The mutualistic interaction between plants and arbuscular mycorrhizal fungi

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1   **The mutualistic interaction between plants and arbuscular mycorrhizal fungi**

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13 **Summary**

14 Mycorrhizal fungi belong to several taxa and develop mutualistic symbiotic associations with

15 over 90% of all plant species, from liverworts to angiosperms. While descriptive approaches

16 have dominated the initial studies of these fascinating symbioses, the advent of molecular

17 biology, live cell imaging and 'omics' techniques have provided new and powerful tools to

18 decipher the cellular and molecular mechanisms that rule mutualistic plant-fungus interactions.

19 In this chapter we focus on the most common mycorrhizal association, arbuscular mycorrhiza

20 (AM), which is formed by a group of soil fungi belonging to Glomeromycota. AM fungi are

21 believed to have assisted the conquest of dry lands by early plants around 450 million years

22 ago, and are found today in most land ecosystems. AM fungi have several peculiar biological

23 traits, including obligate biotrophy, intracellular development inside the plant tissues,

24 coenocytic multinucleate hyphae and spores, as well as unique genetics - such as the putative

25 absence of a sexual cycle -, and multiple ecological functions. All of these make the study of

26 AM fungi as intriguing as it is challenging, and their symbiotic association with most crop

27 plants is currently raising a broad interest in agronomic contexts for the potential use of AM

28 fungi in sustainable production under conditions of low chemical input.

29

AN OVERVIEW OF MYCORRHIZAL INTERACTIONS

30

31 Mycorrhizal fungi are a heterogeneous group of diverse taxa, associated with the roots of over

32 90% of all plant species, from liverworts to angiosperms. Although they can spend part of

33 their life cycle in the rhizosphere, mycorrhizal fungi always associate with the roots of plants,

34 including forest trees, wild grasses and many crops, and colonize environments such as alpine

35 and boreal zones, tropical forests, grasslands and croplands. Both partners benefit from the

36 relationship: mycorrhizal fungi improve the fitness of their host plants, by influencing mineral

37 nutrition and water absorption and by increasing tolerance to biotic and abiotic stresses. The

38 host plant rewards the fungal symbiont with carbon compounds derived from the

39 photosynthetic process (1).

40 Irrespectively of their taxonomic position, mycorrhizal fungi develop an extensive hyphal

41 network in the soil, the aptly named wood-wide web, which can connect whole plant

42 communities and potentially grant the horizontal transfer of nutrients. Such underground web

43 has caused a paradigm shift in our knowledge of plant ecology, by introducing the key role of

44 below ground microbes and opening the discussion on how they influence the composition

45 and fitness of plant communities (2).

46 The term mycorrhiza is derived from the Greek words for 'fungus' and 'root': a beautiful

47 linguistic rendering of such biological synergies. This does not in any way imply that

48 mycorrhizal fungi only colonize roots; in fact, so-called basal plants lacking true roots also

49 rely on mycorrhizal fungi for their nutrition and host the symbionts in other organs, such as

50 the liverwort thallus. This feature is an evolutionary reminder of the first known mycorrhizal

51 interactions. Four hundred and fifty million year-old fossils of some of the first land plants

52 display characteristic symbiotic fungal structures in their simple, prostrate shoots. In short, the

53 evolution of mycorrhizal symbioses predates the appearance of roots, even if mycorrhizal

54 fungi are currently restricted to roots in the vast majority of extant plants.

55   Mycorrhizal fungi can be divided into two major groups: aseptate endophytes such as

56   Glomeromycota, and septate Asco- and Basidiomycota. Usually, the classification of

57   mycorrhizal interactions is based on plant anatomical traits and identifies two broad

58   categories (Fig. 1), ectomycorrhizae (EM), where the fungus is restricted to the intercellular

59   spaces of outer root tissues, and endomycorrhizae, where fungi penetrate the living host cells

60   (3). In ectomycorrhizae, which colonise trees and shrubs, hyphae remain extracellular,

61   inducing important changes to root morphogenesis, while their presence leads to subtle

62   modifications in epidermal or cortical cells. In endomycorrhizae, i.e. ericoid, orchid and

63   arbuscular mycorrhizae (AM), hyphae penetrate the root cells to establish an intracellular

64   symbiosis. While AM are common to diverse plant taxa, ericoid and orchid mycorrhizae are

65   restricted to the order Ericales and the family Orchidaceae, respectively. Such taxonomic and

66   morphological diversity has stimulated the study of the colonization process in numerous host

67   plants since the start of the nineteenth century. Indeed new information generated by

68   genomic sequencing, the use of mutants affected in their symbiotic capabilities and live cell

69   imaging has deeply changed our view of the colonization process (4).

70   The arbuscular mycorrhizal (AM) symbiosis is the most widespread plant-fungus mutualistic

71   association. AM fungi are found in association with all plant phyla, including agriculturally

72   relevant species, and play a crucial ecological role in the functioning of low-input

73   environments. All AM fungi belong to the Glomeromycota phylum and are very specialized

74   symbionts, as revealed by their obligate biotrophy. Currently there is broad interest in AM

75   fungi for their potential to support a reduction in the use of chemical fertilizers and pesticides,

76   and to positively impact sustainable crop production systems to feed a globally growing

77   human population (5, 6). For these reasons this chapter will focus on the AM symbiosis,

78   providing an overview of the current knowledge of the molecular mechanisms controlling this

79   ancient and deeply intimate plant-fungus association.

ARBUSCULAR MYCORRHIZAE

AM fungi form a mutualistic symbiosis with the roots of ~80% of plant species in natural and agricultural systems, and are considered a central component of the plant microbiota.

AM fungi have the unusual ability to grow in two extremely different niches: the soil and the lumen of plant cells. In soil, the extensive hyphal network has been estimated to reach a density of 100 m/cm^3 (7). This hyphal network can acquire water and nutrients with great efficiency and reach a volume of soil that is inaccessible to roots alone (8). The presence and activity of AM fungal mycelia also directly influence the physico-chemical properties of soil (9). Inside their host plant, AM fungi penetrate single cells of the root cortex, where they develop a structure that is the distinctive feature of this association: the arbuscule (Fig. 2). Named from the Latin word for bush, or small tree, each arbuscule results from repeated branching of a single hypha in the lumen of a parenchymatic cell from the inner root cortex (10). With their impressive surface/volume ratio, arbuscules are considered the major site of nutrient exchange (11).

As obligate biotrophs, AM fungi strictly depend on their plant hosts for both growth and reproduction (3, 12). Up to 20% of the photosynthesis products of terrestrial plants is consumed by AM fungi (13). Interestingly, the carbon flow from the plant to the fungus seems to be proportional to the amount of phosphate that the fungus returns to its host (14). Consequently, while the beneficial effects of AM fungi become evident when the nutrient and water supply are limited, root colonization decreases in soils with abundant nutrients. The AM interaction also supports important ecological services such as an increase of soil quality (15) and of biodiversity of the associated plant communities (16). Additionally, many studies have highlighted the positive influence of the AM symbiosis on plant responses to biotic (17, 18) and abiotic stresses such as drought, salinity and heavy metal contaminants (6, 19, 20).

Classification and phylogeny of AM fungi

AM fungi have existed and coevolved with plants for at least 450 million years (21) as fossils records witness (22). Due to their importance in nutrient transfer they are supposed to have played a crucial role during land colonization by plants. The widespread occurrence of AM fungi in plants from the most parts of the world, particularly in the tropics, was acknowledged in the nineteenth century (23, 24). In 1844, the Tulasne brothers described the first AM fungal species, *Glomus microcarpum* and *Glomus macrocarpum* (25). In 2001 Schüßler and colleagues grouped AM fungi within a new monophyletic phylum, the Glomeromycota, distinct from the Zygomycota where they had previously been placed (26).

The phylum Glomeromycota is currently represented by about 250 described species (4) although molecular analyses suggest a broader diversity (27). A recent survey of the global distribution of these plant symbionts showed that AM fungal communities reflected local environmental conditions and the spatial distance between sites. However, despite AM fungi apparently possessing limited dispersal ability, 93% of taxa were found on multiple continents and 34% on all of the six surveyed continents (28).

Before the advent of molecular techniques, the identification of AM fungi was based on the microscopic examinations of spores. These are roundish, from about 30 to 400 μm in diameter and with features with taxonomic value like colour, dimension, cell wall structure, presence of septa and connection to sustaining hypha. However, morphological similarities do not always reflect phylogenetic relationships. The development of PCR-based approaches and advances in molecular analyses led to novel identification rules (26, 29). Since then, the taxonomy of Glomeromycota has been subjected to several changes and nowadays it is still largely open to discussion. On the basis of rRNA gene phylogeny Glomeromycota were described as a sister group of Ascomycota and Basidiomycota (26, 29). However, phylogenetic reconstructions based on mitochondrial (30, 31, 32) or protein-coding nuclear (33) sequences suggest a closer

6

130 relationship with Mortierellales or Mucorales (Mucoromycotina). Recently, on the basis of

131 the complete genome sequence of *Rhizophagus irregularis* (34, 35), Glomeromycota were

132 again phylogenetically placed closer to Mucoromycotina (see later in this chapter). Indeed

133 Mucoromycotina, a basal group of fungi also characterized by a coenocytic mycelium, is now

134 considered a sister group of Glomeromycota (36). Interestingly, Mucoromycotina form

135 functional mycorrhiza-like associations with basal plant lineages (37) suggesting that the

136 symbiotic options available to the first plants emerging onto dry land were more varied than

137 previously thought (38). It now appears likely that the last common ancestor of both fungal

138 groups thrived in primeval soils long before plants colonised the land (36).

139

140 **Biological features, genome organization and genetics of AM fungi**

141 AM fungi are also intriguing from a cellular and genetic point of view. They display many

142 unusual biological features beside their obligate biotrophism: spores and coenocytic hyphae

143 contain thousands of nuclei in a common cytoplasm and no uninucleate life stage is known to

144 occur. This makes classical genetic approaches challenging (12, 36). Depending on the

145 species, a single spore contains from 800 to about 35000 nuclei (39). Single spores are

146 populated by flows of unrelated nuclei streaming from the mycelium, rather than by the

147 replication of one or few nuclei within the developing spore (40).

148 The concept of species is poorly defined in this group of organisms, since they show a high

149 degree of genetic variability. For example, the intrasporal variability of the ITS (Internal

150 Transcribed Spacer) ribosomal region can range between 6% for *Gigaspora margarita* (41),

151 18% for *Glomus intraradices* (42) and over 20% in other *Glomus* '*intraradices*-like' strains

152 (43).

153 In spite of being among the oldest living terrestrial organisms, AM fungi surprisingly appear

154 to have lost sexual reproduction, as no sexual cycle has ever been described.

155   The genetic organization of coexisting nuclei in the Glomeromycota (i.e. coenocytic

156   organisms) has been a major source of debate for the past 15 years, with two opposite

157   hypotheses being supported within the research community: heterokaryosis (44) and

158   homokaryosis (45), which support, respectively, the presence or the absence of internuclear

159   genetic divergence (46). Furthermore, the possibility that AM fungi contain a uniform

160   population of nuclei characterized by intranuclear polymorphism has also been proposed (47).

161   Heterokaryosis may originate by hyphal anastomosis between genetically different mycelia or

162   by the accumulation of mutations. Hyphal anastomosis and the exchange of nuclei has indeed

163   been observed in a few fungal taxa (48, 49), nevertheless such events have only been

164   observed between distinct isolates of the same species from one sampling area (50), but not

165   between isolates from different geographical areas (51). These opposing hypotheses have

166   been reconciled by recent analyses of complete genome sequences of AM fungi (next

167   paragraph).

168   A further increase in the genetic complexity of AM fungi is given by the presence of

169   endobacteria and viruses. Many AM fungi harbor endobacteria in their hyphae and spores:

170   two types of endobacteria are known in Glomeromycota: rod-shaped Gram-negatives, limited

171   in distribution to members of the Gigasporaceae family and coccoid Mollicutes-related

172   endobacteria, distributed across different lineages of AM fungi (52). The genomes of a few

173   endobacteria have been sequenced: all are characterized by a limited size (0.5 - 1.8 Mb),

174   which is consistent with complete dependence on their fungal host and their inability to grow

175   in pure culture (53, 54, 55). So far, the biological role has only been investigated for

176   *Candidatus* Glomeribacter gigasporarum, the rod-shaped bacterium hosted by *Gigaspora*

177   *margarita*. The endobacterium enhances fungal sporulation, bioenergetic capacity, ATP

178   production and ability to detoxify reactive oxygen species. Endobacteria also appear to

179   enhance fungal responsiveness to strigolactones, root-exudated signals that act as hyphal

180 branching factors for AM fungi. Overall, this endosymbiosis improves the fungal ecological

181 fitness by priming mitochondrial metabolic pathways and giving the AM fungi more tools to

182 face environmental stresses (56, 57).

183 Our knowledge of mycoviruses of AM fungi is still very limited (58, 59). In one case a

184 biological function has been reported: the presence of the virus led to the production of a

185 higher number of spores and increased stimulation of plant growth (58). All together these

186 studies underline the importance of these additional genetic components of AM fungi, as such

187 components can contribute to the symbiosis.

188

189 **The first genome project dedicated to an AM fungus**

190 Extensive efforts were made to sequence the first genome of an AM fungus. Two independent

191 research groups (34, 35) published the genome sequences of the same *Rhizophagus*

192 *irregularis* isolate DAOM197198 (60). By sampling the extraradical hyphae of mycorrhizal

193 hairy root cultures, Tisserant and colleagues obtained an assembly of 101 Mb out of an

194 estimated genome size of 153 Mb (34). This assembly, although highly fragmented, is

195 believed to include almost all the protein-coding genes (23,561 high-confidence gene

196 models), and places the genome of *R*. *irregularis* among the largest fungal genomes

197 sequenced to date, along with those of obligate biotrophic powdery mildews (61) and the

198 ectomycorrhizal symbiont *Tuber melanosporum* (62).

199 In order to specifically address the issue of the heterokaryotic nature and to determine the

200 extent to which nuclei differ from each other, Lin and colleagues performed a *de novo*

201 genome sequencing of individual nuclei collected from crushed spores (35). Their

202 comparative analysis revealed a surprisingly low level of polymorphism: > 99.97% of the

203 aligned genome sequences were identical in different nuclei. The genome organization of this

204 strain is therefore considered basically homokaryotic. By contrast, within a single nucleus, the

205   45S rDNA repeat unit - and in particular the ITS region - turned out to be highly diverged and

206   the relative abundance of alleles appeared to differ between nuclei. Genome annotation

207   resulted in 27,392 protein-coding gene models representing 30,003 putative transcripts with a

208   high level of putative/predicted (retro-)transposable elements (35), findings consistent with

209   those of Tisserant et al. (34).

210   The gene repertoire of *R. irregularis* mostly overlaps with the repertoire of sequenced

211   Mucoromycotina species (34, 35) and phylogenomic analyses demonstrated that

212   Glomeromycota are more closely related to Mucoromycotina than to their postulated sister

213   Dikarya (35).

214   The obligate biotrophy of AM fungi is not mirrored by any drastic loss of metabolic

215   complexity in central metabolism. One striking genomic feature is anyway the lack of genes

216   encoding plant cell wall degrading enzymes; the same situation has been observed in obligate

217   biotrophic pathogens (61) and ectomycorrhizal symbionts (62).

218   More recently, the *in silico* genome analysis of five different *R. irregularis* isolates (63) has

219   highlighted substantial genetic diversity among isolates. Two isolates contained a stable

220   population of two dominant divergent haploid nuclei (a unique dikaryon-like condition) while

221   the other three isolates only displayed one dominant genotype (63). *R. irregularis* therefore

222   appears to develop both homokaryotic and heretokaryotic mycelia, even if the modes by

223   which this differential genomic pattern arises remain unclear. In addition, the identification of

224   a putative mating type locus suggests the existence of cryptic sex-related processes (34, 63).

225   Beside reconciling the previous contrasting results, such novel data indicate that conventional

226   modes of reproduction - including mating - may exist in this lineage which can suggest

227   approaches to deliver genetic improvement of AM strains.

228

229   **The colonization process**

230   The establishment of the AM symbiosis requires a succession of well characterized

231   developmental steps (Fig. 3) (3). In advance of direct plant-fungus contact - the so called

232   presymbiotic stage - diffusible molecules mediate reciprocal recognition between the two

233   symbionts. Then, the fungal hyphae contact the root epidermis, where they form adhesion

234   structures called hyphopodia; these precede root entry and mark the initiation of the symbiotic

235   phase. In response to these chemical and physical stimuli, the contacted epidermal cell

236   develops an intracellular accommodation structure, called prepenetration apparatus (64),

237   which drives fungal penetration and guides hyphal passage across the epidermal cell and

238   towards the inner root tissues. Within the root, hyphae grow inter- and intracellularly to reach

239   the deepest cortical cells where arbuscules develop (65). As root colonization proceeds, the

240   AM fungus explores the soil developing an extensive hyphal network, the extraradical

241   mycelium, which also can produce a new generation of asexual spores.

242   Host plants control each step of symbiosis development, leading to a precise synchronization

243   of fungal and plant developmental processes (65, 66). Over the last decade, the molecular

244   components controlling AM colonization have been intensively studied in angiosperms (11,

245   65) and more recently in basal land plants (67, 68).

246

247   **Presymbiotic signaling**

248   AM fungi perceive the vicinity of a host *via* root-exuded molecules that induce spore

249   germination and hyphal branching (69, 70). The most studied plant symbiotic signals are

250   carotenoid-derived phytohormones called strigolactones (SL) (71), which have a primary role

251   in plant development (72). AM sense SLs in root exudates at concentrations as low as 10 nM.

252   Fungal responses to GR24, a synthetic molecule commonly used to study SL actions, include

253   the enlargement of mitochondria, a rapid increase in ATP and NADH and nuclear

254   proliferation (73, 74, 75, 76). GR24 exposure also causes a sharp increase in Ca^{2+}

255   concentration in the fungal cytoplasm (77). Although fungal SLs receptors remain unknown,

256   such observations suggest that SLs are perceived *via* a Ca^{2+}-mediated signaling pathway and

257   trigger a cellular and molecular prelude to root colonization (74, 75, 78).

258   AM fungi also release signal molecules that trigger plant symbiotic responses (79), including

259   transcriptional regulation, nucleus-associated Ca^{2+} signals, starch accumulation in roots and

260   lateral root formation (80, 81, 82, 83, 84, 85, 86). Repeated oscillations in nuclear Ca^{2+}

261   concentration (spiking) have been observed in the root epidermal cells contacted by AM

262   fungal hyphopodia, but also when the same cells were treated with exudates from germinated

263   AM fungal spores (83). Similarly, the expression of the early symbiotic gene *ENOD11* in *M*.

264   *truncatula* is upregulated upon both fungal contact (87) and the perception of fungal exudates

265   (80).

266   Different N-acetylglucosamine oligosaccharides have been characterized in AM fungal

267   exudates as bio-active molecules responsible for such plant responses. They include tetra- and

268   penta-chito-oligosaccharides (CO4 and CO5) (86) as well as lipo-chito-oligosaccharides

269   (LCOs), which are very similar to nodulation (Nod) factors released by nitrogen-fixing

270   rhizobia (85). When applied as purified molecules, such chitin derivatives mimic the

271   perception of fungal exudates in the host roots, including nuclear Ca^{2+} spiking (85, 86) and

272   the regulation of symbiosis-related genes (80, 85, 88). Interestingly, the release of CO4 and

273   CO5 in *Rhizophagus irregularis* exudate is boosted upon GR24 treatment (86), suggesting the

274   existence of a positive loop between plant and fungal signal perception and production of

275   these oligosaccharides.

276   The study of plant signaling mechanisms involved in the perception of AM fungal signals has

277   been developed in legumes such as *Medicago truncatula*, largely following the research on

278   rhizobial Nod factors signaling. Such comparative investigations have revealed the existence

279   of a so-called 'common symbiotic signalling pathway' (CSSP), which includes several genes

280   that are essential for both symbioses (89, 90). Evidence that the same genes are also involved

281   in diverse symbiotic, pathogenic and parasitic plant interactions is accumulating (91).

282   The CSSP starts on the plant cell membrane, with a malectin-like domain (MLD) leucine-rich

283   repeat (LRR) receptor-like kinase (known as SYMRK, in *Lotus japonicus*). SYMRK forms a

284   complex with Nod factor receptors NFR1 and NFR5 and is believed to also interact with the

285   so-far unidentified receptor(s) for AM fungal signals (90). In its cytoplasmic domain,

286   SYMRK also interacts with a MAP kinase kinase (92), and HMGR1, a 3-hydroxy-3-

287   methylglutaryl-CoA reductase involved in mevalonate synthesis. Indeed, mevalonate has

288   recently been demonstrated to trigger downstream symbiotic responses such as nuclear Ca^{2+}

289   spiking and ENOD11 expression (93).

290   All the remaining CSSP proteins that have currently been identified are localised to the

291   nucleus. They include three nucleoporins - NUP85, NUP133, and NENA (94, 95, 96) -

292   possibly involved in nuclear targeting of CSSP actors, as well as the ion channel

293   CASTOR/POLLUX and the SERCA-type Ca^{2+}-ATPase MCA8. Both these latter proteins

294   localize to the nuclear envelope (97, 98) and are essential for nuclear Ca^{2+} spiking (98, 99,

295   100, 101). The nuclear envelope lumen is considered the site where Ca^{2+} is stored and

296   released from during symbiotic Ca^{2+} signaling. The Ca^{2+}-and-calmodulin-dependent protein

297   kinase CCaMK localizes to the nucleoplasm and is composed of a serine/threonine kinase

298   domain, a calmodulin binding domain and three Ca^{2+}-binding EF-hand domains (102, 103).

299   Upon an increase in Ca^{2+} concentration, CCaMK is subject to a complex conformational

300   change (102, 104, 105). When active, CCaMK regulates gene expression through its

301   interacting partner, CYCLOPS (106, 107, 108) and the action of transcription factors such as

302   NSP1 and NSP2 (109, 110, 111, 112), NIN (113, 114) and RAM1 (89, 115).

303   The exchange of chemical signals that mediate reciprocal recognition probably becomes more

304   intense as soon as a hyphopodium adheres to the surface of the root epidermis. Hyphopodium

305 differentiation depends on plant cell wall-bound signals as shown by the seminal studies of

306 Giovannetti et al. (116) and Nagahashi and Douds (117). Only recently, though, monomeric

307 cutin has been proposed to be responsible for hyphopodium differentiation. This deduction

308 comes from the observation that RAM1 activation increases the expression of RAM2, a

309 glycerol3-phosphate acyl transferase involved in the biosynthesis of cutin precursors (115,

310 118).

311 Interestingly, in a recent paper Gutjahr et al. (119) identified loss of responsiveness to AM

312 fungi in a rice mutant, which was also mirrored by the absence of physical contact and of

313 characteristic transcriptional responses to AM fungal diffusible signals. The gene responsible

314 for the loss of symbiosis, *DWARF 14 LIKE* (*D14L*), encodes an alpha/ beta-fold hydrolase,

315 that is a component of an intracellular receptor complex involved in the detection of the

316 smoke compound, karrikin. Thus D14L seems to be required to support initial colonization

317 events by AM fungi. Overall, these results reveal a novel plant recognition strategy for AM

318 fungi and envisage the existence of an additional signaling molecule, the D14L ligand.

319

320   **Host cell colonization**

321 As soon as a hyphopodium develops on the root surface, the nucleus of the underlying

322 epidermal cell moves toward the fungal contact site, then migrates to the opposite side of the

323 cell traversing the lumen and partially displacing the vacuole. Concurrently, a broad,

324 columnar cytoplasmic aggregation assembles between the nucleus and the fungal contact site.

325 This aggregate is rich in endoplasmic reticulum, cytoskeleton, Golgi stacks and secretory

326 membranes, and constitutes the so-called the prepenetration apparatus, or PPA (Fig. 3) (64, 66,

327 120). Such features characterize the PPA as a broad exocytotic process finalized at the

328 construction of the novel membrane domain - in fact an extension of the host plasmalemma -

329 where the fungus will be hosted: the perifungal membrane which envelops all intracellular

330   fungal structures (120). Only after the PPA is fully deployed, a penetrating hypha develops

331   from the hyphopodium, crosses the epidermal cell wall and enters the cell lumen, strictly

332   following the PPA route (64). PPAs are not observed in plants that lack CSSP genes such as

333   *dmi2* or *dmi3* (64). Furthermore constitutive expression of an active CCaMK variant induces

334   cytoplasmic aggregates that resemble a PPA (121). Consequently, one key function of the

335   CSSP is the activation of the cellular program responsible for fungal hosting (11).

336   PPA formation is not limited to epidermal cells, where the fungus starts its intracellular

337   development, but is also observed in outer and inner cortical cells, in preparation for

338   arbuscule formation (66). Cortical cells that are preparing to harbor an arbuscule display the

339   most extensive PPAs: here, the cell membrane invagination does not envelop a single hypha,

340   but progressively expands to line each of the fine branches that can fill up most of the cell

341   lumen (10). The perifungal membrane - around intracellular hyphae - or periarbuscular

342   membrane (PAM) around arbuscules (65) outlines the so-called symbiotic interface, the novel

343   cell compartment where the fungus is hosted and where most of the signal and nutrient

344   exchanges are believed to occur (123, 124). In line with this, the PAM comprises a specific

345   sub-set of membrane-associated proteins (122). Though the signal that induces branching and

346   differentiation of arbuscules is currently unknown, several plant genes required for arbuscule

347   development and/or function have been identified, including Vapyrin (125), two Vesicle-

348   Associated Membrane Proteins (126), EXO70I (127), proteases (128, 129), a proton ATPase

349   (130, 131), ATP-binding cassette (ABC) transporters, Stunted Arbuscule (STR) and STR2

350   (132) and phosphate transporters (133, 134). Interestingly, trafficking of the symbiotic

351   phosphate transporters and ABC transporters to the PAM requires gene expression coincident

352   with arbuscule branching (135), leading to the hypothesis that PAM construction is achieved

353   by synchronizing two cellular and molecular processes: the massive reorientation of

354   exocytosis towards the developing PAM, and the transcription of specific genes encoding for

355 PAM-resident proteins (11, 135). As transcriptional control seems crucial to ensure the

356 correct protein composition of the PAM, plant transcription factors active in the AM

357 symbiosis have been also characterized. So far, transcription factors required for AM

358 symbiosis include CYCLOPS (108, 136), the gibberellin repressor protein DELLAs (137,

359 138), Reduced Arbuscular Mycorrhiza1 (RAM1; 115), Required for Arbuscule Development1

360 (RAD1; 139), MtERF1 (140), and DELLA-Interacting Protein1 (DIP1; 141). Recent results

361 suggest a model where DELLA proteins regulate arbuscule development through modulation

362 of RAM1 and RAD1 that in turn regulate genes required to support arbuscule branching (142).

363 Arbuscules are ephemeral structures that collapse and degenerate approximately two to three

364 days after maturity (11, 143), while the host cell regains its previous organization and can

365 undergo a new round of colonization.

366 The correct formation and functioning of an arbuscule is also expected to be under fungal

367 control; however, the functional studies on the fungal partner are very few. Kloppholz et al.

368 (144), discovered the first AM fungal effector, named secreted protein 7 (SP7). Effector

369 proteins are secreted by plant-colonizing microbes and are generally thought to promote

370 compatibility or to suppress plant defense responses by interfering with metabolism or

371 signaling pathways (145; chapter XX of this book). In particular, SP7, which is secreted into

372 the host cell and localizes to the plant nucleus, counteracts the plant immune response by

373 interacting with the pathogenesis-related transcription factor Ethylene Response Factor 19

374 (144). Although their mechanisms of action have not been elucidated yet, two additional

375 fungal genes have been recently identified with a putative role in accommodation of fungal

376 structures in the root (146, 147).

377 While such targeted investigations start shedding light on the cellular and molecular

378 mechanisms that act inside each host cell, transcriptomic and genomic studies suggest that

379 these are just the first steps into the characterization of fungal effectors and their function in

380   AM (148, 149).

381

382   **The transfer of nutrients**

383   Nutrient uptake and transfer to the host plant are the most documented roles of AM fungi. The

384   extraradical mycelium acts as an extension of the root system, taking up phosphate (P),

385   nitrogen (N), sulfur (S), and trace elements from the soil, and delivering them to the host plant

386   *via* the intraradical mycelium (8). The PAM is considered the site where this symbiotic

387   transfer occurs: mineral nutrients released in the interface compartment are captured by PAM-

388   bound plant transporters that translocate them to the host cell cytoplasm (150).

389

390   **Phosphorus**

391   Two pathways contribute to inorganic phosphate (Pi) uptake in mycorrhizal plants: a direct

392   pathway by the root epidermal cells and a mycorrhizal pathway *via* AM fungi (151, 152). AM

393   fungi are capable of significantly improving the uptake of Pi ions, which are characterized by

394   low mobility and availability in soil. By using radioactive P, Smith and Smith (8) found that

395   in mycorrhizal plants most of the P delivered to the plant came from the fungus and that the

396   direct pathway was almost inactive. Indeed, depending on the plant and fungal species, AM

397   fungi can contribute from 20 to 100% of the plant P uptake (153).

398   The mycorrhizal pathway involves initially the fungal uptake of soluble Pi from the soil. This

399   is mediated by $Pi:H^+$ transporters, which have been described in *Diversispora epigaea* (154),

400   *Rhizophagus irregularis* (155) and *Funneliformis mosseae* (156). However, a role remains to

401   be clarified for the putative Pi:Na+ transporters RiPT1 and RiPT2, recently identified in the *R.*

402   *irregularis* genome (152).

403   Within the extraradical mycelium, Pi can supply the metabolically active Pi pool (used for the

404   biosynthesis of phospholipids, DNA, RNA and proteins) or rapidly accumulate in vacuoles in

405   the form of long-chain or short-chain polyphosphates (polyP) (157, 158), presumably through

406   the action of the polyP polymerase/vacuolar transporter chaperone complex (148). PolyP is

407   considered the major Pi store in hyphae as well as the main form of Pi translocation over long

408   distances within hyphae (159). Indeed, polyP accumulation in the extraradical mycelium

409   mirrors an equivalent Pi uptake from the soil (160). Interestingly, polyP translocation towards

410   the host is mediated by the activity of a fungal aquaglyceroporin, which is highly expressed in

411   the intraradical mycelium and is responsible for water transport across the plasma membrane

412   (161). These findings provide novel insights on the mechanisms involved in the directional P

413   transport towards the roots, and they highlight a key role of host transpiration and fungal

414   aquaporins. PolyP degradation in the intraradical mycelium, possibly by vacuolar

415   endopolyphosphatase and exopolyphosphatase activities, sustains Pi flux from the fungus to

416   the apoplastic interface compartment (148, 162).

417   On the plant side the activation of the mycorrhizal pathway is mirrored by the downregulation

418   of plant Pht1 (H^+-dependent) transporters located in root epidermal cells, such as *Medicago*

419   *truncatula* phosphate transporters MtPT1 and MtPT2 (163) and the upregulation of

420   mycorrhiza-inducible Pth1 transporters (152 and references therein). Some of them are mostly

421   or exclusively expressed in arbusculated cells. Among them, MtPT4 localizes to the PAM

422   surrounding the arbuscule branches (164, 122). Interestingly, the two mycorrhiza-inducible Pi

423   transporters, *MtPT4* and *LjPT4*, are expressed in the root tips of non-colonized plants,

424   suggesting they play a role in the Pi-sensing machinery of root tips (165).

425

426   **Nitrogen**

427   Although the impact of AM symbiosis on plant N uptake is not as clearly defined as that of Pi,

428   there is increasing evidence for the existence of a N pathway through the fungal hyphae to the

429   host plant, in spite of the contribution of AM fungi to the plant total N nutrition varying

430   considerably depending on the context (8, 158, 166). Nitrogen is found in soil in both organic

431   and inorganic compounds and plants use all of them. The former include simple molecules

432   such as urea, amino acids, amines and peptides, and complex ones, such as proteins, while

433   inorganic N compounds are mainly represented by nitrate (NO_3^-) and ammonium (NH_4^+). In

434   soils where N is limited or poorly mobile, due to drought or acidity, the contribution of the

435   AM fungus to plant N nutrition can be considerable (152, 167) ranging between 24 and 42%

436   of a plant total N content (168).

437   A few mechanisms of N uptake and transfer in the AM symbiosis have been recently

438   described (166). Extraradical hyphae preferentially take up NH_4^+, which is energetically less

439   costly than alternative N sources such as NO_3^- and amino acids. López-Pedrosa et al. (169)

440   demonstrated that *GintAMT1*, a gene encoding for a high-affinity NH_4^+ transporter (AMT) in

441   the AM fungus *R. irregularis*, is expressed in the extraradical mycelium. A second *R.*

442   *irregularis* AMT, has been characterized (170). *GintAMT1* and *GintAMT2* are differentially

443   expressed during the fungal life cycle and in response to N. In contrast to *GintAMT1*,

444   *GintAMT2* transcript levels are higher in the intraradical than extraradical hyphae.

445   Inside the extraradical mycelium, N compounds are converted into amino acids, mainly

446   arginine (171, 172). Arginine is then translocated to the intraradical hyphae within tubular

447   vacuoles and then reconverted into inorganic N compounds by the sequential enzymatic

448   activitiy of arginase and urease: NH_4^+ is the most likely form of N transferred from fungus to

449   plant (173, 174). It has been proposed that arginine binds to the negatively charged poly-P

450   and both could move together within the hyphae (171, 175). The eventual transfer of NH_4^+

451   from the apoplast to the plant cells probably relies on NH_4^+ transporters sitting on the PAM

452   (176, 177, 178). Remarkably, mutations in AM-specific Pi and NH_4^+ transporters have an

453   impact on intraradical fungal development and arbuscule lifespan (179, 180). It has been

454   speculated that these transporters not only deliver nutrients to the plant cells, but also trigger

455   signaling processes that control arbuscule maintenance (180).

456   Even though the flux of mineral nutrients within the periarbuscular space is assumed to be

457   directed towards the plant cell, fungal Pi (181, 182) and NH_4^+ transporters (170) are expressed

458   in arbuscules. This finding suggests that the fungus may recover nutrients from the

459   periarbuscular interface, as a mechanism to control the amount of nutrients delivered to the

460   host.

461   As well as taking up inorganic N, AM fungi also appear to obtain N from complex organic

462   material (183, 184, 185, 186). Such a process probably involves, among other transporters, an

463   amino acid permease (AAP). A fungal APP (GmosAAP1) has been characterized in *F*.

464   *mosseae*. Since *GmosAAP1* is expressed in the extraradical mycelium and upregulated upon

465   exposure to organic nitrogen (187), this gene may play a role in the first steps of amino acid

466   acquisition from the soil. Since short peptides can represent a greater proportion of N in soils

467   than free amino acids, it is notable that AM fungi also possess functional dipeptide

468   transporters such as RiPTR2 (188). In yeast complementation assays, RiPTR2 allowed the

469   uptake of several dipeptides such as Ala-Leu, Ala-Tyr, Tyr-Ala. *RiPTR2* is expressed in the

470   extraradical hyphae, suggesting a role in the uptake of organic N from soil; however, a

471   stronger expression is consistently observed in the intraradical phase (188). This finding

472   points to a function for this transporter in the mobilization of organic N in mycorrhizal roots.

473   Despite all the interesting findings, many critical questions about N transport through the fungal

474   hyphae and across the mycorrhizal interface are still unanswered and should be addressed in

475   future studies (166).

476

477   **Other mineral nutrients**

478   In addition to the improvement of plant N and P nutrition, physiological studies also have

479   highlighted a role for AM fungi in enhancing the absorption of other ions such as sulphur,

480   potassium or different secondary macro- and microelements (1, 152).

481   Sulphur (S) is a key macronutrient for plant growth, development and response to several

482   stresses. Casieri et al. (189) observed an increased S content in mycorrhizal compared to non-

483   mycorrhizal plants and the up-regulation of two S transporters (*MtSULTR1.1* and

484   *MtSULTR1.2)* in *Medicago truncatula*. More recently, a *Lotus japonicus* transporter

485   (LjSultr1;2), specifically involved in sulphate uptake from arbuscules, has been identified

486   (190).

487   Despite the importance of potassium (K^+) for plant growth, the contribution of AM symbiosis

488   to plant K^+ nutrition has only occasionally been studied. It appears that plant K^+ nutrition is

489   improved by mycorrhization, especially under K^+ limiting conditions. Moreover, this

490   improvement could affect abiotic stress tolerance, P homeostasis maintenance, or exclusion of

491   soil contaminants such as radiocaesium (191 and reference therein). The characterization of

492   genes involved in the transport and metabolism of K^+ and other mineral nutrients is required

493   before a comprehensive map of the transportome of arbuscular mycorrhizae can be developed.

494

495   **Carbon**

496   In exchange for the improved access of plants to nutrients, AM fungi take advantage of

497   carbon compounds of plant origin, consuming between 10 to 30% of the plant photosynthates

498   (192). The transcriptional regulation of genes involved in sucrose transport has been reported

499   in several plant-fungus combinations (152 and references therein), although more efforts are

500   required in order to clarify which plant sucrose transporters and regulatory mechanisms are

501   active in sucrose partitioning during mycorrhization. On the fungal side, only one high-

502   affinity monosaccharide transporter (MST), probably responsible for C uptake from the

503   interface compartment, has been described (193). The gene *MST2* is highly expressed in

504   arbuscules and intercellular hyphae. The high affinity and transport capability for xylose

505   residues suggest that the use of derivatives from plant cell wall polymers could be an

506   additional source of C. Interestingly, *MST2* is also expressed in extraradical hyphae, which

507   can take up glucose and xylose, suggesting a partial metabolic independence of AM fungi

508   from host plants.

509   For full mutualism to occur, a functional linkage between C and P exchange, under a fine

510   control by both partners, is likely to be in place. Recent studies have demonstrated that a

511   strategy of reciprocal rewards rules AM interactions: in the presence of multiple partners, the

512   most beneficial one is rewarded with the majority of resources (14, 194). A similar

513   mechanism also appears to regulate C and N exchange (175). These striking results support

514   the idea that biological market dynamics ensure the stable regulation of resource exchange in

515   the evolution of AM symbiosis. However, other evidence suggests that reciprocal regulation

516   represents only a fraction of the forces determining resource exchange in the AM symbiosis

517   and such reciprocity is only found in a subset of symbionts under specific conditions (195).

518

519   **The impact of AM symbiosis on above ground organs of the host plant**

520   The impact of the AM symbiosis goes beyond the root apparatus and involves distal parts of

521   the plant through a fine shaping of the whole plant physiology. The first molecular evidence

522   of a systemic effect was observed at the level of gene expression profiles: changes in

523   transcript pattern, which were not a mere consequence of an improvement in P nutrition, were

524   observed in shoots of *M. truncatula* upon root colonization by AM fungi (196). A

525   transcriptional reprogramming was also reported for other plants such as tomato and maize

526   (198, 199, 200). Gerlach and colleagues (200) also performed parallel ionomic and

527   metabolomic analyses showing drastic changes in leaf elemental composition, a general

528 increase in C *versus* N metabolism and an accumulation of secondary metabolites. The AM

529 symbiosis therefore influences the physiological status of plant leaves.

530 The systemic effect of the AM symbiosis was recently shown to also extend to fruits with the

531 potential to increase their nutritional values. Lycopene, carotenoid and volatile compound

532 contents were significantly increased in fruits of mycorrhizal tomato plants compared to those

533 of non-mycorrhizal plants (201, 202). An overall increase in fruit yield of mycorrhizal tomato

534 plants, as well as qualitative and quantitative changes in amino acid profile accompanied

535 phenological modifications as an accelerated flowering and fruiting time (203, 204). Not only

536 vegetative but also reproductive traits are therefore under the influence of the AM symbiotic

537 interaction. This situation has major ecological and agronomical implications.

538 Such systemic effects have been proposed to depend, at least to some extent, on the action of

539 phytohormones, which are also involved in AM establishment and functioning (205, 206,

540 207). The levels of several hormones such as salycilic acid (SA), jasmonic acid (JA), abscisic

541 acid (ABA), auxin and ethylene, are altered during AM colonization (208, 209, 210, 211). In

542 addition, SA, ethylene and cytokinins are considered negative regulators of fungal penetration

543 and root colonization (205). At later stages, arbuscule development is suppressed by

544 biologically active gibberellins and promoted by DELLA gibberellin repressors (137, 205,

545 212). By contrast, ABA and auxins positively regulate arbuscule development and

546 functionality (213, 214), while contrasting effects have been described for jasmonates (215).

547 The alteration of transcriptional profiles and hormonal balance in mycorrhizal plants may also

548 have an impact on the plant response to abiotic and biotic stresses (6, 18, 216, 217, 218). AM

549 symbiosis often reduces the damage caused by soil-borne pathogens, while the effect on pests

550 and pathogens attacking from aboveground are more variable and are highly dependent on the

551 combination of AM fungus, plant and attacker (18). The effect of AM at both local and

552 systemic scale strongly suggests that the bioprotective role of mycorrhization is not simply

553 related to improved mineral nutrition, changes in the root apparatus and/or in the microbial

554 rhizosphere communities, but rather to the activation of systemic defense responses (218, 219,

555 220, 221, 222, 223). In support of this hypothesis, stress and defense-related genes are up-

556 regulated in mycorrhizal plants, which in turn show increased tolerance to foliar bacterial

557 pathogens (196, 199).

558 In this context, the combined action of plant hormones and gene regulation may contribute to

559 the generation of a primed status in the plant, allowing a more efficient activation of defence

560 mechanisms in the case of a subsequent attack (200) The identification of the full set of

561 defence regulatory elements deployed by mycorrhizal plants and indirectly driven by AM

562 fungi, will have important practical implications regarding the effectiveness of the AM

563 symbiosis in biological control and integrated management of pests and diseases.

564

565 **The common mycorrhizal network**

566 AM fungi can also influence plant community dynamics and plant-plant interactions; this has

567 major implications for natural and agricultural systems. A fascinating feature of AM fungi is

568 the ability of their extraradical mycelium to inter-connect individual plants of the same or

569 different species in 'common mycorrhizal networks' (CMNs; 224). CMNs are very common

570 in terrestrial ecosystems, where they are thought to play key roles. Plants invest between 10

571 and 30% of their photosynthetic products in their fungal symbionts, and receive in exchange

572 up to 90% of their mineral requirements (4, 192). CMNs represent possible pathways for the

573 movement of soil-derived nutrients and plant-derived carbon within the network and between

574 CMN-interconnected plants. However, the knowledge on how C, N and P (as well as other

575 nutrients) are exchanged and redistributed *via* the CMNs is still limited. The transfer of C *via*

576 CMNs has been demonstrated from autotrophic to achlorophyllous (non-photosynthetic)

577 plants, yet its transfer between autotrophic plants remains more controversial (225, 226**,** 227).

578   Similarly, the role of CMNs on mineral (e.g. N) transport between plants is not so clear (228).

579   Moreover, the terms of trade, that is the relationship between the investment of a given plant

580   into a CMN (amount of assimilated C), and the return of investment in terms of mineral

581   nutrients provided by the CMN, are unresolved. Different co-cultivated plants benefit

582   differently from their CMN, depending on the AM fungal species involved (229, 230, 231).

583   To address such questions, Walder et al. (232) set up an elegant microcosm experiment with a

584   pair of plants (flax and sorghum) interlinked by a CMN of either *R*. *intraradices* or *F*.

585   *mosseae*. Fluxes of C, P and N were then monitored through C stable isotope tracing and ^{15}N

586   and ^{33}P labeling. Depending on the fungal species, a strong asymmetry was observed in

587   resource exchange: flax invested little C but obtained up to 94% of the N and P provided by

588   the CMN. Furthermore, the overall biomass was larger when the plants were grown together

589   than in monoculture. Overall, CMNs appear to contribute to the productivity increase that is

590   often observed in intercropping compared with conventional monocropping systems (233).

591   These findings clearly challenge the "biological market" model where the most beneficial

592   partners are favoured (14) and suggest that resource exchange in the AM symbiosis is

593   determined by more complex factors (195).

594   Recently, a new role for CMNs was discovered. Plants can exploit CMNs to transfer defense

595   signals to neighboring individuals. The first demonstration of interplant signalling *via* CMNs

596   was in tomato plants attacked by the foliar necrotrophic fungus *Alternaria solani:* six defense-

597   related genes were upregulated in uninfected plants that were only connected to the infected

598   individuals by CMN (234). The CMN-mediated transfer of defense signals was also observed

599   between insect-attacked plants and healthy neighboring plants (235, 236). Altogether, CMNs

600   seems to act as a belowground interplant defense communication system. Nevertheless, the

601   nature and the mechanism of signal transfer through the fungal mycelium as well as the

602   ubiquity and ecological impact of interplant signaling in nature remain to be fully understood

603 (237).

604

605 **CONCLUDING REMARKS**

606 Our understanding of the AM interaction at multiple levels, from cells to ecosystems, is

607 increasingly benefiting from the developments and advances of investigation tools such as

608 'omics' technologies, live cell imaging, stable isotope tracking and genetic manipulation.

609 Already genome sequencing and analyses has accelerated our understanding of enigmatic

610 aspects of the genetics and biology of AM fungi. It is likely that mycorrhizal research will

611 more and more rely on multidisciplinary approaches and combinations of analytical

612 techniques - in both controlled and natural conditions - to answer numerous questions

613 concerning the evolution, ecology and functioning of this fascinating interaction. The holistic

614 knowledge generated by such approaches will be crucial to boost the fruitful application of

615 the AM symbiosis in sustainable agronomical practices to face current challenges in global

616 food production and security.

617

622

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Figure legends

Figure 1. Root colonization in ectomycorrhizal (blue) and arbuscular mycorrhizal (pink)

interactions. Ectomycorrhizal fungi envelop root tips with a thick mycelial mantle. From this

mantle, intercellular hyphae generate the so-called Hartig net around epidermal cells. In the

case of arbuscular mycorrhizae, the root tip is usually not colonized; hyphae developed from a

germinated spore produce a hyphopodium on the root epidermis. Intraradical colonization

proceeds both inter- and intracellularly, culminating with the development of highly branched

arbuscules inside inner cortical cells. Reprinted from *Nature Communications* (3) with

permission of the publisher.
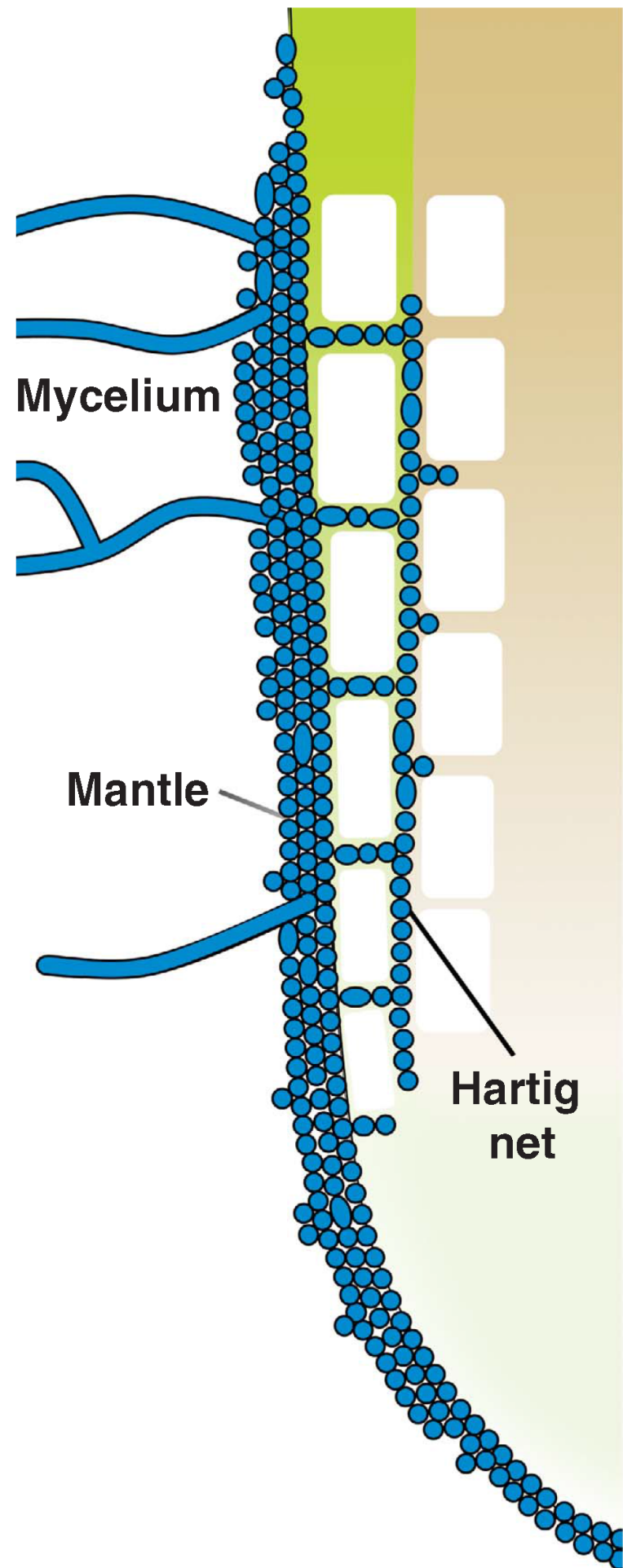
Figure 2. Fluorescence micrographs of different stages in the life cycle of the AM fungus

Gigaspora gigantea. A spore (S) and the germination hyphae (GH) show strong cytoplasmic

autofluorescence (a). Hyphopodia (arrows) on the surface of a host root (b) give rise to single

infection units, with several arbuscules (A) in the inner root cortex (c). A high magnification

from a root longitudinal section is presented in d, showing two arbuscules in adjacent cortical

cells. Bars = 100 µm (a-c); 25 µm (d); fungal fluorescence was excited with 380 nm UV light.

Figure 3. Root colonization by AM fungi. Spore germination generates a short explorative

mycelium. The perception of root exudates induces repeated hyphal branching, increasing the

probability of a direct contact between the symbionts. Concurrently, fungal exudates are also

released and activate the Common Symbiotic Signaling Pathway in root cells. Signal

transduction includes nuclear-associated calcium signals (spiking) and leads to the activation

of cellular and transcriptional responses (green cells and nuclei). Plant-fungus contact is

followed by the formation of an adhering hyphopodium on the root surface. The contacted

epidermal cell then assembles a prepenetration apparatus (PPA), a broad cytoplasmic

1399   aggregation (yellow) responsible for the exocytotic biogenesis of the symbiotic interface

1400   compartment, where the intracellular hypha is hosted. Root colonization proceeds through the

1401   epidermis into the inner cortical cells with a PPA-like process. Intercellular hyphae can also

1402   develop along the root axis. Eventually, highly branched arbuscules develop in the lumen of

1403   inner cortical cells, deploying an extensive surface for nutrient exchange. Reprinted from

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Ectomycorrhiza

Arbuscular mycorrhiza

Mycelium

Mantle

Hartig net

Hyphopodium

Arbuscules

Spore