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REVIEW

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Proteomics as a tool to monitor plant-microbe endosymbioses in the rhizosphere

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Abstract In recent years, outstanding molecular approaches have been used to investigate genes and functions involved in plant-microbe endosymbioses. In this review, we outline the use of proteomic analysis, based on two-dimensional electrophoresis and mass spectrometry, to characterize symbiosis-related proteins. During the last decade, proteomics succeeded in identifying about 400 proteins associated with the development and functioning of both mycorrhizal and rhizobial symbioses. Further progress in prefractionation procedures is expected to allow the detection of symbiotic proteins showing low abundance or being present in certain cell compartments.

Keywords Mycorrhiza · Nitrogen-fixing symbiosis · Proteins · Two-dimensional electrophoresis · Mass spectrometry

Introduction

In recent years, outstanding molecular approaches have been used for the identification of genes and functions involved in plant-microbe endosymbioses. Following the first completion of genome sequencing projects, biological research has developed high throughput genetic programs with multiparallel analyses of gene transcripts, proteins and metabolites, that are now tentatively transposed to the world of mycorrhizal and rhizobial symbioses (Franken and Requena 2001; Colebatch et al. 2002a, 2002b). Major advances have been realised thanks to the use of plant mutants, isolated following ethyl methane sulfonate treatment, gamma-ray irradiation, insertion mutagenesis, or screening for natural variants (Sagan et al. 1995; Borisov et al. 1999; Penmetsa and Cook 2000). The mutants studied are defective in defined steps of the symbiosis, helping to dissect the sequence of events leading to a mycorrhiza or a nodule (Catoira et al. 2000; Walker et al. 2000; Marsh and Schultze 2001). Interestingly, common steps in the early symbiotic pathways were deduced from the analysis of mycorrhizal and rhizobial mutants (Marsh and Schultze 2001). A common feature of mutants sharing this pathway is the lack of extensive root hair deformation; however, genes need yet to be assigned to these genetic loci, in order to get an insight into the precise functions involved. Recently, the molecular genetic linkage map of *Medicago truncatula* was published (Thoquet et al. 2002). Based on large-insert bacterial artificial chromosome libraries, map-based cloning has lead to the isolation of two candidate genes encoding for a basic leucine zipper-type zinc-finger protein and a receptor kinase with a leucine-rich repeat region in the putative external domain (Stougaard 2001).

Genomic approaches have mainly been initiated in *Lotus japonicus* (Cyranoski 2001) and *M. truncatula* (Journet et al. 2001, 2002; Frugoli and Harris 2001; Oldroyd and Geurts 2001). These model legume systems have received increasing attention during recent years, due to their simple diploid genome, short life cycle, ease of transformation and regeneration (Barker et al. 1990; Handberg and Stougaard 1992). However, by comparison to *Arabidopsis thaliana*, *L. japonicus* and *M. truncatula* bear relatively large genomes (~500 Mb). Although an international genome sequencing project has recently been launched for *M. truncatula* (http://www.noble.org/press_release/medicago/NewsConference2001/Medicago Sequencing Project.htm) available genomic information currently lies essentially under the form of expressed sequence tags (ESTs) (Kawasaki and Murakami 2000; Bell et al. 2001). A regularly updated database gathering

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more than 150,000 *M. truncatula* ESTs, representing roughly 29,000 clustered sequences, is held by the Institute for Genomic Research, Rockville, Maryland (Quackenbush et al. 2000). In France, a joint program between the Genoscope (Evry), the CNRS-INRA LBM-RPM (Toulouse) and INRA-Université de Bourgogne BBCE-IPM (Dijon) laboratories, produced 24,000 ESTs from control plants, nodules and mycorrhizas, leading to about 6,400 distinct genes (Journet et al. 2002). Concerning the microsymbionts, complete genome sequencing was achieved for *Mezorhizobium loti* (Kaneko et al. 2000) and *Sinorhizobium meliloti* (Galibert et al. 2001). In addition, the 536-kb symbiotic plasmid of *Rhizobium* sp. NGR234 (Freiberg 1997) and a 410-kb region of the chromosome of *Bradyrhizobium japonicum* (Gottfert et al. 2001) were sequenced and annotated. Arbuscular mycorrhizal fungi bear large and highly repeated genomes, a feature that completely hinders the development of genomic analyses. Due to their obligate symbiotic status, the amount of accessible mRNA material is very limited; however, many limitations have been overcome with the help of polymerase chain reaction (PCR)-based methods (Franken and Requena 2001) and in vitro monoxenic cultures (St-Arnaud et al. 1996). Several EST libraries have thus been constructed using activated spores of *Gigaspora rosea* (Stommel et al. 2001; Tamasloukht et al. 2003), *Glomus mosseae* (Requena et al. 2002), presymbiotic mycelium of *Gigaspora margarita* (Lanfranco et al. 2000) or *Glomus inraradices* (Lammers et al. 2001) and extraradical hyphae of *Glomus intraradices* (Sawaki and Saito 2001). Besides bioinformatic annotation, functions now need to be assigned to these sequences. *In silico* approaches can identify candidate differentially expressed genes; however, these must be confirmed by quantifying their expression at the molecular level through transcriptomics. In parallel, proteomics will allow one to quantify the expression of the gene products.

Studies of gene expression at the transcript level were initially led by differential screening methods. Differential display reverse transcription PCR (Liang and Pardee 1992), differential screening of cDNA libraries and suppressive subtractive hybridization helped to identify genes involved both in mycorrhizal (Tahiri-Alaoui and Antoniw 1996; Martin-Laurent et al. 1997; Krajinski et al. 1998; Lapopin et al. 1999; van Buuren et al. 1999, 2000; Delp et al. 2000; Roussel et al. 2001; Requena et al. 2002; Brechenmacher et al. 2003; Tamasloukht et al. 2003; Wulf et al. 2003) and rhizobial (Gamas et al. 1996; Lievens et al. 2001) symbioses. With the increasing number of available EST sequences, the RNA accumulation of numerous genes will now be possible through the use of cDNA arrays (Duggan et al. 1999). In particular, micro- and macro- arrays will be soon available to the *M. truncatula* European scientific community (Journet et al. 2002).

Proteomics: why and how?

Knowledge of where and when proteins are expressed is essential for understanding biological processes. Indeed, RNA and protein amounts might not always correlate as demonstrated by analyses in yeast (Gygi et al. 1999b; Santucci et al. 2000) and mammalian cells (Gerner et al. 2000). Post-transcriptional mechanisms include control of translation rate and protein turnover (Pradet-Balade et al. 2001). Most often, proteins may also be regulated by post-translational modifications, more than 200 different types of which have been reported, including glycosylation, phosphorylation, ribosylation, palmitoylation or sulphation (Gooley and Packer 1997). Originally coined in 1995 by Marc Wilkins, the term "proteome" describes the "protein complement of the genome" (Wilkins et al. 1995). Proteomics is thus the large-scale analysis of proteins. Traditionally, this technology is based on the combination of two-dimensional electrophoresis (2DE), allowing the separation of denatured protein polypeptides according to their isoelectric points and molecular weights, and mass spectrometry identification methods, either by peptide mass fingerprinting or de novo sequencing.

Protein extraction process and 2DE

Sample preparation is the most critical step in 2DE since a proteome consists of many proteins differing according to their tissue localization, abundance, sub-localization and chemical properties including solubility. Many sample preparation methods have therefore been developed (Cordwell et al. 2000, Görg et al. 2000). Interestingly, in the case of endomycorrhizal symbiosis, more recent methodological improvements were aimed at carrying out proteomic and transcriptomic approaches on the same biological material (Dumas-Gaudot et al. 2003a).

Since it was first introduced in 1975 (O'Farrell 1975), 2DE has evolved at different levels. The isoelectrofocusing first dimension which was originally based on carrier ampholyte gradients, is now based on immobilized pH gradients (IPGs; Bjellqvist et al. 1982) allowing higher resolution and reproducibility, as well as higher loading capacities, an asset for the realization of micropreparative gels (Bjellqvist et al. 1993; Blomberg et al. 1995; Görg et al. 1998). The principle of IPG technology lies in the use of immobilines, acrylamide derivatives which are copolymerized within the gel matrix, overcoming the hindrance of gradient instability. When immobilines carrying groups with varying pKs are mixed in various proportions, any pH scale can be formed, from wide (Görg and Weiss 1998) to ultra narrow, allowing one to focus on specific isoelectric points while keeping a high resolution (Wildgruber et al. 2000; Tonella et al. 2001). The second dimension is based on the discontinuous sodium dodecyl sulphate gel system described by Laemmli (1970). The polyacrylamide percentage is generally fixed but may also be a gradient in order to gain resolution in the display

of polypeptides (Görg and Weiss 1999). Additionally, the TRIS-tricine method designed by Schagger and von Jagow (1987) may be employed to focus on low molecular weight polypeptides (Jan et al. 2001). With these improvements in the resolution power of 2DE, there has been increasing interest in the preparation of the protein sample. Prefractionation methods such as affinity chromatography (Geng et al. 2001), chromatofocusing (Herbert and Righetti 2000) or subcellular fractionation (van Wijk 2000) aim at enriching protein fractions, thereby increasing the number of proteins resolved. Moreover, solubilization methods have improved due to the use of novel detergents such as sulphobetaines (Rabilloud et al. 1997) helping in particular to separate and display membrane proteins (Molloy et al. 1998; Santoni et al. 2000). After separation, proteins can be detected either by autoradiography for labelled proteins, or after Western blotting, by staining the membrane or incubating it with specific antibodies, or directly, by gel staining. Among gel staining methods, silver staining is most traditionally employed due to its high sensitivity (Rabilloud 1999); however, colloidal Coomassie blue is preferred when subsequent mass spectrometry analyses are needed (Neuhoff et al. 1988). Recently, fluorescent Sypro dyes have emerged as the ideal alternative, showing high sensitivity and linearity over 5 orders of magnitude, and minimal background in mass spectrometry (Patton 2000). After digitalization, gels are analysed by computer-assisted image analysis, allowing the automatic detection of protein spots, quantification and comparison between different gel images (Jia et al. 2001).

Mass spectrometry identification methods

After proteins of interest have been mapped, micropreparative gels are realised (Bjellqvist et al. 1993). The protein spots are excised and digested with an endoprotease, generally trypsin, directly within the gel matrix (Rosenfeld et al. 1992). Peptides are then analysed by mass spectrometry (MS), allowing one to obtain either a peptide mass fingerprint of the protein, or internal amino acid sequences (Corthals et al. 2000). Peptide mass fingerprinting (PMF) is based on the computer-driven matching between experimentally obtained masses of peptides, and *in silico* calculated masses of protein sequences deposited in databases (Mann et al. 1993). This approach is ideally suited to genetically well-characterized organisms, for which the entire genome has been sequenced such as, for example, *Arabidopsis thaliana* or *Oryza sativa* (Millar et al. 2001; Fukuda et al. 2003), or for which numerous ESTs are available, provided those are long enough. However, it is not adapted to cross-species identification, although specific softwares have been designed to overcome this problem (Wilkins and Gooley 1998). Sequencing peptides is possible by mass spectrometry, through the generation of peptide ladders, in which individual peptides differ in length by one amino acid. These peptides are generated by fragmentation according to a model defined by Biemann (1990). Fragmentation of the peptide bonds will produce *a*, *b* , and *c* series ions when the positive charge remains at the peptide N-terminus, and *x*, *y* , and *z* series ions when the charge remains at the peptide C-terminus. Mass spectrometers generally consist of three components: a ionization source, a mass analyser, and a detector, among which various combinations can be made. MS analyses of proteins and peptides take advantage of two "soft" ionization methods: matrix-assisted laser desorption ionization (MALDI; Karas and Hillenkamp 1988) and electrospray ionization (ESI; Fenn et al. 1989). In MALDI, peptides are first crystallized with a small molecular aromatic "matrix" on a metallic slide, which is then inserted into the apparatus. Under high vacuum and voltage conditions, a laser beam is directed onto the slide, resulting in the desorption and sublimation of the matrix crystals. ESI consists in spraying the peptides under atmospheric pressure and high voltage, from the tip of a fine capillary. As the highly charged droplets evaporate, peptide ions with one or more charged protons are ejected into the gas phase. Three mass analysers are generally employed to characterize the ionized peptides: ion trap (IT), triple quadrupole (Q3) and time-of-flight (TOF). Hence, MALDI-TOF, ESI-Q3 and ESI-IT are the mass spectrometers most commonly used for protein and peptide analyses. However the introduction of "hybrid" instruments such as ESI-Q-TOF and more recently, MALDI-Q-TOF has revolutionized the technology (Shevchenko et al. 2000). MALDI-TOF is the simplest mass spectrometer both conceptually and in design. The mass over charge (m/z) ratios of ionized peptides are measured with high accuracy and automatization allows one to analyse hundreds of samples per day. This instrument is traditionally used to generate peptide mass fingerprints; however, sequence information may be obtained by post-source decay, a process in which ions acquire excess energy causing their fragmentation (Kaufmann et al. 1994). ESI-Q3 are extremely versatile instruments based on a first quadrupole mass filter, allowing the selection of ions with a certain m/z ratio, while a second quadrupole is used for collision induced dissociation (CID), and a third to record the m/z of the fragment ions. In ESI-IT, peptide ions are trapped by a radio-frequency field in the quadrupole ion trap, and by applying a small voltage, become unstable, are ejected and detected. This feature also allows CID and MS^n experiments, helping in the detailed structural analysis of components, such as glycopeptides. The ESI-Q-TOF is a hybrid mass spectrometer showing enhanced resolution by comparison to the other ESI-based instruments, and intermediary sensibility between those and MALDI-TOFs. The presence of a collision cell allows CID fragmentation and subsequent sequence analysis of peptides. Two types of MS data are thus generated for protein identification by correlation with sequence databases: accurate peptide masses or/and fragmentation spectra. Several software tools, based on different algorithms, are available on the web for comparing these experimental

Table 1 On-line protein identification algorithms for mass spectrometric data. *PMF* Peptide mass fingerprinting, *FIS* fragment-ion search

Algorithm	URL	Identification mode
PeptIdent	http://us.expasy.org/tools/peptident.html	PMF
Mascot	http://www.matrixscience.com/	PMF, FIS
MS-Fit	http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm	PMF
MS-Tag	http://prospector.ucsf.edu/ucsfhtml4.0/mstagfd.htm	FIS
PepFrag	http://prowl.rockefeller.edu/PROWL/pepfragch.html	FIS
Profound	http://129.85.(19.(192/profound_bin/WebProFound.exe	PMF
PepSea	http://(195.41.108.38/PepSeaIntro.html	PMF
Lutefisk	http://www.immunex.com/researcher/lutefisk/	De novo sequencing

Table 2 Proteomic studies in the field of plant-microbe symbioses

Biological models	Proteins characterized by 2 DE	Identified proteins	References
Ectomycorrhizal symbiosis			
Eucalyptus globulus / *Pisolithus tinctorius*	15	0	Hilbert et al. (1999)
Betula pendula / *Paxillus involutus*	35	0	Simoneau et al. (1993)
Eucalyptus grandis / *Pisolithus tinctorius*	26	0	Burgess et al. (1995
E. grandis / *P. tinctorius*	26	0	Burgess and Dell (1996)
Pinus sylvestris / *Suillus bovinus*	9	9	Tarkka et al. (2000)
Tuber borchii	23	5	Vallorani et al. (2000)
Endomycorrhizal symbiosis			
Allium cepa / *Glomus mosseae*	15	0	Garcia-Garrido et al. (1993)
Nicotiana tabacum / *G. mosseae* / *Glomus intraradices*	34	0	Dumas-Gaudot et al. (1994)
Lycopersicon esculentum / *G. intraradices*	5	0	Simoneau et al. (1994)
Pisum sativum / *G. mosseae*	42	0	Samra et al. (1997)
L. esculentum / *G. mosseae*	44	0	Benabdellah et al. (1998)
L. esculentum / *G. mosseae*	14	0	Dassi et al. (1999)
L. esculentum / *G. mosseae*	26	1	Benabdellah et al. (2000)
Triticum aestivum / *G. mosseae*	1	1	Fester et al. (2003)
Medicago truncatula / *G. mosseae*	55	8	Bestel-Corre et al. (2002)
P. sativum / *G. mosseae*	7	6	Repetto et al. (2003)
M. truncatula / *G. mosseae*	34	6	Bestel-Corre (2002)
Ri T-DNA *Daucus carota* / *G. intraradices*	8	2	Bestel-Corre (2002)
G. intraradices	450	6	Dumas-Gaudot et al. (2003b)
Acaulospora laevis / *Gigaspora rosea* / *Scutellospora castanea* / *G. mosseae*	12	0	Samra et al. (1996)
Rhizobial symbiosis			
Vigna unguiculata / *Rhizobium* sp. *NGR234* / *R. fredii* / *Sinorhizobium meliloti*	16	0	Krause and Broughton (1992
Glycine max / *Bradyrhizobium japonicum*	12	1	Winzer et al. (1999)
G. max / *B. japonicum*	17	17	Panter et al. (2000)
Melilotus alba / *S. meliloti*	600	100	Natera et al. (2000)
Trifolium subterraneum / *Rhizobium leguminosarum*	16	10	Morris and Djordjevic (2001
R. leguminosarum	4	12	Guerreiro et al. (1997)
R. leguminosarum	22	5	Guerreiro et al. (1998)
S. meliloti	52	23	Guerreiro et al. (1999)
B. japonicum	19	15	Munchbach et al. (1999)
B. japonicum	32	28	Dainese-Hatt et al. (1999)
S. meliloti	189	52	Chen et al. (2000a)
S. meliloti	60	11	Chen et al. (2000b)
M. truncatula / *S. meliloti*	51	7	Bestel-Corre et al. (2002)
M. truncatula / *S. meliloti*	41	11	Bestel-Corre (2002)
S. meliloti	41	41	Bestel-Corre (2002)

results to theoretical mass data derived from sequence databases (Fenyo 2000, Table 1). When the protein sequence is represented in a database, the identity can thus be rapidly established. Conversely, de novo sequencing has to be performed by interpreting fragmentation data, which is slower and requires more operator input (Corthals et al. 2000).

Proteomics of plant-microbe symbiotic interactions

Although 2DE was used quite early to study mycorrhizal and rhizobial symbioses, very few proteins were identified, due to the limitation of both electrophoretic and identification methods (Table 2). Concerning mycorrhizas, pioneering studies were achieved with ectomycorrhizas in the early 1990s (Hilbert et al. 1991; Simoneau

et al. 1993; Burgess et al. 1995; Burgess and Dell 1996) allowing researchers to detect symbiosis-related (SR) polypeptides, up-regulated or newly induced in mycorrhizal roots, as well as down-regulated polypeptides, by comparison to control roots and mycelium extracts. Only very recently were some SR proteins identified by mass spectrometry and N-terminal sequencing (Tarkka et al. 2000), as well as mycelial proteins (Vallorani et al. 2000). A large-scale proteomic project is now an ambition in order to match these data with EST data obtained for *Pinus sylvestris* (Tagu, personal communication). For arbuscular mycorrhizas, a similar progression was followed, from descriptive studies (Garcia-Garrido et al. 1993; Dumas-Gaudot et al. 1994; Simoneau et al. 1994; Samra et al. 1997; Benabdellah et al. 1998; Dassi et al. 1999) to the first identifications of a vacuolar H^+-ATPase and a Myk15 protein with an unknown function (Benabdellah et al. 2000; Fester et al. 2003). Additionally, protein profiles of both dormant and germinated spores of several fungi were compared (Samra et al. 1996). Now, proteome analysis is used as a powerful tool to reveal more and more proteins involved in mycorrhiza development and functioning including proteins involved in defence response, root physiology and the respiratory pathway (Dumas-Gaudot et al. 2001; Bestel-Corre et al 2002). Proteomics of the rhizobial symbiosis also started a decade ago (Krause and Broughton 1992), and there was renewed interest in the technique several years after, with studies focussing either on nodule proteins (Winzer et al. 1999; Panter et al. 2000; Natera et al. 2000; Morris and Djordjevic 2001) or on the isolated bacteria (Guerreiro et al. 1997, 1998, 1999; Munchbach et al. 1999; Dainese-Hatt et al. 1999; Chen et al. 2000a, 2000b). Lately, a proteome reference map of *M. truncatula*, the model system used to study root symbioses, was established (Mathesius et al. 2001). In our laboratory, several proteomic studies were completed recently, focussing on mycorrhizal and rhizobial symbioses, challenged with different pollutants. Concerning mycorrhization, 73 newly induced protein spots were detected in *M. truncatula* - *G. mosseae* and *Ri T-DNA Daucus carota* - *G. intraradices* interactions, among which 16 proteins were analysed, with homologies found for 14 of them. Besides proteins belonging to previously reported categories (Bestel-Corre et al. 2002), proteins involved in signalisation and gene regulation processes were also identified (Bestel-Corre 2002). Additionally, putative amino acid sequences were obtained for nine proteins of *G. intraradices* extraradical mycelium, among which homologies were found for six proteins including enzymes from central metabolism (Dumas-Gaudot et al. 2003b). For the first time, a systematic proteome analysis of mycorrhization has been initiated, opening up the possibility of directly identifying the functions taking place in this symbiotic process. Concerning nodulation, 74 newly induced proteins were detected in the *M. truncatula* - *S. meliloti* interaction, among which 16 proteins were analysed and identified (Bestel-Corre 2002). Proteomics also helped to study the impact of sewage sludges polluted with heavy metals or polycyclic aromatic hydrocarbons, on mycorrhization and nodulation, both on the interactions, and on the isolated microsymbionts. Although a control sludge showed positive effects towards *M. truncatula* plants non-inoculated or inoculated with *G. mosseae* or *S. meliloti*, the polluted sludges exhibited clear negative effects on plant growth and root symbioses. A clear correlation was established between some symbiosis-related proteins and the levels of mycorrhization and nodulation, revealing a potential use of this technology for environmental studies (Bestel-Corre 2002). Sewage sludge-related proteins were also identified in mycorrhized or nodulated *M. truncatula* roots (Bestel-Corre 2002), and in cultured *S. meliloti* cells (Bestel-Corre 2002), thus giving some supplementary information when these data were compared to physiological data. Similarly, variations in the pea (*Pisum sativum* L.) root proteome were identified in response to cadmium stress, during the symbiotic interaction with *G. mosseae* (Repetto et al. 2003). This targeted proteomic approach enabled workers to reveal two cadmium-induced proteins (a short-chain alcohol dehydrogenase and an UTP-1-phosphate uridylyltransferase) which were mycorrhiza-regulated.

Future prospects

In the near future, further protein identifications will be possible in the field of plant-microbe endosymbioses. First, attempts should be made to identify those proteins which were detected in the early steps of the symbioses. To this end, considerable information may be gained by studying the proteome of symbiosis-defective mutants (Marsh and Schultze 2001). The use of Ri T-DNA transformed roots may help researchers to collect material at the initial stages of the symbiosis, and this may be realised with mycorrhizal carrot roots (Fortin et al. 2002) or with *M. truncatula* -transformed roots, inoculated with one or the other symbiont (Boisson-Dernier et al. 2001). In particular, a method for isolating *Colletotrichum* appressoria was published, which may be applied to arbuscular mycorrhizal fungi (Hutchison et al. 2000). Then, subcellular fractionation could be used to enrich root extracts with specific proteins. This has already been used successfully for a few cases in endomycorrhizal and rhizobial symbioses, respectively (Benabdellah et al. 2000; Winzer et al. 1999; Panter et al. 2000). In particular, membrane proteins may be further addressed by taking advantage of the more recent significant advances that have been achieved concerning their extraction (Seigneurin-Berny et al. 1999; Ferro et al. 2000) and solubilization (Rabilloud 1998; Chevallet et al. 1998; Molloy 2000). More precisely, plasma membrane proteins may be purified after aqueous two-phase partitioning of the microsomal fraction (Santoni et al. 1998) and special attention may be paid to the perisymbiotic membrane, surrounding the arbuscules or the bacteroids. This approach is being developed in soybean and *Lotus*

japonicus nodules (Wienkoop and Saalbach 2003) and might similarly be applied to arbuscule-containing cells. Indeed, a technique has been published, which allows one to obtain root cell fractions enriched with arbuscule-containing cells (Fester et al. 1999). Likewise, intraradical hyphae may be isolated (Saito 1995) in order to focus on fungal proteins; however, considerable amounts of material should be collected to achieve this aim, representing a long and tedious task. Then, improvements can be applied to the 2DE technique, by fractionating protein samples into narrower pH ranges (subproteomics). This can be achieved with narrow-range first-dimension IPG gels (Wildgruber et al. 2000; Tonella et al. 2001) and ideally, protein samples should be prefractionated with a chromatofocusing device such as the multicompartment electrolyzer (Herbert and Righetti 2000). In order to focus on specific proteins, prepurification may also be realised, for instance by chitin affinity chromatography to analyse chitin-binding proteins (Slezack et al. 2001). Supplementary information may also be obtained by specifically focussing on phosphorylated or glycosylated proteins. Indeed, phosphoproteome analysis has previously been performed to study plant responses to bacterial and fungal elicitors (Lecourieux-Ouaked et al. 2000; Peck et al. 2001) and glycoproteins can also be easily visualized after 2DE (Packer et al. 1998). Finally, more refined proteomic tools have recently emerged, that could be applied to the study of symbioses. The fluorescence-based differential in gel electrophoresis technique allows the differential analysis of two samples concurrently run within the same gel, circumventing reproducibility problems and image analysis (Unlu et al. 1997). More revolutionary are the multidimensional protein identification (Washburn et al. 2001) and isotope-coded affinity tag (Gygi et al. 1999a) methods, which by-pass 2DE by directly analysing protein mixtures by mass spectrometry.

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