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Rapid Paper

OsSYMRK Plays an Essential Role in AM Symbiosis in Rice (*Oryza sativa*)

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Arbuscular mycorrhizal (AM) fungi establish mutualistic symbiosis with a wide range of terrestrial plants, including rice. However, the mechanisms underlying the initiation of AM symbiosis are yet to be elucidated, particularly in nonleguminous plants. We previously demonstrated that chitin elicitor receptor kinase 1 (OsCERK1), a lysin motif receptor–like kinase essential for chitin-triggered immunity, also plays a key role in AM symbiosis in rice. However, the mechanisms underlying the regulation of switching between immunity and symbiosis by OsCERK1 are yet to be fully elucidated. SYMBIOSIS RECEPTOR–LIKE KINASE (SYMRK)/DOES NOT MAKE INFECTIONS 2 (DMI2) is a leucine-rich repeat receptor–like kinase associated with both root nodule symbiosis and AM symbiosis in legumes. The homolog of SYMRK in rice, OsSYMRK, has a shorter form than that in legumes because OsSYMRK lacks a malectin-like domain (MLD). The MLD reportedly contributes to symbiosis in *Lotus japonicus*; however, the contribution of OsSYMRK to AM symbiosis in rice remains unclear. Phylogenetic analyses indicated that the MLD of SYMRK/DMI2 is widely conserved even in mosses and ferns but absent in commelinids, including rice. To understand the function of OsSYMRK, we produced an *Ossymrk* knockout mutant using genome editing technology. AM colonization was mostly abolished in *Ossymrk* with a more severe phenotype than *Oscerk1*. Ca^{2+} spiking against chitin tetramer was also diminished in *Ossymrk*. In contrast, comparable defense responses against chitin heptamer to the wild type were observed in *Ossymrk*. Bimolecular fluorescence complementation studies demonstrating an interaction between OsSYMRK and OsCERK1 indicate that OsSYMRK may play an important role in switching from immunity to symbiosis through the interaction with OsCERK1 in rice.

Keywords: AM symbiosis • Chitin-triggered immunity • Malectin-like domain • OsCERK1 • Rice (*Oryza sativa*) • SYMRK/DMI2

Editor-in-Chief's Choice

Introduction

Plants are able to accurately recognize symbionts and establish mutualistic symbiosis despite the presence of numerous pathogenic and nonpathogenic microbes in the natural environment. Arbuscular mycorrhizal (AM) fungi are known symbionts across a wide range of hosts (>80%) and promote phosphate uptake in host plants. Despite the importance of AM fungi to ecosystems and agriculture, the mechanisms underlying the initiation of AM fungi symbiosis still remain largely unknown.

We and another group previously revealed that a lysin motif (LysM) receptor–like kinase, chitin elicitor receptor kinase 1 (OsCERK1), plays an important role in AM symbiosis (Miyata et al. 2014, Zhang et al. 2015). The *Oscerk1* knockout mutant resulted in severe delays in AM colonization. In contrast, OsCERK1 is known to function as a pattern recognition receptor with essential roles in chitin-triggered immunity (Shimizu et al. 2010). These results indicate that OsCERK1 is involved in both immunity and symbiosis. OsCERK1 dimerizes with chitin elicitor binding protein (CEBiP) to form a receptor complex in which CEBiP preferably recognizes longer-chain chitin oligosaccharides (COs) such as heptamer/octamer (Kaku et al. 2006, Hayafune et al. 2014). Interestingly, *cebip* knockout mutants do not have a mycorrhizal phenotype despite the inability of the *cebip* mutant to function in chitin-triggered immunity. Because CEBiP is essential for defense response but not symbiosis, we hypothesized that the receptor complex containing OsCERK1 changes its partner depending on when functioning in defense responses or AM symbiosis. Therefore, we aimed to identify proteins able to form a receptor complex with OsCERK1 and contribute to AM symbiosis in rice. The role of the NOD FACTOR RECEPTOR 5 (OsNFR5)/MYC FACTOR RECEPTOR 1 (OsMYR1)/LysM containing receptor-like kinase 2 (OsRLK2) receptor in AM symbiosis has previously been evaluated (Miyata et al. 2016, He et al. 2019). Complementation

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analysis using *ljnfr5* indicated that OsNFR5 may contribute to the induction of root nodule (RN) symbiosis; however, the mycorrhizal phenotype of *Osnfr5* knockout mutants was weaker than that of *Oscerk1*, with no significant difference compared to the wild type (WT) (Miyata et al. 2016). Although recent studies have provided some insight, the mechanisms underlying the initiation of AM symbiosis are yet to be fully elucidated, particularly in nonleguminous plants.

To understand the initiation of AM symbiosis in rice, we focused on a *SYMBIOSIS RECEPTOR–LIKE KINASE* (*SYMRK*)/*DOES NOT MAKE INFECTIONS 2* (*DMI2*) ortholog gene, *OsSYMRK*. *SYMRK*/*DMI2* in *Lotus japonicus*/*Medicago truncatula* encodes a membrane protein containing a malectin-like domain (MLD), a leucine-rich repeat (LRR) and a kinase domain. In legumes, SYMRK/DMI2 reportedly plays essential roles in RN symbiosis and AM symbiosis (Endre et al. 2002, Stracke et al. 2002). The presence of a common pathway controlling RN symbiosis and AM symbiosis, termed the common symbiosis pathway, is thought to support the concept that the RN symbiosis pathway originated from the AM symbiosis pathway (Parniske 2008). SYMRK/DMI2 is considered a member of the common symbiosis pathway in addition to calcium/calmodulin-dependent protein kinase, nucleoporins and CYCLOPS (Oldroyd 2013). The presence of a SYMRK/DMI2 homolog in rice has been reported in several studies (Parniske 2008, Nakagawa and Imaizumi-Anraku 2015). However, the length of the OsSYMRK and SYMRK homolog amino acid sequences in maize is shorter than that in legumes due to the lack of an MLD (Markmann et al. 2008). The MLD of SYMRK/DMI2 reportedly plays an important role in initiating symbiosis. In *L. japonicus*, a single amino acid substitution in the conserved sequence motif, GDPC motif, of *symrk-14* abolished MLD cleavage and prevented nodulation and AM colonization (Kosuta et al. 2011, Antolin-Llovera et al. 2014). As OsSYMRK does not contain an MLD, the contribution of OsSYMRK to AM symbiosis remains unclear. However, OsSYMRK has been shown to contribute to the formation of root nodules in a *symrk* mutant of *L. japonicus* partially (Markmann et al. 2008) and fully (Li et al. 2018). These findings indicate that OsSYMRK plays a potential role in symbiosis. Despite these findings, direct evidence for the involvement of OsSYMRK in AM symbiosis in rice is yet to be reported. We therefore produced *Ossymrk* mutant lines using Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) techniques and found that OsSYMRK plays an important role in initiating AM symbiosis in rice.

Results

Structural characterization of OsSYMRK

The homolog of SYMRK/DMI2 in rice, OsSYMRK, is annotated as Os07g0568100 in the Rice Annotation Project Database, LOC_Os07g38070 in the rice genome annotation project and XP_015646949.1 in National Center for Biotechnology Information (NCBI). The amino acid length of OsSYMRK is shorter than that of leguminous SYMRK/DMI2 as OsSYMRK lacks an MLD (Markmann et al. 2008, Nakagawa and Imaizumi-Anraku 2015, Li et al. 2018). Due to the absence of the large MLD sequence in OsSYMRK, the protein sequences of the extracellular domains of LjSYMRK and OsSYMRK share 27.4% identity and 40.4% similarity. However, the extracellular domain of LjSYMRK without the MLD has 53.95% identity and 67.10% similarity with OsSYMRK. The intracellular domains of LjSYMRK and OsSYMRK have high identity (64.5%) and similarity (76.6%) with each other (**Fig. 1A, B**). The presence of two LRR domains in OsSYMRK was detected by analyzing the NCBI Conserved Domains (CD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) as reported previously (Markmann et al. 2008). However, LRR domains were not detected by SMART (http://smart.embl-heidelberg.de/). OsSYMRK has a large deletion between the second LRR domain (LRR2) and the third LRR domain (LRR3) corresponding to the region of LjSYMRK containing three LRR domains (**Fig. 1C**). The first LRR domain (LRR1) is well conserved between OsSYMRK and LjSYMRK, with 42.8% identity and 66.6% similarity.

Expression of OsSYMRK is induced by low phosphate conditions

To evaluate the contribution of OsSYMRK to AM symbiosis, we measured expression levels of OsSYMRK in a range of conditions using TENOR (https://tenor.dna.affrc.go.jp/) (Mizuno et al. 2010, Oono et al. 2011, 2014). Expression levels of *OsSYMRK* in the root were increased under low phosphate conditions and suppressed under high phosphate conditions (**Supplementary Fig. S1A**). Furthermore, the result of our quantitative real-time PCR (qRT-PCR) analysis showed that *OsSYMRK* expression was significantly increased in the 0-mM phosphate condition compared to the 1-mM phosphate condition (**Supplementary Fig. S1B**). In addition, expression levels of *OsSYMRK* were higher in root samples than in leaf samples (**Supplementary Fig. S2**). These data indicate that OsSYMRK may be involved in AM symbiosis in rice.

The MLD of OsSYMRK is not conserved in commelinids

Based on the structural differences among SYMRK homologs observed in various species and the complementation analysis using a *symrk* mutant of *L. japonicus*, the presence of an MLD in SYMRK/DMI2 in legumes is thought to have contributed to the evolution of RN symbiosis (Markmann et al. 2008). However, the evolutional trajectory of SYMRK structures remains unclear. To identify the ancestral type of SYMRK, we compared the structure of SYMRK ortholog genes in *Marchantia paleacea*, a moss, which can be a host in AM symbiosis, and *Selaginella moellendorffii*, a fern, which is also a potential host. *Chara braunii*, which is the origin of angiosperm, and *M. polymorpha*, which is a non-AM host moss, were used as an out-group. The most similar LjSYMRK amino acid sequence

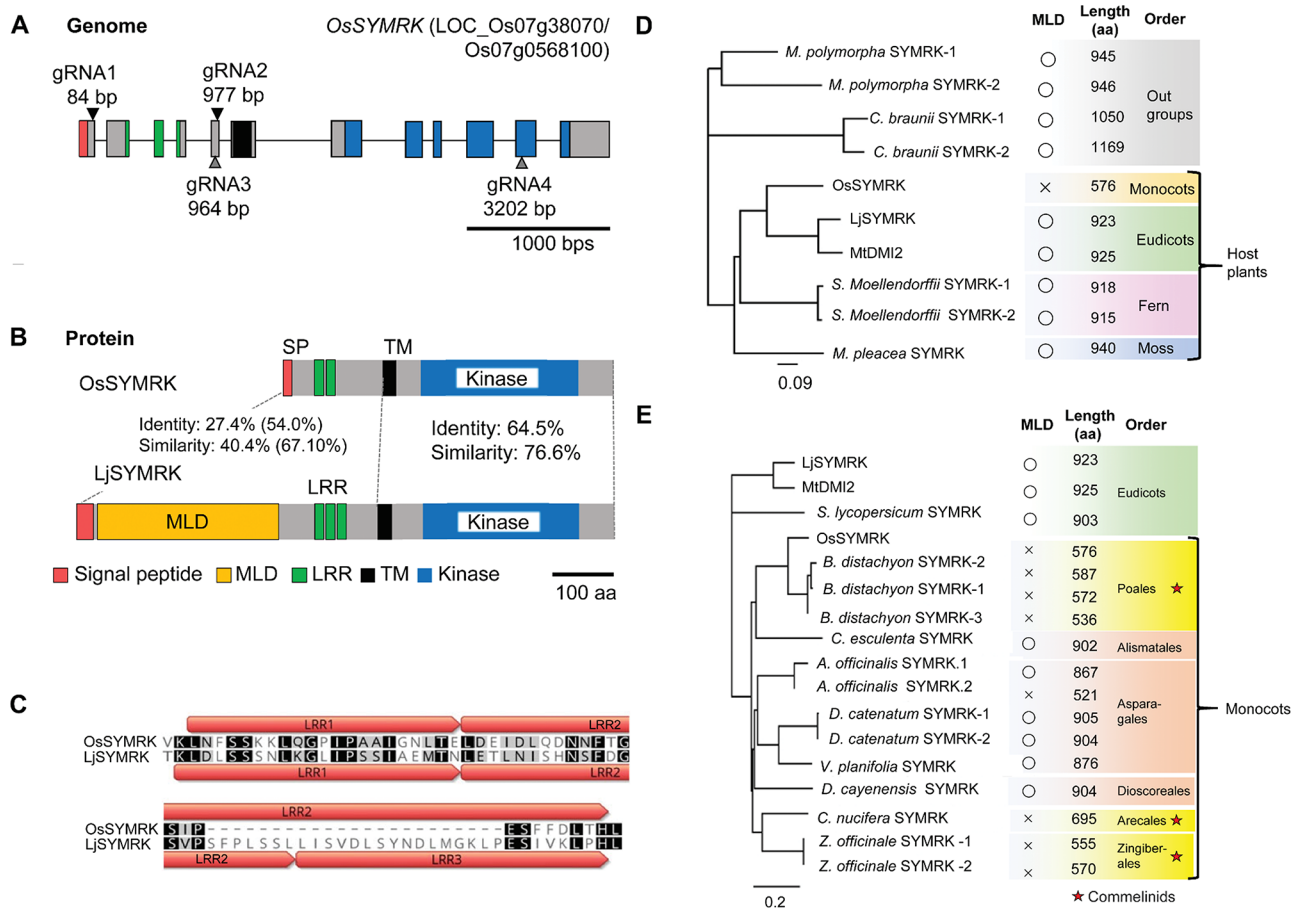

Fig. 1 Structural features of OsSYMRK and phylogenetic analysis of SYMRK homologs in various plant species. (A) Gene sequence of OsSYMRK showing exons (boxes) and introns (lines). Sequences encoding domains are highlighted in colors as follows: red, signal peptide (SP); green, LRR; black, transmembrane (TM) and blue, kinase domain. Triangles indicate the four target positions of gRNAs to generate the *Ossymrk* mutant. Numbers indicate the position of cleavage sites from the start of the ORF. (B) Comparison of OsSYMRK and LjSYMRK proteins. The identity and similarity of extracellular domains without MLD are presented: orange, MLD of LjSYMRK; red, signal peptide; green, LRR; black, TM and blue, kinase domain. (C) Conservation of LRR domains between LjSYMRK and OsSYMRK. The positions of LRR domains were conformed using NCBI CD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). (D) Phylogenetic tree of OsSYMRK, LjSYMRK, MtDMI2 and SYMRK homologs in early diverging land plants: *C. braunii*, *M. polymorph*, *M. paleacea* and *S. moellendorffii*. (E) Phylogenetic tree of OsSYMRK, LjSYMRK, MtDMI2 and *S. lycopersicum* SYMRK and SYMRK homologs in monocots plants: *B. distachyon*, *Z. officinale*, *C. nucifera*, *C. esculenta*, *A. officinalis*, *D. catenatum*, *V. planifolia* and *D. cayenensis* subsp. *rotundata*. Stars indicate commelinids. The presence of an MLD, length of amino acid sequences and orders are shown. (D, E) Annotation numbers of homologs are provided in **Supplementary Table S1**.

in each plant was selected from the NCBI database. Domain searching using SMART revealed homologs in *M. paleacea* and *S. moellendorffii* contained an MLD. Similarly, *C. braunii* SYMRK and *M. polymorpha* SYMRK-1 and SYMRK-2 also contained an MLD. These results indicate that ancestral SYMRKs contained an MLD prior to the acquisition of AM symbiosis (**Fig. 1D**).

To determine the timing of the evolutional event leading to the loss of the MLD from OsSYMRK, we evaluated the structure of SYMRK orthologs in the following Poales species and peripheral orders in monocots (**Fig. 1E**): *Brachypodium distachyon*, *Zingiber officinale*, *Cocos nucifera*, *Colocasia esculenta*, *Asparagus officinalis*, *Dendrobium catenatum*, *Vanilla planifolia* and *Dioscorea cayenensis* subsp. *rotundata*. The SYMRK homologs in Poales, *C. nucifera* and *Z. officinale* lacked an MLD. Conversely, SYMRK homologs in *A. officinalis*, *D. catenatum* and *V. planifolia* in Asparagales and *C. esculenta* in Alismatales contained an MLD. The orders in which SYMRK orthologs did not contain an MLD, namely, Poales, Arecales and Zingiberales, belong to commelinids, a large group proposed by the Angiosperm Phylogeny Group IV system (Byng et al. 2016). Based on these findings, the MLD of SYMRK was likely lost at the origin of commelinds. In the case of *A. officinalis*, two splicing variants with and without an MLD were identified.

The GDPC motif is a highly conserved four-amino acid sequence that is known to be important for MLD cleavage in legumes (Kosuta et al. 2011, Antolin-Llovera et al. 2014). Kosuta

et al. demonstrated that this motif is widely conserved in various plants, including a moss, *P. patensm* (Kosuta et al. 2011). We found that SYMRK homologs in a wide range of terrestrial plants, including *M. polymorpha* and *M. paleacea*, also contain a GDPC motif. As *M. polymorpha* and *P. patensm* are not hosts of AM symbiosis, this GDPC motif does not appear to be related to the evolution of AM symbiosis (**Supplementary Fig. S3A**). Rather, the lack of a conserved GDPC motif among SYMRK homologs in *C. braunii* (**Supplementary Fig. S3B**) implies that the GDPC motif was conserved in terrestrial plants but not in the plants before terrestrialization. Although the MLD motif is not present in SYMRK homologs in commelinids, all SYMRK homologs we evaluated had a conserved GDPC motif except for *B. distachyon* (**Supplementary Fig. S3A**). The *B. distachyon* SYMRK homologs were found to have a single amino acid substitution changing from GDPC to EDPC. These results indicate that commelinid plants lost MLD and related processes during evolution, while the GDPC motif has been conserved in most land plants for MLD cleavage.

As shown in **Fig. 1C**, OsSYMRK has a deletion between LRR2 and LRR3 compared to the LjSYMRK sequence. Most monocot species were found to have the same deletion. The SYMRK sequence in tomato also had a deletion between LRR2 and LRR3 (**Supplementary Fig. S4A**). However, the SYMRK homologs of *C. braunii* and the mosses (*M. polymorpha*, *M. paleacea* and *S. moellendorffii*) contain LRR2 and LRR3 as in the SYMRK sequence in *L. japonicus* (**Supplementary Fig. S4B**). The region between LRR2 and LRR3 in the *M. paleacea* SYMRK homolog had high similarity with LjSYMRK, 38.2% identity and 74.4% similarity. These results indicate that LRR2 and LRR3 in LjSYMRK originated from LRRs in ancestral plants and were lost in monocots and tomatoes.

OsSYMRK is essential for AM symbiosis

To determine the contribution of OsSYMRK to AM symbiosis in rice, we produced an *Ossymrk* knockout mutant using the pZK_gYSA_MMCas9 vector (Mikami et al. 2015). To measure Ca^{2+} spiking, WT seeds overexpressing the Yellow Cameleon (YC) were used for this transformation (Carotenuto et al. 2017). Two guide RNAs (gRNA), gRNA1 and gRNA2, were designed with the cleavage positions at 84 and 977 bp from the start of ORF, respectively (**Fig. 1A**).

Two independent lines, lines 1 and 7, with gRNA2 mutations were obtained. Both lines 1 and 7 had the same single-base insertion of adenine in the gRNA2 region in the T_0 generation. Line 1 was a heterozygote, and line 7 was a homozygous mutant. This mutation generates a stop codon at 152 aa from the start codon due to a frameshift (**Fig. 2A**, **Supplementary Fig. S5A**). No gRNA1 mutations were observed in any of the generated lines, including lines 1 and 7 (**Supplementary Fig. S5B**). We named these mutants *Ossymrk-1* lines 1 and 7.

To examine the phenotype of *Ossymrk-1* line 1, which had a heterozygous mutation in the T_0 generation, we examined the mycorrhizal phenotype of T_1 seeds. To determine the genotype of T_1 plants, we designed modified primers that generated HindIII recognition sites in the *Ossymrk-1* mutation (AAGCTT) but not in the WT (CAGCTT) (**Fig. 2B**). We examined the band pattern of HindIII-digested or undigested PCR products from T_0 line 1 (heterozygote), line 7 (homozygous mutant) and WT. Band sizes from all samples not treated with HindIII and WT samples treated with HindIII were the same (90 bp), whereas bands from *Ossymrk-1* line 7 samples treated with HindIII had smaller bands (28 and 62 bp) as designed (**Fig. 2C**). In principle, treatment of heterozygotes with HindIII should result in two bands; however, the band patterns of WT and heterozygotes were indistinguishable under our experimental conditions.

We determined the genotype of samples using this method and examined the mycorrhizal phenotype of 17 plants in the T_1 generation of *Ossymrk-1* line 1. Genotype analysis demonstrated that three plants (nos. 2, 7 and 8) were homozygous mutants (**Fig. 2D**). Furthermore, these plants exhibited no colonization at 3 weeks postinoculation (wpi) with *Rhizophagus irregularis* spores, although other plants and WT demonstrated root colonization (**Fig. 2E**). AM fungi were able to form hyphopodia, but hyphae were unable to enter epidermal cells in *Ossymrk-1* line 1 no. 2 (homozygous mutant; **Fig. 2F**), whereas internal hyphae and arbuscules were produced in *Ossymrk-1* line 1 no. 12 (revertant; **Fig. 2G**).

The other line, *Ossymrk-1* line 7, also showed no AM colonization, as observed for *Ossymrk-1* line 1 (**Fig. 4A, B**). Because both *Ossymrk-1* mutant lines 1 and 7 have the same single-base insertion although they are independent lines, we designed a further gRNA in a different region to obtain another mutant to affirm the involvement of OsSYMRK in AM symbiosis. Two gRNAs, gRNA3 and gRNA4, were designed at 964 and 3202 bp from the start of the ORF, respectively (**Fig. 1A**). Three independent lines (lines 2, 5 and 8) contained a biallelic mutation in T_0 generation. Lines 2 and 8 had the same single-base insertion of adenine and thymine at the gRNA4 locus with no mutations at the gRNA3 locus (**Supplementary Fig. S6A, B**). The mutation in *Ossymrk-2* generated a stop codon at 447 or 452 aa from the start of the ORF. We named these lines *Ossymrk-2* lines 2 and 8, respectively (**Fig. 3A**). The other line, line 5, contained a different biallelic mutation and was named *Ossymrk-3*. The single-base deletion of guanine at 1339 bp from the start codon of cDNA generated a stop codon at 457 aa. The single-base insertion of thymine, also present in lines 2 and 8, generated a stop codon at 447 aa in *Ossymrk-3* line 5. Evaluation of the mycorrhizal phenotypes of T_1 generation seeds of *Ossymrk-2* lines 2 and 8 and *Ossymrk-3* line 5 demonstrated almost no AM colonization, similar to findings in *Ossymrk-1* (**Fig. 3B, C**). The colonization ratio in *Ossymrk-2* and *Ossymrk-3* was remarkably low, with the hyphae of AM fungi unable to enter the epidermal cells of *Ossymrk-2* and *Ossymrk-3* despite the formation of hyphopodia. These results indicate that OsSYMRK plays a key role in AM symbiosis in rice.

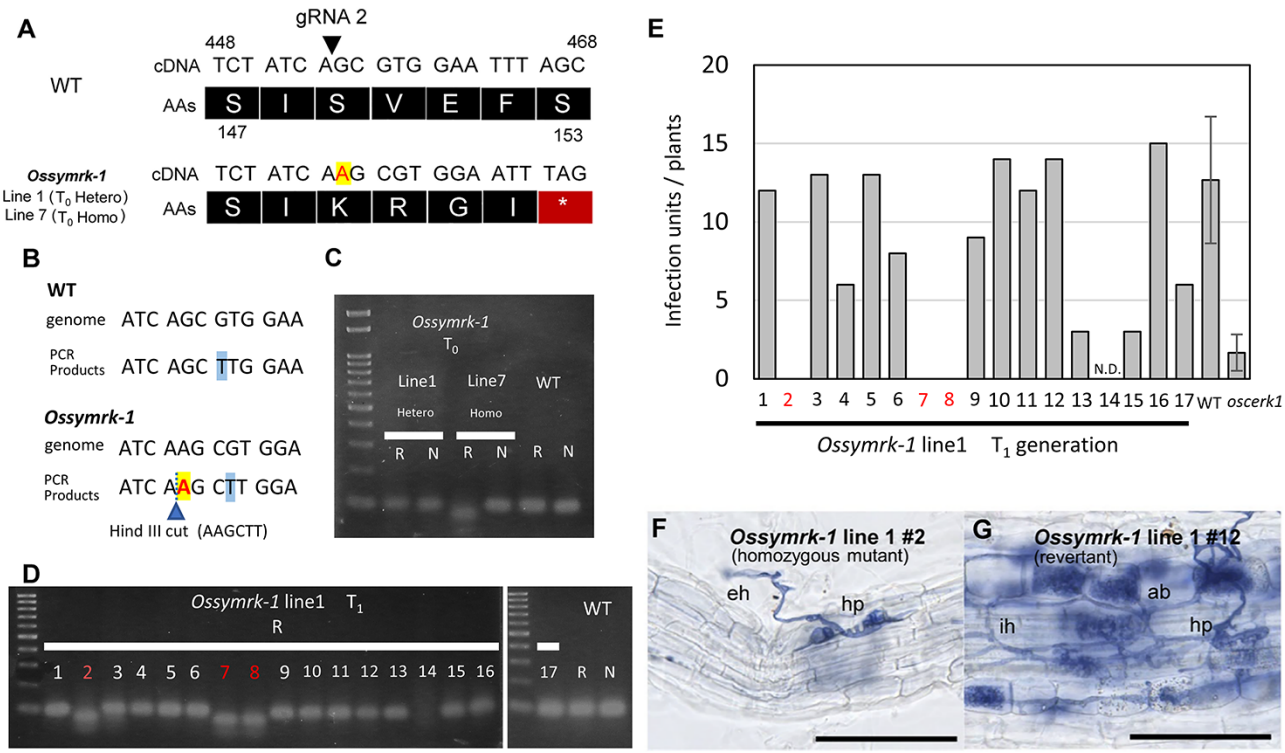

Fig. 2 AM symbiosis is diminished in *Ossymrk-1*. (A) cDNA sequence and expected amino acid sequence of *Ossymrk-1* and WT. The single-base insertion of adenosine (red letter highlighted in yellow) generated a stop codon at 153 aa in *Ossymrk-1* line 1 (heterozygote) and line 7 (homozygote). Numbers indicate the number of bases or amino acids from the start of the ORF. (B) Detection of *Ossymrk-1* mutants. A single-base substitution was introduced using PCR, and a HindIII site was introduced into the *Ossymrk-1* sequence but not into the WT sequence. The triangle indicates the HindIII target site. Letters highlighted in pale blue show primers used to introduce the single-base substitution and HindIII site into the *Ossymrk-1* sequence. (C) Electrophoresis demonstrating the band pattern of PCR products of line 1 (heterozygote), line 7 (homozygote) and WT with (R) or without (N) Hind III digestion. Digestion of the homozygous mutant of *Ossymrk-1* with HindIII generated a 28-bp product and a 62-bp product, whereas WT PCR products not digested with HindIII had a 90-bp product. (D) The band pattern of single-base substitution PCR products from 17 T_1 generation plants of *Ossymrk-1* line 1 and WT treated with HindIII. Nos. 2, 7 and 8 (red letters) indicate the homozygote mutant. No. 14 did not grow well, and DNA could not be obtained. (E) Number of root colonizations per plant. Candidate plants were inoculated with 4,800 *R. irregularis* spores per plant. WT plants (nos. 2, 7 and 8; red letters) had no colonization. N.D., no data. (F, G) Trypan blue staining of homozygous (F) *Ossymrk-1* line 1 no. 2 and (G) *Ossymrk-1* line 1 no. 12 at 3 wpi. eh, extraradical hyphae; ih, internal hypha; hp, hyphopodia; ab, arbuscules. Scale bars = 100 um.

The mycorrhizal phenotype of *Ossymrk-1* is more severe than that of *Oscerk1*

As we previously reported, the *Oscerk1* mutant has a significantly lower colonization ratio compared to the WT; however, arbuscules can be observed in *Oscerk1* after prolonged incubation (Miyata et al. 2014, 2022). Therefore, the phenotype of *Oscerk1* can be described as severely delayed colonization. Under our experimental conditions, the number of infection units was substantially lower in *Oscerk1* compared to the WT, although the *Oscerk1* mutant formed a small number of arbuscules at 3 wpi (**Fig. 2E**). Conversely, *Ossymrk-1* homozygous mutants demonstrated almost no colonization (**Fig. 2E, F**) at 3 wpi. In *Ossymrk-1*, AM colonization typically stopped at the stage of hyphopodia formation (**Fig. 4A**, **Supplementary Fig. S7**). These results demonstrate the differences in mycorrhizal phenotypes between *Oscerk1* and *Ossymrk-1*. To verify this phenotypical difference between *Oscerk1* and *Ossymrk-1*, we examined mycorrhizal phenotypes at 2, 3 and 5 wpi using the T_1 generation of homozygous mutant lines *Ossymrk-1* line 7, *Oscerk1* and vector control (VC); VC was generated by the transformation of YC seeds with empty pZH_OsU6gRNA_ MMCas9 vectors. Arbuscles were clearly observed in VC at 2 wpi, with degradation starting at 3 wpi under our experimental conditions. Subsequently, most arbuscules had collapsed and vesicles could be observed in VC at 5 wpi. On the contrary, AM colonization was rarely observed in the T_1 generation of *Ossymrk-1* line 7 at all tested time points (**Fig. 4A**). The *Oscerk1* mutants did not initiate AM colonization at 2 wpi but formed arbuscles at 3 and 5 wpi, although at a lower frequency compared to VC.

To evaluate phenotypical differences, the root colonization ratio was compared among *Ossymrk-1*, *Oscerk1*, VC, YC and WT at 3 wpi. Because *Ossymrk* mutants were produced using YC seeds, both YC and VC (YC with empty vector of pZK_gYSA_MMCas9 vector) were used as a control. No

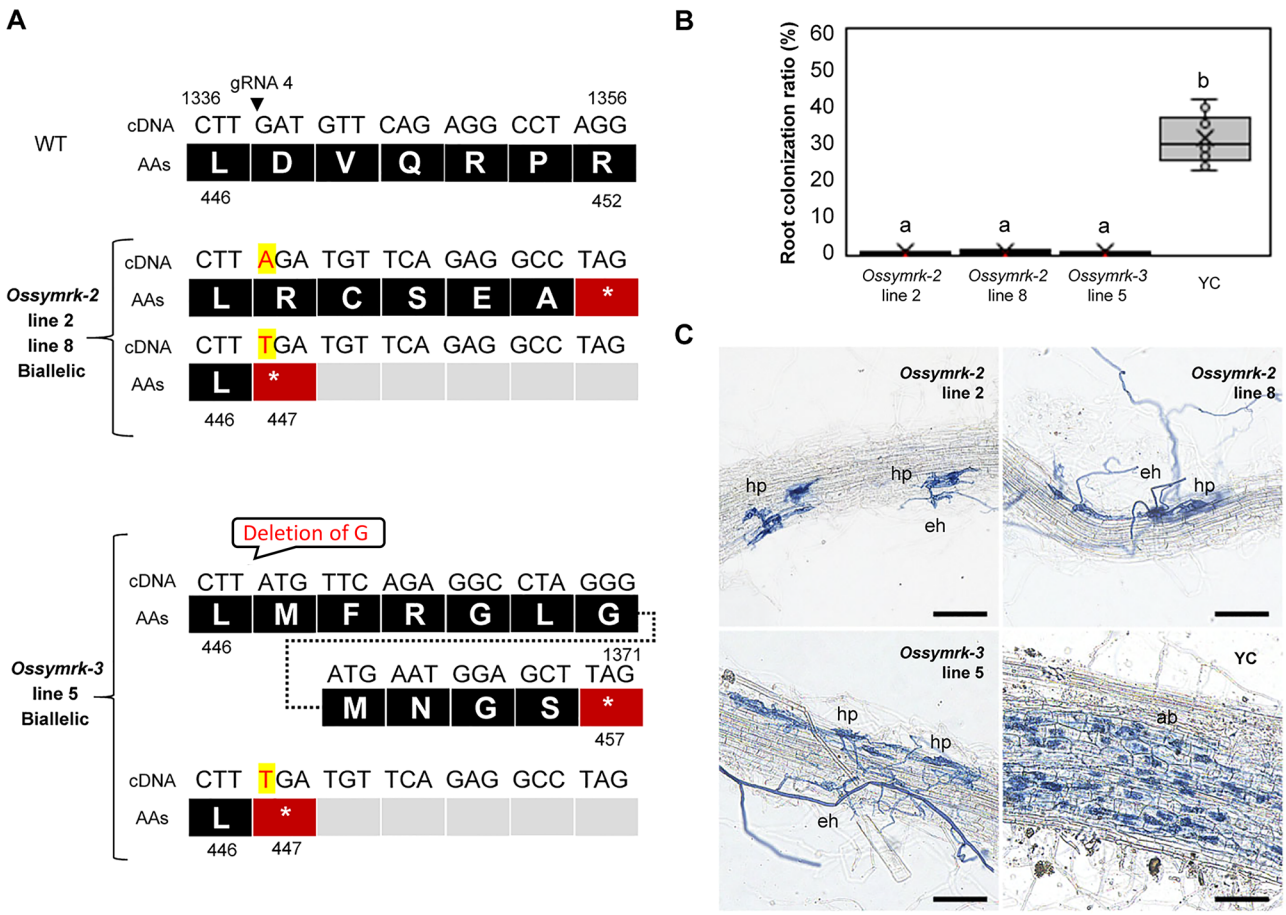

Fig. 3 Further *Ossymrk* mutants, *Ossymrk-2* and *Ossymrk-3*, were unable to establish AM symbiosis. (A) The position of cDNA mutations and expected amino acid sequences in *Ossymrk-2* and *Ossymrk-3* mutants. Biallelic mutations at the gRNA4 position were observed in these mutants. Two independent mutants, *Ossymrk-2* lines 2 and 8, contained the same biallelic mutations (A or T insertion) and generated a stop codon at 447 and 452 aa from the start of the ORF, respectively. The other biallelic mutation was named *Ossymrk-3* line 5 and contained a single-base insertion of T and one-base deletion of G, resulting in a stop codon at 447 and 457 aa from the start of the ORF, respectively. (B) Root colonization in *Ossymrk-2* lines 2 and 8, *Ossymrk-3* line 5 and WT at 3 wpi after treatment with 2,400 *R. irregularis* spores per plant ($n \geq 4$). Lowercase letters denote statistical significance based on one-way ANOVA and Tukey's post hoc test ($P > 0.05$). (C) Trypan blue staining of *Ossymrk-2* and *Ossymrk-3* at 3 wpi. eh, extraradical hyphae; ih, internal hypha; hp, hyphopodia; ab, arbuscules. Scale bars = 100 um.

significant difference in root colonization ratio was observed among the three controls (VC, YC and WT), indicating that the transformation of the empty vector for genome editing and YC expression had no effect on the mycorrhizal phenotype. In contrast, the root colonization ratio was significantly lower in *Oscerk1* and *Ossymrk-1* compared to controls. As shown in **Fig. 3A**, AM colonization was almost never observed in *Ossymrk-1* line 7 and the colonization ratio of all the tested *Ossymrk-1* plants was 1.00% on average under our experimental conditions. Conversely, the *Oscerk1* mutant gradually started colonization at 3 wpi with the root colonization ratio reaching approximately 20%, although this ratio remained lower than VC in which AM colonized >50% of all roots (**Fig. 4B**). Increased colonization after prolonged incubation was not observed for the *Ossymrk* mutant. These results demonstrate that the *Ossymrk-1* has a much more severe mycorrhizal phenotype than *Oscerk1*.

To verify the phenotypes of *Ossymrk-1* and *Oscerk1*, we measured expression levels of genetic markers of AM, *AM1* and *AM3*, in *Ossymrk-1*, *Oscerk1* and VC plants inoculated with or without spores at 25 d after inoculation (dpi) (**Fig. 4C, D**). Expression levels of *AM1* and *AM3* were lower in *Ossymrk-1* than in *Oscerk1* and VC. These results also support the finding that *Ossymrk-1* has a more severe phenotype than *Oscerk1*.

Although *Ossymrk-1* mutants lost the ability to form AM symbiosis, AM fungi rarely formed arbuscules in the roots of *Ossymrk-1* under our experimental conditions. AM fungi entering the host plant cells were able to produce arbuscules as in WT plants (**Supplementary Fig. S8**). This unusual colonization in *Ossymrk*-1 may explain the moderately increased expression levels of *AM1* and *AM3* in *Ossymrk-1* following treatment with mycorrhizal fungus spores (**Fig. 3C, D**).

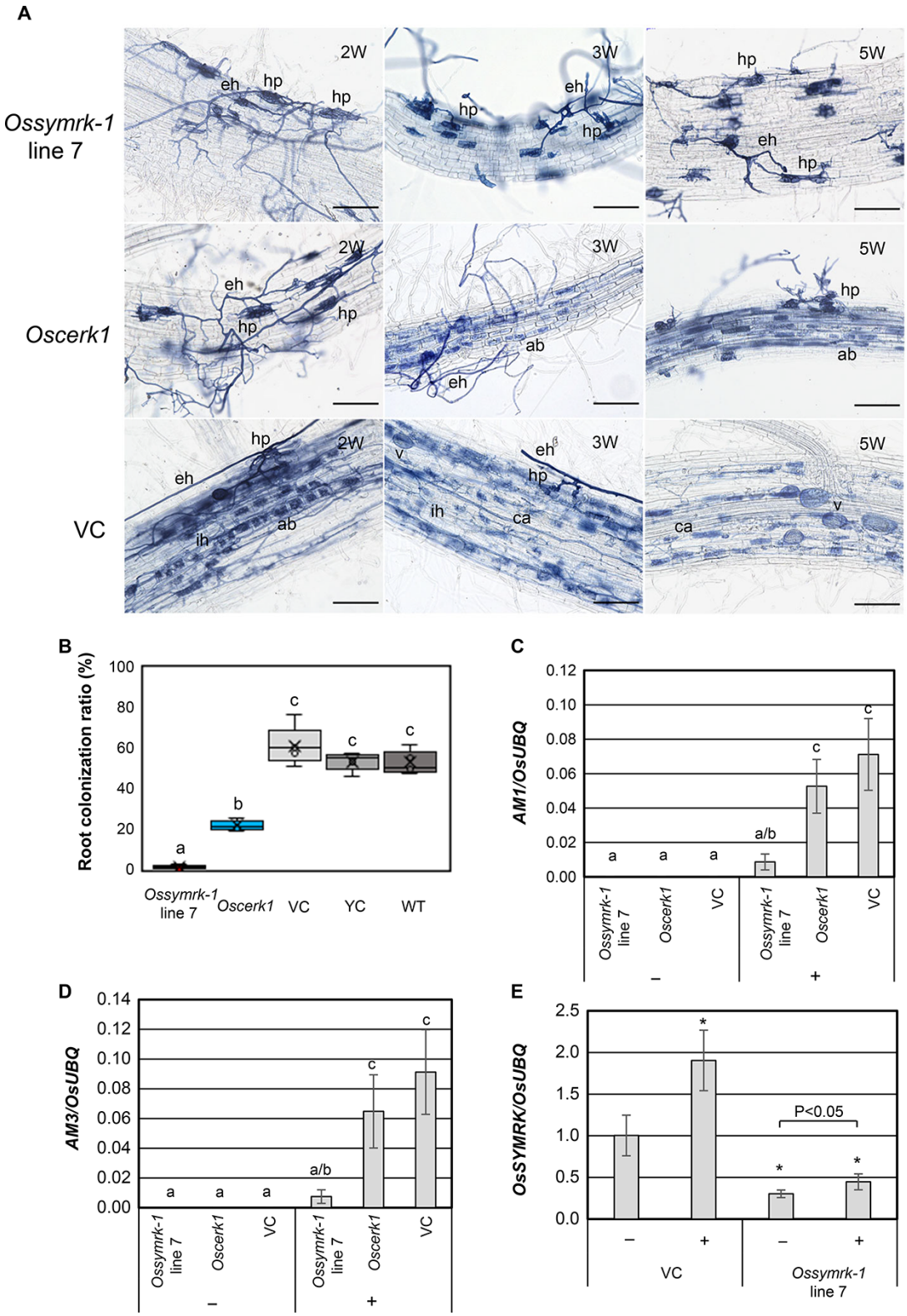

Fig. 4 Mycorrhizal phenotypes of *Ossymrk-1* and *Oscerk1*. (A) The mycorrhizal phenotypes of *Ossymrk-1*, *Oscerk1* and VC at 2, 3 and 5 wpi after treatment with 4,800 *R. irregularis* spores per plant. Roots were stained with trypan blue. eh, extraradical hyphae; ih, internal hypha; hp, hyphopodia; ab, arbuscules, ca, collapsing arbuscules; v, vesicles. Scale bars = 100 mm. (B) Root colonization ratio in *Ossymrk-1*, *Oscerk1*, VC, YC and WT at 3 wpi ($n = 5$). (C–E) Expression levels of symbiotic marker genes, *AM1*, *AM3* and *OsSYMRK*, in *Ossymrk-1*, *Oscerk1* and VC at 25 dpi after treatment with or without 4,800 *R. irregularis* spores per plant ($n = 3$). (C, D) Lowercase letters denote statistical significance based on one-way ANOVA and Tukey's post hoc test ($P > 0.05$). (E) Asterisks indicate a significant difference from VC samples treated with distilled water (DW) by Welch's *t*-test ($P < 0.05$). No statistical difference was observed between inoculated and uninoculated *Ossymrk-1* by Welch's *t*-test ($P > 0.05$). Data are presented as the mean of three biological replicates, and error bars indicate the standard deviation.

AM colonization induces *OsSYMRK* expression

SYMRK/DMI2 proteolysis is reportedly important for RN symbiosis in *M. truncatula*. Pan et al. demonstrated that changes in DMI2 protein levels during nitrogen-fixing symbiosis and DMI2 protein stability are controlled by the proteasome pathway (Pan et al. 2018). The MLD of SYMRK is also known to undergo proteolytic cleavage in *L. japonicus* (Antolin-Llovera et al. 2014). The *symrk-14* mutant, which has a single amino acid substitution in the GDPC motif that abolishes MLD release, is unable to establish RN symbiosis and AM symbiosis (Kosuta et al. 2011). The presence of lower amounts of SYMRK without an MLD compared to full-length SYMRK indicates that the release of the MLD induces rapid degradation of SYMRK (Antolin-Llovera et al. 2014).

Although MLD cleavage by proteolysis is important for symbiosis in *L. japonicus*, OsSYMRK does not have an MLD. Accordingly, the mechanisms underlying the regulation of OsSYMRK levels during symbiosis are yet to be elucidated. Accordingly, we measured expression levels of *OsSYMRK* in the root of VC plants inoculated with or without *R. irregularis* spores at 25 dpi. Expression levels of *OsSYMRK* in VC plants inoculated with AM spores were approximately 2-fold higher than those in uninoculated plants (**Fig. 4E**).

Low levels of *OsSYMRK* expression were detected in the *Ossymrk-1* line 7 mutant. However, expression levels were decreased more than 2-fold compared to VC plants not inoculated with AM fungus. The detection of low *OsSYMRK* expression levels may be attributable to the presence of a corresponding region of mRNA after the stop codon despite the insertion mutation causing a frameshift. Regardless, significant induction of *OsSYMRK* gene expression in response to AM symbiosis was not detected in the *Ossymrk-1* mutant (**Fig. 4E**). These results indicate that, unlike legumes, the amounts of OsSYMRK are regulated by the transcriptional level rather than the protein level in rice.

Chitin tetramer–induced Ca^{2+} spiking is largely diminished in *Ossymrk-1*

Nuclear-associated Ca^{2+} oscillation, the so-called Ca^{2+} spiking, is thought to be a downstream mediator of the initiation of symbiosis. In the case of RN symbiosis, nod factor perception by LysM receptors has been shown to induce Ca^{2+} spiking (Ehrhardt et al. 1996, Kosuta et al. 2008, Sieberer et al. 2012). In rice, short COs, such as chitin tetramer (CO4), are thought to trigger AM symbiosis, with treatment with CO4 shown to induce Ca^{2+} spiking (Genre et al. 2013). In addition, the lack of response of *Oscerk1* to CO4 indicates that CO4 perception through OsCERK1 induces Ca^{2+} spiking in rice (Carotenuto et al. 2017).

To evaluate the possible involvement of OsSYMRK in Ca^{2+} spiking, seedlings of YC and *Ossymrk-1* line 7, a mutant of YC seeds used in the previous report, were treated with CO4 (Carotenuto et al. 2017). YC seedling treated with 10^{-6} M CO4 displayed clear and sharp peaks of Ca^{2+} spiking, with

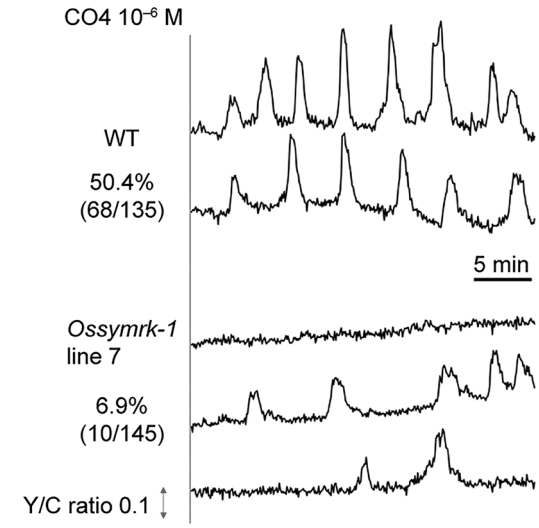

Fig. 5 Reduced Ca^{2+} spiking in the *Ossymrk-1* mutant. Ca^{2+} spiking responses to 10^{-6} M CO4 in *Ossymrk-1* and YC at 7 d after germination. Numbers indicate the total number and proportion of nuclei with Ca^{2+} spiking. The frequency of the appearance of Ca^{2+} oscillation in *Ossymrk-1* was statistically different compared to YC by Pearson's chi-squared test ($P < 0.0001$).

50.4% of the nuclei demonstrating Ca^{2+} spiking. In contrast, CO4-treated *Ossymrk-1* had a greatly diminished Ca^{2+} spiking response, with a ratio of nuclei with a Ca^{2+} spiking response found to be 6.9% (**Fig. 5**). The difference in the frequency of Ca^{2+} spiking response between YC and *Ossymrk*-1 was statistically significant. Furthermore, the shape of Ca^{2+} peaks was different and the appearance of the peaks was arrhythmic in *Ossymrk-1* mutants compared to YC. These results indicate that OsSYMRK is involved in CO4-induced Ca^{2+} spiking in rice.

OsSYMRK may interact with OsCERK1

The above results indicated that OsCERK1 and OsSYMRK play key roles in AM symbiosis in rice. We therefore used bimolecular fluorescence complementation (BiFC) to determine the potential physical interaction between these two membrane proteins. OsCERK1 and OsSYMRK attached to split Venus fragments at the C-terminal were coexpressed in *Nicotiana benthamiana* leaves and observed under a fluorescence microscope.

As OsCERK1 is known to form a homodimer (Shimizu et al. 2010; Hayafune et al. 2014), we used OsCERK1-Venus^N and OsCERK1-Venus^C as a positive control, with fluorescence observed at the plasma membrane in plants expressing these proteins (**Supplementary Fig. S9**). OsCERK1-Venus^N and OsSYMRK-Venus^C resulted in fluorescence at the plasma membrane, similar to the positive control. OsCERK1-Venus^C and OsSYMRK-Venus^N also resulted in similar observation. Conversely, single inoculation with OsCERK1-Venus^N, OsCERK1-Venus^N, OsSYMRK-Venus^N or OsSYMRK-Venus^C resulted in no observable fluorescence (**Supplementary Fig. S9**). These results indicate that OsCERK1 and OsSYMRK may physically

interact in vivo, although the speculation is based on the results obtained from a single experimental approach gained in a heterologous system.

Furthermore, we examined the ability of OsSYMRK to form dimers using BiFC. We observed fluorescence at the plasma membrane with OsSYMRK-Venus^{N} and OsSYMRK-Venus^{C}, indicating that OsSYMRK is able to form dimers similar to OsCERK1.

OsSYMRK does not contribute to chitin-triggered immunity

The potential interaction between OsSYMRK and OsCERK1 implies that OsSYMRK may be involved in switching between AM symbiosis and chitin-triggered immunity. To determine the involvement of OsSYMRK in chitin-triggered immunity, we measured expression levels of *OsKS4*, the momilactone biosynthesis gene (Shimura et al. 2007), and *OsDTC2*, the gene responsible for oryzalexin biosynthesis (Nemoto et al. 2007), in YC and *Ossymrk-1* line 7 roots treated with 10^{-5} M CO4, chitin heptamer (CO7) or H_2O for 6 h.

CO7 increased the expression of *OsKS4* and *OsDTC2* by more than 2-fold in the root of YC; however, CO4 had no effect on *OsKS4* and *OsDTC2* expression levels. This finding indicates that CO7 induced a strong defense response, whereas CO4 did not, as the results of a previous study (Yamada et al. 1993). Increased expression of *OsKS4* and *OsDTC2* was also observed in response to CO7 in *Ossymrk-1* line 7 (**Fig. 6A, B**).

To evaluate chitin-triggered immunity using a different method, reactive oxygen species (ROS) production in response to CO7 was examined in the callus of *Ossymrk-1*, *Oscerk1; Oscerk2* and YC plants treated with 10^{-9} M CO7 or H_2O. Increased ROS production following treatment with CO7 was observed in both *Ossymrk-1* and YC (**Fig. 6C, D**). Conversely, no ROS production was observed in *Oscerk1;Oscerk2* (**Fig. 6E**). These results indicate that OsSYMRK is not involved in chitin-triggered immunity.

Discussion

In legumes, mutation of SYMRK/DMI2 leads to abortion of RN symbiosis at an early stage and prevents AM colonization (Endre et al. 2002, Stracke et al. 2002). However, direct evidence for the requirement of OsSYMRK in AM symbiosis is yet to be demonstrated. In the present study, we demonstrate that OsSYMRK is essential for AM symbiosis in rice. Furthermore, ROS assays and defense response gene expression analyses show that OsSYMRK is not involved in chitin-triggered immunity.

OsSYMRK and OsCERK1 are receptor-like kinases required for AM symbiosis that appear to have distinct functions. OsSYMRK regulates AM symbiosis only, whereas OsCERK1 is associated with both immunity and symbiosis. The mechanism by which OsCERK1 regulates switching between these opposing processes remains unclear. As CEBiP is involved in chitin-triggered immunity as a chitin receptor but not in AM symbiosis, we hypothesized the presence of an alternative molecule that is involved in AM symbiosis but not in chitin-triggered immunity. The lack of AM colonization in *Ossymrk-1* and the interaction between OsSYMRK and OsCERK1 imply

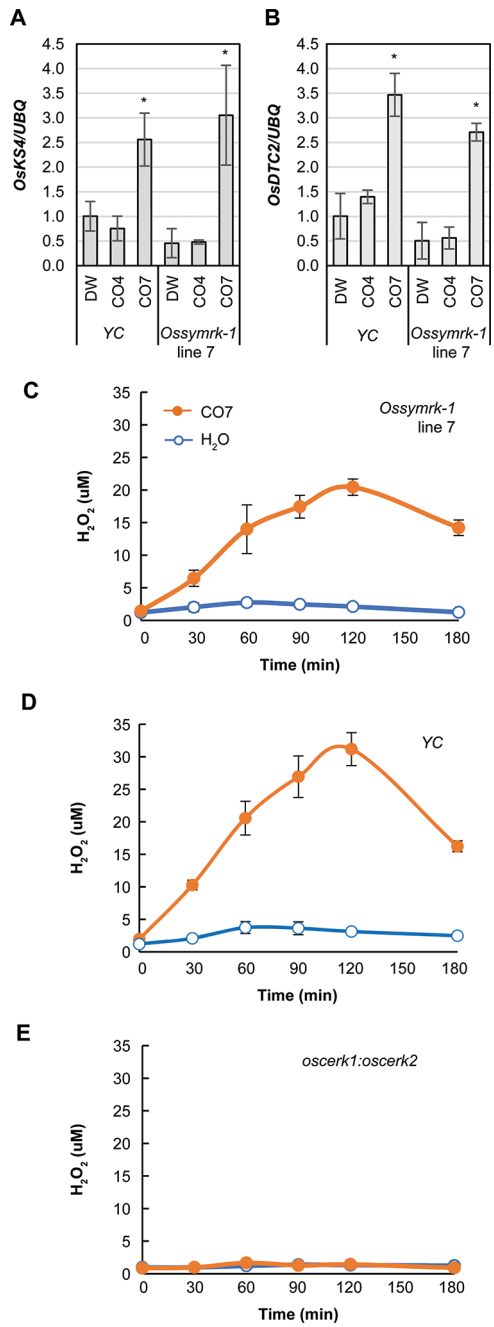

Fig. 6 Induction of chitin-triggered immunity in *Ossymrk-1*. (A, B) Expression levels of (A) *OsKS4* and (B) *OsDTC2* in *Ossymrk-1* and YC treated with 10^{-5} M CO7, 10^{-5} M CO4 or H_2O. The mean and SD are shown for three independent plants. Asterisks indicate a significant difference between the treated and untreated samples in each strain by Welch's *t*-test ($P < 0.05$). Data are presented as the mean of three biological replicates, and error bars indicate a standard deviation. (C–E) ROS production by the calluses of (C) *Ossymrk-1*, (D) YC and (E) *Oscerk1;Oscerk2* treated with 10^{-9} M CO7 or distilled water (DW).

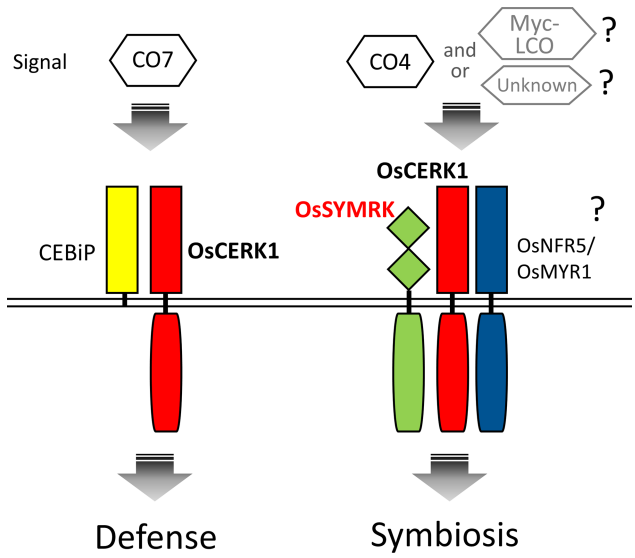

Fig. 7 A hypothetical model of the ligand-dependent receptor complex formation that regulates immunity and symbiosis in rice.

that OsSYMRK may represent a candidate partner of OsCERK1 in AM symbiosis, similar to CEBiP in chitin-triggered immunity (**Fig. 7**).

Although both OsCERK1 and OsSYMRK are required for AM symbiosis, the mycorrhizal phenotypes of *Oscerk1* and *Ossymrk* differed substantially, with the *Ossymrk* mutant shown to have a more severe phenotype than *Oscerk1*. The mycorrhizal phenotype of *Ossymrk* is more similar to that of *Osccamk* than *Oscerk1*, with no arbuscules observed even at 5 wpi (Miyata et al. 2022). The redundant function of other molecules could be the reason for the weak phenotype in *Oscerk1*. Recently, we demonstrated that OsCERK2, which is the most similar LysM receptor–like kinase to OsCERK1, potentially functions in AM symbiosis and chitin-triggered immunity. However, in contrast to our expectations, the *Oscerk1;Oscerk2* double-knockout mutant had the same phenotype as the *Oscerk1* mutant (Miyata et al. 2022). In light of this finding, OsCERK2 redundancy does not appear to explain the weak phenotype of *Oscerk1*. Besides OsCERK2, there are eight further unevaluated LysM receptor–like kinases in rice and redundancy of these receptors may explain the observed phenotype of *Oscerk1*. Alternatively, as OsCERK1 regulates chitin-triggered immunity, a weakened immune response in *Oscerk1* may explain the phenotype of *Oscerk1*. In addition to these hypotheses, OsSYMRK may function in integrating signals from OsCERK1 and other unknown pathways. In *M. truncatula*, lipochitin oligosaccharides (LCOs) and COs reportedly induce AM symbiosis in a synergistic manner (Feng et al. 2019). Both CO4 and CO8 were shown to be involved in the initiation of both AM symbiosis and chitin-triggered immunity, with LCO able to suppress defense response and induce AM symbiosis. The authors posited that the recognition of chitin by CERK1 and LYR4 and LCO by NOD FACTOR PERCEPTION is responsible for AM symbiosis in *M. truncatula*, implying that a similar LCO-responsive pathway exists in rice. In contrast, our previous studies found no response to Myc-LCO in rice (Miyata et al. 2014). Currently, there is a lack of evidence for the involvement of LCO in AM symbiosis in rice. Hence, Myc-LCO with different modifications or other unknown signals could be involved in the induction of AM symbiosis via a receptor complex involving OsSYMRK.

The *OsSYMRK* gene contains sequences corresponding to LRR domains despite a large deletion among the regions corresponding to LRR2 and LRR3 of LjSYMRK. In particular, LRR1 appears to be highly conserved in terrestrial plants and may therefore be essential for the function of SYMRKs. Generally, LRR receptor–like kinases are responsible for the recognition of protein ligands. As an example, FLAGELLINSENSING-2 (FLS2) functions in recognizing flagellin to trigger defense responses (Gómez-Gómez and Boller 2000). Another LRR receptor–like kinase, BRI1-ASSOCIATED RECEPTOR KINASE 1, acts as a coreceptor of other receptors such as FLS2 (Sun et al. 2013) or BRASSIN OSTEROID INSENSITIVE1 (BRI1), which is the receptor for brassinosteroid signaling (Nam and Li 2002). Whether SYMRKs recognize protein ligands directly or act as coreceptors is currently unclear. Furthermore, the LRR domain of SYMRKs may not be essential for function despite being well conserved.

The mechanisms underlying the perception of CO4 via OsCERK1 during AM symbiosis are yet to be fully elucidated. To date, a direct interaction between OsCERK1 and COs has not been established, whereas CEBiP is known to bind chitin oligosaccharide (Kaku et al. 2006, Shinya et al. 2012). The fact that OsSYMRK lacks LysM domains suggests that OsSYMRK does not bind COs. As a knockout mutant of *cebip* has been shown to establish AM symbiosis normally (Miyata et al. 2014), the perception of Myc-LCO and CO4 is unlikely to involve CEBiP. In contrast, He et al. reported that OsNFR5/OsMYR1 is able to bind CO4 (He et al. 2019), indicating that OsNFR5/OsMYR1 may be a candidate receptor for CO4 and should be studied further.

Regarding the evolutional trajectory of SYMRK/DMI2, the structural differences between OsSYMRK and LjSYMRK reported in previous studies, including the presence and absence of MLD and LRR domains, have been explained by stepwise domain acquisition, which ultimately contributed to the evolution of RN symbiosis (Markmann et al. 2008). Contrary to this widely accepted hypothesis, our phylogenetic analyses revealed that the MLD of SYMRK homologs was conserved even in moss and fern, which are not hosts of AM symbiosis. In contrast, the lack of an MLD was specific to species belonging to commelinids, including Posales, Arecales and Zingiberales. These findings indicate that ancestral SYMRK contains an MLD and that commelinids lost the MLD in SYMRK proteins during evolution while maintaining the ability to form AM symbiosis.

In legumes, the MLD of SYMRK undergoes proteolytic cleavage in *L. japonicus* and this cleavage is thought to be important for signal transduction and turnover of SYMRK. A *symrk* mutant, *symrk-14*, with a single-base substitution in the GDPC motif has been shown to be unable to undergo MLD cleavage, with plants unable to establish RN symbiosis (Antolin-Llovera

et al. 2014) or AM symbiosis (Kosuta et al. 2011). The GDPC motif is highly conserved in a wide range of plant species, including moss and rice (Kosuta et al. 2011). Conversely, the GDPC motif is also present in the SYMRK homolog of *M. polymorpha*, which is not a host of AM symbiosis, indicating that the acquisition of the GDPC motif is not necessarily associated with AM symbiosis and appears to originate from more primitive, non-AM plants. The finding that the SYMRK homolog of an alga thought to be an ancestor of angiosperms, *C. braunii*, does not contain a GDPC motif indicates that the GDPC motif may have been obtained during terrestrialization. Once the plants obtained the GDPC motif in SYMRK homologs, including OsSYMRK, it was highly conserved in terrestrial plants. However, the SYMRK homologs in *B. distachyon* are an exception and have a single amino acid substitution changing the GDPC motif to an EDPC motif. Similar to OsSYMRK, SYMRKs in *B. distachyon* do not have an MLD. If a GDPC motif is required for MLD release, the presence of a GDPC motif in SYMRK homologs in commelinids may not be required and mutation of the GDPC motif may not be evolutionarily disadvantageous. Future studies are required to determine the importance of a GDPC motif for AM symbiosis.

SYMRK homologs in moss and fern have three LRR domains. The LRR2 and LRR3 regions of LjSYMRK and SYMRK homologs in *M. paleacea* have high similarity, indicating that the three LRR domains in the SYMRK homologs of legumes originated from these ancestral plants. On the contrary, a large deletion in the region corresponding to LRR2 and LRR3 was observed in all monocots. The fact that three LRR domain sequences are conserved in eurosids I, including legumes, and eurosids II, including *Arabidopsis* (Markmann et al. 2008), indicates that this sequence is highly conserved in the eudicots. Besides monocots, tomato also has a deletion in the LRR2 and LRR3 regions. However, the position of the deletion in the SYMRK homolog of tomato differed from that of monocots. In addition, the fact that Solanales and Poales are evolutionarily distant indicates that the deletion of LRR domains may have occurred independently.

The results of the present study indicate that OsSYMRK and other SYMRK homologs in commelinids have undergone unique structural changes during evolution, with the loss of MLD while conserving their function in AM symbiosis. The presence of MLD is critical for the regulation of SYMRK protein levels in legumes; thus, the lack of MLD in OsSYMRK implies the presence of alternative regulatory mechanisms in rice. The increased expression levels of *OsSYMRK* induced by AM colonization indicate that the regulation of OsSYMRK at the level of gene expression is important in rice, although other regulatory mechanisms may be present. Further studies of AM symbiosis in nonleguminous plants, such as rice, and comparison with legumes may increase our understanding of mechanisms underlying the initiation of AM symbiosis and possible diversification in these mechanisms during evolution. These studies may also increase our understanding of the ability of plants to distinguish pathogens from symbionts in natural environments.

Materials and Methods

Protein sequence of OsSYMRK and phylogenetic analysis

The structures of OsSYMRK and LjSYMRK were analyzed using SMART (http://smart.embl-heidelberg.de/) (Letunic et al. 2021) and NCBI CD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Lu et al. 2020). Structure homology was evaluated by identity, and similarity was calculated using Geneious (Biomatters Ltd. Auckland, New Zealand). Similarity was calculated using BLOSUM75 with a threshold of 1. The sequences of LjSYMRK homolog genes were obtained from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Annotation numbers are provided in **Supplementary Table S2**. We generated phylogenetic trees using the Geneious tree builder (Biomatters Ltd). The presence of MLDs was determined using SMART.

Vector construction for CRISPR/Cas9 genome editing

gRNAs were designed using CRISPR-P ver. 2.0 (http://crispr.hzau.edu.cn/CRISPR2/) (Liu et al. 2017). gRNA sequences are presented in **Supplementary Table S2**. The oligonucleotides of designed gRNA sequences were annealed and ligated using T4 ligase (Nippongene, Chiyoda-ku, Tokyo, Japan) into the pZK_OsU6-gRNA vector digested with BbsI. The pZK_OsU6-gRNA vector for gRNA1 and gRNA3 was digested with EcoRV (Nippongene) and AscI (New England Biolabs, Ipswich, MA, USA). The pZK_OsU6-gRNA vector for gRNA2 and gRNA4 was digested with PvuII (New England Biolabs) and AscI, and fragments containing the U6 promoter were extracted from agarose gel using NucleoSpin Gel and PCR Clean-up (Takara Bio, Kusatsu, Siga, Japan). The digested vector for gRNA1/gRNA3 and extracted fragments for gRNA2/gRNA4 were combined using T4 ligase (Nippongene). The pZK_OsU6-gRNA vector containing the two gRNA sequences was digested with PvuII and AscI and ligated into the pZK_gYSA_MMCas9 vector digested with PmlI and AscI. Information regarding all vectors used in the present study is described in a previous study by Mikami et al. (2015).

Transformation for genome editing in rice was performed as described previously (Ozawa 2009, Ozawa et al. 2012). In brief, the *Agrobacterium tumefaciens* strain EHA-105 was used for transformation. YC seeds used in a previous study (Carotenuto et al. 2017) were sterilized with sodium hypochlorite solution and Tween 20 before incubation on N6D medium for 1 month. Generated YC calluses were used for transformation. As a selection marker, 35 μg/ml G-418 (Enzo Life Sciences, Inc., NY, USA) was added to growth medium. 12.5 μg/ml meropenem hydrate (Sumitomo Pharma, Chuoku, Tokyo, Japan) was used to remove agrobacteria after co-inoculation. Genomic DNA was extracted from leaves of regenerated plants according to the method described previously (Miyata et al. 2016). The target region was amplified by PCR using Go-taq (Promega, Madison, WI, USA), and the primers are presented in **Supplementary Table S2**. Bands were extracted from agarose gel using NucleoSpin Gel and PCR Clean-up (Takara Bio). PCR products were sequenced using a 3130X Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA).

DNA extraction and treatment with restriction enzymes for detection of the *Ossymrk1* homozygous mutant

Genomic DNA extraction was performed as previously described (Miyata et al. 2016). Genomic DNA from T_0 or T_1 generation *Ossymrk-1* plants was amplified by PCR using the single-base change primer shown in **Supplementary Table S2**. PCR products were digested with HindIII (Nippongene) for 3 h. Band patterns were assessed using electrophoresis.

Plant materials and growth conditions for inoculation analyses

Seeds were sterilized with 70% ethanol and sodium hypochlorite solution and then immersed in sterilized water for 7 d at 28°C. Germinated seeds were inoculated with or without *R. irregularis* spores as previously described (Miyata et al. 2014). Plant roots were stained with trypan blue, the number of infection units per plant was counted and the root colonization ratio was calculated. The root colonization ratio was calculated according to a previously described method (Miyata et al. 2022). In brief, stained roots were spread on 2-mm grid dishes, and the presence of arbuscules was determined using a binocular stereomicroscope at crossing points. The whole root (>100 locations) of each plant was observed to calculate the root colonization ratio. Plants were visualized using a BZX 800 microscope (Keyence, Japan). For the evaluation of defense responses, the roots of YC were preincubated for 1 h and *Ossymrk-1* seedlings were treated with 10^{-5} M CO4 and CO7 (Yaizu Suisankagaku Industrial Co., Japan) in a small dish. After 6-h treatment with CO4, CO7 or H_2O, roots were removed and immediately frozen in liquid nitrogen. For the analysis of phosphate-dependent expression of *OsSYMRK*, *Oryza sativa* cv. Nipponbare BL no. 2 (WT) seedlings at 5 d after germination were used. Seedlings were incubated for 10 d in the Hoagland solution with or without phosphate (Hoagland and Arnon 1950). As the source of phosphate and control, 1 mM $NH_4H_2PO_4$ (1 mM phosphate) and 1 mM NH_4Cl_2 (0 mM phosphate) were used, respectively. Frozen roots were later used for RNA extraction.

RNA manipulation and real-time RT-PCR

Total RNA was extracted using the Cetyl trimethyl ammonium bromide method as previously described (Nakagawa et al. 2011) and reverse-transcribed using ReverTra Ace qPCR RT master mix (TOYOBO, Kita-ku, Osaka, Japan) with gDNA remover according to the manufacturer's instructions. Aliquots of the resulting cDNA were subjected to real-time PCR analyses using a 7500 Fast Real-Time PCR System (Applied Biosystems). *Oryza sativa* ubiquitin (*OsUBQ*) was used as a reference standard. SYBR green was used for qRT-PCR of *AM1*, *AM2* and *OsUBQ*. TaqMan qRT-PCR was used for *OsKS4* and *OsDTC2*. All primer sets used in the expression analysis are listed in **Supplementary Table S1**.

Ca^{2+} spiking analysis

For calcium spiking analysis, nuclear-localized YC 2.60 (NLS-YC) (Nagai et al. 2004) was introduced into *Ossymrk-1* mutants by crossing with WT rice expressing NLS-YC (Carotenuto et al. 2017). Surface-sterilized NLS-YC seeds were placed on 0.8% water agar plates and grown in a growth chamber at 30°C under dark conditions for 5–7 d. Germinated roots were treated with 10^{-6} M CO4 in Buffered Nodulation Media (Ehrhardt et al. 1996). Calcium imaging was performed using a Nikon microscope TE2000-U equipped with 20× dry objectives and imaging systems (Photometrics CoolSNAP DYNO, Ludl MAC5000). YC fluorescence images of root cells were acquired every 5 s with Fluorescence Resonance Energy Transfer (FRET) (Cyan Fluorescent Protein (CFP) ex. 440 nm/Yellow Fluorescent Protein (YFP) em. 535 nm) and CFP (CFP ex. 440 nm/CFP em. 480 nm) filter sets. FRET and CFP fluorescence intensities were measured with the ratio of intensities (FRET int./CFP int.) calculated using NIS-Elements AR (Nikon, Minatoku, Tokyo, Japan).

BiFC analysis

BiFC was performed according to a previously described method (Miyata et al. 2016). OsCERK1 and OsSYMRK were cloned into nEYFP/pUGW2 (35S pro, C-nEYFP) and cEYFP/pUGW2 (35S pro, C-cEYFP) vectors, respectively, provided by Dr. Tsuyoshi Nakagawa. Plasmids were introduced into the LBA4404 *A. tumefaciens* strain. Bacterial cultures were collected and resuspended in a medium containing 10 mM MES-KOH (pH 5.6), 10 mM $MgCl_2$ and 100 mM acetosyringone (Sigma-Aldrich). Bacterial cultures were infiltrated into 3-week-old leaves of *N. benthamiana*. YFP fluorescence was measured at 3 d postinfiltration. A Leica Stellaris microscope was used for microscopic analyses.

Chitin-induced ROS assay

CO7 was kindly supplied by Yaizu Suisankagaku Industrial Co. (http://www.yskf.jp/yskfen/index.html) and reacetylated prior to use. Suspension cultures of *Ossymrk-1*, *Oscerk1*, *Oscerk2* and YC were maintained as previously described (Yamada et al. 1993). Rice calluses were incubated as previously described (Hayafune et al. 2014). Analyses were performed using 40 mg of calluses at 5 d after transfer to a new medium. After preincubation for 30 min, calluses were treated with 10^{-9} M CO7 or sterilized H_2O and incubated for 30, 60, 90, 120 or 180 min at 25°C. The concentration of ROS species in the reaction mixture was determined using a luminol-dependent chemiluminescence assay (Schwacke and Hager 1992).

Supplementary Data

Supplementary data are available at *PCP* online.

Data Availability

The nucleotide sequence reported in this paper has been submitted to the database as following accession numbers—OsSYMRK: LOC_Os07g38070 (rice genome annotation project) and Os07g0568100 (RAP-DB). The homologs of OsSYMRK are shown in **Supplementary Table S1**.

All other data underlying this article will be shared upon reasonable request to the corresponding author.

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Disclosures

The authors have no conflicts of interest to declare.

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