Review

Plants and arbuscular mycorrhizal fungi: an evolutionary-developmental perspective

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Arbuscular mycorrhizas (AMs) are widespread symbiotic associations that are commonly described as the result of co-evolution events between fungi and plants where both partners benefit from the reciprocal nutrient exchange. Here, we review data from fossil records, characterizations of AM fungi in basal plants and live cell imaging of angiosperm colonization processes from an evolutionary-developmental perspective. The uniformity of plant cell responses to AM colonization in haploid gametophytes and diploid sporophytes, in non-root organs, and throughout many seed plant clades highlights the ancient origin of the interaction and suggests the existence of common molecular and cellular processes. The possibility that pre-existing mechanisms involved in plant cell division were recruited by plants to accommodate AM fungi is discussed.

Arbuscular mycorrhizas: the outcome of a successful colonization process

In the multifaceted world of symbioses, arbuscular mycorrhizas (AMs) represent a unique interaction between two eukaryotes – an obligate biotrophic fungus and its host plant – leading to an overall improvement of the fitness of the interacting partners [1]. AMs improve plant nutrient uptake thanks to the fine exploration of the rhizosphere by the hyphae, which in return receive plant carbohydrates that are essential for the completion of the fungal life cycle. AM fungi belong to the Glomeromycota [2]: these ancient fungi have coevolved with plants for the last 400 million years, assisting the colonization of dry lands and of most ecosystems by higher plants [3]. Evidence in support of these evolutionary aspects is mostly based on the original descriptions of fossil records [4–6], reviewed together with paleobotanical data [7,8], and phylogenetic analyses based on DNA sequences [9,10].

AM fungi are considered to be intractable organisms by taxonomists because they are asexual, obligatorily biotrophic, multinucleate and unculturable microbes; therefore species definition and recognition remain open to discussion [11]. However, advances in molecular analyses have allowed their identification also in basal groups of plants [12–14] and stimulated original views on the roles that AM fungi might play in ecosytems [15,16].

The aim of this review is to illustrate the process of plant colonization by AM fungi from an evolutionary-developmental (evo-devo) perspective. To provide insights into the potential plant mechanisms that allowed the diffusion of the symbiosis, we summarize observations from fossil records and extant basal plants and focus on the colonization process of angiosperms, which has been elucidated by recently published information on the cellular and molecular dynamics regulating these events. Our final goal will be to verify whether the time is right for an integrative framework linking the knowledge bases of descriptive and ecological studies to novel indications coming from phylogenetic trees, genome sequencing projects and functional analyses.

Lessons from fossils

In 1975, Pirozynski and Malloch [3] proposed that a mutually beneficial symbiosis between fungi and plants had assisted the original invasion of plants into the harsh terrestrial environment. The claim was largely founded on the discovery of the 400 million year old fossils from the

Glossary

Arbuscule: highly branched structure produced by arbuscular mycorrhizal fungi inside the cell lumen of their host. Arbuscules are considered to be the key element of the symbiotic nutrient exchanges between the plant and the fungus.

Embryophytes: plants where the embryo is retained within the maternal tissue. They include basal groups, which are characterized by the lack of vascular tissues (mosses, hornworts, liverworts), and vascular plants or tracheophytes (ferns, horsetails, gymnosperms and angiosperms).

Gametophyte: multicellular structure or individual that produces gametes through mitosis. It is composed entirely of haploid cells and corresponds to the haploid generation of a plant life cycle.

Hyphopodium: swollen and usually highly branched hypha of an AM fungus that is attached to the host plant epidermis and that initiates intraradical colonization. In the field of AM interactions, the term 'appressorium' has often been used as a synonym for hyphopodium.

Perifungal interface: thin apoplastic compartment that surrounds each intracellular fungal structure inside the plant tissues. The interface consists of plant cell-wall components and is bordered by an invagination of the plant plasma membrane.

Phragmosome: complex of cytoplasmic trans-vacuolar strands organized in highly vacuolated plant cells in preparation for mitosis. Phragmosome strands develop between the nucleus and the pre-prophase band and progressively broaden and fuse until the central vacuole is split. Eventually the nucleus is repositioned at the centre of the cell and enters mitosis.

Sporophyte: multicellular structure or individual that produces spores by meiosis. It represents the diploid generation of a plant life cycle.

Tracheophytes: embryophytes that possess differentiated vascular tissues. They include club mosses, ferns, horsetails, gymnosperms and angiosperms.

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sedimentary rocks of the Rhynie Chert, in Scotland, which provided a wealth of information not only on Early Devonian land plants [4], but also on the fungi that inhabited this paleoecosystem [5,6]. Aseptate hyphae, vesicle-bearing hyphae and spores were detected inside rhizome tissues of *Nothia aphylla*, a vascular plant [6], suggesting that the endophytes might be related to the modern-day AM fungi. By contrast, in another Early Devonian land plant, *Aglaophyton major*, fungal structures consisting of a basal trunk bearing a bush-like tuft of repeatedly branched hyphae within the plant cell were found [5,17], which are structurally identical to the arbuscules (see Glossary) of today's AMs [18]. This provided morphological evidence that AMs were already present 400 million years ago [17]. After the discovery of Ordovician fossil communities, Redecker *et al.* [19] reported on a 460 million year old Glomeromycota-like fungus, although it was not associated with plant remains. This finding offered a calibration point for the Glomeromycota stem lineage in phylogenetic trees [8] and moved the first record of AM-like fungi back to a period when land flora was likely to have consisted of plants akin to the modern-day groups of mosses, liverworts and hornworts (Figure 1).

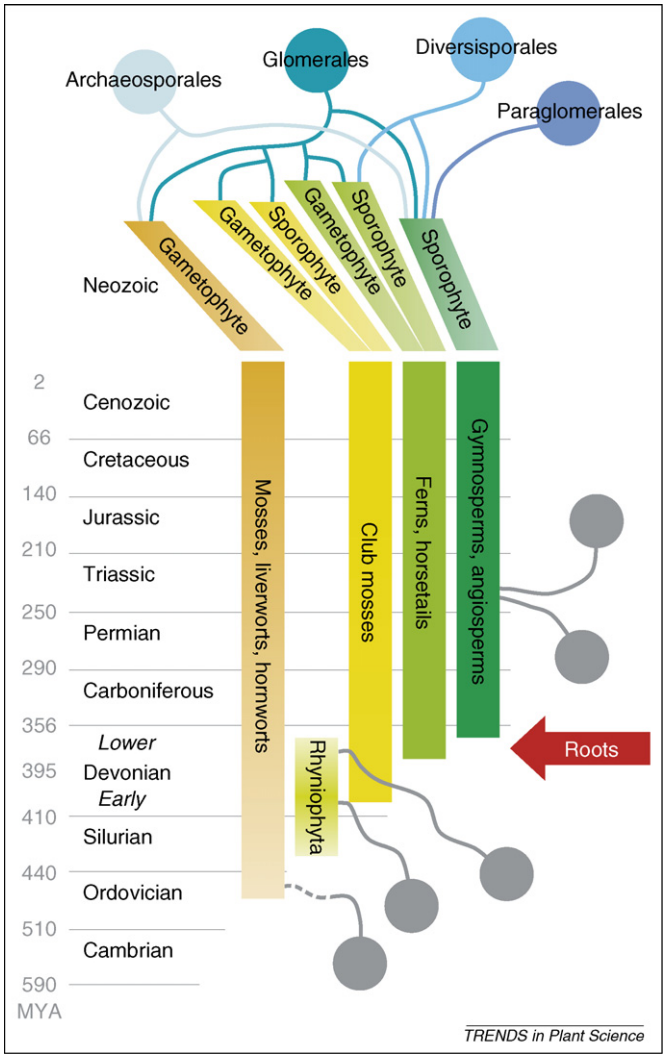

Figure 1. Summary of the current knowledge on AM fungal distribution throughout geological periods, plant taxa, gametophytic and sporophytic generations. The distribution of extant AM fungal families (Archaeosporales, Glomerales, Diversisporales and Paraglomerales [2]) throughout haploid and diploid generations in the present flora is illustrated in the top part of the chart on the basis of findings reported in Refs [12–14] and reviewed in Refs [8,11,15,16]. The lower part localizes the currently known AM fossils on a time scale, side by side with plant evolution. The broken grey line indicates the speculative association between the AM-like fossil fungi and the plant clades existing in the Ordovician [19]. The solid grey lines illustrate AM-like fungi associated to the Devonian Rhyniophyta [4–7,17] or fossil tracheophytes [7]. The scheme also highlights how AMs pre-dated the appearance of roots (red arrow). MYA = million years ago.

A model of AM evolution can be outlined by combining morphological and structural evidence from fossil records with phylogenetic analyses and divergence-time estimates that reconstruct the evolutionary lineages of these Devonian plants [8] and fungi [10].

Plant ploidy and AM symbiosis

All plants undergo a life cycle that takes them through both haploid (gametophytic) and diploid (sporophytic) generations. One of the most important changes that occurred during the first 100 million years after plant colonization of dry land was the progressive rise of taxa with dominance of the diploid phase in their life cycle (Figure 1). The fossil AM samples discussed in the previous paragraph involve plants that are mostly ascribed to the sporophytic generations. However, within extant plant species (i.e. liverworts and hornworts as well as basal tracheophytes, such as club mosses and ferns) the presence of AM fungi in gametophytes has been known for a long time [20]. In the 1980s, ultrastructural investigations led to extensive descriptions of AM-like fungi in liverwort and fern gametophytes, as well as in many vascular sporophytes [18,21,22]. More recently, the direct comparison of sporophytic and gametophytic infected tissues from the same fern, *Psilotum*, confirmed that the colonization process was remarkably similar [23], and DNA analyses of ribosomal genes identified the endophytes in many of these basal groups [12–14] as belonging to the Glomeromycota (Figure 1).

The transition from the haploid to diploid status seems therefore to have had little effect on the ability of an organ to be colonized by extant AM fungi. The genetic basis of such a fundamental transition is still poorly understood [24]. Nonetheless, the finding that the gene *AtRHD6* (*ROOT HAIR DEFECTIVE 6*), which promotes root hair development in *Arabidopsis*, also controls rhizoid morphogenesis in the gametophyte of *Physcomitrella* argues that genes with gametophyte-related functions in ancestral land plants were conserved when the sporophytes took over [25]. In the context of AM development, this finding is of particular interest: Glomeromycota can use rhizoids as an entry site into liverworts and ferns [23] and root hairs for entry into higher plants [26], thereby exploiting plant structures with a different origin but with an analogous function.

In conclusion, AM symbiosis has managed to persist smoothly across one of the major metamorphoses of plant development that led to the present dominant position of diploid sporophytes over haploid gametophytes.

Not just roots: the diverse targets of AM fungi

The word mycorrhiza literally describes 'a fungus-associated root'. However, the analysis of fossil records and present-day plant materials consistently suggests that

roots as such are not a prerequisite to successful AM colonization.

Fossil plants from the Rhynie Chert do not possess true roots [8], which in fact appeared as developmentally and anatomically distinct organs in the sporophytes of early vascular plants in Lower Devonian times [27]. This was ~15 million years after the appearance of tracheophytes and 50 million years after the earliest embryophytes. In short, fossil records provide evidence that fungal organisms that entered into mycorrhizal-like symbioses existed before the appearance of true roots (Figure 1).

In *Aglaophyton major* [17], the symbiotic niche of AM fungi was in fact not a root, but rather derived from a shoot tissue. Even in today's ecosystems, AMs do not necessarily involve roots. For example, in *Psilotum*, AM fungi grow in rhizomes [23]. Strictly speaking, the adventitious roots of higher plants originate from shoot cells: many experiments have demonstrated that adventitious roots, for example from onion and leek, are heavily colonized [28,29]. AM fungi thus appear to be more versatile than is generally considered, with regard to the host organs they target. This plasticity is not unique to AM fungi: a more striking and extreme example is that of *Magnaporthe oryzae*, the rice blast agent, which is usually considered to be a leaf pathogen; *M. oryzae* also prospers in roots [30], without major alterations to its developmental program.

Although root architecture is not essential for AM colonization, multicellular tissue organization seems to be mandatory for this process. Isolated or cultured cells are never colonized, even under strong fungal pressure such as in laboratory experiments. A recent study [31] demonstrated that soybean cultured cells respond to AM fungal exudates by modulating intracellular calcium concentration and the expression of symbiosis-related genes, thus showing the ability to perceive diffusible fungal signals. However, they are never recognized by the fungus as a potential colonization target [32].

Not all cell types in a host organ can be colonized. The fungus preferentially develops inside the inner tissues of the host, commonly described as 'cortical tissues'. In liverworts, Glomeromycota proliferate in the internal parenchyma [13], particularly along the thallus midrib. The fungus usually penetrates through the rhizoids and then enters the parenchyma cells beyond the lower thallus epidermis, mostly following an intracellular colonization strategy (Figure 2). Large coiled hyphae spread from cell to cell and originate intercalary arbuscule-like structures with thick trunk hyphae.

The preferential niche for AM fungi is also represented by cortical tissues when they colonize the roots of higher plants. After a very limited growth across the epidermis (usually involving one single cell), they spread into the root cortex through a variety of colonization strategies (Figure 3). By contrast, root meristems and differentiating tissues are never infected, and neither are the endodermis, the vascular tissues or specialized cells such as those accumulating phenols. The determinants of such a specificity remain obscure, partly because our understanding of the genetic mechanisms and of the master genes that regulate root development is restricted to seed plants [33] and mainly to *Arabidopsis* [34], which is unfortunately one of the few non-mycorrhizal plants. Large-scale transcriptomic analyses of some model mycorrhizal plants, such as rice and *Medicago* [35,36], have so far failed to

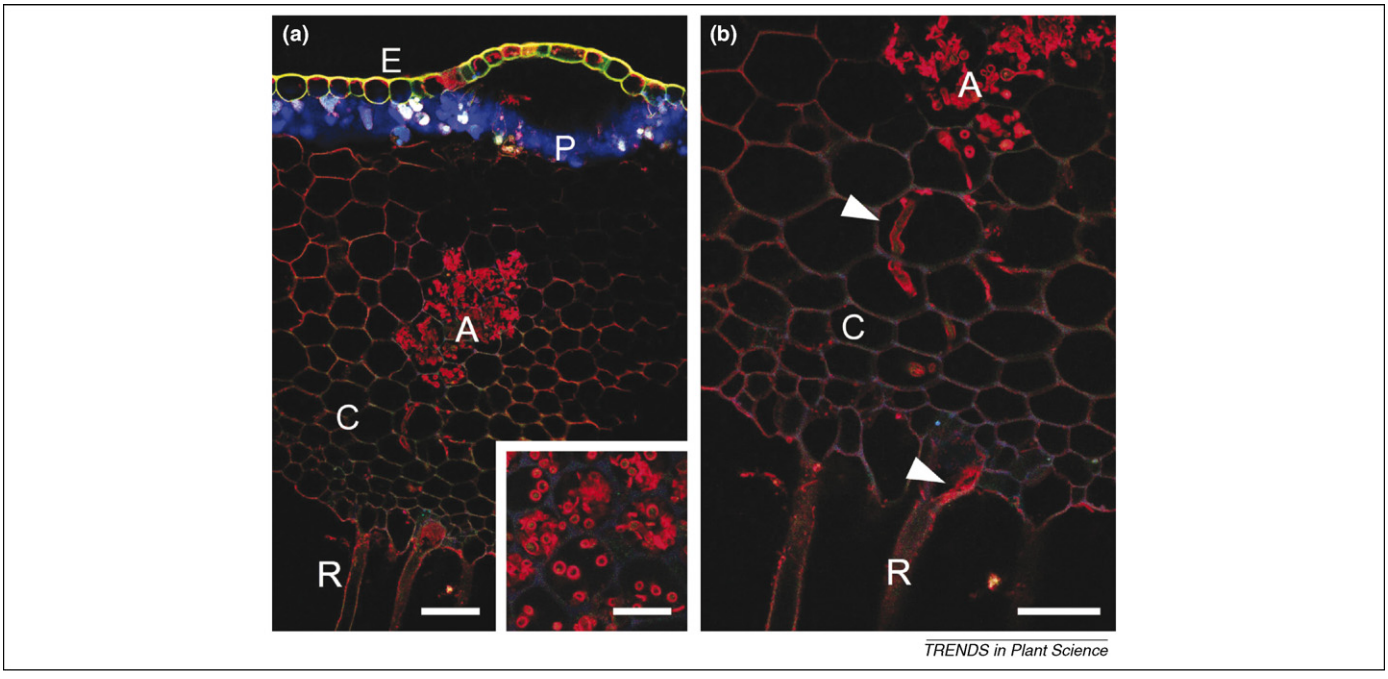

Figure 2. Confocal images showing AM colonization in a cross section of a *Marchantia polymorpha* gametophyte. Plant cell wall autofluorescence outlines the rhizoids (R) and the non-photosynthetic cortical tissues (C) in dark red, as well as the epidermis (E) in yellow. The palisade parenchyma (P) is marked by chlorophyll autofluorescence, false-coloured in blue. Fungal cell walls are labelled in light red by tetramethylrhodamine-conjugated wheat germ agglutinin. **(a)** The arbuscule-like structures (A) develop in the central region of the cortical tissue. The inset shows a higher magnification of colonized cells: the absence of intercellular hyphae indicates that the fungus follows an intracellular route similar to that shown in Figure 3b. **(b)** Penetrating hyphae (arrowheads) grow from the rhizoids through the outer cell layers (arrowheads) to reach the cortical tissues (C). Scale bars represent 100 μm in (a); 50 μm in (b); 25 μm in inset.

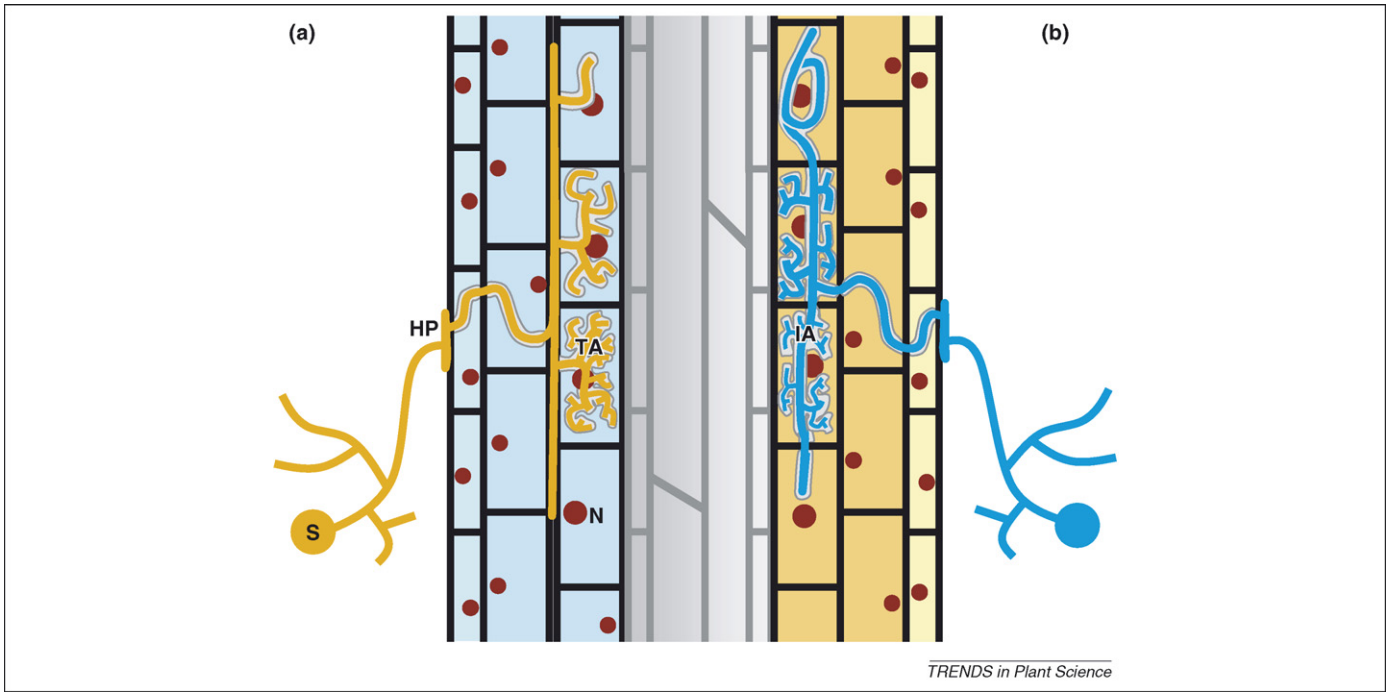

Figure 3. Root colonization by AM fungi. The scheme summarizes the main features of AM fungal development and root colonization patterns in angiosperms. The spontaneous germination of soil-borne AM spores (S) originates a short germination mycelium. The fungus then recognizes its host plant through an only partially understood chemical dialogue before initiating the development of infection units. Hyphal growth and branching are known to be stimulated by root exudate molecules [44]. This increases the chances of contact with root epidermal cells, where swollen hyphopodia (HP, also known as appressoria) are formed. After this event, the symbiotic phase initiates with the development of a penetrating hypha, which in most cases follows an intracellular route across epidermal and outer cortical cells. Once the inner cortex is reached, infection can either proceed via **(a)** intercellular hyphae that in turn originate terminal arbuscules (TA), as in most legumes, or via **(b)** intracellular hyphal coils that subsequently differentiate into intercalary arbuscules (IA), as in carrot. The two colonization patterns were first described by Gallaud at the beginning of the last century [29] and recently revisited in the so-called 'Arum-Paris continuum' model, which describes the possible range of intermediate patterns in relation with the identity of the fungal and plant partners. In all cases, intracellular hyphae are surrounded by the perifungal interface: an apoplastic compartment limited by an invagination of the plant plasma membrane and containing a thin layer of cell-wall materials of plant origin. In addition to this, arbusculated cells show nuclear (N) enlargement and repositioning close to the fungal branches.

reveal whether genes that control root development and cell-type specification in roots, for example *SCARECROW* or *SHORTROOT* [34], are involved in AM interaction.

Scaling down to the cellular level, AM-colonized cells of liverwort and fern gametophytes [13,23] show the same ultrastructural features as analogous cells from fern sporophytes. Large hyphae cross the plant cell wall with limited signs of wall degradation and ramify to produce branched arbuscule-like structures. A very similar pattern is present in gymnosperms such as *Ginkgo biloba* [37] or in angiosperms such as *Daucus carota* (Figure 4).

Arbuscule accommodation changes the host cell architecture to a great extent. The nucleus of arbuscule-containing cells moves from the periphery to a central location, the vacuole is fragmented, plastids change their morphology to avoid starch accumulation and a novel apoplastic compartment is produced by plasma membrane proliferation and cell-wall deposition all around the fungus [38]. Irrespective of the plant clade, all arbusculated cells in fact develop a very intimate, intracellular, contact with the fungus (Figure 4b), although this is still physically separated from the cytoplasm by the perifungal membrane and a thin interfacial material [38,39]. These features represent a landmark for biotrophic associations and are strikingly uniform. Morphological differences reported in literature (arbuscule positioning, intensity of branching, absence or presence of intercellular hyphae) seem to be secondary aspects that reflect the structural diversity characteristic of the vast majority of plant clades.

In conclusion, AM fungi can gain access to inner plant tissues via single-celled rhizoids, root hairs or epidermal cells, but irrespective of the entry route, they find in cortical tissues a niche where arbuscules develop. Our knowledge on the molecular and genetic mechanisms regulating plant responses to AM fungi is currently restricted to a few model plants, some legumes and rice [35,36,40,41]. It will be of great interest to understand whether such responses are conserved in all AM host plants. A recent study on AM fungi living in Lycopsid and Psilotaceae lineages where sporophytic and gametophytic phases are independent [14] demonstrates that most fungal species are present in both achlorophyllous gametophytes and photosynthetic sporophytes, suggesting that carbon transfer from the latter to the former might be mediated by the shared symbiotic mycelium. This of course raises the question whether, in spite of the structural uniformity discussed above, the role played by the fungus is different inside a gametophytic or sporophytic cell [16]. As an example, it would be crucial to understand whether the fungal and plant transporters for carbon and phosphate, which are the functional markers for an efficient mycorrhiza [42,43], also operate in basal plants.

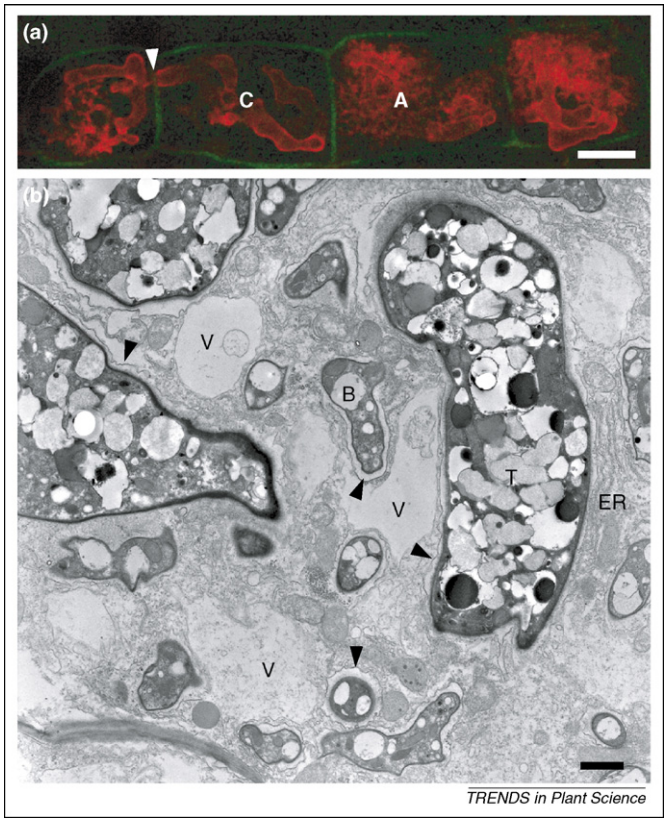

Figure 4. Intercalary arbuscules of *Gigaspora margarita* in carrot roots. **(a)** Confocal image showing a series of hyphal coils (C) and arbuscules (A) in a file of inner cortical cells after fungal wall labelling with tetramethylrhodamine-conjugated wheat germ agglutinin (red). This plant–fungus association is characterized by an intracellular colonization pattern, where hyphae grow from cell to cell (arrowhead) across the plant cell wall (weak green autofluorescence). Scale bar represents 20 μm. **(b)** Transmission electron microscopy image showing the ultrastructural details of an arbusculated cell. Thinner branches (B) originate from the huge trunks of the fungus (T). Irrespective of their size, all the hyphae are in intimate contact with the plant cytoplasm, being surrounded by the perifungal interface (arrowheads) and adjacent to sheets of endoplasmic reticulum (ER). Owing to the massive fungal development, the central vacuole appears in the form of vacuolar lobes (V). Scale bar represents 1.5 μm.

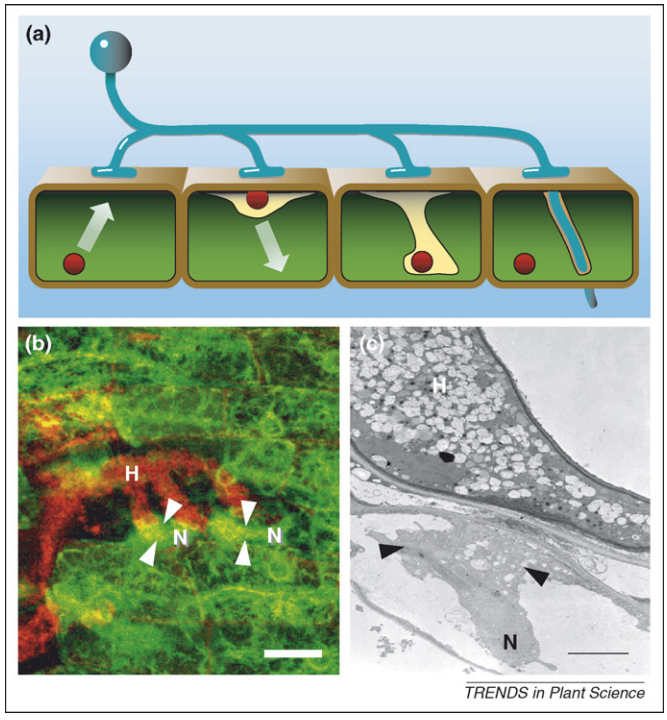

Figure 5. The prepenetration apparatus (PPA) in root epidermal cells. **(a)** The scheme illustrates the process of PPA development. Fungal contact triggers nuclear repositioning and the assembly of a localized cytoplasmic aggregation underneath the hyphopodium. Subsequently, the nucleus initiates a second migration across the cell, associated with the assembly of a broad cytoplasmic bridge, the PPA, predicting the trajectory of intracellular hyphal development. Finally, fungal penetration is associated with PPA disassembly, and the intracellular hypha is surrounded by the host plasma membrane and a thin layer of cell-wall materials. **(b)** Confocal image showing two PPAs assembled inside two carrot root epidermal cells between their nucleus (N) and the *Gigaspora gigantea* hyphopodium (H) contact points. The PPAs (arrowheads) are visualized by labelling the plant endoplasmic reticulum with GFP-HDEL (green), and fungal autofluorescence is shown in red. Scale bar represents 20 μm. **(c)** Transmission electron microscopic image presenting the same situation, with a swollen hyphopodium (H) contacting the root epidermis and the underlying epidermal cell showing a cytoplasm-rich PPA (arrowheads) between the contact point and the nucleus. Scale bar represents 7 μm.

Hosting AM fungi inside a plant cell: a co-optation of pre-existing cellular mechanisms?

The constancy of several basic traits in AM (i.e. plant cell responses, fungal growth and accommodation) offers a starting point for researching the development of this symbiosis. AM fungi do not simply colonize their host tissues by contacting their surface or sneaking between their cells: as a result of complex and still largely unknown signalling mechanisms [44], they directly enter the plant cell lumen with an ease that many pathogenic fungi have never achieved [45].

The general organization of colonized cells and the concept that the perifungal interface and membrane are built by the plant have been acknowledged since the first pioneering studies dating back to the late 1970s [18]. Conversely, the subcellular dynamics leading to the assembly of this unique apoplastic compartment have only recently been investigated by combining *in vitro* mycorrhizal root organ cultures with confocal and electron microscopy [46,47].

This approach has led to the description of the prepenetration apparatus (PPA) [46], an ephemeral intracellular structure, composed of an aggregation of cytosol, cytoskeleton and organelles, including the nucleus (Figure 5). This aggregation develops as a cytoplasmic bridge across the epidermal cell, predicting the track of the penetrating hypha a few hours before the fungus actually enters the cell lumen.

More recently [47], root cortical cells have also been demonstrated to respond in advance to fungal penetration via PPA-like assemblies where the nucleus again has a major involvement. The organization of such cortical PPAs is finely modulated depending on the fungal structure (hypha, arbuscule) that the cell will eventually host. Confocal and TEM observations have revealed that the PPA contains cytoskeleton elements, endoplasmic reticulum (ER), numerous Golgi bodies, plastids and mitochondria, and it is particularly rich in proliferating membranes, thus supporting its role in the synthesis of the perifungal interface. The organization of such membranous compartments closely recalls the vesicular-tubular pattern observed during cell plate deposition at the end of cell division.

In addition to this ultrastructural clue, several morphological and functional analogies can be drawn between PPA assembly and cell division events. Both processes directly involve the whole exocytotic machinery: ER, Golgi

apparatus, secretory vesicles and cytoskeleton. In both cases, *de novo* cell-wall deposition occurs within the cell lumen rather than along the pre-existing wall. Cell division is not exclusive to meristematic cells: differentiated cells can also undergo mitosis. When this happens, a specialized cytoplasmic organization develops in the cell, the so-called phragmosome [48]. Phragmosome formation involves nuclear repositioning and the appearance of broad transvacuolar cytoplasmic strands, which precede mitosis and cell-wall deposition within the cell lumen.

Similarly, the cells that respond to fungal colonization are differentiated, crossed by cytoplasmic strands and build a new cell wall compartment within their cell lumen. The thin apoplastic space separating the fungal cell wall from the host perifungal membrane is, in fact, made of cell wall components of plant origin and is very similar in composition to the cell plate [39]. In addition, several cell-wall-synthesis-related plant genes (namely *MtCel1*, which has been associated with cellulose synthesis, and *Mt-XHT1*, which encodes a xyloglucan endotransglucosylase-hydrolase involved in the construction of xyloglucan polymers in plant cell walls, as well as several genes encoding hydroxyproline-rich glycoproteins, expansins and arabinogalactan-proteins, which are important wall components) are known to be upregulated in mycorrhizal roots [39,49].

Cellular and molecular data suggest that the cells involved in the perifungal interface construction can co-opt pre-existing molecular mechanisms (e.g. gene networks) to perform similar functions in a new context, as suggested for evo-devo events in higher plants [50]. In particular, primordial, basic mechanisms, such as cell plate deposition and phragmosome development, might have been recruited and modulated when the necessity for assembling new cell-wall materials within the cell lumen as an intracellular niche for the AM symbiont arose. This hypothesis might explain the existence of common molecular and genetic determinants for AM interactions over the plant taxa.

The modulation of flexible pre-existing mechanisms through an evolutionary tinkering [50] is not a novelty in cell biology and in plant symbiosis evolution in particular. Mechanisms well established upon AM colonization might have been further co-opted to install nitrogen-fixing symbiosis, a hypothesis that is soundly supported by the identification of a common set of plant genes required for invasion by AM fungi and rhizobia [32,38,40,41,51].

Concluding remarks

Fossil records, recent phylogenetic data and new identifications of AM fungal partners in lower plants, together with detailed studies of the colonization process, blend to form novel ideas about the ecological and evolutionary relevance of AMs. The broad inventory of AM presence in the plant kingdom, and the constancy of AM phenotypes in ancient and modern plant lineages, as well as in both gametophytes and sporophytes, suggests that the molecular and cellular mechanisms that underlie fungal accommodation [46,47] were already present in the most basal taxa and were operating in haploid genomes.

The innate immunity and resistance of plants to pathogens are also primitive traits [45]. Understanding how AM fungi have eluded these mechanisms is an exciting field of research: experimental evidence, based on comparative genomics approaches, is required to test the validity of the hypothesis that plant cells modulated their basic mechanisms to orchestrate fungal interactions. In addition, comparing the genomes of saprobic, pathogenic and symbiotic fungi will help to decipher their evolution in the framework of their plant interactions: the sequencing of an AM fungal genome will be crucial for achieving this goal [52].

Paleontology, developmental biology and molecular genetics have already been fruitful in some branches of animal biology [53], whereas the conceptual integration of plant evolution from the molecular to the ecological level [50] is still a young field. Such analyses applied to AMs in the framework of evo-devo approaches present exciting opportunities for elucidating the genetic determinants of plant–AM fungi compatibility.

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