Colonization of Mutualistic Mycorrhizal and Parasitic Blast Fungi Requires OsRAM2-Regulated Fatty Acid Biosynthesis in Rice

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Accepted 20 December 2021.

Arbuscular mycorrhizal (AM) fungi form a mutual association with the majority of land plants, including most angiosperms of the dicotyledon and monocotyledon lineages. The symbiosis is based upon bidirectional nutrient exchange between the host and symbiont that occurs between inner cortical cells of the root and branched AM hyphae called arbuscules that develop within these cells. Lipid transport and its regulation during the symbiosis have been intensively investigated in dicotyledon plants, especially legumes. Here, we characterize *OsRAM2* and *OsRAM2L*, homologs of *Medicago truncatula* *RAM2*, and found that plants defective in *OsRAM2* were unable to be colonized by AM fungi and showed impaired colonization by *Magnaporthe oryzae*. The induction of *OsRAM2* and *OsRAM2L* is dependent on *OsRAM1* and the common symbiosis signaling pathway pathway genes *CCaMK* and *CYCLOPS*, while overexpression of *OsRAM1* results in increased expression of *OsRAM2* and *OsRAM2L*. Collectively, our data show that the function and regulation of *OsRAM2* is conserved in monocot and dicot plants and reveals that, similar to mutualistic fungi, pathogenic fungi have recruited RAM2-mediated fatty acid biosynthesis to facilitate invasion.

Keywords: AM symbiosis, arbuscular mycorrhiza, *M. oryzae* infection, mycorrhizal interactions, *OsRAM2*, rice

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H. Dai, X. Zhang, and B. Zhao contributed equally to this article.

Funding: Support was provided by the National Science Foundation (31870218, 32088102, 31730103, 31825003, and 32050081), National Key R&D Program of China (2019YFA0904703 and 2016YFA0500502), and the Strategic Priority Research Program “Molecular Mechanism of Plant Growth and Development” of the Chinese Academy of Sciences (XDB27040207).

*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary table and supplementary figures are published online.

The author(s) declare no conflict of interest.

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The arbuscular mycorrhizal (AM) symbiosis is an ancient and ubiquitous beneficial mutualism between fungi belonging to subphylum Glomeromycotina and over 80% of terrestrial plants, including important crops such as maize (*Zea mays*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*) (Brundrett and Tedersoo 2018; Genre et al. 2020). The AM association can facilitate plant uptake of soil phosphorus and nitrogen as well as increase plant local and systemic disease resistance (Liu et al. 2007; Parniske 2008; Smith and Read. 2008) whereas, in exchange, plants spare up to 20% of photosynthate for AM fungi for their growth and proliferation (Chiu and Paszkowski 2019; Genre et al. 2020; Graham et al. 1996; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017; Wang et al. 2017). This nutrient exchange takes place through tree-shaped, subcellular structures called arbuscules that are formed in cortical cells of the host root (Choi et al. 2018; Genre et al. 2005, 2008; Gutjahr and Parniske 2013; Wang et al. 2017).

The symbiosis is established through reciprocal signaling and recognition between the host plant and AM fungi. Plant-derived strigolactones increased the fungal release of short-chain chitooligosaccharides (CO4 and CO5) and lipo-chitooligosaccharides (Akiyama et al. 2005; Besserer et al. 2006; Genre et al. 2013; Maillet et al. 2011), called mycorrhizal factors. In rice, the lysin motif (LysM)-containing receptor kinase OsMYR1 was shown to bind CO4, which promotes it to interact with OsCERK1 (Zhang et al. 2015; He et al. 2019) to activate the common symbiosis signaling pathway (CSSP) shared by AM symbiosis and nodulation (Oldroyd 2013; Zipfel and Oldroyd 2017). Central to the activation of symbiosis signaling is the induction of nuclear calcium oscillations which are decoded by a calcium- and calmodulin-dependent serine/threonine protein kinase (CCaMK) and its interacting protein CYCLOPS (Gutjahr et al. 2008; Horváth et al. 2011; Lévy et al. 2004; Messinese et al. 2007; Ovchinnikova et al. 2011; Singh and Parniske 2012; Singh et al. 2014; Yano et al. 2008). The lone DELLA homolog in rice (slender rice 1), a suppressor of gibberellic acid signaling (Sun 2011), can interact with CYCLOPS and promote CCaMK-CYCLOPS complex formation to regulate symbiosis (Floss et al. 2013; Jin et al. 2016; Yu et al. 2014). As a GRAS family member, DELLA can form a complex with other two GRAS proteins, DELLA INTERACTION PROTEIN 1 and REDUCED ARBUSCULE MYCORRHIZAL 1 (RAM1) to directly or indirectly regulate expression of mycorrhizal-associated genes such as *EXO70I*, *FatM*, *RAM2*, *STR*, and *PT4* and, thus, control mycorrhizal colonization (Bravo et al. 2017; Floss et al. 2013; Foo et al. 2013;

Gobbato et al. 2012, 2013; Müller et al. 2020; Park et al. 2015; Rich et al. 2015; Xue et al. 2015; Yu et al. 2014).

RAM1 is regulated by *DELLA*, *CYCLOPS*, and *WRI5a* as well as *OsPHRs* (Jiang et al. 2018; Pimprikar et al. 2016; Shi et al. 2021; Yu et al. 2014), and it is required in mycorrhizal infection, arbuscule development, and senescence in *Medicago truncatula*, *Lotus japonicus*, *Petunia* spp., *Brachypodium distachyon*, and rice (Gobbato et al. 2013; Müller et al. 2020; Park et al. 2015; Pimprikar et al. 2016; Rich et al. 2015; Shi et al. 2021; Xue et al. 2015; Yu et al. 2014). In turn, *RAM1* can regulate the AM symbiosis-induced genes *WRI5a*, *RAM2*, *FatM*, and *STR*, which function in lipid accumulation and transfer from the plant to AM fungi (Bravo et al. 2017; Gobbato et al. 2012, 2013; Gutjahr et al. 2012; Jiang et al. 2017, 2018; Keymer et al. 2017; Luginbuehl et al. 2017; Müller et al. 2020).

RAM2, a glycerol-3-phosphate acyl transferase, is required for synthesis of 2-monoacylglycerols, which might be transported from the plant root to the AM fungus by *STR* and *STR2* (Bravo et al. 2017; Gobbato et al. 2013; Jiang et al. 2017, 2018; Keymer et al. 2017; Luginbuehl et al. 2017; Wang et al. 2012; Zhang et al. 2010), and production of cutin monomers (Wang et al. 2012). *MtSTR* encodes a periarbuscular membrane-localized ABC transporter that is regulated by the AP2/EREBP transcription factor *WRI5a* and GRAS protein *RAM1* (Jiang et al. 2017, 2018; Park et al. 2015; Zhang et al. 2010). *RAM2* was reported as a direct target of *RAM1* (Gobbato et al. 2012). In *M. truncatula*, *ram2* was defective in hyphopodia formation and arbuscule formation by AM fungi as well as in oomycete pathogen infection (Bravo et al. 2017; Wang et al. 2012). However, the function of *RAM2* in monocots remains unknown. In this study, we identified *RAM2* homologs in rice named *OsRAM2* and *OsRAM2L* and found *OsRAM2* is required for mycorrhizal colonization and infection by the fungal parasite *Magnaporthe oryzae*.

RESULTS

OsRAM2 and *OsRAM2L* are induced upon AM colonization.

We identified two rice proteins that grouped in the same phylogenetic clade as *Medicago truncatula* *RAM2* in *O. sativa*, which were named *OsRAM2* and *OsRAM2L* (Fig. 1A). To study their expression patterns in rice, we collected roots, stems, and leaves from AM-inoculated and mock-inoculated plants. Unlike in stems, leaves, and mock-inoculated tissues, *OsRAM2* and *OsRAM2L* were substantially expressed in roots colonized by AM fungi, with *OsRAM2* showing higher expression (Fig. 1B and C). The same patterns were reported with RNA sequencing of mycorrhizal-colonized rice roots (Shi et al. 2021). *OsRAM2* and *OsRAM2L* were induced in roots after AM colonization, with colonization levels increasing over time (Fig. 2A and B; Supplementary Fig. S1A), and with *OsRAM2* having consistently over 30-fold higher expression than *OsRAM2L* in AM-colonized roots. The transcription levels of *OsRAM2* and *OsRAM2L* were reduced to approximately 30% under high-phosphate conditions that attenuate mycorrhizal symbiosis by up to 50% (Fig. 2C and D; Supplementary Fig. S2). Taken together, these results indicate that *OsRAM2* expression is highly correlated with mycorrhiza.

To precisely visualize *OsRAM2* expression in the AM symbiosis, we generated a reporter construct containing a 3-kb *OsRAM2* promoter driving β -glucuronidase (*GUS*) and evaluated *GUS* expression in mycorrhizal roots. We found that *GUS* was specifically expressed in cortical cells containing arbuscules (Fig. 2E), consistent with what was observed for *RAM2* in *M. truncatula* (Gobbato et al. 2013), further suggesting that *OsRAM2* might be involved in the AM symbiosis.

Mycorrhizal colonization was impaired in *Osram2* and *Osram2/Osram2l*.

To further investigate the function of *OsRAM2* and *OsRAM2L* in the mycorrhizal symbiosis, we designed clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 constructs to individually target *OsRAM2* and *OsRAM2L* and used these constructs to generate transgenic plants. The *OsRAM2* and *OsRAM2L* of the transgenic lines were successfully edited by their respective guides. An *Osram2* allele with a single-nucleotide insertion, resulting in premature stop codon and producing a predicted truncated protein of 50 amino acids, was isolated (Fig. 3A). A single-nucleotide insertion in *OsRAM2L* led to a frame shift from the 60th amino acid and stop at 3' untranslated region, producing a mutated protein of 544 amino acids (Fig. 3B). The functional domains of *OsRAM2* and *OsRAM2L* are localized in the C termini, suggesting that the truncated or mutated proteins are non-functional. We then obtained an *Osram2/Osram2l* double mutant through hybridization.

Mycorrhizal infected roots of *Osram2* and *Osram2/Osram2l* were collected at 6 weeks postinoculation (wpi). Compared with a 50% colonization level in the wild type (WT), *Osram2* roots displayed dramatically reduced colonization, which was limited to the occasional formation of intercellular hyphae and stunted arbuscules (Fig. 3C and D). In rare cases, the hyphae were able to penetrate into cortical cells but could not branch normally (Fig. 3C). Consistent with the phenotype observed for *Osram2*, hyphae and arbuscules were rarely seen in *Osram2/Osram2l* roots (Fig. 3C and D). No obvious differences in phenotype between *Osram2/Osram2l* and *Osram2* were observed, suggesting that *OsRAM2L* makes a limited contribution to mycorrhizal symbiosis. We then assayed the expression of *OsRAM2* and *OsRAM2L* in *Osram2* and *Osram2/Osram2l* mutants in mycorrhizal colonized and mock-inoculated roots. *OsRAM2* expression was almost abolished in both mutants, while *OsRAM2L* expression was reduced to approximately 30% in *Osram2* and 50% in *Osram2/Osram2l* mutants after mycorrhizal colonization (Fig. 3E and F).

In addition, to further validate the requirement of *OsRAM2* and *OsRAM2L* in the symbiosis, we surveyed the expression of several AM marker genes in *Osram2* and *Osram2/Osram2l*. The transcript levels of five marker genes, including two early-stage mycorrhizal-specific marker genes, *OsAM1* and *OsAM3* (Gutjahr et al. 2008; Yu et al. 2014; Zhang et al. 2015), and three late-stage marker genes, *OsAM14*, *OsHA1*, and *OsPT11* (Gutjahr et al. 2008; Wang et al. 2014; Yu et al. 2014; Zhang et al. 2015), were reduced in *Osram2*- and *Osram2/Osram2l*-colonized roots (Fig. 4), consistent with the reduction in mycorrhizal colonization. Taken together, we concluded that *OsRAM2* is involved in mycorrhizal fungal infection and colonization in the cortex cell as well as arbuscule development.

Induction of *OsRAM2* and *OsRAM2L* are dependent on *OsRAM1*, *CCaMK*, and *CYCLOPS*.

RAM1, a GRAS-domain transcription factor, was proven to be involved in AM symbiosis in dicots and monocots (Gobbato et al. 2012, 2013; Müller et al. 2020; Park et al. 2015; Pimprikar et al. 2016; Rich et al. 2015; Shi et al. 2021; Xue et al. 2015; Yu et al. 2014). In *M. truncatula*, *RAM1* directly regulates *RAM2* expression through associating with the *RAM2* promoter (Gobbato et al. 2012). In *B. distachyon*, similar to *MtRAM2* in *M. truncatula*, *BdRAM1* expression increased over time with increasing mycorrhizal colonization (Müller et al. 2020). *BdRAM1* overexpression constitutively elevated *BdRAM2* expression in both the shoot and roots (Müller et al. 2020). To further identify whether *OsRAM2* is regulated by *OsRAM1*, we examined the expression pattern of *OsRAM2* and *OsRAM2L* in the *Osram1* mutant and *OsRAM1*-overexpressing rice roots. We found that the transcript levels of *OsRAM2* and *OsRAM2L*

Fig. 1. *Oryza sativa* orthologs of *RAM2* are induced in roots colonized by *Rhizophagus irregularis*. **A**, Phylogenetic tree of *MtRAM2* with homologs from *Medicago truncatula* (Mt), *Glycine max* (Gm), *Oryza sativa* (Os), and *Arabidopsis thaliana* (At) was constructed using a maximum-likelihood algorithm in MEGA-X with 1,000 bootstraps value. **B**, *OsRAM2* expression in the roots, stems, and leaves of arbuscular mycorrhizal (AM)-colonized (AM+) or noncolonized (AM-) plants at 6 weeks postinoculation (wpi). A higher abundance of *OsRAM2* transcripts was detected in mycorrhizal roots relative to noninoculated roots. **C**, *OsRAM2L* expression in the roots, stems, and leaves of AM-colonized (AM+) or noncolonized (AM-) plants at 6 wpi. Expression levels were normalized to the geometric mean of housekeeping gene *OsCyclophilin2* (B and C). The RNA was extracted from three individual plants. Error bars represent standard deviation ($n = 3$, where n denotes the number of technical repetition). Asterisks indicate significant difference (Student's t test; * and ** indicate $P < 0.05$ and 0.01 , respectively; ns = not significant).

Fig. 2. *OsRAM2* is exclusively expressed in rice arbusculated cells. **A**, *OsRAM2* gene expression is induced in roots colonized by *Rhizophagus irregularis* compared with mock-inoculated roots (white bars). Plants were harvested 4, 5, and 6 weeks postinoculation (wpi). *OsRAM2* gene expression increases over time. **B**, *OsRAM2L* gene expression in *R. irregularis*-colonized and mock-inoculated roots over time. Low phosphate (LP, 0 μM KH_2PO_4) increases expression of **C**, *OsRAM2* and **D**, *OsRAM2L* in arbuscular mycorrhizal (AM) symbiosis; high phosphate (HP, 200 μM KH_2PO_4) reduces this induction. Expression levels were normalized to the geometric mean of housekeeping gene *OsCyclophilin2* (A to D). The RNA was extracted from three individual plants. Error bars represent standard deviation ($n = 3$, where n denotes the number of technical repetition). Asterisks indicate significant difference (Student's t test; * and ** indicate $P < 0.05$ and 0.01 , respectively; ns = not significant). **E**, Bright field (upper panel) and corresponding WGA AF-488-stained (bottom panel) images of rice roots colonized with *R. irregularis* reveals β -glucuronidase staining in cortical arbuscular cells driven by the *OsRAM2* promoter. Bar = 50 μm .

were reduced to less than 10 and 20% of WT levels in both AM-colonized and mock-inoculated *Osram1* mutant roots, respectively (Fig. 5A to D) and increased up to 30- to 50-fold in AM-colonized roots overexpressing *OsRAM1* (Fig. 5B and D). The increase in *OsRAM2* and *OsRAM2L* expression was proportionally much higher than the increase of mycorrhizal colonization in the same roots (Supplementary Fig. S3), indicating that *OsRAM1* regulation of *OsRAM2* expression is only partly correlated with mycorrhizal colonization.

In *L. japonicus*, CYCLOPS, a CSSP protein which forms a complex with CCaMK and DELLA, directly regulates *RAM1*

expression through association with an *AMCYC-RE* cis-element in its promoter (Pimprikar et al. 2016). In order to check whether the *OsRAM2* expression pattern relies on CSSP genes, we analyzed *OsRAM2* and *OsRAM2L* expression in *ccamk-2* and *cyclops-3* mutants, which have defects in cortical mycorrhizal colonization (Supplementary Fig. S3) (Gutjahr et al. 2008). The transcripts of *OsRAM2* and *OsRAM2L* were reduced to less than 1 and 15% of WT levels in *ccamk-2* and *cyclops-3* mutants, respectively, in AM-colonized plants (Fig. 5B and D). These results support the notion that *OsRAM2* and *OsRAM2L* function downstream of CSSP components.

Fig. 3. Mycorrhizal colonization is impaired in *Osram2* and *Osram2/Osram2L* mutants. DNA alignments of *OsRAM2* or *OsRAM2L* and corresponding coding and protein sequences for **A**, *Osram2* and **B**, *Osram2l* mutants and wild-type (WT) plants. Target sequences of the guide RNAs are indicated in bold. CRISPR/Cas9-induced insertions are labeled in blue. Numbers indicate nucleotide or protein position in the WT sequence. Single-nucleotide insertion results (blue) in a frameshift (red) and premature stop codon (red asterisk) in *OsRAM2* (A) and protein sequence alteration in *OsRAM2L* (B). **C**, Trypan blue staining of the fungus to reveal the arbuscule morphology in WT, *Osram2*, and *Osram2/Osram2l* roots colonized by *Rhizophagus irregularis*. Scale bar = 100 μ m. **D**, Quantification of *R. irregularis* root-length colonization in WT, *Osram2*, and *Osram2/Osram2l* roots. Bar graphs show the mean, with error bars representing standard deviation. The experiment was repeated three times with similar results. Each plant analyzed is plotted as a black dot. Asterisks indicate significant difference (Student's *t* test; * and ** indicate $P < 0.05$ and 0.01 , respectively; ns = not significant). **E**, *OsRAM2* expression is induced after arbuscular mycorrhizal (AM) inoculation and decreased in *Osram2* mutants. **F**, *OsRAM2L* expression is obviously upregulated upon AM inoculation but the expression level is reduced in *Osram2* and *Osram2/Osram2L* mutants. Expression levels were normalized to the geometric mean of housekeeping gene *OsCyclophilin2* (E and F). The RNA was extracted from three individual plants of WT or mutants. Error bars represent standard deviation ($n = 3$, where n denotes the number of technical repetition). Asterisks indicate significant difference (Student's *t* test; * and ** indicate $P < 0.05$ and 0.01 , respectively; ns = not significant).

OsRAM2/OsRAM2L participate in *Magnaporthe oryzae* infection.

Considering the similarities in the signaling events and morphological changes that occur inside host cells during infection of the biotrophic pathogen of *Magnaporthe oryzae* and colonization by mycorrhizal fungi (He et al. 2019; Marcel et al. 2010; Paszkowski. 2006; Sesma and Osbourn. 2004; Zhang et al. 2015; Zhang et al. 2021), we assayed whether *OsRAM2* plays a role in the plant-*M. oryzae* interaction. Interestingly, we found that *OsRAM2* was dramatically induced upon infection by *M. oryzae* (Fig. 6A and B), implying that *OsRAM2* participates in *M. oryzae* colonization of rice roots. *M. oryzae* typically invades plant roots, although it is a more severe pathogen in rice leaves (Marcel et al. 2010; Sesma and Osbourn. 2004; Zhang et al. 2021). We inoculated rice roots with *M. oryzae* and measured infected lesion size in the WT and *Osram2* and *Osram2/Osram2L* mutants. We observed obvious pathogen-induced browning and root growth inhibition in the WT at 2 wpi (Fig. 6C). Intriguingly, the growth

of the WT roots was severely impaired upon *M. oryzae* invasion, whereas the root lengths of *Osram2* and *Osram2/Osram2L* mutants were only slightly affected (Fig. 6D). The length of non-inoculated *Osram2* and *Osram2/Osram2L* mutants roots was comparable with that of the WT (Fig. 6C; Supplementary Fig. S4). In addition, the infection ratio, which represents the ratio of lesion length to root length, was lower in *Osram2* and *Osram2/Osram2L* mutant roots (Fig. 6E). Consistent with this, the relative fungal growth biomass reflecting the amount of fungus on the roots was also lower in *Osram2* and *Osram2/Osram2L* mutant roots than in those of the WT (Fig. 6F), as revealed by the ratio of expression of *M. oryzae* *Pot2* versus rice *ubiquitin* (Zhang et al. 2021).

We also found higher expression of the defense marker *OsCHITINASE* in infected *Osram2* and *Osram2/Osram2L* mutants compared with infected WT roots, while *OsPR10* was slightly upregulated in *Osram2/Osram2L*-colonized roots (Fig. 6G and H). In summary, enhanced defense-related signaling appears to have suppressed the invasion of rice blast in *Osram2*

Fig. 4. Expression of arbuscular mycorrhizal (AM) marker genes. AM marker genes including *OsPT11*, *OsAM1*, *OsAM3*, *OsAM14*, and *OsHA1* decreased expression in *Osram2* and *Osram2/Osram2L* mutants compared with that in the wild type (WT) upon mycorrhizal colonization. Expression levels were normalized to the geometric mean of housekeeping gene *OsCyclophilin2*. The RNA was extracted from three individual plants of WT or mutants. The experiment was repeated three times with similar results. Error bars represent standard deviation ($n = 3$, where n denotes the number of technical repetition). Asterisks indicate significant difference (Student's t test; * and ** indicate $P < 0.05$ and 0.01 , respectively; ns = not significant).

Fig. 5. Expression of *OsRAM2* and *OsRAM2L* is dependent on common symbiosis signaling pathway. **A**, Transcript level of *OsRAM2* is reduced in the noninoculated *Osram1* mutant roots. **B**, *OsRAM2* displays induced expression in the *OsRAM1* overexpression roots during arbuscular mycorrhizal (AM) symbiosis. In *Osram1*, *ccamk-2*, and *cyclops-3* roots, the induction of *OsRAM2* by AM inoculation is decreased relative to the AM-colonized wild type (WT). **C**, Transcript level of *OsRAM2L* is reduced in the noninoculated *Osram1* mutant roots, reminiscent of *OsRAM2*. **D**, *OsRAM2L* is induced in symbiosis and *OsRAM1* overexpression facilitates *OsRAM2L* expression. Expression of *OsRAM2L* is almost abolished in *Osram1*, *ccamk-2*, and *cyclops-3* roots. Expression levels were normalized to the geometric mean of housekeeping gene *OsCyclophilin2* (A to D). The RNA was extracted from three individual plants of the WT or mutants. Error bars represent standard deviation ($n = 3$, where n denotes the number of technical repetition). Asterisks indicate significant difference (Student's t test; * and ** indicate $P < 0.05$ and 0.01 , respectively; ns = not significant).

and *Osram2/Osram2l* mutants. In addition to this, CSSP gene mutants, including *ccamk-2* and *cyclops-3* roots, did not show altered response to fungal inoculation compared with WT plants (Supplementary Fig. S5), indicating that *OsRAM2*'s participation in the *M. oryzae* infection process is independent of CSSP components.

DISCUSSION

AM fungi are characterized as fatty acid auxotrophs due to the lack of fatty acid synthase genes in the genome. Several studies showed that host-derived fatty acids are directly delivered to the AM fungi (Bravo et al. 2017; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017). With the addition of the fatty acids myristate and palmitate to the media, AM fungi were able to grow extensively and produce infection-competent secondary spores in the absence of a host plant (Kameoka et al. 2019; Sugiura et al. 2020), further supporting the importance of fatty acids as an energy source for fungal growth. In legumes, *RAM2* is required for the accumulation of fatty acids in mycorrhizal roots, which are then likely transferred to the fungi by STR and STR2 (Bravo et al. 2017; Jiang et al. 2017, 2018; Wang et al. 2012). Here, we show that the rice *RAM2* homolog *OsRAM2* was exclusively expressed in arbuscular cells, and both *Osram2* and *Osram2/Osram2l* displayed defects in mycorrhizal infection and arbuscule development, indicating that *OsRAM2*-mediated fatty acid biosynthesis is important for mycorrhizal colonization in rice. *Osram2* and *Osram2/Osram2l* double mutants also showed less severe *M. oryzae* infection; thus, *OsRAM2* plays an important role in both AM symbiosis

and pathogen colonization, and this role is conserved in dicotyledon and monocotyledon plants and *OsRAM2* is a potential target to restrain *M. oryzae* infection in agriculture.

OsRAM2 and *OsRAM2L* showed decreased expression in the mycorrhiza-colonized roots of CSSP gene *ccamk-2* and *cyclops-3* mutants, indicating that *OsRAM2* and *OsRAM2L* might act downstream of CSSP in response to symbiotic fungi. Furthermore, we showed that the transcript levels of *OsRAM2* and *OsRAM2L* decreased in the *Osram1* mutant. In addition, overexpressing *OsRAM1* can active expression of *OsRAM2* and *OsRAM2L*, consistent with what was reported in *B. distachyon* (Müller et al. 2020). Thus, the regulation of *RAM2* by *RAM1* is conserved in rice, *Medicago truncatula*, and *B. distachyon* (Gobbato et al. 2012, 2013; Müller et al. 2020; Wang et al. 2012).

In addition to symbionts, the phytopathogenic fungus *Golovomyces cichoracerum* also recruits the fatty acid biosynthesis program during invasion, as indicated by the increased levels of fatty acids and reduced fungal invasion in *Arabidopsis* leaves of fatty acid biosynthetic gene mutants (Jiang et al. 2017). In *M. truncatula*, the oomycete pathogen *Phytophthora palmivora* can barely form appressoria in *ram2* roots (Wang et al. 2012). In contrast to AM fungi, the genes involved in fatty acid biosynthesis are present in the genomes of *G. cichoracerum*, *Phytophthora* spp., and *Magnaporthe oryzae* (bin Yusof et al. 2014; Liang et al. 2018; Rodenburg et al. 2018), suggesting that host lipids could serve as an alternative energy source for these parasites (Liang et al. 2018). In this study, we found that *OsRAM2* is induced in roots colonized by *M. oryzae*. Interestingly, the fatty acid biosynthesis genes including pyruvate kinase (*OsPK*, *LOC_Os01g47080*) and ketoacyl-acyl carrier protein reductase

Fig. 6. *OsRAM2* participates in *Magnaporthe oryzae* colonization. **A and B**, *OsRAM2* and *OsRAM2L* expression upon *M. oryzae* infection. **C**, Growth phenotype of *M. oryzae*-inoculated and noninoculated rice roots. Infection of rice blast fungal pathogen in *Osram2* and *Osram2/Osram2l* roots was weaker than that in the wild type (WT). Lesions were photographed at 2 weeks postinoculation (wpi). The experiment was repeated three times and showed the same result. Bar = 10 mm. **D**, Root length of *Osram2*, *Osram2/Osram2l*, and WT at 2 wpi. **E**, Disease resistance to rice blast in *Osram2*, *Osram2/Osram2l*, and WT, measured by the ratio of lesion length to root length. **F**, Disease resistance to rice blast in *Osram2*, *Osram2/Osram2l*, and WT roots at 2 wpi by real-time PCR. **G and H**, Relative expressions of defense-related marker genes *OsPR10* and *OsCHITINASE* in *Osram2*, *Osram2/Osram2l*, and WT roots at 2 wpi by real-time PCR. Expression levels were normalized to the geometric mean of housekeeping gene *OsCyclophilin2* (A, B, G, and H). The RNA was extracted from three individual plants of the WT or mutants. Error bars represent standard deviation ($n = 3$, where n denotes the number of technical repetition). The experiment was repeated three times with similar results. Asterisks indicate significant difference (Student's *t* test; * and ** indicate $P < 0.05$ and 0.01 , respectively; ns = not significant).

(*OsKAR*, *LOC_Os12g13930*) was upregulated by *M. oryzae* infection (Supplementary Fig. S6). Rice roots lacking *OsRAM2* showed more resistance to rice blast fungi, reminiscent of *Mtram2* resistance to an oomycete pathogen infection in *Medicago truncatula*, suggesting that the fatty acid program is also recruited by parasitic pathogen in monocotyledon plants.

Interestingly, it has been reported that *Magnaporthe oryzae* might use an invasion strategy similar to that of mycorrhizal fungi in plant roots (Marcel et al. 2010). However, *ccamk-2* and *cyclops-3* showed WT levels of resistance to *M. oryzae* (Supplementary Fig. S5). The same results were also observed in *dmi3* (homolog of *CCaMK*) mutants in *Medicago truncatula* inoculated by *P. palmivora* (Rey et al. 2015). These different lines of evidence suggest that *RAM2* is regulated separately in response to symbiosis and pathogenic fungi in both monocots and dicots. However, the mechanisms underlying its regulation in phytopathogen invasion requires further exploration.

MATERIALS AND METHODS

Plant material and growth conditions.

O. sativa subsp. *japonica* 'Nipponbare' was used as the WT for the *Osram2*, *Osram2/Osram2l*, *Osram1*, *ccamk-2*, and *cyclops-3* mutants, as well as the *OsRAM1* overexpression rice line. *Osram2* and *Osram2l* mutants were generated using the CRISPR-CAS9 mediated editing methods in Nipponbare (He et al. 2019). The *Osram2/Osram2l* double mutant line was generated by crossing *Osram2* and *Osram2l* plants. The alleles of *ccamk* and *cyclops* mutants were *ccamk-2* and *cyclops-3*, kindly provided by professor Uta Paszkowski (Gutjahr et al. 2008). *Osram1* mutants were reported previously (Shi et al. 2021).

Plant growth and *Rhizophagus irregularis* inoculation.

Rice seeds were surface sterilized with 75% ethyl alcohol for 1 min and 25% sodium hypochlorite for 30 min, followed by five washes with sterile water. The seeds were then transferred to half-strength Murashige and Skoog (1/2 MS) medium. Healthy seedlings were transplanted into a vermiculite, sand, and perlite (3:1:1) mixture containing *Rhizophagus irregularis* spores and cultured in a greenhouse at 28°C under conditions of 16 h of light and 8 h of darkness. The spores were produced by an *R. irregularis*-leek coculture and diluted to a final concentration of 400 spores/plant (Zhang et al. 2021). For phosphate treatment, 10 ml of Hoagland solution with 0 μ M KH_2PO_4 (low phosphate [LP]) or 200 μ M KH_2PO_4 (high phosphate [HP]) were applied to the plants every 2 days from the third week until harvest. When the rice plants were inoculated with *R. irregularis* in the substrate (vermiculite, sand, and perlite [3:1:1] mixture), proper amounts of water were supplied in the first 2 weeks for plant growth. Subsequently, 10 ml of Hoagland solution with 0 μ M KH_2PO_4 (LP) or 200 μ M KH_2PO_4 (HP) were applied to the plants every 2 days from the third week until harvest.

Root staining and mycorrhizal colonization quantification.

For quantification of arbuscular mycorrhizal colonization, arbuscular mycorrhizal fungi colonized roots were stained with ink as follows: 10% KOH for 12 min at 95°C, rinsing by water, and staining with ink for 6 min at 95°C. The mycorrhizal colonization level was examined and quantified using a gridline intersect method, as described previously (Zhang et al. 2015); each point represented a single plant. For arbuscules structure images, mycorrhizal fungi-colonized roots were stained with trypan blue as follows: 10% KOH for 30 min at 90°C, 10% H_2O_2 for 10 min at room temperature, and staining with trypan blue for 20 min at 90°C. Images were taken using a Zesis Axio Scope A1 microscope.

Rice blast infection assay.

Magnaporthe oryzae root infections were carried out as previously described (Zhang et al. 2021). *M. oryzae* cultures for root infection assays were grown on potato dextrose agar for 8 days at 22°C, and mycelial plugs were taken from the colony margin. The diameter of mycelial plugs was slightly smaller than the tubes to be used later. The 50-ml plastic centrifuge tubes were filled with 35 ml of sterile vermiculite containing 10 ml of sterile water. The mycelial plug was placed on top of this and covered with a further 5 ml of vermiculite. Mock-inoculated tubes were placed with sterile agar plugs. Three surface-sterilized seeds were placed on top of the second vermiculite layer, followed by an additional 5 ml of vermiculite and 5 ml of sterile water. Five replicate tubes were set up for each treatment. Tubes were covered with the covers and incubated at 28°C under conditions of 16 h of light and 8 h of darkness for 2 to 3 weeks. The seedling roots were then washed in water and measured for lesions using a ruler.

Measurement of relative fungal growth.

In total, 2-cm infected roots centered on the lesion were cut, and total DNA was extracted using a cetyltrimethylammonium bromide method (Zhang et al. 2021). The relative expression of the *M. oryzae* *Pot2* gene and the rice *ubiquitin* gene were measured by real-time PCR. The primer sequences for *Pot2* and *ubiquitin* and the formula for computing relative fungal growth were previously described (Zhang et al. 2021).

Histochemical GUS analysis.

The AM fungi colonized transgenic plant roots were soaked in the GUS staining buffer (10 mM EDTA, 100 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.5 mM $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$, $\text{K}_3\text{Fe}(\text{CN})_6$, 0.1% Triton X-100 and 0.5 mM X-Gluc) follow by a vacuum treatment for 30 min. The root samples were then incubated at 37°C overnight. The staining was terminated by immersing the root samples in 50% ethanol. Then the plants carrying *proOsRAM2:GUS* were immersed into 1xphosphate buffered saline solution containing 0.2 μ g/ml WGA-Alexa Fluor 488 overnight for WGA staining. Images of roots stained with WGA-Alexa Fluor 488 (Yu et al. 2014) were taken with a Zesis Axio Scope A1 microscope.

Plasmid construction and rice transformation.

For gene editing in rice, target sequences were selected using the CRISPR website. For single-gene editing, the target sequences were synthesized and inserted into the subgenomic RNA through the *BsaI* site and then cloned into the Ph-Ubi-cas9-7 vector using the LR recombination reaction. For promoter analysis, 3-kb *OsRAM2* promoters were cloned into the modified plasmid pCambia1300-GUS using *SalI* and *BamHI*. For *OsRAM1* overexpression in rice, the full-length cDNA of *OsRAM1* without the stop codon was cloned into pDONR207, then inserted into pCambia1301 by LR recombination. The constructs were introduced into *Agrobacterium* strain EHA105 and transferred to Nipponbare as described previously (Zhang et al. 2015). Primers used for cloning are listed in Supplementary Table S1.

RNA extraction and gene expression.

For quantitative real-time PCR (qRT-PCR), each biological replicate comprised three individual roots. Total RNA was extracted from 100 mg of well-ground root tissue of each sample using Trizol reagent. Reverse transcription was carried out with 1 μ g of total RNA by using the PrimeScript RT Reagent Kit (Takara Bio) according to the manufacturer's instruction. qRT-PCR was performed using a Bio-Rad CFX Connect real-time detection system and the SYBR Green Premix Ex Taq (Takara Bio). For the gene expression analysis, the relative transcript levels of three technical repeats per sample were

normalized to the geometric mean of amplification of house-keeping genes *OsCyclophilin2* (LOC_Os02g02890) and $2^{-\Delta C_t}$ was used to analyze the data. The presented data are from three independent biological repeats. Sequences of real-time PCR primers are given in Supplementary Table S1.

Phylogenetic analyses.

MtRAM2 was used as query to identify its orthologs in *Medicago truncatula*, *Glycine max*, *O. sativa*, and *Arabidopsis thaliana* with BLASTP with standard parameters in phytozome V13 and the rice genome annotation project. The homologs with protein identity higher than 55% were selected. All of the amino acid sequences were aligned using MEGA-X. The phylogenetic tree was constructed using the maximum-likelihood method in MEGA-X with 1,000 bootstrap repetitions. The other parameters are default.

AUTHOR-RECOMMENDED INTERNET RESOURCES

CRISPR website: <http://crispr.hzau.edu.cn/CRISPR2/>
phytozome V13: <https://phytozome-next.jgi.doe.gov/pz/portal.html>
Rice genome annotation project: <http://rice.uga.edu/>
RiceXPro: <https://ricexpro.dna.affrc.go.jp/>

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