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Corresponding Author: Dr T. Kojima; National Agriculture and Food Research Organization (NARO) Institute of Livestock and Grassland Science, 768 Senbonmatsu, Nasushiobara, Tochigi, 329-2793 Japan, kojima@naro.affrc.go.jp; Tel, +81-287-37-7208.

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Isolation and phenotypic characterization of *Lotus japonicus* mutants specifically defective in arbuscular mycorrhizal formation

Tomoko Kojima[1,8*], Katsuharu Saito[2, 8], Hirosuke Oba[1,6], Yuma Yoshida[2], Junya Terasawa[2], Yosuke Umehara[3], Norio Suganuma[4], Masayoshi Kawaguchi[5] and Ryo Ohtomo[1,7]

[1]National Agriculture and Food Research Organization (NARO) Institute of Livestock and Grassland Science, Nasushiobara, Tochigi, 329-2793 Japan

[2]Faculty of Agriculture, Shinshu University, Minamiminowa, Nagano, 399-4598 Japan

[3]National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, 305-8602 Japan

[4]Department of Life Science, Aichi University of Education, Kariya, Aichi, 448-8542 Japan

[5]National Institute of Basic Biology, Okazaki, Aichi, 444-8585 Japan

[6]Present address: The University of Tokyo, Nikko, Tochigi, 321-1435 Japan

[7]Present address: NARO Hokkaido Agricultural Research Center, Sapporo, Hokkaido, 062-8555 Japan

[8]These authors contributed equally to this work

[*]Corresponding author: E-mail, kojima@naro.affrc.go.jp; Tel, +81-287-37-7208; Fax, +81-287-36-6629.

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

Footnotes: The nucleotide sequence of *LjRAM2* and *LjSTR* have been submitted to the DNA Data Bank of Japan under the accession number AB830530 and AB830531, respectively.

Abstract

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*-homologue 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 with arbuscules was observed in the two mutants. The responsible genes 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

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 gymnosperm, angiosperm, fern, and bryophyte (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 more than 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 (Bonfante and Solesse 2010, Brundrett 2004). 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, Garcia-Garrido et al. 2009). Meanwhile, host plants percept 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 Schultze 2001, Barker and Larkan 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 *SYMRK* (Stracke et al. 2002), two ion channels (*CASTOR* and *POLLUX*; Imaizumi-Anraku et al. 2005), three nucleoporins [*NUP85* (Saito et al. 2007), *NUP133* (Kanamori et al. 2006) and *NENA* (Groth et al. 2010)], calcium and calmodulin-dependent kinase (*CCaMK*; Lévy et al. 2004, Mitra et al. 2004, Tirichine et al. 2006) and *CYCLOPS* (Yano et al. 2008) in *L. japonicus*

62 (Stougaard 2001, Kistner and Parniske 2002, Parniske 2008). RN symbiosis occurred approximately
63 60 million years ago (Doyle 2011), much later than AM symbiosis. Therefore, it has been postulated
64 that RN symbiosis recruited a part of the genetic system in AM (Parniske 2008).

65 The common symbiosis mutants affect distinct steps of AM fungal accommodation (Kistner et
66 al. 2005). In the wild type of *L. japonicus*, AM fungal hyphae attach to the root surface and form
67 hyphopodia from which penetrating hyphae emerge (Bonfante et al. 2000). The penetrating hyphae
68 separate two adjacent epidermal cells and pass into these epidermal cells through a prepenetration
69 apparatus (PPA) that is derived from host as a tunnel-like structure (Genre et al. 2005). This hyphal
70 penetration is followed by hyphal extension in the intercellular space of the cortex and arbuscule
71 formation in the inner cortical root cells. In symbiotic mutants of *symrk*, *castor*, *pollux*, *cyclops* and
72 three nucleoporin genes, AM fungal hyphae separate the two adjacent epidermal cells, but cannot
73 penetrate into the epidermal cells, where the hyphae swell and terminate further extension (Wegel et al.
74 1998, Bonfante et al. 2000, Senoo et al. 2000, Novero et al. 2002, Demchenko et al. 2004, Kistner et al.
75 2005, Saito et al. 2007, Groth et al. 2010). In contrast, a *ccamk* mutant has been shown to block
76 hyphal penetration at the root surface (Senoo et al. 2000, Demchenko et al. 2004, Kistner et al. 2005).
77 Occasionally, the mutants of the common symbiotic genes allow AM fungal accommodation, especially
78 in the late growth stage (Novero et al. 2002, Demchenko et al. 2004). However, in *cyclops* and *ccamk*
79 mutants, arbuscule formation was almost completely blocked, indicating that CYCLOPS and CCaMK
80 are required for establishment of intracellular accommodation of AM fungi (Demchenko et al. 2004,
81 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. The *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 *STRs* (Zhang et al. 2010, Gutjahr 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 M3 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” (Table S1).

In order to re-evaluate the mycorrhizal phenotype of the mutant candidates, we performed a second screening using the M3 lines. A total of 32 M3 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 (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 (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 versus 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 *L. 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 ($\chi^2 = 0.244$, $P = 0.621$). 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 (Figs. 1 A, D). The hyphae emerged from the hyphopodium transverse to the root epidermal cells (Fig. 1 D), and this was followed by the extension of intraradical hyphae in the intercellular space of the cortex (Figs. 1 D, E) and arbuscule formation in the inner cortical cells (Figs. 1 B, E, F). Vesicles were often observed in the cortex colonized with *R. irregularis* (Figs. 1 B, 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. 1 C). 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. 2 A and 3 A). The hyphopodia localized on the boundary of two adjacent epidermal cells along the longitudinal axis (Figs. 2 B, D and 3 B, D, E). Penetrating hyphae emerging from the hyphopodia entered between two adjacent epidermal cells, but terminated their growth and caused abnormal swelling in the epidermis (Figs. 2 D, E and 3 D, E). 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. 2 F and 3 F). 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. 2 F and 3 F). 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. 4 G). Notably, arbuscular and vesicular colonization of ME2329 mutant were much lower than that in wild-type plants (Figs. 4 H, I), and the mutants showed abnormal arbuscule formation (Figs. 4 A, 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 (Figs. 1 E, F). In the ME2329 mutant, AM fungal hyphae penetrated the epidermis and spread between cortical cells, as in the wild type (Figs. 4 D, E). The hyphae then penetrated inner cortical cells and formed thick trunk hyphae (Fig. 4 F); however, the development of fine branches from the trunk hypha was arrested. In the cortical cells containing stunted arbuscules, starch granules stained with iodine were often observed (Figs. 4 B, 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 (Figs. S1 A-D). The number of infection threads per plant was not significantly different among these plant lines (Fig. S1 E). The ME778, ME966 and ME2329 lines formed pink nodules (Figs. S2 A, E, I, M) that contained both infected and uninfected cells, as seen in the wild type (Figs. S2 B, F, J, N). The infected cells were filled with numerous bacteroids (Figs. S2 C, G, K, O) whose ultrastructure was not different between the wild type and the three mutants based on transmission electron microscopic analysis (Figs. S2 D, H, L, P).

Linkage mapping

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

192 Mapping analysis of ME966 indicated that a mutated locus is located south of chromosome 2,
193 near the TM0504 and TM0889 markers (Fig. 5 B). We noticed that the *NENA* gene, which encodes for
194 SEH1-like nucleoporin and is required for infection by AM fungi and rhizobia, is located near this region.
195 A *nena* mutant was originally isolated as a mutant affected in AM development, but later was found to
196 be also arrested in nodulation (Groth et al. 2010). Therefore, it is possible that ME966 carries a
197 mutation in *NENA* gene. However, one co-segregating marker, TM0796 of the *NENA* gene, was
198 located outside the target region of ME966. Furthermore, no mutation was found in the genomic
199 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. 5 C). Notably, the orthologue 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*-homologue 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 an *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 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 upregulated. *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 upregulated in inoculated roots of the ME2329 mutants to similar levels as in wild-type plants.

220 **Discussion**

221 Genetic studies on AM development in leguminous plants have been increasing since the late 1990s
222 (Barker and Larkan 2002). As a result of these studies, the common symbiosis pathway involved in
223 both AM and RN formation has been identified, as have several symbiotic genes (Parniske 2008).
224 However, knowledge of the specific pathways involved in AM symbiosis remains limited (Gobbato et al.
225 2012, Wang et al. 2012, Groth et al. 2013). In this study, we successfully isolated three mutants
226 specifically defective in AM symbiosis from EMS-mutagenized plants (Table 2). Among the isolated
227 mutants, ME778 and ME966 were severely arrested at an early stage of mycorrhizal development. To
228 the best of our knowledge, the previously identified AM-specific genes related to the early stage are the
229 GRAS-type transcription factor *RAM1* (Gobbato et al. 2012) and the glycerol-3-phosphate
230 acyltransferase *RAM2* (Wang et al. 2012) in *M. truncatula*. RAM1 functions in the activation of gene
231 expression in an AM-specific pathway downstream of the common symbiosis pathway by interacting
232 with NSP2, which is also a GRAS-type transcription factor required for both AM and RN symbiosis
233 (Lauressergues et al. 2012). The mutated genes in the ME778 and ME966 lines may be involved in
234 this early stage of mycorrhization. Segregation of the Myc^- phenotype in the F2 population of ME778
235 and in the wild type was not significantly different from the 9:7 (wild type versus mutant) ratio, which
236 seems to indicate that ME778 has a digenic trait. However, genotyping of ME778 did not clearly
237 indicate two loci of mutated genes, though at least one mutated locus was estimated to occur on the
238 short arm of chromosome 2. Consequently, we cannot rule out the possibility that the ME778 mutant
239 shows complicated patterns of inheritance of the Myc^- phenotype. In that case, the standard genetic

240 mapping procedures used in the present study might be limited in their ability to detect the causative
241 genes of ME778. ME966 revealed a monogenic recessive trait located on the long arm of chromosome
242 2. Notably, *NENA*, which is required for both AM and RN symbiosis (Groth et al. 2010), was found to be
243 located around the identified target region of ME966; however, further mapping analysis indicated that
244 *NENA* was located outside this region. In addition, we were unable to detect any mutation in *NENA* of
245 ME966. Finally, sequencing of *LjRAM2* in the ME966 line did not reveal any mutation (data not shown).
246 These results indicate that the ME966 line may have a mutation in a novel locus that is required for AM
247 formation, although further mapping analysis and allelism tests are required to elucidate whether the
248 causative gene is novel. Once the causative genes of ME778 and ME966 have been identified, they
249 may improve our knowledge of AM-specific molecular mechanisms.

250 Microscopic observation of ME778 and ME966 revealed blocking of epidermal penetration by
251 AM fungi. In the two mutants, AM fungi formed abnormal balloon-like swollen hyphae between
252 epidermal cells, and terminated hyphal growth there. Occasionally, hyphal colonization with normal
253 arbuscules was observed, especially at later stages of AM development. This phenotypic
254 characteristics of the two mutants were similar to those of the phenotype of certain mutants of common
255 symbiosis genes, such as *symrk*, *castor*, *pollux* and nucleoporins, but not *ccamk* (Kistner et al. 2005).
256 Three categories of AM common symbiosis mutants have been recognized (Parniske 2008): type I
257 mutants are characterized by impaired epidermal opening, type II mutants by impairment of
258 intracellular passage through the outer layer, and type III mutants by impaired arbuscule formation.
259 The common symbiosis mutants *symrk*, *castor*, *pollux*, and nucleoporins are categorized into type II;

the impairment of intracellular passage in these mutants occurs when the fungal hyphae form a swelling structure between two adjacent epidermal cells and cannot penetrate the epidermal cells (Bonfante et al. 2000, Demchenko et al. 2004, Kistner et al. 2005, Saito et al. 2007). The ME778 and ME966 mutants are also categorized into type II, indicating that their causative genes are indispensable for the early step of 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. Thus, the causative genes of the two mutants are in an AM-specific pathway, although it remains unclear whether this pathway is located upstream, downstream or parallel to the common symbiosis pathway.

In contrast to ME778 and ME966, the ME2329 line revealed a defect in arbuscule development. To date, several genes, such as *Medicago PT4* (Javot et al. 2007) and *STR* (Zhang et al. 2010), have been shown to be involved in arbuscule formation or maintenance. A mutated locus of ME2329 was mapped at the south end of chromosome 4 near an orthologue of *STR*. Furthermore, ME2329 showed a nonsense mutation in the *STR* gene. The *Medicago* and rice *STR* genes encode a half-size ABC transporter in the ABCG subfamily and are essential for arbuscule development (Zhang et al. 2010, Gutjahr et al. 2012). It can be inferred, therefore, that the formation of stunted arbuscules in ME2329 is caused by a mutation in the *Lotus STR* gene. Six AM marker genes tested in this study were induced in response to *R. irregularis* infection even in ME2329 mutant. However, the transcript levels of arbuscule-related genes *LjPT4*, *LjAMT2;2* and *LjSTR* were extremely low compared to those of wild type. This may be due to severely impaired arbuscule formation of ME2329 mutant. *SbtM1*, *SbtM4* and *SbtS* are upregulated during the early stages of AM formation, but the three subtilase

genes show distinct expression patterns in symbiosis (Takeda et al. 2009). Expression of *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 were diminished in ME2329, which was similar to the arbuscule-related genes *LjPT4*, *LjAMT2;2* and *LjSTR*. In contrast, transcript level of *SbtM4* in ME2329 mutant was not different from that in 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, the *SbtM4* expresses at sites of rhizobial infection such as epidermal cells near the infection threads (Takeda et al. 2009). ME2329 mutant showed epidermal penetration by AM fungi and intraradical hyphal spreading in the intercellular spaces of cortex. The induction of *SbtM4* in ME2329 mutant appears to be triggered by the fungal infection in roots independent of arbuscule formation. 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 (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 (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 much 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.

Materials and Methods

Initial screening of the arbuscular mycorrhizal mutants

Seeds of *L. japonicus* MG-20 were mutagenized by EMS-treatment. The second generation (M2) plants were used for mutant screening. *L. 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 sand paper or concentrated sulfuric acid to promote germination. Next, the seeds were sunk in 5% sodium hypochlorite solution for surface sterilization and rotated for 10 min, then washed with distilled water several times. A plastic box (6.25 × 6.25 × 9.5 cm^3) was filled with 250 ml vermiculite (size: 3 mm; Asahi-kogyo) and inoculated with 6,000 spores of *R. irregularis* DAOM197198, previously known as *Glomus intraradices* (Mycorise, Premier Tech, Canada).

Additionally, approximately 15-20 seeds per mutant line were seeded into a plastic box filled with 250 ml of vermiculite and fertilized with 65 ml of modified Hornum solution with a low phosphate concentration of 0.25 mM NaH_2PO_4 (Pajuelo and Stougaard 2005) (batch culture system). They were cultivated in a growth chamber (day: 16 h, 25°C; night: 8 h, 22°C) for 4 weeks, after which the roots were washed and the plants were cut into roots and shoots. The roots were used for staining of AM colonization by a modified method using 24-well plates (Brundrett et al. 1996). The roots of each plant were cleared in 1 ml of 10% (w/v) KOH in separated wells. The roots in the plates were maintained at room temperature overnight, and then incubated for 1 h at 80°C, after which 1 ml of 3 M HCl was added to each well. Next, the solution in each well was replaced with 0.5 ml of 0.05% trypan blue, and the roots in the plates were incubated at 80°C for 30 min. After removing the trypan blue, the roots were washed once with distilled water, and 200 µl of water was added to each well for observation under a dissecting microscope. The shoots were placed in water before moving to the next step of screening. If a plant showed no or low AM colonization compared with the wild type, the shoot was first cultivated in a small plastic box with watered vermiculite for one week to promote root growth, then planted in a glass tube (2.5 cm ϕ × 10 cm in height) filled with 40 ml 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 M3 generation were harvested.

Second screening of the arbuscular mycorrhizal mutants

For the second screening, 16 seedlings of the M3 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 one week to promote root growth, after which they were planted in vermiculite inoculated with *M. loti* Tono (Kawaguchi et al. 2002). The *M. loti* was prepared as follows. One colony on YM agar medium (Keele et al. 1969) was inoculated in 60 ml of YME medium (Sherwood 1970) and incubated at 28°C for 4 days with rotation at 160 rpm. The cells were collected by centrifugation (4,000×*g*, 10 min) and washed twice with sterilized distilled water. The bacterial pellet originating from the 120-ml culture was 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 in eachplastic box. After cultivation for 4 weeks, the number of nodules was counted and candidate mutant lines whose phenotype was Myc$^-$ or Nod$^+$ were selected for the next analysis.

Segregation analysis of the candidate mutants

The M3 or M4 generation plants of the candidate mutant lines were back-crossed with *L. japonicus* MG-20. At least 47 back-crossed F2 plants (Table 2) were inoculated with approximately 2,000 spores of *R. irregularis* and grown in the batch culture system. AM fungal colonization was checked under a dissecting microscope after the roots were stained with trypan blue as described above.

Microscopic observation of AM fungal colonization

L. 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} NH_4NO_3, 0.027 g L^{-1} KH_2PO_4, 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 hyphal, arbuscular and vesicular colonization of trypan blue-stained roots was determined as the percentage of root length colonization using a magnified intersection method at ×200 (McGonigle et al. 1990). For WGA staining, roots were fixed in a solution of ethanol and acetic acid at a 3:1 ratio. The roots were rinsed with pure water and incubated in 10% KOH for 10 min at 90°C in a water bath. After rinsing twice with phosphate buffered saline (PBS), the roots were incubated in PBS overnight 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 that were stained with trypan blue as described above were incubated overnight in a solution containing 1% iodine and 1% KI at room temperature. After rinsing with pure water, the roots were observed using a light microscope.

398

399 **Infection thread formation**

400 In order to observe infection thread formation, *L. japonicus* MG-20, ME778, ME966 and ME2329 were
401 transplanted into pots filled with vermiculite and inoculated with *M. loti* MAFF303099 constitutively
402 expressing DsRed (Maekawa et al. 2009). The plants were grown in a growth chamber at 26°C on a
403 16-h light/8-h dark cycle. The plants were supplied with N-free B&D solution. Roots were harvested at
404 2 weeks after inoculation. Fluorescence of DsRed was observed using an Axio Imager D1
405 epifluorescence microscope (Carl Zeiss). Digital images were captured with an AxioCam digital CCD
406 camera (Carl Zeiss) operated with AxioVisio software (Carl Zeiss).

407

408 **Observation of root nodule development**

409 Plant roots were harvested 4 weeks after the inoculation of *M. loti* MAFF303099. Nodules cut from
410 roots were fixed in 2% paraformaldehyde and 2% glutaraldehyde in HEPES buffer (pH 7.0) at room
411 temperature for 2 h. After rinsing with the HEPES buffer, samples were post-fixed in 2% OsO_4 in
412 HEPES buffer for 1 h at room temperature and washed three times with pure water, dehydrated in an
413 ethanol series (50, 70, 80, 90, 95 and 100%) and substituted with propylene oxide for 5 min twice. The
414 nodules were then infiltrated with Spurr resin (Polysciences). The resin was polymerized for 12 h at
415 70°C. Semi-thin sections (approximately 0.2 μm) were cut and stained with 0.1% toluidine blue O for
416 optical microscopic observation with an Axio Imager D1 light microscope. Ultra-thin sections

(approximately 80 nm) were stained with Ti blue (NisshinEM) followed by lead citrate and observed by TEM (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 to 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 rpm, 10 sec, 3 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 rpm, 5 min), 200 µl of distilled water was added to each pellet to dissolve the extracted DNA. After centrifugation, 100 µl supernatant was used for PCR amplification as a template DNA.

The genetic linkage map was constructed by using single sequence repeat (SSR) markers developed by the Kazusa DNA Research Institute (http://www.kazusa.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; chromosome 6: TM0302, TM0331, 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

L. 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 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 (Table S2) from genomic DNA. The PCR products were Sanger-sequenced using a BigDye Terminator Kit, version 3.1 (Applied Biosystems).

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Table

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.

Lines	Hyphal colonization %	Arbuscular colonization %	Vesicular colonization %
Wild type (MG-20)	72 ± 17*	72 ± 17	62 ± 16
ME778	< 1	< 1	< 1
ME966	2 ± 4	2 ± 4	2 ± 3

* mean ± SD.

Table 2 Phenotypic characterization and segregation analysis of mycorrhizal mutants of *Lotus japonicus*.

Mutant lines	Phenotype of M3 plant	F2 segregation (Wild type: mutant)	*P* value (χ^2 test*)
ME778	Blocking hyphal penetration at epidermis	61:43	0.000
ME966	Blocking hyphal penetration at epidermis	71:21	0.630
ME2329	Stunted arbuscule	62:28	0.181

* Expected frequencies are calculated based on 3:1 ratio.

Figure legends

Fig. 1 Arbuscular mycorrhizal colonization in the wild type of *Lotus japonicus*. (A-C) Bright field images of roots stained with trypan blue. Hyphae of *Rhizophagus irregularis* DAOM197198 attached to the root surface and entered into the root (A). Arrow shows entry point of the hyphae. The hyphae spread into the intercellular space of the cortex and then formed arbuscules and vesicles (B). *Gigaspora margarita* MAFF520054 also formed arbuscules but not vesicles in the roots (C). (D-F) Fluorescent images of *R. irregularis* hyphae stained with WGA conjugated with Oregon Green 488 and observed using a confocal laser scanning microscope. The plant cell wall was stained with propidium iodide (red). *R. irregularis* formed hyphopodia, penetrated the epidermis (arrowhead), spread into the intercellular space and formed mature arbuscules (D and E) which consisted of trunk hyphae and fine branches (F). a, arbuscule; e, extraradical hypha; f, fine branch of arbuscule; h, hyphopodium; i, intraradical hypha; s, spore; t, trunk hypha of arbuscule; v, vesicle. Bars = 50 µm (A-C) and 20 µm (D-F).

Fig. 2 Arbuscular mycorrhizal colonization in the ME778 mutant. (A-C) Bright field images of roots stained with trypan blue. Extraradical hyphae of *R. irregularis* (A and B) and *G. margarita* (C) attached to the root surface but could not enter into the root. Arrow shows the fungal entry blocked in the epidermis. (D-F) Fluorescent images of *R. irregularis* hyphae stained with WGA-Oregon Green 488 and observed using a confocal laser scanning microscope. The plant cell wall was stained with propidium iodide (red). Fungal hyphae attached to the root surface, formed swollen hyphal structures from

hyphopodia and terminated their growth between two adjacent epidermal cells (asterisks) (D and E). Once it had penetrated the epidermis, *R. irregularis* formed arbuscules in cortical cells (F). a, arbuscule; e, extraradical hypha; h, hyphopodium; i, intraradical hypha. Bars = 50 µm (A-C) and 20 µm (D-F).

Fig. 3 Arbuscular mycorrhizal colonization in the ME966 mutant. (A-C) Bright field images of roots stained with trypan blue. Extraradical hyphae of *R. irregularis* (A and B) and *G. margarita* (C) attached to the root surface but could not enter into the root. Arrow shows the fungal entry blocked in the epidermis. (D-F) Fluorescent images of *R. irregularis* hyphae stained with WGA-Oregon Green 488 and observed using a confocal laser scanning microscope. The plant cell wall was stained with propidium iodide (red). Two focal planes of the root surface: the surface of the epidermis (D) and cell layer of the epidermis (E). The fungus formed a hyphopodium that emerged from an extraradical hypha on the root surface and entered between two adjacent epidermal cells (asterisks). However, the hypha stopped its growth there and formed a swollen structure (arrowhead). Occasionally, arbuscules were formed in cortical cells (F). a, arbuscule; e, extraradical hypha; h, hyphopodium; i, intraradical hypha; v, vesicle. Bars = 50 µm (A-C) and 20 µm (D-F).

Fig. 4 Arbuscular mycorrhizal colonization in the ME2329 mutant. (A-C) Bright field images of roots stained with trypan blue. *R. 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. *R. 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. 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 (closed circles), and ME2329 mutant (open circles) inoculated with *R. irregularis*. Error bars show the standard error of the means (n = 3).

Fig. 5 Genetic mapping of mutated loci. (A) Segregation of genotypes in the F2 population of ME778 (MG-20 background) crossed with wild type B-129 on chromosomes 1 to 6. Molecular markers are indicated on the horizontal axis. Numbers shown in parentheses are genetic distance (cM) on the linkage group. Mutated loci of ME966 (B) and ME2329 (C) were located on chromosomes 2 and 4, respectively. The names of the molecular markers and AM-related genes are indicated above the horizontal lines. The numbers of recombination events that occurred between the mutated locus and the molecular markers are indicated below the lines. Molecular markers near the mutated loci are shown in bold. Scales indicate genetic distances (cM).

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

Fig. 1

MG20

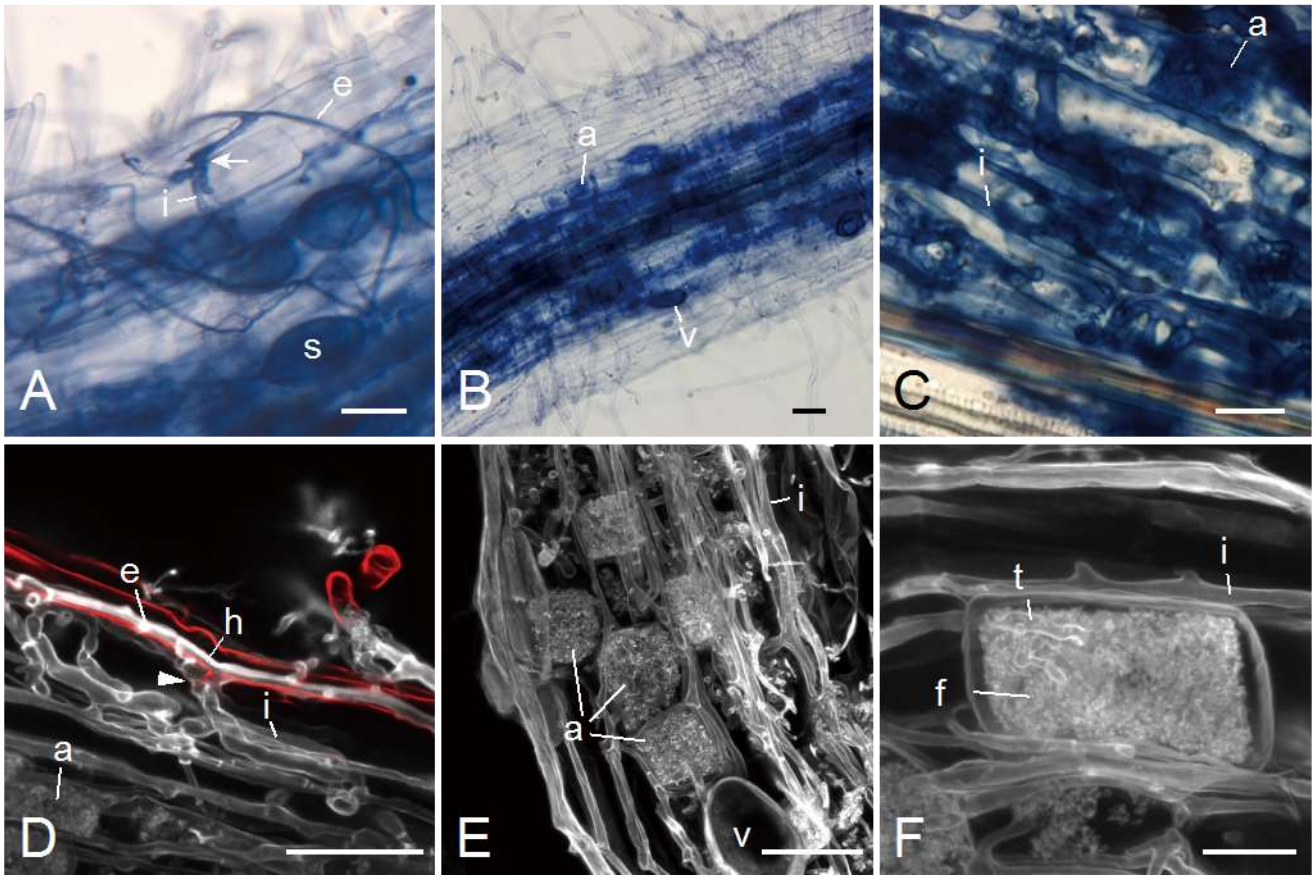


Fig. 2

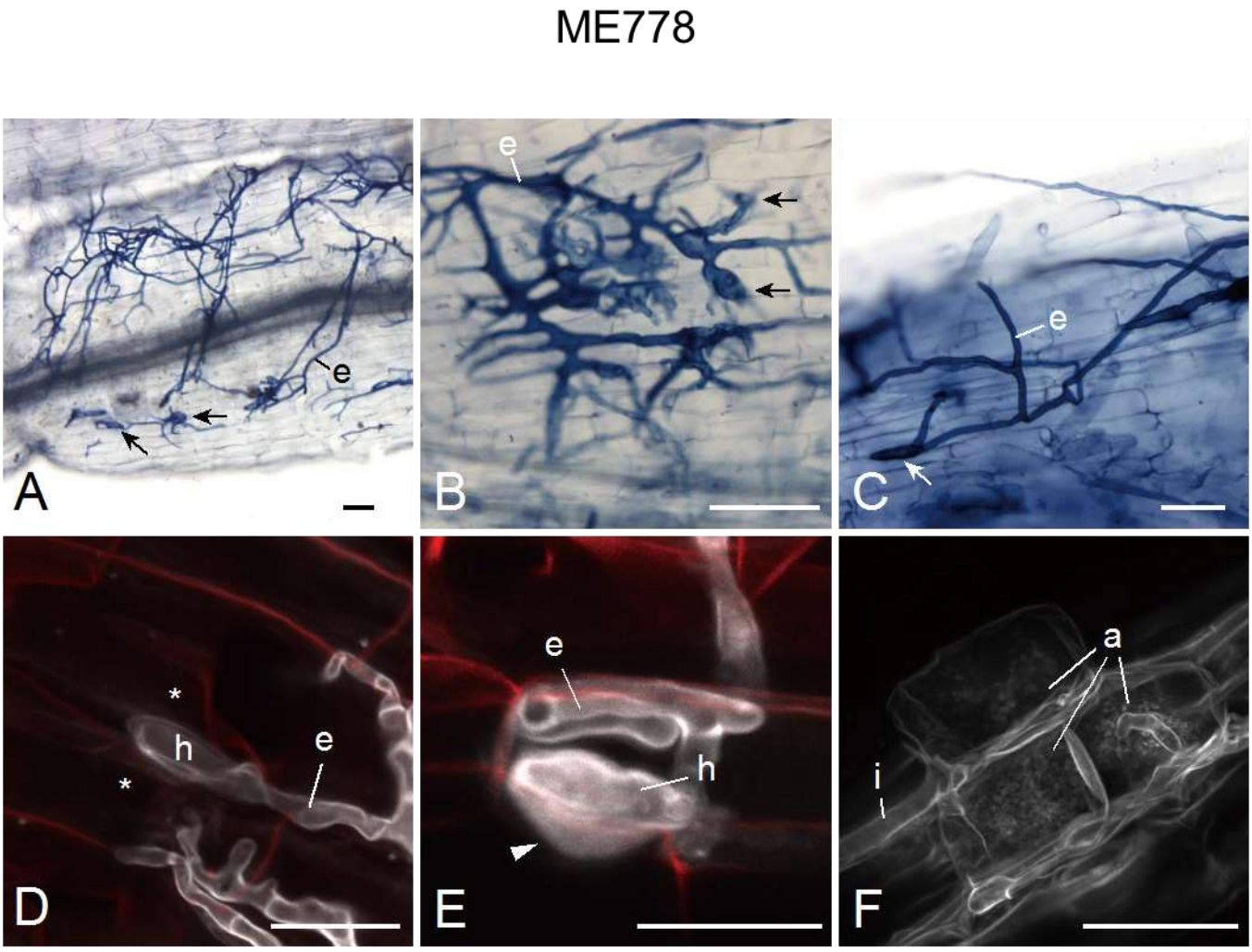


Fig. 3

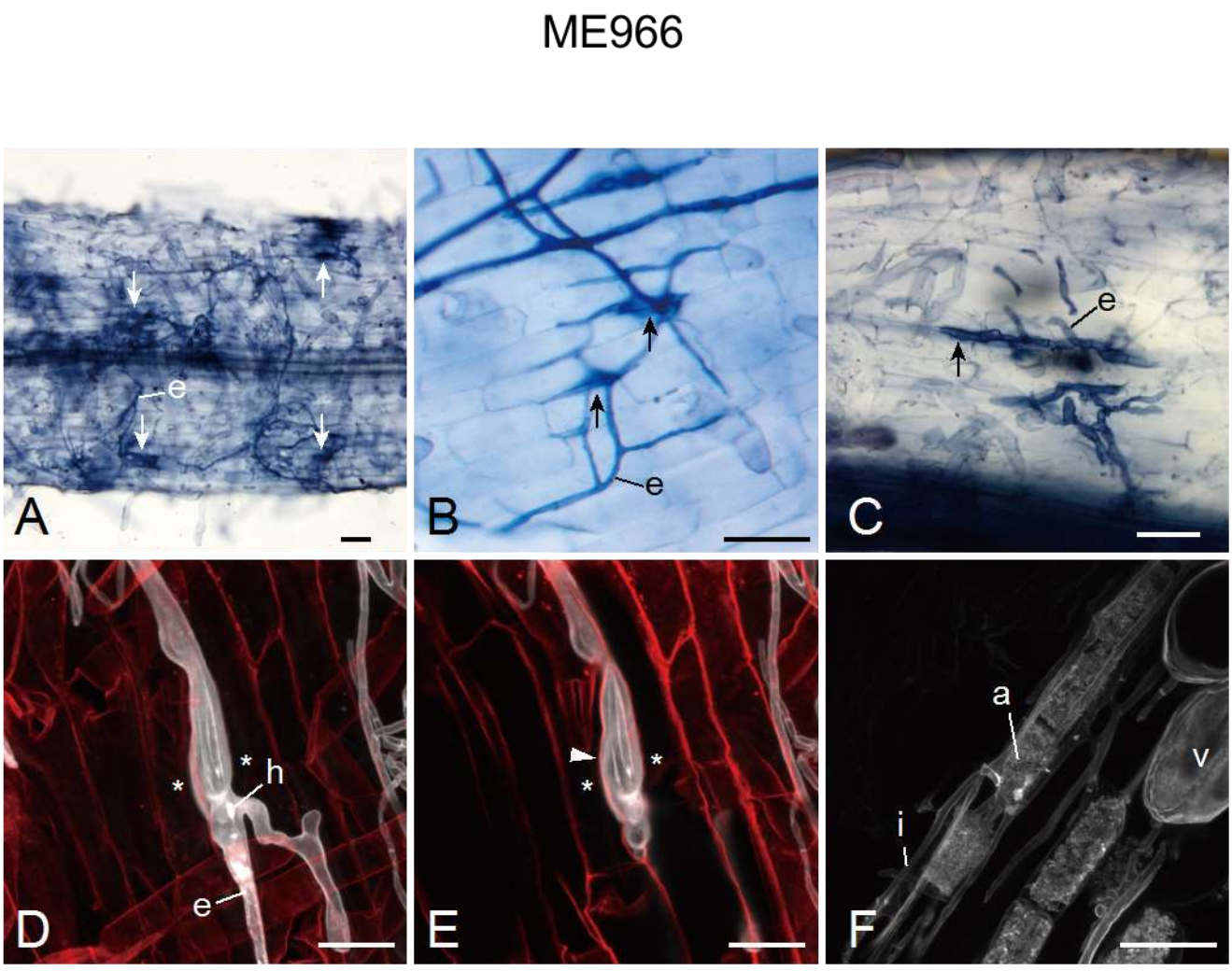


Fig. 4

ME2329

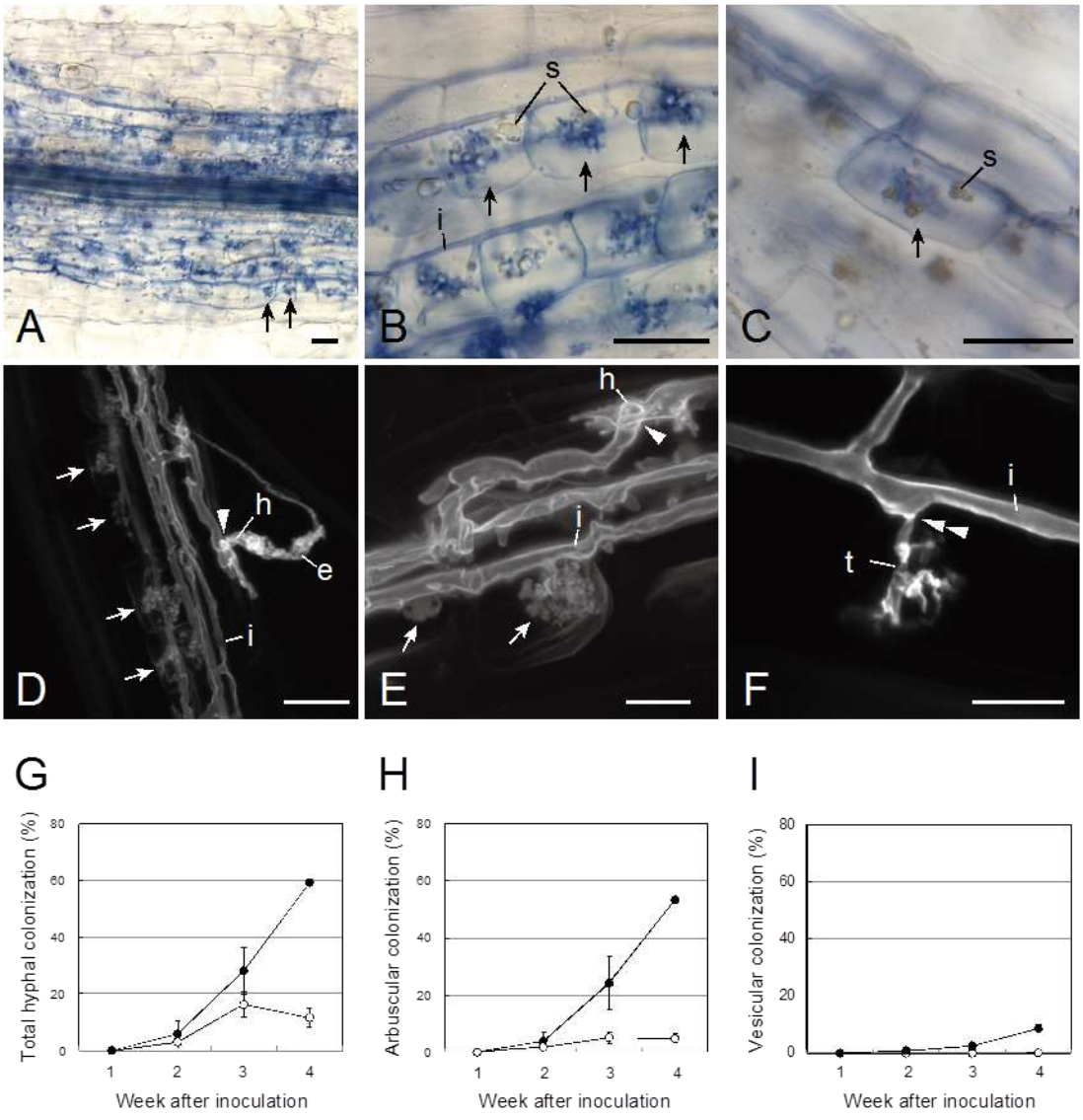

A
B
s
i
C
s
D
h
e
i
E
h
i
F
i
t
G
Total hyphal colonization (%)
0
20
40
60
80
1
2
3
4
Week after inoculation
H
Arbuscular colonization (%)
0
20
40
60
80
1
2
3
4
Week after inoculation
I
Vesicular colonization (%)
0
20
40
60
80
1
2
3
4
Week after inoculation

Fig. 5

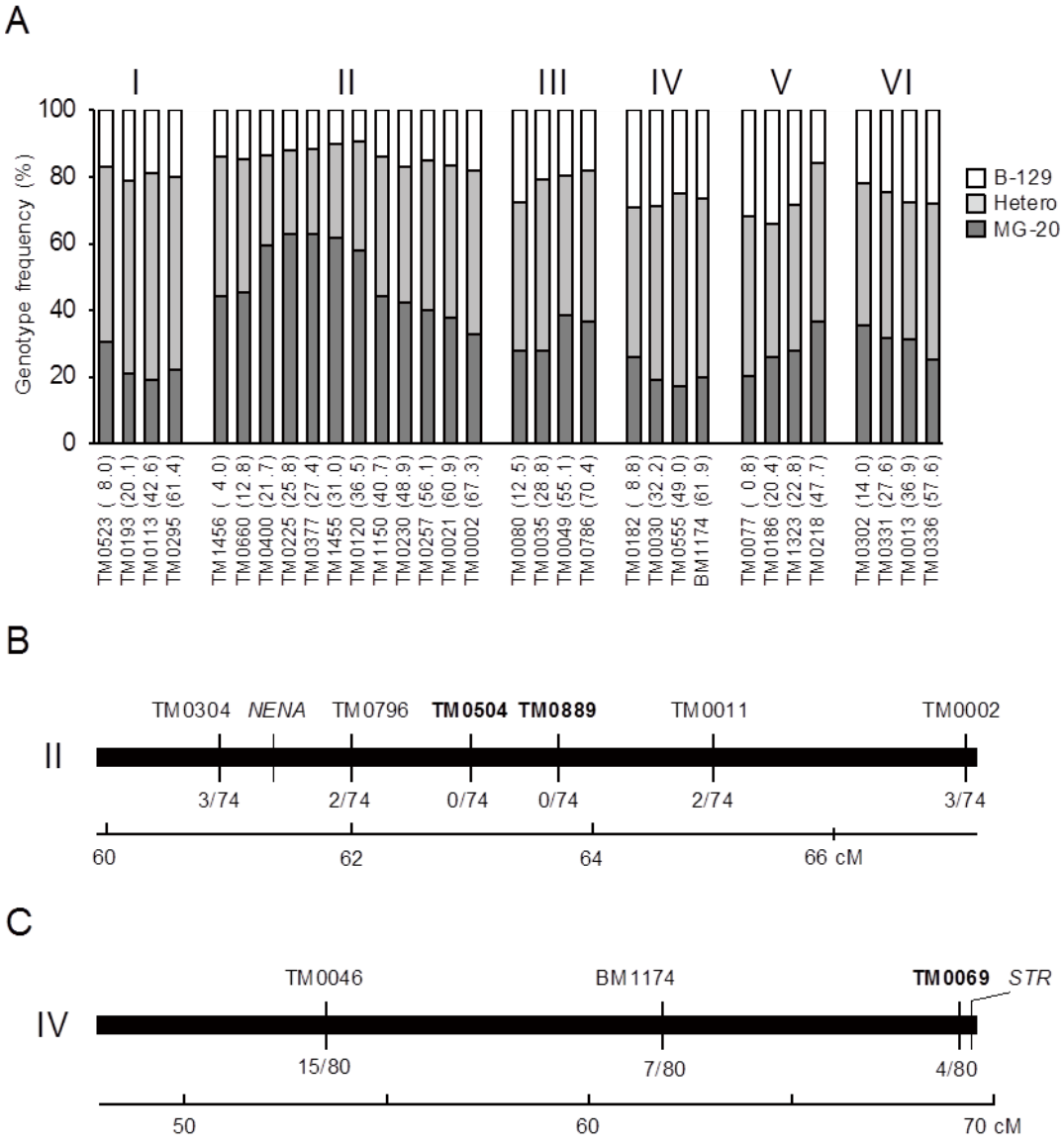

A
I
II
III
IV
V
VI
100
80
60
40
20
0
Genotype frequency (%)
B-129
Hetero
MG-20
TM0523 (8.0)
TM0193 (20.1)
TM0113 (42.6)
TM0295 (61.4)
TM1456 (4.0)
TM0660 (12.8)
TM0400 (21.7)
TM0225 (25.8)
TM0377 (27.4)
TM1455 (31.0)
TM0120 (36.5)
TM1150 (40.7)
TM0230 (48.9)
TM0257 (56.1)
TM0021 (60.9)
TM0002 (67.3)
TM0080 (12.5)
TM0035 (28.8)
TM0049 (55.1)
TM0786 (70.4)
TM0182 (8.8)
TM0030 (32.2)
TM0555 (49.0)
BM1174 (61.9)
TM0077 (0.8)
TM0186 (20.4)
TM1323 (22.8)
TM0218 (47.7)
TM0302 (14.0)
TM0331 (27.6)
TM0013 (36.9)
TM0336 (57.6)
B
TM0304
NENA
TM0796
TM0504
TM0889
TM0011
TM0002
II
3/74
2/74
0/74
0/74
2/74
3/74
60
62
64
66 cM
C
TM0046
BM1174
TM0069
STR
IV
15/80
7/80
4/80
50
60
70 cM

Fig. 6

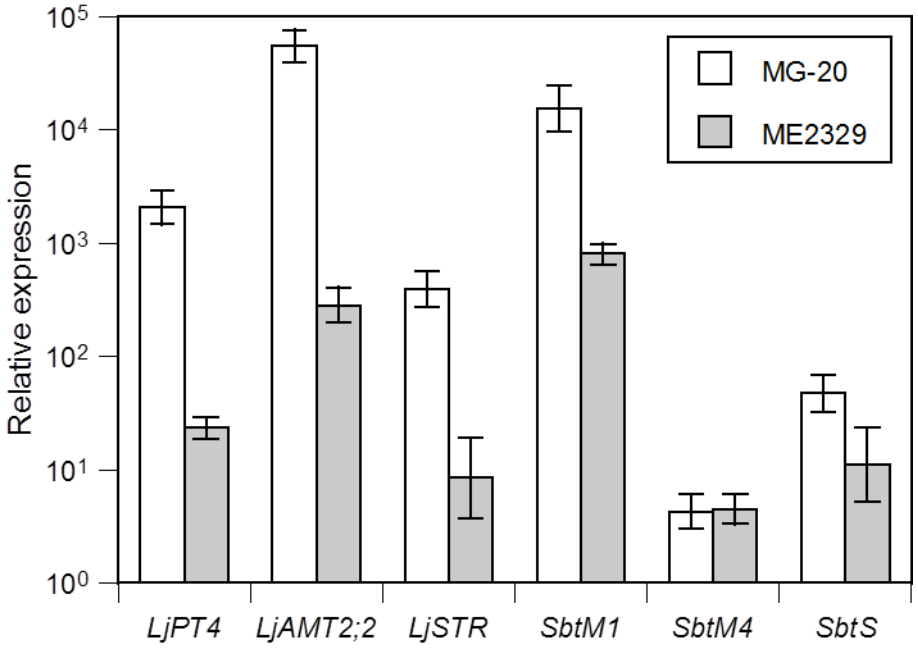
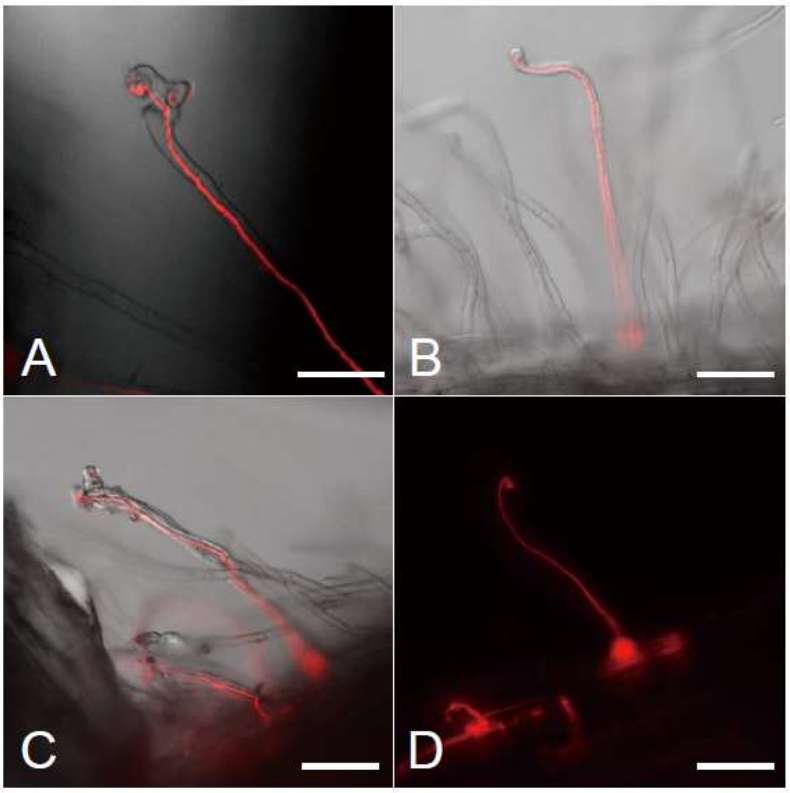

Relative expression
10^5
10^4
10^3
10^2
10^1
10^0
MG-20
ME2329
LjPT4
LjAMT2;2
LjSTR
SbtM1
SbtM4
SbtS

Table S1 Isolated mutant candidates defective in mycorrhization.

Lines	Phenotype	
	Mycorrhization	Nodulation
Wild type (MG-20)	+	+
ME181	-	-
ME356	-	-
ME459	-	-
ME733	-	-
ME778	-	+
ME781	-	-
ME797	-	-
ME798	-	-
ME801	-	-
ME823	-	+
ME833	-	+
ME843	-	-
ME930	-	-
ME966	-	+
ME989	-	-
ME1473	-	-
ME1748	-	-
ME1935	-	+
ME1949	-	-
ME2032	-	-
ME2177	-	+
ME2329	-	+
ME2555	-	+
ME2568	-	+

Table S2 Primers used for sequencing.

Target	Primer name	Direction	Sequence	Reference
PCR and sequencing				
Nena	N158m	Forward	ATGGCGAAGGAGGTGTTGAC	Groth et al. 2010 modified
	N168	Reverse	AGAAGTGGGTTCAAATGCAGCCT	Groth et al. 2010
LjRAM2	RAM2f1	Forward	TGGCCAATCCTCGTTTACAT	This study
	RAM2r1	Reverse	AGGGAGCATCAGTGAGCCTA	This study
	RAM2f2	Forward	AGCACACAGTGGTTGCTGAC	This study
	RAM2r2	Reverse	AAGTGCGGAGAATCTGAGGA	This study
	RAM2f3	Forward	CCAAAGCCCATAATCTTCCA	This study
	RAM2r3	Reverse	CTCGCTCTTGGCGTGAGTAT	This study
LjSTR	STRf1	Forward	GGCACCAACAACCAAAGAGT	This study
	STRr1	Reverse	TGTTGCTTGCAATCTGGTTC	This study
	STRf2	Forward	TTTTCCTTCTTGCCCCTTCT	This study
	STRr2	Reverse	AGCCAAAGAGTCTGGGAATG	This study
	STRf3	Forward	CTGGACAAGATCACCGTCCT	This study
	STRr3	Reverse	CAGGTGTCCATCCTGGAGTT	This study
	STRf4	Forward	TTACCCACGTTTGGCTTCTC	This study
	STRr4	Reverse	GGTTGTGGCAATGACAACTG	This study
	STRf5	Forward	CTCGTGGCGAACATTACTCA	This study
	STRr5	Reverse	GGTTACAGAAACGGGAACGA	This study
Expression analysis				
LjUBC		Forward	ATGTGCATTTTAAGACAGGG	Lohmann et al. 2010
		Reverse	GAACGTAGAAGATTGCCTGAA	Lohmann et al. 2010
LjPT4		Forward	TCCGGGCTCTCCTTTGG	This study
		Reverse	AGAAGCATAGCGTTCCCATCA	This study
LjAM2;2		Forward	ACACATGCTTGCACTGCTACC	Guether et al. 2009
		Reverse	CTGCCCATCCTTGAACAACCC	Guether et al. 2009
LjSTR		Forward	CTATATTGGTGACGAGGGAAGG	This study
		Reverse	GTCCTGAGGTAGGTTCATCCAG	This study
LjSbtM1		Forward	TGTATGCTGCTGCTGAAAAAAACAACT	Takeda et al. 2009
		Reverse	CTTCTTGACCTTTTGCAATAAATGGGATTC	Takeda et al. 2009
LjSbtM4		Forward	ATGTAAGCTATGCTGCTGGAATAGAG	Takeda et al. 2009
		Reverse	ATGCAACAGCAGGGGCTAG	Takeda et al. 2009
LjSbtS		Forward	ATTGATCACAATGCCAGAGATG	Takeda et al. 2009
		Reverse	TGTTGGGAAGATTGTAGCA	Takeda et al. 2009



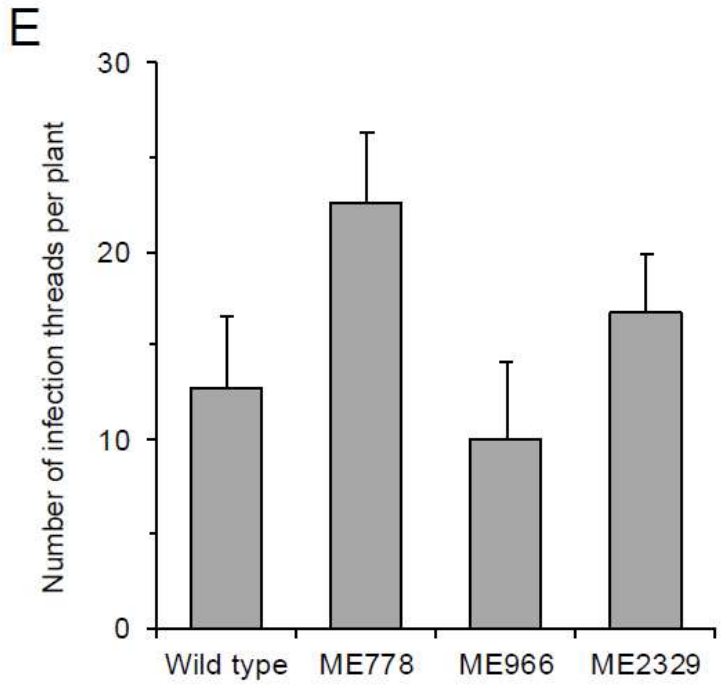


Fig. S1 Infection threads in *Lotus japonicus* wild type MG-20 (A), ME778 (B), ME966 (C) and ME2329 (D) colonized with *Mesorhizobium loti* MAFF303099 constitutively expressing DsRed. Fluorescent images of *M. loti* (red) were observed using an epifluorescence microscope. Bars are 50 μm. (E) Number of infection threads at 2 weeks after inoculation. The number of infection threads was not significantly different between the plant lines (ANOVA). Error bars show the standard error of the means (n = 4-5).

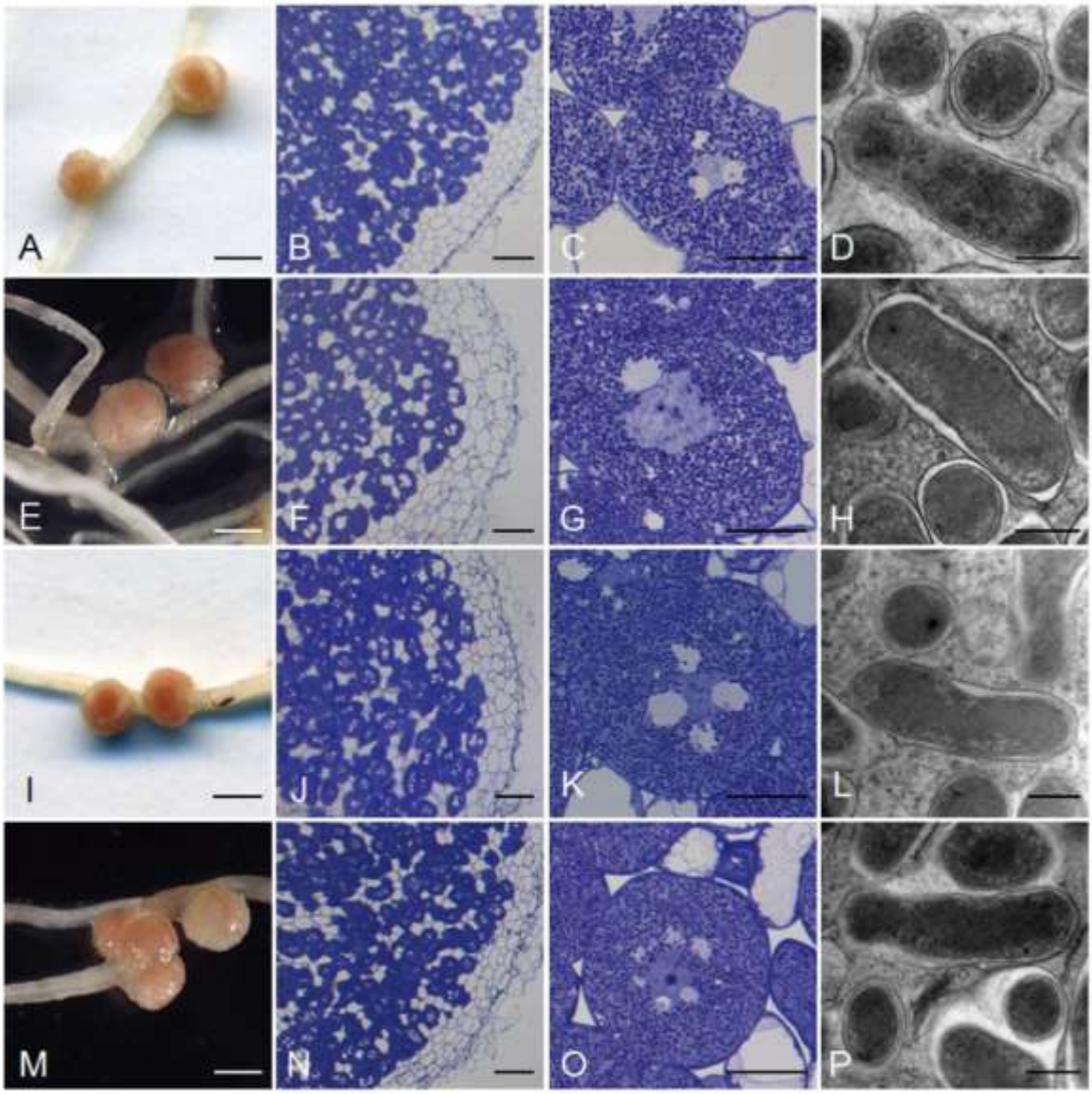


Fig. S2 Root nodule development in *L. japonicus* wild type MG-20 (A-D), ME778 (E-H), ME966 (I-L) and ME2329 (M-P) colonized with *M. loti* MAFF303099. Appearance of root nodules (A, E, I, M). Bars are 1 mm. Sections of root nodule stained with toluidine blue show infected cells and uninfected cells in the wild type (B, C), ME778 (F, G), ME966 (J, K) and ME2329 (N, O). Bars = 100 μm (B, F, J, N), = 20 μm (C, G, K, O). Transmission electron micrographs of bacteroids show that the morphology of bacteroids in ME778 (H), ME966 (L) and ME2329 (P) did not differ from that in wild type (D). Bars are 500 nm.