defence responses. On the other hand, XB3 from rice is probably a soluble protein that interacts with the kinase domain (located in the cytoplasm) of the R protein Xa21 and is essential for the signal transduction initiated from Xa21 (Wang et al., 2006).

OsRHC1 is structurally distinct from both EL5 and XB3. To verify that OsRHC1 is related to a plant defence signalling pathway that commonly exists in different plant species, gain-of-function tests were performed in the model plant A. thaliana. Several lines of evidence support the notion that the expression of OsRHC1 can enhance disease resistance in transgenic A. thaliana. Constitutive induction of four defence marker genes was observed without pathogen attack (Fig. 6A). These defence marker genes include PDF1.2 and Thi2.1, which are involved in the defense response. The results suggest that OsRHC1 plays a role in the plant defence response, possibly by modulating the expression of these defence marker genes.

Fig. 7. Expression of defence marker genes PR1 (A), PR2 (B), PDF1.2 (C), and Thi2.1 (D) when treated with MG132 (a 26S proteasome inhibitor). MG132 treatment was performed using 6-week-old A. thaliana seedlings as described in the Materials and methods. The whole seedlings were harvested 4 h after treatment to extract total RNA. Subsequent reverse transcription and real-time PCR experiments were performed as described in Fig. 6 and the Materials and methods. Col-0 with mock treatment was used as the reference for comparison (expression level set to 1).
genes represent the defence signals initiated from different phytohormones. \( PRL \) and \( PR2 \) are induced by the salicylic acid (SA) pathway (Thomma \textit{et al.}, 1998; Thibaud \textit{et al.}, 2004). \( PDF1.2 \) and \( Thi2.1 \) are induced by the jasmonate/ethylene (JA/ET) pathway (Thomma \textit{et al.}, 2001) and the JA pathway (Ellis \textit{et al.}, 2002), respectively. Since the expression of \( OsRHC1 \) led to the induction of defence marker genes related to different defence phytohormones (SA, JA, and ET), the function of this gene may help to mediate signals from multiple pathways.

When subjected to pathogen challenge, the transgenic lines exhibited: (i) a reduction of disease symptoms (Fig. 5B); (ii) a lowering of pathogen titres (Fig. 5C); and (iii) an induction of several defence marker genes (Fig. 6B). In \( A. thaliana \), the expression of \( PR1 \) and \( PR2 \) are known to be induced by the challenge of \( Pst \) DC3000 (Uknes \textit{et al.}, 1992). Similar observation was obtained in this study (see Supplementary Fig. S4 at \textit{JXB} online). Interestingly, a further induction of \( PR1 \) and \( PR2 \) was observed in the transgenic lines, when compared to the wild type Col-0 (Fig. 6B). This result suggested that pathogen-derived signals cannot saturate the expression of \( PR1 \) and \( PR2 \) in the presence of the \( OsRHC1 \) signal. While \( Pst \) DC3000 inoculation did not induce the expression of \( Thi2.1 \) in Col-0 in this study (see Supplementary Fig. S4 at \textit{JXB} online), a higher expression of \( Thi2.1 \) was maintained in the transgenic lines (Fig. 6B).

Therefore, additional defence mechanisms may result from expressing \( OsRHC1 \). The higher expression of \( PR1 \), \( PR2 \), and \( Thi2.1 \) in the transgenic lines may explain their stronger resistance toward \( Pst \) DC3000. By contrast, \( PDF1.2 \) probably did not play a significant role in the enhanced resistance against \( Pst \) DC3000 exhibited by the transgenic lines, since \( PDF1.2 \) was repressed by pathogen-derived signals in these lines, similar to the case in Col-0 (Fig. 6B). In \( A. thaliana \), the activation of the SA pathway by pathogen challenges will suppress the JA pathway (and hence repress the expression of \( PDF1.2 \)) (Spoel \textit{et al.}, 2003).

Many RING zinc finger proteins are members of E3 ubiquitin ligases (Callis and Vierstra, 2000). For instance, XB3 (Wang \textit{et al.}, 2006), EL5 (Takai \textit{et al.}, 2002) and several ATL family members (Serrano \textit{et al.}, 2006) belong to this category. Besides these proteins, the involvement of RING zinc finger containing E3 ubiquitin ligases in defence responses have been frequently reported in both plants (Devoto \textit{et al.}, 2003; Goritsching \textit{et al.}, 2007) and animals (Ben-Neriah, 2002). Ubiquitin-mediated protein degradation via the 26S proteasome is an important regulatory step in many signalling processes (Callis and Vierstra, 2000). In \( A. thaliana \), there is only two E1 proteins (ubiquitin activating enzyme), over 40 E2 proteins (ubiquitin conjugating enzyme), and more than 1000 genes encoding E3 proteins (ubiquitin ligase) (Vierstra, 2003). E3 proteins are important to determine the specificity of target proteins for degradation (Callis and Vierstra, 2000; Vierstra, 2003). To test if the ubiquitin-mediated protein degradation is essential for the protective functions conferred by \( OsRHC1 \), the effects of the 26S proteasome inhibitor MG132 were studied. When treated with MG132, no induction in the expression of defence marker genes was observed in the transgenic lines (Fig. 7), suggesting a link between the function of \( OsRHC1 \) and the 26S proteasome. The membrane localization of \( OsRHC1 \) (Fig. 1B) may help to recruit membrane associated targets and bring them in close proximity to the special E2 protein recognized by the RING zinc finger domain (Fig. 1A).
Due to the availability of a large collection of mutants in A. thaliana, the defence signal transduction networks in A. thaliana have been well established. On the other hand, the corresponding system in rice is still under construction. NPR1 is a key component regulating defence responses in A. thaliana, which is participating in both SA and JA pathways (Cao et al., 1994; Ryals et al., 1996; van Wees et al., 1999, 2000). In rice, the JA pathway may play a significant role in defence responses since the level of SA is constitutively high (Silverman et al., 1995). The observation that OsRHC1 can induce defence marker genes for both SA and JA pathways (see above) suggests a distinct role of this protein in rice defence responses. The homologue of NPR1 has been identified in rice (NH1) and overexpression of NPR1 (Cao et al., 1998) and NH1 (Chern et al., 2005) in rice enhances the resistance toward bacterial pathogens. We tried to position OsRHC1 in relation to NPR1 using the npr1-3 mutant of A. thaliana. Our results suggested that the activity of NPR1 is a prerequisite for the functioning of OsRHC1. Therefore, OsRHC1 is either upstream from the NPR1 in the signal transduction pathway or it acts on a negative regulator of the NPR1 pathway.

Supplementary data

Supplementary figures are available online. These non-essential data provide additional information for data interpretation as depicted in the text.

Fig. S1. A loading control of total membrane protein extracted from leaf tissues. Leaf tissues about 6–8 mm away from the wounding site were collected at 2, 4, and 6 d after clipping. Membrane bound protein samples were prepared as described in the Materials and methods. Protein samples were stained with Coomassie Brilliant Blue R-250 (Bio-Rad 161-0400).

Fig. S2. Specificity testing of the anti-OsRHC1 antibody. Membrane protein extracted from 6-week-old A. thaliana Col-0 and the OsRHC1 transgenic A. thaliana H-2-9 line seedlings was subjected to western blot analysis (detailed in the Materials and methods). A single dominant band was observed in the protein sample from the transgenic line, while that from Col-0 exhibited no background signals.

Fig. S3. Pathogen inoculation test of transgenic A. thaliana expressing OsRHC1 or the empty vector. The expression of the transgene OsRHC1 in the transgenic line was verified using real-time PCR. Treatment conditions were as described in the legend to Fig. 5 and the Materials and methods.

Fig. S4. Expression of the four defence marker genes (PR1, PR2, PDF1.2, and Thi2.1) in Col-0 inoculated with Pseudomonas syringae pv. tomato DC3000. Pathogen inoculation, sample preparation, and real-time PCR experiments were performed as described in Figs 5 and 6 and the Materials and methods. For each defence marker gene, Col-0 with mock inoculation was used as the reference for comparison (expression level set to 1).

Fig. S5. Expression of defence marker genes (PR1, PR2, PDF1.2, and Thi2.1) in the mutant npr1-3 background without (A) or with (B) the challenge of Pst DC3000. Sample preparation and real-time PCR experiments were performed as described in Fig. 6 and the Materials and methods. Col-0 was used as the reference for comparison (expression level set to 1).

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**Expression of a RING-HC protein improves resistance**


