Protein Identification and Quantification by Mass Spectrometry-Based Analysis: Applications in Plant-Pathogen Interactions Studies

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Abstract: Mass spectrometry (MS) has become an essential technology for proteomics applications in biological sciences. Advances in this technique have been possible owing to improvements in MS instrumentation, new experimental strategies in sample preparation, and development of bioinformatics tools for data analyses. In recent years, complementary strategies to the classical two-dimensional gel electrophoresis approaches (2-DE) have been developed. These techniques are based on multidimensional peptide separation coupled to tandem MS (also referred as "second generation proteomics"), enabling protein expression analysis and high throughput protein identification studies. New methods such as Multidimensional Protein Identification Technology (MudPIT) and stable isotope labeling of protein/peptide samples (either by chemical, metabolic, or enzymatic methods), among others, are powerful tools for large-scale studies on characterization and expression of proteins in complex biological systems. Hence, these techniques can be very useful in the study of plant-pathogen interactions, aiding to detect and characterize both plant proteins concerned in defense reactions and pathogen proteins involved in pathogenicity and/or virulence. But these techniques have been implemented in these biological systems just recently. We will examine here how MS-based proteomics approaches are helping to better understand the multifaceted phenomena underlying plant-pathogen interactions.

Keywords: Mass spectrometry, proteomics, plant-pathogen interaction, protein identification, protein quantification.

INTRODUCTION

Remarkable technological advances in biological sciences over the past few years have forged a new era of research, even contributing to the appearance of emerging disciplines such as systems biology. Although the understanding of living organisms at the molecular level is still in its infancy, it is evident that comprehensive investigations of the "omics cascade", with genomics, transcriptomics, proteomics, and metabolomics as building blocks, will play a pivotal role in current biological scientific investigations [1].

Proteomics is an essential discipline in protein characterization research. Profiling whole proteins, identifying their modifications and interactions, and providing information on their abundance are crucial for a comprehensive understanding of cell functions and the molecular mechanisms underlying biological process [2]. Advances in this field have been possible due to improvements in mass spectrometry (MS) instrumentation, experimental strategies, and bioinformatics tools. MS enables protein identification and quantification at a large scale, and hence has become a powerful tool in protein analysis and the key technology in proteomics. For instance, the study of protein-protein interactions *via* affinity-based isolations at both small and proteome-wide scales, the mapping of numerous organelles and cell proteomes, and the generation of quantitative protein profiles from diverse species have been greatly benefited from MS-based proteomics [3]. Numerous and excellent reviews compiling general concepts on MS-based methodologies are available [3-6], and will not therefore detailed here.

Proteomics are generally classified into: 1) "classical proteomics", for individual protein identification which mainly utilizes 2-DE and MS; and 2) "second generation proteomics" or "MS-based proteomics", for the characterization of whole proteomes and their functions. 2-DE produces maps of proteins, separated by pI and molecular weight in polyacrylamide gels, that can be compared (classical quantitative proteomics). The identification of the proteins detected in these maps is carried out by excising the spot of interest, and protein digesting with a suitable enzyme. Finally, resulting peptides are then used for protein identification by MS. Although 2-DE is a valuable and broadly-used approach, it has some limitations. Besides occasional problems of reproducibility, it poses serious restrictions for the analysis of membrane proteins, large proteins or proteins with extreme pI´s. However, the major drawback is its limited dynamic range which hinders the identification of proteins when their relative concentration in a sample is low. [7]. It should also be noted that this approach is not suitable for the systematic identification of posttranslational modifications at exact sites [8]. MS-based proteomics are based in the digestion of protein extracts in solution without previous separation. Complex samples of peptides are further fractionated using multidimensional chromatography coupled to automatic high-throughput peptide identification by MS. The final step is the analysis by bioinformatics tools using protein/DNA se-

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quences databases [9]. Because of this methodological scheme, the term "peptide level proteomics" has been proposed for these techniques [10]. The development of these high-performance strategies over the past decade has been tremendous. Indeed, just ten years ago, the sequencing of a protein was an outstanding achievement, whereas the identification of hundreds of proteins in a single experiment has nowadays become a routine event [11].

Plant pathogens, their hosts and the interactions between them have been extensively studied using classical biochemical, genetic, molecular biological and plant pathology approaches [12]. However, systems biology approaches such as genomics and proteomics can provide global information about genes and proteins networks which are crucial to understand molecular processes taking place during a given plant-pathogen interaction.

Plants are endowed with diverse and sophisticated mechanisms to protect themselves against pathogens attacks. Whereas some of these defence mechanisms are innate and provide physical and chemical barriers hampering pathogen infection, others are only induced upon pathogen recognition. Pathogen-induced defences include, for instance, the so-called gene-for-gene resistance (also known as vertical, monogenic, race-specific resistance or effector-triggered immunity). This defence response is triggered after a plant resistance gene (R-gene) product recognizes a specific gene product (effector) of a given microbial pathogen. This recognition unleashes a hypersensitive response (HR) in the host plant leading to a (nearly) complete restriction of the pathogen's spread to the original sites of infection [13]. In addition to R-gene-mediated defence response, plants rely on recognition of general elicitors, also called pathogen-associated molecular patterns (PAMPS), to detect potential pathogens and to activate a basal defence response (also known as broad-spectrum, horizontal, polygenic or general resistance) that confers effective resistance to the invasion of the majority of pathogens [13]. Despite plants have a wide battery of defence mechanisms, phytopathogens are also able to deploy a broad range of weapons to interfere or suppress such defences. These arms enable them to colonize plant tissues, to break the host resistance barriers, and to induce a disease state in the infected plant [2].

Because proteins are involved in multiple and critical processes during plant-pathogen interactions, proteomics is an essential tool for an in-depth understanding of both defence mechanisms of plants and pathogenicity/virulence strategies employed by pathogens. 2-DE coupled with MS is the most classical proteomics approach employed in plant-pathogen interactions studies. Indeed, it has been extensively used to characterize cellular and extracellular virulence and pathogenicity factors produced by diverse phytopathogens as well as to identify changes in protein levels in plants upon infection (reviewed by [2, 12, 14]). However, proteomics is a continuously-evolving discipline which constantly provides novel and improved techniques aiming to large-scale identification and characterization of proteins in complex biological systems. In this review, we will mainly focus on the recent "second generation proteomics" studies performed in order to better understand the molecular mechanisms governing different plant-pathogen interactions.

PROTEIN IDENTIFICATION METHODS AND THEIR LIMITATIONS IN PLANT-PATHOGEN INTERACTIONS STUDIES

As aforementioned, 2-DE and MS are the most widely-used analytical techniques for the profiling and identification of proteins in plant-pathogen interaction studies [12]. The fact that MS does not require pathogen- or plant-specific reagents (for instance, specific antibodies) to analyze specific peptides or proteins masses makes it an attractive tool for protein identification. MS-based proteomics approaches also help in acquiring details about the signalling cascades involved in plant-pathogen interactions. However, it is worth mentioning that *in planta* studies of pathogens can also pose difficulties in proteomics because of considerable biological variation, which may arise, for instance, from microenvironment differences, small genotypic variations within a heterozygous organism, and posttranslational modifications of proteins [15]. Furthermore, proteomics studies of plant-pathogen interactions face additional difficulties because of the intrinsic complexity of these interactions. Indeed, the intimate contact established between the two organisms makes it difficult to distinguish proteins that are differentially expressed by the plant in response to the pathogen from those ones expressed by the pathogen [14].

To overcome these difficulties simple study models can be used, focusing in the analysis of either the pathogen or the host plant proteins. For example, to analyze the rice (*Oryza sativa* L. cv. Jinheung) secretome during its interaction with the rice blast fungus (*Magnaporthe grisea*) and its glucose equivalent elicitor, Kim and co-workers [16] employed *in vitro* suspension-cultured rice cells co-cultivated with the incompatible race KJ401 of the pathogen and /or treated with 50 μg/ml (glucose equivalent) elicitor during 24 h. Through a comparative analysis employing 2-DE, 21 differential protein spots were identified and linked to *M. grisea* and/or elicitor treatments. Subsequently, MALDI-TOF-MS and μLC-ESI-MS/MS mass spectrometers were used to identify proteins of interest. Most of them, such as chitinases, an expansin, a 533-kDa secretory protein (DUF 26), and germins/oxalate oxidases were involved in plant defense. Moreover, changes in the secreted proteins abundance were further correlated at the transcriptional level in both suspension-cultured cells and *in planta* pathogen infection experiments by semi-quantitative RT-PCR analysis. This is the first secretome analysis providing clues to understand the rice defense response against *M. grisea* and its elicitor, and suggests that suspension-cultured cells are an excellent tool to investigate the secretome during plant-pathogen interactions [16].

A high-throughput MS/MS analysis was used to identify proteins secreted by the pathogenic fungus *Fusarium graminearum* during its *in vitro* growth in 13 different liquid media and when infecting wheat (*Triticum aestivum*) heads [17]. A comparative study was performed after collecting by centrifugation proteins secreted at *in vitro* growth and by vacuum filtration proteins secreted *in planta* conditions. As a result, 289 fungal proteins (229 *in vitro* and 120 *in planta*) were identified by LC-ESI-MS/MS, demonstrating that MS-based proteomics is an effective method to survey proteins secreted by a filamentous fungus under different growth

conditions. Interestingly, 49 proteins were exclusively found *in planta*. Thus, this work also demonstrated that fungal proteins can be identified by MS in the infected host, despite the wide dynamic range of protein abundance (i.e. a low ratio of fungal protein to plant biomass) found in this study model, a well-known limiting factor when carrying out analysis of whole proteomes.

Other example on the use of a simplified and easy-to-manipulate model system is the interesting study carried out to investigate the shift from a dormant to an infective phase in *Botrytis cinerea* [18]. In this work, a total of 126 secreted proteins were identified by growing the pathogen in liquid medium supplemented with highly-esterified pectin, partially-esterified pectin, or sucrose (used in the constitutive expression control) as sole carbon sources. In this case, authors used a simple shotgun proteomics approach based on: 1) separation of the secreted proteins in each growth condition by SDS-PAGE; 2) gel lines horizontally cut into 3 slices and in-gel digestion, and 3) analysis of the resulting peptides by LC-MS/MS. Changes in secreted proteins profiles were observed, indicating the adaptability of *B. cinerea* to different nutrient availability conditions. However, despite this model system aimed to simulate the interaction of the pathogen with the expected nutrient sources from the host (i.e. cell wall components), no major differences in protein secretion patterns were found regardless the fungus was grown in highly- or partially-esterified pectin-amended medium. Therefore, the activation of *B. cinerea* from the dormant state to the infective stage did not seem to solely depend on changes in the esterification degree of the pectin component of the host plant cell wall.

For all significant advances in MS and in protein/peptide fractionation technologies, large dynamic concentration range of proteins in biological extracts is the greatest obstacle for full proteome coverage. To reduce protein concentration range, innovations are then required to improve the performance of the currently-available separation procedures of complex peptides mixtures [19]. Combining multiple separation procedures, as fractionation of cellular compartments, protein isolation using affinity properties and protein/peptide separation with multidimensional liquid chromatography can increase the coverage of protein identification [20] and get a deeper insight into a proteome. MudPIT (Multidimensional Protein Identification Technology) is the most widely-used technique, based on protein digestion in solution and subsequent peptide mixture separation by HPLC with SCX columns. Fractions collected from SCX are then analyzed by RP-HPLC coupled on-line to a mass spectrometer [21-23]. Kaschani and co-workers [24] have recently employed two distinct approaches to examine a particular set of proteins in the study model *Botrytis cinerea*/*Arabidopsis thaliana*. In particular, they focused on activities of serine hydrolases in *B. cinerea*-infected *A. thaliana* plants by activity-based protein profiling with fluorophosphonate (FP)-based probes. On the one hand, these proteins were subjected to on-bead digestion and MudPIT analysis, and on the other hand they were separated by SDS-PAGE and then subjected to in-gel digestion followed by MS/MS analysis. In both approaches LC-ESI-MS/MS was used for protein identification. In total, they could identify over 50 *A. thaliana* and *B. cinerea* serine hydrolases in the (infected) leaf proteome.

Another strategy to increase the dynamic range of protein concentration is to include a cellular fractionation step previous to MS analysis. By doing so, Lee and co-workers [25] monitored changes in the bean (*Phaseolus vulgaris* cv. Early Gallatin) leaf proteome in a time-course infection bioassay with the rust fungus *Uromyces appendiculatus*. In this study, *P. vulgaris* leaves were inoculated with a liquid suspension of either an avirulent (49) or a virulent (41) race of *U. appendiculatus*. Proteins from crude cell wall, organelle/membrane, and soluble subcellular fractions were analyzed by high-throughput LC-MS/MS. After separating each fraction by SCX followed by RP, 12 elution fractions were electrosprayed directly into a mass spectrometer. Some 3,000 plant and pathogen proteins were identified (false-positive rate < 5%) and, in order to maximize the detection of a wide variety of proteins, LC-MS/MS was repeated. In addition, authors showed a statistically comparative study to quantify proteins in a single plant variety (susceptible or resistant to infection depending on the *U. appendiculatus* race used) which enabled them to define basal and *R*-gene-mediated plant defences at the proteomics level [25].

An additional limitation in proteomics studies of plant-pathogen interactions is the low number of available completely-sequenced plant and pathogen genomes [14, 26]. Fortunately, there are a considerable number of pathosystems for which sequencing of the genomes of both partners in the interaction are currently in progress [14]. Hence, proteomics studies of these pathosystems will have great advantage with respect to unambiguous protein identification and to assignment the identified proteins to a organism (host or pathogen) with any degree of certainty [14]. However, currently-available databases are fraught with redundancies; inconsistencies in nomenclature, fused genes, inappropriately-translated introns or, finally, multiple names are erroneously assigned [27]. A plausible alternative in the meantime the complete genome of an organism of interest is available is the use of either data collections from different groups or EST (Expressed Sequence Tag) databases [19]. Although many genes may not be represented in EST databases, numerous studies on protein identification combine this option with homology searches against non-redundant protein sequences databases from similar related organisms [28-31]. In the case of species for which no genome sequences information are available, protein analysis must be performed by *de novo* sequencing, which consists in direct reading of the amino acid sequence of a peptide obtained from its MS/MS spectrum. The main advantage of this approach over database search methods is that it allows the identification of spectra for which the exact peptide sequence is not deposited in sequence databases. This is particularly true for peptides containing sequence polymorphisms and post-translational modifications [32]. On the contrary, *de novo* analysis is computationally intensive and requires high-quality fragment ion spectra. Thus, a more effective strategy may be to use simply model systems for which protein sequence database are available, to obtain an spectral identification by sequence database searching, and apply *de novo* sequencing tools to the remaining unassigned high quality spectra [32].

PROTEIN QUANTIFICATION IN PLANT-PATHOGEN INTERACTION STUDIES

The development of strategies for accurate protein quantification is currently one of the most challenging areas in proteomics [33]. An essential goal of proteomics is the ability to quantify changes in protein expression in response to a wide range of physiological situations, including responses to biotic and abiotic stresses. An understanding of protein expression changes taking place during a plant-pathogen interaction is key to comprehend the molecular mechanisms underlying it. The majority of the studies in this research field are focussed either on changes in the plant defence responses upon pathogen and/or elicitors challenge, or on protein expression changes in the pathogen during its interaction with the host or in a situation that mimics it. Therefore, relative quantification approaches are pivotal in proteomics analysis of plant-pathogen interactions.

The classical method employed to analyze changes in protein abundance in differentially-treated samples is 2-DE. Some of the 2-DE limitations related to low reproducibility in protein quantification and gel-to-gel variations can be overcome by employing Difference Gel Electrophoresis (DIGE) technology [34]. In DIGE, proteins of each different sample are labeled with different fluorophores, mixed, and finally run in the same 2-DE gel. This enables samples comparison under the same electrophoretic conditions. Although 2-DE is a valuable technique, the complexity of biological samples often exceeds the resolution of this approach. Therefore, the development of advanced MS-based quantitative strategies enabling the simultaneous identification and quantification of proteins at large scales has been an important breakthrough in the high-throughput screening of proteomes [35]. Despite the great potential of MS-based quantitative strategies, relative quantification analysis in proteomics studies of plant-pathogen interactions is predominantly performed by 2-DE (reviewed by [2, 12, 16, 36-44]). However, the use of gel-free quantitative procedures has considerably increased during the last few years. These techniques can be grouped in stable isotope labeling and label-free quantification methods. Descriptions of these strategies as well as their advantages and limitations have been comprehensively reviewed elsewhere [20, 33, 45-48]. We will briefly describe how these approaches have been implemented in plant-pathogen interactions studies, reviewing the most recent contributions (2006-2010) (Summarized in Table **1**).

Stable Isotope Labeling Techniques

Labeling strategies are based on the incorporation of different isotopic mass tags into proteins/peptides of the samples to be compared. Differential isotopic labels can be introduced metabolically (*in vivo* labeling) or enzymatically or chemically (*in vitro* labeling), and depending on the used method, at either the peptide or the protein level. Introduction of tags produces a change in the protein/peptide mass without affecting their analytical or biochemical properties [33]. Once labeling is performed, different samples are combined in equal ratio and jointly processed by single or multidimensional LC-MS/MS analysis. Relative quantification of proteins is based on the ratio of signal intensities or peaks areas of heavy and light peptides.

In Vivo labeling

In vivo or metabolic labeling involve the incorporation of stable isotopes during protein biosynthesis in cells or in entire organisms growing in medium supplemented with an amino acid (or any other nutrient) labeled with a stable isotope. The consequence of this process is a homogeneous labeling of the whole proteome [45]. An organism or cell under different treatments can therefore be differentially labeled, processed and, finally, their proteome changes be compared. This strategy minimizes experimental variations between samples, allowing highly-accurate relative protein quantification [45].

Metabolic labeling of plant cells appeared unfeasible due to their autotrophic nature Therefore, an efficient incorporation of exogenously-supplied labeled amino acids is hindered. Nevertheless, the use of SILAC (Stable Isotope Labeling by coded Amino acids in Cell culture) strategy [57] has allowed the efficient labeling of *A. thaliana* cell cultures with stable isotopes [58]. Indeed, incorporation of [$^{13}C_6$]-arginine into the proteome of *A. thaliana* suspension cells was achieved with 80% efficiency. To the best of our knowledge, this is the only application of SILAC strategy in plant proteome studies. Metabolic labeling based in ^{15}N-enriched ammonium salts in growth medium seems to be a better choice for autotrophic organisms such as plants. Thus, ^{15}N labeling has been successfully used in relative protein quantification experiments performed with both whole plants [59-62] and plant suspension cells [49, 63-65], achieving in some cases up to 98 % incorporation.

The early recognition and signalling events that occur in plant cells in response to pathogen attacks need to be extremely rapid, reliable and specific to prevent further proliferation and extensive damage [2]. A rapid and reversible process that greatly increases protein dynamics in the cell is the post-translational modification of proteins. Amongst post-translational modifications, protein phosphorylation has been particularly demonstrated to play an important role in triggering the activation of defense signalling pathways in plants. For instance, ^{15}N labeling has been employed to identify phosphorylated proteins involved in early signalling during defense response of *A. thaliana* cells after perception of general elicitors (bacterial flagellin [flg22] or fungal xylanase) [49]. Using the $^{14}N/^{15}N$ labeling strategy combined with selective purification of phosphopetides and LC-MS/MS detection, these authors performed a large scale protein analysis and compared differential phosphorylation patterns of membrane proteins in mock-treated and elicitor-treated *A. thaliana* cells. Thus, 1,172 phosphopeptides, including 1,011 not yet described were identified. Nearly 60% of the phosphopeptides identified were found in both flg22- and xylanase-treated cells, therefore suggesting that both elicitors might share the same signalling pathway. This study allowed the identification of quantitative changes in phosphorylation defence-related protein as well as in membrane-associated proteins. Although the functional analysis of these proteins remains to be done, these differentially-phosphorylated proteins are potential new signal transduction components or early-response regulators candidates in *Arabidopsis* cells [49]. However, since a signalling process such as protein phosphorylation/desphosphorylation is tem-

Table1. Summary of Gel-free Quantitative Proteomics Strategies Employed in Plant-pathogen Interaction Studies

Gel-Free Quantitative Proteomic Strategy	Interaction/Organism	Purpose	Reference
In vivo labeling			
$^{14}N/^{15}N$ labeling	*Arabidopsis thaliana*-bacterial elicitors flagelin (fgl22) or fungal xylanase	Comparative analysis of the phosphoproteome of plasma membrane of *A. thaliana* cells in response to elicitors treatment	[49]
$^{14}N/^{15}N$ labeling	*Nicotiana tabacum-Phytophthora* spp. elicitor cryptogein	Characterization of proteins associated to tobacco DMR microdomains after elicitor treatment	[50]
In vitro labeling			
iTRAQ	*A. thaliana-Pseudomonas syringae* pv. tomato	Relative quantification of differentially-expressed phosphoproteins during diverse defence responses (compatible interaction, incompatible interaction [HR], and basal resistance)	[51]
iTRAQ	*A. thaliana*-bacterial elicitor flagellin (fgl22)	Quantitative analysis of the phosphoproteome of plasma membrane of *A. thaliana* cells in response to elicitor treatment	[52]
iTRAQ	*A. thaliana- P. syringae* pv. tomato	Analysis of changes in the extracellular proteome (secretome) during diverse defence responses (compatible interaction, incompatible interaction [HR], and basal resistance)	[53]
iTRAQ	*Picea abies* –fungal elicitor chitosan	Analysis of the proteome changes produced in Norway spruce cells by the fungal cell wall elicitor chitosan	[54]
iTRAQ	*Fusarium graminearum* grown in liquid medium under conditions conducive to trichothecene production	Study of pathogenicity factors of *F. graminearum*	[55]
Label-free			
	Lycopersicon esculentum- Cladosporium fulvum	Phosphoproteome analysis of tomato plants during HR response	[56]
	Botrytis cinera-media supplemented with host plant extracts	Comparative analysis of the *B. cinerea* secretome	[30]
	Phaseolus vulgaris- Uromyces appendiculatus	Proteome analysis of bean plants infected by virulent and avirulent races of the obligate rust fungus pathogen	[25]

porary and reversible, it would be interesting to study the dynamics of the identified phosphorylation sites at different time-points during longer periods in the quantitative analysis.

Recently, Stanislas and co-workers [50] also employed differential $^{14}N/^{15}N$ labeling to compare protein content associated to tobacco Detergent-Resistant Membranes (DRMs) extracted from cultured cells treated or not with cryptogein, an elicitor of defence reaction secreted by diverse species of the oomycete genus *Phytophthora*. In yeasts and animal cells, the association of particular proteins to DRM microdomains seems to play a central role in the regulation of crucial physiological cell processes [66]. In plants, for instance, a proteomics analysis of tobacco DRMs led to identify proteins involved in signalling response to biotic stress, cellular trafficking and cell wall metabolism, suggesting the participation of DRM microdomains in these processes [67]. The fact that most of the earliest events following cryptogein treatment of tobacco cells involved proteins located on the plasma membrane led to analyze the DRMs proteome in response to this elicitor [50]. Metabolic labeling of tobacco BY-2 cells and isolation of DRMs in combination with LC-MS/MS analysis enabled the identification of 305 proteins, most of them involved in signalling and response to biotic and abiotic stresses. The utilization of a more powerful analyzer (LTQ-Orbitrap mass spectrometer) and a search in a Solanaceae-specific database made it possible to identify

twice the number of proteins previously found using a nanospray LCQ Deca XP Plus ion trap mass spectrometer [67]. Although the relative abundance of the vast majority of proteins associated to DRMs was not significantly modified upon cryptogein treatment, four proteins related to cell trafficking (dynamins) were less abundant, whereas one signalling protein (a 14-3-3 protein) was increased in elicited cells compared to (non-treated) control cells. This suggested that the dynamic association of these proteins to DRM microdomains could be involved in the regulation of the signalling response triggered by this *Phytophthora* spp. elicitor [50].

In Vitro Labeling

In vitro labeling strategies are based in the introduction of isotopic tags into proteins or their respective proteolytic peptides by chemical or enzymatic reactions. These techniques are applicable to a broad range of biological samples. In Isotope-Coded Affinity Tags (ICAT), cysteine residues of proteins from two distinct samples are differentially-labeled with either light or heavy isotopes [68]. Thereafter, samples are mixed and subjected to protease digestion followed by affinity purification of cysteine-labeled containing peptides. An important limitation for ICAT is that most proteins contain a low number of cysteine residues. Therefore, despite the labeling strategy simplify the peptide mixture, the analysis excludes proteins which do not contain cysteine. Moreover, the number of quantificable peptides for each protein is limited when using ICAT, what reduces the reliability of the measurement [33]. The ICAT strategy has been used for comparative analysis of plant proteomes in different biological situations [69-74]. However, to the best of our knowledge, ICAT has not been employed in plant-pathogen interactions research.

A similar strategy to ICAT that targets reactive residues more abundantly found in proteins than cysteine has been recently developed. The iTRAQ (isobaric Tag for Relative and Absolute Quantification) technique is based on the chemical tagging of primary amino groups of peptides generated from proteins digests [75]. iTRAQ reagents consist of a reporter group, a balance group and a amino-reactive group that specifically reacts with the side chain of lysine and the N-terminal amine of peptides. The reporter group is a tag with various masses based on diverse combinations of isotopic elements. The balance group also varies in mass to ensure that the combined mass of the reporter and balance groups remains constant. As such, peptides labeled with different isotopes are isobaric in MS. Quantification using iTRAQ strategy is based on the observation of different reporter group ions in the low-mass region that are released from the tag during MS/MS fragmentation. Currently, two sets of up to four (4-plex) or up to eight (8-plex) distinct isobaric variants are available for iTRAQ strategy, which allows multiplexing (up to four or eight samples) of different treatments in a single LC-MS/MS analysis. Besides the capacity to compare multiple samples simultaneously, labeling in iTRAQ is performed at the peptide level. Because every tryptic peptide should be labeled, multiple peptides can be detected for the same protein, thus increasing the protein identification confidence level. Therefore, possibilities to identify and quantify low-abundance proteins in complex biological samples are significantly enhanced [33]. On the contrary, iTRAQ presents several disadvantages such as variability in labeling efficiency, and in the initial protein digestion, and the high cost of reagents [33]. Despite these limitations, iTRAQ has great potential and the number of reports employing this technique in plant proteomics studies has considerably increased in the last years [51-54, 76-81].

The first study reporting the use of iTRAQ in a plant-pathogen interaction was that of Jones and co-workers [51] to investigate early responses of *A. thaliana* to three *Pseudomonas syringae* pv. tomato DC3000 strains. Relative quantification of differentially-expressed phosphoproteins during diverse defence responses (compatible interaction, incompatible interaction [HR] and basal resistance) was analyzed. Affinity enrichment of phosphorylated proteins coupled to iTRAQ enabled the identification of five *A. thaliana* proteins which showed significant changes in their abundance depending on the inoculation treatment (either inoculated with each of the *P. syringe* strains or non-inoculated). Four of the proteins were identified as a dehydrin, a putative p23 co-chaperon, a heat shock protein (HSP81), and a plastid associated protein (PAP)/fibrillin, which are known to be phosphorylated or harbour potential phosphorylation sites. However, phosphopeptides could not be accurately detected from these putative phosphoproteins. In this study, phosphorylated proteins were enriched from the total soluble leaf proteins fraction using affinity columns, and subsequently labeled by iTRAQ [51]. iTRAQ labeling of peptides rather than that of phosphorylated proteins, followed by a phophopeptide enrichment step, may significantly improve identification of the latter ones [82]. This strategy was followed by Nühse and co-workers [52] in a quantitative analysis of the phosphoproteome of *A. thaliana* plasma membranes. They studied the regulatory mechanism of the plant innate inmune response after treatment with the bacterial elicitor flagellin (flg22). Novel phosphorylation sites of eight proteins involved in flg22 signalling were identified, and their fold induction in response to this elicitor was quantified. Moreover, a specific role in the signalling process was determined for phosphorylation sites present in ATPases and an NADPH oxidase (RbohD) of the plasma membrane. It is worth mentioning here that RobhD mediates the rapid production of reactive oxygen species (ROS) in response to elicitors and pathogens [52].

Kaffarnakik and co-workers [53] have recently extended the study on the *A. thaliana-P. syringe* pv tomato interaction previously described [51], analyzing changes in the extracellular proteome (secretome) of this pathosystem. Indeed, the multiplexing potential of iTRAQ was utilized to simultaneously compare the secretomes of non-inoculated and inoculated *A. thaliana* cells with three *P. syringe* strains that induce different defence responses in this model host plant (see above). Authors identified 45 *A. thaliana* proteins present in the three different interactions examined. These proteins displayed variable accumulation patterns depending on the type of interaction (compatible interaction, incompatible interaction [HR], or basal resistance). Remarkably, the virulent strain (triggering a susceptible interaction) caused the extracellular accumulation of numerous host proteins predicted to be cytosolic (i.e. lacking the common signal peptide for secretion), suggesting that the pathogen may ma-

nipulate the host protein secretion pattern to promote a successful invasion of plant tissues [53].

To our knowledge, the few studies which have employed iTRAQ for comparative proteome analyses in plant-pathogen interactions were focussed on the model plant *A. thaliana* defence responses either upon phytopathogenic bacteria attacks [51, 53], or upon treatment with the bacterial elicitor flagellin [52]. Regarding to non-model plants, Lippert and co-workers [54] employed iTRAQ strategy to study early changes in the proteome of Norway spruce (*Picea abies*) suspension cells induced by the fungal cell wall elicitor chitosan. The experimental design allowed the simultaneous comparison of five different time points after chitosan treatment, although the analysis was limited to the four-plex iTRAQ reagents available at the time this study was carried out. Through comparison of elicitor-induced proteome and transcriptome responses in Norway spruce culture cells, these authors identified proteins and transcripts functionally related with both calcium-mediated signalling and oxidative stress responses not observed in a previous study on the response of intact trees to fungal attack [54].

When a pathogenic fungus starts the infection process of a host plant, both secreted and intracellular proteins change their expression patterns to facilitate the attack. To understand this, several proteomics studies were carried out using a 2D-approach (reviewed by [83]). However, the work of Taylor and co-workers [55] provides a very recent example on the use of iTRAQ to study pathogenicity factors. The pathogen under study was the ascomycete *F. graminearum*, causing devastating problems in maize, wheat and other cereals. *F. graminearum* produces trichothecene mycotoxins which contribute to fungal virulence [55]. Changes in the proteome of *F. graminearum* grown in liquid medium under conditions conducive to trichothecene production and in the absence of the host plant were investigated. This simplified model was chosen on the basis of results from previous proteomics studies. These results showed that pathogen proteins were scarcely identified in *F. graminearum*-infected plants at early stages of the infection, presumable due to their very low relative concentrations compared to plant host proteins [55]. The new study allowed the identification of 435 proteins, 130 of them displaying significantly changes in their expression pattern along the sampling time-course. Functional analysis revealed that proteins potentially implicated in *F. graminearum* virulence were up-regulated, whereas down-regulated proteins corresponded to enzymes involved in primary metabolism, protein chaperones, or proteins involved in the cellular translational machinery [55]. Interestingly enough, 99% of the transcripts related to proteins which were found to be significantly up-regulated by iTRAQ analysis, were also expressed *in planta* under conditions favourable to pathogen infection, as revealed by an earlier microarray analysis [84] Therefore, the combined use of a simplified system simulating a plant-pathogen interaction along with the large-scale proteome analysis provided by iTRAQ technology constitute a valuable approach for the screening of putative pathogenicity factors. However, the remarkable correspondence between *in vitro* and *in vivo* responses found by Taylor and co-workers [55] could not be found in other pathosystems, and extrapolations from *in vitro* results should always be taken with caution.

Proteomics approaches have also been employed to dissect signal transduction pathways involved in phytopathogenicity traits of fungi [83]. For instance, the G protein a-subunit (Gna1) of *Stagonospora nodorum* is a key regulatory element in the pathogenicity of this fungus in wheat [85]. Several Gna1-regulated proteins were identified when wild type and *gna1* mutant proteomes were compared by 2-DE coupled to LC-MS/MS analysis [85]. A more in-depth dissection of the GnaI signal transduction pathway has been recently conducted using iTRAQ and 2D-LC-MALDI-MS/MS [86]. While 2-DE analysis only identified abundance changes in seven proteins [85], the iTRAQ approach uncovered 49 proteins whose abundances were significantly altered in the *gna1* mutant compared with the wild type [86]. The results of this study suggested that Gna1 has a regulatory role on primary metabolic pathways, mannitol metabolism and several stress-related proteins. Moreover, a short-chain dehydrogenase (Sch3) significantly less abundant in the *gna1* mutant was further characterized by gene disruption. Indeed, functional characterization of a *sch3* mutant revealed its inability to sporulate *in planta*, therefore implicating Gna1 signalling in the asexual reproduction of *S. nodorum* [86].

Label-Free Quantification

Quantification of changes in protein abundance can also be achieved by label-free proteomic approaches. Because of certain advantages with regard to isotopic labeling, there is a trend towards the use of label-free quantification methods. Among other advantages, the latter strategies do not need high-cost labeling reagents and the number of samples that can be simultaneously compared is not limited by the number of available tags, as occurs in isotope-labeling techniques [33, 48]. Moreover, it seems suitable to all types of biological samples and allows relative quantification over a higher dynamic range than some isotope-labeling procedures [35]. However, these techniques require a better reduction of interferences potentially leading to suppression effects [33] as well as a high reproducibility of the entire experimental procedure in order to obtain reliable comparative analyses [35]. Unlike to isotopic-labeling approaches, samples in label-free quantitative methods are individually analysed by LC-MS/MS. This poses a disadvantage since parallel sample processing could introduce errors. Label-free protein quantification is generally based on two alternative types of measurements. The first one is the measurement of ion intensity changes such as peptide peak area or peak height in a chromatogram. The second is based on the spectral counting of identified proteins after MS/MS analysis. Peptide peak intensity or spectral count is measured for individual LC-MS or LC-MS/MS runs. Then, relative changes in protein abundance are calculated *via* direct comparison of peak intensity of the same peptide or the spectral count of the same protein [81]. Comparative studies showed a strong correlation between two quantitative methods when the peptides with high signal-to-noise ratio in the extracted ion chromatogram were used in the analysis [87]. Moreover, spectral counting-based quantification was more reproducible, and covered a larger dynamic range, than the peptide ion chromatogram-based quantification [87].

Relative quantification by label-free approaches has been introduced in the field of plant-pathogen interaction just recently [25, 30, 56]. Stulemeijer and co-workers [56] conducted a functional phosphoproteome analysis in tomato (*Lycopersicon esculentum*) plants expressing both the *Cf-4* resistance gene and the *Avr4* avirulent gene of the leaf mold fungal pathogen *Cladosporium fulvum*. HR is induced in tomato plants after a temperature drop by the Cf-4 resistance protein that mediates recognition of the Avr4 fungal elicitor [88]. Through phosphopeptide affinity enrichment combined with LC-MS/MS 48 phosphoproteins were identified, most of which have not been described in tomato before. Furthermore, relative quantification based on peptide ion peak area enabled the identification of differentially-phosphorylated peptides in Cf-4/Avr4 tomato plants with respect to control tomato plants (consisting of a mixture of *Cf-4*- and *Avr4*-expressing parental seedlings that were exposed to the same treatment). The result was informative about changes induced in the phosphoproteome upon HR triggering [56]. Relative quantification by spectral counting has also been employed to compare the secretome of *B. cinera* grown in mock-infecting media supplemented with host plant extracts [30], and to define basal and *R*-gene-mediated plant defences at the proteomic level in the pathosystem bean-rust fungal pathogen (*Uromyces appendiculatus*) [25].

CONCLUDING REMARKS

Proteomics is a powerful tool currently contributing to better understand the complex molecular networks operating during the interaction between plants and pathogens. Although 2-DE and MS is a classical proteomics approach most frequently used by researchers in this field, important limitations such as wide dynamic range, poor sensitivity and low reproducibility are inherent to it. Nevertheless, improvements in MS technology and instrumentation have allowed both protein identification and quantification at a large-scale, hence assisting in the advance of this research area. An additional important restriction for an in-depth analysis of plant and pathogens proteomes is a limited number of available full-sequenced genomes. However, numerous plants and phytopathogens genome sequencing projects are in progress what undoubtedly will facilitate proteomics studies of an increasing number of pathosystems. Moreover, as it was shown in this review, several MS-based identification/quantification approaches are now available to perform proteomics studies of plant-pathogen interactions. However, we think that important considerations must be taken into account in order to decide which method is going to be implemented: number of treatments to evaluate, availability of adequate data analysis software, the mass spectrometer performance, the most-suitable statistical analysis, the complexity of the biological sample and its source, and whether the experiment will be carried out in cell culture or in a complete plant. The choice of the best strategy in proteomics analysis is key in the study the dynamic proteome and to provide robust results. Undoubtedly, proteomics studies will be very soon essential for an in-depth understanding on how plants resist pathogen attacks, and to identify which are the pathogenicity factors deployed by phytopathogens counteracting plant defense responses. Exciting advances expected to be achieved by proteomics studies in the field of plant-pathogen interactions will provide fundamental knowledge to develop novel strategies for an effective control of plant diseases.

ABBREVIATIONS

ESI	=	Electrospray ionization
HPLC	=	High performance liquid chromatography
LC	=	Liquid chromatography
MALDI	=	Matrix-assisted laser desorption/ionization
MS/MS	=	Tandem mass spectrometry
PAGE	=	Polyacrylamide gel electrophoresis
pI	=	Isoelectric point
RP	=	Reverse phase
SCX	=	Strong cation exchange
SDS	=	Sodium dodecyl sulphate
TOF	=	Time of flight
2-DE	=	Two-dimensional gel electrophoresis

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