Isolation and Phenotypic Characterization of *Lotus japonicus* Mutants Specifically Defective in Arbuscular Mycorrhizal Formation

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Several symbiotic mutants of legume plants defective in nodulation have also been shown to be mutants related to arbuscular mycorrhizal (AM) symbiosis. The origin of the AM symbiosis can be traced back to the early land plants. It has therefore been postulated that the older system of AM symbiosis was partially incorporated into the newer system of legume–rhizobium symbiosis. To unravel the genetic basis of the establishment of AM symbiosis, we screened about 34,000 plants derived from ethyl methanesulfonate (EMS)-mutagenized *Lotus japonicus* seeds by microscopic observation. As a result, three lines (ME778, ME966 and ME2329) were isolated as AM-specific mutants that exhibit clear AM-defective phenotypes but form normal effective root nodules with rhizobial infection. In the ME2329 mutant, AM fungi spread their hyphae into the intercellular space of the cortex and formed trunk hyphae in the cortical cells, but the development of fine branches in the arbuscules was arrested. The ME2329 mutant carried a nonsense mutation in the *STR*-homolog gene, implying that the line may be an *str* mutant in *L. japonicus*. On the ME778 and ME966 mutant roots, the entry of AM fungal hyphae was blocked between two adjacent epidermal cells. Occasionally, hyphal colonization accompanied by arbuscules was observed in the two mutants. The genes responsible for the ME778 and ME966 mutants were independently located on chromosome 2. These results suggest that the ME778 and ME966 lines are symbiotic mutants involved in the early stage of AM formation in *L. japonicus*.

**Keywords**: Arbuscular mycorrhizal fungi • EMS • *Lotus japonicus* • Symbiotic mutants.

**Abbreviations**: AM, arbuscular mycorrhiza; EMS, ethyl methanesulfonate; LCO, lipochitooligosaccharide; PBS, phosphate-buffered saline; RN, root nodule; SSR, simple sequence repeat; WGA, wheat germ agglutinin.

The nucleotide sequences of *LjRAM2* and *LjSTR* have been submitted to the DNA Data Bank of Japan under the accession numbers AB830530 and AB830531, respectively.

**Introduction**

Arbuscular mycorrhizal (AM) fungi belong to the phylum Glomeromycota and they can colonize the roots of many terrestrial plants (Smith and Read 2008). In general, the AM fungi do not have host specificity and can colonize various plant species, including gymnosperms, angiosperms, ferns and bryophytes (Smith and Read 2008). The AM fungi provide host plants with phosphate taken up from the soil, and in return receive carbon from the host plants. This symbiosis was already thought to be present >400 million years ago when the terrestrial plants appeared on earth, as certified by fossil and phylogenetic studies (Simon et al. 1993, Remy et al. 1994, Taylor et al. 1995, Redecker et al. 2000). It is generally considered that AM symbiosis was established earlier than the other mycorrhizal symbioses (Brundrett 2002, Bonfante and Solesse 2010). For example, ectomycorrhizal symbiosis associated with woody plant species might have appeared about 130-plus million years ago (Smith and Read 2008). Therefore, because AM symbiosis is one of the earliest symbioses established between microorganisms and terrestrial plants, it is important to understand its mechanism.
For AM colonization into plant roots, first, the fungal spores in soil must germinate. The spores can germinate in the absence of host plants (Giovannetti and Sbrana 1998). Then, the germinating hyphae branch near the host roots, and this branching is induced by a branching factor, strigolactone, secreted by plants (Akiyama et al. 2005, Besserer et al. 2006). This molecule is also involved in the infection of host plants by parasitic plants (Cook et al. 1996, García-Garrido et al. 2009). Meanwhile, host plants perceive diffusible molecules secreted by AM fungi, and initiate preparation for fungal accommodation (Bonfante and Requena 2011). One of the diffusible fungal molecules is a lipochitooligosaccharide (Myc-LCO), which has a structure similar to the Nod factors produced by rhizobia (Maillet et al. 2011). In root nodule (RN) symbiosis, Nod factors are recognized by LysM receptor kinases of legume plants (Limpens et al. 2003, Madsen et al. 2003, Radutoiu et al. 2003). Although plant receptors for the signal molecules released by AM fungi have not been identified, the Myc-LCO might also be received by a member of the LysM domain-containing receptor family (Op den Camp et al. 2011).

The signaling transduction mechanism in the early phase of AM symbiosis after signal molecule recognition has been extensively analyzed in legume plants (Parniske 2008). In early works, it was reported that several non-nodulating pea mutants were defective in AM formation (Duc et al. 1989). Thereafter, legume model plants, Medicago truncatula and Lotus japonicus, were used for genetic studies of symbiotic mutants (Sagan et al. 1995, Bonfante et al. 2000, Senoo et al. 2000, Solaiman et al. 2000, Marsh and Schultz 2001, Barker and Larkin 2002, Jacobi et al. 2003a, Jacobi et al. 2003b, Demchenko et al. 2004, Boisson-Dernier et al. 2005, Morandi et al. 2005). These studies have demonstrated that several symbiotic genes of plants required for RN symbiosis were also essential for the AM symbiosis. The genes that are required for both nodulation and mycorrhization are currently known as components of a common symbiosis signaling pathway comprising the leucine-rich repeats receptor kinase SYMK (Stracke et al. 2002), two ion channels (CASTOR and POLLUX; Imaiizumi-Anraku et al. 2005), three nucleoporins (NUP85, NUP133, and NENA; Groth et al. 2010), calcium and calmodulin-dependent kinase (CCaMK; Levy et al. 2004, Mira et al. 2004, Tirichine et al. 2006) and CYCLOPS (Yano et al. 2008) in L. japonicus (Stougaard 2001, Kistner and Parniske 2002, Parniske 2008). RN symbiosis occurred approximately 60 million years ago (Doyle 2011), much later than AM symbiosis. Therefore, it has been postulated that RN symbiosis recruited a part of the genetic system in AM (Parniske 2008).

The common symbiosis mutants affect distinct steps of AM fungal accommodation (Kistner et al. 2005). In the wild type of L. japonicus, AM fungal hyphae attach to the root surface and form hyphopodia from which penetrating hyphae emerge (Bonfante et al. 2000). The penetrating hyphae separate two adjacent epidermal cells and pass into these epidermal cells through a pre-penetration apparatus (PPA) that is derived from the host as a tunnel-like structure (Genre et al. 2005). This hyphal penetration is followed by hyphal extension in the intercellular space of the cortex and arbuscule formation in the inner cortical root cells. In the symbiotic mutants symrk, castor, pollux, cyclops and of three nucleoporin genes, AM fungal hyphae separate the two adjacent epidermal cells, but cannot penetrate into the epidermal cells, where the hyphae swell and terminate further extension (Wegel et al. 1998, Bonfante et al. 2000, Senoo et al. 2000, Novero et al. 2002, Demchenko et al. 2004, Kistner et al. 2005, Saito et al. 2007, Groth et al. 2010). In contrast, a cccamk mutant has been shown to block hyphal penetration at the root surface (Senoo et al. 2000, Demchenko et al. 2004, Kistner et al. 2005). Occasionally, the mutants of the common symbiotic genes allow AM fungal accommodation, especially in the late growth stage (Novero et al. 2002, Demchenko et al. 2004). However, in cyclops and cccamk mutants, arbuscule formation was almost completely blocked, indicating that CYCLOPS and CCAmK are required for establishment of intracellular accommodation of AM fungi (Demchenko et al. 2004, Kistner et al. 2005, Yano et al. 2008).

As genes downstream of the common symbiosis signaling pathway, RAM1 and RAM2 were identified in M. truncatula, and shown to be indispensable for the AM formation (Gobbato et al. 2012, Wang et al. 2012). The GRAS-type transcription factor RAM1 specifically functions in Myc factor signaling, but not in Nod factor signaling. RAM1 regulates the transcript level of RAM2, which codes for a glycerol-3-phosphate acyltransferase that enhances cutin production to promote fungal hyphopodia formation on the root surface. Furthermore, several genes required for the formation of mature arbuscules have been identified. Mutations in an AM-specific phosphate transporter led to premature death of the arbuscules (Javot et al. 2007, Yang et al. 2012). The ABC transporter STR genes (Zhang et al. 2010, Gutjar et al. 2012), Vapyrin (Feddermann et al. 2010, Pumplin et al. 2010) and SNAREs (Ivanov et al. 2012, Lota et al. 2013) are also involved in the development of mature arbuscules. Two of these genes, Vapyrin and SNAREs, are required for both RN symbiosis and arbuscule formation (Murray et al. 2011, Ivanov et al. 2012). Recently, another two mutants that impaired arbuscule development were isolated by genetic screening of the EMS-mutagenized population (Groth et al. 2013). However, the genetic basis for AM symbiosis is still not totally understood.

In the present study, we screened for AM-specific mutants from EMS-mutagenized L. japonicus to clarify the genetic mechanism in host plants underlying the establishment of AM symbiosis. For the isolated mutants, we further analyzed their phenotypic characterization and determined the map positions of the causative genes.

Results

Screening for L. japonicus mutants defective in AM, but not RN symbiosis

We used an EMS-mutagenized M2 population originating from L. japonicus MG-20 for the mutant screening. A total of 34,459
plants originating from 2,113 M₁ lines were inoculated with *Rhizophagus irregularis*, and 22,935 plants were then evaluated for AM fungal colonization. As a result, 95 plants that originated from 67 M₁ lines and that appeared to show less colonization than the wild type under a dissecting microscope were isolated as potential AM-defective mutants (phenotype: Myc⁻). However, only 47 of these plants survived in the M₂ generation because some of the M₂ plants were dwarf, weak or sterile plants. The surviving mutant lines were given names consisting of the prefix ‘ME’ plus the line identification number, e.g. ‘ME778’ (Supplementary Table S1).

In order to re-evaluate the mycorrhizal phenotype of the mutant candidates, we performed a second screening using the M₂ lines. A total of 32 M₂ lines originating from 32 different M₁ lines were further tested with respect to mycorrhizal development. Some lines could not germinate at all or were too small for their mycorrhizal development to be investigated. Thus, 24 mutant lines were confirmed to have the Myc⁻ phenotype, which exhibited lower mycorrhizal colonization than the wild type (Supplementary Table S1). To select AM-specific mutants from among these lines, nodule formation was evaluated after inoculation with *Mesorhizobium loti* TONO. Finally, nine lines were isolated as mutants specifically defective in AM formation (Supplementary Table S1).

Among the isolated mutants, the ME778 and ME966 mutants of the back-crossed F₃ generation showed very low levels of AM colonization (Table 1). In contrast to these two mutants, the other seven lines showed some level of colonization with *R. irregularis*, although all colonization levels were lower than that of the wild type (data not shown). For example, the ME823 mutant showed 21 ± 9% (mean ± SD) hyphal colonization and 19 ± 8% arbuscular colonization vs. corresponding levels of 54 ± 10% and 52 ± 11%, respectively, in the wild type. The ME2329 mutant also showed a low level of AM colonization but differed from other mutants in its small and stunted arbuscules (Table 2; Fig. 4).

### Segregation of the AM phenotype

The ME778, ME966 and ME2329 lines that exhibited a clear AM phenotype in the mutant screening were back-crossed with *Lotus japonicus* MG-20 in order to investigate segregation of the mutant phenotype in the F₂ population (Table 2). The ratio of segregation of the AM phenotype in the ME966 and ME2329 mutant lines was approximately 3:1 (wild type: mutant), indicating that the mutant phenotype is segregated as a monogenic recessive trait. In the ME778 mutant, segregation of the Myc⁻ phenotype in the F₂ population deviated from the monogenic recessive 3:1 ratio (Table 2), but was not significantly different from the 9:7 ratio for two genes (*χ² = 0.244, *P* = 0.621). It seems likely that ME778 carries mutations in two genes present on separate loci, although we cannot rule out the possibility of a segregation distortion or other hereditary pattern.

### AM colonization in the ME778, ME966 and ME2329 mutants

We further investigated AM colonization in the ME778, ME966 and ME2329 mutants, which exhibited a clear AM phenotype in the mutant screening. In the wild-type plants, the hyphae of *R. irregularis* attached to the root surface and formed hyphopodia (Fig. 1A, D). The hyphae emerged from the hyphopodium transverse to the root epidermal cells (Fig. 1D), and this was followed by the extension of intraradical hyphae in the intercellular space of the cortex (Fig. 1D, E) and arbuscule formation in the inner cortical cells (Fig. 1B, E, F). Vesicles were often observed in the cortex colonized with *R. irregularis* (Fig. 1B, E). The AM fungus *Gigaspora margarita* also showed a colonization pattern similar to that of *R. irregularis* but did not form vesicles in the roots (Fig. 1C). Both ME778 and ME966 exhibited similar phenotypic characteristics on AM colonization. The AM fungus *R. irregularis* formed hyphopodia at the root surface of the two mutants (Figs. 2A, 3A). The hyphopodia localized on the boundary of two adjacent epidermal cells along the longitudinal axis (Figs. 2B, 3B, 4B). Penetrating hyphae emerging from the hyphopodia entered between two adjacent epidermal cells, but terminated their growth and caused abnormal swelling in the epidermis (Figs. 2D, 4D, 3D, 3E, 4E). The *R. irregularis* hyphae did not progress inside the root. Similarly, *G. margarita* formed hyphopodia at the root surface and attempted to penetrate the epidermis of ME778 and ME966 mutants, but did not enter the roots (Figs. 2F, 3F). In rare cases, some AM fungal hyphae penetrated the epidermis, extended their intraradical hyphae and formed normal arbuscules in the inner cortical cells of ME778 and ME966 mutants (Figs. 2F, 3F). In contrast to the ME778 and ME966 mutant lines, in ME2329, AM fungal hyphae penetrate the epidermis; however, the rate of hyphal colonization was significantly lower than that in wild-type plants (Fig. 4G). Notably, arbuscular and vesicular colonization of the ME2329 mutant was much lower than that in wild-type plants (Fig. 4H, I), and the mutants showed abnormal arbuscule formation (Fig. 4A, B). In the wild type, arbuscules consisting of trunk hyphae and fine branches emerged from the intraradical hypha and fully filled the interior of cortical cells (Fig. 1E, F). In the ME2329 mutant, AM fungal hyphae penetrated the epidermis and spread between cortical cells, as in the wild type (Fig. 4D, E). The hyphae then penetrated inner cortical cells and formed thick trunk hyphae (Fig. 4F); however, the development of fine branches from the trunk hypha was

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**Table 1** Mycorrhizal colonization in *Lotus japonicus* wild type MG-20 and mycorrhizal mutants, ME778 and ME966, at 4 weeks after inoculation of *Rhizophagus irregularis* DAOM197198

<table>
<thead>
<tr>
<th>Lines</th>
<th>Hyphal colonization, %</th>
<th>Arbuscular colonization, %</th>
<th>Vesicular colonization, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (MG-20)</td>
<td>72 ± 17*</td>
<td>72 ± 17</td>
<td>62 ± 16</td>
</tr>
<tr>
<td>ME778</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>ME966</td>
<td>2 ± 4</td>
<td>2 ± 4</td>
<td>2 ± 3</td>
</tr>
</tbody>
</table>

* Mean ± SD.
arrested. In the cortical cells containing stunted arbuscules, starch granules stained with iodine were often observed (Fig. 4B, C).

Nodule formation of ME778, ME966 and ME2329 mutants

In the mutant screening, we had observed that ME778, ME966 and ME2329 formed mature nodules when inoculated with M. loti. Therefore, we carried out a detailed phenotypic characterization of M. loti infection and root nodule formation in these three mutants. Plants of the wild type, ME778, ME966 and ME2329 lines formed normal infection threads in their root hairs (Supplementary Figs. S1A–D). The number of infection threads per plant was not significantly different among these plant lines (Supplementary Fig. S1E). The ME778, ME966 and ME2329 lines formed pink nodules (Supplementary Fig. S2A, E, I, M) that contained both infected and uninfected cells, as seen in the wild type (Supplementary Fig. S2B, F, J, N). The infected cells were filled with numerous bacteroids.
Supplementary Fig. S2C, G, K, O) whose ultrastructure was not different between the wild type and the three mutants based on transmission electron microscopic analysis (Supplementary Fig. S2D, H, L, P).

Linkage mapping

To determine the map positions of mutated loci in the mutant lines ME778, ME966 and ME2329, we generated a genetic linkage map of the mutant phenotype using simple sequence repeat (SSR) markers. Based on the segregation analysis described above, it is inferred that ME778 mutants have a digenic trait. When the ME778 line (MG-20 background) (having two causative genes, 'aa' and 'bb') was crossed with ecotype B-129 (having genes 'AA' and 'BB' at the same loci), the segregation ratio of genotypes at one locus (locus A) in the F2 population of AM-defective mutants is theoretically MG-20 (aa): hetero (Aa): B-129 (AA) = 4:2:1 (aabb + 2 × aAB + aABb + 2 × AAbb: AAbb). The TM0225 and TM0377 markers on chromosome 2 exhibited a good approximation of the theoretical segregation ratio (Fig. 5A). This implies that at least one causative gene of ME778 may locate around these molecular markers. However, it is also possible that the ME778 mutant has more complicated patterns of inheritance of the Myc phenotype than a simple digenic inheritance.

Mapping analysis of ME966 indicated that a mutated locus is located south of chromosome 2, near the TM0504 and TM0889 markers (Fig. 5B). We noticed that the NENA gene, which encodes SEH1-like nucleoporin and is required for infection by AM fungi and rhizobia, is located near this region. A nena mutant was originally isolated as a mutant affected in AM development, but later was found to be also arrested in nodulation (Groth et al. 2010). Therefore, it is possible that ME966 carries a mutation in the NENA gene. However, one co-segregating marker, TM0796 of the NENA gene, was located outside the target region of ME966. Furthermore, no mutation was found in the genomic sequence of the NENA gene in ME966 (data not shown).

The mutant phenotype of the ME2329 line was mapped at the south end of chromosome 4, near marker TM0069 (Fig. 5C). Notably, the ortholog of MtSTR required for arbuscule
formation (Zhang et al. 2010) is located in this region. To confirm whether a mutation is present in the STR homolog in ME2329, we sequenced the LjSTR gene of the mutant and found a nonsense mutation (C1240T, Q414Stop) in the middle region of the coding sequence of the LjSTR gene. This implies that ME2329 may be a str mutant in L. japonicus, although we cannot absolutely exclude the possibility that other mutations brought about by the EMS treatment contribute to the ME2329 phenotype.

Marker gene expression in ME2329 mutant

The expression levels of AM marker genes were examined by real-time reverse transcription–PCR (RT–PCR) (Fig. 6). In wild-type plants inoculated with R. irregularis, arbuscule-related genes LjPT4 and LjAMT2;2 (Guether et al. 2009) were strongly up-regulated. LjSTR and three subtilase genes, SbtM1, SbtM4 and SbtS (Takeda et al. 2009, 2011), were also induced in mycorrhizal roots. In inoculated ME2329 roots, all six marker genes were induced by AM fungal colonization, but the abundance of LjPT4, LjAMT2;2, LjSTR, SbtM1 and SbtS transcripts was dramatically reduced compared with the inoculated wild type. SbtM4 was up-regulated in inoculated roots of the ME2329 mutants to similar levels to those in wild-type plants.

Discussion

Genetic studies on AM development in leguminous plants have been increasing since the late 1990s (Barker and Larkan 2002). As a result of these studies, the common symbiosis pathway involved in both AM and RN formation has been identified, as have several symbiotic genes (Parniske 2008). However, knowledge of the specific pathways involved in AM symbiosis remains limited (Gobbato et al. 2012, Wang et al. 2012, Groth et al. 2013). In this study, we successfully isolated three mutants specifically defective in AM symbiosis from EMS-mutagenized plants (Table 2). Among the isolated mutants, ME778 and
ME966 were severely arrested at an early stage of mycorrhizal development. To the best of our knowledge, the previously identified AM-specific genes related to the early stage are the GRAS-type transcription factor RAM1 (Gobbato et al. 2012) and the glycerol-3-phosphate acyltransferase RAM2 (Wang et al. 2012) in *M. truncatula*. RAM1 functions in the activation of gene expression in an AM-specific pathway downstream of the common symbiosis pathway by interacting with NSP2.

**Fig. 4** Arbuscular mycorrhizal colonization in the ME2329 mutant. (A–C) Bright field images of roots stained with trypan blue. *Rhizophagus irregularis* formed stunted arbuscules (arrows) in the roots (A). Arbuscules did not fully develop in cortical cells (B). Starch granules stained brown with iodine were visible in the cortical cells containing stunted arbuscules (C). (D–F) Fluorescent images of *R. irregularis* hyphae stained with WGA–Oregon Green 488 and observed using a confocal laser scanning microscope. *Rhizophagus irregularis* colonized in epidermis (arrowhead) and spread their intraradical hyphae in roots, but stunted arbuscules (arrows) were formed in cortical cells (D and E). The fungal hyphae penetrated cortical cells (double arrowheads) and formed thick trunk hyphae in the cells but formed few fine branches generated from them (F). e, extraradical hypha; h, hyphopodium; i, intraradical hypha; s, starch granule; t, trunk hypha of arbuscule. Scale bars = 50 μm (A–C) and 20 μm (D–F). The percentage of AM fungal total colonization (G), arbuscular (H) and vesicular colonization (I) of the *L. japonicus* wild type MG-20 (filled circles), and ME2329 mutant (open circles) inoculated with *R. irregularis*. Error bars show the SEMs (n = 3).
which is also a GRAS-type transcription factor required for both AM and RN symbiosis (Lauressergues et al. 2012). The mutated genes in the ME778 and ME966 lines may be involved in this early stage of mycorrhization. Segregation of the Myc− phenotype in the F2 population of ME778 and in the wild type was not significantly different from the 9 : 7 (wild type vs. mutant) ratio, which seems to indicate that ME778 has a digenic trait. However, genotyping of ME778 did not clearly indicate two loci of mutated genes, though at least one mutated locus was estimated to occur on the short arm of chromosome 2. Consequently, we cannot rule out the possibility that the ME778 mutant shows complicated patterns of inheritance of the Myc− phenotype. In that case, the standard genetic mapping procedures used in the present study might be limited in their ability to detect the causative genes of ME778. ME966 revealed a monogenic recessive trait located on the long arm of chromosome 2. Notably, NENA, which is required for both AM and RN symbiosis (Groth et al. 2010), was found to be located around the identified target region of ME966; however, further mapping analysis indicated that NENA was located outside this region. In addition, we were unable to detect any mutation in NENA of ME966. Finally, sequencing of LjRAM2 in the ME966 line did not reveal any mutation (data not shown). These results indicate that the ME966 line may have a mutation in a novel locus that is required for AM formation, although further mapping analysis and allelism tests are required to elucidate whether the causative gene is novel.

Microscopic observation of ME778 and ME966 revealed blocking of epidermal penetration by AM fungi. In the two mutants, AM fungi formed abnormal balloon-like swollen hyphae between epidermal cells, and terminated hyphal growth there. Occasionally, hyphal colonization with normal arbuscules was observed, especially at later stages of AM formation.
The south end of chromosome 4 near an ortholog of the common symbiosis genes have been shown to be involved in arbuscule formation or mycorrhization, as in the case of common symbiosis genes. The ME778 and ME966 mutants formed root nodules at almost the same level as the wild type. This may be due to severely impaired arbuscule formation of the ME2329 mutant. SbtM1, SbtM4 and SbtS are up-regulated during the early stages of AM formation, but the three subtilase genes show distinct expression patterns in symbiosis (Takeda et al. 2009). Expression of the SbtM1 gene is specific to AM symbiosis and involved in arbuscule formation, while SbtM4 and SbtS are also expressed in RN (Takeda et al. 2009, Takeda et al. 2011). Induction of SbtM1 and SbtS genes by AM fungal colonization was diminished in ME2329, which was similar to the case of the arbuscule-related genes LjPT4, LjAMT2;2 and LjSTR in the ME2329 mutant. In contrast, the transcript level of SbtM4 in the ME2329 mutant was not different from that in the mycorrhizal wild type. SbtM4 is activated via signaling through the common symbiosis pathway and the transcripts accumulate in both AM and RN (Takeda et al. 2011). In RN, SbtM4 is expressed at sites of rhizobial infection such as epidermal cells near the infection threads (Takeda et al. 2009). The ME2329 mutant showed epidermal penetration by AM fungi and intraradical hyphal spreading in the intercellular spaces of the cortex. The induction of SbtM4 in the ME2329 mutant appears to be triggered by the fungal infection in roots independent of arbuscule formation. The ME2329 mutant showed low AM colonization, but hyphal colonization was not affected. In the ME2329 mutant, we observed starch granules in the cortical cells containing stunted arbuscules. In the wild type, in contrast, starch granules were almost absent from arbuscule-containing cells (Gutjahr et al. 2009) while AM colonization increases the import of photosynthetic carbohydrates into roots (Wang et al. 1989). This may be due to rapid consumption of carbohydrates in the arbuscule-containing cells for fungal growth. Because ME2329 mutants are defective in arbuscule development, it is likely that nutrient exchange between ME2329 mutants and AM fungi is arrested, which may result in an accumulation of excess carbohydrates in the form of starch granules in the cortical cells. The ME2329 mutant could be used to analyze the development and function of arbuscules, especially with respect to nutrient exchange between host and fungal symbionts.

Isolated mutants other than ME778, ME966 and ME2329 showed low hyphal colonization of less than half that of the wild type (Supplementary Table S1), although the structures of their intraradical hyphae and arbuscules were normal, as seen in the wild type (data not shown). The low hyphal colonization of the mutants may have been caused by a reduction of entry events into roots by AM fungi or a decrease in the growth rate of intraradical hyphae in the cortex. Inevitably, the hyphal colonization rate of the mutants varied among the experiments.

FIG. 6 Gene expression analysis in L. japonicus MG-20 and the ME2329 mutant. Real-time RT-PCR was performed to determine expression of AM marker genes LjPT4, LjAMT2;2, LjSTR SbtM1, SbtM4 and SbtS in mycorrhizal roots 4 weeks after inoculation with R. irregularis and non-mycorrhizal roots 2 weeks after transplant that did not show phosphorus deficiency symptoms. Expression levels are normalized on the basis of the amount of LjUBC and expressed relative to non-mycorrhizal roots of L. japonicus MG-20. Values are the means of three biological replicates. Error bars show the 95% confidence interval.
Characterization of AM-specific mutants in *L. japonicus*

Materials and Methods

Initial screening of the arbuscular mycorrhizal mutants

Seeds of *L. japonicus* MG-20 were mutagenized by EMS treatment. The second-generation (*M*₂) plants were used for mutant screening. *Lotus japonicus* MG-20 and castor-11 mutants (*Myc*⁻ and *Nod*°, MG-20 background; Imaizumi-Anraku et al. 2005) were also used as controls. Approximately 16–20 seeds from each mutant line were treated with sandpaper or concentrated sulfuric acid to promote germination. Next, the seeds were suspended in 180 ml of twice-concentrated N-free B&D medium (Broughton and Dilworth 1971). A 10 ml aliquot of bacterial suspension was inoculated per 250 ml of vermiculite and fertilized with modified Hornum solution. The plant was inoculated with 4,000 spores of *R. irregularis* and cultivated for 4 weeks. If no or low colonization in the plant roots was observed again, the seeds of the next *M*₃ generation were harvested.

Second screening of the arbuscular mycorrhizal mutants

For the second screening, 16 seedlings of the *M*₃ generation of each line screened as *Myc*⁻ mutant candidates were grown with AM inoculation in the batch culture system as described above. When the plants showed low or no colonization in their roots, nodule formation was checked as follows. Several shoots from each mutant line were planted in vermiculite and incubated for 1 week to promote root growth, then planted in a glass tube (2.5 cm φ × 10 cm in height) filled with 40 ml of vermiculite and fertilized with modified Hornum solution. The plant was inoculated with 4,000 spores of *R. irregularis* and cultivated for 4 weeks. If no or low colonization in the plant roots was observed again, the seeds of the next *M*₄ generation were harvested.

Segregation analysis of the candidate mutants

The *M*₃ or *M*₄ generation plants of the candidate mutant lines were back-crossed with *L. japonicus* MG-20. At least 47 back-crossed *F*₂ plants (*Table 2*) were inoculated with approximately 6,000 spores of *R. irregularis* and grown in the batch culture system. AM fungal colonization was checked under a (data not shown). Several common symbiosis mutants allow AM fungal accommodation with progression of the growth stage (Novero et al. 2002, Demchenko et al. 2004). A possible reason for the variability of hyphal colonization of the isolated mutants may be the influence of plant growth stage.

In the present study, we constructed an efficient screening method for *Myc*⁻ mutant isolation and developed a method for staining AM roots using a 24-well plate to screen AM-specific mutants more efficiently. One advantage of this staining method is that AM fungal colonization could be rapidly assessed in many samples. Using this method, each root sample was processed in one well of the 24-well plate throughout the staining. The stained root samples kept in the 24-well plate were then quickly assessed under a dissecting microscope. This method will allow for the isolation of more AM-defective mutants in future studies, which is important because there are far fewer studies on the genetic screening of AM formation than studies on nodulation.

For the screening of *L. japonicus* mutants, we isolated three mutant lines that specifically showed an AM-defective phenotype. Two of the isolated mutant lines, ME778 and ME966, are defective at an early step of mycorrhization, while the ME2329 mutant showed defective development of arbuscules and had a mutation in the *LjSTR* gene, implying that the line may be an *str* mutant in *L. japonicus*. Genetic studies on AM-specific pathways are limited (Zhang et al. 2010, Wang et al. 2012, Groth et al. 2013); future studies should focus on cloning and functional analysis of the causative genes of the AM-specific mutants in order to better understand the genetic mechanism of AM formation and the molecular evolution of plant–microbe symbiosis.
dissecting microscope after the roots were stained with trypan blue as described above.

**Microscopic observation of AM fungal colonization**

*Lotus japonicus* MG-20 and the back-crossed F₃ plants of ME778, ME966 and ME2329 were transplanted into 50 ml pots filled with sand (size: 0.5–2.0 mm) and inoculated with 500 spores of *R. irregularis*, or into 250 ml pots filled with sand and Akadama soil in a 1:1 ratio (containing 0.53 g l⁻¹ NH₄NO₃, 0.027 g l⁻¹ KH₂PO₄ and 0.107 g l⁻¹ KCl) inoculated with 150 spores of *G. margarita* MAFF520054. The plants inoculated with *R. irregularis* were supplied with half-strength Hoagland’s solution containing 100 µM Pi twice a week.

Roots were harvested at 4 weeks after inoculation and stained with trypan blue or wheat germ agglutinin (WGA). The total colonization using a magnified intersection method at a 3:1 ratio. The roots were rinsed with pure water and incubated in 10% KOH for 3 h at room temperature, and then stained with 5 µg ml⁻¹ WGA conjugated with Oregon Green 488 (Invitrogen) in PBS. The stained roots was determined as the percentage of root length with trypan blue or WGA staining. The total root length with trypan blue or wheat germ agglutinin (WGA) was determined as the percentage of root length colonization using a magnified intersection method at a 3:1 ratio. The roots were rinsed with pure water and incubated in 10% KOH for 3 h at room temperature, and then stained with 5 µg ml⁻¹ WGA conjugated with Oregon Green 488 (Invitrogen) in PBS for several hours at room temperature. After rinsing with PBS, the roots were stained with 5 µg ml⁻¹ propidium iodide. After rinsing with PBS, the roots were mounted on a cover glass and observed using a confocal laser scanning microscope (FV1000-D; Olympus). Optical sections were projected using an FV-10 ASW (Olympus). To stain starch granules with iodine, roots were fixed in a solution of ethanol and acetic acid at a 3:1 ratio. The roots were fixed in a solution of ethanol and acetic acid at a 3:1 ratio. The roots were rinsed with pure water, incubated in 10% KOH for 3 h at room temperature, and then stained with 5 µg ml⁻¹ WGA conjugated with Oregon Green 488 (Invitrogen) in PBS for several hours at room temperature. After rinsing with PBS, the roots were stained with 5 µg ml⁻¹ propidium iodide. After rinsing with PBS, the roots were mounted on a cover glass and observed using a confocal laser scanning microscope (FV1000-D; Olympus). Optical sections were projected using an FV-10 ASW (Olympus). To stain starch granules with iodine, roots were fixed in a solution of ethanol and acetic acid at a 3:1 ratio. The roots were fixed in a solution of ethanol and acetic acid at a 3:1 ratio. The roots were rinsed with pure water, incubated in 10% KOH for 3 h at room temperature, and then stained with 5 µg ml⁻¹ WGA conjugated with Oregon Green 488 (Invitrogen) in PBS for several hours at room temperature. After rinsing with PBS, the roots were stained with 5 µg ml⁻¹ propidium iodide. After rinsing with PBS, the roots were mounted on a cover glass and observed using a confocal laser scanning microscope (FV1000-D; Olympus). Optical sections were projected using an FV-10 ASW (Olympus). To stain starch granules with iodine, roots were fixed in a solution of ethanol and acetic acid at a 3:1 ratio. The roots were fixed in a solution of ethanol and acetic acid at a 3:1 ratio. The roots were rinsed with pure water, incubated in 10% KOH for 3 h at room temperature, and then stained with 5 µg ml⁻¹ WGA conjugated with Oregon Green 488 (Invitrogen) in PBS for several hours at room temperature. After rinsing with PBS, the roots were stained with 5 µg ml⁻¹ propidium iodide. After rinsing with PBS, the roots were mounted on a cover glass and observed using a confocal laser scanning microscope (FV1000-D; Olympus). Optical sections were projected using an FV-10 ASW (Olympus). To stain starch granules with iodine, roots were fixed in a solution of ethanol and acetic acid at a 3:1 ratio. The roots were fixed in a solution of ethanol and acetic acid at a 3:1 ratio. The roots were rinsed with pure water, incubated in 10% KOH for 3 h at room temperature, and then stained with 5 µg ml⁻¹ WGA conjugated with Oregon Green 488 (Invitrogen) in PBS for several hours at room temperature. After rinsing with PBS, the roots were stained with 5 µg ml⁻¹ propidium iodide. After rinsing with PBS, the roots were mounted on a cover glass and observed using a confocal laser scanning microscope (FV1000-D; Olympus). Optical sections were projected using an FV-10 ASW (Olympus).

**Infection thread formation**

In order to observe infection thread formation, *L. japonicus* MG-20, ME778, ME966 and ME2329 were transplanted into pots filled with vermiculite and inoculated with *M. loti* MAFF303099 constitutively expressing DsRed (Maekawa et al. 2009). The plants were grown in a growth chamber at 26°C and 70% r.h. with a 16 h light/8 h dark cycle. The plants were supplied with N-free B&D solution. Roots were harvested at 2 weeks after inoculation. Fluorescence of DsRed was observed using an Axio Imager D1 epifluorescence microscope (Carl Zeiss). Digital images were captured with an AxioCam digital CCD camera (Carl Zeiss) operated with AxioVisio software (Carl Zeiss).

**Observation of root nodule development**

Plant roots were harvested 4 weeks after the inoculation of *M. loti* MAFF303099. Nodules cut from roots were fixed in 2% paraformaldehyde and 2% glutaraldehyde in HEPES buffer (pH 7.0) at room temperature for 2 h. After rinsing with the HEPES buffer, samples were post-fixed in 2% OsO₄ in HEPES buffer for 1 h at room temperature and washed three times with pure water, dehydrated in an ethanol series (50, 70, 80, 90, 95 and 100%) and substituted with propylene oxide for 5 min twice. The nodules were then infiltrated with Spurr resin (Polysciences). The resin was polymerized for 12 h at 70°C. Semi-thin sections (approximately 0.2 µm) were cut and stained with 0.1% toluidine blue O for optical microscopic observation with an Axio Imager D1 light microscope. Ultra-thin sections (approximately 80 nm) were stained with Ti blue (NissinEM) followed by lead citrate and observed by transmission electron microscopy (JEM-1400; JEOL) at an accelerating voltage of 80 kV.

**Linkage mapping**

Among the candidate mutants, the ME778, ME966 and ME2329 lines were crossed with *L. japonicus* B-129 to construct a genetic linkage map of the causative genes. The F₂ plants were inoculated with *R. irregularis* spores. After 3–4 weeks cultivation, plants showing low or no AM colonization or the presence of small and stunted arbuscules were selected. Genomic DNA was prepared from leaves of the F₂ plants. Briefly, one leaf from each plant was crushed using a multi-beads shocker (Yasui Kikai) (1,000 r.p.m., 10 s, three times) in 50 µl of extraction buffer (200 mM Tris–HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS). Then 100 µl of ethanol was added to the extract. After centrifugation (16,000 r.p.m., 5 min), 200 µl of distilled water was added to each pellet to dissolve the extracted DNA. After centrifugation, 100 µl of the supernatant was used for PCR amplification as a template DNA.

The genetic linkage map was constructed by using SSR markers developed by the Kajusa DNA Research Institute (http://www.kajusa.or.jp/lotus/markerdb_index.html). To identify the chromosome on which a mutated locus of ME778, ME966 or ME2329 is located, 39 SSR markers were used (chromosome 1, TM0523, TM0193, TM0113 and TM0295; chromosome 2, TM1456, TM0660, TM0400, TM0225, TM0377, TM1455, TM0120, TM1150, TM0230, TM0257, TM0021, TM0304, TM0796, TM0504, TM0889, TM0011 and TM0002; chromosome 3, TM0080, TM0035, TM0049 and TM0786; chromosome 4, TM0182, TM0030, TM0555, TM0046, BM1174 and TM0069; chromosome 5, TM0077, TM0186, TM1323 and TM0218; and chromosome 6, TM3020, TM3031, TM0013 and TM0336). The primers of each SSR marker were labeled with FAM or HEX and used for PCR amplification. Two PCR products labeled with FAM and HEX were mixed and analyzed by polyacrylamide gel (6% acrylamide and 7 M urea in TBE buffer) electrophoresis. DNA bands were analyzed using a Molecular Imager Pharos FX Plus System (BioRad).

**Gene expression analyses**

*Lotus japonicus* MG-20 and the ME2329 mutant were inoculated with 500 spores of *R. irregularis* in 50 ml pots filled with sand supplied with half-strength Hoagland’s solution containing 100 µM Pi. Total RNA of the roots was extracted using...
RNAiso Plus (TAKARA) and was treated with TURBO DNA-free DNase (Life Technologies) following the manufacturer’s instructions. First-strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). The gene-specific primers for quantitative real-time PCR are listed in Supplementary Table S2. Real-time PCR was performed using a StepOne Real-Time PCR System (Life Technologies) with a Power SYBR Green PCR Master Mix (Life Technologies). Expression levels were normalized on the basis of LjUBC quantity. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. All reactions were performed with three biological replicates.

Sequencing

Fragments of NENA (Groth et al. 2010), LjSTR (Zhang et al. 2010) and LjRAM2 were amplified with the primers (Supplementary Table S2) from genomic DNA. The PCR products were Sanger-sequenced using a BigDye Terminator Kit, version 3.1 (Life Technologies).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


