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A Rice GRAS Gene Has an Impact on the Success of Arbuscular Mycorrhizal Colonization

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Abstract

Arbuscular mycorrhiza (AM) is one of the most spread symbiosis established between 80% of land plants and soil fungi belonging to the Glomeromycota. Molecular determinants involved in the formation of arbuscular mycorrhizas are still poorly understood. It has been demonstrated that in both Legumes and rice plants, several GRAS transcription factors are directly involved in both mycorrhizal signaling and colonization, namely NSP1, NSP2, RAM1, DELLA, DELLA-interacting protein (DIP1) and RAD1. Here, we focused on a rice GRAS protein, named *Arbuscular Mycorrhizal* 18 (*OsAM18*), previously identified as specifically expressed in rice mycorrhizal roots, and considered as an AM-specific gene. Phylogenetic analysis revealed that OsAM18 had a peculiar amino acid sequence, which clustered with putative SCARECROW proteins, even though it formed a separate branch. Allelic *osma18* mutant displayed a drastic reduction in mycorrhizal colonization intensity and in arbuscule abundance, as mirrored by *OsPT11* expression level. Non-mycorrhizal *osam18* plants displayed a comparable plant development and root apparatus compared with the WT, while mycorrhizal *osam18* mutants showed a reduction of plant biomass compared with mycorrhizal WT plants. The results suggest that OsAM18 is a rice protein, which is likely to have an impact not only on the colonization process and AM functionality, but also on the systemic effects of the AM symbiosis.

Keywords

Rice, Arbuscular Mycorrhizal Symbiosis, GRAS Proteins, Transcription Factors

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1. Introduction

The arbuscular mycorrhizal (AM) symbiosis is established between fungi of the Glomeromycota phylum and more than 80% of land plant species, being therefore the most widespread terrestrial symbiosis [1]. The most characteristic feature of the symbiosis is a highly branched fungal structure called arbuscule, which is located inside root cortical cells [2]: here nutrient exchanges between the two partners occur. The AM fungus provides the plant with mineral nutrients, such as phosphate, nitrogen and sulphur [3]-[5], while, in return, it receives carbon compounds essential for the completion of its life cycle [6]. The massive reorganization described in AM colonized plants requires a unique transcriptional program [7] which induces changes associated with metabolic pathways controlling nutritional exchanges, cell wall modifications, secondary metabolism, signal transduction, and protein turn-over [8]-[14]. On the basis of a RNAseq analysis, Xue and colleagues showed that 45 genes encoding transcriptional factors (TFs) or transcriptional regulators were significantly up-regulated in *Lotus japonicus* mycorrhizal roots when compared with non-mycorrhizal roots [15]. These genes belong to the main TFs families and include GRAS, AP2/ERF, NO APICAL MERISTEM, ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR, CUP-SHAPED COTYLEDON (NAC)-domain, MADS, MYB, C2H2 zinc finger, bZIP, ARF, WRKY DNA binding domain, NIN-like protein and LOB domain. These results indicate how mycorrhizal symbiosis also leads to deep change in transcription regulation. Some of these TFs have already been reported to be induced during the AM symbiosis [10] [16] [17] and in some cases have also been characterized [18]-[20].

GRAS proteins belong to a huge family which has been divided in several subfamilies. The name derives from three family members, GAI (GIBBERELLIC ACID INSENSITIVE), RGA (REPRESSOR of GAI) and SCR (SCARECROW) [21]. Plant GRAS TFs are involved in diverse biological processes, such as root and shoot development, regulation of phytohormone signalling pathway, abiotic stress [22]-[25] and nodule development [26], and they have been reported to play a role also in arbuscular association [18]. GRAS proteins are well characterized in legumes, where NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2 are considered essential for nodule development [26] [27] and involved in AM colonization process [28] [29], where they cause a decrease of root colonization. Other GRAS TFs, such as *Required for Arbuscular Mycorrhization* 1 (RAM1), *Required for arbuscule development* (RAD1) and DELLA protein, were recently identified in *L. japonicus* and *Medicago truncatula*. These genes have a functional role in arbuscule development [15] [18] [30]. The homologues of NSP1 and NSP2 have also been studied in rice, where at least 60 GRAS genes have been identified [31]. In detail, OsNSP1 and OsNSP2 are capable to fully restore the phenotype of the corresponding *L. japonicus nsp*1 and *nsp*2 mutants [21], suggesting a conservative role of these genes in both Legumes and monocots. Moreover, both genes have been identified as indispensable for strigolactone biosynthesis [32]. Rice lines mutated in SLR1/DELLA were shown to be unable to form AM symbiosis [33]. Other GRAS components have been identified as crucial for AM establishment in rice: among them, DELLA Interacting Protein 1 (DIP1), which interacts both with DELLA and OsRAM1 [33]. All these GRAS-domain proteins form a complex that has been proposed to be directly associated with mycorrhizal gene expression [33].

Here we focused our study on the biological function of a rice GRAS protein, named *Arbuscular Mycorrhizal* 18 (*OsAM18*), previously identified as exclusively induced in mycorrhizal roots during transcriptomic analysis [34] [35]. To assess whether this protein played a role during AM association, we took advantage of the availability of an *osam18* mutant rice line. A drastic reduction in mycorrhizal colonization intensity and in arbuscule abundance was observed in the root system of the *osam18* mutant. Despite this alteration, the arbuscule morphology displayed a normal phenotype. In addition, the mycorrhizal *osam18* mutants showed a decrease of fresh weight both in shoots and roots compared with mycorrhizal WT plants, whereas such a reduction of plant development was not observed in non-mycorrhizal mutants and WT plants. This result suggests that *OsAM18* is likely to have an impact not only on the colonization process, but also on the systemic effects of the AM symbiosis.

2. Materials and Methods

2.1. Biological Material and Growth Conditions

Rice (cv Nipponbare) line carrying Tos17 insertion into *osam18* coding sequence (line NC5532) was selected from public databases (http://signal.salk.edu/cgi-bin/RiceGE) and provided by the Rice Genome Resource Center of the National Institute of Agrobiological Sciences (RGRC-NIAS), Japan. To isolate homozygous mutants,

DNA extraction [36] and two rounds of PCR were carried out. Wild-type and mutated loci were distinguished by the use of specific primer pairs: combining an insertion-specific primer (Tos17: ATTGTTAGGTTGCAAGTTAGTTAAGA) with a gene-specific primer allowed to identify the mutant allele (OsAM18f: ATTCCCTCAGCAACAACCAC), while two gene-specific primers spanning the insertion amplified the wild-type allele (OsAM18f: ATTCCCTCAGCAACAACCAC; OsAM18r: TATGGTCGGAAGCCTGACTC). The primers were provided from public databases (http://signal.salk.edu/cgi-bin/RiceGE). Only seeds of the identified homozygous plants were used for the subsequent experiments.

Seedlings of *Oryza sativa* ssp. japonica cv. Nipponbare wild-type and of *osam18* mutant line were inoculated with *Funelliformis mosseae* Gerd. & Trappe BEG12 (MycAgro Lab, France, www.mycagrolab.com) by mixing the inoculum with sterile quartz sand (30% v/v) [11]. Plants were grown in a growth chamber under a 14 h light (24˚C)/10 h dark (20˚C) regime, and watered once a week with tap water, and once a week with a modified Long-Ashton solution containing a low phosphorus concentration (3.2 µM $Na_2HPO_4 \cdot 12H_2O$) [37]. Plants were grown in 15-cm-high and 3-cm-diameter tube-pots and maintained in a growth chamber, as described above, until harvesting [28 days post-inoculation (dpi)].

Fresh weight of root and shoot tissues of the control (c) and mycorrhizal (myc), mutant and wild-type, rice plants was evaluated. Each tissue was isolated and the fresh weight was measured by means of analytical balance.

2.2. *In Silico* Analyses

OsAM18 nucleotide sequence was obtained from the Rice genome Annotation Project (http://rice.plantbiology.msu.edu/), corresponding to the annotated "GRAS family transcription factor containing protein, expressed" (LOC_Os03g40080). The position of exons and introns was predicted using a program available at http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/spideyweb.cgi.

The GRAS domain was identified by ScanProsite and NCBI Blastp tools [38]. Leucine-rich nuclear export signals (NESs) were predicted using NetNES server [39]. pSORT server was used to predict the protein localization.

All the rice sequences containing a GRAS domain were retrieved from the Rice Genome Annotation Project website (http://rice.plantbiology.msu.edu/index.shtml) [40]. *Medicago truncatula* and *Lotus japonicus* sequences containing a GRAS domain and belonging to the SCARECROW family were obtained from their respective databases.

On the basis of the HMM (Hidden Markov Model) logo of the GRAS family (PF03514) available at Pfam website (http://pfam.xfam.org/family/PF03514#tabview=tab4), a custom Perl script was developed to find and isolate the most conserved part of the GRAS domain from each of the selected proteins. Only domains matching the following signature pattern H-[I,V]-[I,V]-D(84,260)-W(44,98)-W-x(10)-W were used for the alignment and the subsequent phylogenetic analysis. Alignment was done by ClustalX v. 2.1 [41] and phylogenetic trees were generated by Neighbor-joining algorithm in R using "ape" v. 3.2 [42], "seqinr" v. 3.1-3 [43] and "ade4" v. 1.7-2 [44] packages.

2.3. Morphometric Analysis

In order to describe the root system architecture, the length of Crown Roots (CRs) and the number of Large Lateral Roots (LLRs) were annotated manually for each plant. The branching index (BI = n LLR/cm CR) was calculated as described by Vallino and colleagues (2014) [45].

Portions of mycorrhizal roots were stained with cotton blue and the level of mycorrhizal formation was assessed according to Trouvelot and colleagues (1986) [46]. Over 80 root cm for six biological replicates were considered for each condition. Statistical tests were carried out through one-way analysis of variance (One-way Anova) and Tukey's *post hoc* test, using a probability level of $P < 0.05$.

To assess the arbuscule phenotype, WT and *osam18* mutant roots were treated for 1 h in phosphate buffer, pH 7, containing 3% (w/v) paraformaldehyde. After washing in phosphate buffer, roots were embedded in 8% low melting agarose and sectioned with a series 1000 Microtome Sectioning System (Vibratome, St. Louis, MO, USA). Two hundred µm thick-vibratome sections were treated for 5 min in 0.5% commercial bleach, diluted in phosphate buffer, washed again, and then incubated for 2 h with wheat germ agglutinin-fluorescein isothiocyanate (WGA-TRITC) (Sigma-Aldrich, Milan, Italy), at a final concentration of 10 µg/mL, to detect fungal cell

walls. Working conditions for the Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) and for observations and image acquisition were used as described by Volpe and colleagues (2013) [20].

To examine the presence of aerenchyma, 25 different portions of the sampled LLRs were embedded and sectioned as described above. Cross sections, 100 µm thick, were observed under a light microscope [Primo Star Zeiss (Carl Zeiss MicroImaging, Göttingen, Germany) with a Leica DFC425 digital camera (Leica Microsystems, Wetzlar, Germany) attached].

2.4. Nucleic Acid Extraction and cDNA Synthesis

Genomic DNA was extracted from *F. mosseae* sporocarps. Approximately 50 sporocarps were added to 50 µl of 10X Red Taq (Sigma) buffer and crushed with a sterile pestle. The sample was heated at 95˚C for 15 min and centrifuged at 12,000 g for 5 min. The supernatant was transferred to a new tube and stored at −20˚C. Fungal genomic DNA was used to test each primer pair used for real-time PCR to exclude cross hybridization.

Total RNA was extracted from rice roots of mycorrhizal and non-mycorrhizal plant grown via the sandwich method using the Plant RNeasy Kit (Qiagen), according to the manufacturer's instructions. Samples were treated with TURBO™ DNase (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The RNA samples were routinely checked for DNA contamination by means of RT-PCR (OneStepRT-PCR, Qiagen) analysis, using *OsRubQ1* [34].

For single-strand cDNA synthesis, about 700 ng of total RNA was denatured at 65˚C for 5 min and then reverse-transcribed at 25˚C for 10 min, 42˚C for 50 min and 70˚C for 15 min. The final volume was 20 µl and contained 10 µM random primers, 0.5 mM dNTPs, 4 µl 5× buffer, 2 µl 0.1 M DTT, and 1 µl Super-Script II (Invitrogen, Carlsbad, CA, USA).

2.5. Real-Time Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed using an iCycler apparatus (Bio-Rad, Hercules, CA, USA). Each PCR reaction was carried out in a total volume of 20 µl containing 1 µl diluted cDNA (about 20 ng), 10 µl 2X SYBR Green Reaction Mix and 3 µl of each primer (3 µM).The following PCR program was used: 95˚C for 90 s, 50 cycles of 95˚C for 15 s, 60˚C for 30 s. A melting curve (80 steps with a heating rate of 0.5˚C per 10 s and a continuous fluorescence measurement) was recorded at the end of each run to exclude the generation of non-specific PCR products [47]. All reactions were performed on at least three biological and three technical replicates.

Baseline range and Ct values were automatically calculated using iCycler software. Transcript levels were normalized to the Ct value of *OsRubQ1* (OsRubQ1f: GGGTTCACAAGTCTGCCTATTTG; OsRubQ1r: ACGGGACACGACCAAGGA) [34] (OsPT11f: GAGAAGTTCCCTGCTTCAAGCA; OsPT11r: GAGAAGTTCCCTGCTTCAAGCA) [34]. Only Ct values leading to a Ct mean with a standard deviation below 0.5 were considered [48]. Statistical tests were carried out through one-way Anova and Tukey's *post hoc* test, using a probability level of $P < 0.05$. All statistical elaborations were performed using PAST statistical package (version 2.16) [49].

3. Results and Discussion

GRAS proteins belong to a family of transcriptional regulators unique to plants. They play important regulatory roles in a number of plant processes including signaling, development, abiotic stress, and symbiosis [22] [27]. Recent works demonstrated a direct involvement of several GRAS transcription factors in both mycorrhizal signaling and colonization, namely NSP1, NSP2, RAM1, DELLA, DELLA-interacting protein (DIP1) and RAD1 [15] [18] [29] [30] [32] [33] [50].

Here, we focused on a rice GRAS protein previously identified by a whole transcriptome analysis as being specifically expressed in rice mycorrhizal roots and silent in response to mock treatment [34]. No further functional characterization of this GRAS protein, named OsAM18, is available so far.

3.1. Gene Isolation and Phylogenetic Analysis of *OsAM18*

The *OsAM18* cDNA sequence is 2334 nt long. The corresponding genomic sequence spans a region around

4653 bp on chromosome 3, and comprises six introns (**Figure 1(a)**). The predicted protein sequence is 777 amino acids long, with predicted molecular mass and pI of 88.3 kDa and 5.53 respectively. GRAS proteins have highly variable N-termini that differ in length and sequence, but share significant homologies throughout their C-termini. A putative GRAS domain was identified in *OsAM18* using NCBI database and ScanProsite software. Both programs identify the GRAS domain between amino acids 334 and 680 (**Figure 1(b)**). No predicted nuclear localization signal (NLS) was identified in the amino acid sequence while a pSORT prediction indicated a potential cytoplasmic localization. Therefore, we hypothesize it could play a regulatory role at cytoplasmic level probably through protein-protein interaction. Moreover, like NSP2 and RAD1, OsAM18 has no predicted DNA-binding domains [15].

When aligned to GRAS domains of other rice proteins, OsAM18 clustered with putative SCARECROW proteins, remaining, however, as a separate branch (**Figure 2(a)**). A similar topology also arose when the analysis was done considering only SCARECROW proteins from rice, *L. japonicus* and *M. truncatula*: OsAM18 was part of a well-supported cluster, but formed a separate branch (**Figure 2(b)**).

The last 20 amino acids of OsAM18 were, in fact, poorly aligned to the other GRAS domains of the SCARECROW cluster, although the terminal part of the GRAS signature pattern W-x(10)-W was conserved (**Figure 3**).

It seems that OsAM18 has a peculiar sequence, which differentiates it from the other SCARECROW proteins.

3.2. Analysis of *osam18* Mutant Plants

Since the structure of the GRAS proteins does not reveal much about the biochemical function [25], mutant analysis was used to shed light on its physiological and regulatory function. To understand the role of OsAM18 protein in rice plants and AM symbiosis, we took advantage of the *osam18* mutant generated by the endogenous retrotransposon *Tos*17 insertion into the corresponding single copy gene LOC_Os03g40080 (**Figure 1**).

osam18 mutants showed a normal plant development, with shoot and root morphologies comparable to those of the WT plants (**Figure 4(a)**). No significant differences was recorded between *osam18* mutants and WT in the fresh weight of both apparati (**Figure 4(b)**) and in the branching index (BI = nLLR/cmCR—**Figure 4(c)**), which describes the degree of the root apparatus branching [45] [51]. Rice is derived from a semi-aquatic ancestor, therefore its roots may form aerenchyma tissues. Observations by light microscope revealed no differences in the formation of aerenchyma tissues in the LLRs of WT and *osam18* mutants (**Figure 4(d)**).

3.3. *osam18* Mutants Display Impaired Mycorrhization

To better investigate the role of OsAM18 during the presence of the AM fungus, we colonized WT and *osam18* mutant plants with *Funelliformis mosseae*. Mycorrhizal *osam18* mutant plants showed a significant reduced

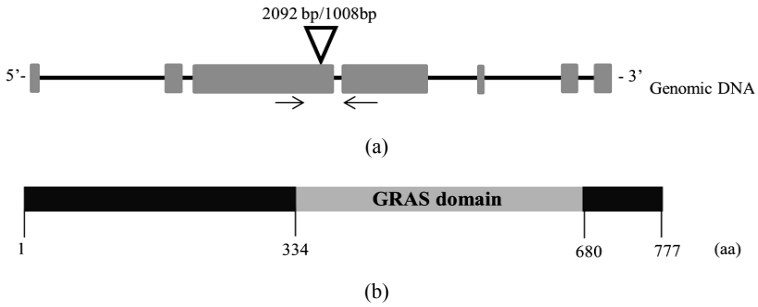
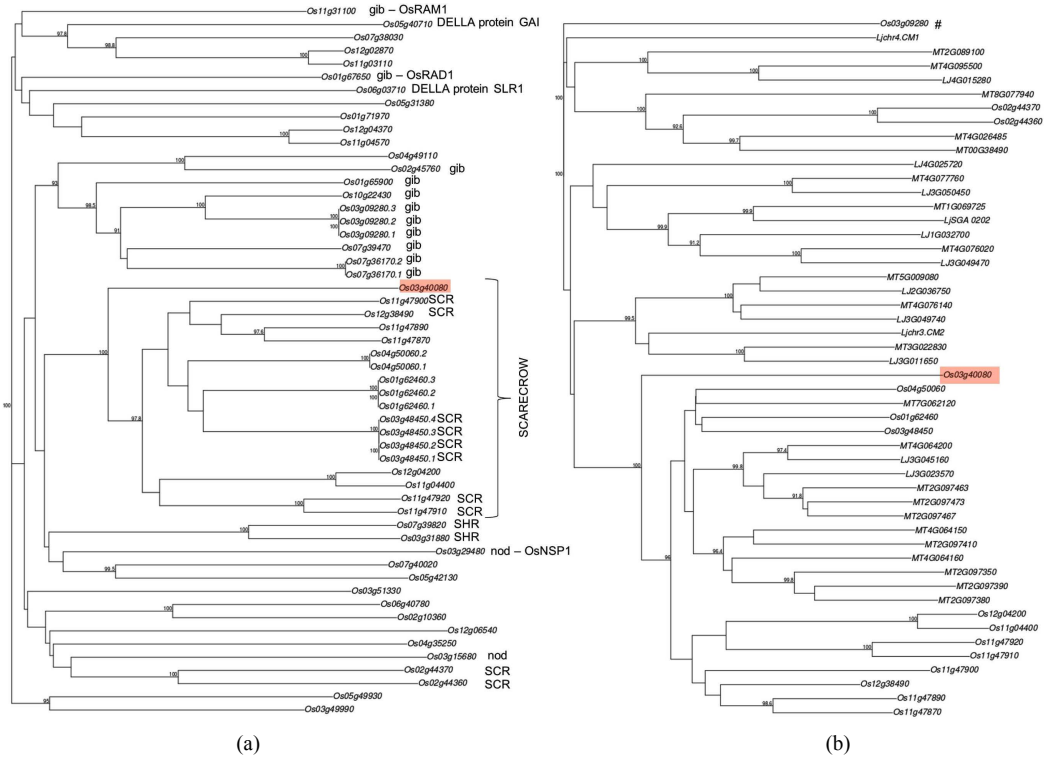


Figure 1. Features of genomic and protein sequence of OsAM18. (a) *OsAM18* genomic DNA is composed of 7 exons and 6 introns (grey boxes and black bars, respectively). Arrows indicate the position of primers used for the mutant screening. Insertion site of *Tos*17 in the NC5532 mutant line is indicated by a triangle. The insertion is in the third exon (in position +2092 bp from start codon if considering genomic sequence, or +1008 if considering cds), at the beginning of the GRAS domain; (b) OsAM18 protein sequence is 777 amino acids (aa) long with the putative GRAS domain positioned between the amino acids 334 and 680 of the protein.



(a) (b)

Figure 2. Phylogenetic analysis of OsAM18. (a) Unrooted phylogenetic tree of the rice (*Oryza sativa*) GRAS proteins. Os03g40080* (highlighted in red) is OsAM18. gib: gibberellin-responsive protein; SCR: SCARECROW protein; SHR: short root protein; nod: nodulation signaling pathway protein; (b) Rooted phylogenetic tree of putative SCARECROW GRAS proteins of *Oryza sativa (Os*), *Lotus japonicus* (*Lj*) and *Medicago truncatula* (Mt). Os03g40080 (highlighted in red) is OsAM18. # indicate the outgroup.

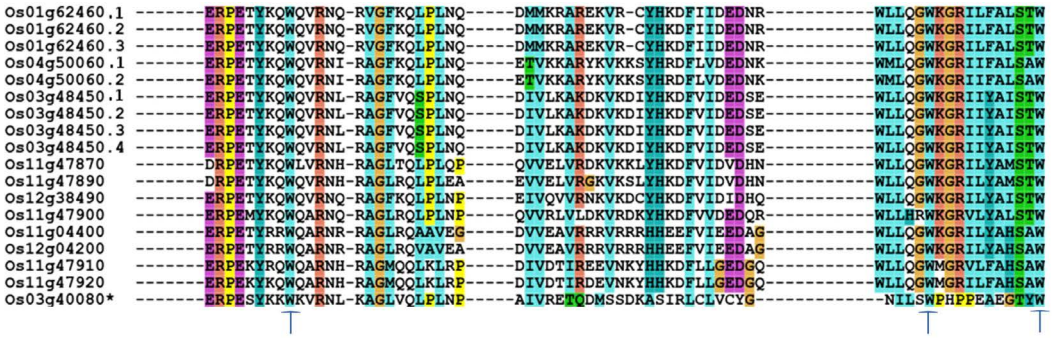


Figure 3. Alignment of GRAS domains belonging to the SCARECROW cluster. The figure shows an excerpt from the alignment of all the GRAS domains represented in the unrooted NJ tree. In particular, proteins belonging to the SCARECROW cluster are represented in the final region of the alignment, in which it is possible to recognize the terminal part of the GRAS signature pattern W(44,98)-W-x(10)-W (indicated with arrows). Os03g40080 (indicated with an asterisk) is OsAM18.

shoot and root fresh weight compared to mycorrhizal WT plants (**Figure 5(a)**), indicating a negative impact on plant development. The colonized roots were assessed at 28 dpi accordingly to Trouvelot and colleagues (1986) [46]: *osam18* mutant roots showed a statistically significant reduction (about 50%) of percentage of mycorrhizal intensity (M%) and arbuscules abundance (A%) in the root system compared to the WT (**Figure 5(b)**). The lower abundance of arbuscules was confirmed by the measurements of *OsPT11* expression level, since this plant phosphate transporter is considered a molecular marker for arbusculated cells. As shown in **Figure 5(c)**, *osam18* mutants showed a statistically significant lower *OsPT11* mRNA abundance compared to the WT (about

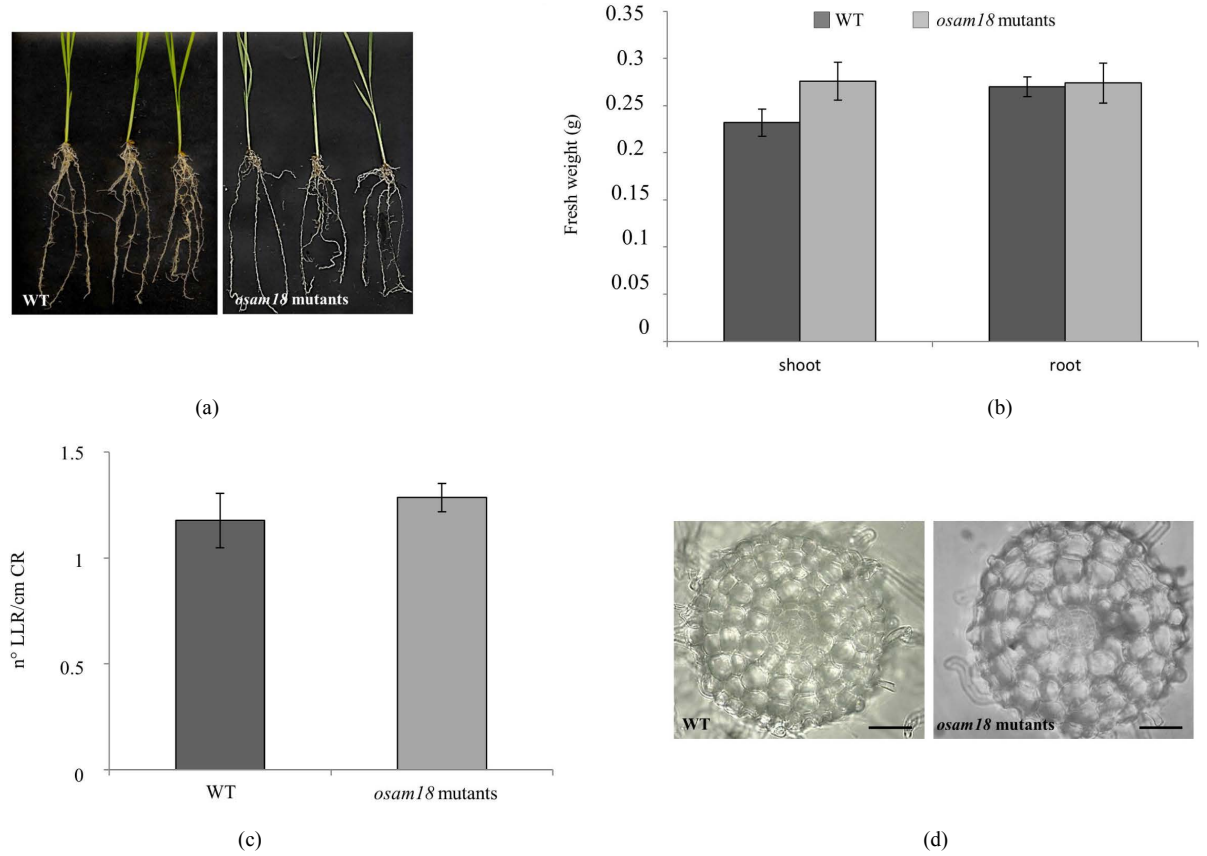


Figure 4. Phenotyping of *osam18* mutants. (a) WT and *osam18* mutant plants showed a similar macroscopical phenotype, with a normal shoot and root development in three independent biological replications; (b) fresh weight of shoot and root of *osam18* mutants and WT plants. Data are the average of six plants and ± SE are represented by bars; (c) *osam18* mutants and WT plants showed no significant difference in the branching index (BI = nLLR/cmCR). Data are the average of five biological replications and ± SE are represented by bars; (d) LLRs cross section of WT and *osam18* mutant plants: no aerenchyma tissue is evident. Scale bars: 33 µm.

50%) corroborating the reduction of AM colonization level. To investigate whether *OsAM18* knock-out also affected arbuscule morphology, WT and *osam18* mutant roots were stained with WGA-TRITC which detects the fungal cell wall and reveals the arbuscule morphology. Differently to the severe mycorrhizal phenotype detected in other mutated AM-marker GRAS genes, the *osam18* mutant fungal structures had the same morphology observed in WT. Indeed, *osam18* mutant displayed fully developed and highly branched arbuscules (**Figure 5(d)**).

4. Conclusions

Taken together, these results demonstrated that the knock-out of *OsAM18* gene led to a significantly reduced degree of mycorrhization but had no effect on the arbuscule morphology.

As a first hypothesis, the reduced colonization level could be related to the peculiar root system of rice, where a different susceptibility to AM fungi had been demonstrated [45] [51]. However, in the *osam18* mutants, the density of LLR, considered as the preferential niche for AM fungi [45] [51], was similar to the WT plants. Therefore, the reduced colonization level detected in *osam18* mutant does not seem to be a consequence of a reduced root branching. AM colonization in rice also depends on specific tissues. It has in fact been demonstrated that the aerenchyma increase is negatively correlated with AM colonization [45]. However, no change in aerenchyma production was detected in *osam18* mutant LLR. Lastly, since the arbuscule morphology proved to be normal, we hypothesized that the perifungal membrane was regularly assembled around the thin arbuscular branches [4] [52] leading to a correct expression of *OsPT11*. We found indeed a lower *OsPT11* mRNA level, which probably mirrored the lower arbuscule number.

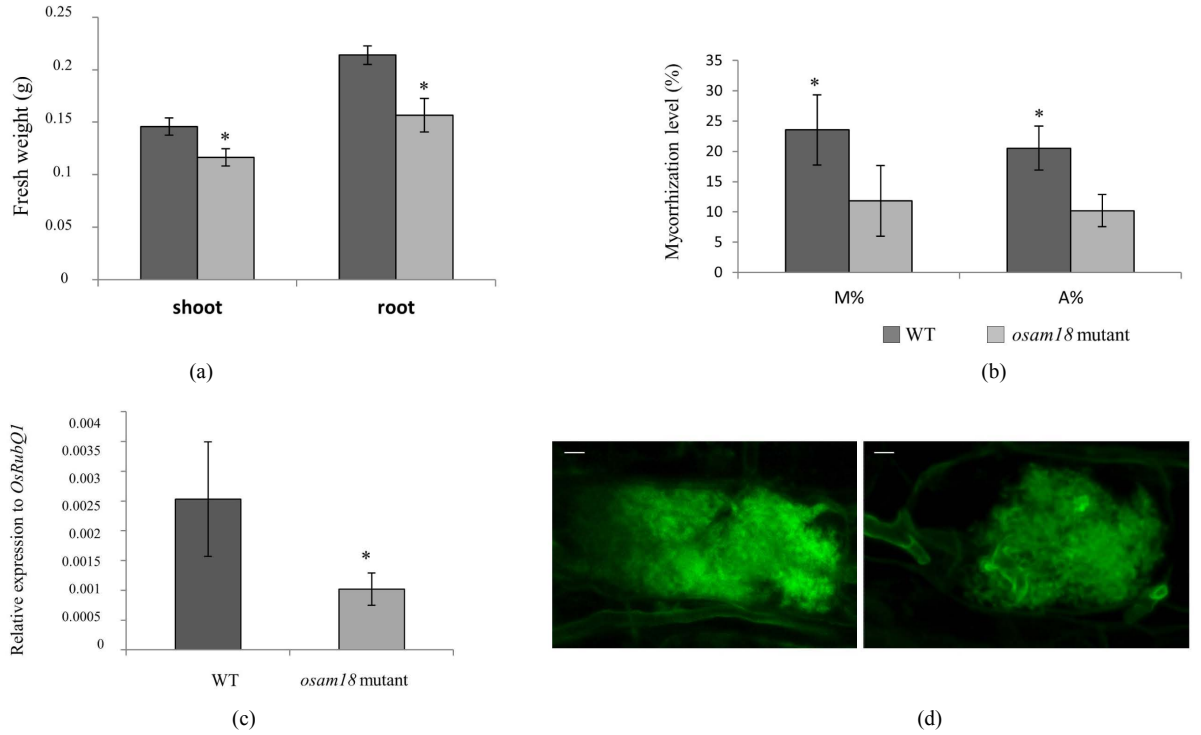


Figure 5. Phenotyping of *osam18* mutants colonized by the AM fungus *Funelliformis mosseae*. (a) Fresh weight of shoot and root of colonized *osam18* mutants and WT plants. Data are the average of at least four plants and ± SE are represented by bars (Kruskal Wallis test). Asterisks indicate significant differences (Kruskal Wallis test, $P < 0.05$); (b) Degree of colonization expressed as mycorrhizal intensity (M%) and arbuscule abundance (A%) in the root system of both WT and *osam18* mutant plants. Data are the average of five biological replicates and ± SE are represented by bars. Asterisks indicate significant differences (One-way Anova test, $P < 0.05$); (c) Quantitative expression of *OsPT11* by qRT-PCR in mycorrhizal roots of WT and *osam18* mutant plants. Data are the average of at least three biological replicates and the bars represent ± SE. Asterisk indicates significant differences, with a P value < 0.05; (d) Cortical cells from WT and *osam18* mutant plants containing arbuscules: green fluorescence is due to wheat germ agglutinin-fluorescein isothiocyanate. Scale bars: 10 µm.

The reason why the GRAS knock-out leads to a less successful symbiosis in the *osam18* mutant remains elusive. On one hand the fungal growth is partially inhibited leading to a decreased presence of both hyphae and arbuscules; on the other hand the mutation has an impact on AM functionality not only at the level of cortical cells (decreased transcripts of *OsPT11*), but also at systemic level (decreased size and weight of shoots and roots from mycorrhizal mutant plants). Since the mutation in itself does not cause any clear change in the phenotype of the non-colonized plants, it seems that *OsAM18* may act as a novel positive regulator of AM symbiosis: it could interact with some of the metabolic pathways which control plant growth response to AM fungi, like hormonal pathways or nutrient flow regulation. Moreover, we suggested that OsAM18 could be a component of the GRAS-domain proteins complex which is involved in the elicitation of AM symbiosis.

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