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# Rice *OsACDR1* (*Oryza sativa* Accelerated Cell Death and Resistance 1) Is a Potential Positive Regulator of Fungal Disease Resistance

**Jung-A Kim, Kyoungwon Cho[1], Raksha Singh, Young-Ho Jung, Seung-Hee Jeong, So-Hee Kim, Jae-eun Lee, Yoon-Seong Cho, Ganesh K. Agrawal[2], Randeep Rakwal[2,3], Shigeru Tamogami[4], Birgit Kersten[5], Jong-Seong Jeon[6], Gynheung An[7], and Nam-Soo Jwa***

**Rice *Oryza sativa* accelerated cell death and resistance 1 (*OsACDR1*) encodes a putative Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK). We had previously reported upregulation of the *OsACDR1* transcript by a range of environmental stimuli involved in eliciting defense-related pathways. Here we apply biochemical, gain and loss-of-function approaches to characterize *OsACDR1* function in rice. The OsACDR1 protein showed autophosphorylation and possessed kinase activity. Rice plants overexpressing *OsACDR1* exhibited spontaneous hypersensitive response (HR)-like lesions on leaves, upregulation of defense-related marker genes and accumulation of phenolic compounds and secondary metabolites (phytoalexins). These transgenic plants also acquired enhanced resistance to a fungal pathogen (*Magnaporthe grisea*) and showed inhibition of appressorial penetration on the leaf surface. In contrast, loss-of-function and RNA silenced *OsACDR1* rice mutant plants showed downregulation of defense-related marker genes expressions and susceptibility to *M. grisea*. Furthermore, transient expression of an OsACDR1:GFP fusion protein in rice protoplast and onion epidermal cells revealed its localization to the nucleus. These results indicate that *OsACDR1* plays an important role in the positive regulation of disease resistance in rice.**

## INTRODUCTION

Although lacking specialized somatic immune cells, plants have developed highly sophisticated mechanisms to cope with a range of adverse environments and stresses, including pathogen attacks. The mitogen-activated protein kinase (MAPK) cascade is a critical component of the plant self-defense mechanism (Akira et al., 2006; Chisholm et al., 2006). Perceiving external stimuli, the MAPK cascade is activated by three sequential phosphorylation events affecting a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAPK) (Ligterink and Hirt, 2001; Nakagami et al., 2005). Activation of the MAPK cascade is known to trigger a series of reactions involving the generation of reactive oxygen species (ROS), lignifications of cell walls, callose deposition, phytoalexin accumulation and the induction of pathogenesis-related (PR) genes, which culminates in a hypersensitive response (HR)-like cell death (Asai et al., 2002; Dangl and Jones, 2001; Pedley and Martin, 2004; Pieterse and Dicke, 2007).

In the genome of *Arabidopsis thaliana*, 60 MAPKKKs, 10 MAPKKs and 20 MAPKs have so far been assigned (Ichimura et al., 2002). The most heterogeneous and largest group of those, the MAPKKKs have been divided into two subgroups (Nakagami et al., 2005): i) the family of MEKK-like kinases including AtMEKK1 and AtANP1 in *Arabidopsis*, NtMAPKKK$\alpha$ and NtNPK1 in tobacco, LeMAPKKK$\alpha$ in tomato, and ii) the family of Raf-like kinases including AtCTR1 (Constitutive Triple Response 1) and AtEDR1 (Enhanced Disease Resistance 1) in *Arabidopsis*, and LeCTR1 and LeCTR2 in tomato. The *Arabidopsis edr1* mutant showed an enhanced resistance to bacterial and fungal pathogens, accelerated senescence in response to ethylene treatment and spontaneous HR-like lesion development under drought stress in the absence of a pathogen. This suggests that AtEDR1 functions as a negative regulator of disease resistance and ethylene-induced senescence (Frye and Innes, 1998; Frye et al., 2001; Tang et al., 2005). In tomato, the overexpression of the LeCTR2 *N*-terminus resulted in altered growth patterns, increased ethylene responses and enhanced susceptibility to a fungal pathogen (Lin et al., 2008). Furthermore, using a yeast two-hybrid system, it was shown

Department of Molecular Biology, College of Natural Science, Sejong University, Seoul 143-747, Korea, [1]Environmental Biology Division, National Institute for Environmental Studies (NIES), Tsukuba 305-8506, Ibaraki, Japan, [2]Research Laboratory for Biotechnology and Biochemistry, GPO Box 8207, Kathmandu, Nepal, [3]Health Technology Research Center (HTRC), National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8569, Ibaraki, Japan, [4]Laboratory of Biologically Active Compounds, Department of Biological Production, Akita Prefectural University, Akita 010-0195, Japan, [5]Max Planck Institute for Molecular Plant Physiology, GabiPD Team, Bioinformatics, D-14424, Potsdam-Golm, Germany, [6]Graduate School of Biotechnology and Plant Metabolism Research Center, Kyung Hee University, Yongin 446-701, Korea, [7]National Research Laboratory, Department of Life Science and Center for Functional Genomics, Pohang University of Science and Technology, Pohang 790-784, Korea
*Correspondence: nsjwa@sejong.ac.kr

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that LeCTR2 interacts preferentially with ETR1-type ethylene receptors such as LeETR1 and LeETR2. LeCTR2 was also shown to be involved in ethylene signaling and negative regulation of the disease resistance pathway (Lin et al., 2008).

Within the rice genome (*Oryza sativa* L.), at least 1 putative MAPKKK (Kim et al., 2003), 8 MAPKKs and 17 MAPKs are known so far. To our knowledge, the total number of MAPKKKs in rice remains unknown. Previously, we reported the cloning of rice MAPKKK, named *OsEDR1*, based on a structural similarity to AtEDR1 in *Arabidopsis* and LeCTR2 in tomato with 46.5% and 46% amino acid similarity (Kim et al., 2003). The transcriptional profiling of *OsEDR1* against diverse environmental stresses including elicitors involved in plant defense responses showed its upregulation at the mRNA level against these stimuli. Among these elicitors, jasmonic acid (JA), salicylic acid (SA), ethephon generating ethylene, and chitosan are well known for their involvement in regulating plant defense responses (Bari and Jones, 2009; Jwa et al., 2006; Wasternack, 2007). This preliminary work (Kim et al., 2003) led us to hypothesize a possible role for *OsEDR1* in rice plant defense mechanisms.

In the present study, we have performed a functional analysis of the *OsEDR1* gene using biochemical, gain and loss-of-function approaches. Our results suggest that *OsEDR1* is potentially involved in lesion development and functions as a positive regulator of disease resistance in rice. Based on our findings, we have also renamed *OsEDR1* as *Oryza sativa* accelerated cell death and resistance 1 (*OsACDR1*).

## MATERIALS AND METHODS

### Plant materials and growth

Rice plants (*Oryza sativa* L. japonica-type cvs. Hwayoungbyeo, Dongjinbyeo, Nipponbare, Ilmibyeo, Choocheongbyeo, Nampyungbyeo and Ohdeabyeo) were grown in commercial rice soil in pots and maintained in a greenhouse (under natural light and temperature conditions) for growth and seed harvest. Selected cultivars were also grown in a growth chamber under white fluorescent light (wavelength 390-500 nm, -150 $\mu$mol.m$^{-2}$.s$^{-1}$, 12 h photoperiod) at 28°C and 70% relative humidity, and the temperature was shifted to 25°C that is optimum for inoculation with rice blast fungus (Jung et al., 2006).

### Mutant analysis

The *OsACDR1* mutant of cv. Hwayoungbyeo was screened from 100,000 mutant lines of cvs. Dongjinbyeo and Hwayoungbye. These mutant lines were maintained by the Pohang University of Science and Technology (Korea). The *osacdr1* KO mutant plants and corresponding wild-type (WT) (cv. Hwayoungbyeo) were grown in a greenhouse for phenotypic observations and seed harvesting. These plants were also grown in a growth chamber for inoculation with *M. grisea*.

### Vector constructs and rice transformation

*Agrobacterium*-mediated transformation technique was used for Hwayoungbyeo and other rice cultivars. *Agrobacterium* strain LBA 4404 was co-cultured with rice callus using standard protocol (Hiei et al., 1994). Full cDNA clones of *OsACDR1,* 3311 bp (Kim et al., 2003) including 3051 bp of open reading frame (ORF) in the pBluescript SK(-) vector were digested with *Not*I and blunt ended with Klenow enzyme redigested with *Kpn*I. The fragment was inserted into a *Sac*I blunt ended and *Kpn*I digested multicloning site of pCAMBIA 1300 vector (http://www.cambia.org/daisy/cambia/585.html) for constitutive over-expression under the control of the CaMV 35S promoter. The resulting overexpression construct was termed *OsACDR1*-OE. The GATEWAY system (Invitrogen) was used to generate the *OsACDR1*-RNAi construct. For this, a 450-bp fragment of *OsACDR1* cDNA of the 3′ end was amplified using primers Rmap-F (forward) and Rmap-R (reverse) and subcloned into pGEM-T (Promega, http://www.promega.com), resulting in the vector *OsACDR1* pGEM-T. Upon sequence confirmation, the *OsACDR1* PGem-T vector was amplified using primers attB1 and attB2, and the resulting attB-PCR products were subcloned into the Gateway pDONR cloning vector by a BP clonase reaction (Invitrogen, http://www.invitro-gen.com). These entry clones were inserted into the destination vector, pB7GWIWG2 (II) (VIB-Ghent University, Belgium) using LR clonase reactions (Invitrogen). The resulting RNAi construct was named *OsACDR1*-RNAi. To construct KO complementation vectors, full-length cDNA fragments of *OsACDR1* were digested with *Xho*I and *Not*I and inserted into the same sites of the pBluescript SK(-) vector. The 1,970 bp promoter was digested with *Bam*HI and promoter fragments were inserted into the *Xho*I sites of previously prepared full-length cDNA of *OsACDR1* in pBluescript SK(-) vector. The additional extra 109 base pairs were deleted with site-directed mutagenesis using the primer OsACDR1-FUS for recovering WT 5′-UTR and translational start codon. The 1,240 bp promoter and full length ORF of *OsACDR1* was digested first with *Not*I, Klenow treated and digested with *Spe*I. The resulting product was inserted into *Hin*dIII and *Xba*I sites of pCAMBIA 3301 vectors, resulting in the complementation construct *OsACDR1*-Com. *OsACDR1*-OE transgenic plants were selected for hygromycin resistance, whereas *OsACDR1*-RNAi and *OsACDR1*-Com plants were selected for phosphinotricin resistance. The genotypes of regenerated plants were confirmed by PCR using resistance specific primers: Bar-F and Bar-R for the *BAR* gene, and HPT-F and HPT-R for *HPT* gene. All primer combinations are given in Table 1.

### Semi-quantitative RT-PCR analysis, phytoalexin determination and immunoblot analysis

RNA isolation and RT-PCR analysis were performed as described previously (Lee et al., 2006; Rakwal et al., 2008). Briefly, first-strand cDNA was synthesized in a 50 $\mu$l reaction mixture using a StartaScript RT-PCR Kit (Stratagene, USA) with 10 $\mu$g total RNA isolated from the leaves of rice plants. Specific primers were designed from the 3′-UTR regions (forward and reverse primer sequences are provided in Table 1) of each of the genes used in this study by comparison and alignment with all available related genes in the databases, NCBI and KOME (Knowledge-based Oryza Molecular Biological Encyclopedia, http://cdna01.dna.affrc.go.jp/cDNA/). The rice phytoalexins sakuranetin and momilactone A in leaves were analyzed and concentrations estimated using a liquid chromatography-tandem mass spectrometry technique as previously established and described (Tamogami et al., 1997). Three independent biological replicates from VC and OE1 (without and with lesions) plants were used for phytoalexins estimation.

### Pathogen infection

The *M. grisea* strain PO6-6 (compatible) was obtained from Kyung Hee University, Korea. The KJ401 (89-010) strain (incompatible) was provided by the Rural Development Administration (RDA), Korea. Culturing of fungal pathogen, spore production and inoculation, and assessment of disease severity was performed as described previously (Jung et al., 2006). Disease severity was measured according to the rice blast disease index along with the student's test ($P < 0.05$) which was used for statistical analysis of the results. Two-week-old rice plants (cvs. Hwayoungbyeo, Dongjinbyeo, Nip-

**Table 1.** The primer combinations used in this study

| Forward primer | | Reverse primer | | No. of PCR cycles |
|---|---|---|---|---|
| Primer name (gene) | Nucleotide sequence (5′-3′) | Primer name | Nucleotide sequence (5′-3′) | |
| RJSR343 (*OsAPX1*) | GACAAGAAACCCTCTGCAGTTT | RJSR343 (*OsAPX1*) | GTAGTCTGCTGGTTCACACTGG | 30 |
| RJSR345 (*OsAPX2*) | GTCCAACCCTTTGATCTTTGAC | RJSR345 (*OsAPX2*) | CCACGACAGTCTTGATCGTACT | 30 |
| RJSR349 (*OsPAL1*) | GAGGAGTGCAACAAGGTGTTC | RJSR349 (*OsPAL1*) | CTATAAACCAAACGGCAACGA | 30 |
| RJSR29 (*OsPR1b*) | GGAGAAGGGCTCCTACGACTAC | RJSR29 (*OsPR1b*) | GCGCATATATATCTACYGAGAGCA | 30 |
| RJSR63 (*OsPR10a*) | ATGTCCTAAAGTCGGATGTGCT | RJSR63 (*OsPR10a*) | ACATTTCTGCGGCTCTCATTAT | 30 |
| *OsActin*-F | TCCATCTTGGCATCTCTCAG | OsActin-R | GTACCCGCATCAGGCATCTG | 25 |
| Rmap-F | AAAAAGCAGGCTCTTGTGATTGGTGAAAGGATTGG | Rmap-R | AGAAAGCTGGGTGCAATTAGGACGGTGAAGGATCT | 30 |
| attB1 | GGGGACAAGTTTGTACAAAAAAGCAGGCT | attB2 | GGGACCACTTTGTACAAGAAAGCTGGGT | 30 |
| Bar-F | CATCGCAAGACCGGCAACAGGATTCAA | Bar-R | GCTCCACTGACGTTCCATAAATTCCCC | 30 |
| HPT-F | GTAAATAGCTGCGCCGATGG | HPT-R | TACTTCTACACAGCCATCGG | 30 |
| *OsACDR1*-F | GCCATTGATACCACCTTACAGG | *OsACDR1*-R | ATGTATGGTGTATCAGTGCGC | 25 |
| OsACDR1-FUS | AGCGGAGACGTGGTTGACGAGATGAAGAATCTGTTCAAGAGT | | | |
| *rMAP-B-SDM* | *GGGCCGCAAATTCGGGTCTTTCTGCCAGCA* | | | |
| *OsACDR1*-KpnI-F | GGGTACCATGAAGAATCTGTTCAAGA | *OsACDR1*-*Bam*HI-R | TGGATCCAGGGGTGGAAGAATTCACC | 30 |

ponbare and other transgenic lines) were used to perform the pathogenicity test. Pathogenicity tests were repeated more than 10 times.

**Microscopic observation**

Phenolic compounds, spore germination, hyphal growth visualization and GFP fluorescence in leaves were observed according to previously described procedures (Jung et al., 2006).

**Solid-phase kinase assay**

A previously prepared full-length cDNA of *OsACDR1* in the pBluescript SK(-) vector was used for expression vector construction. An internal *Bam*HI site 130 bp from *OsACDR1* stop codon was deleted using site-directed mutagenesis with the rMAP-B-SDM primer. The full length *OsACDR1* ORF was amplified using primers with *Kpn*I and *Bam*HI adaptors (OsACDR1-KpnI-F and OsACDR1-*Bam*HI-R) and cloned into pBluescript SK(-) vector. The *OsACDR1* full cDNA clone was digested with *Bam*HI and inserted into the *Bam*HI site of the pcDNA3 vector containing a HA-tag. It was then transfected into mammalian cells (293FT) for recombinant protein expression. Recombinant HA-OsACDR1 protein was then subjected to immunoblotting using an anti-HA antibody and affinity purified using protein G agarose beads. The affinity purified fusion protein, HA-OsACDR1 (100 kDa) was incubated at 30°C for 30 min in a 25 μl reaction mixture containing kinase buffer (25 mM Tris-HCl, pH 7.5/0.2 mM EDTA/5 mM $MgCl_2$/4 mM 2-mercaptoethanol), 0.1 mM [$\gamma^{-32}$P] ATP(2,000-5,000 cpm/pmol), 2 g myelin basic protein [MBP (Invitrogen)] as an artificial substrate. Phosphorylation was initiated by adding [$\gamma^{-32}$P]ATP, and terminated by placing the mixture on ice. Proteins were fractionated on 12% SDS-PAGE and stained with Coomassie brilliant blue (CBB). The level of $^{32}$P incorporation was determined by autoradiography on an X-ray film.

**OsACDR1 protein localization analysis**

A GFP fusion protein was constructed using the full-length *OsACDR1* cDNA clone with an *N*-terminal fusion of the EGFP clone under the control of CaMV 35S promoter. For rice protoplast preparation, leaves from the two-week-old WT was cut into 0.5-1 g, 5-10 $mm^2$ pieces by razor blade. Cut pieces were vacuum infiltrated for 2 min at 15 mm Hg and then incubated in a cell wall degrading enzyme mixture (0.5 M mannitol, 2% cellulose-RS, YAKULT HONSHA, Japan, 0.05% pectolyase Y-23, KYOWA, Japan, pH 5.8). Cell wall degradation was carried out in the dark at 32°C for 1-2 h with gentle rotation (50-75 rpm). The reaction solution was filtered through 100 μm mesh (Sigma, USA) for protoplast separation. Transformation of the protoplasts was carried out as described previously by Baur et al. (2005) using 30 μg of DNA per transformation. Plasmid DNAs were transformed into onion (*Allium cepa*) epidermal cells by a helium biolistic particle delivery system (Bio-Rad) following a previously described protocol (Lee et al., 2006). After incubation for 12-48 h at 28°C, the subcellular distribution of the EGFP fusion protein was examined with a fluorescence microscope (Zeiss, Germany, FITC-BP:450-490 nm, FT:510 nm, LP:515 nm).

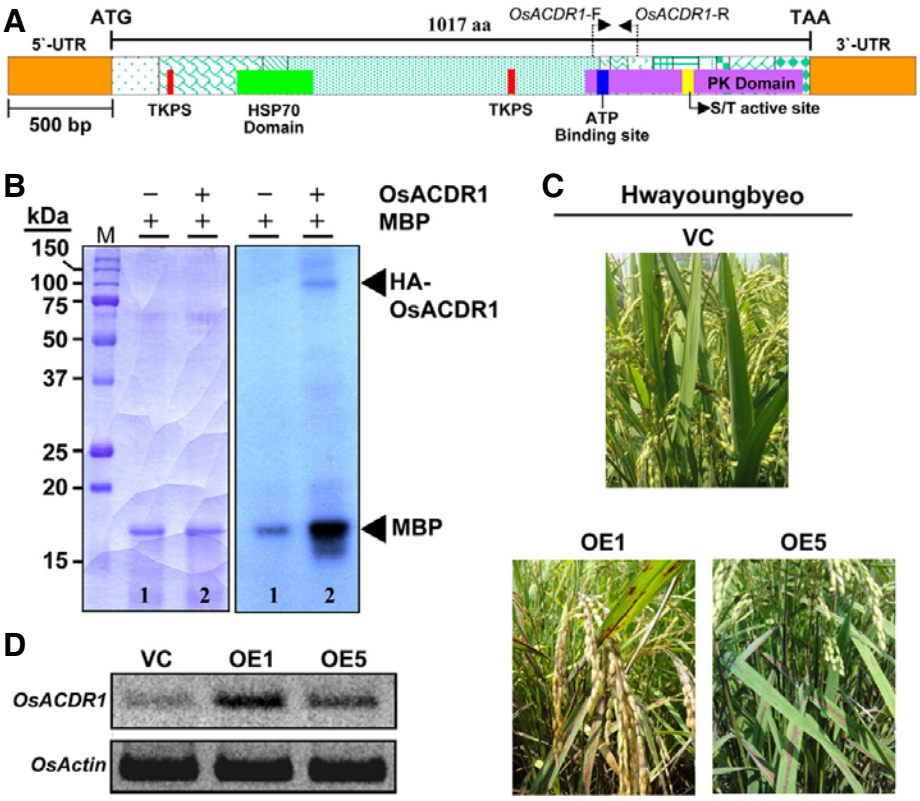


**Fig. 1.** Structural features of *Os ACDR1*, kinase activity and *Os ACDR1* overexpression in rice. (A) Schematic representation of the cDNA encoding *OsACDR1*. Individual exons are depicted by boxed regions with different patterns. The 5′- and 3′-UTRs are shown along with the start (ATG) and stop (TAA) codons. See also at GabiPD, The GABI Primary Database: http://www.gabipd.org/database/cgibin/GreenCards.pl.cgi?BioObjectId=1996960&Mode=ShowBioObject. (B) Solid-phase kinase assay. MBP was used as substrate of affinity-purified fusion protein *HA-OsACDR1*. Left and right gel images represent CBB staining and autoradiograph of the gel, respectively. +/- symbols represent the presence or absence of the respective substrate. (C) Transgenic rice (cv. Hwayoungbye) plants overexpressing *OsACDR1* under CaMV 35S promoter display HR-like lesion phenotype. OE1 and OE5 lines (75-80 days after planting) were selected as representative for strong and mild lesion phenotypes, respectively. The presence of lesions in almost all leaves of mature OE1 and OE5 can be seen. (D) *OsACDR1* transcript in overexpression lines. Semi-quantitative RT-PCR analysis was used to determine OsACDR1 transcript levels in OE1 and OE5 plants using the *OsACDR1F* and *OsACDR1R* primers. *OsActin* was used as internal control. Abbreviations: CBB, coomassie brilliant blue; OE, overexpression; HSP, heat shock protein; M, molecular marker; MBP, myelin basic protein; PK, protein kinase; S/T, serine/threonine; TKPS, tyrosine kinase phosphorylation sites; UTR, untranslated region; VC, vector control.

## RESULTS

### Kinase activity and structural features of OsACDR1

The *OsACDR1* gene is composed of 13 exons and 12 introns (Fig. 1A), similar to the exon/intron numbers in the genomic structure of *AtEDR1* and *LeCTR2* (Lin et al., 2008). Phylogenetically, OsACDR1 is closely clustered with AtEDR1 and LeCTR2 having 46.5% and 46% homology at the amino acid level, respectively. The *N*-terminal region of OsACDR1 contains a HSP (heat shock protein) 70 domain (green), which is a conserved domain, known as a CN box in the CTR1-like proteins (Huang et al., 2003). The 2$^{nd}$ and 4$^{th}$ exons contain predicted tyrosine kinase phosphorylation sites (labeled TKPS; red). The C-terminal region contains an ATP binding site (blue) as well as a serine/threonine protein kinase active site (yellow) within the protein kinase domain (violet).

To test whether OsACDR1 possesses kinase activity, a solid-phase kinase assay was performed using MBP as a generic substrate (Fig. 1B). To express the OsACDR1 protein, an *OsACDR1* full-length cDNA clone was transfected into a mammalian cell line (293FT) under the control of a cauliflower mosaic virus (CaMV) 35S promoter. This was necessary because the *E. coli* system failed to express the OsACDR1 protein. The influenza virus hemagglutinin recombinant protein HA-OsACDR1 was immunoprecipitated with anti-HA antibodies followed by a kinase assay. Results revealed a strong band for MBP (17 kDa) and a weak band for OsACDR1 (100 kDa) in the presence of both MBP and purified recombinant OsACDR1 protein. In the absence of recombinant OsACDR1 protein, only a weak band for MBP was observed. These results indicate that the OsACDR1 protein has kinase activity with low autophosphorylation. The OsACDR1 expression was difficult to visualize by staining the gel with CBB. Therefore, Western blot analysis was used to confirm the identity with anti-HA antibodies (Supplementary Fig. 1).

### Functional characterization of *OsACDR1*

Three independent molecular approaches were used to investigate function of the *OsACDR1* gene in rice. These techniques comprised overexpression of full-length *OsACDR1* cDNA, search for loss-of-function rice mutants in a T-DNA population using reverse genetics, and silencing of the *OsACDR1* gene using RNAi. The Korean rice cultivar ‘Hwayoungbyeo’ was used for all of these approaches. Several other rice cultivars, including the Japanese cv. Nipponbare, were used to generate *OsACDR1* overexpression lines in order to analyze the functional conservation among cultivars.

### Overexpression of the *OsACDR1* gene results in HR-like lesions

Six homozygous $T_3$ transgenic lines of cv. Hwayoungbyeo were generated. Each line carrying a single T-DNA insertion in *OsACDR1* was confirmed by Southern blot analysis (Supplementary Fig. 2). The commonly observed phenotype among these independent lines was the spontaneous development of typical HR-like lesions on leaves in 4-week-old seedlings com-

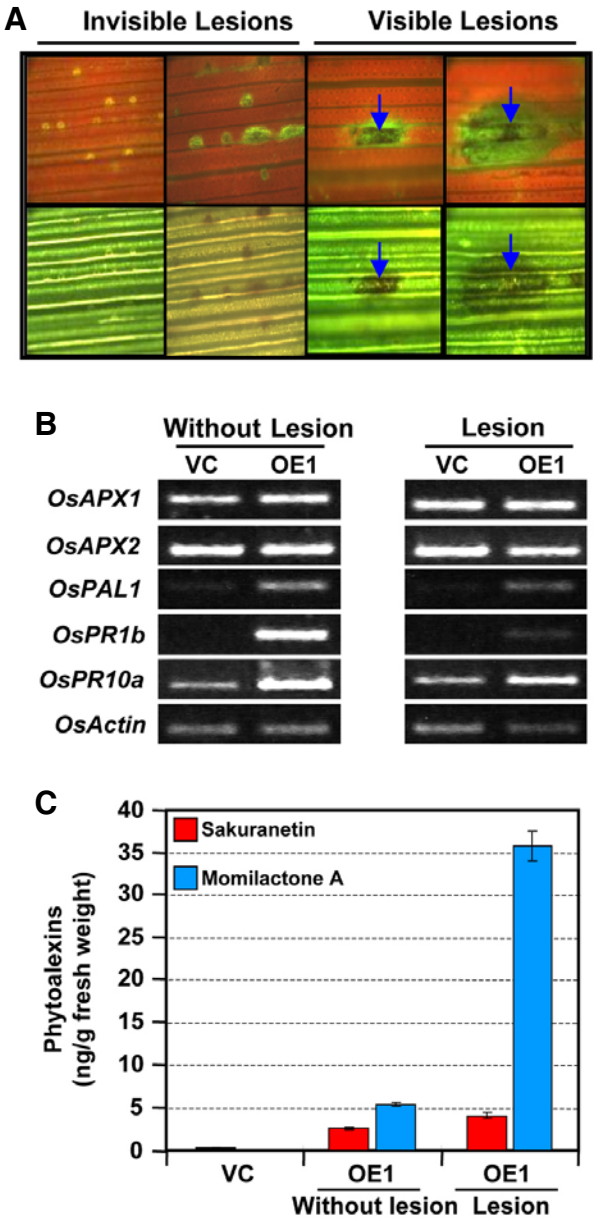


**Fig. 2.** Overexpression of *OsACDR1* up-regulates defense-related responses. (A) Accumulation of phenolic compounds. Fluorescence around invisible or visible lesions on the leaves represents accumulation of phenolic compounds. Blue arrows indicate lesions. (B) Expression analysis of defense-related marker genes in leaves. Total RNA isolated from leaves without and with lesions was used to monitor changes in gene expression using RT-PCR. (C) Phytoalexins determination. Phytoalexins were extracted and quantified as described in "Materials and Methods". Bars represent the mean and standard deviation (SD) of values obtained from three biological replicates.

pared to an absence of lesions in transgenic plants carrying the vector control (VC) (data not shown). The lesion phenotype was stably expressed and genetically inherited into the next generation. Lesion severity varied from line to line, so we divided these lines into strong and mild lesion phenotype categories. Two independent lines are shown in Fig. 1C. *OsACDR1* overexpressing line 1 (OE1) showed strong lesion development, whereas OE5 showed mild lesions in almost all leaves 75-80 days after planting, compared to healthy green leaves in the VC. Of note, OE1 and OE5 lines showed slightly different growth and developmental patterns as is expected between the transgenic lines. In general, these transgenic plants showed slight growth retardation, less tillering and consequently lower yields as compared to the VC. Compared to the mild lesion mimic transgenic lines, the strong lesion mimic transgenic lines gave lower yields. It is likely, that, the lesion mimic phenotype resulted in the induction of constitutive defense response requiring energy consumption that should have been otherwise used for growth, and subsequently grain production and yield. The HR-lesion phenotype was also observed in other rice cultivars overexpressing the *OsACDR1* gene. These rice cultivars included Nipponbare, Ilmibyeo, Choochungbyeo, Nampyungbyeo, and Ohdaebyeo for 42, 22, 13, 9, and 4 OE homozygous independent lines, strongly suggesting that *OsACDR1* overexpression causes the HR-like lesions. Semi-quantitative RT-PCR analysis of these OE lines showed higher expression (approximately 2-fold) of *OsACDR1* in OE1 compared to weak expression in OE5 (Fig. 1D).

### *OsACDR1*-OE plants accumulate phenolic compounds and secondary metabolites, and express enhanced levels of defense-related marker genes

HR-like lesions on leaves are known to be generally accompanied by an alteration in defense-related responses, such as the accumulation of phenolic compounds and changes in the expression of pathogen-related (PR) genes and secondary metabolites (Dangl and Jones, 2001; Jwa et al., 2006). Both OE1 and OE5 homozygous $T_3$ transgenic lines showed enhanced defense responses with respect to phenolic compound accumulation, upregulation of defense-related marker genes and phytoalexin production in the leaves. The results obtained from the OE1 plants are presented in Fig. 2. To examine whether overexpression of *OsACDR1* affects defense-related pathways, phenolic compounds, defense-related marker genes and phytoalexin accumulation were measured in leaves with and without lesions in VC and OE1 transgenic plants. Under UV light, pale green fluorescence was observed around necrotic lesions on the leaves, suggesting the accumulation of phenolic compounds within and around the affected areas (marked by arrows, Fig. 2A). Semi-quantitative RT-PCR analysis was performed on leaves (with and without apparent lesions) of VC and OE1 plants for phenolic compound-related (*OsPAL1*), oxidative stress-related (*OsAPX1* and *OsAPX2*), and PR (*OsPR1b* and *OsPR10a*) genes. Results showed that the transcripts of *OsPAL1*, *OsPR1b* and *OsPR10a* were strongly enhanced in OE1 compared to VC plants (Fig. 2B). Little or no change was observed in the transcript levels of oxidative stress-related *OsAPX1* and *OsAPX2* genes. No change was observed in actin gene expression, which was used as an internal control. Furthermore, sakuranetin and momilactone A, two major rice phytoalexins (van Etten et al., 1994), also significantly accumulated in OE1 plants (Fig. 2C). Negligible amounts of sakuranetin were detected in VC plants, whereas momilactone A was not detected in VC plants. Sakuranetin levels were increased by approximately 2-fold in OE1 plants before lesion formation, and by 4-fold after lesion formation. The production of momilactone A was also increased by more than 5-fold in OE1 plants before lesion formation and 32-fold after lesion appearance. Together these results indicate that *OsACDR1* overexpression influences multiple defense-related pathways causing induced defense responses in OE rice plants. Notably, these defense responses

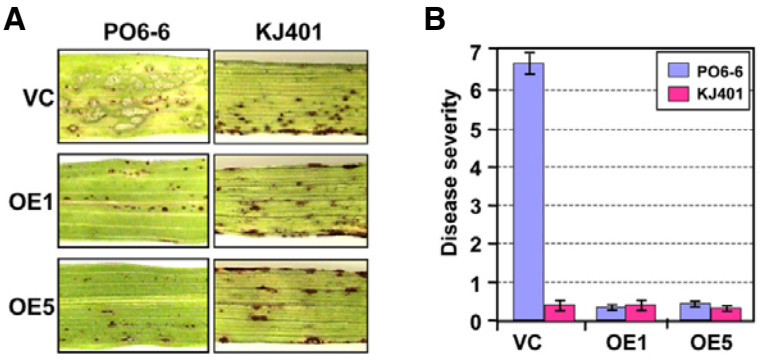


**Fig. 3.** *OsACDR1* overexpression confers broad-spectrum resistance to blast pathogen. (A) Resistance to fungal pathogen *M. grisea*. PO6-6 and KJ401 (89-010) are compatible and incompatible pathogens to cv. Hwayoungbyeo. Photographs and disease severity scores were taken 5 DPI. A total of eight $T_3$ OE lines were tested, and results of disease symptoms on a single leaf from one VC and two independent OE lines are shown as representatives. A typical disease symptom was observed on VC leaves, whereas OE1 and OE5 leaves manifested HR-like symptoms. (B) Disease severity. Error bars indicate SDs obtained from ten independent measurements.

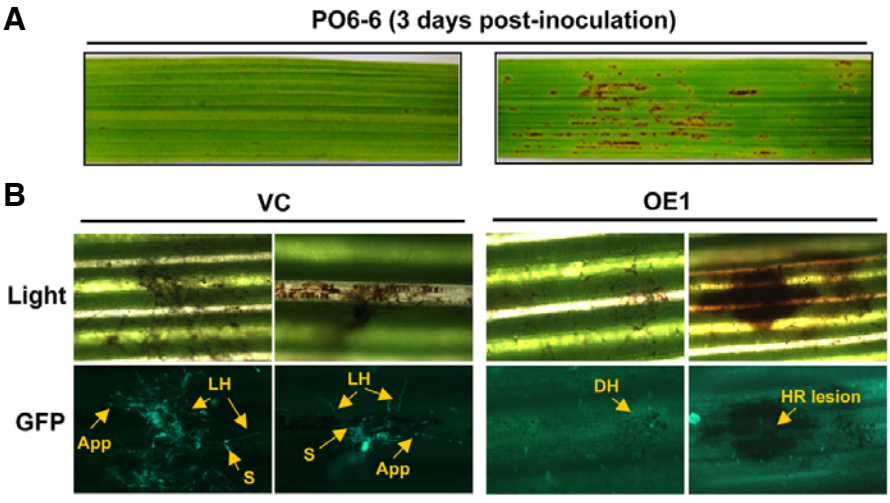


**Fig. 4.** Rice blast fungus fails to successfully colonize on the leaves of *OsACDR1*-OE1 plants. (A) Rice blast symptoms produced by green fluorescent protein (GFP) expressed *M. grisea*. Three-week-old rice seedling leaves inoculated with actively growing GFP expressed *M. grisea* PO6-6 spores. Photographs were taken 3 DPI. Typical disease progression and HR-like symptoms on the leaves of VC and OE1, respectively, are shown as representatives. Left leaf is Hwayoungbyeo WT and right leaf is OE1. (B) Monitoring penetration from appressoria of fungal spores using microscope. UV light was used to visualize invasive hyphae of actively growing GFP expressed *M. grisea* (PO6-6) after 96 h development within VC rice leaves; but no viable hyphae were detected on OE1 leaves. Abbreviations: App, appressorium; DH, dead hyphae; LH, live hyphae; S, spore.

are observed in the absence of any pathogenic signal.

### *OsACDR1*-OE plants exhibit enhanced disease resistance to rice blast pathogen *Magnaporthe grisea*

Both compatible (PO6-6) and incompatible KJ401 (89-010) races of *M. grisea* were used to determine disease resistance in *OsACDR1*-OE plants (OE1 and OE5). It is important to mention here that rice plants at 3-4 weeks-old stage were used for the inoculation with *M. grisea* spores, because at this stage the rice plants (OE1 and OE5 lines) do not show any lesion mimic phenotype. Disease severity was evaluated at 5 days post-inoculation (DPI). The compatible P06-6 race caused typical blast disease symptoms on leaves in VC, but these symptoms were significantly reduced in both OE1 and OE5 plants (Fig. 3A). In the compatible interaction, disease severity decreased by approximately 6-fold in OE1 and OE5 plants (Fig. 3B). Similar levels of disease resistance were manifested in other rice cultivars (Nipponbare, Ilmibyeo, Choochungbyeo, Nampyungbyeo, and Ohdeabyeo) overexpressing *OsACDR1*. We therefore propose that *OsACDR1* overexpression enhances disease resistance against *M. grisea* in a range of different rice cultivars.

### Inhibition of fungal penetration into plant cells

In order to investigate *OsACDR1* conferred disease resistance, the penetration ability of germinated *M. grisea* spores was monitored by spraying GFP-tagged PO6-6 spores (compatible race) on leaves of OE1 and VC plants. Germinating spores and appressoria appeared normally on the leaf surface of both OE1 and VC plants, as visualized by fluorescence microscopy (data not shown). However, GFP fluorescence was markedly reduced at 3 DPI in leaves of OE1 plants with no appearance of appressorial hyphae, coinciding with the appearance of HR-like lesions (Fig. 4). Conversely, VC plant leaves showed actively growing fluorescent hyphae when no lesions were present. These results suggest that *OsACDR1* overexpression directly or indirectly inhibits fungal penetration into the plant cell wall after spore germination.

### *Osacdr1* knock-out and OsACDR1-RNAi plants exhibit lesions and susceptibility to *Magnaporthe grisea*

A single T-DNA knock-out (KO) mutant line of the *OsACDR1* gene was acquired in a mutant screen of 100,000 lines of cvs. Dongjinbyeo and Hwayoungbyeo. PCR analysis showed T-DNA insertion in the 6$^{th}$ exon, with the same orientation as the *OsACDR1* gene (Fig. 5A). Loss-of-function of *OsACDR1* in *Osacdr1* mutant plants also caused lesion phenotype, which tightly co-segregated with *OsACDR1* (Fig. 5B). This lesion phenotype was fully recovered when the full-length *OsACDR1* cDNA was introduced into the homozygote *Osacdr1* progenies under the control of an *OsACDR1* native promoter, indicating that the new lesion phenotype is due to the loss-of-function of *OsACDR1*. *OsACDR1*-RNAi plants ($T_3$ homozygous) also

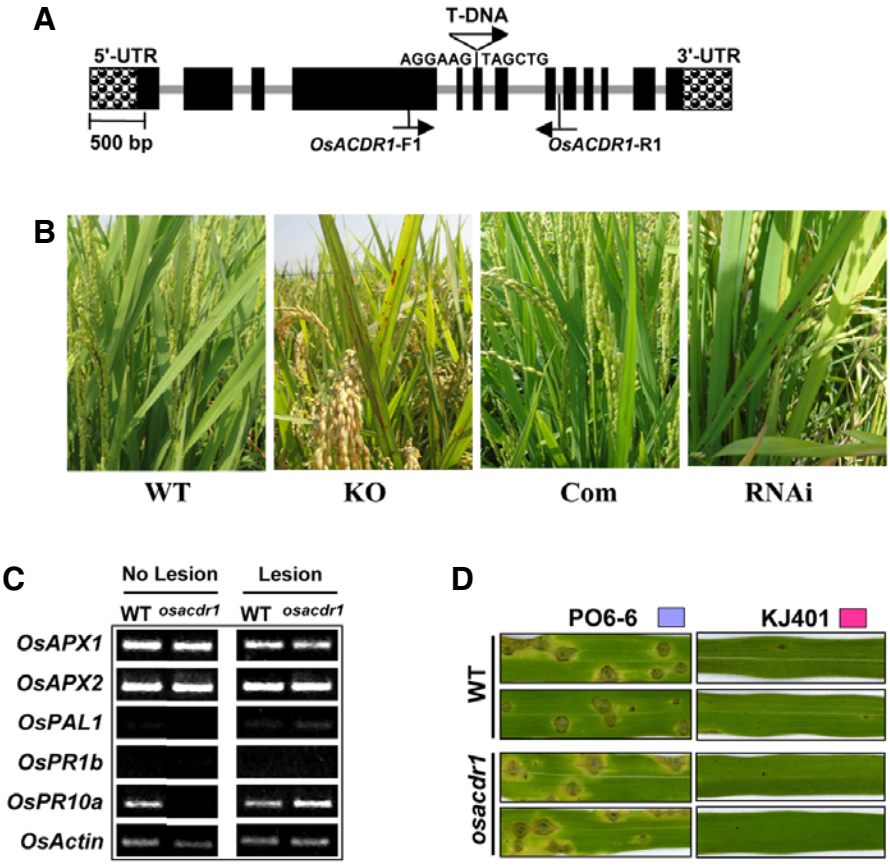


**Fig. 5.** *Osacdr1* knock-out (KO) mutant plants display enhanced susceptibility to fungal pathogen. (A) Genomic organization of *OsACDR1* and T-DNA insertion site. T-DNA is inserted into the 6th exon. The arrow indicates the direction of the T-DNA insertion, but does not reflect the actual size of the T-DNA (12 kb). A few nucleotides surrounding the mutation position have been presented to designate it. (B) Lesion appearance on leaves of WT, KO mutant, complemented plant (Com), and silencing plant (RNAi); lesions on the leaves of mature plants (75-80 days after planting) are visible in the KO and RNAi plants. (C) Alterations in expression levels of five defense-related marker genes in leaves analyzed by RT-PCR. Total RNA was isolated from leaves without and with lesions. *OsAPX1* & *OsAPX2*, *OsPAL1*, and *OsPR1b* & *OsPR10a* are the same as described in Fig. 2B. (D) Enhanced susceptibility of *osacdr1* mutant plants to a compatible race (PO6-6) of *M. grisea*. Three-week-old mutant and wild-type (WT) plants were inoculated with *M. grisea* PO6-6. Photographs were taken 5 DPI.

showed lesion phenotype similar to that revealed by *Osacdr1* plants. The RT-PCR data of *Osacdr1 OE1 and OE5 lines*, *OsACDR1*-RNAi lines (RNAi1-4), KO lines 1 and 2, and its complemented line (Com1-3) are shown in Supplementary Fig. 3. Unlike the *OsACDR1*-OE plants, no considerable change was observed in the transcript levels of defense-related marker genes in leaves of *Osacdr1* plants compared to the corresponding WT plants (Fig. 5C). Specifically, transcript levels of *OsAPX1/2*, *OsPAL1*, *OsPR1b* and *OsPR10a* were unchanged in leaves without lesions in *Osacdr1* plants. No change was also observed in the expression of the actin gene control. Although the *Osacdr1* plants also developed spontaneous lesions of leaves, enhanced levels of defense responses were not observed in the absence of any pathogen. A compatible interaction of *Osacdr1* plants with *M. grisea* race PO6-6 showed disease susceptibility (Fig. 5D). No significant differences were observed between *Osacdr1* and WT plants infected with incompatible pathogen KJ401 using both enhanced disease susceptibility and disease severity indexes, suggesting that incompatible interaction signaling is not controlled by *OsACDR1*-mediated defense signaling. These results combined with the overexpression data indicate *OsACDR1* involvement in cell death and positive regulation of disease resistance in rice.

**OsACDR1 localizes to nucleus**

Rice protoplast and onion (*Allium cepa*) epidermal cells were used to determine the localization of OsACDR1 with a reporter gene encoding EGFP fused to *OsACDR1* under the CaMV 35S promoter (*35S::EGFP::OsACDR1*) (Fig. 6). GFP fluorescence of the EGFP::OsACDR1 chimeric protein in each transgenic cell was observed with a fluorescence microscope equipped with a FITC filter. GFP fluorescence in transgenic rice protoplasts showed clear localization of the EGFP::OsACDR1 chimeric protein to the nucleus (N), when compared with autofluorescence (blue) in the nucleus and cell wall in the DAPI channel (Fig. 6A). A similar result was observed when GFP fluorescence (green) and chlorophyll autofluorescence (red) were merged in transgenic onion epidermal cells (Fig. 6B). These parallel experiments indicate that OsACDR1 localizes to the nucleus *in vivo*.

## DISCUSSION

Like other plants, rice possesses a number of MAPKKKs. However, the total number of MAPKKs remains to be determined. OsACDR1 has been identified as a putative MAPKKK (Kim et al., 2003), and in that study, its transcript expression patterns were investigated against a variety of abiotic environmental stimuli. In this study, we have conducted a functional characterization of OsACDR1 using overexpression and loss-of-function mutants. OsACDR1 shows some similarity to the functionally characterized AtEDR1 and LeCTR2, which have been shown to mediate defense signaling (Lin et al., 2008; Tang et al., 2002; 2005). Despite overall low similarity among OsACDR1, AtEDR1, and LeCTR2 at amino acid level, the predicted structural domains HSP70, ATP-binding sites and serine/threonine protein kinase active site were found to be highly conserved. The HSP70 domain (CN box) is conserved in the *N*-termini of OsACDR1 and LeCTR2. This domain has been suggested to selectively interact with the histidine kinase domain of ETR1-type ethylene receptors (Lin et al., 2008). The Gly354 residue in the HSP70 domain of AtCTR1 is reported to

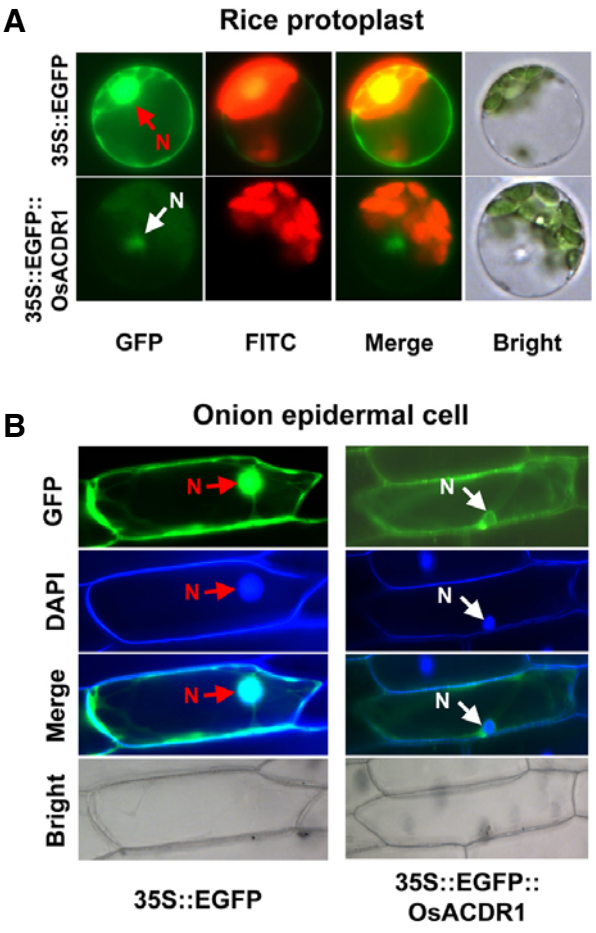


**Fig. 6.** Nuclear localization of OsACDR1. (A) Localization of OsACDR1 (35S::EGFP::OsACDR1) to the nucleus (N) region of rice protoplast. (35S::EGFP) and (35S::EGFP::OsACDR1) constructs were expressed transiently in the protoplasts of rice and examined under a fluorescence microscope after 12 h of incubation. The protoplasts were imaged using bright, GFP and FITC channels of a fluorescence microscope. Merge is the combination of GFP and FITC. (B) Nuclear localization of OsACDR1 in onion epidermal cell. Onion epidermal cells were bombarded with 35S::EGFP and 35S::EGFP::OsACDR1 constructs and incubated at 28°C for 12 h. The epidermal cells were imaged using bright, GFP, DAPI channels of a fluorescence microscope.

be essential for interaction with ethylene receptors, and therefore may also be involved in signal perception (Clarke et al., 1998; Huang et al., 2003). This residue is also conserved in the HSP70 domain of OsACDR1 suggesting that the OsACDR1 *N*-terminus might also participate in signal perception. Furthermore, the OsACDR1 C-terminus (amino acids 724-994) includes an ATP binding site and a serine/threonine protein kinase active site, with 90 and 92% similarities with the C-terminus of AtEDR1 (658-928) and LeCTR2 (690-960), respectively. These have been shown to autophosphorylate as well as phosphorylate MBP as a substrate *in vitro* (Lin et al., 2008; Tang and Innes, 2002). The results suggest that their enzymatic activities could be similar. Indeed, OsACDR1 was also shown to autophosphorylate as well as phosphorylate the generic protein kinase substrate MBP (Fig. 2), suggesting that this protein probably participates in a phosphorylation cascade.

Spontaneous HR-like lesion formation on leaves was observed as a characteristic phenotype conferred by *OsACDR1* in overexpression, loss-of-function and silencing (RNAi) rice plants. However, there were distinct differences in lesion type between plants overexpressing *OsACDR1* (long spreading-type cell death lesions all over the plant) and *Osacdr1* KO (round necrotic-type lesions, which are milder as compared to the OE1 and OE5 lines) plants. The HR-like response is known to result in lesion appearance and is considered as one of the more prominent defense mechanisms (Dangl and Jones, 2001; Lam et al., 2001). Plants capable of developing such spontaneous lesions are referred to as "lesion-mimic mutants (LMMs)" and have been identified in a variety of plants (reviewed in Lorrain et al., 2003). Not all LMMs are known to manifest resistance to pathogens. For example, Yin et al (2000) reported nine *spotted leaf* (*spl*) mutants in rice (*spl1, spl2, spl3, spl4, spl5, spl6, spl7, spl9* and *spl11*), which develop spontaneous lesions. Of these, only four LMMs (*spl1, spl5, spl9* and *spl11*) were found to confer enhanced resistance to the rice blast pathogen *M. grisea*. Although alteration in *OsACDR1* expression in rice was tightly associated with the development of HR-like lesions, plants overexpressing *OsACDR1* manifested enhanced expression of defense-related markers and resistance to compatible blast pathogen in contrast to the opposite effect seen with *Osacdr1* KO or *OsACDR1*-RNAi plants. Therefore, *OsACDR1* mediates defense signaling leading to defense responses, which might eventually culminate in cell death. These results suggest that *OsACDR1* is a molecular switch controlling HR-like cell death downstream of pathogen recognition in rice. Based on our results, it is reasonable to hypothesize that *OsACDR1* mediated defense signaling is probably different from that mediated by *AtEDR1* and *LeCTR2*.

In conclusion, use of multiple approaches for the functional characterization of *OsACDR1* in rice indicates that *OsACDR1* is a positive regulator of fungal disease resistance. This finding is in contrast to known functions of *AtEDR1* and *LeCTR2* as negative regulators of disease resistance in *Arabidopsis* and tomato, respectively (Frye and Innes, 1998; Frye et al., 2001; Lin et al., 2008). Our results suggest that *OsACDR1*-mediated defense responses inhibit successful infection over 3 days at the leaf surface, preventing any further growth of the fungal pathogen. We have also shown OsACDR1 localization to the nucleus, highlighting a possible role in the regulation of multiple defense signaling pathways. However, the mode of action and mechanism by which OsACDR1 confers resistance against *M. grisea* remains to be elucidated. Identification of OsACDR1 interacting proteins may help in revealing its MAPK cascade, and other down- and up-stream components. Given present and previous findings on regulation of *OsACDR1* expression by biotic (this study) and abiotic signals (Kim et al., 2003), it means that OsACDR1 is responsible for physiological responses in rice plants against both biotic and abiotic stresses. The question arises how OsACDR1 discriminates between different signals thus leading to (mediating/resulting in) stress-specific responses.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

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