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Chapter 4

Symbiotic Proteomics — State of the Art in Plant–Mycorrhizal Fungi Interactions

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Additional information is available at the end of the chapter

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Abstract

Mycorrhizae are symbiotic associations between soil fungi belonging to diverse taxa and the roots of about 90% of all terrestrial plant species. The mutualistic nature of these symbioses is based on the nutritional exchanges between the partners. However, the benefits to the plant partner are not limited to an improved mineral nutrition because they also include a general increase in stress tolerance and health. Because of these benefits, mycorrhizae are of great interest in sustainable agriculture and forestry. In the past few years, the development of high-throughput molecular tools, in addition to the advancements in microscopy techniques, has allowed us to gain a deeper insight on the molecular mechanisms underlying the establishment and functioning of these symbioses. In this chapter, we focus on the use of proteomic tools to better understand the molecular bases of cell communication and the regulation of developmental and metabolic pathways in mycorrhizal associations.

Keywords: Proteomics, mycorrhizal associations, laser microdissection

1. Introduction

Plants cannot move away from unfavourable environments, or run away from hungry eaters, or escape from detrimental microorganisms. Fortunately, not all environments and all organisms are a threat to plants, and plants have also evolved strategies to survive adverse environmental conditions. In fact, plants have adapted to most environments, they have learned how to avoid risky relationships with detrimental microorganisms, how to be unconcerned by neutral microorganisms and how to develop intimate affairs with beneficial partners.

The latter type of interaction is referred to as a 'mutualistic symbiosis'. Symbiosis was first defined at the end of the nineteenth century by Anton De Bary as a term that simply described the regular coexistence of taxonomically different organisms [1]. A mutualistic symbiosis means more than 'regular coexistence', as it includes all relationships in which both partners can benefit from the association, and where benefits can be measured in terms of fitness and nutrient exchange.

Among mutualistic symbioses, the association of plants with nitrogen-fixing rhizobia [2] and with mycorrhizal fungi, in particular, are the result of a long co-evolution and co-operation between plants and soil microbes [1]. Different types of mycorrhizae have been found in nature: ectomycorrhiza (ECM) is predominant in forest soils and is characterized by the fact that the fungal hyphae remain outside the plant cell; endomycorrhiza comprises orchid, ericoid and arbuscular mycorrhiza (AM) and derives its name from the fact that the fungal hyphae are able to enter into the plant root cells [3].

ECM fungi have evolved from wood- and litter-decaying fungal ancestors, without any obvious reversal to saprotrophy [4]. Although the oldest ECM root fossils date back to 50 million years ago (MYA) [5], molecular analyses place the origin of ECM fungi in the Cretaceous [6] and suggest that they probably played a role in the migration of plants from the tropics to the poorer temperate regions [7]. ECM fungi mostly belong to Basidiomycota and Ascomycota, and they form symbioses with a relative small number of plant species [4]. They play an important role in forest establishment and in the successful reforestation of harsh environments, such as saline areas [8]. Moreover, ECM fungi can form fruiting bodies which have an important economic impact, such as truffles.

The AM symbiosis involves the majority of crop plants and results from the successful interaction between fungi in the Glomeromycota and the roots of about 80% of terrestrial plants [9]. This symbiosis is one of the oldest biotrophic interactions, dating back 400–450 MYA and is thought to have played a pivotal role in the water-to-land transition during plant evolution [10] (Figure 1). AM fungi have become so intimately dependent on plants that they are obligate biotrophs.

The evolutionary success of mycorrhizal symbioses likely derives from the bidirectional nutrient exchange that takes place between the two partners in most associations: fungi deliver mineral nutrients to the plants, while receiving sugars in return. It has been estimated that up to 20% of the photosynthesis-derived compounds of terrestrial plants (approximately 5 billion tons of carbon per year) are consumed by symbiotic fungi [11]. On the other hand, for example, 70% of the overall Pi acquired by arbuscular mycorrhizal rice plants is delivered *via* the symbiotic route [12]. Mycorrhizal plants benefit from their interaction with symbiotic fungi not only in terms of improved mineral nutrition, with an increased biomass production, but also in better protection against pathogens and abiotic stresses [13].

Because of the importance of mycorrhizal symbioses in plant health, several studies have focused on the biology, evolution and biodiversity of mycorrhizal associations [14]. In particular, the recent development of high-throughput molecular tools has allowed us to gain deeper knowledge on the molecular mechanisms governing the plant–fungus interaction [14],

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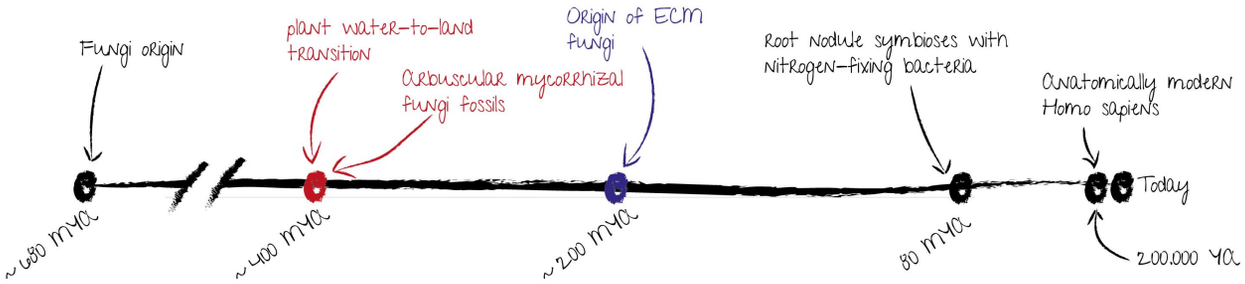

Figure 1. Schematic timeline of the root symbiosis development.

providing useful information for the application of these beneficial fungal agents to optimize plant health, nutrition and yields in sustainable agriculture and forestry.

Although examples will be given for all mycorrhizal types, this chapter mainly focuses on AM associations due to the following reasons. First, fossil and molecular records indicate for AM fungi a very long co-evolution with plants, with an unchanged morphology over 400 million years [15]. This observation opens several interesting questions such as: when has this symbiosis evolved? Has the molecular machinery that regulates this symbiosis evolved over time, or are we looking at the same situation fixed millions years ago? Understanding the biology of this obligate biotrophic interaction is a scientific challenge, but it would allow us to unravel the molecular mechanisms of the oldest known symbiosis [16]. The second reason is the high relevance of AM symbiosis for crop plants; better knowledge of these associations would have agro-environmental applications, with consequent economic and social impact.

2. Plant–symbiotic fungi interactions

The plant AM fungal interaction starts in the soil surrounding the plant roots, a region termed rhizosphere, where both plants and fungi release chemical signals in a pre-symbiotic molecular dialogue [17]. Among their root exudates, plants release in the rhizosphere signals such as strigolactones and cutin monomers, which elicit hyphal branching in AM fungi as well as apical growth of fungal hyphae towards the root surface, following the gradient of plant molecules (Figure 2A). Although the fungal receptors for these plant molecules remain unknown, it has been proved that they are perceived by the fungus, causing a signal cascade.

Fungal signal molecules have been identified in the past few years as being lipo-chito-oligosaccharides (LCOs) [18], the same type of signal molecules produced by rhizobia when interacting with legume plants, and chito-oligosaccharides (COs) [19]. Although the plant receptors for the fungal signal molecules have not been identified yet, large families of receptors are predicted to potentially bind these molecules.

Thanks to the exchange of these plant and fungal signal molecules, the plant and the AM fungus recognize each other and begin a more intimate phase of the interaction, with the fungus starting root colonization. The plant paves the way for fungal colonization by building up the so-called pre-penetration apparatus (PPA) [20], a transient assembly that defines the

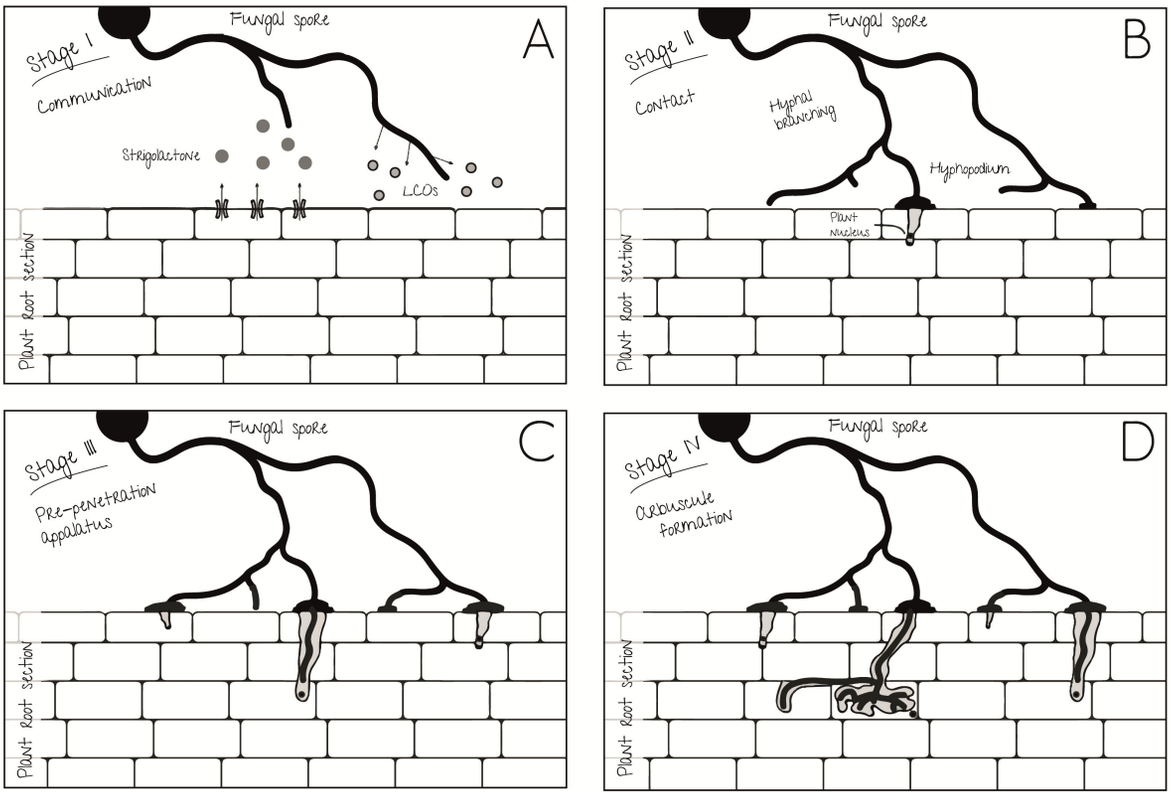

Figure 2. The boxes represent the four phases of formation of the plant–fungus association in the AM symbiosis. (A) Plant roots exude strigolactones and induce hyphal branching, while the fungus releases LCOs, perceived by the plant. (B) The AM fungus contacts the root surface and forms hyphopodia. (C) Epidermal plant cells produce a pre-penetration apparatus; the AM fungus starts to grow inside the plant and reaches the cortex. (D) The AM fungal hypha branches inside the cortex cells and form the arbuscules.

path followed by the fungal hyphae toward the inner root layers (Figure 2B–C). The AM fungus follows the path created by the PPA until the root cortex, where it starts to form a tree-like structure called 'arbuscule' (Figure 2D). The arbuscule is the core of a functional AM symbiosis, shaped as a highly branched structure where each hyphal branch is surrounded by the plant cell membrane. The contact surface between the plant and the AM fungus greatly increases around the arbuscule, thus increasing the area of nutrient exchanges. After few (ca. 4–5) days, the arbuscule collapses [21] and is replaced by a new one in the same or in another cortical cell. During AM fungal colonization, 'early stage' indicates the phase occurring prior to and during the initial contact between the two symbionts. This stage ends with the formation of the arbuscules that mark the transition to the 'late stage' of the symbiosis. However, fungal colonization of plant roots occurs at many access points not normally synchronized and the mycorrhizal symbiosis is highly dynamic, meaning that when new access points are created, arbuscules are forming and collapsing. Therefore, early and late stages can be really distinguished only after the first contacts between plant and fungus. The intracellular accommodation of unbranched hyphae (during the early stage) and of arbuscules (at a later stage) is a coordinated developmental process between the plant and the fungal cells: it involves an intricate and largely unexplored signal exchange, intense secretory activity related to the biogenesis of the perifungal membrane and an overall reorganization of the cell architecture.

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Several plant genes are known to be required for the establishment and functioning of the AM symbiosis. Some of them encode proteins that are components of the so-called 'SYM pathway' and are essential for early signalling and root colonization [10]. Other genes are likely involved in nutrient exchange during arbuscule functioning, such as the *Medicago truncatula* gene coding for a phosphate transporter (PT4) specifically induced in arbuscule-containing cells [22]. However, despite molecular and cellular evidence of the expression of these genes in arbuscule-containing cells, the corresponding proteins have not been identified through proteomic approaches until very recently [23], most likely because of their accumulation in a small subpopulation of root cells, those harbouring the arbuscules, and because of technical difficulties with membrane protein extraction.

Whereas the main signal molecules involved in the AM fungus–host plant dialogue have been identified, little information is so far available on the recognition events and on the long-maintenance factors involved in the ECM symbiosis (Garcia et al. 2015), although auxin and ethylene have been identified as some of the signals exchanged between the two partners in ECM [24,25]. By contrast, nothing is known concerning this aspect in the ericoid and the orchid mycorrhiza. The colonization steps have a very different morphology in ECM and AM symbioses. During the symbiotic phase, ECM fungi form a fungal sheath (the mantle) that develops outside the root. From the inner layers of the mantle, some hyphae penetrate between the epidermal and the outer cortical cells to form an intercellular hyphal network (the Hartig net) inside the root tissues [1]. Mycorrhiza-induced small secreted proteins (MiSSPs) are fungal proteins known to be involved during the formation and maintenance of the symbiosis between ECM fungi and their host plant [26,27].

The identification of the key molecular players in mycorrhizal symbioses is mandatory to understand the complex interactions between the symbiotic partners and the ways to improve and fully exploit their symbiotic potential in sustainable crop and forest management. Genome sequences of several mycorrhizal fungal species are now available and provide a great opportunity to increase our knowledge on the mycorrhizal lifestyle, on the metabolic capabilities of these symbioses and on the molecular dialogue between the two symbiotic partners [28].

3. The symbiotic proteomics of mycorrhizal interactions

Proteomics is the large-scale study of proteins from a specific proteome in order to understand cellular processes, and includes assessment of protein abundance, protein modifications, along with identification of interacting partners and networks. As the aim of proteomics is the identification of proteins, the molecular components actually taking part in cellular processes, rather than their genetic information, proteomics could be the main technique to unravel the key players of mycorrhizal symbioses. However, when the number of proteomics studies is compared with those using genomics, transcriptomics or microscopy, the gap is very significant (Table 1). One of the reasons is that the methods for protein identification are based, nowadays, mainly on mass spectrometry, a more complex and expensive technology than

high-throughput DNA or RNA sequencing. Protein identification is made by matching the peptides masses to corresponding masses calculated by the software on proteins or translated gene sequences available in databases. If sequences are not found in databases, protein identification fails. Over the years, many loopholes have been found, among them the easiest was to use sequences from other species. Concerning the identification of plant proteins, the best-studied and well-sequenced plant is *Arabidopsis thaliana*. Unfortunately, this plant is not able to form any type of mycorrhizal symbiosis. In the past few years, DNA sequencing has become cheaper and almost a routine technique, allowing the genome sequence of many organisms to become available.

Another aspect that has hindered the use of proteomics in the study of mycorrhizal interactions is the fact that the mycorrhizal symbiosis involves a small percentage of plant root cells, that may contain fungal structures at different developmental stages and with different putative roles [29]. For example, arbuscules are limited to the root cortical cells in the AM symbiosis, a tissue where not all plant cells are colonized. In addition, the majority of key proteins are likely to be membrane proteins. Taken together, this means that protein extraction from AM roots will lead to a very small percentage of proteins expressed in symbiosis. This 'dilution effect' has made it very hard to identify the key proteins directly involved in plant–fungus interactions.

In summary, the lack of sequence databases of reference organisms and the difficulties in protein extraction have characterized the first decade of proteomics applications to mycorrhizal symbiosis, and explain the limited results obtained.

4. Proteomics in action

The first proteomic investigation of the plant–mycorrhizal fungus symbiosis was published by Dumas et al. (1990) and used mono-dimensional polyacrylamide gel electrophoresis (PAGE) to separate soluble proteins from non-mycorrhizal roots and from roots infected by different AM fungi [30]. After this pioneering study, and because of improvements in sample extraction, sample purification and in the technological performance of the equipment, many studies have aimed to identify the key players involved in mycorrhizal interactions (Figure 3). Many strategies have been set up, depending on the target mycorrhizal type, on the symbiotic stage of interest and subcellular localization [30].

4.1. Proteomics on the early stages of the mycorrhizal symbiosis

The studies on the early stages of the symbiosis coincide with the earlier studies in symbiotic proteomics. Burgess and collaborators set up a complex experiment to identify proteins either induced, enhanced or inhibited during the early stages of ECM development [31]. They compared, over a time-course, the profiles of proteins expressed in roots inoculated with three different isolates of *Pisolithus tinctorius* showing different degrees of root colonization: isolate H2144 exhibited a very high infectivity, isolate 441 showed moderate infectivity, while isolate H506 was not able to induce ECM [31]. They used two-dimensional electrophoresis (2DE)-

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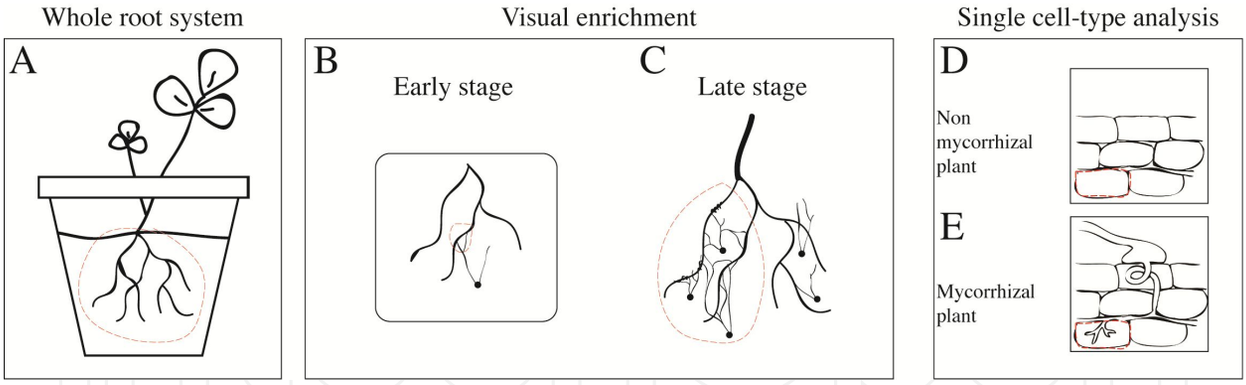

Figure 3. The figure represents different approaches that can be used to collect biological material for AM roots proteomics. (A) Collection of the whole root is the easiest and quickest approach, but it has a drawback due to the fact that the amount of proteins involved in the symbiosis is a very small percentage of the all extracted proteins. The visual enrichment approach allows the collection only of the roots in contact with the fungus. (B) The root organ culture system allows to study the early stages of colonization by following the root and hypha growth until they contact each other and then collect only the root pieces reached by the hyphae. (C) By microscope inspection, roots with the higher percentage of fungal hyphae in contact can be selected. (D–E) Using a laser microdissection (LMD) approach, it is possible to select non-colonized cells (D) and colonized cells (E) from root sections, and to collect them separately, thus avoiding the dilution effect caused by the heterogeneous situation of a mycorrhizal root.

PAGE, a gel electrophoresis technique introduced in the middle 1970s by O'Farrell and Klose and able to separate with high-resolution proteins by two orthogonal properties: iso-electrical point and molecular weight [32]. With this approach, Burgess et al. (1995) found that the morphological changes observed in the inoculated plant roots were linked with massive changes in protein composition, and claimed that these changes commenced at the time of contact between the two partners [31]. It was later discovered [33,34] that these morphological changes during the establishment of ECM were not caused by the contact between plant and fungus, because molecular signals released in the rhizosphere were sufficient to trigger them. The work by Burgess et al. (1995) was nevertheless important because it revealed some plant and fungal symbiosis-related polypeptides and demonstrated that their upregulation was tightly correlated with fungal infectivity.

Five years later, another paper on the early stage of the ECM symbiosis was published by Laurent et al. (1999). They used the same methodological approach, 2DE-PAGE separation, but they focused their attention on the cell wall polypeptides, in order to identify cell surface proteins involved in ECM symbiosis development. It was a huge sampling effort not only because they analyzed the early stage of the symbiosis but also because they had to enrich samples for cell wall polypeptides (CWP). One of the main results was the observation of the enhanced synthesis of several immunologically related 31- and 32-kDa fungal polypeptides, called symbiosis-regulated acidic polypeptides (SRAPs) [35]. As gene expression studies were also carried out, these proteomic data also highlighted the fact that expression of SRAP-32 was regulated at transcriptional level, suggesting that the synthesis of new hyphal proteins is an important process during symbiosis formation [35].

In AM symbiosis, the phase between the first contact and the formation of the first arbuscule, a period ranging between few hours and 1–2 weeks after inoculation, is normally consid-

ered as an early stage of the interaction [36]. Focus on this particular phase is important to understand the cross-talk between the partners and how the proteomes of the two organisms change during the colonization events. A study by the group of Dumas-Gaudot [37] focused on the early stages of the AM symbiosis in three different genotypes of the model plant *M. truncatula*: the wild-type (J5), a mycorrhiza-defective (TRV25, dmi3) and an autoregulation-defective (TR122, sunn) genotype. The study was aimed at investigating changes in the root proteome elicited in response to appressorium formation by *Glomus intraradices*. For this purpose, the authors compared by 2DE-PAGE the root proteome from non-inoculated roots and from roots synchronized for appressorium formation by *G. intraradices*. The authors showed that proteins that responded to appressorium formation were differentially expressed in different genotypes. This paper was important because it also reported, for the first time, the identification of plant root proteins involved in mycorrhizal symbioses by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry. This technique revealed appressorium-responsive proteins that were previously unknown on the basis of transcriptome analyses, demonstrating that proteomics and transcriptomics are complementary approaches [37].

The early stage of the mycorrhizal symbiosis represents a challenging, but also a very attractive, stage for proteomics. The sampling time and sampling method are crucial for the experiment's outcome, and synchronization of the root colonization events would enrich root samples in the proteins of interest. Lopez-Meyer and Harrison (2006) first proposed a system to synchronize AM fungal spore germination and root penetration events [38]. Despite this technical improvement, the amount of proteins involved in the plant response to the AM fungus and in the PPA formation, as compared to the amount of proteins in the whole root, is expected to be very low and at the limit of detectability. Therefore, new strategies for sample preparation and experimental designs are required to reveal the key components of this fundamental phase of the mycorrhizal symbiosis.

4.2. Proteomics on late stage

Late stage of the AM symbiosis commonly indicates the phase in which the fungus has already build up the arbuscules. In nature, the symbiosis is highly dynamic and very complex because, while arbuscules are forming, new penetration events occur.

Bestel-Corre et al. [39] reported the first mass spectrometry (MS)-based identification of mycorrhiza-related proteins. These authors studied the response of *M. truncatula* inoculated either with the AM fungus *Glomus mosseae* (current name *Funneliformis mosseae*) or with the nitrogen-fixing bacterium *Sinorhizobium meliloti*. Proteins were separated by 2DE-PAGE and image analyses, with precise quantification of spots volume performed to identify differentially expressed protein spots. Those spots were excised from the gels and analyzed by mass spectrometry. Notably, only plant or bacterial proteins were identified, may be due to difficulties in extracting fungal proteins. The authors identified several proteins related to defence responses, root physiology and respiratory pathway. However, none seemed to be a key protein in the mycorrhizal symbiosis.

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The proteomic analysis of the late stage of the AM symbiosis using the whole root system as starting material never allowed very good results. To overcome this problem, many studies on the late stage of the symbiosis focused their attention on a specific sub-cellular compartment. With this approach, the same group published two other papers few years later, using sub-cellular fractionation methods, reporting more remarkable results described in the next paragraphs.

Mycorrhizal systems different from ECM and AM have been seldom investigated with proteomic approaches. However, recent results have been published for orchid mycorrhiza by Valadares et al. (2014). For orchids, the association with symbiotic fungi is required for seed germination and seedling development, when the plant relies on the fungus also for carbon supply (a strategy termed mycoheterotrophy). Recently, 2D-LC-MS/MS (two-dimensional liquid chromatography MS/MS) coupled to isobaric tagging for relative and absolute quantification has been used to identify proteins with differential accumulation in the orchid species *Oncidium sphacelatum* at different stages of plant development after seed inoculation with a *Ceratobasidium* sp. fungal isolate. Eighty-eight proteins, including proteins putatively involved in energy metabolism, cell rescue and defence, molecular signalling and secondary metabolism, have been identified and quantified. These results suggest profound metabolic changes during the development of mycorrhizal orchids, likely related to a switch from the fully mycoheterotrophic to the photosynthetic stages [40].

4.3. Proteomics on sub-cellular compartment

Although 30% of naturally occurring proteins are predicted to be embedded in biological membranes [41], comprehensive membrane proteomics is technically difficult due to the hydrophobicity, heterogeneity and lower abundance of membrane proteins. In mycorrhizal symbioses, membrane proteins are very important because they likely include the receptors that control the fungal–plant dialogue as well as the transporters that mediate nutrient exchange. For these reasons, many authors have focused on membrane proteins using different enrichment protocols.

The first study on sub-cellular fractionation of membrane proteins was conducted by Benabdellah et al. (1998). They isolated the microsomal protein fraction from colonized tomato roots, where they found several differentially expressed proteins [42]. Few years later, the same group identified for the first time a protein related to mycorrhizal symbiosis by Edman N-terminal sequencing, after plasma membrane enrichment and 2DE-PAGE protein separation [43].

The years between 2000 and 2010 saw the rapid increase of mass spectrometry as the main proteomic technique for protein identification and quantification, replacing other techniques previously used. The difficulties and the low number of proteins identified with the Edman N-terminal sequencing were overcome with the advent of mass spectrometry, and new intriguing possibilities were opened.

Valot et al. (2005) also used a sub-cellular proteomic approach to monitor membrane-associated protein modifications during the AM symbiosis [44]. 2DE-PAGE of root microsomes

revealed some mycorrhiza-responsive proteins including 15 induced, 3 up-regulated, and also 18 down-regulated proteins. Among those 36 regulated proteins, 25 were identified using the MALDI-TOF. Except for an acid phosphatase and a lectin, none of them was previously reported as being regulated during the AM symbiosis. This sub-cellular proteomic approach allowed for the first time the identification of fungal proteins expressed *in planta*. In their final conclusion, the authors pinpointed the next challenge: the identification of membrane proteins located in and around the arbuscule, the mycorrhizal symbiosis-specific fungal structure.

Arbuscules are ephemeral structures that form continuously and collapse at the end of a short life-span. The identification of proteins temporarily present in a sub-set of cell types remains, at the present time, a technical challenge for quantitative proteomics. Moreover, membranes associated with the arbuscules are significantly less, relative to the overall root membranes, as also suggested by the low amount of fungal RNA found in extensively colonized AM roots, maximally reaching 12% of the total RNA extracted [45]. Despite these considerations, the same group attempted to enrich samples for plasma membranes using a discontinuous sucrose gradient method [46]. In this chapter, two complementary proteomics methodologies for protein fractionation and identification were applied for the first time to the plant–AM fungus symbiotic association: an automated 2D liquid chromatography-tandem mass chromatography (LC-MS/MS) using a strong cation exchange and reverse phase chromatography, and SDS-PAGE combined with a systematic LC-MS/MS analysis. The enrichment for plasma membrane proteins helped to reduce the sample complexity, and both proteomic approaches involved a pre-fractionation step before MS analysis, another step that reduced further sample complexity. Only proteins consistently retrieved with the two methodologies were taken into account, resulting in the identification of 78 proteins. Of those proteins, 56% were predicted to contain one or more transmembrane domains, while 30% were already known to be localized on the plasma membrane. Very stringent criteria were applied to detect only proteins that were exclusively found in the plasma membrane of mycorrhizal plants. Only two proteins passed this severe threshold: a plasma membrane proton-efflux P-type ATPase (Mtha1) and a blue copper-binding protein (MtBcp1). Considering the highly stringent criteria, Valot et al. (2006) concluded that these two proteins were biologically relevant and deserved further investigations. The importance of these proteins was in fact revealed in subsequent studies. Even though they did not identify the specific function of MtBcp1, Pumplin and Harrison (2009) suggested the presence of at least two distinct domains in the peri-arbuscular membrane (PAM): an 'arbuscule branch domain' that contains the symbiosis-specific phosphate transporter, MtPT4, and an 'arbuscule trunk domain' that contains MtBcp1 [47]. Concerning the other protein specifically induced in arbuscule-containing cells, Wang et al. (2014) showed that H+-ATPases are required for enhanced proton pumping activity in membrane vesicles. Functional impairment of this gene led to impairment in the host plant nutrient uptake through the mycorrhizal symbiosis, whereas its overexpression increased both phosphate uptake and plasma membrane potential, suggesting that this H+-ATPase plays a key role in energizing the peri-arbuscular membrane, thereby facilitating nutrient exchange in arbusculated plant cells [48].

In the past few years, proteomics has seen great technical advances, especially in the mass spectrometry equipment and bioinformatics resources, with the development of new separa-

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tion techniques, new multi-dimensional procedures, new searching algorithms, new mass spectrometers and the availability of more databases. Owing to these new technologies, Abdallah et al. (2014) were able to identify 1,226 root membrane proteins and to report for the first time the proteomic identification of several symbiosis marker genes: MtPt4, a mycorrhiza-specific phosphate transporter [22], the AM-inducible ammonium transporter GmAMT4.1 in soybean [49], STR half-ABC transporters [50] and vesicle-associated membrane proteins VAMP721d/e [51].

In the experiments of Abdallah et al. (2014), proteins were quantified by label-free counting. Protein quantification in label-free experiments is generally based on two types of measurements: peptide peak intensity and spectral count. These parameters are measured for individual LC-MS/MS or LC/LC-MS/MS runs and changes in protein abundance are calculated *via* a direct comparison between different analyses [52]. The spectral counting strategy used by Abdallah et al. (2014) suggests that accommodation of AM fungi within root cortical cells implies both a dynamic reorganization of the root membrane proteome and the *de novo* synthesis of AM-related proteins [23]. This study, beside the identification of proteins corresponding to key genes already identified in mycorrhizal symbiosis, also reported new proteins, many of which support the importance of membrane trafficking during mycorrhiza colonization.

In summary, sub-cellular and peptide fractionations led to the identification of many key proteins involved in AM symbiosis. Despite the recent contributions of proteomics to the study of the plant–fungus mycorrhizal interactions were substantial, the role of many of them as actors in the symbiosis is still to be fully understood.

4.4. Proteomics to identify fungal proteins

Most proteomic studies on the AM interaction have focused on plant proteins. Identification of fungal proteins is more challenging due to the impossibility to grow AM fungi in axenic cultures, to the lower amount of fungal biomass, as compared to plant material, and to the more scanty sequence information. However, pioneering studies have been carried out in France by Dumas-Gaudot and Recorbet [53,54].

Dumas-Gaudot et al. (2004) used the root organ culture method [55] to enrich for proteins expressed in the extra-radical mycelium of the AM species *G. intraradices*. They successfully produced, for the first time, a 2DE reference map for the extra-radical proteome of an AM fungus. After the selection of the most intense protein spots, they tried to identify them by mass spectrometry. Unfortunately, only very few proteins from filamentous fungi were known and present in public databases, and the only available genome sequence, at that time, was from *Neurospora crassa*, phylogenetically very far from Glomeromycota. In spite of that, identification was possible for 8 proteins out of the 14 analyzed, and homologies were found for 4 of them.

Few years later, the same group attempted again the identification of fungal proteins expressed in the AM association [54]. They maintained the same experimental system, but they used the GeLC-MS/MS method and could identify 92 different fungal proteins. GeLC-MS/MS approach

combines a mono-dimensional gel (1D-PAGE) and a nano-scale capillary liquid chromatography-MS/MS. Briefly, after the 1D-PAGE separation, the mono-dimensional gel is cut in several pieces; in-gel digestion results in different protein fractions that are separated and analyzed by LC-MS/MS. Using the MetaCyc database, a collection of more than a thousand metabolic pathways [57], these authors grouped those proteins in 11 pathways that span energy, metabolism and cell rescue processes. These data, together with previous identifications of putative homologues of cell-cycle gene in *G. mosseae* and *G. intraradices* [56,57], suggest that signalling pathways known in model species may also operate in AM fungi. Although the GeLC-MS/MS strategy opened the possibility to large-scale proteomics of mycorrhizal fungi, no further data have been published with this technique.

Secreted fungal proteins play key roles in host plant colonization and symbiosis development in ECM interactions. Vincent et al. (2012) have identified the extracellular proteins secreted in the growth medium by the free-living mycelium of the ECM fungus *L. bicolor* using 2-DE, IPG-IEF shotgun (IPG strip was cut into fractions and tryptic peptides were eluted from the each fraction) and SDS-PAGE shotgun, with the aim to validate predicted secreted proteins and identify putative novel effectors of the symbiosis. Among the 224 proteins identified, there were carbohydrate-active enzymes (CAZymes), probably involved in cell wall remodelling during hyphal growth, as well as secreted proteases. Additionally, the involvement of some of these proteins in the establishment of the mycorrhizal symbiosis was supported by transcriptomic analyses of ECM roots [58].

5. Mycorrhizal fungi and heavy metals

In plants, stress tolerance to soil pollution can be increased by their interaction with mycorrhizal fungi [59]. Six out of ten of the most polluted soils in the world are contaminated by heavy metals [60], and mycorrhizal symbioses have been found to reduce metal toxicity to the host in soils with potentially toxic amounts of soluble and insoluble metals. Phytoremediation, the plant-mediated reclamation of polluted soils, is receiving increasing attention as a natural method to restore the biological features of the soil. Mycorrhizal fungi can have an important part in this process. Many studies have been carried out on the benefits of mycorrhizal plant–fungus interaction in heavy-metals-polluted soils, but only few of them have used a proteomic approach to identify the key proteins potentially involved in mycorrhiza-mediated stress tolerance. Researches on this topic have analyzed different plant organs, like leaves or root, but also focused on the symbiotic fungus.

Bona et al. (2010) studied the leaf proteome of the arsenic hyperaccumulator fern *Pteris vittata* inoculated with two fungi (*Glomus mosseae* and *Gigaspora margarita*), with and without arsenic treatment [59]. The symbiosis with both fungi decreased arsenic concentration compared with non-mycorrhizal plants, indicating the protective effect of mycorrhizal fungi. Interestingly, the plant protein expression profile was different when the plant was inoculated with *G. mosseae* or *G. margarita*. Although they studied a different biological system, Cangahuala-Inocente and colleagues (2011) identified instead a core of 25 proteins, supporting the

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existence of conserved plant responses to *Glomus irregulare* and *Glomus mosseae*, at least in a woody perennial species such as grapevine [61].

A study on root proteomics has been conducted by Aloui et al. [62]. They reported, using a 2DE approach followed by MS/MS, the protective effect conferred by *G. intraradices* to the model legume *M. truncatula* in the presence of Cd. They identified 36 mycorrhiza-related proteins, but only 6 displayed changes in abundance upon Cd exposure. These proteins – a cyclophilin, a guanine nucleotide-binding protein, an ubiquitin carboxyl-terminal hydrolase, a thiazole biosynthetic enzyme, an annexin, a glutathione S-transferase (GST)-like protein and a S-adenosylmethionine (SAM) synthase – seem to have a function in oxidative stress alleviation [62]. The authors also suggested that antioxidant enzymes and non-enzymatic antioxidants could be probably involved both in arbuscule senescence [63] and in plant protection against oxidative damage caused by Cd.

In addition to plant proteomics, mycorrhizal fungi have been also investigated for changes in their protein profiles when exposed to heavy metals. Adaptive metal tolerance has been reported for mycorrhizal fungi isolated from polluted soils [64], although the underlying cellular and molecular mechanisms have been seldom identified [57].

Chiapello et al. (2015} used gel-based and gel-free techniques as a complementary approach to study the proteome of *Oidiodendron maius* Zn, an ericoid mycorrhizal fungus isolated from a polluted soil [65] and showing adaptive tolerance to zinc and cadmium [66]. *O. maius* Zn can establish endomycorrhizal symbiosis with the roots of ericaceous plants also in heavily contaminated soils [67]. The aim of the study was to understand the response of this metal-tolerant fungus to Cd and Zn ions and to reveal common and/or specific cellular and molecular mechanisms to counteract heavy metal stress caused by these to metals. The authors concluded that Cd and Zn induce common as well as specific responses. Among the common induced proteins, agmatinase, an enzyme involved in polyamines biosynthesis, represents a novel finding in relation to heavy metal responses in fungi.

6. Conclusion and future perspective

Proteomics has allowed us to identify proteins expressed and regulated during the development and functioning of mycorrhizal symbioses, therefore contributing to a better understanding of the events occurring at the cellular level.

Protein identification is strongly dependent on gene and protein sequences available in databases, and the constant increase in the number of sequenced genomes in the past decade, together with improvement of mass spectrometry technology, has helped scientists to obtain more reliable data. In the past few years, a specialized fungal genomics portal, called MycoCosm (http://genome.jgi.doe.gov/fungi), has been created by the US Department of Energy (DOE) Joint Genome Institute (JGI), offering an access point to the data from all the sequencing genome project managed by the DOE JGI [68,69]. Starting from the three first genome projects on *Laccaria bicolor*, *Tuber melanosporum* and *Rhizophagus intraradices* [70,71,72], several more

genomes from symbiotic fungi, including ECM, AM, orchid and ericoid fungi, have been recently sequenced, with the aim to determine the diversity of the molecular processes involved in the interaction [28]. Despite the more powerful techniques and wider reference datasets for protein identification, current limitations exist in the application of proteomics to the study of plant–microbe interactions. In particular, new extraction methods, microsomal studies, sub-cellular enrichment, gel-free separation methods, pre-fractioning separations and new mass spectrometry are still far from being fully explored [73].

In order to identify proteins from a very small subset of target cells, Gaude et al. (2012) combined laser capture microdissection (LCM) and LC-MS/MS [74]. Laser microdissection permits the rapid isolation, from sections of a heterogeneous tissue, of a selected cell population in a manner compatible with the extraction of DNA, RNA or proteins [29]. Using LCM, arbuscule-containing cortical cells and cortical cells from non-mycorrhizal *M. truncatula* roots were isolated. Proteomic analyses on these cells revealed a number of proteins involved in lipid metabolism, most likely related to the synthesis of the PAM. This targeted analysis on a specific subset of colonized cells, those harbouring the arbuscules, curiously did not identify known PAM marker proteins, thus suggesting that either sample preparation or instrument capability were not sensitive enough. Although this first use of LCM in the proteomic investigation of the AM mycorrhizal symbiosis did not identify known marker proteins, it highlighted the PAM as an important carbon sink. The LCM technique coupled with MS/MS techniques could be a powerful combination to investigate the protein profiles of specific cells at specific time-points. Moreover, LCM can help to overcome the problem of asynchronous fungal development and arbuscule maturation in mycorrhizal roots. To be able to combine LCM samples of synchronous arbusculated cells with sub-cellular enrichment, peptide pre-fractionation and analysis with powerful MS instruments such as Orbitrap Velos (Thermo Fischer company) may reveal an unexpected specificity during the development of this symbiotic structure.

Novel MS/MS techniques developed in the past few years in other research fields could also be applied to investigate plant–fungus symbiotic interactions. For example, selected reaction monitoring (SRM) is a targeted MS technique used to complement untargeted shotgun methods. SRM is used to measure across multiple samples – in a consistent, reproducible and quantitatively precise manner – a set of candidate proteins involved in a particular cellular process [75]. Based on known data from the literature or previous experiments, a set of target peptides that optimally represent the protein are selected and after their validation they are used for protein quantification. Unfortunately, the sensitivity of SRM is limited and it cannot cover the entire proteome of an organism. Nevertheless, this technique is really promising for the fine protein quantification in different cell types or conditions. Taylor et al. (2014) used SRM in plant science to confirm protein abundance in *Arabidopsis* mutant lines, even when discrimination between very similar proteins was needed [76]. However, the application of this technique for the identification of OsPT11, homologue to MtPT4, from wild-type and mutant lines did not work, probably due to the method's sensitivity (Chiapello, 2013, unpublished data). Another promising technique to further investigate the proteome of arbuscule-containing cells is the single-cell imaging mass spectrometry (IMS), a powerful technique used to map the distribution of endogenous biomolecules with subcellular resolution [77].

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	Year	Plant	Fungus	Separation technique	Identification technique	Reference
ECTO	1995	*Eucalyptus grandis*	*Pisolithus tinctorius*	2DE-PAGE	---	Burgess et al., 1995
	1999	*Eucalyptus globulus subsp. bicostata*	*Pisolithus tinctorius*	2DE-PAGE	---	Laurent et al., 1999
	2007	---	*Boletus edulis*	2DE-PAGE	ESI-Q-TOF MS	Liang et al., 2007
	2012	---	*Laccaria bicolor*	IPG-IEF / 1-DE and 2-DE -PAGE	LCQ IT	Vincent et al., 2012
AM	2000	*Lycopersicon esculentum*	*Glomus mosseae*	2DE-PAGE	N-terminal sequencing	Benabdellah et al., 2000
	2001	*Medicago truncatula*	*Glomus mosseae*	2DE-PAGE	MALDI-TOF	Bestel-Corre et al., 2001
	2004	---	*Glomus intraradices*	2DE-PAGE	Q-Tof2	Dumas-Gaudot et al., 2004
	2005	*Medicago truncatula*	*Glomus intraradices*	2DE-PAGE	MALDI-TOF	Valot et al., 2005
	2006	*Medicago truncatula*	*Glomus intraradices*	2DE-PAGE	MALDI-TOF	Amiour et al., 2006
	2006	*Medicago truncatula*	*Glomus intraradices*	1-DE and 2-DE -PAGE / 2D-LC	LCQ Deca XP+	Valot et al., 2006
	2009	---	*Glomus intraradices*	1-DE-PAGE	ESI-Q-TOF MS	Recorbet et al., 2009
	2009	*Medicago truncatula*	*Glomus intraradices*	2DE-PAGE	LCQ Deca XP+	Aloui et al., 2009
	2010	*Pteris vittata*	*Glomus mosseae* / *Gigaspora margarita*	2DE-PAGE	QSTAR XL hybrid quadrupole-TOF	Bona et al., 2010
	2011	*Vitis vinifere*	*Glomus mosseae* / *Glomus intraradices*	2DE-PAGE	MALDI-TOF	Cangahuala-Inocente et al., 2011
	2012	*Populus alba*	*Glomus intraradices*	2DE-PAGE	ESI-Q-TOF MS	Lingua et al., 2012
	2014	*Medicago truncatula*	*Rhizophagus irregularis*	1-DE-PAGE	LTQ XL ion trap	Abdallah et al., 2014
Others	2015	---	*Oidiodendron maius*	2DE-PAGE / 2D-LC	MALDI-TOF / QSTAR MS/MS	Chiapello et al., 2015
	2014	*Oncidium sphacelatum*	*Ceratobasidium* sp. Isolate	2D-LC	ESI-Q-TOF MS	Valadares et al., 2014

Table 1. List of papers in which proteomics has been applied to study mycorrhizal symbiosis. For simplicity, AM fungi have been indicated with the names used in the original articles, despite the relatively recent taxonomic revision (Redecker D1, Schüssler A, Stockinger H, Stürmer SL, Morton JB, Walker C. 2013. An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (Glomeromycota). Mycorrhiza 23:515-31).

The ability to analyze a single-cell proteome is exciting, but also extremely challenging. The first difficulty is the sensitivity, both correlated with the sample itself and with the mass

spectrometry detection capability. Every single cell can contain proteins in a range of few to million copies per cell. However, mass spectrometers are now really powerful, and even with an attomole detection limit, only the most abundant proteins are detectable [78]. The estimated number and concentration of proteins in a single mammalian cell is 33 attomole/cell for the most abundant and 830 yotomole/cell for the less abundant [77]. The second challenge is the inherent limitation associated with the imaging modality itself. Even if further development is needed to obtain the combined resolution and sensitivity required, IMS stands up as a very promising technique to analyze specific cell types or conditions. By employing IMS, Ye et al. (2013) detected a large array of organic acids, amino acids, sugars, lipids, flavonoids in roots and root nodules of *M. truncatula* during nitrogen fixation [79]. They demonstrated that IMS can obtain unique information on the identity and spatial distribution of plant metabolites, although high-resolution MALDI-MS is required to fully resolve the metabolic differences in nodule chemistry.

In conclusion, similarly to other 'omics' approaches, proteomics has also made rapid progress in the recent year, thus making this approach a very useful one to complement information on gene expression in mycorrhizal tissues. At this speed of technological developments, methods that allow us to easily assign proteins up-regulated during symbiosis to specific cell types and sub-cellular compartments may not be too far ahead. These proteomic techniques will be powerful tools to unravel the molecular component involved in plant–mycorrhizal fungal interactions.

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