Myristate as a carbon and energy source for the asymbiotic growth of the arbuscular mycorrhizal fungus *Rhizophagus irregularis*

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Abstract

Arbuscular mycorrhiza (AM) is one of the most widespread mutualistic symbioses, which is formed between the majority of land plants and soil-borne fungi belonging to Glomeromycotina. AM fungi are obligate symbionts that cannot complete their natural life cycle without a host. Recent evidence suggests that lipids synthesized by a host are transferred to AM fungi that possess no fatty acid synthase genes in their genome and that mutations in lipid biosynthesis-related genes of the host lead the symbiotic interaction to fail (1-3). We hypothesized that lipids derived from plants are crucial for AM fungal growth and reproduction. In this study, we evaluated whether AM fungi can grow on medium supplied with fatty acids under asymbiotic conditions without the host. Myristate led to an extensive hyphal growth of *Rhizophagus irregularis* and an increase in biomass production. Other examined fatty acids showed no effect on biomass production. Myristate also induced secondary spore formation. The myristate-induced spores can germinate, colonize carrot hairy roots, and form the next generation of mature daughter spores. A fluorescently labeled fatty acid probe was taken up by branched hyphae of AM fungi. Tracer experiments using ^{13}C-labeled myristic acid showed that myristate and its metabolites were utilized for the synthesis of triacylglycerol and cell wall components of AM fungi. Furthermore, myristate activated ATP generation in the fungal hyphae. Here we demonstrate that myristate is utilized as a carbon and energy source for biomass production and

37 sporulation under asymbiotic conditions.

38

39 **Introduction**

40 Arbuscular mycorrhizal (AM) fungi belonging to the subphylum Glomeromycotina (4) form
41 symbiotic associations with over 70% of land plant species (5). AM fungi provide hosts with
42 minerals taken up via hyphal networks in soil and in return receive carbon sources, such as
43 sugars and lipids derived from plant photosynthates. This is regarded as an obligate symbiotic
44 relationship, where the life cycle of AM fungi is completed through colonization with their host.
45 Only a few reports have been published on AM fungal culture without hosts. One report showed
46 that the co-cultivation of the AM fungus *Glomus intraradices* with strains of *Paenibacillus*
47 *validus* separated from each other with dialysis membranes induced secondary spore formation
48 of the AM fungus (6). Another study reported that some fatty acids, including palmitoleic acid
49 and (*S*)-12-methyltetradecanoic acid, induced fertile secondary spores in asymbiotic cultures of
50 AM fungi (7). These results suggest that AM fungi may be cultured independently from host
51 plants under artificial conditions. In nature, the life cycle of AM fungi proceeds as follows.
52 Resting spores of AM fungi germinate, and then germ tubes emerge from the spores and
53 elongate into the soil. After colonization into plant roots, AM fungi form a highly branched
54 hyphal structure, called an arbuscule, in plant cortical cells, which is a site of nutrient exchange

55 between AM fungi and their hosts. After receiving carbon sources from their hosts, AM fungi
56 activate the formation of extraradical hyphal networks in soil and form spores on their hyphae.
57 A key step of AM fungal growth and reproduction is to obtain carbon sources from their hosts
58 for the production of energy and the carbon skeleton of the fungal cell components. Tracer
59 experiments and NMR analyses show that hexoses transfer to AM fungi as a carbon source (8-
60 10). Several AM fungal monosaccharide transporters have been identified (11-13). Recently,
61 lipids derived from plants have been proven to be another carbon source (1-3, 14, 15). No fatty
62 acid biosynthesis activity in extraradical hyphae has been detected, so lipids were assumed to
63 be synthesized in intraradical hyphae and transferred to extraradical hyphae and spores (10, 16).
64 However, AM fungi have no cytosolic fatty acid synthases involved in *de novo* fatty acid
65 biosynthesis in their genomes, indicating that AM fungi cannot produce long chain fatty acids
66 by themselves (17-20). During AM symbiosis, plants activate fatty acid biosynthesis and
67 transfer lipids, presumably 16:0 β-monoacylglycerol (β-MAG), to AM fungi via arbuscules (1-
68 3, 14, 15). In plant mutants that are defective in fatty acid biosynthetic genes, AM fungi cannot
69 develop arbuscules and reduce colonization in roots. These results indicate that AM fungi
70 require external fatty acids for their growth. Once AM fungi take up fatty acids, they can utilize
71 them using fatty acid desaturase and elongase genes that are present in their genomes and
72 expressed in intraradical hyphae (16-18, 20-23). Thus, we examined whether AM fungi can

73 grow and reproduce fertile spores through the application of fatty acids under asymbiotic
74 conditions.
75
76 **Results**
77 **Myristate activates AM fungal growth**
78 Initially, we screened several fatty acids and β-MAGs to identify chemicals that promote AM
79 fungal growth under asymbiotic conditions. These compounds were added to the modified
80 *Saccharomyces cerevisiae* synthetic complete (SC) medium (*SI Appendix*, Table S1) with
81 different dissolving methods: adding an aqueous solution of fatty acid salts, fatty acids and β-
82 MAGs dissolved in ethanol, or fatty acids conjugated with bovine serum albumin (BSA). Fatty
83 acid–BSA conjugates were dissolved to a final concentration of 0.5 mM, whereas potassium
84 salts of fatty acids were converted into an insoluble form due to the formation of metal soap
85 with Ca^{2+} and Mg^{2+} in SC medium. Moreover, fatty acids and β-MAGs were aggregated in the
86 solid culture (*SI Appendix*, Fig. S1). In the absence of fatty acids, no biomass increase was
87 detected in *R. irregularis* (Fig. 1*A* and *B*). After spore germination, *R. irregularis* generated a
88 thick and short subtending hypha that soon branched to produce straight-growing thick hyphae
89 (hereafter referred to as runner hyphae), with several thin lateral hyphae with a low level of
90 ramification (Fig. 1*D* and *E*). The hyphal elongation ceased within one or two weeks after

91 germination. By contrast, myristates (C14:0) among the tested chemicals substantially
92 increased fungal biomass regardless of the methods used for adding myristate (Fig. 1*A*). Fungal
93 biomass continued to increase in the culture depending on the concentration of myristate (Fig.
94 1*B*). As a consequence, the total dry weight of the two-month-old colony at 1 mM potassium
95 myristate doubled that of inoculated spores. Surprisingly, 16:0 β-MAG and palmitate (16:0),
96 candidate compounds released from arbusculated host cells during symbiosis (14, 15), did not
97 increase biomass production. During cultivation with myristate, vigorous hyphal development
98 and subsequent differentiation of secondary spores were observed (Fig. 1*F–P* and *SI Appendix*,
99 Fig. S1). After germination, *R. irregularis* differentiated a few densely packed coil (DPC)-like
100 structures from the runner hyphae in the vicinity of an inoculated spore (Fig. 1*G*). DPC is an
101 extensively branched hyphal structure, which was first observed in *G. intraradices* co-
102 cultivated with *P. validus* (24). *R. irregularis* elongated its runner hyphae as generating
103 branched hyphae similar to branched absorbing structures (BAS) (25) and expanded its habitat
104 by generating new runner hyphae (Fig. 1*H–J*). The short-branched hyphae (hereafter referred
105 to as BAS) were formed at short intervals (Fig. 1*K*). Interestingly, at the beginning of the
106 cultivation supplemented with potassium myristate, numerous precipitates of myristate salt
107 were observed throughout the growth medium; however, the precipitates around actively
108 growing hyphae were solubilized (Fig. 1*H*, *I*, and *L*). Myristate induced secondary spores,

109 which were frequently observed along the runner hyphae in the vicinity of the inoculated spores
110 (Fig. 1*M*). Myristate-induced spores also occurred apically or intercalary along the lateral
111 branches of the extensively growing runner hyphae (Fig. 1*N* and *O*). The size of the spores was
112 approximately 50 μm in diameter, which is almost half the size of inoculated spores (Fig. 1*F*,
113 *M*, and *P*). In the presence of palmitoleic acid (C16:1 Δ9) in the medium, extensive hyphal
114 branching and secondary spore formation was observed as reported by Kameoka et al. (7),
115 although this was not associated with an increase in biomass (Fig. 1*A* and *SI Appendix*, Fig.
116 S1*B*). When lauric acid (C12:0) was applied as a lauric acid–BSA conjugate, *R. irregularis*
117 showed an active runner hyphae elongation with few DPC, BAS, or secondary spores (*SI*
118 *Appendix*, Fig. S1*C*). However, this conjugate did not significantly increase fungal biomass (Fig.
119 1*A*). As the lauric acid–BSA conjugate was effective on hyphal elongation even at low
120 concentrations (1 and 10 μM), lauric acid is not likely to be utilized as a macronutrient for *R.*
121 *irregularis* (*SI Appendix*, Fig. 1*D*).

122 We further developed culture systems to promote fungal growth under asymbiotic
123 conditions. First, we examined a liquid culture of the modified SC medium containing
124 potassium myristate. The liquid culture was also effective for fungal biomass production (*SI*
125 *Appendix*, Fig. S2*A*). After germination, precipitates of metal soaps attached to the hyphal
126 surface (*SI Appendix*, Fig. S2*B*). The fungal hyphae continued to elongate and became tangled.

127 During fungal growth, the metal soaps on the surface gradually disappeared. Typical BAS was
128 not recognized due to the aggregation of the fungal hyphae, although highly branched fine
129 hyphae were observed in the presence of myristate (*SI Appendix*, Fig. S2*C*). In contrast to the
130 solid culture, very few secondary spores formed in the liquid culture. Next, an immobilized cell
131 culture system was tested, in which an inoculum of *R. irregularis* spores embedded in the center
132 of a Phytagel tablet was incubated in modified SC liquid medium (*SI Appendix*, Fig. S3*A*). This
133 culture system can prevent the aggregation of fungal hyphae and facilitate the diffusion of
134 medium components in the Phytagel tablets. Notably, AM fungal growth in the immobilized
135 cell culture system with potassium myristate was increased at 3–4-fold compared with that in
136 the solid and liquid cultures (Fig. 1*A* and *C*; *SI Appendix*, Fig. S2*A*). The hyphal elongation was
137 very active, and some hyphae spread out to the Phytagel tablets and continued to grow (Fig. 1*Q*
138 and *R* and *SI Appendix*, Fig. S3*B–D*). The elongation pattern was similar to that in the solid
139 culture. For instance, DPC-like structures, runner hyphae, BAS, and myristate-induced spores
140 were observed (*SI Appendix*, Fig. S3*E–I*). The myristate-induced spores were still smaller than
141 the inoculated spores (*SI Appendix*, Fig. S3*J*).

142 As monosaccharides are utilized by AM fungi as carbon sources during symbiosis (8-
143 10), we examined whether monosaccharides are effective on fungal growth in an immobilized
144 cell culture system. In the absence of potassium myristate, neither glucose nor xylose promoted

145 biomass production (Fig. 1*C*). Similarly, no additional effect of the monosaccharides to increase
146 biomass production was detected in the combination with potassium myristate.
147
148 **Myristate-induced spores have infection capability**
149 Myristate-induced spores began to differentiate from two weeks after inoculation in the solid
150 medium. The number of myristate-induced spores increased with time and concentration of
151 myristate and finally reached 1.4 spores per inoculated spore at 8 weeks post-inoculation (Fig.
152 2*A*). However, the spores were still half the size of the inoculated spores (Fig. 2*B*). In the
153 immobilized cell culture system, the number of myristate-induced spores was equal to that in
154 the solid culture, and the spores were further generated up to 2.5 per inoculated spore in the
155 combination with xylose (Fig. 2*C* and *SI Appendix*, Fig. S4*A*). Although the spore size and
156 thickness of spore walls were small (*SI Appendix*, Fig. S4*B* and Fig. S5*A*), the morphology of
157 the myristate-induced spores resembled that of the symbiotically generated spores (*SI Appendix*,
158 Fig. S5*A*). Both myristate-induced and symbiotically generated spores contained a large number
159 of nuclei (*SI Appendix*, Fig. S5*A*). We further evaluated the capability of the myristate-induced
160 spores as propagules. Forty-six percent of the myristate-induced spores were able to germinate
161 (*SI Appendix*, Fig. S5*B*). The abundance of germ tubes from the induced spores was less than
162 that from the symbiotically generated spores, possibly due to the small spore size (*SI Appendix*,

163 Fig. S5*C*). To test whether the myristate-induced spores can colonize plant roots, a single spore
164 generated in the immobilized cell culture system with potassium myristate and xylose was
165 inoculated to carrot hairy roots. In total, 245 spores were examined in six independent
166 experiments. Some induced spores had infectivity to the hairy roots; and as a result, mature
167 spores of the next generation were produced on the extraradical hyphae that emerged from the
168 roots (Fig. 2*D*–*G*). Approximately half of the germinated spores can colonize hairy roots and
169 produce daughter spores, albeit large variations in the germination and infectivity of spores
170 were observed among the trials due to the experimental manipulations (*SI Appendix*, Table S3).
171

172 **AM fungi utilize myristate as a carbon and energy source**

173 To address the use of fatty acids by AM fungi under asymbiotic conditions, we analyzed fatty
174 acid uptake using a fluorescent derivative of fatty acid, C_1-BODIPY 500/510 C_{12}, in the
175 immobilized cell culture system. The fluorescent probe was absorbed by the BAS (not the
176 runner hyphae) within 10 min of the exposure (Fig. 3*A*). The probe was first taken up from the
177 hyphal tips of BAS (Fig. 3*A*), and then the fluorescent signals were observed in lipid body-like
178 structures (26, 27) in the runner hyphae over time, which were translocated by cytoplasmic
179 streaming (*SI Appendix*, Movie S1). Long exposure of the probe showed that the signals were
180 localized in the myristate-induced spores as well as BAS, runner hyphae, and DPC, but

181 inoculated parent spores were rarely labeled (*SI Appendix*, Fig. S6). Translocation of the
182 fluorescent fatty acid probe to the myristate-induced spores prompted us to examine whether
183 myristate is utilized as a component of the major storage lipid, triacylglycerol. We cultured *R.*
184 *irregularis* in the medium supplemented with [1-^{13}C]myristic acid. After extraction of the lipids
185 from the fungal materials, triacylglycerol was purified through preparative TLC and analyzed
186 by ^{13}C-NMR. In the spectrum, two peaks at 173.1 and 173.4 ppm (corresponding to the carboxyl
187 carbons of acyl chains at the α and β positions, respectively) were observed at a much higher
188 intensity compared with those of triacylglycerol prepared from monoxenically cultured *R.*
189 *irregularis* (Fig. 3*B*). This finding shows that exogenous myristate is incorporated into
190 triacylglycerol, especially as acyl carboxyl components. To further examine whether myristate
191 provides the carbon skeleton for fungal cell components, we applied [1-^{13}C]myristate to the
192 solid culture medium and analyzed cell wall components. After the extraction of fungal cell
193 walls, chitin and chitosan in the cell walls were converted into glucosamine through acid
194 hydrolysis. LC-MS analysis of the extracted glucosamine demonstrated that the relative ion
195 intensity of M+1 was significantly higher in AM fungi supplemented with [1-^{13}C]myristate
196 (mean 14.6%) than in those supplemented with non-labeled myristate (6.5%). This finding
197 indicates that external myristate was taken up by *R. irregularis* and utilized for the biosynthesis
198 of chitin and chitosan of the fungal cell walls (Fig. 3*C*). To evaluate myristate as an energy

199 source, ATP production was measured after the application of myristate. We applied myristate
200 to germinating hyphae and measured ATP content at 12 h after application. ATP content
201 increased by 2.4-fold in the presence of myristate compared with that in the absence of myristate
202 (Fig. 3*D*). When carbonyl cyanide m-chlorophenylhydrazone (CCCP), an inhibitor of
203 depolarization of mitochondrial membrane potential, was simultaneously applied to the hyphae,
204 ATP content did not increase independently of fatty acid application. We also confirmed that
205 potassium myristate activated gene expression involved in β-oxidation, the glyoxylate cycle,
206 gluconeogenesis, and the TCA cycle at 3 h after the application (Fig. 3*E*). Interestingly, a gene
207 encoding *N*-myristoyl transferase (NMT), which catalyzes the myristoylation of proteins was
208 upregulated by myristate.
209

210 **Discussion**

211 AM fungi have an obligate biotrophic lifestyle, so these fungi cannot possibly grow and survive
212 without host-derived nutrients. Recently, AM fungi have been shown to receive lipids via
213 arbuscules from the host (1-3, 14, 15); however, the availability of lipids as nutrient is largely
214 unknown. Here we show that myristate (C14:0) is used for the hyphal growth of *R. irregularis*
215 as a carbon and energy source under asymbiotic conditions, which provides the first evidence
216 of the increase of AM fungal biomass in a pure culture system. Furthermore, we confirmed that

217 myristate-induced spores are infective propagules capable of generating symbiotic daughter
218 spores. Based on lipid profile analyses of plant mutants defective in arbuscule formation, 16:0
219 β-MAG or its related chemicals are transferred to AM fungi from the host under symbiotic
220 conditions (1-3, 14, 15). Recently, palmitoleic acid, a monounsaturated C16 fatty acid, has been
221 shown to induce hyphal branching and fertile spores in the absence of a host (7). However, fatty
222 acids and β-MAGs except for myristate were ineffective in biomass production in our culture
223 conditions. From our result that the fluorescently labelled fatty acid derivative C_1-BODIPY
224 500/510 C_{12} was accumulated in AM fungal hyphae, fatty acids are likely to be non-specifically
225 taken up. Rather, myristate seems to be essential for biological processes in AM fungi via a
226 myristate-specific metabolism. Myristate is used for the lipid modification of proteins, called
227 protein *N*-myristoylation, in a variety of eukaryotes (28-30). Protein *N*-myristoylation is
228 catalyzed by NMT, which transfers myristate from myristoyl-CoA to the N-terminal glycine
229 residue of the target proteins (31). *N*-myristoyl proteins are involved in diverse cellular
230 processes such as protein phosphorylation and signal transduction. Disruption of the *NMT* gene
231 causes recessive lethality in several fungal species (32-34). In the present study, the *R.*
232 *irregularis NMT1* gene was upregulated by the application of myristate. Protein *N*-
233 myristoylation may be activated in *R. irregularis* by externally supplied myristate, which allows
234 it to proliferate.

235 Under conditions where myristate was supplied in the absence of a host, AM fungi
236 formed DPC-like structures after germination, explored the media by developing runner hyphae
237 in association with BAS formation, and subsequently differentiated secondary spores. We found
238 that *R. irregularis* has the capability to absorb fatty acids through BAS and DPC. Absorbed
239 fatty acids or their metabolites are likely to be stored in lipid bodies or glyoxysomes and
240 translocate in runner hyphae as previously observed in symbiotic extraradical hyphae (27, 35,
241 36). A part of the fatty acids or lipids is delivered to newly formed spores, which may be used
242 for spore germination and subsequent hyphal elongation.

243 The observation of incorporating ^{13}C in triacylglycerols and cell wall components of
244 *R. irregularis* when [1-^{13}C]myristate is supplied indicates that myristate is utilized as a carbon
245 source to synthesize cellular components. During symbiosis, β-oxidation, the glyoxylate cycle,
246 and gluconeogenesis are active in AM fungi, and these metabolic pathways have been proposed
247 to play a crucial role in the generation of carbohydrates from lipids (10, 35-37). Based on our
248 observation that major genes involved in β-oxidation, the glyoxylate cycle, gluconeogenesis,
249 and the TCA cycle were induced by the application of myristate, absorbed myristate is likely
250 metabolized via such metabolic pathways and resulting carbohydrates are used to build the
251 fungal cells. Furthermore, ATP elevation through myristate addition indicates that myristate is
252 also used as an electron donor for the respiration of AM fungi. In our culture systems, hexoses

253 did not give rise to an increase in fungal biomass, although xylose in combination with myristate
254 induced a great number of secondary spores. This finding means that AM fungi are hardly able
255 to use sugars for carbon sources under these conditions. However, hexoses derived from the
256 host are taken up via fungal monosaccharide transporters in intraradical hyphae and/or
257 arbuscules during symbiosis (9). In addition, the monosaccharide transporter *MST2* is expressed
258 even in BAS formed on the medium in a carrot hairy root system (23) and induced in
259 extraradical hyphae applied with xylose (12). Thus, we cannot rule out the possibility that AM
260 fungi can utilize external sugars for their growth under asymbiotic conditions.

261 In conclusion, we proved that AM fungi are fatty acid auxotrophs, which leads to the
262 possibility of generating a pure culture of biotrophic AM fungi. Although myristate initiates
263 AM fungal growth and sporulation, the size of myristate-induced spores remains small
264 compared with that of symbiotically generated spores. Similar results were obtained with
265 palmitoleic acid-induced spores (7). As smaller spores show low germination rates and
266 infectivity (38), spore maturation in the absence of the host represents an exciting future
267 challenge for the pure culture of AM fungi. Our findings have shed new light on the cellular
268 and molecular biology of AM fungi, and have important implications for the genetic
269 transformation and the development of new strategies for the inoculum production of these
270 organisms.

271

272 **Materials and Methods**

273 More details of study methods are provided in *SI Appendix*, *Extended Methods*.

274

275 **Biological materials.** Sterile spore suspension of the AM fungus *R. irregularis* DAOM197198

276 (or DAOM181602, another voucher number for the same fungus) was purchased from Premier

277 Tech. Contaminated hyphae in the spore suspension were removed by density-gradient

278 centrifugation using gastrografin as described in *SI Appendix*, *Extended Methods*.

279

280 **Asymbiotic culture.** Approximately 300–400 spores of *R. irregularis* were inoculated on 0.3%

281 Phytagel (Sigma-Aldrich) plates containing modified SC medium (1.7 g l^{-1} yeast nitrogen base

282 [MP Biomedicals], 2.0 g l^{-1} complete SC mixture [Formedium], 5 mM ammonium sulfate, 1

283 mM glycerol, 5 mg l^{-1} thiamine hydrochloride, 5 mg l^{-1} nicotinic acid, 5 mg l^{-1} pyridoxal

284 phosphate, and appropriate amounts of fatty acids) and then covered with 0.3% Phytagel

285 dissolved in 3 mM magnesium sulfate in a 12-well culture plate. For the liquid culture, Phytagel

286 was removed from the medium. Three types of fatty acids were added to the medium: fatty acid

287 salts, fatty acids in an organic solvent, or fatty acids conjugated with BSA (*SI Appendix*,

288 *Extended Methods*). The plates were incubated at 28 ºC in the dark. Hyphal elongation was

289 observed under a dissecting microscope and light microscopes.

290

291 **Immobilized cell culture.** An overview of the immobilized cell culture system is represented

292 in the *SI Appendix* (Fig. S3*A*). Thirty-five ml of 0.75% Phytagel containing 3 mM magnesium

293 sulfate was poured into a 90 mm Petri dish and solidified. A Phytagel tablet (6 mm high and

294 17.5 mm wide) with a circular incision (3 mm deep and 11.5 mm wide) was cut out using a

295 sterile double cork borer (inner diameter: 11.5 and 17.5 mm, respectively). The gel within the

296 circular incision on the top side of the gel tablet was removed to 3 mm deep using a spatula or

297 disposable pipette tip connected to an aspirator to prepare a hole for spore inoculation. To flatten

298 the bottom of the hole, a small amount of 0.75% Phytagel was added. Approximately 300–400

299 spores were placed in the hole and covered with 0.75% Phytagel containing 3 mM magnesium

300 sulfate. The Phytagel tablets containing spores were transferred into a six-well culture plate.

301 Each well was filled with 5 mL of full- or half-strength modified SC liquid medium with or

302 without 0.5 mM potassium myristate and monosaccharides (glucose and xylose). AM fungi

303 were grown at 28 ºC in the dark. During the culture, the medium was changed once a month.

304

305 **Measurement of fungal biomass.**

306 Fungal materials were recovered from gels in wells of a culture plate by melting the gels in

307 citrate buffer and weighed using a micro analytical balance (*SI Appendix*, *Extended Methods*).
308 The number of inoculated spores in the well was counted in advance under a dissecting
309 microscope. The standardized growth increment of *R. irregularis* was calculated by dividing
310 the total fungal dry weight in a well by the number of inoculated spores and subtracting the
311 mean dry weight of an inoculated spore.
312
313 **Spore morphology and germination assay.** Myristate-induced spores were produced by an
314 immobilized cell culture system in the presence of 0.5 mM potassium myristate in a half-
315 strength modified SC medium for approximately three months. Myristate-induced and
316 symbiotically generated spores were mounted with polyvinyl alcohol–lactic acid–glycerol
317 (PVLG) or Melzer's reagent for microscopic observation. Spores were incubated with 10 μM
318 SYTO 13 green fluorescent nucleic acid stain (Thermo Fisher) for 2 h and observed by
319 epifluorescence microscopy. For spore germination assay, myristate-induced and symbiotically
320 generated spores were placed onto 0.3% Phytagel containing 3 mM magnesium sulfate and
321 incubated at 28 ºC for one week. Spore germination was examined under a dissecting
322 microscope.
323
324 **Single spore inoculation.** A single myristate-induced spore produced by an immobilized cell

325 culture system in the presence of 0.5 mM potassium myristate and 5 mM xylose in a half-
326 strength modified SC medium was placed onto carrot hairy root plates using a pipette. The
327 production of daughter spores on extraradical hyphae emerging from hairy roots was observed
328 under a dissecting microscope. AM fungal colonization was confirmed by trypan blue staining.
329

330 **Fatty acid uptake.** Fatty acid uptake was evaluated using a fluorescent fatty acid analogue,
331 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid (C_1-BODIPY 500/510
332 C_{12}, Thermo Fisher Scientific). *R. irregularis* was grown in an immobilized cell culture system
333 with modified SC medium containing 0.5 mM potassium myristate without sugars for 4 or 6
334 weeks. Fungal hyphae were stained with 0.5 mM C_1-BODIPY 500/510 C_{12} in modified SC
335 medium. After a 10-minute the incubation, fungal hyphae protruding outside a Phytagel tablet
336 were observed using a laser scanning confocal microscope. For the samples incubated for 1 or
337 5 days in the medium containing the fluorescent probe, a Phytagel tablet containing fungal
338 materials was melted by adding citrate buffer. Fluorescent signals were observed under an
339 epifluorescence microscope.
340

341 **LC-MS analysis of glucosamine.** [1-^{13}C]Myristic acid (Taiyo Nippon Sanso) and non-labeled
342 myristic acid were dissolved in 200 mM potassium hydroxide to 100 mM final concentration

343 of fatty acids. *R. irregularis* was cultured in a modified SC solid medium supplied with 1 mM
344 neutralized myristate for two months. Approximately 1–2 mg dry fungal materials per sample
345 were recovered from the solid medium as described above. Extraction of glucosamine derived
346 from fungal biomass and LC-MS analysis was described in *SI Appendix*, *Extended Methods*.
347 The relative intensities of the molecular ion peaks of glucosamine ($[M+H]^+$, *m/z* 180.19;
348 $[M+1+H]^+$, *m/z* 181.19; and $[M+2+H]^+$, *m/z* 182.19) were monitored. The relative fraction of
349 M+0, M+1, and M+2 in the glucosamine standard solution were 92.9%, 7.1%, and 0%,
350 respectively.
351
352 **^{13}C-NMR analysis of triacylglycerols.** *R. irregularis* was cultured in a modified SC solid
353 medium supplied with 1 mM neutralized [1-^{13}C]myristate (Cambridge Isotope Laboratories,
354 Inc.) for 2.5 months as described above. Approximately 43 mg of wet fungal materials were
355 recovered from solid medium. Extraction of lipids from fungal biomass, purification of
356 triacylglycerol, and ^{13}C-NMR analysis were described in *SI Appendix*, *Extended Methods*.
357
358 **Determination of ATP content.** Five hundred spores of *R. irregularis* were incubated in 100
359 μL of sterilized water at 28 ºC for 5 days. Potassium myristate was added to the germinating
360 spores at a final concentration of 0.5 mM. For the control, the protonophore CCCP was

361 simultaneously added at a final concentration of 50 μM. The germinating spores were incubated
362 for 12 h. After centrifugation at 3,500 rpm for 10 min, 400 μL of phosphate-buffered saline
363 (PBS; pH 7.4) was added to the pellet. The spore suspension was transferred to a 2 mL tube
364 containing a metal crusher (TAITEC) and placed on ice. The sample was crushed with a bead
365 crusher (μT-12, TAITEC) at 2,200 rpm for 10 s three times. The crushed fungal materials were
366 transferred to a 1.5 mL tube and centrifuged at 8,000×*g* for 2 min at 4 ºC. The supernatant was
367 recovered and used for determination of ATP and protein concentration. ATP concentration was
368 measured using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) following
369 the manufacturer's instructions. Protein concentration was assayed using the Qubit Protein
370 Assay Kit (Thermo Fisher Scientific). ATP content in the germinating spores was calculated as
371 pmol per mg protein.
372
373 **Quantitative RT-PCR.** *R. irregularis* was grown in an immobilized cell culture system with
374 modified SC medium containing 0.5 mM potassium myristate without sugars for 3 weeks.
375 Subsequently, Phytagel tablets containing fungal materials were incubated in a modified SC
376 medium without fatty acids for 11 days to induce fatty acid starvation. During the first three
377 days of starvation, the culture medium was exchanged every day. After the starvation treatment,
378 half of each sample was added to 100 mM potassium myristate to a final concentration of 0.5

379 mM, and the remaining half of each sample was added the same amount of sterilized water.
380 After a 3 or 12 h incubation, fungal hyphae protruding outside a Phytagel tablet were recovered
381 using forceps and immediately immersed in 500 μL of RNAiso Plus (Takara Bio). The fungal
382 hyphae were crushed using the bead crusher μT-12 with a metal crusher at 2,200 rpm for 10 s
383 three times with cooling on ice. RNA purification, cDNA synthesis, and semiquantitative PCR
384 was conduct as described in *SI Appendix*, *Extended Methods*.
385
386 **Statistical analysis.** All statistical analyses were performed using R version 3.5.2. To examine
387 the differences among experimental groups, data were analyzed with Student's t-test or Tukey
388 HSD test, as appropriate. Differences at $P < 0.05$ were considered to be significant.
389
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395

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522

523 **Figure legends**

524

525 **Fig. 1.** Asymbiotic culture of *R. irregularis* in the presence of fatty acids. (*A*) Standardized
526 growth increment (see Materials and Methods) during 8 weeks in the modified SC solid medium
527 supplemented with potassium salts of fatty acids, fatty acids and β-MAGs, or fatty acid–BSA
528 conjugates (n = 3–4). (*B*) Time course of biomass production at different concentrations of
529 potassium myristate (n = 5–6). (*C*) Biomass production in an immobilized cell culture system.
530 Immobilized fungal spores were incubated in half-strength modified SC medium supplemented
531 with combinations of potassium myristate and sugars at 8 weeks after inoculation (n = 6). For
532 each boxplot, the boxes show the first, median, and third quartiles; the whiskers extend to 1.5×
533 interquartile range, and data points for each treatment are displayed. The same lowercase letter
534 indicates no significant difference (Tukey's test, $P < 0.05$; *A–C*). Fungal growth in the solid
535 medium without fatty acids (*D* and *E*) or with potassium myristate (*F–P*). (*D*) Germinating
536 spore. Hyphal elongation (*E*) without fatty acids and (*F*) with potassium myristate at 8 weeks
537 after inoculation. (*G*) DPC-like structures formed around an inoculated spore. (*H*) Radial
538 growth of fungal mycelium. (*I*) Elongation of runner hyphae. (*J*) Branching of runner hyphae
539 and the formation of BAS. (*K*) Magnified image of BAS. (*L*) Front line of elongated mycelium.
540 The medium around the fungal hyphae became transparent, indicating that the precipitates of
541 metal soaps were solubilized. (*M*) Myristate-induced secondary spores generated around the

542 inoculated spores. (*N* and *O*) Myristate-induced spores formed along the runner hyphae. (*P*)
543 Magnified image of a myristate-induced spore. Fungal growth in the immobilized cell culture
544 system (*Q*) without fatty acids and (*R*) with potassium myristate at 8 weeks after inoculation.
545 See *SI Appendix* (Table S2) for sample details. BAS, branched absorbing structure; BH,
546 branching hypha; DPC, densely packed coil; MS, myristate-induced spore; P, precipitate of
547 metal soaps; PS, parent spore (inoculated spore); PT, Phytagel tablet; RH, runner hypha; and
548 SH, subtending hypha. Scale bars: 200 μm (*D*–*G* and *I*–*P*) and 1,000 μm (*H*, *Q*, and *R*).
549
550 **Fig. 2.** Spore formation of *R. irregularis* under asymbiotic conditions. (*A*) Number and (*B*)
551 diameter of myristate-induced spores generated in the solid medium containing different
552 concentrations of potassium myristate. Spore diameter was measured at 8 weeks after
553 inoculation. (*C*) Number of myristate-induced spores in an immobilized cell culture containing
554 0.5 mM potassium myristate and 5 mM monosaccharides at 8 weeks after inoculation. (*D*–*G*)
555 Inoculation of a single myristate-induced spore to carrot hairy roots. (*D*) Inoculated spore that
556 was produced in the immobilized cell culture containing potassium myristate and xylose. (*E*)
557 Germination of the inoculated spore. (*F*) Daughter spores (DS) on extraradical mycelia (ERM)
558 emerged from the carrot hairy roots. (*G*) Colonization of AM fungi in the carrot hairy roots.
559 Arrows indicate an inoculated myristate-induced spore. A, arbuscule; and V, vesicle. See *SI*

560 *Appendix* (Table S2) for sample details. Scale bars: 500 μm. Bars in *A* are standard errors of
561 means (*A*). For each boxplot, the boxes show the first, median, and third quartiles; the whiskers
562 extend to 1.5× interquartile range, and data points for each treatment are displayed. The same
563 lowercase letter indicates no significant difference (Tukey's test, $P < 0.05$, $n = 3–6$ (*A*) and $n =$
564 6 (*C*)).
565
566 **Fig. 3.** Utilization of fatty acids by *R. irregularis* under asymbiotic conditions. (*A*) Uptake of
567 the fluorescently labeled fatty acid derivative C_1-BODIPY 500/510 C_{12}. A cluster of BAS
568 (upper panels), magnified BAS (middle panels), and a single hypha of BAS (lower panels).
569 Optical sections captured using a confocal laser scanning microscope were projected.
570 Fluorescence images and superimposed bright field images were shown. BAS, branched
571 absorbing structure; and RH, runner hyphae. Scale bars, 200 μm. (*B*) ^{13}C-NMR spectra of
572 triacylglycerol isolated from mycelial extracts of *R. irregularis* grown in monoxenic root organ
573 culture (upper) and asymbiotic culture supplemented with 1 mM potassium [1-$^{13}C_1$]myristate
574 (lower). Inset: Chemical structure of a triacylglycerol. (*C*) Mass isotopomer distribution of
575 glucosamine derived from the fungal cell wall. *R. irregularis* was cultured in the solid medium
576 containing unlabeled or $^{13}C_1$-labeled myristate ($n = 5$). *P* values are based on a *t*-test with the
577 Bonferroni correction. (*D*) ATP content in *R. irregularis* at 12 h after the application of

578 potassium myristate or DW (control) in the presence or absence of CCCP (n = 4). P values are

579 based on a t-test. (E) Gene expression analysis in *R. irregularis* at 3 h after the application of

580 potassium myristate shown by real-time RT-PCR. Relative expression ($\log_2$ fold change) was

581 normalized to water-treated samples (myristate treated: n = 5; water-treated: n = 6). *P < 0.05,

582 **P < 0.01, and ***P < 0.001 (t-test). See *SI Appendix* (Table S4) for gene details. GC,

583 glyoxylae cycle; GNG, gluconeogenesis. For each boxplot, the boxes show the first, median,

584 and third quartiles; the whiskers extend to 1.5× interquartile range, and data points for each

585 treatment are displayed (C and D). Mean ± s.d. (E).

Fig. 1

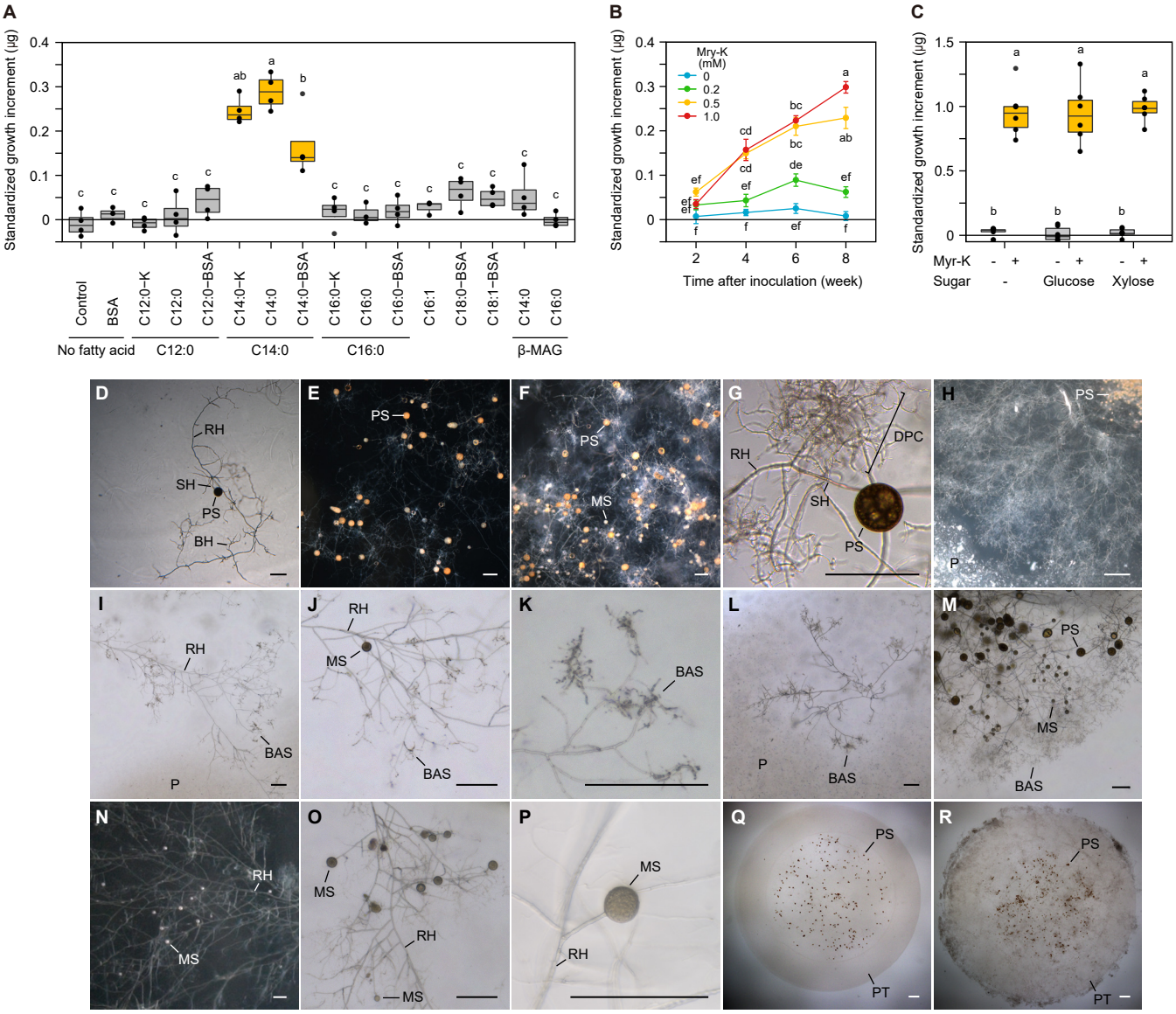

A
Standardized growth increment (µg)
Control
BSA
C12:0−K
C12:0
C12:0−BSA
C14:0−K
C14:0
C14:0−BSA
C16:0−K
C16:0
C16:0−BSA
C16:1
C18:0−BSA
C18:1−BSA
C14:0
C16:0
No fatty acid
C12:0
C14:0
C16:0
β-MAG
B
Mry-K (mM)
0
0.2
0.5
1.0
Time after inoculation (week)
C
Myr-K
Sugar
Glucose
Xylose
D
RH
SH
PS
BH
E
PS
F
PS
MS
G
RH
DPC
SH
PS
H
PS
P
I
RH
BAS
P
J
RH
MS
BAS
K
BAS
L
P
BAS
M
PS
MS
BAS
N
RH
MS
O
MS
RH
MS
P
MS
RH
Q
PS
PT
R
PS
PT

Fig. 2

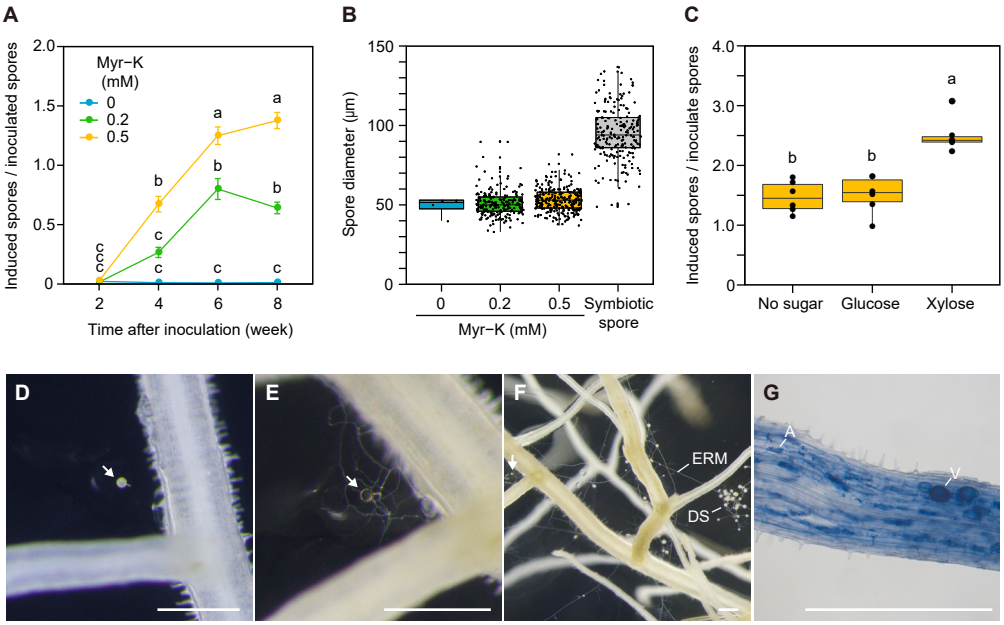
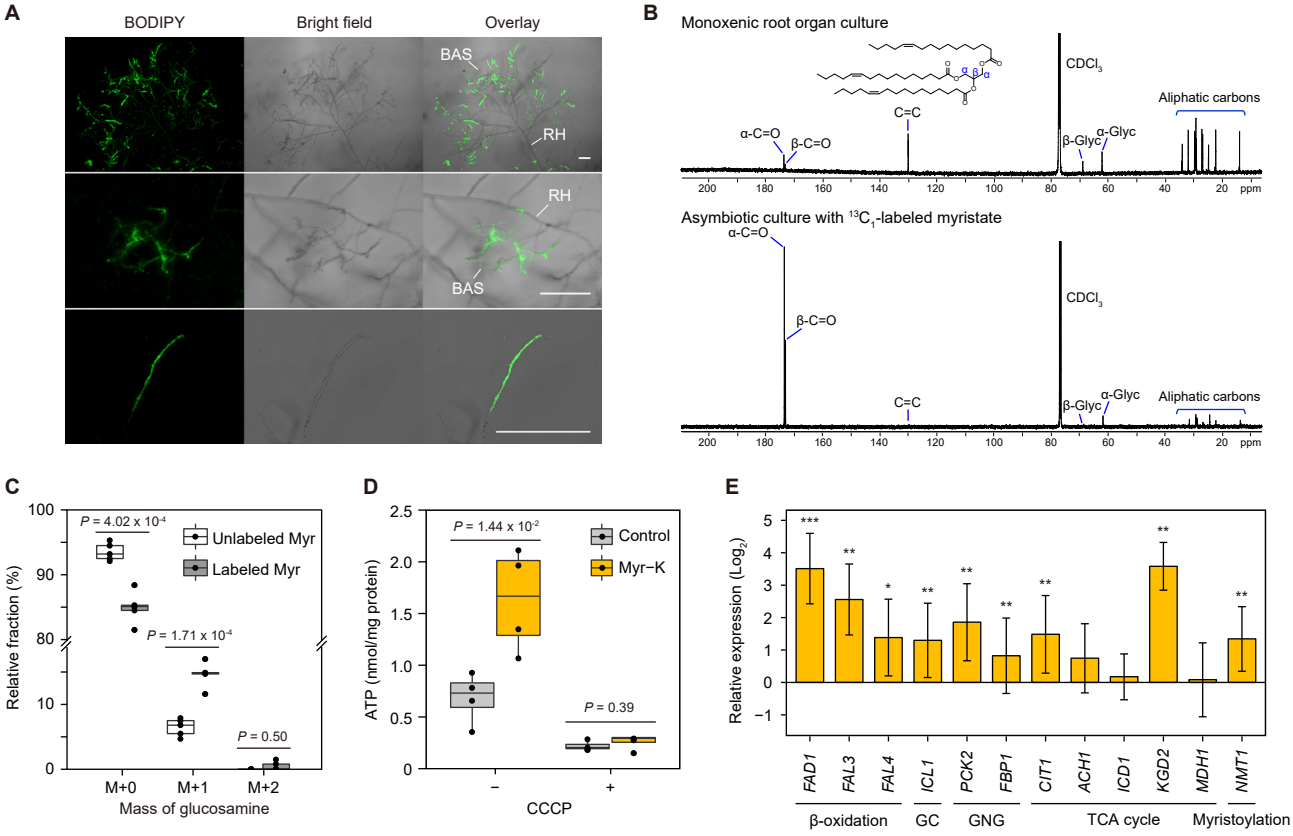

A
Induced spores / inoculated spores
Myr−K
(mM)
0
0.2
0.5
Time after inoculation (week)
B
Spore diameter (μm)
Myr−K (mM)
Symbiotic spore
C
Induced spores / inoculate spores
No sugar
Glucose
Xylose
D
E
F
ERM
DS
G
A
V

Fig. 3


A
BODIPY
Bright field
Overlay
BAS
RH
RH
BAS
B
Monoxenic root organ culture
CDCl3
Aliphatic carbons
C=C
α-C=O
β-C=O
β-Glyc
α-Glyc
200 180 160 140 120 100 80 60 40 20 ppm
Asymbiotic culture with 13C1-labeled myristate
α-C=O
CDCl3
β-C=O
C=C
β-Glyc
α-Glyc
Aliphatic carbons
200 180 160 140 120 100 80 60 40 20 ppm
C
Relative fraction (%)
P = 4.02 x 10-4
Unlabeled Myr
Labeled Myr
P = 1.71 x 10-4
P = 0.50
M+0
M+1
M+2
Mass of glucosamine
D
ATP (nmol/mg protein)
P = 1.44 x 10-2
Control
Myr−K
P = 0.39
−
+
CCCP
E
Relative expression (Log2)
FAD1
FAL3
FAL4
ICL1
PCK2
FBP1
CIT1
ACH1
ICD1
KGD2
MDH1
NMT1
β-oxidation
GC
GNG
TCA cycle
Myristoylation