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

2

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12

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

30 **AN OVERVIEW OF MYCORRHIZAL INTERACTIONS**

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.

80 **ARBUSCULAR MYCORRHIZAE**

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

83 AM fungi have the unusual ability to grow in two extremely different niches: the soil and the
84 lumen of plant cells. In soil, the extensive hyphal network has been estimated to reach a
85 density of 100 m/cm^3 (7). This hyphal network can acquire water and nutrients with great
86 efficiency and reach a volume of soil that is inaccessible to roots alone (8). The presence and
87 activity of AM fungal mycelia also directly influence the physico-chemical properties of soil
88 (9). Inside their host plant, AM fungi penetrate single cells of the root cortex, where they
89 develop a structure that is the distinctive feature of this association: the arbuscule (Fig. 2).

90 Named from the Latin word for bush, or small tree, each arbuscule results from repeated
91 branching of a single hypha in the lumen of a parenchymatic cell from the inner root cortex
92 (10). With their impressive surface/volume ratio, arbuscules are considered the major site of
93 nutrient exchange (11).

94 As obligate biotrophs, AM fungi strictly depend on their plant hosts for both growth and
95 reproduction (3, 12). Up to 20% of the photosynthesis products of terrestrial plants is
96 consumed by AM fungi (13). Interestingly, the carbon flow from the plant to the fungus
97 seems to be proportional to the amount of phosphate that the fungus returns to its host (14).
98 Consequently, while the beneficial effects of AM fungi become evident when the nutrient and
99 water supply are limited, root colonization decreases in soils with abundant nutrients.

100 The AM interaction also supports important ecological services such as an increase of soil
101 quality (15) and of biodiversity of the associated plant communities (16). Additionally, many
102 studies have highlighted the positive influence of the AM symbiosis on plant responses to
103 biotic (17, 18) and abiotic stresses such as drought, salinity and heavy metal contaminants (6,
104 19, 20).

105 **Classification and phylogeny of AM fungi**

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

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

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

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 heterokaryotic 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 Ca²⁺-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 Ca²⁺ signals, starch accumulation in roots and
260 lateral root formation (80, 81, 82, 83, 84, 85, 86). Repeated oscillations in nuclear Ca²⁺
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 Ca²⁺ 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 Mycorrhizal (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. Klopffholz 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 activity 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 salicylic 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 ¹⁵N
586 and ³³P 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

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622

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1373

1374 **Figure legends**

1375 **Figure 1. Root colonization in ectomycorrhizal (blue) and arbuscular mycorrhizal (pink)**
1376 **interactions.** Ectomycorrhizal fungi envelop root tips with a thick mycelial mantle. From this
1377 mantle, intercellular hyphae generate the so-called Hartig net around epidermal cells. In the
1378 case of arbuscular mycorrhizae, the root tip is usually not colonized; hyphae developed from a
1379 germinated spore produce a hyphopodium on the root epidermis. Intraradical colonization
1380 proceeds both inter- and intracellularly, culminating with the development of highly branched
1381 arbuscules inside inner cortical cells. Reprinted from *Nature Communications* (3) with
1382 permission of the publisher.

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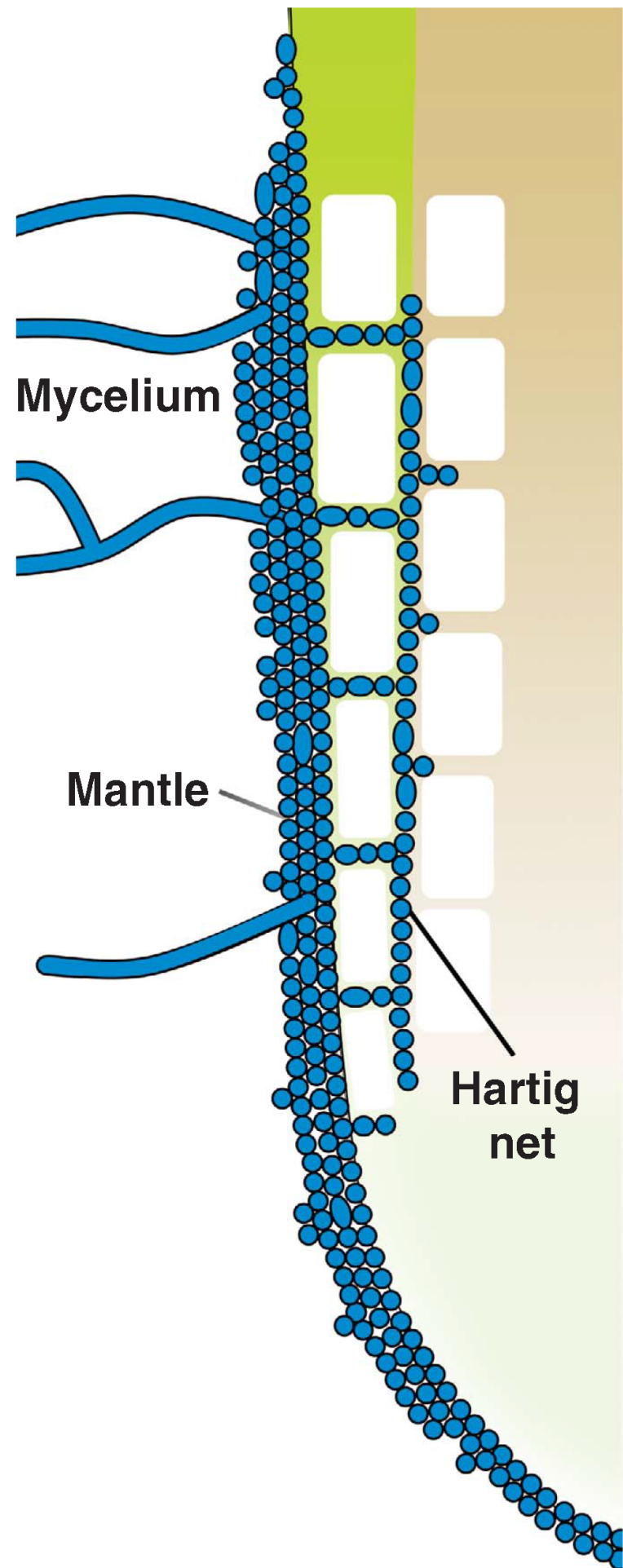
1384 **Figure 2. Fluorescence micrographs of different stages in the life cycle of the AM fungus**
1385 ***Gigaspora gigantea*.** A spore (S) and the germination hyphae (GH) show strong cytoplasmic
1386 autofluorescence (a). Hyphopodia (arrows) on the surface of a host root (b) give rise to single
1387 infection units, with several arbuscules (A) in the inner root cortex (c). A high magnification
1388 from a root longitudinal section is presented in d, showing two arbuscules in adjacent cortical
1389 cells. Bars = 100 μm (a-c); 25 μm (d); fungal fluorescence was excited with 380 nm UV light.

1390

1391 **Figure 3. Root colonization by AM fungi.** Spore germination generates a short explorative
1392 mycelium. The perception of root exudates induces repeated hyphal branching, increasing the
1393 probability of a direct contact between the symbionts. Concurrently, fungal exudates are also
1394 released and activate the Common Symbiotic Signaling Pathway in root cells. Signal
1395 transduction includes nuclear-associated calcium signals (spiking) and leads to the activation
1396 of cellular and transcriptional responses (green cells and nuclei). Plant-fungus contact is
1397 followed by the formation of an adhering hyphopodium on the root surface. The contacted
1398 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

