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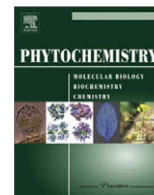

Review

Proteomic approaches to study plant–pathogen interactions

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ABSTRACT

The analysis of plant proteomes has drastically expanded in the last few years. Mass spectrometry technology, stains, software and progress in bioinformatics have made identification of proteins relatively easy. The assignment of proteins to particular organelles and the development of better algorithms to predict sub-cellular localization are examples of how proteomic studies are contributing to plant biology. Protein phosphorylation and degradation are also known to occur during plant defense signaling cascades. Despite the great potential to give contributions to the study of plant–pathogen interactions, only recently has the proteomic approach begun to be applied to this field. Biological variation and complexity in a situation involving two organisms in intimate contact are intrinsic challenges in this area, however, for proteomics studies yet, there is no substitute for *in planta* studies with pathogens, and ways to address these problems are discussed. Protein identification depends not only on mass spectrometry, but also on the existence of complete genome sequence databases for comparison. Although the number of completely sequenced genomes is constantly growing, only four plants have their genomes completely sequenced. Additionally, there are already a number of pathosystems where both partners in the interaction have genomes fully sequenced and where functional genomics tools are available. It is thus to be expected that great progress in understanding the biology of these pathosystems will be made over the next few years. Cheaper sequencing technologies should make protein identification in non-model species easier and the bottleneck in proteomic research should shift from unambiguous protein identification to determination of protein function.

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1. Introduction

Over the last few years, much attention has been given to microarray studies of mRNA expression, and next generation DNA sequencers now permit a truly global analysis of the mRNA complement (via cDNA) of any cell. However, it has long been

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known that gene expression is regulated at different levels, and a number of heritable characteristics are not encoded by DNA. For example, changes in mRNA abundance are not always mirrored by corresponding protein levels and early rapid changes in cell behavior often rely on pre-existing proteins that are either post-translationally modified, have changed their sub-cellular location and/or are degraded. Other nuances that can be biologically relevant include genes encoding differently spliced mRNAs that can give rise to more than one protein. Thus, as more layers of gene regulation, such as epigenetic DNA modifications and the plethora of small non-coding RNAs are uncovered, it becomes clear that many biological questions can only be addressed at the protein level as the presence of either a gene or its mRNA are no guarantee of a role in cellular activity. Therefore, technologies aimed at studying proteins in a cell are a welcome complement. Proteomics is the global study of the protein content of a cell, but this presents new technical challenges due to their greater structural complexity when compared to that of nucleic acids. Technological advances are, however allowing for today's progress in this area. In this way, new and exciting questions can now be tackled, which together with other 'omics' such as genomics and metabolomics, allowed for a new perspective towards understanding how a cell works.

Examples of the types of proteomic studies are varied. While yeast-two-hybrid and yeast-three-hybrid systems (Quirino et al., 2004) can be used to identify cellular proteins able to interact with certain proteins under investigation, mass spectrometry is used to identify cellular proteins and two-dimensional (2-D) gel electrophoresis and thus allows comparison of the types of protein and abundance present in different biological situations.

Contrasting samples in respect to time (e.g., proteins expressed early vs. late during development), space (e.g., proteins exclusively present in roots) or treatment (e.g. dehydrated vs. non-dehydrated tissue) have also been studied using 2-D gel electrophoresis, with differently expressed proteins being observed in early proteomic studies decades ago (Orrick et al., 1973; Nagabhushan et al., 1974).

Today, the advances in mass spectrometry (MS) and their synergism with genomics are revolutionizing biochemistry. A mass spectrometer can now be used to identify either a single separated protein or hundreds of proteins present in a complex mixture with relative ease. In this context, mass spectrometry is an analytical technique that identifies the chemical composition of a compound or sample (Sparkman, 2000). This methodology employs chemical fragmentation of a protein into charged particles (ions) and measurements of charge and mass of the resulting particles. Ionization of molecules is obtained through techniques such as either electrospray (ESI) or matrix-assisted laser desorption/ionization (MALDI) (Siuzdak, 1996). Ionized molecules, which gain or lose their charge by protonation, deprotonation or electron ejection, are electrostatically propelled inside the instrument and detected according to their mass to charge ratio. Furthermore, there are a number of databases with information about protein sequences obtained by the translation of open reading frames. This allows for the comparison of data obtained by mass spectrometry to these protein databases to identify the proteins or peptides present in a sample. In addition, the popularity of traditional as well as the development of new high throughput DNA sequencing technologies enables complete genome sequencing to become possible to many more species.

Another development in biochemistry has been the use of tandem mass spectrometry (MS/MS). In MS/MS, a particular ion is selected with a mass filter/analyzer and then the selected ion is further fragmented and analyzed. Fragmentation can be induced by introducing the ion into a chamber with a collision gas such as argon where collision of ions and argon atoms results in fragmentation. The daughter ion spectrum is then analyzed (Siuzdak, 1996). MS/MS is extensively used in '*de novo*' peptide sequencing.

Many methods for protein analysis, using either gel-based or non-gel based protein separation followed by MS or MS/MS analysis, have also been developed (Fig. 1). As the 2-D PAGE is still the most commonly used method, here we will only cite as examples the analysis of proteomes by MudPIT, DIGE, iTRAQ, SILAC, ICAT and LOPIT. In MudPIT (multidimensional protein identification technology), liquid chromatography (i.e., a strong cation exchange phase followed by a reversed phase chromatography) is directly coupled to tandem mass spectrometry (Washburn et al., 2001). Independent protein separation technologies (i.e., non-gel based) are complementary to gel-based ones and together the different techniques allow for improved proteomic coverage (Washburn et al., 2001; Koller et al., 2002).

There has also been much progress in technologies that allow for quantitative measurements of proteins such as DIGE. In DIGE, a gel-based technology, two protein samples labeled with different fluorescent dyes are run on the same gel and directly compared. In iTRAQ, peptides derived from each sample are derivatized with amine-specific isobaric tags which are indistinguishable by MS but exhibit MS/MS signature ions (Patton, 2002; Ross et al., 2004). This allows for both relative and absolute quantitation of peptides from different samples simultaneously. In SILAC, stable isotopes, such as ^{13}C, are used to label amino acids which are made available to cells in culture (Ong et al., 2003). Mass spectrometry can be used for quantitation of proteins as 'light' and 'heavy' forms of a protein can be distinguished. ICAT is similar to SILAC in that it also uses different isotopes to label proteins of different samples. However, in ICAT, the isotopes are in affinity tags. The ICAT tag is composed of an affinity tag such as biotin which is used to purify the labeled proteins, a linker that will harbor either heavy or light isotopes and a reactive group with specificity toward thiol groups

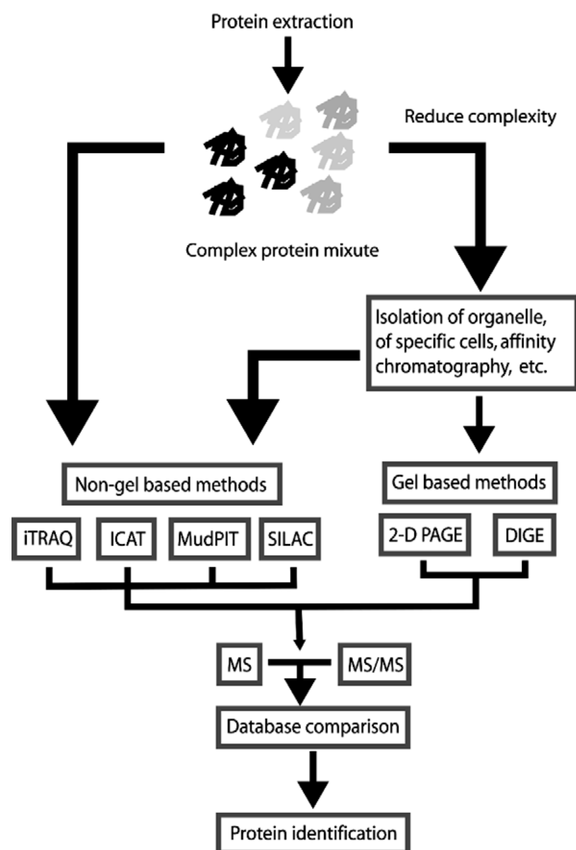

Fig. 1. Schematic representation of the different approaches used in proteomic studies.

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(Gygi et al., 1999). MS/MS analysis allows for determination of the ratio between 'light' and 'heavy' forms of each peptide thereby providing quantitative comparisons between samples. LOPIT or Localization of Organelle Proteins by Isotope Tagging uses an initial partial separation of organelles by density gradient centrifugation followed by analysis of protein distribution in the gradient by ICAT and mass spectrometry. Analysis of the protein distribution in the gradient and grouping with proteins of known cellular localization allows assignment of a protein to an organelle (Dunkley et al., 2004).

The new techniques available for proteomic studies are varied, but this will not be the focus of this review. Those interested in further reading can refer to other reviews (Chen, 2008; Carpentier et al., 2008; Ong et al., 2003). Recent reviews cover plant proteomics in general (Rossignol et al., 2006) and catalogue the proteins that are being identified in plant–pathogen studies through a proteomics approach (Mehta et al., 2008).

Herein, we address the challenges of using a proteomics approach to study plant–pathogen interactions, a biological situation that involves at least two organisms in close contact. We also discuss how genomics is impacting proteomics and future prospects.

2. Proteomic work involving plants

A typical proteomic experiment starts with protein extraction from cells, although such studies with plants can be particularly challenging. This is because plant cells are not only rich in other constituents, such as cell wall polysaccharides and polyphenols, but also have a number of proteases that can degrade samples. In addition, the dominance of certain proteins can make it difficult to study other less abundant proteins. Rubisco (ribulose bisphosphate carboxylase–oxygenase) is the predominant protein in leaves, and storage proteins are also highly abundant in seeds (Chen and Harmon, 2006; Jones et al., 2004). There are various protocols available, each most suited to a specific tissue. Most protocols are based on a trichloroacetic acid (TCA) and/or acetone precipitation of proteins or on phenol extraction, where proteins are solubilized in the phenolic phase and then are precipitated with methanol and ammonium sulfate (Carpentier et al., 2005; Jacobs et al., 2001). The protocols that use phenol have been employed successfully for different plant species.

Two-dimensional gel electrophoresis is currently the most common method used in proteomic studies. In this methodology, the protein samples of interest are initially separated via the isoelectric focusing point (based on protein p*I*) on a strip that has a gradient of pHs (IPG strip) in the first dimension. This is then followed by separation by mass on a SDS–polyacrylamide gel (PAGE) in the second dimension.

Proteins on gels are visualized by staining, this being performed with Coomasie, silver nitrate, or a number of new fluorescent dyes. Although popular, Coomassie Brilliant Blue (R-250) is not as sensitive as silver staining. Colloidal Coomassie (G-250) is an improvement over the standard Coomassie as it is more sensitive. Silver nitrate staining gives excellent results but can interfere with downstream identification of proteins by mass spectrometry. The new fluorescent dyes such as SYPRO-Ruby and Flamingo Pink™ and Krypton™ are very sensitive and compatible with MS technology. SYPRO-Ruby can detect 1–2 ng protein in a band which is a detection limit similar to that of silver staining (Lopez et al., 2000). Unfortunately though, the price of these new dyes still needs to become more competitive. There are also other dyes able to detect specifically modified proteins on SDS–PAGE gels such as phosphoproteins (e.g., Pro-Q™ Diamond) or glycoproteins (e.g., Pro-Q™ Emerald). These dyes allow for a new host of experiments to be performed.

For greater reliability of data, biological (i.e., independently obtained samples) as well as technical replicates (i.e., the same sample run on a different gel) are compared using various software (e.g., PDQuest, Bionumerics, etc.). Gel spots deemed differentially expressed, based on statistical analysis of the gels, are excised and processed for identification by MS analysis. This processing involves a fragmentation process called 'in-gel digestion' in which the protein, still in the matrix, is digested with an enzyme that cleaves at specific points. Trypsin, for instance, cleaves the peptide chain at the carboxyl side of lysine and arginine, except when these are followed by proline. The collection of peptide products is then introduced into the mass analyzer, with the protein either identified through a technique known as peptide mass fingerprinting (PMF) or via tandem MS analysis by '*de novo*' sequencing. In PMF, the absolute masses of the peptides originating from the unknown protein are accurately measured with a mass spectrometer (Clauser et al., 1999). These masses are then compared by bioinformatics to a database containing known protein sequences or the genome of the organism. This is achieved by using computer programs, such as MASCOT (http://www.matrixscience.com/search_form_select.html), Phenyx (http://phenyx.vital-it.ch/pwi/login/login.jsp) and OMSSA (http://pubchem.ncbi.nlm.nih.gov/omssa/). These programs translate genomes into proteins and then theoretically cleave proteins into peptides and calculate the absolute masses of the peptides from each protein. They then compare peptide masses of the unknown target protein to the theoretical peptide masses of each protein deposited in database. PMF is a high-throughput technique, however, it will only work if the protein sequence is present in the database utilized. Therefore, many laboratories prefer to use MS/MS to sequence the peptides.

Although popular, 2-DE gel analysis coupled to MS has many limitations. Overall, it is a laborious technique. Low abundance proteins are particularly hard to detect in complex mixtures of proteins and contaminants present in plant protein samples such as polysaccharides often interfere with gel resolution. Also, on any experiment, one has to make choices of the range of pH gradient to be used in the first dimension, as well as the percentage of acrylamide used for the second dimension gel which limits which proteins will separate well. Therefore, the 2-D gel electrophoresis proteomics approach will always fail to detect certain proteins.

One way to minimize some of the problems of 2-D gel electrophoresis coupled to MS approach is to reduce the complexity of the protein sample. Because certain organelles can be isolated in a highly pure state, this approach is called sub-cellular proteomics. A number of studies have been devoted to examine proteins that are resident in different plant cell compartments, such as the chloroplast (Arai et al., 2008), mitochondria (Brugiere et al., 2004), nucleus (Pandey et al., 2008), peroxisomes (Reumann et al., 2007), vacuole (Jaquinod et al., 2007), tonoplast (Schmidt et al., 2007), endoplasmic reticulum (Maltman et al., 2007) and plant cell-wall (Chivasa et al., 2002). One consequence of the experimental data that sub-cellular proteomics provides is that the signals targeting proteins to particular organelles can be determined with more precision. These studies should also help in development of more accurate programs for the *in silico* prediction of a protein's localization within a cell (Reumann et al., 2007). Proteomics studies at the sub-cellular level are also contributing to the elucidation of new metabolic pathways as well as to the observation of functional differentiation of cells being observed (Majeran et al., 2005; Reumann et al., 2007). Even new roles for organelles are emerging from sub-cellular proteomic studies (Chivasa et al., 2002; Reumann et al., 2007). Based on the presence of proteins, such as ß-glucosidases and myrosinases, previously unknown to be localized to leaf peroxisomes, a new role for these organelles in defense against pathogens and herbivores has been proposed (Reumann et al., 2007).

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Other studies have been able to reduce the complexity of protein samples, by focusing attention on the biology of particular proteins. For example, in two studies the authors proposed to identify proteins able to interact with thioredoxin, a small protein with a disulfide active site involved in redox regulation. To this end, chromatography using a column that contained a mutated thioredoxin was used to trap potential protein targets (Marchand et al., 2006; Wong et al., 2004). Proteins with bound ubiquitin have been identified using a column that contained the ubiquitin binding domain (UBA) polypeptide from the P62 protein bound to agarose beads (Manzano et al., 2008). Proteins that bind RNA were affinity purified with a single stranded DNA column (Masaki et al., 2008) followed by 2-D gel electrophoresis. Other than affinity purification methods, sample complexity was also reduced based on the physical chemical properties of proteins. Hartman et al. (2007) separated proteins present in complexes by differential sedimentation through a rate zonal centrifugation gradient (Hartman et al., 2007). Another approach that has been used with success (in bacteria) is fractionation of proteins by ammonium sulfate precipitation (Park et al., 2008).

3. Plant–pathogen interactions and proteomics

3.1. A late blossoming

Modification of proteins, such as by phosphorylation, have long been known to be important in the signal transduction cascades that trigger plant defenses (Asai et al., 2002; Dóczi et al., 2007; Jones et al., 2006). Both MAP kinases and calcium-dependent protein kinases (CDPKs) can play a part in pathogen recognition and in the downstream events that lead to plant defense (Romeis, 2001). The signaling cascade downstream from the flagellin receptor FLS2, a leucine-rich repeat receptor (LRR) kinase involved in signal recognition in the plant innate immune response, involves a number of phosphorylation events associated with a MAP-kinase cascade (Asai et al., 2002). In other work, Romeis et al. (2001) investigated the role of the calcium-dependent protein kinase NtCDPK2 by virus-induced gene silencing (VIGS) in *N. benthamiana*. CDPK-silenced plants showed a reduced and delayed hypersensitive response in a gene-for-gene response (Romeis et al., 2001). Furthermore, the *Pseudomonas syringae* type III effectors AvrRpm1 and AvrB, that are recognized by the *Arabidopsis* resistance protein RPM1, induce phosphorylation of the protein RIN4, a negative regulator of plant defense (Mackey et al., 2002). Despite the fact that phosphorylation events are central in the cascades involved in plant defense, knowledge about the targets of the phosphorylation is mostly still lacking. Protein cleavage and degradation have also been shown to play a key role in the early events of the hypersensitive response. The type III effector avrRpt2 from *P. syringae* is able to trigger plant defenses through a cascade of events that involves proteasome-mediated RIN4 disappearance (Kawasaki et al., 2005; Kim et al., 2005; Takemoto and Jones, 2005). These examples highlight the need for large-scale methods to identify the proteins that undergo phosphorylation, as well as proteins that disappear when a plant is responding to the presence of a pathogen. However, these are excellent examples of situations where only a proteomics approach (i.e., as compared to other genomics approaches) will best provide useful biological information. A proteomics-based approach will thus facilitate the identification of such proteins and reveal the details about the signaling cascades involved in the interaction between plants and pathogens.

Despite the potential contribution that proteomics can give to plant–pathogen interaction studies, including a number of studies to address how plants react to abiotic stresses (Bindschedler et al., 2008; Dani et al., 2005; Majoul et al., 2004; Yan et al., 2006; Yang et al., 2007), it is only now that proteomics is blossoming in this area. This 'late' blossoming may have several reasons. *In planta*, studies with pathogens can have considerable biological variation and this can be a problem for the proteomics studies as well. Furthermore, proteomics studies of plant–pathogen interactions face intrinsic difficulties because by definition the plant–pathogen interaction is a complex one, involving the protein complement of two organisms. Because of intimate physical contact, it can be hard to distinguish proteins that are differentially expressed by the plant in response to the pathogen from those of the pathogen itself. Despite these problems, there is currently no substitute for *in planta* studies with pathogens. To minimize biological variability, particular attention should be paid to the experimental design. Inoculation in a block design, pooling tissue samples and independent replication of the experiment are all important measures (Jones et al., 2006).

3.2. Approaches to analyze an interaction involving two organisms

As discussed below, there are papers where researchers have focused on the pathogen side of the plant–pathogen interaction and others where the focus was on the plant side. A simplified model of the actual plant–pathogen interaction has been used while others devised interesting experimental set-ups where the partners of the interaction can be separated. Furthermore, there are studies where the proteins were identified but a host or pathogen origin could not be assigned with any degree of certainty. In addition, there are studies of plant defense that use mutant plants, such as lesion-mimic mutants, which do not directly involve a pathogen at all (Jung et al., 2006; Kim et al., 2008; Tsunezuka et al., 2005).

To learn how a pathogen changes its protein expression in response to the plant, many researchers have used host tissue extracts instead of the host itself. This strategy has been used to study how *Xanthomonas axonopodis* pv. *citri* responds to the presence of leaf extracts from a susceptible host plant (sweet orange) as well as a resistant plant (ponkan) and a non-host plant (passion fruit) (Mehta and Rosato, 2001). In other work, Tahara et al. (2003) examined *X. axonopodis* pv. *passiflora* proteins induced in the presence of host plant (passion fruit) leaf extracts. A putative membrane-related protein and a hypothetical protein were novel proteins induced specifically by the host plant extract and an inorganic pyrophosphatase and a hypothetical protein that showed similarity to the *yci*F gene of *Salmonella thyphimurium* were upregulated by the host plant extract. The function these proteins play in the plant–pathogen interaction are, however, unknown as no functional data was presented in the paper.

Separation of the pathogen from the host is sometimes possible, for instance when the host plant has sturdy leaves. To study protein expression of *Xanthomonas campestris* pv. *campestris* in close interaction with the host plant *Brassica oleracea*, Andrade et al. (2008) used a protocol where bacteria were syringe infiltrated into the leaves of the host and later recovered for protein extraction and separation by 2-D gel electrophoresis for proteomic analysis. This paper showed that it was possible, in certain cases, to study the proteins expressed by the bacterial pathogen using live interaction partners. Here again no functional data about the proteins identified was presented.

Reference maps of secreted proteins of different plant pathogens are a benchmark for future studies involving these pathogens and their hosts. Eighty seven proteins secreted by *X. campestris* pv. *campestris* cultivated in minimal medium were identified (Watt et al., 2005). In another study with the soft-rot causing bacterium *Erwinia chrysanthemi*, among the proteins secreted were a cellulase, proteases, endopectate lyases, pectin acetylesterases, a pectin methylesterase, and a polygalacturonase (Kazemi-Pour et al., 2004). Secretome studies have also been performed with plant

pathogenic fungi such as *Sclerotinia sclerotiorum* (Yajima and Kav, 2006).

To specifically target the identification of plant proteins that respond to the presence of the pathogen, elicitors have been used to treat either the host plant or host cell suspension cultures rather than using the pathogen itself (Chivasa et al., 2005; Kim et al., 2003; Rajjou et al., 2006). Peck et al. (2001) used radioactive labeled orthophosphate to pulse-label an *Arabidopsis* cell suspension that was treated with bacterial or fungal elicitors. Although a simplified version of the actual interaction between a whole plant and a pathogen, these types of experiments allowed for an effective comparison between treatments. In another study, Chen et al. (2007) used a suspension culture of rice cells that constitutively express a *Xa21-GFP* fusion. The rice suspension culture was challenged with compatible and incompatible strains of *Xanthomonas oryzae* pv. *oryzae*, the pathogen responsible for bacterial blight. The presence of GFP helped track plasma membrane purification. The plasma membranes were analyzed for proteins that are differentially expressed and eleven proteins with predicted functions in plant defense were identified. These included a H^+-ATPase, a protein phosphatase, a hypersensitive-induced protein and prohibitin.

Other interesting work by Oh et al. (2005) started with a proteomic comparison of the proteins secreted by *Arabidopsis* cultured cells in the presence of salicylic acid (SA). Thirteen different proteins that responded to the SA treatment were identified. Among the proteins identified by MALDI-ToF MS was GDSL LIPASE 1, or GLIP 1, a SA-induced protein that upon further characterization was found to play a role in the defense against the necrotrophic fungus *Alternaria brassicola*. At least two mechanisms were involved in this defense: direct disruption of *A. brassicola* fungal spores and induction of systemic resistance.

In planta work with mutant strains of a pathogen has been used to compare different types of plant–pathogen interaction. In the work by Jones et al. (2004), the model plant *Arabidopsis thaliana* was challenged with different near isogenic lines of the bacterial pathogen *P. syringae* DC3000. The near isogenic lines used were DC3000, DC3000 carrying the avirulence gene *avrRpm1* and DC3000 *hrpA*, a mutant variant that is defective in a protein that forms the pilus apparatus of the type III secretion system (TTSS) that normally delivers bacterial effectors into the plant cell. DC3000 is a strain of *P. syringae* pv. *tomato* that gives a compatible interaction with the Col-5 *Arabidopsis* accession. A compatible reaction involves disease symptom development and establishment of successful parasitism. *AvrRpm1* is an avirulence gene recognized by the plant R-gene *RPM1* present in Col-5, in a typical gene-for-gene interaction, where the hypersensitive response is triggered by TTSS-dependent delivery of AvrRpm1. Because the DC3000 *hrpA* mutant cannot deliver effectors and fails to cause hypersensitive response, this strain allows for the study of basal defense responses. These basal defenses were mediated by the recognition of pathogen associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides and flagellin. Comparison of the proteins expressed after the different treatments and mock-inoculation in a total of 45 gels, allowed for the identification of two subsets of proteins that showed consistent differences: glutathione S-transferases (GSTs) and peroxiredoxins (Prxs). Both enzyme groups may play a role in the regulation of redox conditions in the pathogen infected tissue. In each case, there were multiple spots of each protein due to post-translational modifications. While both groups of proteins were induced by bacterial challenges, individual members of these families responded more specifically. The abundance of GST8a was specifically associated with the hypersensitive response. A truncated form of PrxA-L had its expression reduced following bacterial challenges. Transcriptomic analysis found that for the GST family changes in protein abundance were not paralleled by the transcripts. This result highlights the importance of studies at the protein level.

Plants can exhibit qualitative or quantitative resistance toward a pathogen. The mechanism for quantitative disease resistance is less well understood. This type of resistance is characterized by a continuous variation in the phenotype that is controlled by multiple genes, each with a small phenotypic effect or by major genes with environmental influence (Michelmore, 1995). Proteomic work has been pursued to compare two lines of *Lycopersicon hirsutum* that differ in quantitative resistance to *Clabibacter michiganensis* subsp. *michiganensis.* This plant pathogen causes bacterial canker in tomato plants (Coaker et al., 2004), a serious disease with symptoms that include leaflet necrosis, unilateral leaf wilt, cankers on the stem and plant death (Gleason et al., 1993). The *L. hirsutum* resistant accession LA407 carries two quantitative trait loci, namely Rcm 2.0 and Rcm 5.1, that together are responsible for 68.8 to 79.9% of the variation in disease severity (Coaker and Francis, 2004). Two-dimensional gel electrophoresis was also used to compare a susceptible line to two different lines, one carrying Rcm 2.0 and the other carrying Rcm 5.1 (Coaker et al., 2004). These three lines were closely related genotypes. Three superoxide dismutase enzymes were differentially expressed among the genotypes indicating a role for oxidative stress in response to the pathogen. Furthermore, lines containing Rcm 2.0 and Rcm 5.1 showed different patterns of protein expression suggesting that they confer resistance through different mechanisms.

There are also some publications that address plant–virus interactions. Viruses are the ultimate parasites as they are inert outside the plant cell. Because of their small genome, they encode a limited number of proteins and many viral genomes have been fully sequenced. Using rice (*Oryza sativa*) and Rice yellow mottle virus (RYMV), Brizard et al. (2006) have developed a protocol based on size exclusion chromatography to extract virus-host protein complexes from infected plants. Different proteins were found in the complexes according to the stage of infection. Among the proteins identified by mass spectrometry were proteins involved in plant metabolism (e.g., glycolysis, malate and citrate cycles) possibly to provide energy for viral replication, proteins involved in plant defense (e.g., peroxidase) and proteins involved in protein synthesis (e.g., elongation factors, chaperones). The proteomic approach has also been proposed to be used for the identification of viruses responsible for plant disease (Cooper et al., 2003).

Proteomic work addressing various plant-pathogenic fungus interactions has also been published. Rampitsch et al. (2006) examined the interaction between a susceptible line of wheat and *Puccinia triticina*, a basidiomycete biotrophic fungus that causes leaf rust. The leaf soluble proteome of the wheat cultivars 'Thatcher' (RL6101) and the near isogenic 'Thatcher*Lr1*' (RL6003) that carries the resistance gene *Lr1*, were compared after inoculation with *P. triticina* urediniospores, race BBBD, and after mock-inoculation. The resistant line 'Thatcher*Lr1*' showed no relevant difference between inoculated and mock-inoculated plants. Probably this was due to the highly localized nature of the hypersensitive response, the responding cells being diluted out by the healthy tissue. For the susceptible 'Thatcher' cultivar only up-regulated proteins were found, possibly due to the fact that the initial interaction events with the biotrophic fungus that were studied are more similar to symbiosis than pathogenesis. Peptides from 32 proteins analyzed were tentatively assigned to a plant origin (7), a fungal origin (22) or unknown (3) based on sequence homology to other proteins. Among the proteins identified were metabolic enzymes, structural proteins, and proteins with a role in pathogenesis.

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Proteomic analysis during a plant-pathogenic fungus interaction in parts other than leaves has also been studied. The xylem sap of tomato plants infected with the vascular wilt fungus *Fusarium oxysporum* was investigated. While the PR-5 protein was found in both compatible and incompatible interactions, other PR proteins were associated with compatible interactions only (Rep et al., 2002). Clubroot is a disease that affects different Brassicas and is caused by the obligate biotrophic fungus *Plasmodiophora brassicae*. Using the *A. thaliana*–*P. brassicae* pathosystem, the protein complement of roots and stems of infected and non-infected plants was analyzed (Devos et al., 2006). Proteins associated with cell defense, metabolism and cell differentiation showed altered abundance compared to non-infected controls. Together with other data, the authors suggest that upon *P. brassicae* infection, a new meristematic area is established in the roots and this may act as a sink for host auxin, carbohydrates, nitrogen and energy to maintain the pathogen and start gall development.

Marra et al. (2006) published interesting proteomic work involving a plant–pathogen interaction and a third element, a biocontrol organism. The three-way interaction involved bean plants, a fungal pathogen (*Botrytis cinerea* or *Rhizoctonia solani*) and an antagonistic strain of the fungus *Trichoderma atrviridae*. The proteomes of the partners involved were analyzed alone, in all combinations of two and the complete interaction involving the three partners. To do so the authors grew each fungus on agar plates covered with a cellophane membrane, such that the fungus could be removed from the plates and arranged in a Petri dish over the plant tissue alone or in a plant–fungus–fungus "sandwich". This interesting experimental set up permitted the partners to interact, as the cellophane membrane allowed the diffusion of micro- and macro-molecules, at the same time that the partners could be separated and have their proteins extracted from each separately.

4. Genomics helping proteomics

The first plant to have its genome fully sequenced was *A. thaliana* and this was accomplished in the year 2000 (Initiative, 2000). In addition to that of *A. thaliana* (~120 Mb), three other plant genomes have now been fully sequenced: rice (~450 Mb) (Project, 2005), poplar (~550 Mb)(Tuskan et al., 2006) and grape (~500 Mb)(Jaillon et al., 2007; Velasco et al., 2007). Rice is a staple food in many countries and was adopted as a model organism for monocot plant species. In the rice genome, 37,544 protein-coding genes were identified (Project, 2005). Of these protein-coding genes, 71% had a putative homologue in *Arabidopsis*. Poplar (*Populus trichocarpa*), also known as black cottonwood, is the model species for trees and can be used to make paper, plywood and lumber (Stokstad, 2006). The poplar genome has 45,555 nuclear genes (Tuskan et al., 2006). It can be transformed, regenerated and vegetatively propagated. Experiments involving addition or knocking out of genes can be performed (Brunner et al., 2004). Of the four fully sequenced plant genomes, the grape genome was the last to become available through the efforts of two separate groups. Grapes can be consumed fresh or dried and also can be used to make juice or wine. Grapes and its derived products have large markets worldwide. The grape genome sequenced was that of the variety Pinot Noir containing over 29,000 predicted genes (Velasco et al., 2007). Genomic resources are recently beginning to be developed for this species (Doddapaneni et al., 2008).

From the four plant genomes already sequenced, *Arabidopsis* and rice have the most well studied biology and the greatest number of tools developed. There are available a number of T-DNA insertional mutant collections for both species (e.g., Hsing et al., 2007; Sussman et al., 2000). However, the availability of the poplar and grape genomes should be an incentive for the development of tools to study these plants. In addition to these four completely sequenced plant genomes, there are many ongoing large scale genome sequencing projects (e.g., papaya already released as a draft (Ming et al., 2008), soybean, corn, tomato, potato). Furthermore, there are Expressed sequenced tags (ESTs) available for many more plant species (http://www.ncbi.nlm.nih.gov).

From the pathogen side of the plant–pathogen interaction, a number of bacterial and fungal genomes have been completely sequenced, assembled, annotated and published (Table 1). Many others are in progress and should be added to the list in the near future. The first plant pathogen to have its genome completely sequenced was *Xyllela fastidiosa* (Simpson et al., 2000). This genome sequencing project of the strain 9a5c was undertaken by a Brazilian consortium of labs. Brazil is a great producer and exporter of oranges and *X. fastidiosa* is responsible for citrus variegated chlorosis (CVC). *X. fastidiosa* is transmitted by sharpshooter leafhoppers (Roberto et al., 1996) and affected oranges lose commercial value for they are small and hard. As its name implies, *X. fastidiosa* is a fastidious bacterium and localizes to the xylem. Other strains of *X. fastidiosa* are known to cause disease in other economically important plants such as Pierce's disease that affect grapevines.

Pseudomonas and *Xanthomonas* are the two bacterial genuses for which there is the greatest amount of genome information. The *P. syringae*/*Arabidopsis* pathosystem has given great contributions to the field of plant–pathogen interactions (Quirino and Bent, 2003). *P. syringae* patovars *phaseolicola*, *syringae* and *tomato* that cause halo blight in bean, brown spot disease in bean and bacterial speck in tomato, respectively, have all had their genomes sequenced (Table 1). Recently, a fourth related genome, *P. syringae* pv. *oryzae* that is a pathogen of rice has also been sequenced (Reinhardt et al., 2009). In the literature there is an enormous amount of information on the *P. syringae* pv. *tomato* DC3000 interaction with *A. thaliana*, despite the fact that *Arabidopsis* is not a natural host of this pathogen. Different xanthomonads that are pathogenic to citrus, crucifers, tomato and rice among other plants have also been sequenced: *X. axonopodis* pv. *citri*, *X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria* and *X. oryzae* pv. *oryzae.* The literature involving *Xanthomonas* is prolific and there is quite a lot known about its type III virulence factors (Gurlebeck et al., 2006).

Another bacterium whose genome was fully sequenced is *Ralstonia solanacerum*, a soilborne plant pathogen that has a wide host range including potato, tomato, tobacco, banana and geranium (Hayward, 1994). *Arabidopsis* can also be infected by *R. solanacearum* to produce wilt symptoms that are similar to those found in its natural hosts (Yang and Ho, 1998).

Magnaporthe oryzae (*M. grisea*) is a fungus responsible for rice blast, a serious plant disease worldwide that is difficult to control. It has been estimated that enough rice to feed 60 million people is destroyed by rice blast disease each year (Zeigler et al., 1994). It was the first plant-pathogenic fungus to have its genome completely sequenced (Dean et al., 2005). The genome of two oomycetes *Phytophthora sojae* and *P. ramorum* have also become available in draft form (Tyler et al., 2006). *P. sojae* is a soybean pathogen and *P. ramorum* is responsible for sudden oak death. There are various efforts to sequence the genomes of many other plant pathogenic fungi (e.g., Fungal Genome Initiative, www.broad.mit.edu). Beyond the scope of this review, many other plant pathogenic organisms, such as viruses, viroids and phytoplasma, all of which have small genomes have also been completely sequenced.

Pathogens usually have small genomes compared to their plant hosts and different plant pathogenic bacteria and fungi have been genetically manipulated for a long time. Therefore, for most pathosystems, it is the sequencing of the plant genome and development of tools to study the biology of the plant that limits progress. Fortunately, there are now a number of pathosystems for which the genomes of both interaction partners

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Table 1
Plant pathogenic bacteria and fungi with completely sequenced genomes.[a]

Pathogen	Type	Strain	Examples of hosts	Disease	Reference
Agrobacterium tumefaciens and *A. vitis*	Bacterium	C58	Maize, soybean, cotton, grape	Crown gall disease	Goodner et al. (2001), Wood et al. (2001), Slater et al. (2009)
Clavibacter michiganensis subsp. *michiganensis*	Bacterium	NCPPB382	Tomato	Bacterial wilt and canker	Gartemann et al. (2008)
Clavibacter michiganensis subsp. *sepedonicus*	Bacterium	ATCC 33113	Potato	Bacterial ring rot	Bentley et al. (2008)
Erwinia caratovora subsp. *atroseptica*	Bacterium	SCRI1043	Potatoes	Blackleg and soft rot	Bell et al. (2004)
Leifsonia xyli subsp. *xyli*	Bacterium	CTCB07	Sugarcane	Ratoon stunting disease	Monteiro-Vitorello CB et al. (2004)
Magnaporthe oryzae	Fungus	70–15	Rice	Blast disease	Dean et al. (2005)
Pseudomonas syringae pv. *phaseolicola*	Bacterium	1448A	Bean	Halo blight	Joardar et al. (2005)
Pseudomonas syringae pv. *syringae*	Bacterium	B728a	Bean	Brown spot disease	Feil et al. (2005)
Pseudomonas syringae pv. *tomato*	Bacterium	DC3000	Tomato and *Arabidopsis*	Bacterial speck of tomato	Buell et al., (2003), Reinhardt et al. (2009)
Pseudomonas syringae pv. *oryzae*	Bacterium	DC3000	Rice	Bacterial halo blight of rice	Reinhardt et al. (2009)
Ralstonia solanacearum	Bacterium	GMI1000	Tomato, potatoes, bananas	Bacterial wilt	Salanoubat et al. (2002)
Xanthomonas axonopodis pv. *citri*	Bacterium	306	Citrus plants	Citrus canker	da Silva et al. (2002)
Xanthomonas campestris pv. *campestris*	Bacterium	B100, 8004, ATCC 33913	Crucifers	Black rot	Vorholter et al. (2008), da Silva et al. (2002), Qian et al. (2005)
Xanthomonas campestris pv. *vesicatoria*	Bacterium	85–10	Tomato and peppers	Bacterial spot	Thieme et al. (2005)
Xanthomonas oryzae pv. *oryzae*	Bacterium	PXO99[a], MAFF, KACC	Rice	Bacterial blight	Salzberg et al. (2008), Lee et al. (2005), Ochiai et al. (2005)
Xyllela fastidiosa	Bacterium	9a5c, M12, M23, Temecula 1	Orange trees and grapevines	Citrus variegated chlorosis (CVC) and Pierce's disease	Simpson et al. (2000), Van Sluys et al. (2003)

[a] Only genomes that were completely sequenced, annotated and published in journals were included.

have been fully sequenced, namely, *M. oryzae*/rice, *R. solanacearum*/*Arabidopsis*, *X. oryzae* pv. *oryzae*/rice, *P. syringae* pv. *oryzae*/rice, *X. campestris* pv. *campestris*/*Arabidopsis*, *P. syringae* pv. *tomato*/*Arabidopsis* and *X. fastidiosa*/grapevine. Because there are many more plant and pathogen genomes with sequencing in progress, this list will likely increase in the near future. Proteomics studies with these pathosystems will have great advantage with respect to unambiguous protein identification and to assignment of a particular protein to plant or pathogen origin. Furthermore, most of the organisms involved in these interactions are quite tractable from an experimental point of view. Therefore, even more progress is expected to be made in the understanding of different aspects of plant–pathogen interactions using these pathosystems. For these pathosystems, the gap between plant–pathogen proteomic studies and functional genomics should be filled.

How about proteomics in non-model organisms whose genomes have not been sequenced? For organisms whose genomes have not been sequenced, peptide mass fingerprint (PMF) is not well suited for protein identification (Carpentier et al., 2008). In this case, the chances of finding conserved peptides are considerably reduced and PMF does not work or leads to false protein identification. It is possible to use data banks with group restrictions, an option viable for different softwares such as MASCOT. For the identification of host proteins one can limit the search to *A. thaliana*, *O. sativa* or green plants in databanks. However, when the plant host of interest is from a distantly related phylogenetic group the scores obtained might be low. When working with non-model organisms it is common to obtain a low success rate of identified proteins (Carpentier et al., 2008).

Another option sometimes available is to collaborate with groups that are carrying genomic studies of the plant of interest. However, until a genome is fully sequenced, working with EST databases is a considerable limitation for proteomics work as EST databases can have only partial coding sequences for any one gene and many genes may not be represented in these databases due to the choice of tissue used for library construction or low mRNA abundance. Therefore, in working with non-model organisms, one needs to make an extra effort to identify protein spots that are differentially expressed. The protein isolelectric point and molecular mass obtained from 2-D gels are not enough to allow for easy matches with theoretical numbers for these protein characteristics obtained from EST databases, since several proteins are post-translationally modified, receiving phosphate and carbohydrate groups, which modify their molecular masses and p*I*s (Coaker et al., 2004). In these cases, MS-based '*de novo*' sequencing is crucial because it adds information to the protein isoelectric point and molecular weight obtained by 2-DE.

Another problem that occurs frequently in proteomic studies with non-model organisms whose genome has not been sequenced is that one obtains no hits in BLAST or FASTA 3 searches. This problem is often due to the small length of the sequence obtained by '*de novo*' sequencing or to too few identified peptide ions, which is insufficient to obtain a clear protein identification (reviewed by Carpentier et al., 2008). For these reasons, the sequences from several peptide ions from the same protein are necessary to identify this protein with reliability and accuracy. In summary, in spite of all advances obtained in proteomics and MS, several limitations, especially in peptide identification in non-model organisms have not been completely solved yet. However, with the advances of genomics powered by next generation sequencing machines, the genomes of non-model organisms should also become available. Consequently, we should see an increase in proteomic work with non-model organisms in the next few years.

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5. Concluding remarks

To understand the cellular biology and biochemistry underlying plant–pathogen interactions there is no substitute for studying proteins which are directly responsible for cellular activity. Therefore, despite the enormous amount of data generated by transcriptome analysis, the picture is still incomplete and the proteomic approach offers a new perspective that so far has been lacking. The technical advances in mass spectrometry now allow for relatively easy identification of proteins, particularly for organisms with fully sequenced genomes. In this scenario, the use of a proteomic approach to study plant–pathogen interactions is now becoming more popular. The most commonly used approach in proteomics studies of plant–pathogen interactions has been 2-D gel electrophoresis followed by mass spectrometry. A consensus about the need for biological and technical replicates and statistical analysis is emerging. The new technological advances such as MudPIT, ICAT, DIGE and others will eventually be used to study how plants and pathogens interact. Hitherto, most proteomics papers are still descriptive and end with speculations about the role of the differentially expressed proteins in the plant–pathogen interaction. At least for model organisms this will change with time and speculation will be substituted for hard data from functional genomics experiments.

The number of organisms with a sequenced genome is constantly growing. Today over 1,000 complete genomes including bacteria, archebacteria and eukaryotes have been published (www.genomesonline.org). Next generation high throughput sequencing techniques, such as pyrosequencing, that allow faster sequencing at low cost, should give a boost to ever more ambitious sequencing projects. In the next few years, the number of completely sequenced plant genomes as well as plant pathogenic microorganisms should drastically increase. This should have a favorable impact in protein identification allowing peptide mass fingerprinting to be used for more plant and pathogen species. Therefore, for non-model plants, the bottleneck in proteomics should shift from unambiguous protein identification to determination of protein function. Information from functionally studied *Arabidopsis* and rice genes will continue to benefit studies with other plant species and help the annotation of other plant genomes. The amount of information about different aspects of the biology of these plant models, as well as the many tools available for them and the number of scientists dedicated to their research creates a synergism that puts them at great advantage over other plant species (Quirino et al., 2004). However, an effort must be made to develop easy transformation protocols for different crop plants that will allow the establishment of knockout collections and silencing experiments in other species. Particularly for the field of plant–pathogen interactions, there will be unique features to each pathosystem and work with model organisms will not reveal the whole story.

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