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The Bifunctional Plant Receptor, OsCERK1, Regulates Both Chitin-Triggered Immunity and Arbuscular Mycorrhizal Symbiosis in Rice

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Rapid Paper

Plants are constantly exposed to threats from pathogenic microbes and thus developed an innate immune system to protect themselves. On the other hand, many plants also have the ability to establish endosymbiosis with beneficial microbes such as arbuscular mycorrhizal (AM) fungi or rhizobial bacteria, which improves the growth of host plants. How plants evolved these systems managing such opposite plant–microbe interactions is unclear. We show here that knockout (KO) mutants of OsCERK1, a rice receptor kinase essential for chitin signaling, were impaired not only for chitin-triggered defense responses but also for AM symbiosis, indicating the bifunctionality of OsCERK1 in defense and symbiosis. On the other hand, a KO mutant of OsCEBiP, which forms a receptor complex with OsCERK1 and is essential for chitin-triggered immunity, established mycorrhizal symbiosis normally. Therefore, OsCERK1 but not chitin-triggered immunity is required for AM symbiosis. Furthermore, experiments with chimeric receptors showed that the kinase domains of OsCERK1 and homologs from non-leguminous, mycorrhizal plants could trigger nodulation signaling in legume–rhizobium interactions as the kinase domain of Nod factor receptor1 (NFR1), which is essential for triggering the nodulation program in leguminous plants, did. Because leguminous plants are believed to have developed the rhizobial symbiosis on the basis of AM symbiosis, our results suggest that the symbiotic function of ancestral CERK1 in AM symbiosis enabled the molecular evolution to leguminous NFR1 and resulted in the establishment of legume–rhizobia symbiosis. These results also suggest that OsCERK1 and homologs serve as a molecular switch that activates defense or symbiotic responses depending on the infecting microbes.

Keywords: Arbuscular mycorrhizal fungi • Defense • *Lotus japonicus* • Rhizobia • Rice • Symbiosis.

Abbreviations: AM, arbuscular mycorrhizal; CEBiP, chitin elicitor-binding protein; CERK1, chitin elicitor receptor kinase1; CO4/CO5, chitin tetrasaccharide and pentasaccharaide; CSP, common symbiotic pathway; dpi, days post-inoculation; GN7, *N*-acetylchitoheptaose; KO, knockout; LCO, lipochitooligosaccharide; LYK, LysM receptor kinase; LysM, lysine motif; MAMP, microbe-associated molecular pattern; NF, Nod factor; NFP, Nod factor perception; NFR, Nod factor receptor; ROS, reactive oxygen species.

Introduction

Plants have evolved a sophisticated system for monitoring microbial signal molecules known as microbe-associated molecular patterns (MAMPs) to protect against infection by potential pathogens (Boller and Felix 2009, Dodds and Rathjen 2010). Detection of MAMPs by plant receptors leads to rapid activation of defense responses such as the generation of reactive oxygen species (ROS) and the production of antimicrobial substances. A lysine motif (LysM) receptor-like kinase, CERK1 (chitin elicitor receptor kinase1), is indispensable for the recognition of a fungal MAMP, chitin, in both *Arabidopsis thaliana* and rice (Miya et al. 2007, Shimizu et al. 2010). In *A. thaliana*, CERK1 forms a homodimeric complex and perceives chitin by itself (Liu et al. 2012) whereas rice OsCERK1 requires another LysM protein, CEBiP (chitin elicitor-binding protein), for the recognition of chitin oligosaccharides (Shimizu et al. 2010), indicating the difference in the chitin receptor systems between these model plants. CERK1 was also shown to be required for the recognition of peptidoglycan, a major component of bacterial cell walls (Willmann et al. 2011). Thus, CERK1 plays a central role in MAMP-triggered immunity against both fungal and bacterial pathogens. This conclusion is supported by the observation that both bacterial and fungal pathogens often

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target the CERK1 or chitin signaling pathway by virulence effector proteins for successful infection (Gimenez-Ibanez et al. 2009, de Jonge et al. 2010).

In contrast to hostile microbes, soil bacteria called rhizobia establish a mutualistic symbiosis exclusively with leguminous plants and improve plant growth by supplying nitrogenous nutrients (Kouchi et al. 2010). Plant symbiotic responses to accept rhizobia are initiated by the recognition of bacterial signal molecules, known as Nod factors (NFs). The structure of NFs varies among rhizobial species and determines their host specificity; however, a chitin oligosaccharide backbone with *N*-acylation at the non-reducing end is common for all NFs. Two types of LysM receptor-like kinases, NFR1/LYK3 and NFR5/NFP, are essential for the recognition of NFs in legume plants (Limpens et al. 2003, Madsen et al. 2003, Radutoiu et al. 2003, Arrighi et al. 2006). In a model legume *Lotus japonicus*, loss of function of either LjNFR1 or LjNFR5 completely abolished all known symbiotic responses to *Mesorhizobium loti* and to purified NFs (Madsen et al. 2003, Radutoiu et al. 2003). It has been shown that the kinase activity of LjNFR1 is crucial to trigger intracellular signaling for rhizobial symbiosis, while LjNFR5 lacks kinase activity (Madsen et al. 2011).

Interestingly, the chitin receptor CERK1 is the closest homolog of leguminous NFR1/LYK3 among the cognate LysM receptor-like kinases in Arabidopsis (Zhu et al. 2006, Zhang et al. 2007). In addition, microsynteny analysis suggests that NFR1 and CERK1 are the close descendants from a common ancestor. Thus, there is an apparent evolutionary relationship between NFR1 and CERK1, although the physiological function of these molecules is seemingly opposite. We previously showed that the expression of symbiotic genes, which was inducible by NFs, was also weakly but significantly induced by chitin treatment in *L. japonicus* (Nakagawa et al. 2011). This induction of symbiotic genes by chitin treatment was observed not only in wild-type plants but also in *nfr1* mutants, indicating the involvement of chitin receptor(s) for the induction of these symbiotic genes. A previous finding that the chimeric receptor genes consisting of the extracellular LysM domain of *Lotus* NFR1 (LjNFR1) and intracellular kinase domain of one of the *Lotus* CERK1/NFR1 homologs, LjLys6 or LjLys7, could rescue the symbiotic defects of an *nfr1* mutant, though not completely, may also support the idea that the kinase domain of chitin receptor(s) in leguminous plants has an ability to confer some symbiotic responses. On the other hand, a similar chimeric gene containing the intracellular kinase domain of Arabidopsis CERK1 (AtCERK1) could not rescue the symbiotic defects of *nfr1* mutants (Nakagawa et al. 2011), indicating that the kinase domain of Arabidopsis CERK1 cannot trigger symbiotic responses, unlike the homologs in legumes. These findings led us to speculate that some crucial mutations in the kinase domain of an ancestral CERK1 enabled the descendants in legumes to trigger symbiotic responses after the divergence of legume plants. Further modifications of amino acid residues in the extracellular domain enabled such molecules to recognize NFs and resulted in the evolution of leguminous NFR1. Supporting this notion, we found that a consecutive three amino acid sequence, YAQ, in the kinase domains of NFR1/LYK3 orthologs, which are not present in AtCERK1, plays a critical role in triggering symbiotic signal transduction (Nakagawa et al. 2011). Based on these results, we proposed that the acquisition of YAQ in the kinase domain of ancestral chitin receptors was a key genetic event in shifting the intracellular signaling from defense to symbiosis responses, and thus in the evolution of root nodule symbiosis (Nakagawa et al. 2011). However, a recent database search revealed that YAQ or analogous YAR motifs are also conserved in CERK1 homologs in most non-leguminous plants (**Fig. 1**) except for *Brassica rapa*, which belongs to the same family as Arabidopsis, namely *Brassicaceae*. Also, De Mita et al. (2014) recently reported that the YAQ motif is well conserved in CERK1 homologs in non-leguminous dicots. Such conservation of the YAQ/YAR motifs in most non-leguminous CERK1 homologs suggested the symbiotic function of these molecules in plant–microbe interactions other than legume–rhizobium symbiosis.

In addition to legume–rhizobium symbiosis, plants are also known to establish another type of symbiotic relationship with a group of soil microbes, called arbuscular mycorrhizal (AM) fungi. AM symbiosis is observed in >80% of existing plant families including both flowering and non-flowering plants, and the remnant of AM symbiosis was detected in Ordovician fossils (Redecker et al. 2000) of early land plants, indicating the long history of this symbiotic system. Because rhizobial symbiosis evolved after divergence of the legume family, it is widely accepted that rhizobial symbiosis was established based on AM symbiosis (Parniske 2008). Supporting this, some signaling components downstream of NFRs are involved in both rhizobial and mycorrhizal symbiosis (Endre et al. 2002, Stracke et al. 2002, Ane et al. 2004, Levy et al. 2004, Imaizumi-Anraku et al. 2005, Kanamori et al. 2006, Messinese et al. 2007, Saito et al. 2007, Yano et al. 2008, Groth et al. 2010, Delaux et al. 2013, Takeda et al. 2013) and this is called the common symbiotic pathway (CSP). Also, an AM fungus *Rhizophagus irregularis* (previous name, *Glomus intraradices*) secretes symbiotic signal molecules called Myc-LCOs (lipochitooligosaccharides), the structures of which are very similar to those of rhizobial NFs (Maillet et al. 2011). Application of Myc-LCOs promoted the

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Lotus NFR1      DFGLTKLIEVGNST--LQT---RLVGTFGYMPPEYAQYGDISPKI
Medicago LYK3   DFGLTKLIEVGNST--LHT---RLVGTFGYMPPEYAQYGDVSPKI
Pea Sym37       DFGLTKLIEVGNST--LHT---RLVGTFGYMPPEYAQYGDVSPKI
Poplar          DFGLTRLTEVGSAS--LHT---RLVGTFGYMPPEYAQYGDVSSKI
R.communis      DFGLTKLTEVGSAS--LPT---RLVGTFGYMPPEYAQYGDVSPKV
Tomato          DFGLTKLTEVGSTS--FHT---RLVGTFGYMPPEYAQYGDVSPKV
Grape           DFGLTKLTEVGSSS--LPT---RLVGTFGYMPPEYAQYGDVSPKV
OsCERK1         DFGLTKLTEVGGTS--MPTGT-RVVGTFGYMPPEYARYGDVSPKV
Solghum         DFGLSKLSEIGTTSQSLPS-L-RVVGTFGYMPPEYARYGEVSPKV
Maize           DFGLTKLTEVGNTS--LPT-RG-IVGTFGYMPPEYARYGDVSPKV
AtCERK1         DFGLTKLTEVGGSA------TRGAMGTFGYMAPETV-YGEVSAKV
B.rapa          DFGLTKLTEVGSSA------TRGAMGTFGYMAPEIV-YGEVSAKV
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Fig. 1 Alignment of the various NFR1/CERK1 sequences. Amino acid sequences around the activation loop (indicated by the wavy line) and the 'YAQ' motif (indicated by the solid line) in the kinase domain are shown. Consensus amino acid residues among all sequences are marked by asterisks. Poplar, Potri.011G010000; *Ricinus communis*, 29842.t000038; tomato, Solyc07g049180.2; grape, GSVIVG0103 0482001; *Sorghum*, Sb07g025790; maize, GRMZM2G317770; *Brassica rapa*, Bra031293.

root colonization of *R. irregularis* in both a legume (*Medicago truncatula*) and non-legumes (*Tagetes patula* and *Daucus carota*) and also promoted the lateral root formation in a CSP-dependent manner. Interestingly, a homologous gene of *NFR5/NFP* was shown to be involved in both AM and rhizobial symbioses in *Parasponia*, the only known non-legume plant that establishes rhizobial symbiosis (Op den Camp et al. 2011). These findings suggest that the perception of symbiotic signal molecules in rhizobial and mycorrhizal symbiotic systems shares some common features, though the molecules involved in the perception of mycorrhizal signals are still unknown.

Collectively, these findings prompted us to hypothesize that non-leguminous CERK1 homologs may play a role in AM symbiosis. In this study, we examined such a possibility and found that rice OsCERK1 is required not only for chitin-triggered immune responses but also for AM symbiosis, indicating the bifunctional nature of this receptor-like kinase in the opposite biological processes, defense and symbiosis. Furthermore, the kinase domains of most non-leguminous CERK1 homologs could trigger the symbiotic signaling in legume–rhizobium interactions similarly to NFR1 when they were expressed as a suitable chimeric receptor. These findings reveal the direct connection between defense and these two symbiotic mechanisms in plants and shed new light on the evolutionary process of plant defense/symbiosis receptors during the long history of land plant evolution.

Results

Involvement of OsCERK1 in AM symbiosis

To examine the possible function of non-leguminous CERK1 homologs in AM symbiosis, we focused on rice because OsCERK1 is the best characterized defense receptor among the CERK1 homologs of mycorrhizal plants. In all three independent lines of *oscerk1* mutants (#19, #53 and #117) generated by gene targeting based on homologous recombination, chitin-triggered immunity was completely abolished in leaves or cultured cells (Kouzai et al. 2014a). In addition, we confirmed that several genes up-regulated or down-regulated by chitin oligosaccharides (*N*-acetylchitoheptaose: GN7) in the roots of control plants became unresponsive in *oscerk1* mutant roots (**Supplementary Fig. S1**). Using these *oscerk1* mutants, we investigated symbiotic phenotypes in AM symbiosis. The AM fungus *R. irregularis* formed a penetration apparatus, the hyphopodium, on the root surface, grew hyphae into root tissues and developed the symbiotic structures known as arbuscules inside the root cells in the control plants (**Fig. 2D, E**). Under our experimental conditions, infection of fungal hyphae in root cortex was observed at 12 days post-inoculation (dpi) and arbuscular development was fully established at 15 dpi in wild-type plants (**Supplementary Fig. S2**). In contrast, *R. irregularis* frequently formed a cluster of hyphopodia from one extraradical hypha on the root surface, but no internal hypha or arbuscule was observed in the roots of *oscerk1* mutants at 15 dpi (**Fig. 2A, B**; **Supplementary Fig. S2**). Prolonged cultivation of the *oscerk1* mutants occasionally allowed the penetration of the fungal hyphae into root cortical cells (21 dpi in **Supplementary Fig. S2**) and arbuscular development was observed at 30 dpi, while the old arbuscules that had been formed in the wild-type plants were already senesced and collapsed at the same time point (**Fig. 2C**; **Supplementary Fig. S2**). Statistical analyses also confirmed significant differences in the symbiotic phenotype, arbuscule development between the wild-type plants and *oscerk1* mutants (**Fig. 2F**; **Supplementary Fig. S3**). Therefore, we accordingly concluded that in addition to its well-known function as a defense receptor, OsCERK1 also plays an important role in the symbiosis with AM fungi in rice.

The role of OsCERK1 in AM symbiosis raised the question of whether chitin-triggered defense responses play a role in AM symbiosis. In this context, it is noteworthy that treatment with chitin oligosaccharides did not induce the expression of the symbiotic marker genes (Gutjahr et al. 2008) (**Fig. 3B, D, F, H**) and infection with *R. irregularis* did not induce the expression of defense genes (Kouzai et al. 2014b) (**Supplementary Fig. S4**). Furthermore, we also confirmed that a knockout (KO) mutant of OsCEBiP (Kouzai et al. 2014b), which is also essential for chitin-triggered immunity and forms a receptor complex with OsCERK1 in response to chitin oligosaccharides (Kaku et al. 2006, Shimizu et al. 2010), established mycorrhizal symbiosis normally (**Fig. 4**). Thus, the signaling pathways activated by OsCERK1 are different between AM symbiosis and chitin-triggered immunity, and OsCERK1 but not chitin-triggered immunity is required for AM symbiosis.

OsCERK1 acts upstream of the common symbiotic pathway

As described, it has been revealed that the upstream symbiotic signaling components belonging to the CSP are shared by both AM and rhizobial symbioses (Parniske 2008). In rhizobial symbiosis, the CSP is triggered by NFR1/LYK3 and NFR5/NFP, whereas the corresponding receptors are not clear in AM symbiosis. Thus, our observations suggested an intriguing possibility that OsCERK1 functions as the receptor of the mycorrhizal signal Myc-LCOs, although the function of Myc-LCOs has been examined only in dicotyledonous plants (Maillet et al. 2011). To test this hypothesis, we evaluated Myc-LCO responses in rice by transcriptome analysis using next-generation sequencing. Unlike chitin treatment, Myc-LCO did not induce major changes in gene expression in wild-type rice plants (**Supplementary Fig. S1**). Although a small number of genes were up-regulated or down-regulated by the Myc-LCOs in the roots of wild-type plants, such changes did not seem to be significant for AM symbiosis from the following reasons. First, these genes did not include any known AM symbiosis-associated genes. Secondly, although a small difference was observed when the mock-treated wild-type and *oscerk1* plants were compared, no difference was observed between Myc-LCO-treated wild-type and *oscerk1* plants. Because we observed no significant responses to the Myc-LCOs in rice under our experimental conditions, the relationship between OsCERK1 and Myc-LCOs remains a subject for future study. However, the induction of all known earliest symbiotic genes,

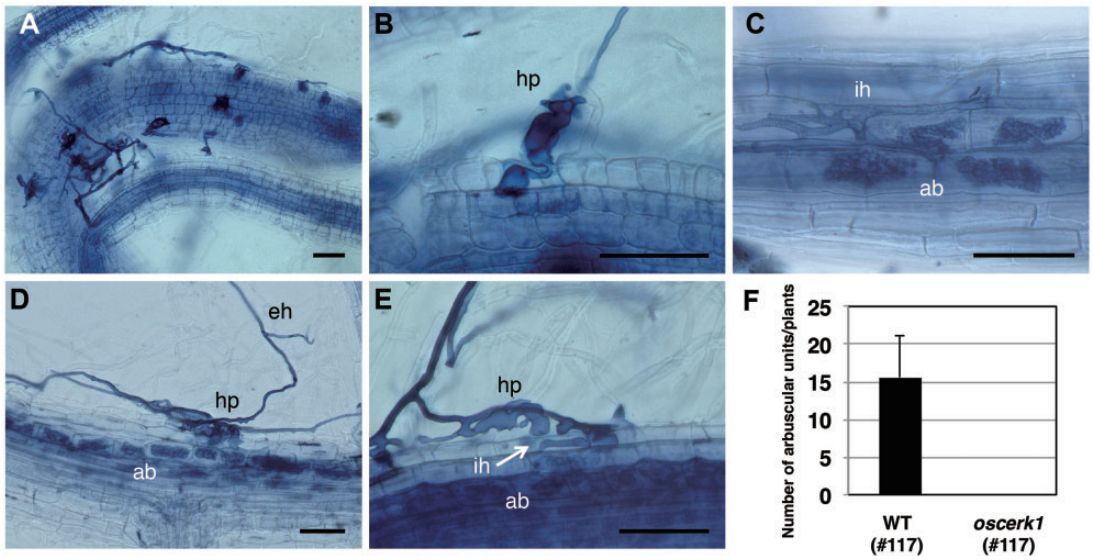

Fig. 2 Arbuscular mycorrhizal symbiosis is severely diminished in *oscerk1* mutants. Homozygous *oscerk1* mutants (A–C) or wild-type siblings (D, E) from segregating T_1 families were inoculated with *R. irregularis* and observed at 15 (A, B, D and E) and 36 dpi (C). eh, extraradical hyphae; ih, internal hypha; hp, hyphopodia; ab, arbuscules. Scale bars = 50 μm. (F) Number of infected units with arbuscules. These statistical analyses were performed at 15 dpi and are shown as the mean and SE for five independent plants.

including both CSP-dependent (AM3 and AM11) and CSP-independent (AM1 and AM2) marker genes (Gutjahr et al. 2008), after the infection of *R. irregularis* was abolished in the *oscerk1* mutant (**Fig. 3A, C, E, G**). These results suggested that OsCERK1 acts upstream of CSP proteins, similarly to NFR1/LYK3.

Kinase domains of non-leguminous CERK1 containing YAQ/YAR motifs can drive the nodulation program required for rhizobial symbiosis

Involvement of OsCERK1 in AM symbiosis in rice indicated that other non-leguminous CERK1 homologs containing YAQ/YAR sequences (**Fig. 1**) may also be involved in AM symbiosis in these plant species. Moreover, the observation that the YAQ motif in the kinase domain of NFR1 plays an important role in triggering CSP and the downstream signaling cascade leading to nodulation in legume–rhizobia symbiosis (Nakagawa et al. 2011) suggested that the kinase domains of these non-leguminous CERK1 homologs also have the ability to drive the nodulation program in legumes. To investigate this possibility, we transformed an *nfr1-4* mutant (Nakagawa et al. 2011) of *L. japonicus*, a model legume, with chimeric constructs comprising the extracellular LysM domain of *Lotus* LjNFR1 and the intracellular kinase domains of CERK1 homologs from tomato, sorghum, *Ricinus communis* or *B. rapa*, and assessed nodulation in the resulting phenotypes. All chimeric constructs except for one bearing the CERK1 kinase from *B. rapa* that lacks the YAQ sequence effectively rescued the defects of symbiotic nodule formation in the *Lotus nfr1-4* mutant (**Supplementary Figs. S5, S6**). In addition, we confirmed that a chimeric construct bearing the kinase domain of rice OsCERK1 complemented the defect of nodulation and enabled the *nfr1-4* mutant to grow under nitrogen nutrient-free conditions (**Fig. 5**; **Supplementary Fig. S7**). These results indicated that non-leguminous CERK1 homologs containing YAQ/YAR motifs conferred symbiotic ability comparable with NFR1 in the legume–rhizobia symbiosis. These findings suggested not only the involvement of these molecules in AM symbiosis in their own plant species but the potential of these non-leguminous CERK1 homologs containing YAQ/YAR motifs to have evolved to NFR1-type symbiotic receptors by adapting the extracellular domains to cognate NFs.

Discussion

In this study, we showed that a well-characterized defense receptor, OsCERK1, also plays an important role in seemingly opposite physiological process, i.e. acceptance of symbiotic AM fungi in rice. In the *oscerk1* mutants, infection of the AM fungus was arrested at the root surface and formation of arbuscules was severely delayed (**Fig. 2**; **Supplementary Fig. S2**). These results indicate that OsCERK1 regulates the acceptance of mycorrhizal hyphae by the root epidermal cells. On the other hand, prolonged cultivation of the *oscerk1* mutants with the AM fungus eventually allowed the penetration of mycorrhizal hyphae into the root cortex and the formation of arbuscules, although the arbuscules formed in the wild-type roots had already been senesced and collapsed at the same time point (**Supplementary Fig. S2**). As the *oscerk1* mutants used in the present study are null mutants (Kouzai et al. 2014a), the contribution of residual OsCERK1 activity to the delayed establishment of mycorrhizal colonization should be excluded. We speculate the following possibilities for the delayed mycorrhization phenotype of *oscerk1* mutants. The first one is the presence of functionally redundant gene(s) of *OsCERK1* in rice. In fact, another LysM receptor kinase, OsRLK10, which shares significant sequence similarity with OsCERK1 is found in the rice

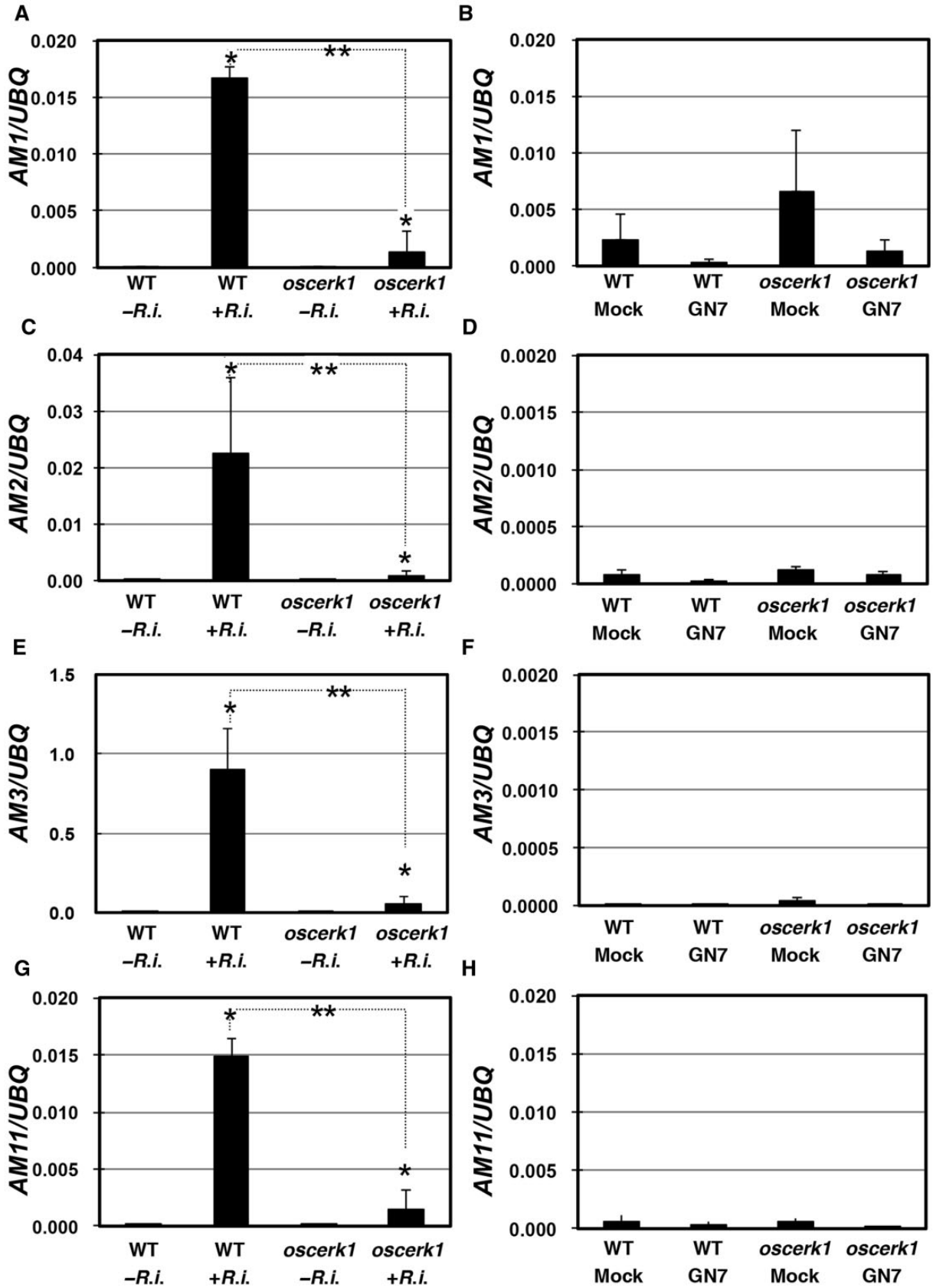

Fig. 3 Molecular phenotype of the o*scerk1* mutant. Expression levels of symbiotic marker genes, *AM1* (A and B), *AM2* (C and D), *AM3* (E and F) and *AM11* (G and H) were determined in the presence or absence of *R. irregularis* (*R.i.*) at 3 weeks post- inoculation (A, C, E, and G) or in response to GN7 treatment for 1 h (B, D, F, and H). Data are expressed as the mean and SE for three biological replicates.

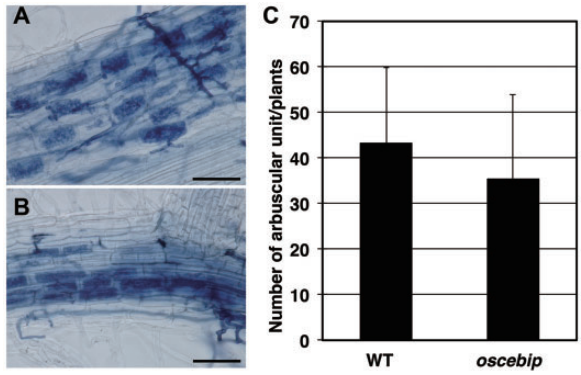

Fig. 4 Arbuscular mycorrhizal symbiosis is normally established in the *oscebip* mutant. Homozygous *oscebip* mutant (A) or wild-type siblings (B) from segregating T_1 families were inoculated with *R. irregularis* and observed at 15 dpi. (C) Number of infected units with arbuscules. These statistical analyses were performed at 15 dpi and are shown as the mean and SE for six independent plants. Scale bars = 50 μm.

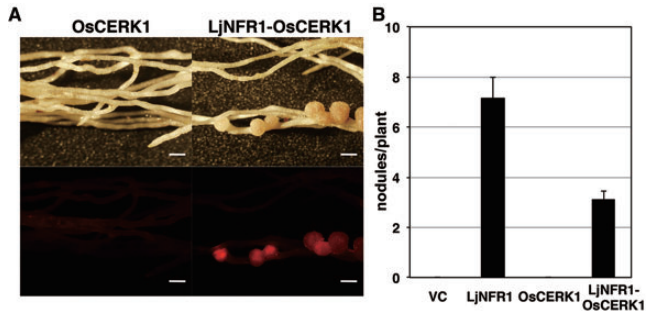

Fig. 5 Complementation tests of the *Lotus nfr1-4* mutant with the vector control (VC), LjNFR1, OsCERK1 or LjNFR1–OsCERK1. (A) The transformed roots are shown as bright-field images (upper panels) and under DsRed fluorescence (lower panels). The images were taken at 28 dpi with DsRed-labeled *M. loti*. Scale bars = 1 mm. (B) Numbers of nodules formed on transformed roots. Data were collected from the roots at 14 dpi and are shown as the mean and SE for >12 independent plants harboring transformed roots.

genome (Shimizu et al. 2010). It might be possible that OsRLK10 weakly compensates the defect of OsCERK1 and enables the delayed mycorrhization in the *oscerk1* mutants. The other possibility is that the role of OsCERK1 in AM symbiosis is auxiliary rather than essential, though the defect of OsCERK1 severely impaired the establishment of AM symbiosis. Future studies with the *oscerk1*/*osrlk10* double mutant or the comparative analyses of symbiotic phenotypes between *oscerk1* and other symbiotic mutants (e.g. CSP mutants) will contribute to extend our understanding of the early signaling mechanism in AM symbiosis (Shimizu et al. 2010).

We also found that the YAQ/YAR motif, a sequence required for the symbiotic ability of the kinase domain of NFR1, is not specific to NFR1 but is also conserved in non-leguminous CERK1 homologs from both dicots and monocots (**Fig. 1**). Together with the recent finding by De Mita et al. (2014), these results suggest that the acquisition of the YAQ/YAR motif pre-dated the establishment of rhizobial symbiosis. The fact that OsCERK1 bearing the YAR motif contributes to AM symbiosis in rice (**Fig. 2**) and the previous finding that the YAQ motif is important for activation of CSP in NFR1 (Nakagawa et al. 2011) suggest that the YAQ/YAR motifs were acquired before or during establishment of AM symbiosis and have been involved in the AM symbiosis in these non-leguminous plants. On the other hand, some non-mycorrhizal plants such as *Brassicaceae* might have lost this YAQ/YAR sequence in the corresponding CERK1 homologs in the course of the loss of AM symbiosis in these plant species.

If so, how do these non-leguminous CERK1 homologs with the YAQ/YAR sequence contribute to AM symbiosis? The finding that NFR1 forms a heteromeric receptor complex with NFR5 and directly binds to NFs (Broghammer et al. 2012) raises an intriguing possibility that OsCERK1 and other CERK1 homologs might also be involved in the perception of mycorrhizal signals, probably forming a receptor complex with NFR5 or a still unidentified counterpart, though we could not observe the Myc-LCO responses in rice (**Supplementary Fig. S1**). Recently, undecorated chitin tetrasaccharide and pentasaccharide (CO4/CO5) were also proposed as fungal symbiotic signals (Genre et al. 2013). In this experiment, CO4 and CO5 were found in the germinated spore exudates of AM fungi and elicited Ca^{2+} spiking, one of the symbiotic physiological responses, in a CSP-dependent manner (Genre et al. 2013). On the other hand, chitin oligosaccharides longer than heptamers triggered immune responses but triggered the Ca^{2+} spiking much less (Kaku et al. 2006, Genre et al. 2013). Because CERK1 was originally identified as a receptor of chitin oligosaccharides, it might be possible that CERK1 is somehow involved in the perception of CO4/CO5 in AM symbiosis. Eventual identification of the CERK1 ligand in AM symbiosis should uncover the early molecular dialogue for establishment of this mutualistic symbiosis.

The AM symbiosis was established during early land plant evolution, and it has been postulated that leguminous plants developed rhizobial symbiosis on the basis of AM symbiosis (Parniske 2008). The finding that the kinase domain of OsCERK1, which is involved in AM symbiosis, could trigger nodulation signaling in the legume–rhizobium interaction provides direct experimental support for such an evolutionary process, at least with respect to intracellular signaling mediated by the kinase domain. The fact that all the kinase domains of CERK1 homologs from non-leguminous plants having AM symbiosis tested here showed the ability to drive the nodulation program in *L. japonicus* further supports such a possibility. Furthermore, requirement for OsCERK1 in both defense and symbiotic responses indicates close evolutionary relationships between these apparently opposite biological responses. The presence of microsyntenies and substantial similarity in the amino acid sequences between CERK1/OsCERK1 and NFR1/LYK3 (Zhu et al. 2006, Zhang et al. 2007) and the observation that the symbiosis receptor, NFR1, triggers not only symbiotic but also defense responses (Nakagawa et al. 2011) support this notion. However, how and when such a bifunctional receptor originated during land plant evolution remains unclear. Functional analyses of CERK1 homologs in various plants, including pteridophytes and bryophytes, will elucidate the

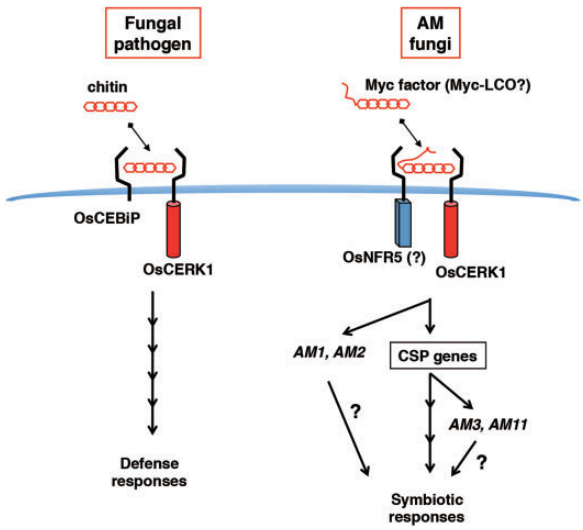

Fig. 6 Model of the roles of OsCERK1 in defense and symbiotic signaling in rice.

origin of such bifunctionality and the relationships between plant defense and symbiosis.

The bifunctionality of OsCERK1 may raise another question of how opposite signaling cascades are regulated by a single receptor. Based on the findings that NFR1/LYK3 form a receptor complex with another LysM receptor kinase, NFR5/NFP, in leguminous plants, and also that a NFR5 homolog in *Parasponia*, the only non-leguminous plant that can establish a rhizobial symbiosis, is required for both rhizobial and AM symbiosis (Op den Camp et al. 2011), it appears an intriguing possibility that OsCERK1 forms a receptor complex with a rice NFR5 homolog for symbiotic signaling. On the other hand, OsCERK1 is known to form a different receptor complex with another LysM receptor protein, OsCEBiP, to perceive chitin oligosaccharides (Kaku et al. 2006, Shimizu et al. 2010). Different receptor partners may enable OsCERK1 to recognize different ligands and trigger opposite physiological signals (**Fig. 6**).

In this study, we have shown that OsCERK1, and probably several other non-leguminous homologs, have dual function in chitin-triggered immunity and AM symbiosis in these plants. After the establishment of AM symbiosis >400 million years ago, terrestrial plants drastically altered their morphology and function through evolution. Concomitantly with this evolutionary process, many genes have diverged and functionally differentiated. Such a long period appears to be sufficient for the separation of the bifacial roles of CERK1 into independent specialized genes in the form of defense and symbiosis receptors. However, most non-leguminous CERK1 homologs, except those in *Brassicaceae*, seem to carry both defense and symbiotic functions (**Fig. 5**; **Supplementary Figs. S5–S7**) (Nakagawa et al. 2011). If the bifunctionality of CERK1 homologs originated from the beginning of AM symbiosis, the present bifacial nature of NFR1/CERK1 may imply that selection pressure is involved in the maintenance of this bifunctionality. It is intriguing to speculate that the bifunctionality of CERK1-type receptors has been advantageous for both mycorrhizal fungi and host plants for the establishment of AM symbiosis. For example, if the Myc factors have high enough affinity compared with chitin oligosaccharides, most of the CERK1 would be mobilized for symbiotic signaling in the presence of mycorrhizal fungi, resulting in automatic shutdown of the futile activation of chitin-induced defense responses. This response would appear to be advantageous even for the host plants, which otherwise would need to halt the activation of chitin-triggered immunity independently to ensure the entry of symbiotic fungi. Confirmation of this possible scenario awaits complete characterization of the ligand–receptor pairs involved in AM symbiosis in various plant species. Further studies will be needed to clarify the origin of such bifunctionality and its significance in regulating 'friend and foe' relationships in plant–microbe interactions.

Materials and Methods

Plant materials and growth conditions

All experimental methods using *L. japonicus* have been previously described (Nakagawa et al. 2011). For chemical treatments, rice seeds (*Oryza sativa* cv. Nipponbare BL no. 2) were surface-sterilized with sodium hypochlorite, washed, and germinated in sterile water. After 2 d, the germinated seedlings were transferred to liquid M medium (Becard and Fortin 1988) and grown for a further 5 d. Seven-day-old seedlings were then transferred to fresh M medium, pre-incubated for 3 h, and incubated in a fresh medium with or without 100 mg ml^{-1} GN7 (Kaku et al. 2006) or a mixture of 10^{-8} M sulfated and 10^{-7} M non-sulfated Myc-LCO-IV [each containing an equal amount of the C16:0 and C18:1 forms (Czaja et al. 2012)] for 1 h. Plants were grown in an artificially lit growth chamber at 30°C for 12 h (light) and at 25°C for 12 h (dark). To evaluate mycorrhizal symbiosis, rice seeds were surface-sterilized with 100% ethanol and immersed in water for 5 d. The germinated seeds were grown in 100 ml pots (D-100; Teraoka) in a greenhouse with a 16 h light and 8 h dark cycle at 28°C. The 100 ml pot contained 20 g (bottom layer) of Akadama soil (Setogahara Kaen) and 45 g (upper layer) of an autoclaved Kanuma soil/Ezo sand/Nippi soil (Nihon Hiryo) mixture (6 : 2 : 1, by weight) containing 5,000 spores of *R. irregularis* (*G. intraradices* DAOM 197198) (Premier Tech). No other nutrients were added except Nippi soil, and water was supplied from the bottom. Roots were stained with trypan blue and the number of arbuscular units per plants were counted 15 d after planting.

Myc-LCO synthesis

N,*N*′,*N*″,*N*‴-Tetraacetylchitotetraose was deacetylated at the non-reducing *N*-acetylglucosamine residue by *Rhizoboum etli* NodB protein (John et al. 1993) expressed in an in vitro cell-free system to give *N*,*N*′,*N*″-triacetylchitotetraose. Selective *N*-acylation of the monodeacetyl chitin tetramer was performed with palmitic (C16:0) or oleic (C18:1Δ9Z) acids using the condensing agent DMT-MM [4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride] (Kunishima et al. 2001) to synthesize LCO-IV (C16:0) and LCO-IV (C18:1Δ9Z), respectively. The non-sulfated Myc-LCOs were sulfated by *Sinorhizobium meliloti* NodH protein expressed in an in vitro cell-free system using PAPS (3′-phosphoadenosine 5′-phosphosulfate) as a sulfate donor (Schultze et al. 1995) to produce LCO-IV (C16:0, S) and LCO-IV (C18:1ΔZ, S). These Myc-LCOs were purified by ODS column chromatography (MeOH/MeCN-H_2O), and the structures were confirmed by electrospray ionization-mass spectrometry (ESI-MS) and/or ^{1}H-nuclear magnetic resonance (NMR).

DNA and RNA manipulation

Extraction of total RNA and construction of the vectors for chimeric cDNAs containing a combination of the extracellular LysM domain (including the transmembrane region) of LjNFR1 and the intracellular domains of various non-legume CERK1s have been previously described (Nakagawa et al. 2011).

Real-time RT–PCR

Total RNAs were reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Aliquots of the resulting cDNA (equivalent to approximately 1 ng of RNA) were subjected to real-time PCR analyses with a 7500 Fast Real-Time PCR system (Applied Biosystems). All the primer sets used in the expression analysis are listed in **Supplementary Table S1**.

Next-generation sequencing

Total RNAs were checked using a 2100 Bioanalyzer (Agilent Technologies) on RNA 6000 NanoRNA chips (Agilent Technologies). The amount of total RNA was quantified using a Quant-iT RiboGreen RNA Assay Kit (Life Technologies) on a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific). The libraries for mRNA sequencing were prepared with a TrueSeq RNA Sample Prep Kit Set (Illumina Inc.). The effective number of molecules for cluster generation during sequencing was titrated by qPCR using a KAPA Library Quantification Kit (Kapa Biosystems) on a Dice TP800 Thermal Cycler (TAKARA BIO INC). The size of the insert was calibrated using a 2100 Bioanalyzer (Agilent Technologies) on DNA1000 or High Sensitivity DNA chips (Agilent Technologies). The libraries were diluted to 7–20 pM to generate approximately 800,000 clusters mm^{-2} on flow cells by cBot (Illumina Inc.) with TruSeq SR Cluster Kits v2 (Illumina Inc.). The flow cells were then mounted on a GAIIx analyzer (Illumina Inc.) and the clusters were sequenced for >70 cycles with a TruSeq SBS Kit v5 (Illumina Inc.). In addition, sequencing was performed using a MiSeq Reagent Kit v2 (Illumina Inc.) and a MiSeq sequencer (Illumina Inc.), when it was necessary to obtain a sufficient number of reads.

The single-end sequencing reads were demultiplexed and the indexed adaptor reads trimmed using CASAVA v1.8.2 software (Illumina Inc.). The raw FASTQ format data sets generated from CASAVA v1.8.2 were assessed for quality using FASTQC v0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Poor-quality reads (Phred score <3) were trimmed from the tail end of each single-end read and only reads with lengths ≥35 bp were subjected to further analysis. All data processing was performed using a combination of UNIX shell commands such as cat, sed, grep and awk, and in-house Shell and Perl scripts using a Linux platform (Scientific Linux 6.4). The quality-trimmed sequencing reads were aligned to the reference IRGSP 1.0 rice transcriptome sequences (http://rapdb.dna.affrc.go.jp/download/archive/irgsp1/IRGSP-1.0_transcript_2013-03-19.fasta.gz) with Bowtie2 v 2.0.1 (Langmead and Salzberg 2012). RPKMs (reads per kilobase of transcript per million mapped reads) of each experiment and statistical significance among data sets were assessed by the Student's *t*-test, Welch's *t*-test, Mann–Whitney U-test and Fisher's exact test using R v. 3.0.2 (Ihaka and Gentleman 1996). Hierarchical clustering with cosine correlation and heat map visualization was performed with the hclust and heat map functions of the R package *amap*. All sequencing data have been deposited in SRA (www.ncbi.nlm.nih.gov/sra). The accession number is PSUB001753-PRJDB1490.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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