Proteomics, a powerful approach towards understanding functional plant root interactions with arbuscular mycorrhizal fungi

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Proteomics, a powerful approach towards understanding functional plant root interactions with arbuscular mycorrhizal fungi

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ABSTRACT

Arbuscular mycorrhiza are symbiotic interactions formed between the roots of more than 80 % of plant species and a limited number of fungi belonging to a single order, the Glomales. The beneficial effects on growth, development and plant health, including for plant species with agricultural interest, have stimulated research to increase our understanding of the mechanisms underlying the development of the symbiosis. In recent years, a significant effort has been made on molecular and genetic analyses to identify arbuscular mycorrhiza-related genes. Together with these approaches, proteome analysis, based on the recent developments of two dimensional gel electrophoresis, mass spectrometry and bioinformatics, offers the possibility of a complementary insight into protein expression and regulation within the symbiosis. After reviewing the past studies dealing with arbuscular mycorrhiza-related proteins, this paper discusses how the new proteomics technology may significantly contribute to an integrated understanding of the processes involved in symbiosis development.

1. INTRODUCTION

Arbuscular mycorrhiza (AM) are symbiotic interactions formed between fungi belonging to the

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order Glomales (Zygomycetes) and the roots of most agriculturally important plant species (1, 2). In natural conditions, these very ancient fungi, thought to have colonized the first land plants during the Devonian (3) today form mycorrhizal associations with more than 80% of plant species (4). The resulting association plays an essential role in the acquisition of mineral nutrients which leads, among other benefits, to enhanced plant growth and health (5, 6). In the intimate interaction within the host roots, these endophytic obligate fungi undergo complex morphogenesis inside the epidermal and cortical cells which ultimates with the formation of arbuscules (7, 8). The arbuscule/cortical cell interface is considered to be the site at which the maximum of carbon and phosphate transfer occurs (9, 10).

Until recently, little was known about the identity of genes expressed in the arbuscules of mycorrhizas, due in part to the difficulty of cloning genes from the tissues of an obligate symbiont. Nevertheless, efforts have been made over the past few years to investigate molecular changes occurring in both the plant and the fungal symbionts during the development of the symbiosis (reviewed by 11, 12). Significant progresses were achieved through the combination of the innovative use of available material such as plant mutants, and some advanced molecular techniques such as differential screening (13, 14, 15), differential display (16, 17, 18, 19) and very recently, suppressive subtractive hybridization (20). Due to the biological system, studies at the protein expression level were still scarce and fragmentary. After providing a tentative overview of the proteins studied up to now, this article will show how the new proteome technology could help towards a more massive identification of proteins involved in arbuscular mycorrhizal symbiosis.

2. Protein analyses in AM symbiosis : state of art and problems encountered

Historically, the most extensively studied proteins in AM symbiosis were those related to plant defence responses, to fungal penetration and to cell metabolism such as phosphorus uptake. All these aspects have been thoroughly reviewed (21, 22, 11). In addition, several works using molecular approaches have revealed the expression of nodulation genes in AM symbiosis (23, 24, 25, 26, 27).

Table 1: Modifications of unknown root proteins reported in arbuscular mycorrhizal symbiosis

Plant species/ AMF	Expression levels
Glycine max/ Glomus fasciculatum	increased (28)
Nicotiana tabaccum/ G. mosseae	increased & new proteins (30)
Pisum sativum/ G. mosseae	increased & new proteins (29)
Trifolium pratense/ G. mosseae	+/- & new polypeptides (31)
Allium cepa/ G. mosseae	+/- & new polypeptides (33)
N. tabaccum/ G. mosseae & G. intraradices	+/- & new polypeptides (32)
Ri-TDNA roots of *Lycopercicum esculentum/ G. intraradices*	+/- & new polypeptides (34)
Myc^+ & Myc^{-1} *Pisum sativum*	+/- & new polypeptides differing in Myc^+ & Myc^{-1} genotypes (35,40)
Lycopersicum esculentum/G. mosseae	+/- & new polypeptides (36)
Medicago truncatula /G. mosseae	+/- & new polypeptides (71,72)

+/- = over and/or down regulation of constitutive polypeptides.

Beside approaches targeted on proteins of known functions, only a few studies (summarized in Table 1) were carried out for searching protein/polypeptides modifications in AM interactions with a non targeted approach. Whatever the plant and the AMF species used, increases in total protein levels and detection of additional protein bands were often reported (28, 29, 30, 31). Soon, changes in protein expression following AM symbiosis were more accurately followed when using two dimensional gel electrophoresis (2-DE) (32, 33). However, data from these studies remained quite scarce. Moreover, they

concerned various different plant species and strains of AM fungi, excluding any comparisons. Several difficulties for identifying symbiosis-related polypeptides were encountered. One was due to the obligate biotrophic status of AMF, which only allows studies from pot culture experiments, thus in non monoxenic conditions. Up to now, only one attempt has been carried out to look for symbiosis-related polypeptides in such conditions, using *Glomus intraradices*-inoculated Ri- T-DNA transformed roots of *Lycopersicon esculentum* (34). Another striking point was related to the problem of identifying the plant or fungal origin of the induced polypeptides. A few attempts were made to, at least, compare the polypeptide patters of mycorrhizas to those of germinated hyphae from pure spores (35, 36) or of extraradical hyphae (34). Nevertheless, fungal polypeptides may only be expressed during the symbiosis. Indeed, differential expression of fungal proteins (37) as well as of metabolic capacities have already been reported (38, 39). From 2-DE studies, it was concluded that most of the AM-induced polypeptides seemed to be of plant origin. Considering that most of these analyses were carried out at late stages of the symbiosis, when the fungus had abundantly developed inter and intra-cellularly into the cortical root cells, the question of efficiency in extracting fungal material from root tissues must be addressed. To highlight symbiosis-related polypeptides, some progress were gained from the comparison of polypeptide patterns of roots in interaction with either pathogenic or mycorrhizal fungi (36). Finally, a major complication was related to the nature of the colonization process. The overlapping occurrence of different stages of colonization renders protein expression studies of any particular stage very difficult. The discovery of several plant mutants with differential capability of penetration by AMF undoubtedly represents an asset for looking for polypeptides specifically involved in precise steps of the symbiosis. Indeed, analyses of wild type pea comparatively to a mycorrhiza-resistant mutant Myc^{-1} allowed to detect some polypeptide modifications during the very early stages of AM symbiosis (40). Nevertheless in all the studies mentioned above, no protein identification was achieved. At this time, progress in the identification of symbiosis-related polypeptides were hampered by poor reproducibility, combined to low capacity of protein loading, when using the conventional 2-DE method based on hand made carrier ampholytes isoelectrofocusing (IEF) gels. Moreover, a classical identification by Edman sequencing was hazardous in the case of AM symbiosis for which, without any pre-purification step, modifications in protein expression were only faintly detected. Some improvements were obtained by sub-fractionating protein extracts. Indeed, when root extracts were separated into soluble and membrane fractions, much more differentially displayed polypeptides were detected in tomato roots as a consequence of the establishment of the AM symbiosis (41). A recent report by the same authors deals with the successful identification by N-terminal micro-sequencing of an AM symbiosis-related H^+-ATPase following the separation of plasma membrane microsomal fractions by 2-DE (42).

3. Proteomic research and AM symbiosis

Although the studies presented above allowed to point out several arbuscular mycorrhiza-related proteins, they are still technically far from massive protein identification.

The term of "Proteomics" was firstly used in 1994 and relates to "the possibility to perform large-scale protein identification in order to help in the elucidation of gene products at the protein level" (43, 44). As recently highlighted by Gevaert and Vandekerckhove (45), it is based on four complementary technologies : 1) high-resolution 2-DE for purifying small amounts of proteins from complex mixtures, 2) generation by mass spectrometry of limited but sufficient structural information from the protein of interest, 3) access to protein or DNA

databases and 4) computer algorithms allowing translation and links between structural information on the protein and DNA sequences. All these techniques taken together have the power to monitor global changes occurring in the protein expression of a tissue or an organism and are now commonly used in biological research (46).

In the field of 2D separation, significant improvements have been obtained through the introduction of immobilized pH gradients (IPG) for IEF (47). This technology allows gel to gel reproducibility and high capacity loading, enabling the detection of proteins expressed at low levels. The application of this technology in plant-*Rhizobium* interactions has already proven its usefulness (48, 49).

Today, mass spectrometric techniques complemented by computer tools represent the method of choice for fast and high-throughput protein identification (50, 51, 52, 53). Most of protein analyses are performed today with two types of instruments : MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometer) and ESI (electrospray ionization) MS. The respective interests of each has been largely described (45, 54, 51). Although MALDI-TOF MS and peptide mass fingerprinting (PMF) analysis is clearly the method of choice for high-throughput protein identification, it strongly depends upon the availability of protein sequences in the databases. The success rate of this approach can be rather low when working with proteins originating from species of which only a limited amount of genomic sequence data is available (45). Moreover, peptide-mass searches are rarely successful against translations of short nucleotides sequences such as ESTs as there is generally insufficient sequence information contained for the multiple peptide matches required for conclusive identification (55). Lastly, using only peptide mass fingerprinting to search in cross species databases is known to be hardly reliable (56). Although additional identification attributes can be obtained in MALDI MS with the possibility of using peptide fragmentation techniques such as MALDI-PSD (45), ESI-MS is generally preferred to achieve peptide sequencing, and more recently, hybrid instruments such as Q-TOF (quadrupole time-of-flight) MS and even MALDI Q-TOF have appeared, rendering the identifications easier.

Proteomics is becoming a necessity in plant biology, as it is in microbiology and medicine. To date, the genome of one plant, *Arabidopsis thaliana*, has been entirely sequenced. The sequencing of the cereal crop *Oryza sativa* is underway and the number of available ESTs of several other plant species is increasing consistently (48). Due to the rapid development of plant genomics and transcriptomics combined with the recent improvements in 2D separation, various applications of proteomics tools in plant genetics and physiology have emerged in the last few years (57).

In the field of plant/micro-organism interactions, since *Arabidopsis thaliana* is not able to form any of the mycorrhizal and rhizobial symbioses, other model plants were requested to study these interactions. Among them, *Medicago truncatula* has soon emerged as a model for legume genetics and genomics (58). This plant species is diploid with a small genome, autogamous, and is easy to transform and regenerate (59, 60). It is nodulated by *Sinorhizobium meliloti*, the genome of which has been extensively studied, and the expression of several symbiosis-related genes has already been reported in this plant (61, 62, 63). Several research programs are now focused on this species, leading to a massive production of ESTs corresponding to various physiological situations including root symbioses with one or the other micro-symbionts. As a complement to the genome and transcriptome *M. truncatula* programs that have been recently initiated, proteome analysis

is also emerging as a powerful strategy. Up to now, it has been more extensively applied to the study of the micro-organisms (64, 65, 48, 66, 67, 68, 69) than to the root/micro-organism interactions (70, 49, 71).

In this paper, we would like to illustrate some problems and solutions met by our group while working in the field of AM proteomics.

We have recently begun to investigate the symbiotic proteome of *M. truncatula*. Plants of the genotype Jemalong J5 were grown in conventional plastic pots and inoculated with the AM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG 12). Differentially displayed proteins in response to mycorrhizal colonization were followed during a time course experiment, in *M. truncatula* roots using analytical silver stained 2D gels (71). In response to AM symbiosis, 12 polypeptides were found up-regulated, 2 were down-regulated and 41 polypeptides were newly induced. Some were only transiently induced while others were present for a longer time. As a preliminary step, we focussed on two newly AM-induced polypeptides to begin mass spectrometry analyses. Following excision from micro-preparative Coomassie blue-stained 2-DE, the spots were first submitted to MALDI-TOF MS. Mass spectra allowed the determination of peptide masses for each polypeptide. However, when these data were used to search in SwissProt and TrEMBL databases, together with the molecular weights and pIs determined from 2D gels, we found uncertain results with a low protein coverage. Indeed, *M. truncatula* and *G. mosseae* sequences are very scarce in databases, and it has recently been reported that there are only very low levels of microsynteny between A. thaliana and *M. truncatula* (72). These two polypeptides were then analysed by tandem MS with a Q-TOF mass spectrometer allowing to obtain some peptide sequences. Following the search in *M. truncatula* EST databases (74), both were found to correspond to ESTs present in the mycorrhiza database (73). These preliminary results clearly show that sequence tagging strongly permits to refine ambiguous identifications.

4. Further prospects for studying the AM symbiotic proteome

In our preliminary study, 41 polypeptides were found newly induced in soluble extracts of *G. mosseae*-colonized roots. This number is significantly increased when compared to our previous reports for other plant species in interaction with the same strain of AM fungus (40, 36). This has to be related to the better ability of separation and higher resolution on immobilized pH gradients (IPG). Moreover, *M. truncatula*, because of its small genome, appears particularly suitable for protein analysis. Nevertheless, new polypeptides related to AM symbiosis are certainly still highly underestimated.

Sub-fractionation into soluble and membrane proteins has already been proved to be very useful for identifying more polypeptide modifications in response to AM symbiosis (41, 42). Proteomic approach appears particularly relevant for studying proteins expressed in special organelles such as the symbiosome in the rhizobial interaction (75). Although the AM symbiosome is much less accessible than that of nodules, further developments combining enrichments in arbuscule-containing cells and optimization of solubilization of membrane proteins (76, 77) could bring more insight into the identification of specific proteins. Another improvement could be achieved by using narrow pH gradients for the first dimension, allowing to construct composite giant 2-DE maps (78). Finally, the use of highest sensitive stains compatible with MS analysis (79) would also improve the capacity for proteomic identification in AM symbiosis.

Nevertheless, even if now we may take benefit from all these technical improvements in both 2-DE and MS analyses, we still have to improve the selection of the biological material

in itself. Because most of the studies dealing with protein identification in AM symbiosis were carried out up to now on plants grown in conventional pot experiments, it cannot be ruled out that some of the observed modifications could be due either to associated microflora or even to secondary contamination. Therefore, AM proteomics should also be undertaken on axenically produced biological material. An interesting alternative of *in vitro* inoculated plants is the possible use of mycorrhizal Ri T-DNA roots, the interest of which has recently been highlighted in several physiological studies (39, 38, 80). Efforts have to be made in order to focus on more precise stages of the symbiosis. This plant/fungus interaction is typically characterized by the asynchronous occurrence of all stages of the symbiosis, making difficult to identify protein expression patterns specific to any of the different developmental steps. For the moment, at least two stages can be more accurately studied : (1) the full symbiosis with numerous arbuscules, (2) the recognition step, with the appressoria formation, considered as the first cellular contact between the two partners (81, 82). It was already stressed above that obtaining subcellular fractions corresponding to arbuscule-containing cells will allow to identify proteins expressed in and around the arbuscules. Another strategy relates to the use of plant mutants deficient for the AM symbiosis (83, 11, 84). This has already been successfully applied both at the protein (40) and gene levels (19). A proteomic analysis of the differentially displayed proteins in early plant mutants inoculated or not with an AM fungus could help in the identification of proteins involved in the appressoria formation. Similarly, studies of differential protein expression following AM fungal inoculation of late mutants, in comparison to wild type genotypes, could strongly support the identification of proteins essential to the AM symbiosis.

5. Conclusions

Despite the ubiquitous occurrence of AM symbiosis, and its importance in the growth and survival of many plant species in both natural habitats and in agroecosystems, our understanding of the molecular mechanisms governing its development and functioning was, until recently, very limited. Over the past few years, the first insights into these mechanisms have been achieved (11, 12). The recent explosion in the development of genetics and genomics tools for the model plant *M. truncatula* (72) has also opened a door to scientists involved in AM symbiosis studies. In complement to transcriptome analyses, which have begun in the frame of several national and international projects, proteomics has to be expanded. Indeed, the use of 2-DE technology together with mass spectrometry identification renders possible the identification and isolation of the genes of an organism via the gene product approach. This is the ideal way of linking molecular genetics with physiology and will be of great interest for researches in the field of plant-microbe interactions. This strategy clearly appears of major interest in AM symbioses, for which only a very limited number of proteins were up to now identified, with tedious and time-consuming purification processes and conventional identification methods (42, 85, 21). In addition, proteomics of the model plant *M. truncatula* would also be suitable for studying at the protein level any responses to environmental conditions such as phosphorus and/or nitrate supplies, dryness etc. This strategy could also be targeted to environmental aspects with the aim of identifying symbiosis-related proteins involved in mycorrhiza stress responses to heavy metals or any other pollutants.

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