CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*

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Chitin is a major component of fungal cell walls and serves as a microbe-associated molecular pattern (MAMP) for the detection of various potential pathogens in innate immune systems of both plants and animals. We recently showed that chitin elicitor-binding protein (CEBiP), plasma membrane glycoprotein with LysM motifs, functions as a cell surface receptor for chitin elicitor in rice. The predicted structure of CEBiP does not contain any intracellular domains, suggesting that an additional component(s) is required for signaling through the plasma membrane into the cytoplasm. Here, we identified a receptor-like kinase, designated CERK1, which is essential for chitin elicitor signaling in *Arabidopsis*. The KO mutants for *CERK1* completely lost the ability to respond to the chitin elicitor, including MAPK activation, reactive oxygen species generation, and gene expression. Disease resistance of the KO mutant against an incompatible fungus, *Alternaria brassicicola*, was partly impaired. Complementation with the WT *CERK1* gene showed *cerk1* mutations were responsible for the mutant phenotypes. CERK1 is a plasma membrane protein containing three LysM motifs in the extracellular domain and an intracellular Ser/Thr kinase domain with autophosphorylation/myelin basic protein kinase activity, suggesting that CERK1 plays a critical role in fungal MAMP perception in plants.

plant immunity | host–pathogen interaction | *N*-acetylchitooligosaccharide

Plants trigger various defense reactions against invading pathogens upon perception of so-called microbe-associated molecular patterns [MAMPs; also known as pathogen-associated molecular patterns (PAMPs)]. MAMP recognition has been implicated to play a major role in the nonhost or basal resistance that makes most plants immune to most potential pathogens (1, 2). In the course of coevolution between hosts and parasites, it seems that pathogens developed various virulence factors to overcome MAMP-mediated defense responses, whereas plants evolved resistance-gene-mediated defense system to detect such factors (3–5). Recent studies also revealed the presence of a striking similarity in the MAMP-mediated immunity between plants and animals (2, 6).

Despite the importance of MAMP-mediated immunity in plants, molecular machineries involved in the perception of MAMPs and their signal transduction have been poorly understood. So far, only the receptor molecules for two bacterial MAMPs, flg22 and EF-Tu, have been identified (7, 8). Both of these receptors, FLS2 and EFR, are receptor kinases with a leucine-rich repeat in the extracellular domain. However, much less information is available for the perception of fungal MAMPs. Chitin and its fragments, chitin oligosaccharides or *N*-acetylchitooligosaccharides, are typical fungal MAMPs that trigger various defense responses in both monocots and dicots, indicating the presence of a conserved machinery to perceive these oligosaccharides in a wide range of plant species (9, 10). A recent finding that chitin also induces immune responses in mice (11) suggests the common presence of a chitin-mediated defense machinery in higher eukaryotes. We recently identified a plasma membrane glycoprotein [chitin elicitor-binding protein (CEBiP)] that plays an important role in the perception of chitin oligosaccharide elicitor in rice (12). CEBiP specifically binds to the chitin oligosaccharide, and its knock-down transformants exhibited the suppression of chitin-induced defense responses, suggesting that CEBiP functions as a receptor for the elicitor. CEBiP contains two extracellular LysM motifs and a transmembrane domain but lacks any obvious intracellular domains for signal transduction, indicating it may require additional factors for signaling through the plasma membrane into the cytoplasm.

Here, we report the identification of CERK1, a receptor-like kinase (RLK) that is critically important for chitin elicitor signaling in *Arabidopsis*. The *CERK1* KO mutants completely lost the ability to respond to the chitin elicitor and initiate defense responses. Resistance against an incompatible fungus, *Alternaria brassicicola*, was also partly impaired in the KO mutant. Complementation with the WT gene supported *CERK1* is responsible for the mutant phenotypes. Similar to CEBiP, CERK1 is a plasma membrane protein with three LysM motifs in the extracellular domain. Interestingly, however, unlike CEBiP, CERK1 contains an intracellular Ser/Thr kinase domain with an autophosphorylation/myelin basic protein (MBP) kinase activity. Together, CERK1 represents a LysM-type receptor-like kinase for the perception of fungal MAMP in plants.

Results

Identification of the Chitin Elicitor Receptor Kinase 1 (CERK1) Gene. Because the rice chitin elicitor-binding protein, CEBiP, contains two LysM motifs that are involved in the recognition of chitin oligosaccharides (12), we tested a series of *Arabidopsis* KO mutants disrupted for the genes encoding LysM containing proteins for loss of response to the elicitor. For this purpose, we used a luminol-based chemiluminescence detection system to measure reactive oxygen species (ROS) (13) upon *N*-acetylchitooctaose $(GlcNAc)_8$ treatment. We found that a Ds-transposon and a T-DNA insertional mutant for a gene encoding a LysM containing receptor-like kinase (*Arabidopsis* Genome Initiative code At3g21630) completely lost the ability to respond to the chitin elicitor. In contrast, two mutants for other LysM

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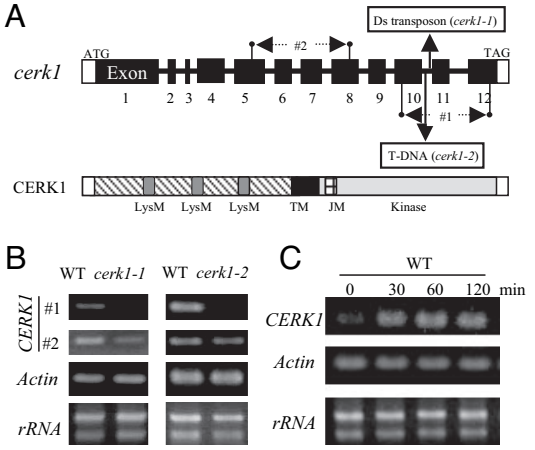

Fig. 1. Characterization of *CERK1* and its mutants. (*A*) Predicted structure of CERK1 protein and the gene model of At3g21630 including the positions of T-DNA and Ds-transposon insertion. LysM, LysM motif; TM, transmembrane domain; JM, juxtamembrane domain. (*B*) Expression of *CERK1* in the Ds (*cerk1-1*, *Left*) and T-DNA (*cerk1-2*, *Right*) insertional mutants. Primers for RT-PCR were designed to amplify the region containing the positions for Ds or T-DNA insertion. (*C*) Induction of the expression of *CERK1* gene by chitin elicitor treatment. The *Arabidopsis* seedlings were treated with 100 μg·ml^{-1} of $(GlcNAc)_8$, taken at the indicated time intervals and used for RT-PCR as described in the text.

receptor-like kinase genes (*Arabidopsis* Genome Initiative codes At1g51940 and At2g33580) showed no difference in the elicitor response (ROS generation), compared with the WT plant. The gene model of At3g21630 as well as the predicted structure of the encoded protein indicates the presence of three extracellular LysM motifs, a transmembrane domain, a juxtamembrane domain, and an intracellular Ser/Thr kinase domain (Fig. 1*A*). We refer to the protein as chitin elicitor receptor kinase 1 (CERK1) and to its mutants as *cerk1-1* (Ds-transposon) and *cerk1-2* (T-DNA) hereafter (Fig. 1*A*). The transposon and T-DNA were both inserted into the same (10th) intron of *CERK1*. RT-PCR with the primer set for the 3′-region of *CERK1* that includes the inserted Ds-transposon or T-DNA in *cerk1-1* or *cerk1-2* amplified the expected fragment only in WT plants, whereas the primers for the 5′-region that does not contain these insertions amplified the corresponding fragment in both WT and mutant plants, indicating the presence of abnormally processed transcripts in these mutants (Fig. 1*B*; the positions of the primers are shown in Fig. 1*A*). Because *CEBiP* is highly induced upon chitin elicitor treatment in rice (12), we investigated whether this is the case for *CERK1*. We found that *CERK1* expression was also up-regulated by treatment with the chitin oligosaccharide elicitor (Fig. 1*C*).

The *cerk1* Mutants Specifically Lost the Ability to Respond to the Chitin Oligosaccharide Elicitor. Fig. 2*A* shows chitin elicitor-induced ROS generation by *Arabidopsis thaliana* seedlings. The values were the average of ROS generation observed for 10 individual seedlings. Although the WT seedlings generated ROS extensively after the treatment with *N*-acetylchitooctaose $(GlcNAc)_8$, as reported (14), the seedlings of the *cerk1* mutants, *cerk1-1* and *cerk1-2*, did not.

To see whether such a deficiency in elicitor response is specific to chitin oligosaccharide, the responses of WT as well as mutant seedlings to another potent elicitor, bacterial lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* (14, 15), was examined. As shown in Fig. 2*B*, both the WT as well as the mutant seedlings responded to the LPS and generated ROS similarly, showing that *cerk1-1* mutation specifically blocked chitin signaling for ROS generation.

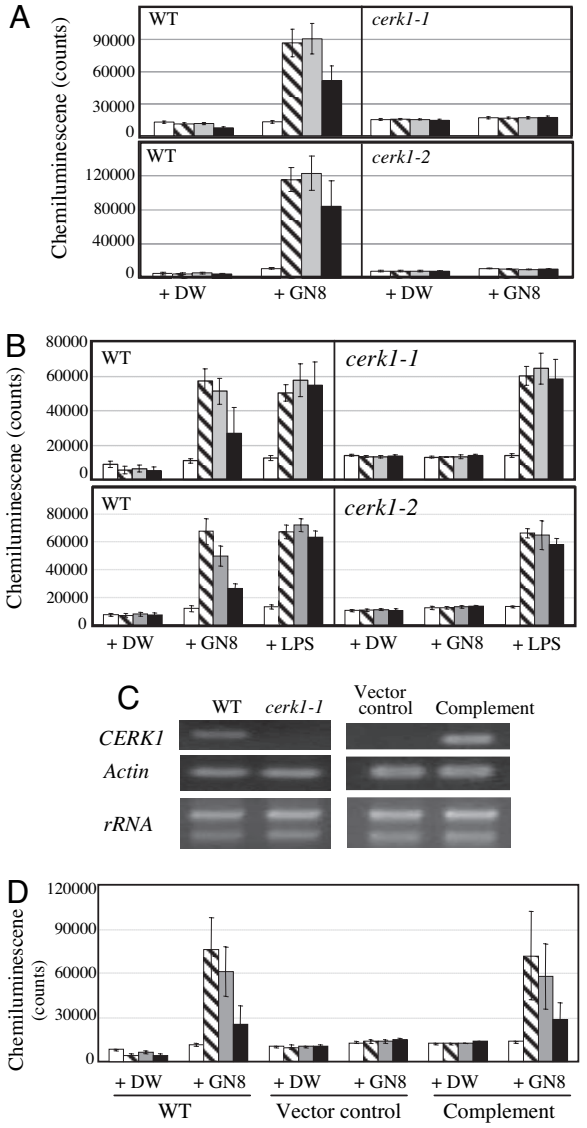

Fig. 2. Specific loss of chitin-induced ROS generation in *cerk1* mutants and the recovery in the transformants complemented with the WT *CERK1* gene. (*A*) ROS generation by *cerk1-1* and *cerk1-2* mutants treated with 100 μg·ml^{-1} of $(GlcNAc)_8$. ROS was analyzed at 0, 30, 60, and 120 min after the elicitor treatment (shown in white, stripe, gray, and black, respectively). WT, WT *A. thaliana*; GN8, *N*-acetylchitooctaose, DW, control seedlings treated with water. (*B*) ROS generation by *cerk1-1* and *cerk1-2* mutants treated with 100 μg·ml^{-1} of *P. aeruginosa* LPS. (*C*) Recovery of *CERK1* expression in the *cerk1-1* mutants complemented with WT *CERK1* gene. (*D*) Recovery of chitin-induced ROS generation in the same transformants.

Complementation of *cerk1-1* Mutation with the WT Gene. To further confirm that the lack of chitin-induced ROS generation was caused by *cerk1* mutation, complementation experiments with the genomic fragments containing the WT *CERK1* gene including 1.1 kb upstream of the starting codon was carried out. Fig. 2 *C* and *D* show that the transgenic T_2 plants successfully recovered the expression of WT *CERK1* gene and also recovered the ability to generate ROS in response to the chitin elicitor. In contrast, those plants transformed with the empty vector retained the mutant phenotype in both *CERK1* expression and ROS response. These results clearly demonstrated that CERK1 is essential for the response to the chitin oligosaccharide elicitor.

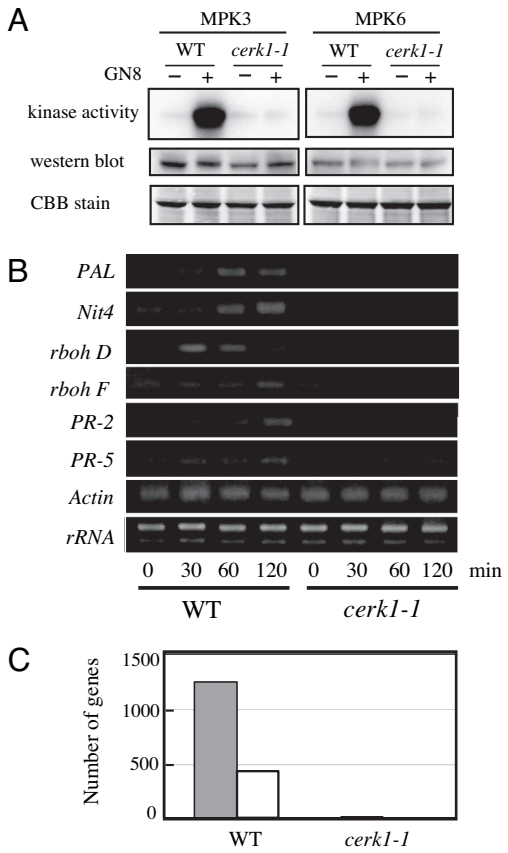

Fig. 3. Loss of chitin elicitor responses in *cerk1-1* mutant. (*A*) Loss of MPK3 and MPK6 activation by $(GlcNAc)_8$ in *cerk1-1* mutant. (*Top*) Immunocomplex kinase assay for monitoring MPK3 and MPK6 activities upon $(GlcNAc)_8$ elicitation. Seedlings (0.1 g) of WT and *cerk1-1* grown in MGRL media for 8 days were transferred on the same media in plastic plates and then incubated for 24 h at 22°C to eliminate mechanical shock. The seedlings were treated with either 100 $\mu g \cdot ml^{-1}$ of $(GlcNAc)_8$ (+) or MGRL media as a mock (−) for 10 min, then frozen in liquid nitrogen. MAPK activity was examined by immunocomplex kinase assay as described in the experimental procedures. (*Middle*) Western blot analysis of MPK3 and MPK6 proteins in WT and *cerk1-1*. The same protein extracts (20 μg) for the immunocomplex kinase assay were used for Western blot analysis. Equal protein loading was confirmed by staining membrane with the Coomassie brilliant blue R-250 (*Lower*). (*B*) Defense gene expression induced by chitin elicitor treatment in WT and *cerk1-1* seedlings. Seedlings were treated with 100 $\mu g \cdot ml^{-1}$ of $(GlcNAc)_8$ and taken at indicated time intervals for RT-PCR analysis. (*C*) Analysis of global gene expression induced by chitin elicitor treatment in WT and *cerk1-1* seedlings. Seedlings were treated with 100 $\mu g \cdot ml^{-1}$ of $(GlcNAc)_8$ for 2 h and used for RNA extraction. Only three genes in *cerk1-1* mutant showed the significant up-regulation by the elicitor treatment compared with 1,222 genes in WT. Similarly, only two genes showed down-regulation in *cerk1-1*, compared with 421 genes in WT.

Chitin Elicitor-Induced Activation of MAPK Was Impaired in *cerk1-1*. Because several different elicitor treatments activate the MAPKs MPK3 and MPK6, which are positive regulators of defense in *Arabidopsis* (16–18), we investigated whether the activation of these MAPKs by the chitin oligosaccharide elicitor was impaired in *cerk1-1* mutant seedlings by using the immunocomplex kinase assay (19) (Fig. 3*A*). As shown in Fig. 3*A*, both MPK3 and MPK6 were activated by $(GlcNAc)_8$ treatment in WT seedlings but not in *cerk1-1* (Fig. 3*A Top*). This was not due to the instability of MAPKs in the *cerk1-1* mutants, because MPK3 and MPK6 protein levels in the mutant seedlings were similar to WT (Fig. 3*A Middle*). We also observed a slight mobility shift of both MPK3 and MPK6 proteins in WT but not in the *cerk1* mutants upon $(GlcNAc)_8$ treatment (Fig. 3*A Middle*). Because the mobility shift of MAPKs is well correlated with their activation (20), these data also showed that MPK3 and MPK6 were not activated in the *cerk1-1* mutants upon $(GlcNAc)_8$ treatment. Thus, CERK1 functions upstream of MPK3 and MPK6 in the chitin signaling pathway.

Genome-Wide Gene Response Induced by the Chitin Elicitor Was Completely Blocked in *cerk1-1*. We and others reported that chitin oligosaccharide elicitors induce the expression of various defense-related genes in both suspension-cultured rice cells and *Arabidopsis* seedlings (14, 21, 22). To investigate whether this response is impaired in *cerk1-1*, we performed RT-PCR experiments (Fig. 3*B*). The expression of typical defense-related genes was up-regulated by $(GlcNAc)_8$ treatment in the WT seedlings, whereas a similar response was not observed in *cerk1-1* mutants. We also reported that the knock-down of *CEBiP* gene in rice resulted in the suppression of gene induction by the chitin oligosaccharide elicitor (12). However, the effect was limited to ≈70% of the up- and 80% of the down-regulated genes normally induced by the chitin oligosaccharide elicitor. To investigate whether this is the case for *cerk1-1* mutants, the global gene expression induced by $(GlcNAc)_8$ treatment was analyzed by using the 44k microarray of *Arabidopsis*. Although $(GlcNAc)_8$ treatment up-regulated the expression of >1,000 genes, including *CERK1* itself as well as other PAMP receptor genes such as *FLS2* (7) and *EFR* (8), and down-regulated several hundred genes in the WT seedlings, practically none of the genes responded in the *cerk1-1* mutant seedlings (Fig. 3*C*). These data showed that CERK1 is the essential positive regulator of genes that are induced by the chitin oligosaccharide elicitor.

The CERK1-GFP Fusion Protein Is Localized in the Plasma Membrane. We showed that CEBiP is localized in the plasma membrane of rice cells. Because CERK1 contains a transmembrane domain, we predicted CERK1 is also localized in the plasma membrane. To test this hypothesis, the CERK1-GFP fusion protein was transiently expressed in onion epidermal cells by using particle bombardment and observed with a confocal laser-scanning microscope. We found that the CERK1-GFP fusion protein was clearly detected in the plasma membrane (Fig. 4*A Upper*), whereas the onion cells transformed with pGWB6-GFP vector (the negative control) exhibited green fluorescence throughout the nucleus and cytoplasm (Fig. 4*A Lower*). These data indicate that CERK1 is localized at the plasma membrane, although the localization may better be confirmed by using similar approach with *A. thaliana* itself.

The CERK1 Intracellular Region Contains a Functional Protein Kinase Domain. Amino acid sequence analysis predicted that the CERK1 intracellular region contains a eukaryotic protein kinase domain (Fig. 1*A*). To examine whether CERK1 is a functional protein kinase, the GST-tagged predicted intracellular region of CERK1 ($GST\text{-}CERK1_{Int}$) and a mutant version lacking the juxtamembrane domain ($GST\text{-}CERK1_{Int}\Delta JM$) were expressed in *Escherichia coli* and purified by affinity purification with the glutathione resin. As a positive control, we included the GST fusion protein with a kinase domain of SYMRK, a key molecule for symbiosis in *Lotus japonicus* (23). The purified GST fusion proteins were incubated in a reaction buffer containing [γ-^{32}P] with or without MBP as an artificial substrate, separated by SDS/PAGE and autoradiographed. We detected autophosphorylation activity of $GST\text{-}CERK1_{Int}$ and GST-SYMRK but neither in GST alone nor in $GST\text{-}CERK1_{Int}\Delta JM$ (Fig. 4*B*). These data indicate that the CERK1 intracellular region contains a functional protein kinase domain. These results also suggest that, consistent with a previous report (24), the juxtamembrane region is essential for protein kinase activity. Furthermore, similar to GST-SYMRK, $GST\text{-}CERK1_{Int}$ was able to phosphor-

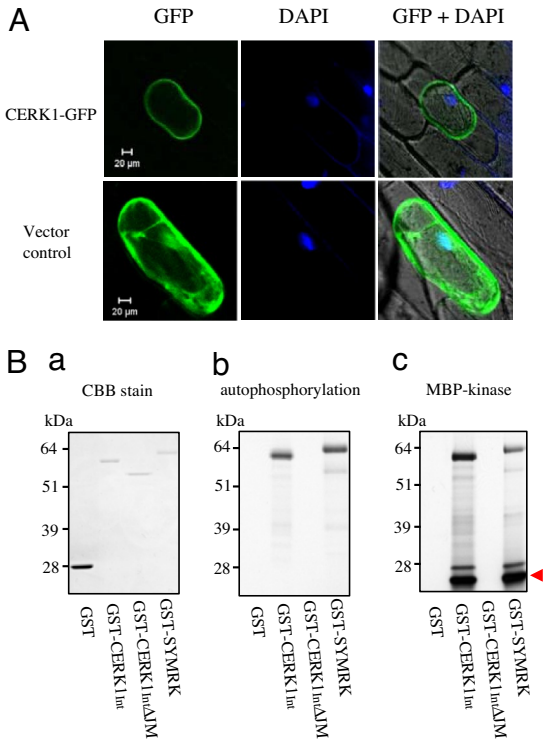

Fig. 4. *CERK1* encodes a functional protein kinase localized at the plasma membrane. (*A*) Detection of CERK1-GFP fusion protein at the plasma membrane. The CERK1-GFP fusion protein was transiently expressed in onion epidermal cells by particle bombardment and observed with a confocal laser-scanning microscope. The vector control expressing GFP was similarly observed. (*B*) Protein kinase activity of intracellular region of CERK1. (*a*) Coomassie brilliant blue (CBB) staining of purified samples. The GST alone, GST-CERK1$_{Int}$, GST- CERK1$_{Int}$ΔJM, and GST-SYMRK were expressed in *E. coli* and purified. The samples (0.5 μg) were separated by 4–12% gradient polyacrylamide gel and stained with CBB R-250. (*b*) Autophosphorylation activity of GST-CERK1$_{Int}$. The samples (0.5 μg) were incubated in a reaction buffer containing [γ-^{32}P] at 30°C for 30 min. The reaction was stopped by addition of the SDS sample buffer. Samples were subsequently separated by SDS/PAGE as described above and autoradiographed. (*c*) MBP-phosphorylation activity of GST-CERK1$_{Int}$. The *in vitro* phosphorylation assay was performed as *b* but in the presence of MBP (5 μg).

ylate MBP *in vitro* (Fig. 4*Bc*). In summary, we concluded that CERK1 is a plasma membrane protein with a functional kinase that mediates chitin signal transduction.

Affinity Labeling with a Chitin Oligosaccharide Derivative Indicated the Significant Difference in the Number of Binding Sites Between the Membrane Fractions of Rice and *Arabidopsis*. Affinity labeling with a biotinylated $(GlcNAc)_8$ derivative, GN8-Bio, did not detect specific binding proteins in the microsomal membrane preparation from the *A. thaliana* Col-0 WT seedlings, whereas the same experiment detected the chitin elicitor-binding protein, CEBiP (12), in the membrane preparation from suspension-cultured rice cells [supporting information (SI) Fig. 5*A*]. Affinity labeling with membrane preparations from leaves of fully grown plants as well as suspension-cultured *Arabidopsis* cells resulted in similar results (Fig. 5*B*). Thus, it is possible that the number of binding sites for the chitin oligosaccharide elicitor is much fewer in the membrane fraction of *Arabidopsis*, compared with rice cells. Alternatively, the efficiency of affinity cross-linking with GN8-Bio is much lower in *Arabidopsis* than in rice.

The *cerk1* Mutation Weakly Affected the Resistance of *Arabidopsis* Against *A. brassicicola*. When challenged with an isolate of *A. brassicicola*, an incompatible fungus, both *A. thaliana* Col-0 WT and *cerk1-2* mutant plants developed small brown necrotic lesions that spread from the inoculated sites at the early stage of inoculation and were then restricted at a later stage. However, lesion development was significantly faster (≈20% larger lesions) in the *cerk1-2* mutant compared with the WT Col-0 plants (Table 1). These observations indicated that the *cerk1* mutation partly impaired the resistance of *A. thaliana* against the incompatible fungus. In contrast, we did not observe a significant difference in severity of disease symptoms between Col-0 and *cerk1-2* plants with a compatible fungus, *Colletotrichum higginsianum*. Thus, the contribution of CERK1 in disease resistance depends on fungi.

Table 1. Lesion development in *cerk1-2* and Col-0 leaves after the inoculation of *A. brassicicola*

Plant	Lesion size, mm
cerk1-2	1.37 ± 0.57
Col-0	1.14 ± 0.56

Three plants were grown in each eight pots and inoculated with *A. brassicicola*. The size of the lesions was measured at 6 days after inoculation. Data shown represent mean ± SD; $n = 86$ for *cerk1-2*, $n = 102$ for Col-0. The difference between the mean value of lesion size of *cerk1-2* and Col-0 was statistically significant ($P < 0.01$).

Discussion

The results described in this article clearly demonstrate that CERK1, a LysM receptor-like kinase, is an indispensable component for the perception and transduction of the chitin oligosaccharide elicitor in *Arabidopsis*. In the *cerk1* mutants, all elicitor-induced responses were completely impaired. The suppression of MAPK activation as well as the elicitor-induced ROS generation indicates that CERK1 locates upstream of the signaling cascade for these responses. The complete loss of the gene responses induced by the chitin elicitor also indicates that CERK1 serves as the "master switch" of the signaling cascade. Furthermore, specific loss of the responses induced by the chitin elicitor, but not by LPS, in *cerk1* indicates that CERK1 is a component of chitin-specific defense signaling.

These results, as well as the localization of CERK1 in the plasma membrane, lead to speculation that CERK1 is involved in the perception of the chitin oligosaccharide elicitor at the cell surface and the transduction of the signal into the cytoplasm via its intracellular serine/threonine kinase activity. Although we have not shown that CERK1 binds to chitin oligosaccharides directly, the finding that both CERK1 and CEBiP, a chitin elicitor-binding protein in rice (12), contain LysM motifs in their extracellular domains suggests that LysM motifs are likely in the chitin-binding domain. The results of the docking simulation of chitotetraose to LysM domains (25) seem to support this prediction. Interestingly, several receptor kinases that contain LysM motifs in the extracellular domain, such as NFR1 and NFR5 in *L. japonicus* (26, 27) and LYK3 in *Medicago truncatula* (28), also play a major role in the perception of Nod factors, derivatives of chitin oligosaccharides essential for nodulation signals. It is of particular interest to know how the structurally related oligosaccharide receptors induce opposite biological responses, defense vs. symbiotic reactions in plants. Further studies of their downstream components are needed to dissect these oligosaccharide signaling pathways.

Identification of a LysM receptor kinase as a potential chitin receptor in *Arabidopsis* leads to an intriguing possibility that rice might contain a similar receptor kinase to make a pair with CEBiP. Similarly, *Arabidopsis* might contain a CEBiP-like molecule as an additional component for the perception of the chitin oligosaccharide elicitor. The results of affinity-labeling experi-

ments, however, indicated that the chitin receptor systems in *Arabidopsis* and rice might be significantly different. That any chitin-binding protein was not detected with the membrane preparations from *Arabidopsis* plants and cultured cells, whereas the previously reported chitin elicitor-binding protein CEBiP was detected with the membrane preparation from rice cells under the same conditions, may indicate that the amount of CEBiP-like molecules in the *Arabidopsis* membrane might be much less comparable to rice, if present. The failure of the detection of the elicitor-binding protein corresponding to the size of CERK1 also indicated that CERK1 itself might not bind to the chitin oligosaccharide, or the amount of CERK1 was not enough to be detected by the present method. It may also be possible, however, that the efficiency of affinity cross-linking with the present ligand is not equal for rice and *Arabidopsis* and made it difficult to detect the binding proteins in the latter plant. More biochemical studies on chitin elicitor-binding proteins in the *Arabidopsis* membrane are thus required to answer these questions.

Infection experiments with the incompatible fungus, *A. brassicicola*, to the WT and *cerk1-2* mutant of *A. thaliana* showed that the *cerk1* mutation caused enhanced necrotic lesions, although the degree was limited. This indicates that chitin perception through the CERK1 receptor kinase at least partly contributes to the resistance of *Arabidopsis* against incompatible fungi. That the *cerk1* mutation has only a limited effect on disease resistance is not surprising, because disease resistance consists of multiple-layered defense machinery including perception of various MAMP molecules (1, 2). Thus, the interruption of a single component of such a multilayered defense system may not result in a drastic change in disease resistance.

Recent finding that chitin induces defense responses not only in plants but also in mice (11) indicates the presence of a common strategy to detect invading pathogens through the recognition of chitin in a wide range of living systems. Moreover, these compounds were shown to be synthesized endogenously by zebrafish and were suggested to be involved in the developmental process of the fish (29). Clarification of the receptor system for chitin oligosaccharide elicitor should also contribute to the study of these systems.

Materials and Methods

Elicitors. Chitosan oligosaccharides were kindly supplied by Yaizu Suisankagaku Industrial Co. and reacetylated as described (30). Lipopolysaccharide from *Pseudomonas aeruginosa* was purchased from Sigma.

Mutants. The Ds-transposon insertion mutant for At3g21630, pst14772 ,was obtained from the RIKEN BioResource Center. The T-DNA KO mutant for At3g21630, 096F09, was obtained from the GABI-Kat. The T-DNA insertion mutants for At1g51940; Salk_079730 and Salk_026814, for At2g33580; Salk_131911, for At4g21410; Salk_069665, Salk_111384 and Salk_146894; and the seeds of ecotype Nossen were obtained from the *Arabidopsis* Biological Resource Center.

Plant Growth and Elicitor Treatment. *Arabidopsis* seeds were surface-sterilized and germinated on Petri plates containing MGRL nutrients supplemented with 0.1% agarose and 1% sucrose (1% glucose in the case of LPS treatment), as reported (14). Seedlings were grown for 8–10 days <16/8-h light/dark cycle at 22°C (light) or 15°C (dark) and used for elicitor treatment. The seedlings were preincubated in fresh MGRL medium in a 48-well microtiter plate for 2 h and then added with 100 $\mu g \cdot ml^{-1}$ of $(GlcNAc)_8$ or LPS solution.

Fungal Culture, Plant Growth, and Inoculations. *A. brassicicola* (isolate O-264) (31) and *C. higginsianum* (MAFF305635) (32) have been described. *Arabidopsis* plants were grown in soil for 28–30 days in a growth chamber at 22°C under a 12-h light–dark cycle. Plants were inoculated by spraying the leaves with a spore suspension (5×10^5 spores$\cdot ml^{-1}$ in distilled water) of *A. brassicicola* and *C. higginsianum.* Alternatively, two 5-μl drops of the spore suspension were placed on each leaf. Inoculated plants were then placed in a growth chamber at 22°C with a 12-h light–dark cycle and maintained at 100% relative humidity. Control plants were treated with only distilled water.

Complementation Experiment. A 4,312-base genomic DNA segment for At3g21630 that contained the 1,084-base upstream sequence and the 3,197-base coding region for *CERK1* was amplified by PCR by using primers 5′-gtgagtctgctcgaggctcttg-3′ and 5′-ttattatctttgacgtgagatgctg-3′. The fragment was first subcloned into pENTR/D-TOPO (Invitrogen) then inserted into binary vector pMDC123 (33) by using the LR clonase reaction following the manufacturer's protocol (Invitrogen). *Agrobacterium tumefaciens* strain C58C1 was transformed with these plasmids and used for infection of flowering plants by the floral-dip method.

Particle Bombardment and GFP Fluorescence Detection. Transient expression of GFP-fusion proteins in onion epidermal cells was performed with a Model PDS-1000/He Biolistic Particle Delivery System (Bio-Rad). Three micrograms of purified plasmids were coated with 1-μm gold particles, as described by the manufacturer. After bombardment, onion epidermal tissue was incubated overnight at 25°C on solid MS medium. The onion cells were floated on 0.5 M sucrose, incubated for 30–60 min, and observed with a confocal laser-scanning microscope (Zeiss LSM 510, excitation wavelength 488 nm by argon laser, emission wavelength 550 nm), and section Z-series of images were collected at different intervals through the specimens to facilitate observation. Twenty to 30 cells were imaged for each experiment. Empty vector pGWB6 was used as a negative control.

Preparation of Purified GST-Fusion Proteins from *E. coli* and *in Vitro* Phosphorylation Assay. The *CERK1* sequence corresponding to the intracellular kinase domain (Tyr_{255} to Arg_{617}) was amplified by PCR and subcloned into pENTR/D-TOPO (Invitrogen) and then into the pDEST15 expression vector for GST fusion protein (Invitrogen) by using the LR clonase reaction. The *CERK1* sequence that does not contain juxtamembrane region (Phe_{322} to Arg_{617}) was similarly introduced into the expression vector as a negative control. The resulting plasmids were designated as pDEST15-$CERK1_{Int}$ and pDEST15-$CERK1_{Int}\Delta$JM, respectively. The pDEST15-$CERK1_{Int}$ (or pDEST15-$CERK1_{Int}\Delta$JM) plasmids were transformed in *E. coli* strain BL21-Al (Invitrogen). The GST-$CERK1_{Int}$ proteins were expressed by adding L-arabinose to a final concentration of 0.2% into LB media and culturing at 23°C for 3 h. The pGEX6P-1 for GST alone and the pGEX4T-1-SYMRK for GST-SYMRK (24) were transformed into Rosetta2 and Rosetta pLacI (GE Health Care Science), respectively. The GST alone and GST-SYMRK proteins were expressed with 1 mM isopropyl 1-thio-β-galactopyranoside by culturing at 23°C for 3 h and 30°C for 2 h, respectively. Purification was performed by using Glutathione Sepharose fast flow (GE Health Care Science) according to the manufacturer's instructions. Purified proteins were desalted, buffer-exchanged by dialysis in 25 mM Tris·HCl pH 7.5 at 4°C, and stored at −80°C until use. Protein concentration was determined by the protein assay kit (Bio-Rad). *In vitro* phosphorylation assay was performed by using 0.5 μg of purified GST-fusion proteins with or without 5 μg of MBP as an artificial substrate in the same buffer for the immunocomplex kinase assay, as described (20), at 30°C for 30 min.

Affinity Biotinylation. Affinity labeling of chitin elicitor-binding protein was performed basically as described (30), although the ligand was changed from the ^{125}I-labeled $(GlcNAc)_8$ derivative to the conjugate of biocytin hydrazide and $(GlcNAc)_8$, GN8-Bio. Ten micrograms of microsomal membrane preparation was mixed with 0.4 μM GN8-Bio and adjusted to a total volume of 300 μl with binding buffer. After incubation for 1 h on ice, 30 μl of 2.5% glutaraldehyde solution was added to the mixture and kept still for 30 min. After the reaction was stopped by the addition of 30 μl of 1 M Tris·HCl, proteins were concentrated by ethanol precipitation with trypsin inhibitor (50 μg) as a carrier for coprecipitation. The precipitate was dissolved in SDS/PAGE sample buffer, boiled for 5 min, and used for SDS/PAGE.

Details of light microscopy, analysis of reactive oxygen species, immunocomplex kinase assay, RT-PCR, microarray analysis, construction of CERK1-GFP fusion vector, membrane preparation, synthesis of biotinylated ligand, SDS/PAGE, and Western blotting are described in *SI Text*.

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