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The structure of arbuscular mycorrhizas: a cell biologist's view

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§a§ Introduction

Arbuscular mycorrhizal fungi are a homogeneous group of soil fungi that are found in most terrestrial ecosystems. They all belong to Glomeromycota a basal fungal taxon which is currently considered phylogenetically related to Mucoromycotina, on the basis of genome sequence data from *Rhizophagus irregularis* (Tisserant et al., 2013; Lin et al., 2014). Glomeromycota are estimated to form symbiotic associations with about 80% of plants, from liverworts and ferns to gymnosperms and angiosperms (Bonfante and Genre, 2008). This ecological success is the result of the major selective advantages that arbuscular mycorrhizas (AM) interactions provide to both the plant and fungus. When lab experiments have compared symbiotic individuals with plants that were grown in the absence of glomeromycetes, striking differences were observed: AM fungi boost plant growth,

improve their capacity to absorb water and mineral nutrients (in particular phosphate, nitrogen) and, through both this enhancement of plant health and a basal triggering of defense responses, protect plants from pathogens (Smith and Read 2008). Beside improving plant overall fitness, AM play a central role in nutrient cycles, soil stability and - last but not least - the survival and diffusion of AM fungi. Similarly to ectomycorrhizal fungi, in fact, glomeromycetes only accomplish their life cycle when growing in association with their plant hosts (although AM fungal reproduction is currently considered strictly asexual); unlike ectomycorrhizal fungi, they cannot be grown for more than a few weeks in the absence of the host, a feature that characterizes AM fungi as obligate biotrophs (Bonfante and Genre, 2010).

The wide diffusion of AM and the remarkably low host specificity of most glomeromycete species appears to be related to the ancient origin of the AM interaction. AM-like structures have been repeatedly identified in 400-450 million year old fossils (Remy et al. 1994; Redecker et al. 2000; Strullu-Derrien et al. 2014, 2016); furthermore, symbiosis-specific genes are found throughout the plant kingdom, including the most basal clades, strongly supporting the hypothesis that AM symbiosis has played a role during the plant conquer of dry lands, and has since undergone minimal modifications (Brachmann and Parniske, 2006).

Today, their application in agricultural practices promises to be a fundamental tool to achieve sustainability in crop production and feed a constantly growing global population (Barrow et al. 2008; Fitter et al. 2011; Rodriguez and Sanders, 2014). These aspects largely account for the increasing interest that AM raise in the scientific community as well as in agro-industrial companies and development agencies. Over the last ten years, substantial advances have been made in our knowledge of many of the cellular and molecular mechanisms that underlie AM associations; the advent of novel technologies such as genome sequencing, high throughput transcriptomics and in vivo confocal microscopy, has opened new ways to explore the hidden world of these fascinating subterranean symbioses. Such advancements have been the subject of many reviews by Smith and Read (2008), Bonfante and Requena (2011), Nadal and Paszkowsky (2013), Gutjahr and Parniske (2013), and Schmitz and Harrison (2014). We here propose a synopsis of the recent literature on the interactions between glomeromycetes and their host plants, focusing on cell organization and nutrient exchange.

§a§ The active role of epidermal versus cortical cells in root colonization

The large multinucleated asexual spores of AM fungi (in some cases reaching 1/2 mm in diameter) can germinate independently of the host plant to develop an asymbiotic mycelium. Its

hyphae are unable to uptake carbon from soil borne organic matter, and are only fed by the extensive nutrient storages accumulated in the spore. Asymbiotic hyphae explore the rhizosphere in search of signals that host plants exude through their roots, such as strigolactones (Akiyama et al., 2005; Besserer et al., 2006) and 2-hydroxy tetradecanoic acid (Nagahashi and Douds, 2011). In the absence of such molecules, mycelial growth is limited to few centimeters; by contrast, the perception of host signals triggers a drastic change in fungal development: the hyphae keep growing and start branching repeatedly, while the whole fungal metabolism is boosted starting from mitochondrial activity (Besserer et al., 2006). As a result of this switch from asymbiotic to presymbiotic growth, bush-like hyphal structures proliferate in the vicinity of the host roots (where signals are more concentrated) and eventually hyphal tips contact the root epidermis (Gutjahr and Parniske, 2013). At the same time as they perceive plant signals, AM fungi also secrete molecules that alert their host of hyphal approach, triggering a number of local and systemic responses ranging from gene regulation to sugar reallocation from the shoot (see section 2 of this book for a complete review of signaling processes in early AM interaction).

From the moment a hypha contacts the wall of an epidermal cell, the interaction steps into the symbiotic phase. Recent evidence (Gobbato et al. 2012; Wang et al. 2012) is strongly

suggesting that cutin monomers (the major components of cuticle in many epigeous organs) play a crucial role in triggering a further developmental switch in glomeromycotan hyphae that rapidly leads to the differentiation of swollen, branched hyphopodia that tightly adhere to the plant cell wall. In most of the studied cases, hyphopodia originate a single penetrating hypha that crosses the epidermal layer penetrating inside the plant cell lumen. In the presence of cracks between epidermal cell files the whole process can take place on the exposed surface of an underlying sub-epidermal cell, but the two cases are structurally comparable. The integrity of penetrated plant cells is maintained by the invagination of the plasma membrane, which proliferates to engulf the developing hypha, and physically separates the fungus from the plant cytoplasm. At the opposite side of the epidermal cell, where the hypha exits the cell lumen, a unique process of plasmalemma-to-plasmalemma fusion takes place: the newly formed perifungal membrane in fact has to fuse with the existing plasma membrane and allow the hyphal tip to reach the apoplast. Outer cortical cells are normally traversed in a similar way, although hyphae often branch within this tissue, spreading the infection along the root axis. Their final targets are the inner cortical cells, where hyphae penetrate and ramify repeatedly to differentiate into arbuscules, the highly branched structures that give their name to this form of mycorrhizas and represent the main site of nutrient

exchange (Parniske 2008; Bonfante and Genre 2010; Harrison 2012).

As intraradical hyphae develop in the host tissues, the extraradical mycelium also proliferates, extending beyond the nutrient depletion zone that surrounds the root system and is responsible for the numerous ecological services attribute to AM fungi (Govindarajulu et al. 2005; Javot et al. 2007).

§a§ The appearance of a novel cell compartment: the symbiotic interface

The AM symbiosis achieves full functionality with the development of an extensive contact surface between the plant and fungal cells, where nutrients and signals are exchanged. This is realized inside the root colonized cells - and in particular those containing arbuscules, where the so-called periarbuscular membrane envelopes each hyphal branch (Fig. 1). This large contact surface is in fact structured as a complex three-dimensional volume, bordered by the fungal and plant membranes, and containing the fungal wall as well as a thin layer of plant cell wall materials. This novel, specialized cell compartment - which is conserved from basal to higher plants - is built through strict coordination between fungal and plant cell development, and is called the symbiotic interface (Scannerini and Bonfante, 1983; Peterson and Massicotte, 2004; Bonfante et al. 2009; Bücking et al. 2007). Being the site of symbiotic nutrient exchange, the interface

has been focussing the interest of AM cell biologists since its discovery and we now know relatively well how it is generated and what it contains (Parniske 2008; Genre and Bonfante, 2010), while its functional aspects - including bidirectional nutrient transport - are currently being unveiled mostly thanks to the development of high-throughput transcriptomic analysis and mutant lines (Schmidt and Harrison, 2014; Gutjahr and Parniske, 2013).

While the symbiotic interface reaches its full complexity and extension in arbusculated cells, the same structure can be found in each of the root cells colonized by intracellular hyphae. In fact the symbiotic interface makes its first appearance in atrichoblasts, the root epidermal cells targeted by presymbiotic hyphae. As soon as a hyphopodium adheres to the epidermis, each contacted plant cell starts to assemble the secretory machinery that will build the intracellular compartment to host the fungus. The cytoplasm aggregates at the contact site, where the nucleus is also moved; then the cytoplasmic aggregation develops into a thick column that crosses the cell predicting the route that the penetrating hypha will follow across the cell lumen (Genre et al. 2005). This cytoplasmic aggregation, called the prepenetration apparatus (PPA), concentrates all the elements of the secretory pathway: abundant endoplasmic reticulum, numerous Golgi bodies and secretory vesicles (Genre et al. 2008; 2012). The process of membrane proliferation has only recently been

understood, following a number of studies highlighting the role of the secretory pathway: a proliferation of Golgi bodies (Pumplin and Harrison, 2009) and plastids (Strack and Fester, 2006; Fester et al. 2001, Lohse et al. 2005) has been described in young arbusculated cells, hinting at a burst in lipid biosynthesis and membrane dynamics; abundant Golgi bodies, modified plastids, secretory vesicles and late endosomes (multivesicular bodies) have also been regularly observed in the PPA cytoplasmic aggregation (Genre et al., 2008). This intense exocytotic activity is aimed at the assembly of the novel membrane which engulfs the penetrating hypha as soon as this crosses the outer cell wall, as revealed by the live imaging of exocytotic markers such as SNARE proteins (belonging to the VAMP 72 family) and exocyst complex members (Exo84). This approach showed the accumulation of such proteins within the PPA and their concentration on a dome-shaped area facing the tip of each developing intracellular hypha in *M. truncatula* and *D. carota* (Genre et al., 2012). On this basis we now envisage interface biogenesis as the result of an accumulation of secretory vesicles all along the PPA, followed by their progressive fusion in front of the advancing hyphal tip: the dome-shaped structure labelled by GFP-tagged SNAREs and Exo84 corresponding to the current site of interface biogenesis (Fig. 1).

The same process is then repeated in each penetrated cell throughout the root cortex, and broad PPAs form prior to and

during arbuscule development, indicating that intracellular fungal accommodation relies on a conserved mechanism that is modulated depending on the symbiotic structure that the colonized cell will harbor (either a simpler trans-cellular hypha or a more complex arbuscule). On this line, RNAi silencing of VTI12 AND VAMP72 SNAREs in *L. japonicus* and *M. truncatula* respectively, resulted in stunted arbuscule development (Lota et al., 2013; Ivanov et al., 2012). Aborted arbuscules and limited epidermal penetration are also displayed by *vapyrin* mutants of *M. truncatula* and *Petunia hybrida* (Murray et al. 2011; Pumplin et al. 2010; Reddy et al. 2007). VAPYRIN is expressed during AM colonization, localizes to vesicle-like subcellular compartments (Feddermann et al. 2010, Pumplin et al. 2010) and its sequence suggests a role in membrane fusion processes and protein-protein interaction (Feddermann and Reinhardt 2011): as a consequence, VAPYRIN is now proposed as another key actor in interface biogenesis (Gutjahr and Parniske, 2013).

The remodelling of the whole cell membrane system has also an impact on the vacuole: ultrastructural observations illustrate how also the tonoplast closely follows the arbuscule development, leading to a flattened but continuous vacuolar system. Aquaporins which are differentially expressed in cortical cells could be good markers for such vacuolar re-organization (Giovannetti et al 2012).

Bricks of the plant wall fill the symbiotic interface. The exocytotic process that takes place in the PPA is not only assembling the perifungal membrane, but also secreting cell wall components into the interface compartment, around the growing intracellular hypha. The major compounds found in the interface matrix are typical components of the plant primary wall, such as cellulose, glucans, polygalacturonans, hemicelluloses, hydroxyproline-rich proteins (HRGP) and arabinogalactan-proteins (Balestrini and Bonfante, 2014). Remarkably, their distribution is not homogeneous within and significant differences can be found between the interface that surrounds large intracellular hyphae, fine arbuscule branches or collapsing arbuscules (Balestrini and Bonfante, 2005). These findings suggest that the composition of the plant-derived interface matrix is developmentally regulated during the symbiosis development.

It is important to underline that the texture of the interface matrix does not appear as structured as the plant primary wall, although most of the 'bricks' are present in both. Even if it was hypothesized it could result from a weak lytic activity by the fungus (Garcia-Garrido et al. 2000), the lack of cell-wall degrading enzymes in *Rhizophagus* genome (Tisserant et al 2013) excludes such a possibility. As an alternative, this could derive from a plant-controlled process that hampers wall assembly mechanisms (Bonfante, 2001). The upregulation of HRGP genes in AM roots and, remarkably,

the concentration of HRGP transcripts in arbusculated cells have been confirmed by gene expression analyses, and genes encoding arabinogalactan proteins (AGPs) - another interface component - are also induced during mycorrhizal development (Balestrini and Bonfante, 2014).

Cell wall-localized enzymes, such as xyloglucan endo-transglycolases (XET), have been isolated from *Medicago truncatula* mycorrhizal roots (Maldonado-Mendoza et al. 2005). Expansins have also been localized in colonized cells (Balestrini et al. 2005) and expansin-related mRNAs were detected by *in situ* hybridization in the epidermal tissue during PPA assembly (Siciliano et al. 2007). These wall-remodeling enzymes could be involved in wall modifications within the interface (possibly maintaining the deconstructed status of the interface matrix), local cell wall weakening related to fungal penetration, or diffuse wall loosening leading to the observed expansion of arbusculated cells (Balestrini and Bonfante, 2014). Lastly, the interface low pH could contribute to the fluidity of the interface materials (Guttenberg, 2000), by facilitating transglycosylation and enzymatic lysis of structural cell wall polysaccharides.

The fungal-derived share of the interface material corresponds to the hyphal wall, although also in this case important modifications have been described. Chitin and glucans have consistently been localized to the fungal wall by affinity techniques (Bonfante, 2001), but a progressive

thinning out of the fungal wall is observed: arbuscule branches display the thinnest and most simplified wall - mostly consisting of amorphous chitin chains - which has been proposed to achieve a more intimate contact with the plant perifungal membrane, possibly facilitating nutrient exchange. At the moment only one piece of experimental evidence supports this hypothesis: PT4 mutants of *Medicago truncatula*, where phosphate transport from the fungus to the plant is strongly affected, develop stunted arbuscules (Javot et al, 2007) where the thin-walled branches are missing.

§§ Genetics at the root of the symbiotic interface

Forward genetics analyses have provided important evidence concerning the genetic control of interface construction: the same plant genes that are responsible for the recognition of presymbiotic fungal signals (see below) are also required for interface development, suggesting that the two processes are strictly connected. The convergence between the signaling pathway that controls AM and the one that controls nodulation has been reviewed in detail in several occasions (Parniske, 2008; Oldroyd et al. 2009; Luginbuehl and Oldroyd, this book; we will only recall here that all of the genes so far identified as controlling AM establishment constitute one signal transduction pathway. This is called the common symbiotic signaling pathway (CSSP) because their gene products are involved in the transduction of both fungal and rhizobial

diffusible signals (Luginbuehl and Oldroyd, this book). Breaking the CSSP by mutation of one of these genes has a direct impact on interface biogenesis in AM. *Castor* mutants of *L. japonicus* show a lack of prepenetration responses in epidermal cells: as a result, the plant cell dies as soon as the fungus breaches into its lumen (Novero et al. 2002). The phenotypes of *dmi1-1*, *dmi2-2* and *dmi3-1* mutants (Ané et al. 2002) have been studied with more detail in *M. truncatula*: the three of them lack PPA formation, which is sufficient to limit fungal colonization to the root surface. Such studies directly relate the success of the symbiotic interaction to interface biogenesis, and also highlight its strict dependency on the genotype of the plant, which therefore appears to have a strong hand in the colonization process (Parniske, 2008).

§a§ Molecular traffic at the symbiotic interface

The transport of nutrients across the symbiotic interface had been postulated since its first descriptions (Scannerini and Bonfante, 1983). Based on the interface structure, it is evident that the two-way exchange of nutrients (Balestrini and Bonfante, 2014) must involve the apoplastic domain as well as both membranes enclosing the interface (the fungal plasma membrane and the plant perifungal membrane).

The first factual hints on the transfer of carbon compounds to the fungus came from pioneering work done on orchid mycorrhizas: Peterson and colleagues (1996) observed a

change in pectin abundance in the matrix surrounding young or senescent 'pelotons' (the equivalent of arbuscules in this type of mycorrhizas) and proposed that cell wall polysaccharides could be the source of carbon for the symbiotic fungus. Indeed, the demonstration of nutrient transfer in mycorrhizal orchid protocorms came with the recent imaging of stable isotope tracers (Kuga et al 2014): through ultra-high resolution secondary ion mass spectrometry (SIMS) imaging of resin-embedded protocorm sections, the authors demonstrated a complex bidirectional flow of C to and from the intracellular hyphae.

While the applicability of this complex experimental set up to AM interactions remains to be tested, a combination of molecular and cellular approaches is providing experimental evidence to the role of the symbiotic interface as the preferential site for plant/fungus exchanges. A fungal hexose transporter has been found to be expressed in intraradical hyphae of *R. irregularis*, strongly suggesting that arbuscules and intracellular hyphae have the capability to upload simple sugars from the interface matrix (Helber et al., 2011). This nicely combines with the observation that *Geosyphon pyriforme*, the only known glomeromycete living in symbiosis with cyanobacteria rather than plants, also relies on a hexose importer for sugar uptake from the bacteria (Schüssler et al. 2006).

Altogether, the idea that fungus-bound carbon reaches the

interface in the form of cell wall polysaccharides looks both realistic and appealing. Anyway, while the arbuscule acts as a sink for carbon compounds derived from plant photosynthesis, it is also a source of mineral nutrients in the plant metabolic balance. The chemical nature of the molecules that are actually transferred to the plant cell, as well as the mechanisms involved in their transport across the interface are still largely hypothetical. Nevertheless, recent studies have identified a few transporters and speculated on their possible cargos.

Most investigations have focussed on model plants such as *M. truncatula* (Liu et al. 2003; Küster et al. 2007; Gomez et al. 2009), rice (Guimil et al. 2005), and *L. japonicus* (Guether et al. 2009a): they all reported significant increases in the expression level of membrane transporters. The mycorrhiza-specific plant phosphate transporter MtPT4, which is localized in the periarbuscular membrane, is essential for active symbiosis in *M. truncatula* (Javot et al, 2007). In *L. japonicus*, 47 putative transporters resulted to be upregulated in mycorrhizal roots, 28 of which may be important for nutrient acquisition. The strongest up-regulated gene of the array is a putative ammonium transporter (Guether et al. 2009b) whose transcripts have been quantified and localized in the arbusculated cells by laser microdissection, although direct evidence of the protein localization in the periarbuscular membrane is still missing. Recently, a

proteomic analysis has confirmed that membranes from the colonized parts of *M. truncatula* roots are indeed enriched in key nutrient transporters (Abdallah et al., 2014).

The aforementioned accumulation of plant cell wall 'bricks', i.e. non-structured wall saccharides, in the symbiotic interface has been envisaged as a possible source for simple sugars to feed the fungus; nevertheless, cell wall oligosaccharides are well known to play a major role in eliciting plant cell defenses (Humphrey et al. 2007). Could analogous signaling processes be also involved in AM? Unfortunately, our capability to locally investigate signal exchange within the interface is extremely limited. Nonetheless, we know that defense responses are elicited to a limited extent during early mycorrhizal colonization (Hause and Fester, 2005); the possibility that such responses are triggered by oligosaccharides chopped from the interface components is very intriguing. It is anyway important to keep in mind that the *Rhizophagus irregularis* genome does not encode any plant cell-wall degrading enzymes, implying that plant wall oligomers - either produced as such or deriving from polymer degradation - are generated by the activity of plant enzymes.

Furthermore, in analogy to what has been observed in pathogenic interactions, the active release of signaling molecules and effectors by intracellular AM hyphae represents an important option. Indeed, the genome sequencing of

Rhizophagus irregularis has revealed an impressive number of small secreted proteins, specifically expressed during the intraradical symbiotic phase, and one of these effectors has been more thoroughly characterized (Kloppholtz et al 2011). Lastly, the very fungal wall can act as a source of signaling molecules: the perception of chitin-related compounds by plant membrane receptors kinases play a major role in pathogenic interactions (Shibuya and Minami, 2001). Chitin is abundant in the wall of glomeromycetes and chitin-based molecules have been identified as AM symbiotic signals at least during the presymbiotic phase, where they activate the CSSP in analogy to the chitin-like Nod factor of nitrogen-fixing rhizobia (Maillet et al. 2011; Genre et al., 2013). In particular, lipo-chito-oligosaccharides stimulating common symbiotic responses - such as root branching or the upregulation of the early nodulin ENOD11 - have been isolated from mycorrhizal roots (Maillet et al., 2011), suggesting that they could have a function in later stages, although their presence and possible role in infected cells remains to be investigated. Altogether, the most recent findings hint at the symbiotic interface matrix as a rather unique apoplastic compartment, whose functions in nutrition and signaling combine with uncommon structural features.

§a§ The plant cell nucleus: a driver of the colonization process

In vivo confocal imaging has revealed the central role of the host cell nucleus throughout AM root colonization, from PPA organization to arbuscule differentiation (Genre et al., 2008). In epidermal cells, the earliest visible response to fungal adhesion is the repositioning of the nucleus at the contact site (Genre et al., 2005). Nuclear repositioning also precedes PPA formation in the outer cortex, well in advance of hyphal contact, suggesting the occurrence of signaling between adjacent cells. In fact, nuclear repositioning is a well-known feature of several plant cell responses to both biotic and abiotic stimuli (Genre and Bonfante, 2007; Hardham et al., 2008; Genre et al., 2009).

In addition, an increase in nuclear size is well documented in arbusculated cells (Balestrini et al., 1992; Genre et al., 2008). This could be explained with the observed increase in nuclear ploidy in mycorrhizal roots (Fusconi et al., 2005; Bainard et al., 2011), suggesting the onset of endoreduplication, but the observation of decondensed chromatin and large, highly organized nucleoli, supports the occurrence of intense transcriptional activity, which could also be at the base of nuclear size change.

The most recent molecular data on differentially expressed genes in arbusculated cells as well the hypothesis that the host nucleus is the preferential target of the fungal effectors, provide further support to the role of nuclear dynamics during mycorrhizal colonization. A challenge for the

next years will be to correlate these morphological changes with variations in chromatin architecture (methylation and acetylation) and the subcellular targeting of fungal small secreted proteins.

Conclusions

The symposium 'Endomycorrhizas' organized in Leeds in 1974 by Sanders, Mosse and Tinkers (Sanders et al., 1975), marked the official kick off for AM research. Forty years later, the power of molecular and genetic tools, genome sequencing of several host plants, advanced imaging techniques, have revealed several details of the colonization events and identified some of the molecular and genetic determinants in AM. However, many of the issues listed in the proceedings from that seminal meeting - embracing physiology, ecology, evolution - remain open.

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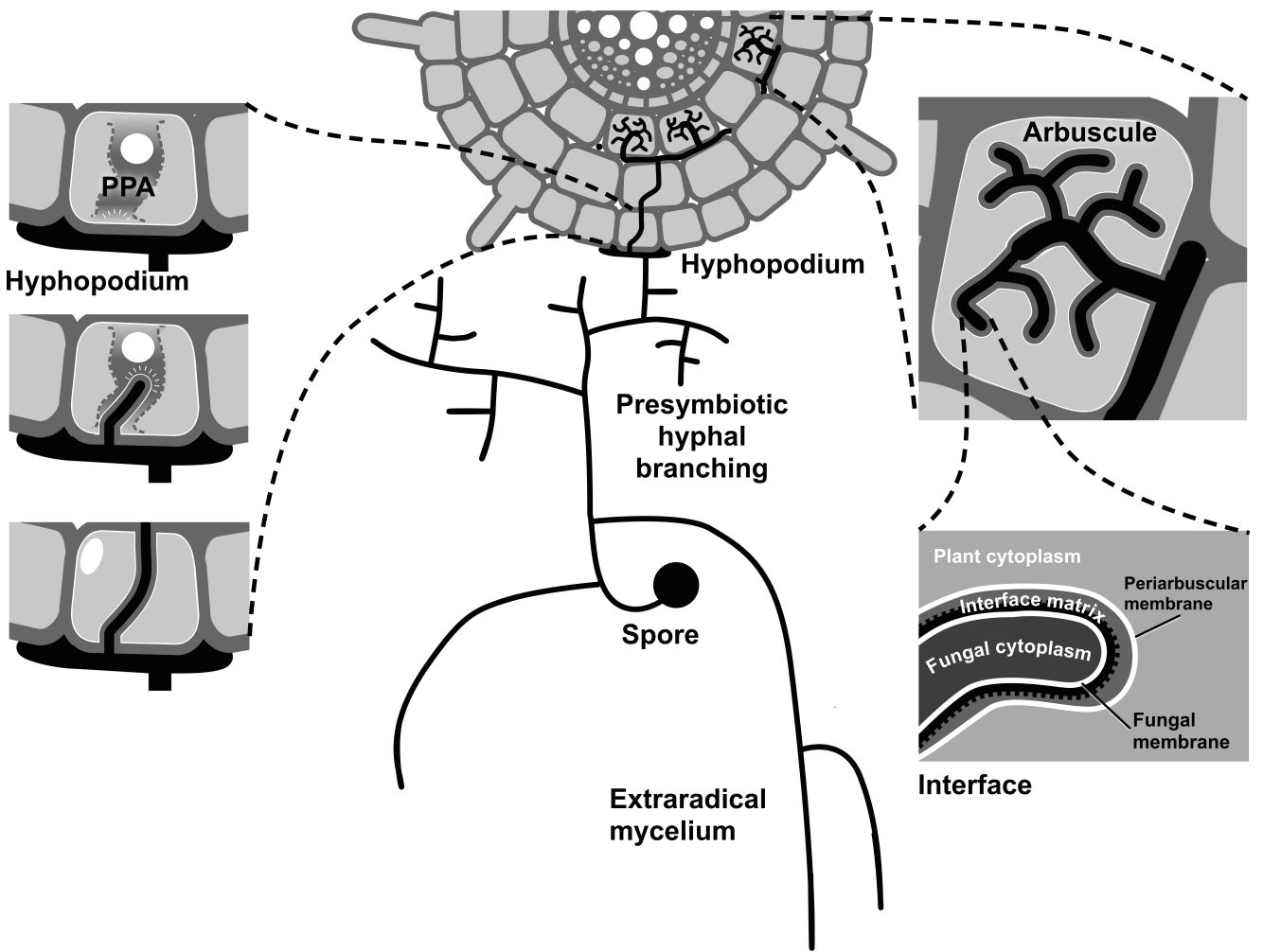


Figure 1. Major structural features of arbuscular mycorrhizas.

Spore germination generates a short asymbiotic mycelium exploring the rhizosphere with a typically negative geotropism. The perception of root exudates triggers presymbiotic hyphal branching, culminating with the formation of a hyphopodium on the root epidermis. This first contact activates pre-penetration responses in the epidermal cell (left panel): the assembly of the prepenetration apparatus (PPA) precedes and directs hyphal penetration; intense exocytosis within the PPA (white lines) assembles the symbiotic interface anticipating hyphal tip growth across the host cell. As the intraradical hyphae reach the inner cortex, arbuscules develop (right panel), where the symbiotic interface achieves its highest complexity (detail): each arbuscule branch is enveloped by the interface matrix and periarbuscular membrane, which mediate nutrient exchange between the symbiotic partners.