OsRAM2 Function in Lipid Biosynthesis Is Required for Arbuscular Mycorrhizal Symbiosis in Rice

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Accepted 31 May 2021.

Arbuscular mycorrhiza (AM) is a mutualistic symbiosis formed between most land plants and Glomeromycotina fungi. During symbiosis, plants provide organic carbon to fungi in exchange for mineral nutrients. Previous legume studies showed that the *required for arbuscular mycorrhization2* (*RAM2*) gene is necessary for transferring lipids from plants to AM fungi (AMF) and is also likely to play a “signaling” role at the root surface. To further explore *RAM2* functions in other plant lineages, in this study, two rice (*Oryza sativa*) genes, *OsRAM2* and *OsRAM2L*, were identified as orthologs of legume *RAM2*. Examining their expression patterns during symbiosis revealed that only *OsRAM2* was strongly upregulated upon AMF inoculation. CRISPR/Cas9 mutagenesis was then performed to obtain three *Osram2* mutant lines (-1, -2, and -3). After inoculation by AMF *Rhizophagus irregularis* or *Funneliformis mosseae*, all of the mutant lines showed extremely low colonization rates and the rarely observed arbuscules were all defective, thus supporting a conserved “nutritional” role of *RAM2* between monocot and dicot lineages. As for the signaling role, although the hyphopodia numbers formed by both AMF on *Osram2* mutants were indeed reduced, their morphology showed no abnormality, with fungal hyphae invading roots successfully. Promoter activities further indicated that *OsRAM2* was not expressed in epidermal cells below hyphopodia or outer cortical cells enclosing fungal hyphae but instead expressed exclusively in cortical cells containing arbuscules. Therefore, this suggested an indirect role of *RAM2* rather than a direct involvement in determining the symbiosis signals at the root surface.

Keywords: arbuscular mycorrhizal symbiosis, CRISPR/Cas9, *OsRAM2*, rice

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Y.-N. Liu and C.-C. Liu contributed equally to this work.

Funding: This research was financed by National Natural Science Foundation of China (grant number 31870203) to B. Wang.

*The e-Xtra logo stands for “electronic extra” and indicates there is supplementary material published online.

The author(s) declare no conflict of interest.

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Arbuscular mycorrhiza (AM) is a mutualistic endosymbiosis formed between most land plants and Glomeromycotina fungi (Smith and Read 2008; Spatafora et al. 2016; Wang and Qiu 2006). Its developmental process involves several phases. Before physical contact, plant roots secrete a variety of compounds, including flavonoids, 2-hydroxy fatty acids, and strigolactones, to trigger nearby AM fungi (AMF) for hyphae elongation and branching (Akiyama et al. 2005; Bécard et al. 1992; Nagahashi and Douds 2011). In response, AMF produce lipochitooligosaccharides and short-chain chitooligosaccharides to activate plant symbiotic signaling pathways in epidermal cells, inducing nuclear Ca^{2+} spiking and early symbiotic gene expression (Genre et al. 2013; Kosuta et al. 2003; Maillet et al. 2011; Sun et al. 2015). After AMF contact the root surface, hyphal tips become swollen and flattened, forming adhesion structures called hyphopodia (Bonfante and Genre 2010). Below hyphopodia, tube-like structures designated as prepenetration apparatuses are assembled and guide fungi to invade the roots (Genre et al. 2005, 2008, 2012). After reaching the inner cortex, fungi spread intercellularly along cortical cells and penetrate inside, developing highly branched, bush-like structures called arbuscules (Genre et al. 2008). Arbuscules are surrounded by a plant-derived plasma membrane called the peri-arbuscular membrane (PAM). Through the interface of PAM and arbuscules, nutrient exchanges occur between symbiotic partners, where plants provide organic carbon to fungi and fungi supply mineral nutrients, especially phosphorus and nitrate, to plants (Smith and Smith 2011; Wang et al. 2017; Wang et al. 2020; Wipf et al. 2019).

Previous studies demonstrated that, during AM symbiosis, plants transfer sugars to fungi (An et al. 2019; Helber et al. 2011; Pfeffer et al. 1999; Shachar-Hill et al. 1995). However, within AMF, carbon is mainly transported and stored as lipids (Bago et al. 2002; Trépanier et al. 2005). Recent genomic studies revealed that AMF lack type I fatty acid synthase and, thus, are unable to synthesize palmitic acid (C16:0) *de novo* (Tang et al. 2016; Wewer et al. 2014). Therefore, these endosymbiotic fungi likely acquire palmitic acid from their plant partners.

Notably, lipid biosynthesis enzymes were found enriched in arbusculated cells (Gaude et al. 2012), and many genes involved in lipid metabolism evolved conservatively within plant species that engage in AM symbiosis (Bravo et al. 2016). One such gene, *fat required for AM symbiosis* (*FatM*), was recently characterized for its role in AM symbiosis. Expressed exclusively in arbusculated cells, *FatM* encodes an acyl carrier protein (acyl-ACP) thioesterase, which preferentially releases palmitic acid in plastids. Free palmitic acid subsequently binds to coenzyme A (CoA) to form palmitoyl-CoA and is transported to the endoplasmic reticulum (ER) for lipid synthesis. In both legume species, *Medicago truncatula* and *Lotus japonicus*, mutated *FatM* led to significantly reduced AMF colonization and higher ratios

of collapsed arbuscules, suggesting that palmitic acid is critical for successful symbiosis (Brands et al. 2018; Bravo et al. 2017; Jiang et al. 2017). In the ER, a *sn*-2 glycerol-3-phosphate acyl transferase (GPAT) enzyme, which is encoded by the *required for arbuscular mycorrhization 2* (*RAM2*) gene, catalyzes palmitoyl-CoA and glycerol-3-phosphate (G3P) to form 2-monopalmitin (C16:0 2-monoacylglycerol [2-MAG]). Mutated *RAM2* in *M. truncatula* and *L. japonicus* results in more severe phenotypes than the *fatm* mutant, where AMF colonization rates dramatically decrease and the rarely observed arbuscules are all defective (Bravo et al. 2017; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017; Wang et al. 2012). Furthermore, two half-size ATP-binding cassette transporters of subfamily G (ABCG), which are encoded by the *stunted arbuscule* (*STR*) and *STR2* genes, also affect arbuscule development in *Medicago* (Zhang et al. 2010). ABCG transporters have been found to export lipids (Bird et al. 2007; Hwang et al. 2016; Pighin et al. 2004), thus, it is hypothesized that the *STR/STR2* heterodimer located on the PAM is involved in lipid transfer from plant cells to peri-arbuscular apoplastic spaces for AMF assimilation (Bravo et al. 2017; Jiang et al. 2017). Collectively, these findings indicate that lipids serve as a major carbon source in plants that support fungal growth, development, and reproduction, during which *RAM2* plays an essential “nutritional” role in transforming palmitoyl-CoA to 2-monopalmitin for late transportation (Bravo et al. 2017; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017).

Additionally, the *M. truncatula* *RAM2* (*MtRAM2*) gene was previously hypothesized to play a “signaling” role at the root surface (Murray et al. 2013; Wang et al. 2012). The close homologs of *RAM2* in *Arabidopsis thaliana* exhibited specific functions of providing precursors for extracellular lipids such as cutin and suberin (Yang et al. 2012). After inoculation by AMF *Rhizophagus irregularis*, significantly reduced hyphopodia numbers were observed on the *Medicago ram2* mutant. Such a phenotype was hypothesized to be caused by lack of a symbiosis “signal” at the root surface, which was supported by observing that the exogenous addition of cutin monomers (e.g., C16 ω -hydroxy fatty acids, 16-OHFA) could rescue the hyphopodia formation phenotype on the *Medicago ram2* roots (Wang et al. 2012). However, it was later reported that, when a different AMF (*Glomus versiforme*) was used, the hyphopodia numbers were not significantly reduced on the *Medicago ram2* mutants, and the reduced colonization levels were explained by a reduction in ability to spread within the root cortex instead of having difficulty in gaining entry to the roots (Bravo et al. 2017). Therefore, it remains uncertain whether the hyphopodia numbers do, indeed, reduce in *ram2* mutants and, if they do, whether this phenotype is controlled by host plants, AMF, or even both. To clarify this issue, we suggest investigating *ram2* mutants in other plant species.

In this study, we aimed to identify *RAM2* orthologs and close homologs in rice, to analyze their expression patterns during AM symbiosis, and, more importantly, to obtain *Osram2* mutants for AM phenotype examinations, including the hyphopodia numbers. The close homologs of *RAM2* in *A. thaliana* (*AtGPAT4*, *AtGPAT6*, and *AtGPAT8*) were all expressed in epidermis cells (Li et al. 2007; Li et al. 2012; Li-Beisson et al. 2009). Also, an *M. truncatula* gene, *ENOD11*, which encodes a putative cell-wall-repetitive, Pro-rich-secreted protein, was expressed specifically in the hyphopodia region at the early phase of symbiosis (Chabaud et al. 2002). Studying the *MtRAM2* promoter with a β -glucuronidase (*GUS*) reporter gene, however, revealed strong activities in arbusculated cells only (Gobbato et al. 2013; Luginbuehl et al. 2017). It remains uncertain whether *RAM2* exhibits transient expression in epidermal cells at the early phase of AM symbiosis, as *MtENOD11* exhibited. Thus, in this study, we also studied the promoter activities

of the *OsRAM2* gene during the early and late phases of AM symbiosis.

RESULTS

Os03g52570 and *Os10g27330* are orthologous to *MtRAM2*.

Using the *MtRAM2* protein sequence, BLASTp searches against the rice proteome identified 16 GPAT homologs (Supplementary Table S1), among which 2 top hits were encoded by *Os03g52570* and *Os10g27330*, and exhibited 86 and 81% overall similarity to *MtRAM2*, respectively, while others showed 50 to 72% similarity. To identify the real orthologs of *MtRAM2* in rice, a phylogenetic tree including 82 GPAT sequences from seven angiosperm species (see Materials and Methods) was reconstructed (Fig. 1; Supplementary Table S1). The tree comprises six highly supported monophyletic subclades with bootstrap values ranging from 65 to 100. To be consistent with a previous work (Washburner et al. 2018), these subclades were named subclades a, b, c, d, e1, and e2. Noticeably, both rice genes (*Os03g52570* and *Os10g27330*) were included in subclade c containing *MtRAM2*, indicating that these genes share a recent common ancestor with *MtRAM2* and are real orthologs (hereafter, we refer to *Os03g52570* as *OsRAM2* and *Os10g27330* as *OsRAM2L*). It seems that *OsRAM2* and *OsRAM2L* originated from a duplication event that occurred in the common ancestor of the Poaceae family. In another surveyed Poaceae species, *Brachypodium distachyon*, an additional duplication event resulted in a third gene in this subclade; however, rice had lost this third gene. In the surveyed Brassicaceae species *A. thaliana* and *Brassica rapa*, no representative gene was found for this subclade, indicating losses of the *RAM2* orthologs in these non-mycorrhizal species. Also, the surveyed species *Phaseolus vulgaris* possessed a single gene, while *Solanum lycopersicum* evolved three genes in this subclade. Subclades a and b formed a sister relationship and these two subclades together showed a sister relationship with subclade c (Fig. 1). Therefore, *Os02g02340* in subclade a and *Os01g63580* and *Os05g37600* in subclade b represent three close paralogs of *MtRAM2* in rice.

OsRAM2 was strongly induced during AM symbiosis.

The transcriptome data from rice roots at 4, 5, and 6 weeks postinoculation (wpi) by *R. irregularis* provided preliminary clues on the expression patterns of *OsRAM2*, *OsRAM2L*, and their close paralogs during AM symbiosis (Fig. 2A). Showing little expression in the mock-inoculated roots, *OsRAM2* was strongly induced upon inoculation by AMF *R. irregularis*. The transcript abundance of *OsRAM2* at 5 and 6 wpi was 10- and 16-times higher than at 4 wpi, respectively, indicating enhanced functioning of *OsRAM2* as mycorrhizal symbiosis develops into the late phase (Fig. 2A). *OsRAM2L*, however, showed a rather low expression level in rice roots and no induction pattern after AMF inoculation at any of the three examined time points. *Os02g02340* from subclade a showed a noninduction pattern similar to that of *OsRAM2L*. As for the genes in subclade b, *Os05g37600* had no detectable transcripts in rice roots, while *Os01g63580* exhibited increased expression at 4 wpi by AMF but not at 5 or 6 wpi (Fig. 2A).

To further verify the expression patterns of these genes, quantitative reverse-transcription (qRT)-PCR assays were performed (Fig. 2B). At 3 wpi, the relative expression levels of *OsRAM2* increased approximately 18-fold in AMF-inoculated roots compared with the control ($P = 0.06$). At 6 wpi, *OsRAM2* expression increased approximately 168-fold in mycorrhizal roots ($P < 0.02$). In contrast, upon inoculation by AMF, the relative expression levels of *OsRAM2L*, *Os02g02340*, and *Os01g63580* did not show statistically significant changes at either 3 or 6 wpi. Together, these results showed us that, within subclades a, b, and c, *OsRAM2* represents the only

GPAT member that is strongly induced upon AMF *R. irregularis* inoculation in rice roots.

Acquiring *Osram2* mutants via CRISPR/Cas9 mutagenesis.

To obtain null *Osram2* mutants in rice, two independent single-guide RNAs (sgRNAs) were designed, with one matching nucleotides 679 to 698 in exon 1 and the other matching nucleotides 1,574 to 1,593 in exon 2 (Fig. 3A). The sgRNAs were independently incorporated into Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 vector pRGEB31

and introduced into rice calli through *Agrobacterium*-mediated transformation (see Materials and Methods). Regenerated rice seedlings were screened for edited *Osram2* alleles. In total, six mutated alleles were detected in the T₀ generation (Supplementary Fig. S1A). Three of these mutated alleles (*Osram2*-1, -2, and -3) were selected for obtaining their homozygotes in subsequent generations, as well as to eliminate the pRGEB31 vector (Fig. 3B; Supplementary Fig. S1). By introducing a nucleotide insertion, the mutant alleles of *Osram2*-1 and -3 had altered reading frames after the insertions. Consequently, their encoded

Fig. 1. A phylogenetic tree consisting of 82 clade III glycerol-3-phosphate acyl transferase (GPAT) sequences from seven representative angiosperms. The tree was built using the maximum-likelihood method, implemented by Mega v7.0 with default parameters settings. In total, 500 bootstrap replicates were performed to obtain support values (%) on each internal branch. The seven plant species include *Oryza sativa* (*Orysa*), *Brachypodium distachyon* (*Bradi*), *Arabidopsis thaliana* (*Arath*), *Brassica rapa* (*Brara*), *Medicago truncatula* (*Medtr*), *Phaseolus vulgaris* (*Phavu*), and *Solanum lycopersicum* (*Solly*). Six monophyletic subclades were revealed, including subclade a (pink), subclade b (cyan), subclade c (green), subclade d (blue), subclade e1 (golden yellow), and subclade e2 (red). *MitRAM2* (blue dot) was found in subclade c along with two rice orthologous genes, *OsRAM2* (red dot) and *OsRAM2L* (red circle). The eight *Arabidopsis* clade III sequences (*AtGPAT1* to *AtGPAT8*) were distributed in five subclades (except subclade c) and also labeled.

Fig. 2. Expression patterns of *OsRAM2*, *OsRAM2L*, and their close paralogs during arbuscular mycorrhiza (AM) symbiosis. **A**, Transcript abundances of *OsRAM2*, *OsRAM2L*, *Os02g02340*, and *Os01g63580* were measured by RNA-Seq. Abundance values are expressed as fragments per kilobase of exon per million (FPKM) reads mapped. Rice wild-type roots were either mock inoculated (negative AM fungi [–AMF]) or colonized by *Rhizophagus irregularis* (+AMF) at 4, 5, and 6 weeks postinoculation (wpi). Different letters indicate significant differences between the groups ($P < 0.05$). **B**, Relative expression levels of these genes were measured by quantitative reverse-transcription PCR at 3 and 6 wpi by *R. irregularis*. Gene expression values were normalized to the rice housekeeping gene *ubiquitin 1* (*OsUBI1*). Statistical analysis was performed using the Student's *t* test with a $P < 0.05$ significance threshold.

Fig. 3. CRISPR/Cas9-mediated *OsRAM2* gene mutagenesis in rice. **A**, Schematic gene structure of *OsRAM2*, which contained two exons (pale red and cyan rectangles, respectively). The two black vertical rectangles represent the targeted sites of designed single-guide RNAs (sgRNAs) within the coding sequence. Target sequences are shown in red letters and protospacer adjacent motif (PAM) sites are shown in green letters. Positions and orientations of the *OsRAM2* edit-F and *OsRAM2* edit-R primers, which were used to detect the editing alleles of *OsRAM2*, are indicated by black arrows. **B**, Nucleotide sequence alignments of the sgRNA target regions showed altered alleles in three T_1 homozygous mutant lines. Acquired mutated alleles are shown below the wild-type (WT) sequences. Black dashes indicate deleted nucleotides and underlined letters indicate inserted nucleotides; – and + indicate deletions and insertions of several nucleotides (nt), respectively.

proteins shared no homology with the wild-type enzyme (467 amino acids [aa]) after amino acid sites 227 and 388, respectively (Supplementary Fig. S2). Because the conserved acyl-transferase domain is located at the C terminus, it can be expected that neither the *Osram2-1* nor *Osram2-3* alleles encode a functional enzyme. Moreover, the mutant allele *Osram2-2* had a proper reading frame but showed a deletion of 12 nucleotides from sites 1,573 to 1,584. Accordingly, 4 aa (PGYV at sites 387 to 390) were missing in the enzyme encoded by *Osram2-2* (Supplementary Fig. S2). The Pro on site 387 and Tyr on site 389 are conserved among different subclades of plant GPATs (Supplementary Fig. S3), and missing these sites could potentially disrupt the OsRAM2 function, which awaits further examination.

To see whether the knockout *OsRAM2* gene would affect the growth and development of rice plants, the three obtained *Osram2* mutant lines were grown together with wild-type rice. As far as we can tell, no abnormal developmental phenotypes

were observed for these mutant lines (Supplementary Fig. S4). This is consistent with the extremely low expression levels of *OsRAM2* gene in rice roots, stems, and leaves under normal growth condition (data not shown).

All *Osram2* mutants displayed severely impaired AM phenotypes after inoculation by *R. irregularis* or *Funneliformis mosseae*.

We then examined the mycorrhizal phenotypes of *Osram2* mutants. After inoculation by *R. irregularis* for 6 weeks, the lateral roots of wild-type rice were heavily infected, with approximately 87% root length colonized by intraradical fungal hyphae and approximately 59% root length forming arbusculated cells for nutrient exchange (Fig. 4A). In contrast, all three *Osram2* mutants showed severely impaired AM phenotypes. Only 7 to 11% of root length of these *Osram2* mutants was colonized by intraradical hyphae and less than 1% of roots formed arbuscules (Fig. 4A). Without fully occupying the spaces of inner cortical

Fig. 4. *OsRAM2* is required for arbuscular mycorrhiza fungi (AMF) colonization by *Rhizophagus irregularis*. **A**, Root length colonization (%) of wild-type (WT) rice and *Osram2-1*, *-2*, and *-3* mutants at 6 weeks postinoculation (wpi) was determined by the gridline intersect method. Different letters indicate statistical differences: analysis of variance, posthoc Tukey, $n = 20$; $F(\text{total})_{3,16} = 638.53$, $F(\text{hyphopodia})_{3,16} = 73.85$, $F(\text{intraradical hyphae [int. hyphae]})_{3,16} = 638.53$, $F(\text{arbuscules})_{3,16} = 489.09$, and $F(\text{vesicles})_{3,16} = 115.65$; $P < 0.001$. **B to E**, Images of the ink-vinegar staining of the hyphopodia in *Osram2* and WT rice roots; hp = hyphopodia. Scale bars = 30 μm . **F to I**, Images of the green fluorescent signals after WGA-Alexa Fluor 488 staining of the fungal cell walls. Laser-scanning confocal images showing arbuscule development in WT and *Osram2* rice roots. Mycorrhizal structures were indicated: a = arbuscules, ih = intraradical hyphae, da = defective arbuscules, and s = septa. Scale bars = 20 μm .

cells as in wild-type rice roots (Fig. 4F), the arbuscules of the three *Osram2* mutants all looked small and distorted, with septa often seen in collapsed fungal hyphae (Fig. 4G to I). Therefore, *OsRAM2* is required for developing normal arbuscules. Consistently, although approximately 18% of wild-type rice roots showed the lipid-storage vesicles, no vesicles could be found in the mutants' roots (Fig. 4A), supporting the idea that the nutrient supply from plants to fungi had been largely, if not completely, blocked. To also see whether *OsRAM2* could potentially play a signaling role at the root surface, the numbers of fungal entry structures (hyphopodia) were counted. Although they could be seen on approximately 7% of wild-type rice roots, rather few (<1%) were detected on the mutant roots ($P < 0.01$) (Fig. 4A). However, compared with those on wild-type rice roots, the hyphopodia formed on mutants showed no morphological difference (at least in our view), and the fungal hyphae below hyphopodia were capable of invading root cells successfully (Fig. 4C to E).

To explore whether a different AMF species may induce altered AM phenotypes (especially the hyphopodia numbers) on *Osram2* mutants, we further inoculated the rice *ram2* mutants with another AMF species, *Funneliformis mosseae*. Likely due to the weak inoculum strength of this species (see Materials and Methods), overall lower colonization rates by *F. mosseae* than by *R. irregularis* were obtained for the wild-type rice plants (Fig. 5). Despite this, similar defective AM phenotypes were observed on *Osram2* mutants (Fig. 5A). Again, the hyphopodia numbers formed on the mutants were significantly fewer than those observed on the wild-type rice (1 versus 10%) (Fig. 5A) but, still, the hyphopodia morphology appeared normal on the mutants (Fig. 5C to E). As with *R. irregularis*, *F. mosseae* also rarely formed arbuscules in mutants' roots (<1%), and they were all defective (Fig. 5G to I). Therefore, for both AMF species used, similar impaired AM phenotypes were observed on all three *Osram2* mutants.

Fig. 5. *OsRAM2* is required for arbuscular mycorrhiza fungi (AMF) colonization by *Funneliformis mosseae*. **A**, Root colonization levels of wild-type (WT) rice and *Osram2* mutants at 6 weeks postinoculation (wpi). Different letters indicate statistical differences: analysis of variance, posthoc Tukey, $n = 20$; $F(\text{total})_{3,16} = 6.30$, $F(\text{hyphopodia})_{3,16} = 5.11$, $F(\text{intraradical hyphae} [\text{int. hyphae}])_{3,16} = 6.30$, and $F(\text{arbuscules})_{3,16} = 4.23$; $P < 0.05$; n.s. = no significance. **B to I**, Images of the ink-vinegar staining of the fungal hyphae showing the normal hyphopodia morphology and defective arbuscules morphology in *Osram2* (C to E and G to I) mutants compared with the WT rice roots (B and F). Mycorrhizal structures were indicated: hp = hyphopodia, a = arbuscules, ih = intraradical hyphae, and da = defective arbuscules. Scale bars = 50 μm .

For several AM marker genes, their relative expression levels were measured by qRT-PCR. At 3 or 6 wpi by *R. irregularis*, the examined AM marker genes (*OsAM1*, *OsAM3*, and *OsPT11*) all showed significantly reduced expression levels in the three *Osram2* mutants (Fig. 6), indicating that symbiosis in these *Osram2* mutants was not established successfully. This is consistent with the observed defective mycorrhizal phenotypes (Fig. 4). For *OsRAM2* itself, the relative transcript abundance was also significantly reduced in the mutants (Fig. 6). To see whether such changes could potentially influence the expression of its close homologs, the transcriptional levels of *OsRAM2L*, *Os02g02340*, and *Os01g63580* were examined in *Osram2-1* and *-3* mutants. Results revealed that the knockout *OsRAM2* gene did not alter the transcript abundances of the three homologs in rice roots (Supplementary Fig. S5).

***OsRAM2* was not expressed in epidermis cells below hyphopodia but exclusively in arbusculated cells during the early and late phases of AM symbiosis.**

To determine the temporal and spatial expression patterns of *OsRAM2*, a 3.1-kb promoter region of *OsRAM2* was fused to a

GUS reporter gene. Stable transgenic rice lines were obtained to establish symbiosis. At 7 to 10 days postinoculation (dpi), expression of GUS proteins was not observed in any parts of the transgenic rice roots, including the epidermis layer (Fig. 7A and D). At 11 dpi, however, blue stains were observed in a few lateral roots and microscopic examination revealed that these blue stains were all located in root cortical cells (Fig. 7B and E). Counterstaining with ink-vinegar and WGA-Alexa Fluor 488 further revealed that the blue stains did not appear in epidermal cells below hyphopodia (Fig. 7F) or outer cortical cells enclosing intraradical hyphae (Fig. 7H and I; Supplementary Fig. S6C) but appeared predominantly in cortical cells containing mature arbuscules (Fig. 7H and I; Supplementary Fig. S6A to D). Noticeably, some cortical cells containing young or small arbuscules showed no blue stains (Fig. 7G to I; Supplementary Fig. S6B), suggesting that *OsRAM2* is not expressed until a late developmental stage of arbuscules. At 21 and 28 dpi, a significant portion of rice lateral roots had blue GUS stains (Fig. 7C; Supplementary Fig. S6E and F). Cross and longitudinal sections of these lateral roots confirmed the presence of blue stains in many cortical cells,

Fig. 6. Arbuscular mycorrhiza (AM) marker gene expression in *Osram2* mutants. Transcript accumulation of *OsPT11*, *OsAM1*, *OsAM3*, and *OsRAM2* were measured by quantitative reverse-transcription PCR in wild-type (WT) and *Osram2* mutants at **A**, 3 weeks postinoculation (wpi) and **B**, 6 wpi by *Rhizophagus irregularis*. Housekeeping gene *OsUBI1* was used for normalization. Different letters indicate levels of statistical significance (analysis of variance, posthoc Tukey); $n = 20$, $P < 0.001$, $F(OsPT11)_{3,16} = 22.33$; $P < 0.001$, $F(OsAM1)_{3,16} = 57.39$; $P < 0.01$, $F(OsAM3)_{3,16} = 5.44$; and $P < 0.05$, $F(OsRAM2)_{3,16} = 4.62$ (A) and $n = 20$, $P < 0.05$, $F(OsPT11)_{3,16} = 3.66$; $P < 0.001$, $F(OsAM1)_{3,16} = 14.08$; $P \leq 0.001$, $F(OsAM3)_{3,16} = 9.66$; and $P < 0.001$, $F(OsRAM2)_{3,16} = 14.23$ (B).

some of which were even close to the epidermis and exodermis layers (Fig. 7J and K).

DISCUSSION

AM symbiosis is an extremely ancient type of mutualism, which has been documented by the fossil records of AM-like associations in some primitive vascular plants, including *Aglaophyton major* and *Horneophyton lignieri* (>407 million year ago) (Remy et al. 1994; Strullu-Derrien et al. 2014; Taylor et al. 1995). Despite its ancient origin and a wide occurrence in extant land plants, our current understanding of the developmental process and underlying mechanisms of AM symbiosis has been largely achieved by studying a few model angiosperm organisms, especially legumes such as *M. truncatula* and *L. japonicus*. Thus far, >60 plant genes regulating AM symbiosis at various developmental phases have been reported (MacLean et al. 2017). For many of these genes, including *RAM2*, their roles in AM symbiosis have only been characterized in legumes, leaving their evolution and functions largely unexplored in other plant lineages. In this study, we aimed to identify *RAM2* orthologs in rice and further investigate their expression patterns and potential roles in AM symbiosis.

MtRAM2 encodes a GPAT enzyme that is required for AM symbiosis (Wang et al. 2012). This type of enzyme has a

specific function in lipid synthesis by transferring acyl group from acyl-CoA onto G3P. In land plants, three separate clades of *GPAT* genes have been reported, in which clades I and II both exhibit a conservative evolutionary pattern, while clade III have diverged into multiple sublineages (Washburger et al. 2018). In the *Arabidopsis* genome, for example, aside from *AtGPAT9* belonging to clade I and *Atlg32200* in clade II, nine *GPAT* genes (*AtGPAT1* to *AtGPAT8* and *At3g11325*) were assigned to clade III (Washburger et al. 2018). Studies on these *Arabidopsis* GPATs revealed that the GPATs of clades I and II both exhibit a *sn-1* acyl transfer regiospecificity onto G3P and are mainly responsible for synthesizing cell membranes and storage lipids, whereas the GPATs in clade III all exhibit *sn-2* acyl transfer regiospecificity and are mainly involved in synthesizing extracellular lipids such as cutin and suberin (Beisson et al. 2012; Yang et al. 2012).

To clarify the evolutionary relationships of *MtRAM2* with its close *GPAT* homologs, we reconstructed a robust *GPAT* clade III gene phylogeny covering 82 sequences from seven representative angiosperm species (Fig. 1; Supplementary Table S1). Six monophyletic subclades (a, b, c, d, e1, and e2) were identified (Fig. 1), revealing that at least six *GPAT* sublineages had evolved in the common ancestor of surveyed angiosperms. Noticeably, *MtRAM2* was found in subclade c along with two rice genes, three *Brachypodium distachyon* genes, one *P. vulgaris* gene, and three

Fig. 7. Promoter activity of *OsRAM2* in *Rhizophagus irregularis*-colonized rice roots. **A**, β -Glucuronidase (GUS) activity was examined from 7 to 10 days postinoculation (dpi) by *R. irregularis*. **B**, *OsRAM2* promoter activity was detected at 11 and 12 dpi by blue GUS staining. Black arrowheads indicate roots containing GUS activity. **C**, More rice roots displayed blue GUS stains at 21 and 28 dpi. **D**, *OsRAM2pro::GUS* roots lacked GUS staining, while **E**, blue stains appeared in root cortical cells at 11 dpi. **F to I**, Counterstaining with ink-vinegar or WGA-Alexa Fluor 488 revealed that blue stains appeared in cortical cells containing developed arbuscules at 11 dpi. Red arrowheads indicate fungal structures: hp = hyphopodia, ih = intraradical hyphae, ya = young arbuscules, and ma = mature arbuscules. **J** and **K**, Images of cross and longitudinal sections of rice lateral roots at 28 dpi. Scale bars = 1 cm (A to C) or 100 μ m (D to K).

S. lycopersicum genes (Fig. 1). No representative sequences from the two surveyed nonmycorrhizal species (*Arabidopsis thaliana* and *Brassica rapa*) were found in this subclade, indicating that a possible function of this sublineage is involved in AM symbiosis. Such a result is consistent with the findings of a previous phylogenomic study (Bravo et al. 2016). According to the resolved gene relationships within this subclade, *OsRAM2* and *OsRAM2L* represent the real orthologs of *MiRAM2* (Fig. 1).

Subclade c lies in an intermediate phylogenetic position between subclades d and a + b (Fig. 1). Previously, in *A. thaliana*, it was demonstrated that *AtGPAT4* and *AtGPAT8* (subclade a) were essential for C16 and C18 cutin monomer formation in the stems and leaves (Li et al. 2007), and *AtGPAT6* (subclade b) was required for incorporating C16 monomers into flower cutin (Li-Beisson et al. 2009). In contrast, *AtGPAT5* (subclade d) was involved in accumulating C22 and C24 suberin monomers in the roots and seed coats (Beisson et al. 2007), and *AtGPAT7* (subclade d) also took part in suberin synthesis in response to wounding (Yang et al. 2012). Like the *AtGPAT4*, *AtGPAT6*, and *AtGPAT8* enzymes, *Medicago* *RAM2* strongly prefers C16 and C18 ω -oxidized acyl-CoA (Wang et al. 2012; Yang et al. 2012). Moreover, unlike *AtGPAT5* (subclade d) and *AtGPAT1* (subclade e1), which possess only acyltransferase activity to generate *sn*-2 lysophosphatidic acids (LPA), *AtGPAT4*, *AtGPAT6*, and *AtGPAT8* (subclades a and b) all have a functional phosphatase domain that remove the phosphate group from LPA to generate 2-MAGs (Yang et al. 2010, 2012). *MiRAM2* in subclade c also possesses the phosphatase domain and mainly synthesizes C16:0 2-MAGs in arbusculated cells during AM symbiosis (Bravo et al. 2017; Jiang et al. 2017; Luginbuehl et al. 2017). When driven by the *MiRAM2* promoter, the *AtGPAT6* gene can fully complement the *ram2* mutant phenotype during AM symbiosis, whereas *AtGPAT5* exhibits only weak complementation (Wang et al. 2012). Therefore, in terms of enzyme function, the GPATs of subclades a, b, and c are analogous to each other.

We then examined the expression patterns of *OsRAM2* and *OsRAM2L* in subclade c, *Os02g02340* in subclade a, and *Os01g63580* and *Os05g37600* in subclade b during AM symbiosis. Four genes, except *Os05g37600*, were expressed in rice roots. Among these genes, only *OsRAM2* was strongly induced upon AMF *R. irregularis* inoculation under our experimental conditions and the transcript abundance level of *OsRAM2L* gene was only approximately 2% of the *OsRAM2* gene at 6 wpi by *R. irregularis* (Fig. 2). This induction pattern of *OsRAM2* is also consistent with the transcriptome data of two previous studies (Fiorilli et al. 2015; Roth et al. 2018).

We then focused on the *OsRAM2* gene and tried to obtain its knockout mutants using the CRISPR/Cas9 mutagenesis technique. Three homozygous mutant lines (*Osram2-1*, *-2*, and *-3*) were obtained (Fig. 3) and their AM phenotypes were further examined. After inoculation by *R. irregularis*, all *Osram2* mutants showed dramatically reduced fungal colonization (Fig. 4). Strikingly, the arbuscules were observed in very few (<1%) of the examined rice roots and they were all defective (Fig. 4G to I). Consistent with such low colonization rates, the expression levels of several AM marker genes (*OsAMI*, *OsAM3*, and *OsPT11*) were significantly reduced in the mutant lines compared with the wild type (Fig. 6). Among them, *OsAMI* and *OsAM3* represent early-expressed AM marker genes and *OsPT11* is a late-expressed gene during symbiosis (Gutjahr et al. 2008). Expression of *OsAMI* was detected in cortical cells containing small arbuscules and cells entrapping intercellularly growing hyphae, while *OsAM3* and *OsPT11* transcripts were activated in arbusculated cells only (Gutjahr et al. 2008). The low expression levels of the three marker genes in the *Osram2* mutants indicate that the knockout of *OsRAM2* would block the

development of AM symbiosis in rice roots. All of these results are consistent with several recent legume studies, which had proposed a nutritional role of *RAM2* in the arbusculated cells (Bravo et al. 2017; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017).

Previously, Wang et al. (2012) observed significantly fewer hyphopodia in the *Medicago ram2* mutant when inoculated by AMF *R. irregularis*. Because close homologs of *MiRAM2* in *Arabidopsis* (the *AtGPAT4*, *AtGPAT6*, and *AtGPAT8* genes) were all involved in synthesizing cutin monomers at the plant surface, it was hypothesized that *MiRAM2* likely plays a signaling role at the root surface and, therefore, affects hyphopodia formation (Wang et al. 2012). However, it was later reported that, when a different AMF species (*G. versiforme*) was used, the hyphopodia numbers were not significantly reduced in the *Medicago ram2* mutants (Bravo et al. 2017). To further clarify this issue, in this work, we used not only *R. irregularis* but also another AMF species, *F. mosseae*, to inoculate rice *Osram2* mutant plants. Pure spores of *R. irregularis* were extracted from transgenic hairy carrot roots, while the *F. mosseae* inoculum was a mixture of granular inoculum propagated with moso bamboo. For both AMF, a significant reduction of the hyphopodia numbers was observed between wild-type rice and *Osram2* mutants (Figs. 4A and 5A), thus supporting the results of earlier work (Wang et al. 2012). Therefore, why were the results of the other work (Bravo et al. 2017) inconsistent with these observations? A very strong AMF inoculum might be used, as indicated by up to 10 hyphopodia numbers per centimeter of colonized roots in the wild-type plants, or the observation was made at a rather early stage (Bravo et al. 2017).

OsRAM2 promoter activities showed no sign of expression in epidermal cells below hyphopodia or outer cortical cells enclosing intraradical hyphae (Fig. 7F, H, and I; Supplementary Fig. S6C) but were strongly expressed in cortical cells containing developed arbuscules (Fig. 7G; Supplementary Fig. S6A, B, and D). Such expression patterns would favor the nutritional rather than signaling hypothesis. The reduction of hyphopodia numbers on mutants could also be explained by the nutritional hypothesis, because a defective nutritional supply in arbusculated cells would limit the spread of both intra- and extraradical fungal hyphae on *Osram2* mutants and, consequently, fewer hyphopodia would be formed. Such an explanation was certainly not in mind when the signaling hypothesis was first proposed. For the pathogenic oomycete *Phytophthora palmivora*, the evidence supporting a signaling role of *RAM2* gene was also reported (Wang et al. 2012). In an accompanying work, Dai and colleagues (H. L. Dai, X. W. Zhang, B. Y. Zhao, N. Yu, and E. T. Wang, unpublished) further showed that, in rice, *OsRAM2* function in lipid synthesis is required for successful colonization by rice blast fungi in roots.

Even without observing the expression of *OsRAM2* in the epidermis, we cannot preclude a possibility that *RAM2* may indirectly affect fatty acid compositions at the root surface. Because some arbusculated cells are very close to the root epidermis and exodermis layers (Fig. 7J and K), accumulated C16:0 2-MAGs in arbusculated cells may be passively diffused or actively transferred from cortical cells toward the root surface, which consequently affects hyphopodia formation. A rice gene encoding a lipid transfer protein (LTP) was previously revealed to be induced at the penetration stage of AMF and specifically expressed in the epidermal root regions where the hyphopodia were formed (Blilou et al. 2000). Moreover, a new study focusing on a liverwort (*Marchantia paleacea*) just revealed that a *RAM2* gene homolog was mainly expressed at the outmost cell layer of thalli upon AMF inoculation (Rich et al. 2021), indicating a shift of expressional position of *RAM2* gene to cortical cells in angiosperms species, including rice and *Medicago*. An

interesting observation was also made in the fossil records of two primitive vascular plants, *A. major* and *H. lignieri*, which both showed abundant AM fungal structures in their aerial stems (Remy et al. 1994; Strullu-Derrien et al. 2014; Taylor et al. 1995), suggesting that, in the early history of land plant evolution, AMF was likely attracted by cutin monomers on the stem surface. It was also revealed that vanillic acid (4-hydroxy-3-methoxy benzoic acid, $C_8H_8O_4$) found in the cell wall extracts of *Daucus carota* roots (an AM host) can efficiently stimulate AMF hyphal growth and branching, while ferulic acid (4-hydroxy-3-methoxy cinnamyl acid, $C_{10}H_{10}O_4$), which is found mainly in *Beta vulgaris* roots (a non-AM host), inhibited AMF growth (Douds et al. 1996). AMF probably relies on recognizing certain cell wall components present in the host root surface to form hyphopodia; however, the exact component and which genes (the exact function of *OsLTP* remains elusive) directly affect its composition at the root surface remain unknown. Therefore, the signaling hypothesis is still intriguing but requires further in-depth investigation.

In summary, in this study, we identified the true orthologs of *MtRAM2* in rice and exploited reverse genetic tools to demonstrate that *OsRAM2* is indispensable for rice mycorrhizal symbiosis. Our findings on the temporal and spatial expression patterns of *OsRAM2* provided further understanding of the early-raised signaling hypothesis of *RAM2*.

MATERIALS AND METHODS

Phylogenetic reconstruction using *RAM2* homologous sequences.

A previous work reported that land plants possess three clades of *GPAT* genes (Waschburger et al. 2018). *MtRAM2* as well as its close homologs in *Arabidopsis* belong to the expanded land-plant-specific clade III (Wang et al. 2012; Yang et al. 2012). To identify *RAM2* orthologs in rice and clarify their relationships to other close *GPAT* homologs, we used the *MtRAM2* protein sequence to perform BLASTp searches against the proteomes of seven representative angiosperm species, including *Oryza sativa*, *Brachypodium distachyon*, *A. thaliana*, *Brassica rapa*, *Medicago truncatula*, *Phaseolus vulgaris*, and *S. lycopersicum* in the Phytosome database v12.1. All identified *GPAT* homologous sequences were aligned by ClustalW and used to reconstruct a phylogenetic tree by the maximum-likelihood method, implemented by MEGA v7.0 (Supplementary Table S1) (Kumar et al. 2016). The robustness of the internal branches was tested by performing a total of 500 bootstrap replicates. The obtained tree was examined to elucidate the relationships of different subclades and the subclade containing *MtRAM2* was identified.

Plant growth and the establishment of AM symbiosis.

Rice (*O. sativa* subsp. *japonica* ‘Nipponbare’) seed were sterilized with 2.5% sodium hypochlorite and 0.1% Tween20 for 15 min and 70% alcohol for 2 min, rinsed three times with distilled water, and germinated on modified Murashige-Skoog (MS) medium (MS basal salts at 2.165 g/liter, sucrose at 20 g/liter, Fe_2 -EDTA at 5 mg/liter, vitamins at 10 mg/liter, and Phytigel at 3 g/liter; pH 6.1 to 6.3). For AM colonization, seedlings were transplanted in pots (diameter = 12 cm) containing an autoclaved sand/perlite (3:1) mixture with three seedlings per pot. AM fungus *R. irregularis* spores were freshly extracted from transgenic hairy carrot root cultures and approximately 200 spores were added to the rhizosphere of each seedling. *F. mosseae* (formerly *G. mosseae*; BGC HK01), obtained from the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences (Beijing, China) and propagated with moso bamboo (*Phyllostachys pubescens*) as a host plant, were used as another mycorrhizal fungus. The granular

inoculum was a mixture of mycorrhizal roots, hyphae, and spores. Inoculum (6 g) containing approximately 100 spores was added uniformly as a layer in each pot at a depth of 20 mm from the soil surface upon planting. Plants were placed in a growth room under a photoperiod of 16 h of light and 8 h of darkness at 28 and 22°C, respectively, and were fertilized twice a week with half-strength Hoagland solution containing 20 μ M phosphates. Plant roots were collected at 3, 4, 5, and 6 wpi, where half of the samples were used for staining and mycorrhizal phenotype examination and the other half were frozen in liquid nitrogen and kept at -80°C for RNA extraction.

RNA extraction, sequencing, and qRT-PCR.

The total RNA of rice roots was extracted by the Trizol method (Invitrogen, Carlsbad, CA, U.S.A.). RNA purity and integrity were examined by RNA electrophoresis in 2.5% (wt/vol) agarose gel. Transcriptome sequencing was conducted on a BGISEQ-500 sequencing platform (BGI, Shenzhen, China) and the data were analyzed by the Hua-Da Gene Technology Co. (Shenzhen, China).

RNA (1 μ g) was treated with DNase I prior to cDNA synthesis using a Revert-Aid First-Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, U.S.A.). qRT-PCR was performed by measuring the intensity of SYBR Green fluorescent dye conjugated to double-stranded DNA molecules using a C1000 Thermal Cycler Real-Time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.). Gene expression values were normalized to the rice housekeeping gene, *ubiquitin 1* (*OsUBI1*) (Chen et al. 2008), and analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Statistical differences were tested via Student's *t* test or analysis of variance followed by Tukey's multiple comparison test ($P < 0.05$). All qRT-PCR primers used in this study are provided in Supplementary Table S2.

Rice transformation.

Callus from the transformed rice cultivar Nipponbare was generated by placing surface-sterilized mature seed on induction medium (N6 basal salts at 3.98 g/liter, sucrose at 30 g/liter, 2,4-dichlorophenoxyacetic acid [2,4-D] at 2.5 mg/liter, vitamins at 10 mg/liter, N-Z-Amine at 0.6 g/liter, L-Proline at 0.3 g/liter, and Phytigel at 3 g/liter; pH 6.2 to 6.4). Plates were sealed with parafilm and cultured in the dark at 26°C for 5 weeks. Before transformation, calli were placed on preculture medium (N6 basal salts at 1.99 g/liter, sucrose at 20 g/liter, Fe_2 -EDTA at 5 mg/liter, 2,4-D at 3 mg/liter, vitamins at 10 mg/liter, N-Z-Amine at 0.6 g/liter, agarose at 7 g/liter, glucose at 10 g/liter, and acetosyringone at 19.6 mg/liter; pH 5.9 to 6.1) for 3 days. Meanwhile, *Agrobacterium* strain EHA105 containing transformed vectors was cultured at 28°C for 2 days on yeast extract broth medium. Bacteria were scraped from the plate and added to approximately 50 ml of suspension liquid medium (N6 basal salts at 1.99 g/liter, sucrose at 20 g/liter, 2,4-D at 2 mg/liter, vitamins at 10 mg/liter, N-Z-Amine at 0.8 g/liter, glucose at 10 g/liter, and acetosyringone at 19.6 mg/liter; pH 5.7 to 5.9) and the optical density at 600 nm was adjusted to 1.0. Precultured calli were transferred to a conical flask containing the suspension liquid medium and shaken gently at 100 rpm for 30 min. Next, the calli were transferred to coculture medium (N6 basal salts at 1.99 g/liter, sucrose at 20 g/liter, Fe_2 -EDTA at 5 mg/liter, 2,4-D at 3 mg/liter, vitamins at 10 mg/liter, N-Z-Amine at 0.8 g/liter, agarose at 7 g/liter, glucose at 10 g/liter, and acetosyringone at 19.6 mg/liter; pH 5.9 to 6.1) for 3 days at 19°C in the dark.

After coculturing, calli were washed with sterile water five times, soaked in a solution containing carbenicillin at 0.5 g/liter, and shaken for 30 min at 200 rpm. Then, calli were placed on selection medium (N6 basal salts at 3.98 g/liter, sucrose at

30 g/liter, 2,4-D at 2.5 mg/liter, vitamins at 10 mg/liter, N-Z-Amine at 0.6 g/liter, agarose at 7 g/liter, carbenicillin at 0.5 g/liter, and hygromycin or other appropriate antibiotics at 50 mg/liter; pH 6.3 to 6.5) and cultured at 26°C in the dark. Every 2 to 3 weeks, vigorous calli were transferred to fresh selection medium for growth. After 5 to 6 weeks, resistant calli that were pale yellow in color were transferred to conical flasks containing regeneration medium (MS basal salts at 4.33 g/liter, sucrose at 30 g/liter, vitamins at 10 mg/liter, 6-benzylaminopurine at 2 mg/liter, kinetin at 2 mg/liter, naphthalene acetic acid at 0.2 mg/liter, indole 3-acetic acid at 0.2 mg/liter, N-Z-Amine at 1 g/liter, and Phytigel at 3 g/liter; pH 6.3 to 6.5) and cultured under a photoperiod of 16 h of light and 8 h of darkness at 28 and 22°C, respectively. After a month, regenerated seedlings were transferred to rooting medium (MS basal salts at 2.165 g/liter, sucrose at 20 g/liter, Fe₂-EDTA at 5 mg/liter, vitamins at 10 mg/liter, and Phytigel at 3 g/liter; pH 6.1 to 6.3) for root growth. After 2 weeks, rice seedlings were transplanted in soil for growth. To identify positively transformed seedlings, genomic DNA was extracted from the leaves using a modified cetyltrimethylammonium bromide method (Murray and Thompson 1980), which was subjected to PCR using specific primers of transformed vectors (Supplementary Table S2).

CRISPR vector construction and obtaining *Osram2* mutant lines.

To generate *Osram* mutants, the CRISPR-Cas9 technique was employed. sgRNA targeting *OsRAM2* was designed using the CRISPR-PLANT and CRISPRdirect online tools (Naito et al. 2015; Xie et al. 2014). The two best-scored sgRNAs (sgRNA1 and sgRNA2) showing the least off-target possibilities were selected and their spacer sequences upstream of the protospacer adjacent motif sites targeted exons 1 and 2 of *OsRAM2*. For each sgRNA, a pair of complementary primers with sticky ends was synthesized, annealed, and ligated with a linear CRISPR vector, pRGEB31, which was predigested by the restriction enzyme *BsaI* (Supplementary Table S2). After transforming rice by *Agrobacterium* containing the pRGEB31-sgRNA1 and pRGEB31-sgRNA2 vectors, the editing alleles of *OsRAM2* were examined through PCR and sequencing with a pair of primers (*OsRAM2* edit-F/R) which cover both editing regions (Fig. 3A; Supplementary Table S2). In subsequent generations, plants carrying desired homozygous mutations without the pRGEB31 construct, which can be detected by *pRGEB31-Cas9-F/R*, were selected for reproduction (Supplementary Table S2). Finally, the T₂ or T₃ generations of the three mutant lines (*Osram2-1*, *Osram2-2*, and *Osram2-3*) were used for AM colonization assays and gene expression studies.

Fungal structure staining and quantification.

For wheat germ agglutinin (WGA) staining (Schaffer and Peterson 1993), fresh rice roots were fixed in 50% ethanol for >4 h and cleared in 20% KOH (wt/vol) for 2 to 3 days at room temperature. Next, the roots were rinsed five times with phosphate-buffered saline (PBS) solution (pH 7.4) and acidified in 0.1 M HCl for 20 min. After incubating overnight in PBS solution containing WGA-Alexa Fluor 488 (Invitrogen, Eugene, OR, U.S.A.) at 1 µg/ml in the dark and washing with PBS, the WGA-stained roots were examined under a confocal Olympus FV10-ASM microscope (Olympus, Tokyo, Japan). For ink-vinegar staining (Vierheilig et al. 1998), fresh rice roots were cleared with 20% KOH for 40 to 120 min at 65°C, acidified by 5% acetic acid for 5 min at room temperature, incubated in ink-vinegar solution for 30 min at 65°C, and decolored in tap water for 14 h. The stained roots were examined under a Nikon E100 microscope (Nikon, Tokyo, Japan). To quantify the fungal colonization rate, a modified gridline intersect method was used to

observe fungal structures (McGonigle et al. 1990), including hyphopodia, intraradical hyphae, arbuscules, and vesicles scored.

Constructing the *OsRAM2pro:GUS* vector and GUS staining.

A 3.1-kb fragment upstream of the *OsRAM2* start codon was amplified by a pair of primers carrying the *SalI* and *BamHI* restriction sites (Supplementary Table S2), then cloned into an intermediate vector, pEASY-Blunt Zero, by TA cloning (Transgene, Beijing, China). The fragment was further digested from the intermediate vector using the *SalI* and *BamHI* enzymes and ligated with a predigested linear binary vector, pBI101.1. Subsequently, the constructed pBI101.1 vector containing the *OsRAM2pro:GUS* reporter unit was introduced to the *Agrobacterium* strain EHA105 and used to transform rice callus. Positively transformed seedlings were identified by PCR using the primers *PBI101.1-F* and *OsRAM2pro-R* (Supplementary Table S2), which were selected for seed propagation. In the T₁ generation, positively transformed offspring were used to examine the expression activities of the *OsRAM2* promoter in rice roots during the early and late stages of AM symbiosis.

GUS staining was performed as previously described (Jefferson et al. 1987). After immergence in a GUS staining solution (100 mM sodium phosphate buffer [pH 7.0], 10 mM Na₂EDTA, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 0.5% [wt/vol] Triton X-100, 20% [wt/vol] methanol, and 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide cyclohexylammonium salt at 0.2 mg/ml), rice plants were vacuum filtrated three times for 5 min each, incubated overnight at 37°C, and placed in 50% ethanol to terminate the reaction. Subsequently, rice roots were fixed by 5% formaldehyde, 5% ethanoic acid, and 50% ethanol; dehydrated with ethanol; and infiltrated and embedded by Paraplast (Sigma-Aldrich, St. Louis, MO, U.S.A.). For the observations, roots were cut into 10-µm-thick sections using a Leica RM2235 microtome (Leica, Wetzlar, Germany).

ACKNOWLEDGMENTS

We thank S. Yang (Nanjing University) for providing the pRGEB31 vector and W. Lan (Nanjing University) for the pBI101.1 vector.

AUTHOR-RECOMMENDED INTERNET RESOURCES

CRISPRdirect: <http://crispr.dbcls.jp>
CRISPR-PLANT: <https://www.genome.arizona.edu/crispr>
Phytozome: <https://phytozome.jgi.doe.gov>

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