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Lessons from arbuscular mycorrhizal fungal genomes

Luisa Lanfranco and Paola Bonfante

Arbuscular mycorrhizal fungi (AMF) have accompanied the majority of land plants since their evolution in the Devonian period with a symbiotic alliance centered on nutrient exchanges. The exploration of AMF genomes is providing clues to explain major questions about their biology, evolution, and ecology. The dynamics of nuclei across the fungal life cycle, the abundance of transposable elements, and the epigenome landscape are emerging as sources of intraspecific variability, which can be especially important in organisms with no or rare sexual reproduction such as AMF. These features have been hypothesized to support AMF adaptability to a wide host range and to environmental changes. New insights on plant-fungus communication and on the iconic function of phosphate transport were also recently obtained that overall contribute to a better understanding of this ancient and fascinating symbiosis.

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Booming knowledge from arbuscular mycorrhizal fungi genomes

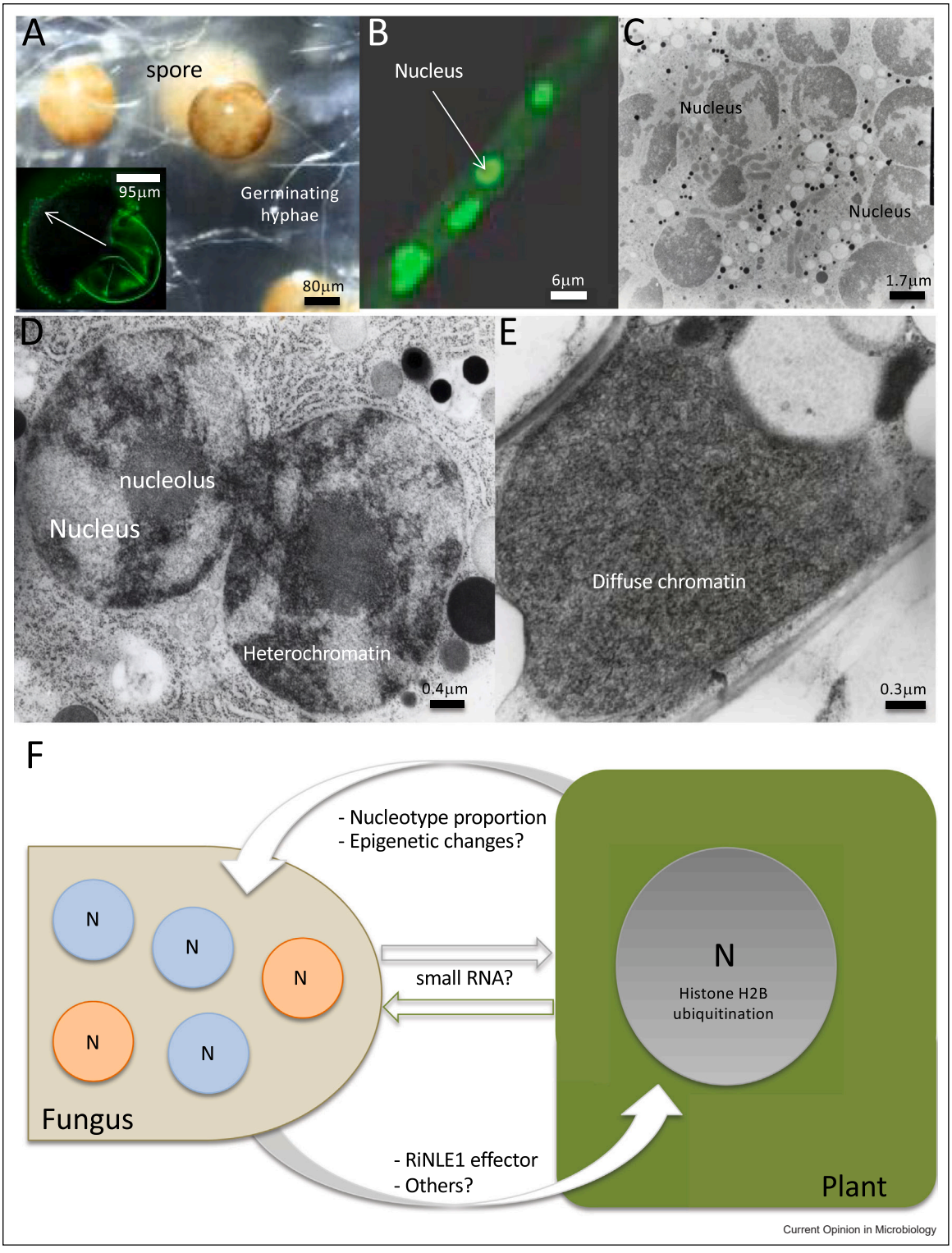
The Arbuscular Mycorrhizal (AM) symbiosis is considered a key biological innovation that was instrumental in land colonization by plants more than 400 million years ago. While there is a bunch of information on how plants evolved the ability to develop arbuscular mycorrhizas [1,2], our knowledge of how the capability to colonize plants originated in AM fungi (AMF) is limited. But, during the last decade, AMF research jumped into the genomics era, leading to studies which offer fresh knowledge on some major questions of AMF biology, evolution, and ecology. They shed light on AMF obligate biotrophism and their capability to adapt to different terrestrial ecosystems colonizing, often simultaneously, a wide range of plant species, and on their evolutionary success in the apparent absence of sexual reproduction.

Genome features and multinuclear status

Genomic data have been obtained so far from representatives of different AMF orders, from *Glomerales* to *Diversisporales* and *Archaeosporales* [3,4], and revealed that these fungi have unusually large genomes (from about 150 Mb of *Rhizophagus* species to 784 Mb of *Gigaspora margarita*, [5]), which are often rich in repetitive sequences. However, the recently sequenced genome of the early diverging AMF *Paraglomus occultum* turned out to be rather small (39.6 Mb) with considerably fewer genes compared to other AMF (10,145 *vs* a mean of 24,000 genes) opening the question of whether this condition was present in the *Glomeromycotina* ancestors or was the result of genome down-sizing processes only involving early diverging AMF [6].

AMF contain thousands of independently dividing nuclei in their spores and coenocytic hyphae (Figure 1) and a long-debated controversy was whether AMF were homokaryotic, harboring multiple identical haploid nuclei, or heterokaryotic, harboring a population of different haploid nuclear genotypes with the heterokaryotic status proposed as a mean to compensate the lack of sexual reproduction. Single nuclei sequencing [4,7] and the more recent exploitation of long-read sequencing and chromatin conformation capture techniques [8–10] helped settle this question. These studies have so far focused on the model species *Rhizophagus irregularis* and have revealed the existence of homokaryotic as well as dikaryotic AMF isolates, that is, isolates that carry thousands of nuclei deriving from two parental strains [10–12]. The dikaryotic status, with two distinct sets of parental chromosomes, could only be clearly revealed by using advanced high-fidelity long reads sequencing coupled to chromatin conformation analyses which were applied to four AMF dikaryons (A4, A5, G1, and SL1) [10]. By contrast, data from conventional long reads sequencing on the C3 dikaryotic strain, from which the A4 is supposed to be clonally derived, seem to not distinguish the two chromosome sets [13]; these findings highlight the need for the most advanced sequencing techniques to correctly describe the AMF genomic organization. Notably, the proportion of the two nucleotypes within a dikaryotic AMF may change in response to abiotic

Figure 1



Morphology of *Gigaspora margarita* and its nuclear complement. **(a)** The huge spores of *G. margarita*, seen under a stereomicroscope possess a pear-like aspect. After squashing, they reveal hundreds of nuclei detected as green fluorescent spots at the edge of the cytoplasm (inset). **(b)** The germinating hyphae reveal a multinuclear structure, after staining with a DNA fluorescent dye. **(c)** A spore seen under a transmission electron

microscope reveals a number of nuclei (N) with electron-dense heterocromatin occurring at the nuclear periphery. **(d)** Two nuclei in the spore have just ended their division. The central nucleolus is surrounded by patches of heterochromatin. **(e)** An intraradical intracellular hypha reveals a nucleus with diffuse and loose chromatin. **(f)** Nuclear interactions between AMF and host plant. The identity of the host plant influences the nucleotype proportion of AMF dikaryotic strains [15] . The two nucleotypes coexisting in the fungal hypha are indicated with different colors. The RiNLE1 fungal effector was shown to interfere in plant cells with histone H2B ubiquitination leading to gene expression regulation to favor colonization [28••]. It has been hypothesized that small RNAs movement may contribute to gene expression regulation through the cross-kingdom RNAi process [22]. N, nucleus.

factors [14] and host plants [15••], highlighting an unexpected link between AMF nuclear dynamics and environmental clues and host plant identity. Moreover, the most abundant haplotype shows higher total gene expression during *in planta* growth [10], having the potential to influence the fungal phenotype and, likely, that of the green partner. Therefore, changes in AMF nucleotype abundance and consequent unbalanced fungal gene expression patterns will also have significant implications for the selection of AMF inoculants more efficient in improving plant health and productivity [16].

In analogy to other organisms, the pangenome concept can be applied to AMF; indeed, a high level of intraspecies variability, mirrored by a high number of lineage-specific genes was found in taxonomically related *R. irregularis* strains [8,17,18]. Notwithstanding the high number of orphan and still uncharacterized genes, this feature has been hypothesized to support AMF adaptability to a wide host range and to environmental changes and to explain the impact of intra-specific variation on the output of the symbiosis [19]. But how is this genetic variability generated in AMF?

The large genome size of AMF is correlated with the abundance of repeated sequences — the majority of which are transposable elements (TEs) — which range from 50% in *R. irregularis* [8,20•] to more than 80% of the whole genome in *G. margarita* [5], with transcriptomic data suggesting ongoing TE activity [5,20•]. It is known that TE mobility represents a source of genetic variability, which can be especially important in organisms with no or rare sexual reproduction such as AMF. Indeed, the physical proximity between some TE (i.e. MULEs) and high copy number genes suggests that TE could drive gene family expansion in *R. irregularis* [20•]. Moreover, in *G. margarita*, a significant spatial association was found between some transposon families (CRYPTON, TIR, and MITE) and candidate small secreted proteins, which may act as effectors to suppress host defense responses [5]. But more studies are needed to clarify the effect of TE mobility on AMF genetic variability and whether, as in pathogens, TEs offer a favorable environment for effectors diversification [18,21].

On the other hand, to keep genome stability, TE silencing is needed. In AMF CG DNA methylation and the RNA interference (RNAi) machinery, which act through small RNAs, seem to contribute to inactivating TEs [20•]. Notably, AMF genomes are characterized by an expansion of key genes involved in RNAi (AGO and RdRp; [22,23]), a feature that is curiously also shared by bdelloid rotifers, another group of asexual organisms [24]. It is tempting to speculate that this expansion in AMF could be the result of a co-evolution between specific anti-TE defense and the diversity and abundance of TE. A big challenge will be to define the role of AGO and RdRp genes and the relevance of TE activity/silencing in the adaptation and evolutionary processes in AMF. Studies on fungal sRNAs open a fascinating hypothesis, a possible cross-kingdom RNAi occurring during AMF root colonization where plant host gene expression could be under the control of AMF small RNAs [22,23]. Indeed, in the pathogen *Botrytis cinerea*, small RNAs derived from TEs were shown to move from fungus to host cells and silence plant defence genes to favor host colonization [25].

The exploration of AMF epigenome and chromatin structure is also an emerging field [8,26]. In line with the presence of a repertoire of genes encoding enzymes involved in DNA methylation, the model AMF *R. irregularis* shows a high degree of 5 methylcytosine (5mC) and a low degree of 6 methyl adenine (6mA) [8,26]. As these epigenetic modifications are associated with variations in gene expression, they may explain phenotypic variations in plant growth that have been observed between genetically identical strains or progenies of the same strain [27]. Two other major advances have been recently obtained by the chromosome-level genome sequencing of *R. irregularis* strains combined with Hi-C analyses. First, the number of chromosomes appears higher (more than 30 [8,9]) than any other described fungus, which is coherent with their large genome size. Second, nuclei are structured into two compartments, a euchromatic compartment, which contains many genes with core functions and a heterochromatic and heavily methylated compartment, which is enriched in repetitive sequences, but also in genes for predicted secreted proteins highly expressed *in planta* [8,10]. This leads to the fascinating hypothesis that the AMF chromatin condensation/accessibility could be, to some extent, under the control of the host plant. In support of this view, transmission electron microscopy shows that AMF nuclei show a different chromatin organization, moving from a dominant heterochromatin structure in the spores to a looser

euchromatin-like morphology in the intraradical hyphae (Figure 1). It is worth noting that epigenetic control could be exerted in the other direction (fungus to plant) as an AMF effector (RiNEL1) was shown to interact with the plant nucleosome protein histone 2B resulting in the downregulation of defence-related gene expression and enhanced mycorrhization [28••].

The genomic basis of the arbuscular mycorrhizal fungi biotrophy

The exploration of AMF genomes also provided clues to unveil key metabolic features. Notwithstanding the different evolutionary stories, all the AMF genomes possess very similar features: a reduced number of plant cell wall degrading enzymes, a lack of fatty acid synthase, and a reduced capacity to synthesize secondary metabolites.

The absence of the fatty acid synthase (FAS) gene allowed the investigation of obligate biotrophy and nutritional dependence on the green host for fatty acid compounds [29,30]. Two *R. irregularis* AMP-binding domain proteins, which were found to be involved in the uptake of myristic acid (14:0) and palmitic acid (16:0) in heterologous expression assays, might be involved in fatty acid import into the fungal arbuscules in colonized roots [31]. These findings boosted attempts to cultivate AMF in asymbiotic conditions in the presence of fatty acids: optimized recipes of growing medium containing myristate, organic nitrogen in combination with the plant hormones SLs and methyl-jasmonate successfully sustain mass production of large size, and root colonization-competent spores [32•]. This knowledge represents a flywheel for applied research where the need for expansion of AMF culture collections runs in parallel with the development of more efficient systems/protocols to isolate AMF [33,34], and with the set-up of a quality control procedure of inocula to guarantee their efficacy in the field [35]. The absence of invertase and thiamine coding genes in all AMF genomes so far sequenced provides further evidence of the biotrophic status.

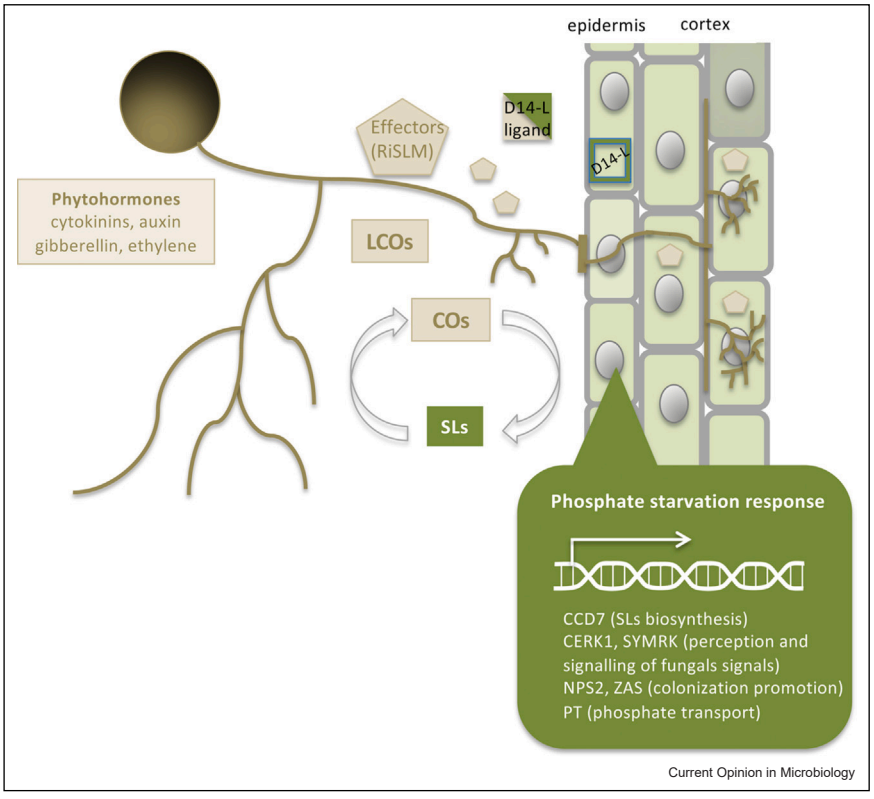
One question genomic data could not shed light on is how the fungus penetrates the plant cell wall during root colonization, as AMF genomes possess few genes encoding Carbohydrate-Active Enzymes (CAZymes) acting towards plant cell wall. Notably the number of CAZymes involved in fungal cell wall dynamics can be high [5]. Local activation of enzymatic and/or non-enzymatic systems from both symbionts, maybe under reciprocal control, that leads to plant cell wall loosening, can be envisaged. For example, *Gigaspora* species possess two gene families containing alcohol oxidases which, in brown rot fungi, participate in Fenton chemistry and contribute to the non-enzymatic degradation of plant cell walls. Such events together with the demonstrated activation of peroxidases, which generate hydroxyl-radicals [5], could provide an explanation of how AMF penetrate plant tissues only eliciting a moderate plant defense.

New insights on the iconic symbiotic function of phosphate transport

The transport of phosphate (Pi) from the fungal hyphae to the plant cells is a key AM symbiotic function, also witnessed by the fact that Pi availability strongly influences the capability of a plant to be colonized by AMF, with high Pi levels strongly inhibiting root colonization [36,37]. Two seminal publications shed light on such regulation as they demonstrated that a plant gene network centered on crucial components (PHR transcription factors [TFs] and SPX proteins) of the so-called Pi starvation response controls mycorrhization level [38••,39••] through the modulation of several plant genes involved in the different stages of the AM symbiosis from the pre-contact molecular dialogue (i.e. SL genes, next paragraph) to the symbiotic functions such as Pi uptake at the peri-arbuscular space (i.e. AM-induced PT). Molecular mechanisms underlying Pi transport in the fungal partner from Pi uptake to polyP synthesis and movement from ERM to IRM have been partially elucidated [40]. Only recently the mechanisms of how AMF release Pi to the symbiotic interface have been unveiled: this process is fulfilled by an SPX (SYG1/Pho81/XPR1) domain-containing phosphate transporter (RiPT7) that was recently characterized by a combination of heterologous expression and reverse genetics assays in *R. irregularis*. RiPT7 is expressed in the intraradical hyphae and its localization at the plasma membrane facilitates the bidirectional Pi transport, according to the Pi gradient. In response to Pi starvation, the SPX domain inhibits Pi transport activity as it drives the RiPT7 targeting to fungal vacuoles [41]. RiPT7-silenced mycorrhizal roots accumulate a large amount of polyP and showed reduced symbiotic Pi transfer compared with control roots, suggesting that RiPT7 loss blocks Pi transfer across the symbiotic interface [41]. Remarkably, RiPT7 inactivation led to the premature degradation of arbuscules in analogy to the phenotype observed in the plant mutant defective of the plant PT responsible for the Pi uptake in arbusculated cells [42]; these findings once again indicate that Pi transfer in both symbiotic partners is a developmental signal to trigger the correct formation of arbuscules.

From an evolutionary perspective, the genome sequencing of many *Glomeromycotina* has revealed strict relationships with their sister taxa, *Mortierellomycotina*, and *Mucoromycotina* [3,4,43], but does not explain their biotrophy *versus* the dominant saprotrophy of these sister groups. Remarkably, the genomes of basal *Glomeromycotina* such as *P. occultum* and the unusual *Geosiphon pyriformis*, the only fungus known to form endosymbiosis with nitrogen-fixing cyanobacteria, also lack FAS genes

Figure 2



Plant-AMF communication. A symbiotic signaling loop between SLs and COs occurs [54••]. A fungal LysM effector (RiSLM) was shown to enhance early symbiotic signaling [56]. Other still uncharacterized molecules may function as AMF symbiotic signals such as the ligand of the plant D14L receptor [57] whose origin (plant or fungal) is unknown. The role of several phytohormones produced by AMF deserves investigation [59]. On the plant side, a gene regulatory network centered on crucial components of the phosphate starvation response controls AM colonization level [38••,39••] through the modulation of several plant genes involved in the different stages of the AM symbiosis.

[44]. These findings suggest that the obligate plant biotrophy is an ancestral trait within *Glomeromycotina*. Other conserved ancestral traits include the lack of genes for thiamine metabolism and sucrose hydrolysis, a reduced number of genes for plant cell wall degradation and the presence of genomic signatures of sexual reproduction (meiosis-related genes and a mating-type MAT locus) even if sexual structures have not yet been detected [44]. On the other hand, possibly as a heritage of an ancient saprotrophic status, AMF maintained the capacity to proliferate in the soil producing an abundant mycelial network.

Beyond the fungal genomes

Plant-fungus communication occurring at the early stages of interaction relies on diffusible molecules released by the plant roots and AMF. The root exudate compounds most active towards AMF are strigolactones (SLs), carotenoid-derived molecules, that, besides acting as plant hormones, when released in soil stimulate fungal metabolism and promote AM establishment [45,46]. Notably, another plant apocarotenoid zaxinone influences the extent of AM colonization acting at early stages as a component of a regulatory network that involves SLs [47]. But how AMF perceive SLs is unknown, while the SLs receptor, D14, an α/β-hydrolase, with the unique feature of dual enzyme and receptor functions, and other SLs signaling components have been characterized in plants [48]. A D14 structural homologue was recently identified in the fungal pathogen *Cryphonectria parasitica*, which is SLs-sensitive and, compared to AMF, also genetically tractable [49]. It would be interesting to further characterize homolog sequences that have been identified in AMF and test their involvement in SLs response.

Upon the perception of SLs/root exudates, AMF release Myc factors — lipochitooligosaccharides (LCO, [50], similar to the Nod factors of the nitrogen-fixing rhizobia) and chitooligosaccharides (CO; [51], simply composed by short (4–5 N-acetyl glucosamine residues) chitin backbones — that activate the so-called common

symbiotic signalling pathway [52] and downstream responses preparing the host plant to symbiosis formation [53]. Recent studies from Andrea Genre's group showed that CO stimulate SL accumulation in host roots, the formation of pre-penetration apparatus, which is a key plant cellular structure during hyphal penetration, and overall root colonization [54••]. These data clearly highlight the occurrence of a symbiotic signaling loop between SLs and CO. Whether this loop exists for LCO deserves to be investigated. The finding that LCO production is common among fungi [55] with a possible role in fungal growth and development raises the question of how plants can distinguish symbiotics from pathogenic or saprotrophic fungi. To make the picture even more complex, slightly longer chitin oligomers (8–9) are well-known elicitors of plant defence which should be avoided by plant-interacting microbes to colonize host tissues. It has been shown that, in analogy to fungal pathogens, AMF produce secreted LysM effectors that can bind chitin oligomers leading to the suppression of plant chitin-triggered defence responses [56]. In particular, the RiSLM from *R. irregularis* is induced by SLs/root exudates and is especially highly expressed in the intraradical mycelium. Notably, the addition of RiSLM to CO tetramers appeared to enhance early symbiotic signalling, suggesting that RiSLM, besides lowering defence responses, may also favor symbiotic signalling possibly interfering with plant Myc factor receptors. Therefore, finely tuned processes involving chitin-based molecules - from the production by AMF, to their sequestration by LysM proteins and to the binding to plant receptors are emerging as crucial issues, although still not completely clarified, for the differential suppression/activation of defence and symbiotic responses. But another most straightforward explanation of how plants distinguish between mutualistic and pathogenic fungi is that other molecules, still uncharacterized, function as additional AMF symbiotic signals. One good candidate is the still uncharacterized ligand of the plant Dwarf14-Like (D14L) receptor that activates a signaling pathway needed for AM establishment [57,58]. The role of several phytohormones, which are produced by germinating spores of AMF [59], should also be analysed.

Conclusions

AMF are still enigmatic organisms, but thanks to the new technologies some curtains went up: their obligate biotrophy is quite ancient trait, mirroring their long co-evolution with plants. However, AMF do not live only with plants! They also establish interactions with other microorganisms, a trait that is crucial for some of their capacities, such as the Pi uptake, which can be improved by the presence of bacteria living in their hyphosphere able to mineralize organic phosphorus, while AMF cannot [60,61••]. In addition to their external microbiota [62•], AMF also possess endobacteria which modulate not only the fungal gene expression, but also the plant metabolism [63••]. Lastly, some AMF possess complex viromes [64], whose functional meaning is still undeciphered. All these additional genomes further raise the level of the genetic complexity of AMF and of their regulatory processes, providing them with the exceptional adaptative capacities to stably interact with most of the land plants since the Devonian times. Figure 2.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

As authors of the manuscript entitled "Lessons from arbuscular mycorrhizal fungal genomes," we declare that we do not have conflicts of interest.

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