Overexpression of Rice (*Oryza sativa* L.) *OsCDR1* Leads to Constitutive Activation of Defense Responses in Rice and *Arabidopsis*

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Submitted 11 May 2009. Accepted 28 July 2009.

Plant aspartic proteases (AP) play key roles in the regulation of biological processes, such as the recognition of pathogens and pests and the induction of effective defense responses. A large number of AP (>400) have been identified in silico in the rice genome. None have previously been isolated and functionally characterized for their involvement in disease resistance. We describe here the isolation and characterization of a gene (*OsCDR1*) from rice which encodes a predicted aspartate protease. Expression of *OsCDR1* was activated upon treatments with benzothiadiazole and salicylic acid, which are signal molecules in plant disease resistance responses. Ectopic expression of *OsCDR1* in *Arabidopsis* and rice conferred enhanced resistance against bacterial and fungal pathogens. The enhanced disease resistance observed in transgenic plants was correlated with induction of pathogenesis-related gene expression and was shown by mutational analysis to be dependent on AP activity of the transgene-encoded product. *OsCDR1* accumulates in intercellular fluids (IF) in transgenic plants. Infiltration of IF from transgenic *Arabidopsis* plants into leaves of wild-type (WT) *Arabidopsis* induced the systemic defense response. These results demonstrate the conservation of CDR1 function between rice and *Arabidopsis* during the disease resistance response.

Plants use complex recognition and response mechanisms to protect themselves from pathogen attack. Major resistance (*R*) genes specify recognition of pathogens carrying the corresponding avirulence (*Avr*) genes, leading to the rapid activation of a battery of inducible defense mechanisms, including reinforcements of the cell wall, synthesis of antimicrobial compounds, and deployment of pathogenesis-related (PR) proteins (Yang et al. 1997; Dangl and Jones 2001). This induced defense is often accompanied by the collapse of challenged plant cells in the specific area showing hypersensitive response (HR), which results from localized cell death to restrict the pathogen at the site of pathogen attack. Some of the effector mechanisms contribute to basal resistance which underpins attempted disease lesion limitation triggered by pathogen-associated molecular patterns (PAMPs) in the absence of *R*-gene-

mediated recognition (Grant and Lamb 2006). A third type of resistance is salicylate (SA)-associated defense (Durrant and Dong 2004), in which local attack by a necrotizing pathogen induces broad-spectrum resistance throughout the plant system against an array of pathogens (Grant and Lamb 2006).

In dicots, several regulatory proteins have been implicated in the transcriptional regulation of defense genes under the control of the SA signaling pathway. The key regulator of SA-mediated resistance, *NPRI*, is functionally conserved in diverse plant species, including rice (*Oryza sativa* L.), which demonstrates that rice has evolved an SA-mediated systemic acquired resistance (SAR) similar to that in *Arabidopsis*. Improved understanding of basal, acquired, and nonhost resistance mechanisms, primarily from studies in *Arabidopsis*, together with demonstrated or anticipated convergence of gene function between monocots and dicots provide an exciting avenue to gain better insights into these resistance mechanisms in rice.

Recently, several proteases have been found to play important roles in plant-microbe interactions. Several *Avr* genes that encode different classes of proteases have been identified (Jia et al. 2000; Orth et al. 2000; Shao et al. 2002). Aspartic proteases (AP), one of the main proteinase classes, play key roles in the regulation of biological processes, including responses to pathogens and pests and the induction of effective defenses (Domsalla and Melzig 2008). The biological functions of plant AP are poorly understood and, for the great majority of plant AP, the biological functions are still hypothetical. In general, plant AP have been implicated in protein processing or degradation in different plant organs, as well as in plant senescence, stress responses, programmed cell death, and reproduction (Simoes and Faro 2004).

Arabidopsis constitutive disease resistance 1 (*CDR1*) is a member of the larger group of the atypical AP and was identified in a screen for gain-of-function mutants displaying enhanced resistance to bacterial pathogens following T-DNA activation tagging (Weigel et al. 2000; Xia et al. 2004). Overexpression of *CDR1* mimics constitutive activation of SAR, including the accumulation of high levels of SA and transcripts of SAR marker genes such as *PR1* and *PR2*. The enhanced resistance of the *CDR1* gain-of-function mutant is likely due to the proteolytic generation of an endogenous peptide elicitor, which might be involved in the activation of SA-dependent local and systemic defense responses (Xia et al. 2004).

Similar to *Arabidopsis*, a large number of AP (>400) have been identified in silico in the rice genome (MEROPS peptidase database). None have previously been isolated and functionally characterized for their involvement in disease resistance. To understand the role of AP in rice disease resistance, we

Professor Chris Lamb passed away on August 21, 2009.

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isolated the rice ortholog *OsCDR1* (GenBank accession number NP_001042193) using published data on the *Arabidopsis* CDR1 (Xia et al. 2004). Here, we report that *OsCDR1* is an apoplast-localized protein and AP activity is essential for its biological function. Further, ectopic expression of *OsCDR1* in *Arabidopsis* and rice led to increased expression of defense-related genes and enhanced disease resistance against bacterial and fungal pathogens, indicating the presence of a similar defense pathway in both species.

RESULTS

Identification of a rice CDR1 homologue.

BLASTP searches were performed using the complete protein sequence of *Arabidopsis* CDR1 (*AtCDR1*) as the query sequence. From the 50 highest-scoring alignments, the 28 *Arabidopsis* and rice sequences were used to create a phylogenetic tree which identified a single rice sequence (gi|115434870) that fell within the same clade as *AtCDR1* (Fig. 1A). This single *OsCDR1* ortholog had 50% similarity and 36% identity with *AtCDR1* at the amino acid level.

The genomic sequence of *OsCDR1*, like *AtCDR1*, has no intron. It contains a predicted open reading frame of 1,362 bp, encoding a protein of 454 amino acid residues with a calculated molecular weight of 45.91 kDa that is predicted to be extracellular by the PSORT algorithm (Nakai and Kanehisa 1992), with a signal peptide cleaved between positions 21 and 22. Like other eukaryotic AP of the pepsin family, *OsCDR1* contains two catalytic aspartic acid residues within two conserved active sites with the sequence motif Asp-Thr/Ser-Gly-Ser/Thr (Fig. 1B).

Responses of *OsCDR1* to chemical treatment.

Quantitative real-time reverse-transcriptase (RT) polymerase chain reaction (qPCR) analysis showed that induced expression of *OsCDR1* occurred as early as 6 h after treatment with SA or with the SA analogue, benzothiadiazole (BTH); peaked at 12 h after treatment; and declined thereafter (Fig. 2). There was no increased transcription of *OsCDR1* after treatment with jasmonic acid (JA).

Generation and characterization of transgenic plants.

To gain further information on the biological function of *OsCDR1*, transgenic *Arabidopsis* and rice lines were produced that ectopically expressed *OsCDR1* under the control of a double *Cauliflower mosaic virus* 35S promoter. Representative homozygous T₃ transgenic *Arabidopsis* lines (*At3*, *At6*, *At8*, and *At17*) and homozygous T₂ rice lines (*R2*, *R6*, *R10*, and *R11*) that displayed varying levels of expression of *OsCDR1* (Fig. 3A) were used in this study. Protein gel-blot analysis using antibodies raised against an *OsCDR1* peptide detected a single polypeptide with an approximate size of 49 kDa in the transgenic lines. The experimentally determined size of plant-expressed *OsCDR1* is greater than the size calculated from its open reading frame (45.91 kDa), indicating that the mature *OsCDR1* protein might be post-translationally modified. As expected, different transformed lines displayed different levels of *OsCDR1* protein (Fig. 3B).

OsCDR1 is an extracellular protein.

Based on the presence of an N-terminal signal sequence, *OsCDR1* was predicted to be an extracellular protein. Protein gel-blot analysis indicated that there was substantial enrichment of *OsCDR1* in intercellular fluids (IF) from transgenic *Arabidopsis* plants. Similar signals were obtained on protein gel blots of total and IF extracts despite there being approximately 25 times more protein in the crude extract (Supplemen-

tary Fig. 1). Furthermore, immunolocalization of *OsCDR1* in tissue sections of transgenic rice leaf tissue showed strong immunofluorescence in the intercellular spaces associated with the cell wall, whereas no immunofluorescence was detected in the wild-type (WT) leaf (Supplementary Fig. 2), further supporting an extracellular location for *OsCDR1*.

Transgenic plants have elevated levels of SA and increased generation of H₂O₂.

To assess possible effects of overexpressing *OsCDR1* on SA accumulation, we measured SA in transgenic lines and the WT. The level of SA was approximately 11-fold higher in transgenic *Arabidopsis* and approximately 1.3-fold higher in transgenic rice compared with the WT. Because SA treatments enhanced H₂O₂ levels in plants (Chen et al. 1993b), we performed diaminobenzidine (DAB) staining which revealed accumulation of H₂O₂ in *OsCDR1*-expressing *Arabidopsis* and rice lines (Supplementary Fig. 3). Previously, H₂O₂ or active oxygen has been shown to be an important pathogen signal in plants (Alvarez et al. 1998).

Overexpression of *OsCDR1* in *Arabidopsis* results in increased expression of defense-related genes.

SA accumulation has been shown to be a requirement for the induction of *PR* gene expression and the establishment of SAR in tobacco and *Arabidopsis* (Gaffney et al. 1993; Delaney et al. 1994). Because elevated levels of SA were detected in transgenic plants, we examined the expression of *PR* genes in *OsCDR1:Arabidopsis* lines. qPCR analysis showed that the transcript levels of both *PR1* and *PR2* were upregulated to varying levels in transgenic *Arabidopsis* lines in the absence of the pathogen (Fig. 4A and B). The *PR1* and *PR2* transcripts were more than 300-fold higher in the uninfected *At17* line than in the equivalent healthy WT. In contrast, *PDF1.2* transcripts, encoding a defensin protein that is induced in *Arabidopsis* by an SA-independent pathway involving JA (Penninckx et al. 1996), were not elevated in uninfected *OsCDR1:Arabidopsis* plants, indicating the activation of SA-dependent responses.

Responses of *OsPR1* genes in transgenic rice.

We examined whether overexpression of the *OsCDR1* gene in rice enhanced the expression of defense genes *PBZ1* (probenazole-inducible gene)/*PR10* and *PR1a* by qPCR. The transcript levels of *PBZ1* and *PR1a* were 23.45 ± 4.99- to 41.35 ± 3.87- and 68.26 ± 2.05- to 133.73 ± 4.81-fold higher, respectively, in uninfected transgenic rice lines compared with that in the WT (Fig. 4C and D). Additionally, the expression patterns of each of the 12 *OsPR1* genes was analyzed by qPCR using specific primers described previously (Mitsuhashi et al. 2008). The transcript levels of the SA-, JA-, and 1-aminocyclopropane-1-carboxylic acid (ACC)-inducible genes *OsPR1#012*, *OsPR1#051*, *OsPR1#074*, and *OsPR1#101*, as well as those of the SA-inducible genes *OsPR1#011* and *OsPR1#052* (Mitsuhashi et al. 2008), were higher in uninfected rice line *R6* compared with those of the WT (Fig. 4E). There was, however, no increase in transcription of *OsPR1#071*, *OsPR1#073*, and *OsPR1#121*, which are upregulated by JA but not by SA (Fig. 4E). These results suggest that *OsCDR1* is likely to be involved in basal defense against pathogens in a manner that is dependent on SA but independent of JA and ACC signaling.

Overexpression of *OsCDR1* in *Arabidopsis* confers enhanced disease resistance to *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*.

The similarity between *AtCDR1* and *OsCDR1* prompted us to test the response of *OsCDR1* overexpressing *Arabidopsis* plants to pathogen challenge. When challenged with *Pseudomonas syringae* pv. *tomato* DC3000, *OsCDR1:Arabidopsis*

supported up to six times less bacterial growth at 2 days post inoculation (dpi) compared with the WT (Fig. 5A). Further, measurement of bacterial titers after 4 dpi indicated that the bacterial population in transgenic *Arabidopsis* lines At8 and At17 leaves were reduced by at least 6- and 10-fold, respectively, compared with that in the WT.

Transgenic *Arabidopsis* lines were further evaluated for disease resistance by the virulent Noco2 isolate of *Hyaloperonospora arabidopsidis*. Compared with the WT, transgenic *Arabidopsis* plants supported up to 10 times less fungal conidia at 5 dpi (Fig. 5B). These results suggest that ectopic expression of *OsCDR1* in transgenic *Arabidopsis* confers disease resistance against *P. syringae* and *H. arabidopsidis*.

Susceptibility of transgenic *Arabidopsis* lines to *Alternaria brassicicola*.

The disease resistance phenotype of *OsCDR1:Arabidopsis* plants against *Alternaria brassicicola*, a necrotrophic fungal pathogen, was also examined. The lengths of lesions were 2.87 ± 0.85 mm in the inoculated WT, whereas they ranged from 3.13 ± 0.16 to 7.0 ± 0.16 mm in the four transgenic lines (Fig. 5C). Lesion lengths in the At17 transgenic plants were significantly higher than that observed in WT plants, whereas there was no significant difference between the other transgenic lines and the WT.

Overexpression of *OsCDR1* in rice confers enhanced disease resistance to *Xanthomonas oryzae* and *Magnaporthe oryzae*.

In order to evaluate the range of protection offered by overexpression of *OsCDR1* in rice, we examined selected trans-

formed lines for resistance against rice blast fungus *Magnaporthe oryzae* and the bacterial blight pathogen *Xanthomonas oryzae*. Following inoculation with *X. oryzae*, the WT plants showed a large diseased area compared with the transgenic lines at 12 dpi. The length of diseased area ranged from 0.90 ± 0.20 to 1.43 ± 0.31 cm in transgenic rice lines compared with 3.4 ± 0.50 cm in the inoculated WT (Fig. 5D).

Further, the transgenic rice lines were evaluated for disease resistance to the blast fungus.

The severity of infection in transgenic plants at 10 dpi was considerably lower than that observed in the WT. The percent diseased leaf area in the four transgenic lines ranged from 21.33 ± 2.08 to $26.33 \pm 0.50\%$ compared with $82.67 \pm 1.53\%$ in the WT plants (Fig. 5E). These results indicate that overexpression of *OsCDR1* in transgenic rice enhances disease resistance against both *X. oryzae* and *M. oryzae*, two major phytopathogens of rice worldwide.

Protease activity is required for *OsCDR1* function.

To determine whether the AP activity of *OsCDR1* was essential for its biological activity, two mutations were made by site-directed mutagenesis (as described below), and these were reintroduced into WT *Arabidopsis*. Unlike plants overexpressing WT *OsCDR1*, plants expressing D119N, where the mutation is in the active site, did not show the expression of *PR* genes. In contrast, plants expressing D233N (where mutation is in aspartate residue away from the active site) showed expression of *PR* genes comparable with that observed in *OsCDR1* transgenic *Arabidopsis* plants (Fig. 6). D119N and D233N overexpressing *Arabidopsis* plants were also analyzed for the expression of *PR1* and *PR2* gene by qPCR analysis. The expression of *PR1* and *PR2* genes in D233N-overexpressing *Arabidopsis* plants was 88- and 128-fold higher than that of the WT plant, whereas the D119N-overexpressing *Arabidopsis* plant did not show any induction of *PR* genes. These results indicated that the AP activity of *OsCDR1* is essential for *PR* gene expression.

Elicitor activity of *OsCDR1*.

In order to determine the elicitor activity of *OsCDR1*, IF were isolated from 2-week-old transgenic *Arabidopsis* (At17) and WT plants grown in liquid Murashige and Skoog (MS) medium. High levels of *PR2* transcripts were observed in WT *Arabidopsis* plants when infiltrated with crude IF, a high molecular weight (HMW) fraction (>10 kDa) or a low molecular weight (LMW) fraction (3 to 10 kDa) of *OsCDR1:Arabidopsis* plants, whereas protein gel-blot analysis showed the presence of *OsCDR1* protein in crude IF and HMW fractions (>10 kDa)

Fig. 2. *OsCDR1* transcripts following benzothiadiazole (BTH) and salicylate (SA) treatments. Rice seedlings were treated with 0.3 mM BTH or 3 mM sodium salicylate. Leaf samples were collected at the indicated time points. Transcript levels were analyzed by quantitative polymerase chain reaction and are expressed relative to the mock treatment at each time point. Bars indicate mean $\pm$ standard deviation.

Fig. 3. Expression of *OsCDR1* in transgenic plants. **A1**, *OsCDR1:Arabidopsis* lines. Expression levels were presented relative to transgenic line At6. **A2**, *OsCDR1:rice* lines. Expression levels were compared with the wild type (WT). Protein-gel blot analysis of **B1**, *OsCDR1: Arabidopsis* lines and **B2**, *OsCDR1:rice* lines. Ponceau S staining of the membrane shows equal loading. Bars indicate mean $\pm$ standard deviation.

only (Fig. 7). No increase in transcript of *PR2* was observed when infiltrated with <3-kDa IF. Further, qPCR analysis showed that crude IFs and HMW and LMW fractions induced *PR2* transcripts in both primary and systemic secondary leaves at 12 and 24 h of infiltration. The LMW fraction, which does not contain OsCDR1 protein, also induced *PR2* transcripts in both the primary and secondary leaves. The response in distant tissues to local inoculation with the LMW fraction was comparable with the distant effects of local inoculation with the HMW fraction, suggesting that the elicitor activity in the LMW fraction makes a substantial contribution to the systemic effects of OsCDR1 activity. Size fractionation analysis of IF demonstrated that the elicitor activity in the LMW fraction is associated with molecules in the 3- to 10-kDa range.

Phenotypic and agronomic characteristics of transgenic plants.

Transgenic plants were further examined for their phenotype. Transgenic *Arabidopsis* lines At3, At6, and At8 appeared normal whereas line At17 showed growth retardation (Fig. 8). No overt growth or developmental differences were observed between the transgenic rice and WT plants in our experimental conditions. Further, transgenic rice lines were evaluated for percent germination, plant height, days for 50% flowering, and test weight. All four transgenic lines showed 100% germination, like the WT. Plant height in transgenics was 77.06 ± 1.50 to 84.25 ± 2.67 cm, while it was found to be 85.12 ± 1.02 cm in the WT (Supplementary Fig. 4A). Days required for 50% flowering ranged from 109.0 ± 1.00 to 128 ± 3.02 days in the

case of transgenics whereas, in the WT, it was 108 ± 2.00 days. Test weight of transgenic seed ranged from 18.41 ± 0.30 to 19.61 ± 0.25 g and was found to be 20.03 ± 0.13 g in the case of the WT. Transgenic rice lines were comparable with the WT plants with respect to agronomic parameters critical from a breeder's point of view.

DISCUSSION

In this study, we explored the potential use of rice gene encoding an aspartate protease for the genetic engineering of enhanced resistance to a spectrum of diseases in rice and *Arabidopsis*. A large number of AP (>400) have been identified in silico in the rice genome (MEROPS peptidase database). However, their physiological roles are largely unknown. A rice CDR1 homologue (OsCDR1) was identified from a phylogenetic tree, generated by neighbor joining, using *Arabidopsis* CDR1 as bait. Our studies showed that *OsCDR1* was transcriptionally activated in response to treatments with BTH or SA but not with JA. Previous studies have demonstrated that BTH is effective in inducing disease resistance and in activating defense responses in rice (Gorlach et al. 1996; Xiong et al. 2001). Unlike most dicot plants, rice plants normally contain relatively high constitutive levels of free SA (Silverman et al. 1995). However, in spite of high endogenous levels, rice leaves have an SA-inducible glucosyl transferase activity (SA-GTase), an enzyme that conjugates SA (Silverman et al. 1995), suggesting that rice leaves can respond to exogenous SA. Whether SA is a signal molecule in rice disease resistance response has not been well established,

Fig. 4. Response of defense-related genes in transgenic plants by quantitative polymerase chain reaction analysis. Fold expression of **A**, *PR1* and **B**, *PR2* genes in transgenic *Arabidopsis* lines. Fold expression of **C**, *PBZ1/PR10* and **D**, *PR1a* in transgenic rice lines. **E**, Response of 12 *OsPR1* genes in transgenic rice line R6 compared with the wild type (WT). Bars indicate mean $\pm$ standard deviation.

although exogenous application of SA to rice plants was found to induce various genes that play important roles in different stress responses (Xu et al. 2006; Li et al. 2008).

Functional validation of *OsCDR1* following identification through bioinformatics was achieved in transgenic *Arabidopsis* lines that ectopically expressed *OsCDR1*. Interestingly, *OsCDR1:Arabidopsis* accumulated high levels of SA and showed several-fold induction of the defense-related genes *PR1* and *PR2* but not of *PDF1.2*, a marker of JA-mediated responses. The accumulation of SA is an important step in the signal transduction pathway leading to SAR, disease resistance, and susceptibility (Delaney et al. 1994). It has been proposed that at least one of the mechanisms of SA effect is through upregulation of levels of active oxygen species such as H_2O_2 (Chen et al. 1993b). Leaves treated with SA have been shown to accumulate H_2O_2 in *Arabidopsis* and rice (Chen et al. 1993a, Ganesan and Thomas 2001). Interestingly, the transgenic plants also exhibited oxidative bursts leading to generation of H_2O_2 which have been previously shown to be associated with *PR1* gene expression at a step in signal transduction upstream of SA (Bi et al. 1995). The high level of *PR* gene expression in *OsCDR1*-transgenic plants suggests a role for *OsCDR1* in SA-mediated disease resistance signaling pathways. This is further supported by the observation that overexpression of *OsCDR1* in transgenic *Arabidopsis* plants enhanced resistance against infection by *P. syringae* and *H. arabidopsidis* but not against *A. brassicicola*. Both *P. syringae* and *H.*

Fig. 6. *OsCDR1* function requires aspartic protease activity. **A**, Immunodetection of *OsCDR1*; reverse-transcriptase polymerase chain reaction analysis of **B**, *OsCDR1* and **C**, *PR1* in *Arabidopsis* transformed with wild-type (WT) *OsCDR1* or with mutated *OsCDR1* in which either an active site aspartate (D119N) or a residue away from the active site (D233N) has been replaced by asparagine.

Fig. 5. Tests of transgenic plants for resistance to pathogens. **A**, Bacterial growth in leaves of wild-type (WT) and transgenic *Arabidopsis* lines infiltrated with virulent *Pseudomonas syringae* pv. *tomato* DC3000 was assayed at 2 days postinoculation (dpi). **B**, Number of conidia in leaves of WT and transgenic *Arabidopsis* lines inoculated with a conidial suspension of *Hyaloperonospora arabidopsidis* at 5 dpi. **C**, Disease severity in WT and transgenic *Arabidopsis* lines challenged with *Alternaria brassicicola* at 5 dpi. **D1**, Challenged transgenic rice lines showed resistance towards *Xanthomonas oryzae* in whole-plant infection assay in greenhouse. **D2**, Length of diseased area was measured at 12 dpi. **E1**, Challenged transgenic rice lines showed resistance toward blast disease in whole-plant infection assay in greenhouse. **E2**, Infected leaf area was measured as percent diseased leaf area (%DLA) at 10 dpi. Bars represent mean $\pm$ standard deviation.

arabidopsidis have biotrophic phases of pathogenicity whereas *A. brassicicola* is a necrotrophic pathogen. Expression of *PR* genes and the SA-dependent signaling pathway are involved in the regulation of defense responses against biotrophic pathogens, while the *PDF1.2* gene and the JA and ethylene signaling pathway are involved in modulating defense responses against necrotrophic pathogens (Gupta et al. 2000; Kunkel and Brooks 2002; Spoel et al. 2003).

In *Arabidopsis*, overexpression of *AtCDR1* causes dwarfing (Xia et al. 2004) whereas, out of four transgenic lines studied, only one line (At17) showed growth retardation and an early leaf senescence phenotype. The leaf senescence phenotype observed in the At17 transgenic line might be due to higher expression of *OsCDR1* or the nature of the T-DNA integration site.

OsCDR1 activation is likely to lead to the generation of an endogenous extracellular peptide elicitor, as has been proposed for *Arabidopsis* *CDR1* (Xia et al. 2004). Indeed, local infiltration of IF from *OsCDR1:Arabidopsis* plants into WT *Arabidopsis* leaves induced the systemic defense response, and an elicitor activity was detected in the IF. The hypothesized elicitor released by *OsCDR1* can rapidly activate basal defense responses in local and systemic leaves, suggesting a high capacity for mobility.

Further, evaluation and extension of this approach to the rice system is of high agronomical importance because rice is susceptible to a broad spectrum of diseases. Hence, transgenic rice plants overexpressing *OsCDR1* were generated and analyzed for the expression of various defense-related genes and for their resistance to *M. oryzae* and *X. oryzae*.

Expression of the different *PR* proteins is commonly used as a marker for SAR. Although activation of *PR* protein-encoding genes by compatible pathogens evidently does not lead to resistance in the plant against the invading pathogen, these genes may be effective if activated before the challenge inoculation occurs. In some cases, constitutive overexpression of *PR* genes

could further enhance resistance to certain pathogens (Jach et al. 1995; Eppele et al. 1997). Hence, we overexpressed *OsCDR1* under a constitutive promoter which leads to activation of defense-related genes, including *PBZ1/PR10* and *PR1*. Although variable levels of *OsCDR1* and defense-related genes were observed among the transgenic lines, there was a correlation between the levels of *OsCDR1* and the degree of induction of defense-related genes. In rice, *OsPR1* genes are induced through the coordinated action of SA-, JA-, and ACC-signaling pathways and underlie the SAR response (Mitsuhara et al. 2008). Expression characteristics of the 12 *OsPR1* genes indicate that the resistance obtained by *OsCDR1*:transgenic rice might be due to SA- but not JA- or ACC-mediated disease resistance signaling.

The fact that overexpression of *OsCDR1* in *Arabidopsis* and rice and previously reported *CDR1* in *Arabidopsis* showed elevated *PR* gene expression and acquired enhanced resistance against phytopathogens suggests the presence of similar defense pathways in *Arabidopsis* and rice. It is important to note that overexpression of *OsCDR1* in rice had no deleterious effects in our experimental conditions. This observation also satisfies the criteria of agricultural genetic engineering which include creating crops that are durable to stresses, require fewer chemical inputs, and do not show unwanted agronomic effects. These findings should greatly facilitate study on disease-resistance pathways in rice and other monocots, and may have a profound impact on controlling diseases in economically important crops such as cereals.

MATERIALS AND METHODS

Plant materials and treatments.

Arabidopsis thaliana ecotype Columbia (Col-0) plants were grown at 23°C under short-day conditions (9-h day length, 120

Fig. 7. Elicitor activity of *OsCDR1*. **A**, Immunodetection of *OsCDR1* in the crude intercellular fluids (IFs) and two fractions of the IFs from At17. **B**, Reverse-transcriptase polymerase chain reaction (PCR) analysis of *OsCDR1* expression in a wild-type (WT) *Arabidopsis* leaf infiltrated with crude IFs and three fractions of the IFs (high molecular weight [HMW] >10 kDa and low molecular weight [LMW] <10 kDa to >3 kDa and <3 kDa). The elicitor in the LMW fraction has a molecular weight of 3 to 10 kDa. **C**, Responses of *PR2* gene expression in a WT *Arabidopsis* leaf infiltrated with crude IFs and two fractions (HMW and LMW) of the IFs. The infiltrated primary (1^o) and untreated secondary (2^o) leaves were collected separately and subjected to quantitative PCR analysis. IFs of the *AtCDR1*-overexpressing plant was kept as a positive control. Bars represent mean $\pm$ standard deviation.

$\mu\text{mol m}^{-2} \text{ s}^{-1}$) for 5 weeks, followed by growth under long-day conditions (16 h of light and 8 h of darkness) until they matured. Indica rice cv. Pusa Basmati 1 (PB 1) was maintained in the greenhouse (28 and 23°C, day and night, respectively). Treatments with BTH and JA (Sigma-Aldrich, St Louis) were performed as described previously (Delaney et al. 1994). For SA treatments, rice seedlings were dipped in 500 ml of 3 mM sodium salicylate with sterilized distilled water as a control.

Plasmid constructions.

OsCDR1 was amplified by PCR from PAC clone P0509B06 using the primers 5'-ATGGCGGCCATGGCGCCTTCCTC-3' and 5'-CTAGGATGAGCCGGCGCAGTCGGCG-3'. The PCR product was gel purified and cloned into the *EcoRV* site of pBluescript KS⁺ to obtain pKSOs*CDR1* and its sequence was verified. The *OsCDR1* insert was excised using *XbaI* and *XhoI* and cloned into pRTdS vector digested with the same enzymes, creating plasmid pD35SOs*CDR1*. The 35SOs*CDR1* gene cassette was excised from pD35SOs*CDR1* using *HindIII* and was cloned into *HindIII* site of the binary vector pCambia-1305.2 to create pD35SOs*CDR1*-T, which was mobilized into *Agrobacterium tumefaciens* strains LBA4404 and GV3101mp90 for rice and *Arabidopsis* transformation, respectively.

OsCDR1 site-directed mutants.

For the construction of *OsCDR1* mutants, pKSOs*CDR1* was used as a template for PCR. For D119N and D233N in which the aspartate residue at positions 119 or 233 was replaced by asparagine, mutation was carried out using the primers 5'-GCCATCGCCACACGGGCA-3' and 5'-CGGTAGCGGTG TGCCCGT-3', respectively. The whole plasmid was amplified with Phusion DNA polymerase (Finnzymes, Espoo, Finland).

Following amplifications, the reaction product was digested with *DpnI* and transformed into *Escherichia coli* (DH5 α). After confirming the mutations by DNA sequencing, mutated pKSOs*CDR1* was excised with *HindIII* and *EcoRI* and cloned into the binary vector pG0229 (Hellens et al. 2000) digested with the same enzymes, creating plasmid pG0229-D119N and pG0229-D233N. Constructs were mobilized into the *A. tumefaciens* GV3101mp90 by electroporation.

Sequence analysis.

Similarity searches on nucleotide and amino acid sequences were carried out using BLAST at the National Center for Biotechnology Information GenBank database. A phylogenetic tree was constructed by the neighbor-joining method based on the genetic distance of the protein sequences by the Clustal X version 1.81.

Generation of transgenic plants.

Transformation of *Arabidopsis* was performed using the floral dip method as described previously (Clough and Bent 1998). Rice calli were used for transformation as described by Hiei and associates (1994).

RNA extraction, RT-PCR, and qPCR.

Total RNA was extracted using Trizol Reagent (Invitrogen Life Technologies, San Diego, CA, U.S.A.) as per the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using superscript II reverse transcriptase (Invitrogen Life Technologies) under conditions recommended by the manufacturers and used as a template for RT-PCR and qPCR. qPCR was conducted using SYBR Green dye either on a LightCycler system for real-time PCR (Roche Applied Science, Indianapolis, IN, U.S.A.) or SYBR Green

Fig. 8. Phenotypes of *OsCDR1:Arabidopsis* lines. Transgenic *Arabidopsis* lines At3, At6, and At8 appeared normal whereas line At17 showed growth retardation compared with the wild type (WT). The pictures were taken 4 weeks after sowing.

JumpStart (Sigma-Aldrich) in a DNA engine Opticon2 (MJ Research, Waltham, MA, U.S.A.) following the manufacturer's instructions. Values were normalized using actin as the internal reference, and fold change in the expression level was calculated (Livak and Schmittgen 2001).

Indirect immunolocalization.

Indirect immunolocalization studies were performed as described by Patkar and Chattoo (2006).

Extraction and quantification of SA.

SA was extracted and analyzed as described (Raskin et al. 1989). Chromatographic separation was achieved by 0.1% formic acid in water versus 0.1% formic acid in methanol using a 3- μ m C18 (2) column (100 by 2 mm; Luna, Phenomenex, Cheshire, U.K.) operated at 23°C, with a 250- μ l/min flow rate. SA concentrations were measured using negative electrospray mass spectrometry on a single quadrupole liquid chromatography-mass spectrometry system (Agilent 1100), measuring in single-ion mode, 137.0 and 141.1 m/z, fragmentor voltage of 70 V; and the spray chamber conditions were 10 liter min⁻¹ drying gas at 350°C, 25 psi nebulizer pressure, 3,500-V capillary voltage.

Detection of reactive oxygen intermediate.

DAB staining was performed as described previously (Alvarez et al. 1998).

Immunoblot analysis.

Anti-OsCDR1 polyclonal antibodies were raised against a synthetic peptide (SSAAAPTQFDPSRSSTYG). Immunoblotting was performed according to standard protocols (Sambrook et al. 1989). The resulting antibodies cross-reacted with the *OsCDR1* product but showed no cross-reactivity with *AtCDR1* product.

Disease assays.

For disease testing, the abaxial side of *Arabidopsis* leaves from 4- to 5-week-old plants were infiltrated using a 1-ml syringe with either 10 mM MgCl₂ (mock) or *P. syringae* DC3000 (10⁶ CFU ml⁻¹ in 10 mM MgCl₂) as previously described (Cameron et al. 1994). *H. arabidopsidis* assays were performed on 4-week-old plants by spraying with a conidial suspension (4 $\times$ 10⁴ conidia ml⁻¹ in sterile water) of *H. arabidopsidis* isolate Noco2 to run-off. Following inoculation, seedlings were incubated at 16°C and conidia were counted at 5 dpi. Inoculation with *Alternaria brassicicola* was done by applying 5 μ l of spore suspension (1 $\times$ 10⁶ spores ml⁻¹) in water on four leaves of 4-week-old plants. Inoculated plants were kept under a transparent cover to maintain high humidity and transferred to a growth chamber (24°C, 12 h of light and 12 h of darkness). Lesions that developed on inoculated leaves were measured at 5 dpi. An in planta *X. oryzae* pv. *oryzae* inoculation assay was done by the "scissors clip-inoculation" method (Kauffman et al. 1973). Plant infection assays with *M. oryzae* (isolate B157; international race IC9) were carried out as described before (Prasad et al. 2008).

IF.

Sterilized (10% bleach, 20 min) At17 *Arabidopsis* seed (approximately 10 mg) were grown in liquid MS medium in 250-ml Erlenmeyer flasks at 100 rpm for 2 weeks. Then, the medium was decanted and the seedlings were washed twice with sterile water and 100 ml of sterile water was added to the flask and swirled overnight at 100 rpm. Following filtration through a 38- μ m filter, the water, containing apoplastic solutes, was fractionated (10-kDa cut-off; Vivaspin 20; Sartorius, Goettingen,

Germany). The filtrate and the retentate were collected and labeled as LMW and HMW fractions, respectively. The LMW fraction was further fractionated using a 3-kDa cut-off filter.

ACKNOWLEDGMENTS

The present work was funded by the U.K. Biotechnology and Biological Sciences Research Council and by a grant from the Indo-UK Collaboration between the Department of Biotechnology, Ministry of Science and Technology, Government of India and the U.K. Government Global Opportunities Fund. We thank MAFF DNA Bank, National Institute of Agrobiological Sciences (NIAS), Japan for providing the PAC clone and L. Hill for assistance with SA assays.

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