Targeted Gene Disruption of *OsCERK1* Reveals Its Indispensable Role in Chitin Perception and Involvement in the Peptidoglycan Response and Immunity in Rice

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OsCERK1 is a rice receptor-like kinase that mediates the signal of a fungal cell wall component, chitin, by coordinating with a lysin motif (LysM)-containing protein CEBiP. To further elucidate the function of OsCERK1 in the defense response, we disrupted *OsCERK1* using an *Agrobacterium*-mediated gene targeting system based on homologous recombination. In *OsCERK1*-disrupted lines, the generation of hydrogen peroxide and the alteration of gene expression in response to a chitin oligomer were completely abolished. The *OsCERK1*-disrupted lines also showed lowered responsiveness to a bacterial cell wall component, peptidoglycan. Yeast two-hybrid analysis indicated that OsCERK1 interacts with the LysM-containing proteins LYP4 and LYP6, which are known to participate in the peptidoglycan response in rice. Observation of the infection behavior of rice blast fungus (*Magnaporthe oryzae*) revealed that disruption of *OsCERK1* led to increased hyphal growth in leaf sheath cells. Green fluorescent protein-tagged OsCERK1 was localized around the primary infection hyphae. These results demonstrate that OsCERK1 is indispensable for chitin perception and participates in innate immunity in rice, and also mediates the peptidoglycan response. It is also suggested that OsCERK1 mediates the signaling pathways of both fungal and bacterial molecular patterns by interacting with different LysM-containing receptor-like proteins.

Plants initiate defense responses upon perceiving structurally conserved molecules, known as microbe-associated molecular patterns (MAMPs). These molecules are perceived by pattern recognition receptors (PRR) (Boller and Felix 2009; Monaghan and Zipfel 2012). The PRR that have been identified to date are the receptor-like protein kinases (RLK) and receptor-like proteins (RLP) localized in the plasma membrane (PM) (Monaghan and Zipfel 2012). Studies have shown that RLK containing leucine-rich repeat motifs in the extracellular domain perceive peptidic MAMPs. In *Arabidopsis thaliana*,

FLS2 and EFR recognize the flg22 peptide derived from bacterial flagella and the elf18 peptide from the bacterial elongation factor Tu, respectively (Gomez-Gomez and Boller 2000; Zipfel et al. 2006). The perception of carbohydrate MAMPs that contain *N*-acetylglucosamine is mediated by PRR with lysin motifs (LysM): LysM-RLK and LysM-RLP (Monaghan and Zipfel 2012).

Chitin, a β -1,4-linked polymer of *N*-acetylglucosamine, is a constituent of fungal cell walls. Its oligomers are a typical carbohydrate MAMP, and they elicit various defense responses in many plant species (Kombrink et al. 2011). In *Arabidopsis*, a LysM-RLK, AtCERK1, is essential for perceiving chitin elicitors (CE) and plays a role in immunity to fungal pathogens (Miya et al. 2007; Wan et al. 2008). AtCERK1 directly binds CE and is sufficient for CE perception (Iizasa et al. 2010; Petutschnig et al. 2010; Shinya et al. 2012). In rice, CEBiP, a LysM-RLP, directly binds CE, whereas OsCERK1, a homolog of AtCERK1, does not (Kaku et al. 2006; Shinya et al. 2012). CEBiP interacts with OsCERK1 in a CE-dependent manner. Reduced expression of either *CEBiP* or *OsCERK1* in RNA interference (RNAi) lines resulted in an impaired response to CE (Kaku et al. 2006; Shimizu et al. 2010), indicating that both of these molecules are required for chitin signaling. Therefore, the CE receptor system in rice is different from that in *Arabidopsis*, which does not require CEBiP-like molecules (Shinya et al. 2012). To further clarify the role of OsCERK1, analysis using *OsCERK1*-disrupted rice plants is desirable because the RNAi lines retained some responsiveness; thus, it is possible that other LysM-RLK are also involved in the response to CE. Moreover, although CEBiP was shown to participate in immunity (Kishimoto et al. 2010; Kouzai et al. 2014), the involvement of OsCERK1 has not been analyzed.

Peptidoglycan (PGN), which contains β -1,4-linked glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid, is a constituent of bacterial cell walls. This polymer elicits defense responses in *Arabidopsis* and rice (Erbs et al. 2008; Gust et al. 2007; Liu et al. 2012). *Arabidopsis* has three LysM-RLP (LYM1 to LYM3). LYM1 and LYM3 bind PGN and participate in the PGN response (Willmann et al. 2011). AtCERK1, which itself does not bind PGN, also acts as a PGN receptor, although biochemical interaction between LYM1 or LYM3 and AtCERK1 has not been reported (Willmann et al. 2011). The homologs of LYM1 or LYM3 in rice, LYP4 and LYP6, also bind PGN. Reduced expression of *LYP4* or *LYP6*

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led to an impaired response to PGN (Liu et al. 2012). Because LYP4 and LYP6 lack intracellular domains, they are assumed to cooperate with LysM-RLK to mediate PGN signaling in rice. However, such a molecule has not been identified.

The subcellular localization of PRR fused to fluorescent protein has been analyzed in cells treated with MAMPs or invaded by filamentous pathogens. After stimulation with flg22, FLS2-green fluorescent protein (GFP) was transferred

from the PM to intracellular vesicles, and then degraded (Robatzek et al. 2006). During infection by *Hyaloperonospora arabidopsidis*, FLS2-GFP was located in the extrahaustorial membrane (Lu et al. 2012), a novel host cell membrane enclosing the haustorium to separate the host cell from the oomycete. Transient expression of OsCERK1-GFP in rice protoplasts showed that OsCERK1 was transported from the endoplasmic reticulum to the PM by vesicle trafficking (Chen et al. 2010). However, the subcellular localization of OsCERK1 in infected rice cells remains unknown.

Although gene disruption mutants are well developed in *Arabidopsis*, it is still difficult to obtain such mutants in other higher plants. In rice, retrotransposon *Tos17*-induced insertion mutants and T-DNA insertion lines are available (Jeon et al. 2000; Miyao et al. 2003) but do not include *OsCERK1*-disrupted lines. Gene disruption through homologous recombination has become a routine technique to investigate gene functions in mammals (Jasin et al. 1996). Terada and associates (2002) first reported targeted homologous recombination in the vicinity of a target gene in rice using a positive/negative (hygromycin phosphotransferase gene [*HPT*]/diphtheria toxin A fragment gene [*DT-A*]) selection system. Recently, Ozawa and associates (2012) improved this technique by optimizing culture conditions, which allowed the production of *CEBiP*-disrupted rice plants (Kouzai et al. 2014).

To elucidate the roles of OsCERK1 in the defense responses and immunity of rice, we generated *OsCERK1*-disrupted rice plants (*oscerk1*) using the *Agrobacterium*-mediated gene-targeting system based on homologous recombination. Here, we show that the response to chitin oligomers in rice completely depends on OsCERK1. Immunity to rice blast fungus was reduced in *oscerk1*, and fluorescence imaging of inoculated rice leaf sheaths revealed that GFP-tagged OsCERK1 was localized around primary infection hyphae. Furthermore, we show that OsCERK1 participates in the response to PGN, probably in cooperation with one or both LYP4 or LYP6.

RESULTS

Generation of *OsCERK1*-disrupted rice plants.

The binary vector used to disrupt *OsCERK1* contains the *HPT* cassette for positive selection, the upper and lower regions of the *OsCERK1* first exon, and two *DT-A* cassettes for negative selection of random integration. Thus, the *OsCERK1* first exon is replaced with the *HPT* cassette, following the occurrence of targeted homologous recombination (Fig. 1A). We screened 1,248 independent hygromycin-resistant calli by polymerase chain reaction (PCR) that amplified 7.1- and 7.0-kb junction fragments (Fig. 1A), and obtained five gene-targeted lines (0.40%). Plants were regenerated from four lines, three of which showed normal growth (lines 19, 53, and 117). A representative result of genotyping of T₁ plants at the *OsCERK1* locus is shown in Figure 1B. A χ^2 analysis of the segregation ratio indicated that the *HPT* cassette in place of *OsCERK1* was inherited by the T₁ generation in a Mendelian manner (Supplementary Table S1).

A genomic DNA-blot analysis indicated that the first exon of *OsCERK1* was deleted in the homozygous *oscerk1* mutant lines, and that *HPT* was integrated into only the target locus (Supplementary Fig. S1A and B). DNA sequencing of the 15-kb fragments covering the *OsCERK1* locus in T₁ seedlings confirmed that the gene replacement occurred at the expected sites in homozygous *oscerk1* plants, and that segregated hygromycin-sensitive plants had the wild-type *OsCERK1* in all three lines. *OsCERK1* transcripts were detected in segregated wild-type lines but not in homozygous *oscerk1* lines (Fig. 1C). The transcript levels of *CEBiP* and other LysM-containing RLK and RLP

Fig. 1. Generation of *OsCERK1*-disrupted rice plants. **A**, Schematic representation of disruption of *OsCERK1* by homologous recombination. Homologous recombination event via double crossovers at hygromycin phosphotransferase (*HPT*)-flanking homologous regions in T-DNA replaces a part of the *OsCERK1* locus (Os08g0538300; 1.1 kb) with the *HPT* cassette (2.1 kb). Black arrowheads indicate position of polymerase chain reaction (PCR) primers used to screen disrupted lines. Fragments of 7.1 and 7.0 kb are amplified only from lines in which the expected homologous recombination occurs. *DT* = diphtheria toxin A fragment gene, *LB* = left border of T-DNA, *RB* = right border of T-DNA. **B**, Representative result of PCR analysis to genotype progeny of gene-targeted line (T₁ generation). *M* = DNA molecular size marker, *Rev* = segregated wild-type, *KO* = knock-out. **C**, Quantitative reverse-transcriptase (RT)-PCR analysis of *OsCERK1* expression. Transcript levels relative to that in nontransformed callus are shown. *WT* = nontransformed callus, *KO* = homozygous *oscerk1* mutant callus, *Rev* = segregated wild-type callus. **D**, RT-PCR analysis of expression of rice LysM-containing receptor-like protein kinase (*RLK*) and receptor-like protein (*RLP*) genes. *WT* = nontransformed callus, *KO* = homozygous *oscerk1* mutant callus, *Rev* = segregated wild-type callus. The expression of *RLK2*, -5, and -8 was not detected even in *WT*.

genes that were expressed in cultured cells were not affected in *oscerk1* or the segregated wild-type lines (Fig. 1D). These data confirmed the *OsCERK1*-specific disruption in the lines created.

Responsiveness to CE is lost in *oscerk1*.

To evaluate the effect of *OsCERK1* disruption on CE perception, we analyzed hydrogen peroxide (H_2O_2) generation and gene expression profiles after CE treatment. When suspension-cultured cells and leaves of *oscerk1* were treated with CE, they could not generate H_2O_2 (Fig. 2A and B). The ability to generate H_2O_2 in response to CE was recovered by introducing *OsCERK1* cDNA, and H_2O_2 was generated in a kinase-activity-dependent manner (Supplementary Fig. S2). Microarray analysis demonstrated that, in nontransformed cells, 1,391 genes were upregulated ($\geq \times 2$) and 617 were downregulated ($\leq \times 1/2$) after 1 h of CE treatment (Fig. 2C; Supplementary Tables S2 and S3). In *oscerk1* cells, none of these genes responded to CE. As a control, we confirmed that the response to a bacterial MAMP, flg22, was not affected in *oscerk1* (Supplementary Fig. S3).

OsCERK1 contributes to innate immunity to *Magnaporthe oryzae*.

Next, we evaluated the immunity to rice blast caused by *Magnaporthe oryzae* in *oscerk1* plants. Spray inoculation of leaf blades with a compatible strain showed no significant dif-

ference in lesion formation between *oscerk1* and nontransformed plants (data not shown). Thus, we evaluated the early infection process of an incompatible strain by scoring hyphal growth in the epidermal cells under a microscope. There was a greater extent of penetration and colonization of infection hyphae in *oscerk1* leaf sheaths than in wild-type ones (Fig. 3).

To analyze the cellular localization of *OsCERK1* in infected cells, we produced transgenic rice plants expressing *OsCERK1-GFP* and observed GFP fluorescence in the leaf sheaths. In uninoculated tissues, *OsCERK1-GFP* was present in the PM, the endoplasmic reticulum-like structures, and vesicle-like organelles (Supplementary Fig. S4). Five independent transgenic lines showed similar fluorescence patterns. As shown in Figure 4, in the cells invaded by infection hyphae of a compatible strain (Ina86-137), GFP signals were detected around the primary infection hyphae as well as in the PM at 30 h postinoculation (hpi). When the infection hyphae penetrated into neighboring rice cells (42 hpi), the GFP signals disappeared from the first-invaded cell, which suggests that the rice cell was dead. In the neighboring cells, GFP signals were present again around the invading hyphae and in the PM.

Involvement of *OsCERK1* in the peptidoglycan response.

Next, we examined the participation of *OsCERK1* in the response to PGN. H_2O_2 generation after treatment with PGN

Fig. 2. Responses to chitin elicitor (CE). **A**, Generation of H_2O_2 in response to CE in cell lines. Suspension-cultured cells (100 mg) were treated with *N*-acetylchitoheptaose at 100 ng ml^{-1} , and H_2O_2 in the culture solution was quantified. WT = nontransformant, KO = two independent homozygous *oscerk1* (lines 53 and 117), Rev = segregated wild-type lines. **B**, Generation of H_2O_2 in response to CE in leaves. Sliced leaf blades (20 mg) floating in water were treated with *N*-acetylchitoheptaose at 100 ng ml^{-1} , and H_2O_2 in the culture solution was quantified. WT = nontransformant, WT (water) = mock-treatment, KO = homozygous *oscerk1*, Rev = segregated wild-type plants. Error bars in A and B represent standard deviation ($n = 3$ independent treatments). **C**, Changes in gene transcript levels in response to CE. Total RNAs were extracted from suspension-cultured cells treated with *N*-acetylchitoheptaose at 100 ng ml^{-1} for 1 h and used for microarray analysis. Bars show number of CE-responsive genes. Up = fold changes $\geq \times 2$, Down = $\leq \times 1/2$, WT = nontransformed cells, KO = *oscerk1* cells (line 53). Only one gene was downregulated specifically in *oscerk1*.

from *Bacillus subtilis* or *Staphylococcus aureus* was partially impaired in the *oscerk1* cell line (Fig. 5A; Supplementary Fig. S5A). Pretreatment of PGN with lysozyme to hydrolyze the glycan chains negated the effect of *OsCERK1* disruption (Fig. 5B), further indicating the involvement of *OsCERK1* in the PGN response. A yeast two-hybrid analysis demonstrated that *OsCERK1* interacted with LYP4 and LYP6, as well as with CEBiP, suggesting that *OsCERK1* may play a role in perceiving PGN along with one or both LYP4 and LYP6 (Fig. 5C).

Fig. 3. Effect of *OsCERK1* disruption on immunity to *Magnaporthe oryzae*. Sixth leaf sheaths of two independent *oscerk1* lines were inoculated with *M. oryzae* (Guy11, an incompatible strain). Degrees of invasion were scored for 100 appressoria per plant under a microscope at 48 h postinoculation and are shown as percentages of total numbers of appressoria in three categories. KO = homozygous *oscerk1*, Rev = segregated wild-type plants. Error bars represent standard deviation ($n = 4$ sibling plants in the T_2 generation). Asterisks indicate significant differences compared with the vector control (VecCon) at $P < 0.05$ (Dunnett's test). Experiments were performed twice; representative data are shown.

To examine the effect of *OsCERK1* disruption on immunity to a bacterial pathogen, we inoculated *oscerk1* leaves with *Xanthomonas oryzae* pv. *oryzae*, the causal agent of rice bacterial leaf blight. We measured lesion area at 14 days postinoculation (dpi) but *OsCERK1* disruption had no detectable effect (Fig. 6A). We also evaluated the bacterial population at 4 dpi using wild-type *X. oryzae* pv. *oryzae* or the *X. oryzae* pv. *oryzae* *hrpX* mutant, which lacks the ability to deliver type III effectors and, thus, pathogenicity, and obtained the same results (Fig. 6B and C).

DISCUSSION

In *OsCERK1*-RNAi cells, the responses to CE were not completely abolished (Shimizu et al. 2010). Therefore, in this study, we first aimed to clarify whether the remaining responsiveness was because of other LysM or low levels of *OsCERK1* transcripts. We generated three independent *oscerk1* lines using an *Agrobacterium*-mediated gene targeting system based on homologous recombination (Ozawa et al. 2012). The frequency of double crossovers at the *OsCERK1* locus was 0.40% in positive/negative selected calli, which was similar to that for the *CEBiP* locus (0.47%; Kouzai et al. 2014). The disruption of *OsCERK1* did not cause gamete or embryo lethality. Using *oscerk1* and *OsCERK1*-complemented lines, we demonstrated that *OsCERK1* with kinase activity is indispensable for the response to CE in rice (Fig. 2). Thus, the contribution of *OsCERK1* to CE perception is the same as that of *AtCERK1* in *Arabidopsis* (Miya et al. 2007), although *OsCERK1* needs *CEBiP* in contrast to *AtCERK1*, which is an “all-in-one” receptor (Shinya et al. 2012). Recently, using a *CEBiP*-disrupted mutant, we showed that *CEBiP* is the major CE-binding protein in rice and almost all of the CE responsiveness in cultured cells is attributable to *CEBiP* (Kouzai et al. 2014). The results of that study and the present study revealed the mechanism of CE perception in rice; almost all CE signals are recognized by *CEBiP* and every CE signal is transmitted into cells via *OsCERK1*.

Fig. 4. Subcellular localization of *OsCERK1*-GFP (green fluorescent protein). Rice leaf sheaths of transgenic plants expressing *OsCERK1*-GFP were inoculated with conidial suspension of *Magnaporthe oryzae* expressing mCherry. GFP = Stacked Z-series confocal GFP fluorescence images, GFP+mCherry = superimposed images of stacked Z-series confocal fluorescence images of GFP (green) and mCherry (magenta), DIC = corresponding differential interference contrast images. Asterisks indicate positions of appressoria (i.e., penetration sites). Bars = 20 μ m.

We also showed that OsCERK1 contributes to immunity against *M. oryzae*. We recently reported a slight but significant reduction in immunity to this fungus in *CEBiP*-disrupted plants (Kouzai et al. 2014). *OsCERK1*-disrupted plants also showed a significant reduction in the early infection stages, suggesting that the perception of CE by the host is a substantial obstacle for the rice blast fungus. The significance of CE perception in rice immunity is also supported by studies on *M. oryzae*. Infection hyphae of *M. oryzae* in the first-invaded rice cell showed only weak staining with a fluorescent probe to detect chitin (Mochizuki et al. 2011), suggesting the presence of a fungal strategy to minimize the detection of cell wall chitin by the host plant during invasion. In fact, α -1,3-glucan that masks the chitin layer in fungal cell walls was shown to be indispensable for successful fungal invasion (Fujikawa et al. 2012). In addition, *M. oryzae* secretes an effector protein, secreted LysM protein 1 (Slp1), to capture CE in competition with *CEBiP* for full virulence (Mentlak et al. 2012). Therefore, suppression of rice

immunity induced by CE is an important stealth mechanism of *M. oryzae* for successful infection. LYP4 and LYP6, *CEBiP*-like proteins in rice, were reported to participate in rice immunity to *M. oryzae* and bind directly with chitin (Liu et al. 2012), although we did not detect any interaction other than *CEBiP* in affinity-labeling analysis using biotinylated CE (Kouzai et al. 2014). Given that this inconsistency in results was not because of differences in experimental conditions, either LYP4, LYP6, or both might play a role in immunity to *M. oryzae* through an as-yet-unknown mechanism other than CE recognition.

Considerable progress has been made in understanding signal transduction through PRR (Kawano and Shimamoto 2013; Monaghan and Zipfel 2012). However, little is known about the dynamic behavior of PRR during the host–pathogen interaction. Transient expression of *OsCERK1*-GFP in rice protoplasts showed that *OsCERK1* was transported from the endoplasmic reticulum to the PM by vesicle trafficking (Chen et al. 2010). Consistent with the observation, *OsCERK1*-GFP was detected

Fig. 5. Responses to peptidoglycan (PGN). **A**, Generation of H_2O_2 in response to PGN in an *oscerk1* line (line 53; KO) and nontransformants (WT). Suspension-cultured cells were treated with PGN at $10 \mu g ml^{-1}$ derived from *Bacillus subtilis* or *Staphylococcus aureus*, and H_2O_2 in the culture medium was quantified. Error bars represent standard deviation ($n = 3$ independent treatments). **B**, Amount of H_2O_2 after treatment with lysozyme-treated PGN in an *oscerk1* line (line 53; KO) and nontransformants (WT). Suspension-cultured cells were treated with PGN at $10 \mu g ml^{-1}$, lysozyme-pretreated PGN (containing lysozyme at $1 \mu g ml^{-1}$) at $10 \mu g ml^{-1}$, or lysozyme at $1 \mu g ml^{-1}$. H_2O_2 in the culture medium was quantified at 2 h. Error bars represent standard deviation ($n = 3$ independent treatments). Different letters above bars indicate significant differences at $P < 0.05$ (Tukey's test). **C**, Interactions between rice LysM-containing receptors. Yeast two-hybrid assay was performed using extracellular domains of *OsCERK1* and *CEBiP* as bait and three *CEBiP*-type receptors (*CEBiP*, *LYP4*, and *LYP6*) as prey. Growth on selective medium (SD/-LWHA) indicated positive interaction. Vector indicates empty vector (control). P.C. = positive control using pGBKT7-53 and pGADT7-T vectors and N.C. = negative control using pGBKT7-Lam and pGADT7-T vectors.

in the PM, the endoplasmic reticulum-like structures, and vesicle-like organelles in leaf sheath cells of stable transformants. In the inoculated cells, OsCERK1-GFP was localized around the primary infection hyphae in the first- and second-invaded rice cells. This observation is consistent with previous reports that primary infection hyphae of *M. oryzae* invaginate the host PM (Koga 1994) and that infection hyphae of *M. oryzae* are encased by a membrane known as the extra-invasive hyphal membrane (EIHM) (Kankanala et al. 2007), which originates from the rice PM (Mentlak et al. 2012). However, OsCERK1-GFP was not detected as encasing the whole invasive hyphae, which was inconsistent with the result using transgenic rice expressing an artificial PM marker gene, LTI6B-GFP (Mentlak et al. 2012). Our observations suggest that CE perception around the infection hyphae might be limited to the early stages, when infection hyphae have just invaded rice cells, and also imply that the EIHM differs from the authentic PM, at least with regard to resident proteins. Similar results have been reported for the extrahaustorial membrane of powdery mildews and oomycetes (Koh et al. 2005; Lu et al. 2012).

In *Arabidopsis*, AtCERK1 also mediates the PGN response but needs the CEBiP-like proteins LYM1 and LYM3, which directly bind with PGN (Willmann et al. 2011). The rice homologs of LYM1 or LYM3, LYP4 and LYP6, also participate in the PGN response (Liu et al. 2012). Our data demonstrate that rice cells respond to two different types of PGN: a meso-diaminopimelate-type from *B. subtilis* and an L-lysine-type from *S. aureus*. In addition, our results indicate that OsCERK1 is involved in the perception of both types of PGN, probably by interacting with one or both LYP4 and LYP6. However, PGN

responsiveness in *oscerk1* cells was not completely abolished, and pretreatment of PGN with lysozyme tended to lower the responsiveness of the *oscerk1* lines. These results imply that other RLK are also involved in perceiving the glycan chains in PGN. The residual responsiveness to lysozyme-treated PGN in both the wild-type and *oscerk1* lines raises the possibility that rice has another one or more receptors for the lysozyme-resistant structure in PGN, or that the PGN preparations used in these experiments were contaminated with another one or more elicitors. Further studies are needed to elucidate the molecular mechanism underlying the PGN recognition in rice.

In the experimental conditions we used, *oscerk1* did not show impaired immunity to *X. oryzae* pv. *oryzae*. This result might be partly ascribed to redundancy among RLK in PGN perception, and also to other perception mechanisms for bacterial MAMPs, including flg22 in rice. Moreover, Yamaguchi and associates (2013) recently reported that Xoo1488, an effector of *X. oryzae* pv. *oryzae*, suppressed the OsCERK1-mediated phosphorylation of a receptor-like cytoplasmic kinase, OsRLCK185, and that overexpression of *Xoo1488* in rice reduced the immunity to *X. oryzae* pv. *oryzae*. The existence of suppression mechanisms targeting the signaling pathways downstream of OsCERK1 might also be a reason why altered immunity to *X. oryzae* pv. *oryzae* was not detected in *oscerk1*. In contrast, reduced expression of *LYP4* or *LYP6* led to impaired immunity to *X. oryzae* pv. *oryzae* (Liu et al. 2012). Given that this inconsistency in results was not caused by differences in experimental conditions, we presume that either *LYP4* or *LYP6*, or both, play other roles besides PGN recognition.

Taken together, our data indicate that OsCERK1 mediates the perception of both fungal and bacterial MAMPs by interacting with different LysM-RLP. The present study also demonstrates the usefulness of a gene-disrupted mutant. The roles of LysM-RLK are an emerging subject in both plant immunity and symbioses (Gust et al. 2012; Nakagawa et al. 2011); therefore, *OsCERK1*-disrupted plants will be useful materials to further investigate plant-microbe interactions.

MATERIALS AND METHODS

Plant and fungus materials and PCR primers.

We used rice (*Oryza sativa* L. *japonica* 'Nipponbare Kanto BL number 2') harboring *Pii* and *Pish*, resistance genes against the pathogen *M. oryzae*. Rice plants were grown hydroponically in nutrient solution under a photoperiod of 14 h of light (28°C) and 10 h of darkness (25°C). Rice calli were cultivated on N6D plates (Toki et al. 2006) containing 0.4% (wt/vol) Gelrite and hygromycin at 50 µg ml⁻¹ and 28°C. The *M. oryzae* strains Guy11 (race 134.1, *avrPish*; provided by M. Nishimura) (Leung et al. 1988) and Ina86-137 (race 007.0; MAFF101511) were cultured on oatmeal agar plates at 26°C to induce conidiation. Sequences of all primers used in this study are listed in Supplementary Table S4.

Gene targeting.

Vector construction and rice transformation for gene targeting were performed as described by Ozawa and associates (2012). A 6.4-kb fragment corresponding to the upper region of the *OsCERK1* promoter and a 5.6-kb fragment corresponding to the lower region from the first exon of *OsCERK1* were PCR-amplified from rice total DNA. Both fragments were cloned into an entry vector containing the *HPT* cassette. The resultant vector and two other entry vectors containing the *DT-A* gene cassette were combined with the destination binary vector using the Gateway system (Invitrogen, Carlsbad, CA, U.S.A.). The T-DNA region of the resultant binary vector is shown in Figure 1A.

Fig. 6. Effect of *OsCERK1* disruption on immunity to *Xanthomonas oryzae* pv. *oryzae* (Xoo). **A**, Lesion area at 14 days postinoculation with *X. oryzae* pv. *oryzae*. **B**, Bacterial population at 4 days postinoculation with *X. oryzae* pv. *oryzae*. **C**, Bacterial population at 4 days postinoculation with *X. oryzae* pv. *oryzae* *hrpX* mutant. Fully expanded upper leaves of glasshouse-grown plants were inoculated by the clipping method. Data are means $\pm$ standard deviation ($n = 4$ plants).

For complementation experiments, the *OsCERK1* cDNA under the control of the rice actin promoter in the binary vector pSTARA R-5 (Inplanta Innovations, Yokohama, Japan) was introduced into *oscerk1* calli derived from T₂ seed of line number 53. The transcript levels of *OsCERK1* in bispyribac-resistant calli were analyzed by quantitative reverse-transcriptase (RT)-PCR. We used two lines with *OsCERK1* transcript levels similar to that in nontransformed calli for complementation tests.

Screening of targeted calli and genotyping.

Genomic DNA was extracted from calli grown on selection medium containing hygromycin at 50 mg liter⁻¹ and subjected to PCR using KOD FX Neo polymerase (Toyobo, Osaka, Japan). We regenerated gene-targeted calli and obtained self-pollinated seed (T₁ generation). Genotyping of the *OsCERK1* locus of T₁ seedlings and calli induced from T₁ seed was performed by PCR.

RT-PCR.

Transcriptional expression was confirmed by RT-PCR using EX Taq (Takara, Ohtsu, Japan). Quantitative RT-PCR was performed using 1× SYBR Premix Ex Taq II (Takara). The relative levels of gene expression were quantified using MX3000P (Stratagene, La Jolla, CA, U.S.A.). The data were normalized to those of the elongation factor gene *eEF-1α* (Jain et al. 2006).

Measurement of H₂O₂ generation.

Suspension-cultured cells (100 mg) were treated with *N*-acetylchitoheptaose at 100 ng ml⁻¹, PGN from *B. subtilis* or *S. aureus* (Sigma-Aldrich, St. Louis) at 10 μg ml⁻¹, or 100 nM flg22 at 28°C with shaking at 800 rpm. PGN (1 mg ml⁻¹) was digested with chicken egg-white lysozyme (Sigma-Aldrich) at 50 μg ml⁻¹ for 16 h at 37°C. H₂O₂ in the culture medium was quantified by the luminol-dependent chemiluminescence assay, as described by Kouzai and associates (2013).

Microarray analysis.

Total RNA was extracted from suspension-cultured cells treated with *N*-acetylchitoheptaose at 100 ng ml⁻¹ for 1 h. Mock treatment was used as the control. All samples were prepared in triplicate. Microarray analyses were performed using rice oligo microarrays with 44k features (Agilent Technologies, Palo Alto, CA, U.S.A.), as described by Takehisa and associates (2012). Data were analyzed using the Subio Platform.

Disease resistance test.

The degree of hyphal growth in leaf sheath epidermal cells was analyzed as described by Tanabe and associates (2009). Briefly, leaf sheaths from the sixth leaves were excised, the inside filled with a conidial suspension (1 × 10⁵ conidia ml⁻¹), and incubated for 48 h at 25°C in the dark. To evaluate immunity to *X. oryzae* pv. *oryzae*, fully expanded upper leaves of glasshouse-grown plants were inoculated by clipping leaf tips with scissors that had been immersed in a bacterial suspension (optical density at 600 nm = 0.3). To quantify the bacterial population, the inoculated leaves (1 cm long) were ground in and diluted with sterile water, then plated on nutrient broth yeast extract medium. After incubation for 2 days at 28°C, the colony numbers were counted.

Preparation of rice and *M. oryzae* transformants for fluorescence imaging.

The coding region of *OsCERK1* was cloned between the *Cauliflower mosaic virus* 35S promoter and *GFP* in the binary vector pGWB5 (Nakagawa et al. 2007), which was introduced into rice plants by *Agrobacterium*-mediated transformation (Toki et al. 2006). The promoter region of the translation elon-

gation factor gene of *Aureobasidium pullulans* (Vanden Wymelenberg et al. 1997) fused to *mCherry* (Clontech, Palo Alto, CA, U.S.A.) was cloned in a binary vector to construct pCambia-TCT, which was introduced into *M. oryzae* Ina86-137 via *Agrobacterium*-mediated transformation (Saitoh et al. 2008).

Confocal microscopy.

OsCERK1-GFP and mCherry fluorescence were observed under a fluorescence microscope (DM6000B; Leica Microsystems, Wetzlar, Germany) equipped with a confocal laser-scanning unit (CSU-X1; Yokogawa Electric, Tokyo), laser units (Sapphire 488 and 561 nm; Coherent Inc., Santa Clara, CA, U.S.A.), a dichroic mirror (DM-405/488/561), and emission filters (GFP, EM-520/35; mCherry, EM617/73). Fluorescence images were collected with an EM-CCD camera (iXon897; Andor Technology, South Windsor, CT, U.S.A.) using a ×40 objective and were analyzed with MetaMorph software (Molecular Devices, Sunnyvale, CA, U.S.A.).

Yeast two-hybrid assay.

A yeast two-hybrid assay was performed using the MATCH-MAKER GAL4 Two-Hybrid System 3 according to the manufacturer's protocol (Clontech). The extracellular regions of *OsCERK1*, *CEBiP*, *LYP4*, and *LYP6* were amplified by PCR and cloned into pGBKT7 or pGADT7, as described by Shimizu and associates (2010).

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