J. Plant Biochem. Biotechnol. (October–December 2017) 26(4):371–386
DOI 10.1007/s13562-017-0402-1

REVIEW ARTICLE

Advances in proteomic technologies and their scope of application in understanding plant–pathogen interactions

N. M. R. Ashwin[1] · Leonard Barnabas[1] · A. Ramesh Sundar[1] · P. Malathi[1] · R. Viswanathan[1] · Antonio Masi[2] · Ganesh Kumar Agrawal[3,4] · Randeep Rakwal[3,4,5]

Received: 1 October 2016 / Accepted: 15 March 2017 / Published online: 10 April 2017
© Society for Plant Biochemistry and Biotechnology 2017

Abstract Proteomics, one of the major tools of 'omics' is evolving phenomenally since the development and application of two-dimensional gel electrophoresis coupled with mass spectrometry at the end of twentieth century. However, the adoption and application of advanced proteomic technologies in understanding plant–pathogen interactions are far less, when compared to their application in other related fields of systems biology. Hence, this review is diligently focused on the advances in various proteomic approaches and their gamut of applications in different facets of phyto-pathoproteomics. Especially, the scope and application of proteomics in understanding fundamental concepts of plant–pathogen interactions such as identification of pathogenicity determinants (effector proteins), disease resistance proteins (resistance and pathogenesis-related proteins) and their regulation by post-translational modifications have been portrayed. This review, for the first time, presents a critical appraisal of various proteomic applications by assessing all phyto-pathoproteomics-related research publications that were published in peer-reviewed journals, during the period 2000–2016. This assessment has revealed the present status and contribution of proteomic applications in different categories of phyto-pathoproteomics, namely, cellular components, host–pathogen interactions, model and non-model plants, and utilization of different proteomic approaches. Comprehensively, the analysis highlights the burgeoning application of global proteome approaches in various crop diseases, and demand for acceleration in deploying advanced proteomic technologies to thoroughly comprehend the intricacies of complex and rapidly evolving plant–pathogen interactions.

Keywords Phyto-pathoproteomics · Plant–pathogen interactions · Global proteome analysis · Targeted proteome analysis

Abbreviations

2DGE–MS	Two-dimensional gel electrophoresis–mass spectrometry
ETI	Effector-triggered immunity
LAPs	Low abundant peptides
PAMPs	Pathogen associated molecular patterns
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
PTM	Post-translational modification

Electronic supplementary material The online version of this article (doi:10.1007/s13562-017-0402-1) contains supplementary material, which is available to authorized users.

✉ A. Ramesh Sundar
rameshsundar_sbi@yahoo.co.in

[1] Plant Pathology Section, Division of Crop Protection, Indian Council of Agricultural Research - Sugarcane Breeding Institute, Coimbatore, Tamil Nadu 641 007, India

[2] Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Padua, Italy

[3] Research Laboratory for Biotechnology and Biochemistry, Kathmandu, Nepal

[4] GRADE (Global Research Arch for Developing Education) Academy Private Limited, Birgunj, Nepal

[5] Faculty of Health and Sport Sciences, and Tsukuba International Academy for Sport Studies (TIAS), University of Tsukuba, Tsukuba, Ibaraki, Japan

Introduction

Plants are being attacked by a wide range of pathogens, viz. fungi, oomycetes, bacteria, viruses, etc. resulting in crop loss worth billions of dollars, every year (Agrios 2005). While pathogens developed their own infection strategies to attack their hosts, plants developed passive and active defense systems to resist the pathogen invasion. During the course of co-evolution, both plants and pathogens acquired their molecular combat systems in a see-saw manner, which ultimately dictated the winner of this arms race. Plants acquired their surveillance system mediated by receptors to recognize the invading enemy's signatures at the cell surface and intracellular level, which initiate the attack against pathogens. On the other hand, pathogens evolved a repertoire of effector proteins for invading and colonizing the tissues by modulating the functions of molecular warriors like resistance proteins (R proteins), which are engaged in host defense (Chisholm et al. 2006; Altenbach and Robatzek 2007). Unravelling these complex interactions by identifying the evolving molecular warriors in individual pathosystems is crucial for understanding pathogenesis and molecular basis of plant disease resistance, which in turn would help in developing efficient crop improvement and protection strategies to reduce crop losses.

With many advances in the field of 'omics', which mainly encompasses genomics, proteomics and metabolomics, the understanding on plant–pathogen interactions is being enriched from time to time. While genomics along with transcriptomics deals with the analysis of genes, regulatory elements and their transcripts, the field of proteomics and metabolomics deals with the analyses of proteins and metabolites, respectively. Recently, two new terms such as proteo-genomics and proteo-metabolomics have been progressively used by researchers to refer to the integrated applications of proteomics with genomics and metabolomics, respectively. Although, the application of genomics and transcriptomics has revolutionized the fundamental concept of plant–pathogen interactions, the application of proteomics that could complement genomics is relatively under-utilized or not exploited to its full potential in most of the crops. For many years, proteomics was viewed as an intricate field, because of variable physical and chemical properties of proteins at different physiological conditions, and difficulties in separation and identification of proteins (Gonzalez-Fernandez et al. 2010; Jorrin-Novo et al. 2015). However, the accumulating genome information of plants and pathogens coupled with the advances in protein/peptide separation technologies and mass spectrometry (MS) analyses, reduced the complexity of proteome analysis and identification of individual proteins.

Over the years, proteomics has emerged as an indispensable approach to understand the systems biology of both prokaryotes and eukaryotes, as evidenced by a great deal of research publications. Nevertheless, the rate of employing proteomics approach for understanding plant–pathogen interactions is not forthcoming, as reflected by just few hundreds of publications. The two major reasons for this slow adoption are the lack of acquaintance on the advancements of proteomic approaches/technologies and how it can be effectively employed to unravel the molecular players that dictate the sequential events of plant–pathogen interactions. In recent years, a handful of review articles have highlighted new developments in proteomics technologies and presented the accumulated proteomic findings in different plant–pathogen interactions, under separate themes. However, till date, there is no deliberation on the status of proteomics applications in specific categories like cellular components, host–pathogen interactions, model and non-model plants, and utilization of different proteomic approaches/technologies. Hence, this review mainly focuses on comprehending the strategies of basic and advanced proteomic approaches/technologies and their scope, and current status of various proteomic applications in deciphering the functional framework of plant–pathogen interactions.

A brief historical perspective of proteomics

Even though the term 'proteome' that refers to the study of all proteins in a system came into use only after 1995 (Wilkins et al. 1995), the field has a dynamic history since the beginning of twentieth century. Precisely, the field has started evolving as early as 1930s, in the area of protein separation (based on isoelectric focusing (IEF), molecular weight, etc.), protein sequencing (Edman degradation—N-terminal amino acid sequencing) and MS. Proteomics has started gaining momentum since 2000, with major technological advancements in two-dimensional gel electrophoresis (2DGE) and MS. Meanwhile, the accumulation of whole genome information of *Arabidopsis* (*Arabidopsis* genome initiative 2000), rice (Goff et al. 2002; Yu et al. 2002), and phytopathogens such as *Xylella fastidiosa* (Simpson et al. 2000), *Agrobacterium tumefaciens* (Goodner et al. 2001; Wood et al. 2001), *Xanthomonas campestris* (Da silva et al. 2002) and *Ralstonia solanacearum* (Salanoubat et al. 2002), have fueled the impetus provided by technological advancements. Thus, the period 1930s–2000 can be regarded as the pre-proteomics era on development of proteomics technology, while the period after 2000 forms the core application era of proteomics in various disciplines of systems biology like phyto-pathoproteomics. The term phyto-pathoproteomics can be defined as the application of proteomic tools to profile the

proteome (total proteins) of pathogen, host and their interactions for understanding different aspects of phytopathology such as pathogenesis, plant disease resistance and molecular interface of plant–pathogen interactions. For a comprehensive history and development of proteomics, the readers can refer elsewhere (Patterson and Aebersold 2003; Thelen 2007; Agrawal et al. 2013).

Technical advances in proteomics

All proteomic approaches ideally have three critical stages viz., sample preparation, gel/column-based protein/peptide separation, and identification of proteins using MS. Sample preparation is the initial and vital step, in which the procedure or methodology of protein extraction may vary from plant to plant/organism to organism based on their cellular composition and organization. For instance, each plant and plant tissues may vary in their rigidity, composition of proteins (in case of membrane/organelles), nucleic acids, polysaccharides, lipids, phenolics, etc. Hence, the extraction procedures like homogenization, inclusion or exclusion of extraction/solubilization buffer components like chaotropes, detergents, reducing agents, and subsequent cleaning-up of contaminants with salts and solvents may largely vary from plant to plant (Bodzon-Kulakowska et al. 2007). However, the protocol using trichloroacetic acid (TCA) and/or acetone precipitation is widely used for sample preparation in many plants/tissues with minor modifications in extraction/solubilization buffers. Standardization of sample preparation methodology is essential, as it can directly influence the extraction of the number of proteins and their abundance. Further, the methodology of sample preparation should be compatible with the downstream proteomic strategies for separation, identification/quantification and analysis (Agrawal et al. 2011).

Over the years, MS has emerged as an indispensable tool for proteomics. MS technology has advanced in many dimensions viz., robustness, accuracy, sensitivity, and selectiveness in identification and quantification of proteins. Similarly, *in silico* tools for MS data analysis have been rapidly refined in many fronts like increased speed and accuracy in matching proteins/peptides from vast database searches. In addition, algorithms for de novo sequencing for organisms that do not have reference genome/proteome database, de novo-assisted database search for highly reliable protein/peptide predictions and quantifications of labelled or label-free proteins/peptides are evolving continuously. Owing to this technological advances, many proteomic approaches have been emerged, which can be broadly classified into global and targeted profiling of proteomes (Liebler and Zimmerman 2013). The strategies to profile a proteome may vary depending on the availability of resources, facilities and specific applications like global profiling or targeted profiling, high throughput analysis of proteins or precise quantification of proteins (Fig. 1). The following sections would focus on one of the major theme of this review, i.e. advances in various proteomic technologies with brief descriptions on the working principle, advantages and disadvantages.

Global proteome analysis

Global proteome profiling is the most commonly used approach to analyze a proteome. Generally, this approach is preferred for comparative analysis of two or more proteome or to establish a reference proteome map. Broadly, global proteome profiling approach can be further classified into gel-based and gel-free/shotgun approaches as illustrated in Fig. 1. Gel-based approaches include 1DGE, 2DGE, 2DE–DIGE (two-dimensional–differential in gel electrophoresis) and 3DGE. On the other hand, gel-free or shotgun approaches include stable isotope labeling with amino acids in cell culture (SILAC), isotope coded affinity tags (ICAT), isobaric tag for relative and absolute quantification (iTRAQ), multidimensional protein identification technology (MudPIT) and deep proteome analysis approaches. In gel-free proteomics approach, the protein mixture would be directly subjected to trypsin digestion and the digested peptides are chromatographically separated and analyzed by MS. The application of gel-free proteomics approach is slowly gaining momentum in model organisms and crops having whole genome information that can be used as a reference proteome database (Song et al. 2012).

Two-dimensional gel electrophoresis (2DGE)

The advent of 2DGE for separation of proteins in 1975 (O'Farrell 1975; Klose 1975; Scheele 1975) has set off the basic rhythm for the emergence of proteomics as a field of specialization. In this method, proteins are first separated based on isoelectric point (pI) by a process called IEF and then by molecular weight. In 2DE-DIGE, an improved method of 2DGE, different protein samples were labelled using different fluorescent dyes, pooled and then resolved in a single gel. This method has overhauled the issue of inherent variability in the migration of protein spots that were observed while comparing large proteome profiles from multiple 2DGE gels (Unlu et al. 1997). However, modern *in silico* analytical tools are improved enough to detect the gel to gel variability among replicates and

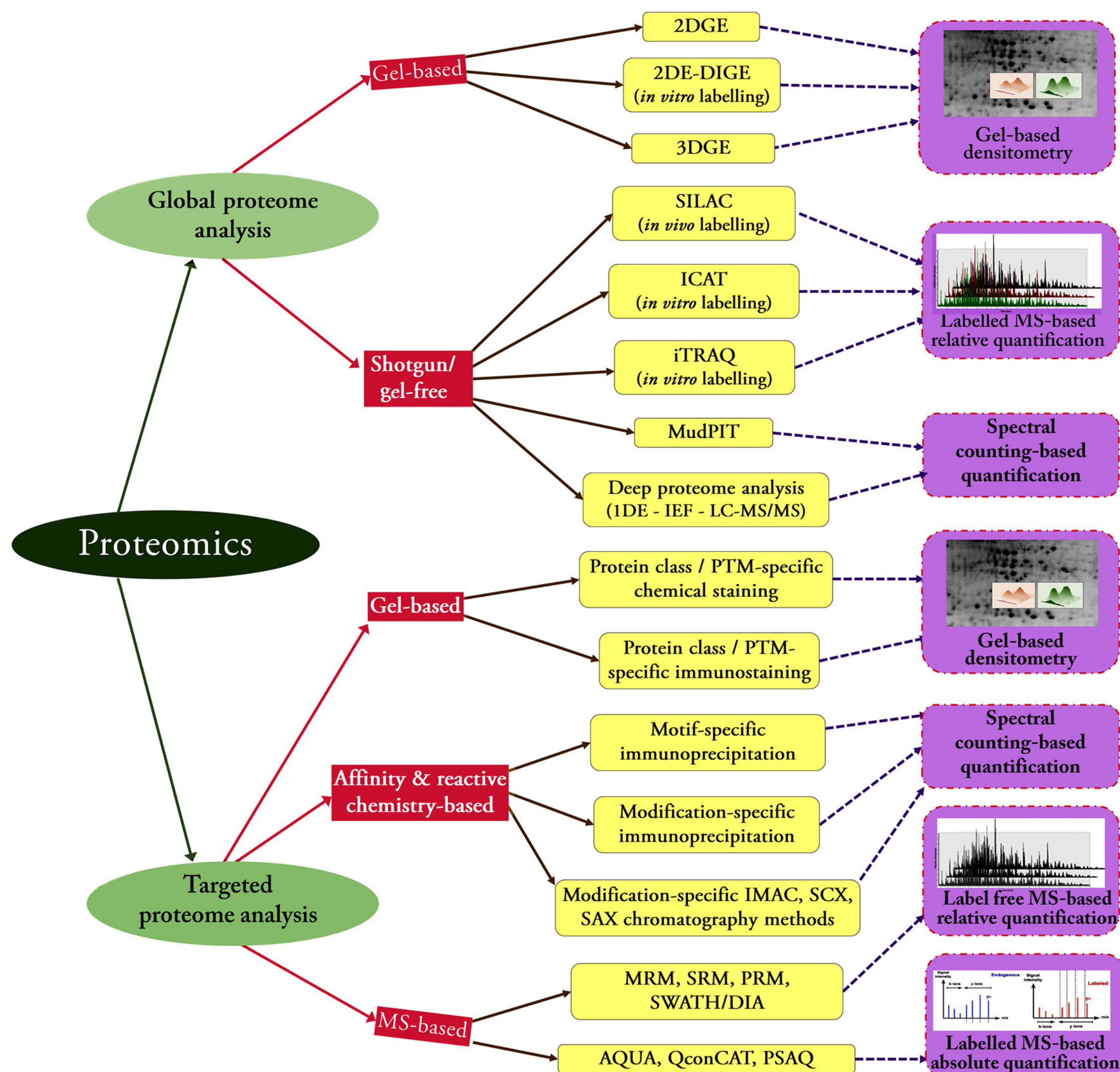

Fig. 1 An overview of various proteomic strategies for analysis, identification and quantification of proteins. *2DGE* two-dimensional gel electrophoresis, *2DE–DIGE* two-dimensional–differential in gel electrophoresis, *3DGE* three-dimensional gel electrophoresis, *SILAC* stable isotope labeling with amino acids in cell culture, *ICAT* isotope coded affinity tags, *iTRAQ* isobaric tag for relative and absolute quantification, *MudPIT* multidimensional protein identification technology, *PTM* post-translational modification, *IMAC* immobilized metal affinity chromatography, *SCX* strong cation exchange, *SAX* strong anion exchange, *MRM* multiple reaction monitoring, *SRM* selected reaction monitoring, *PRM* parallel reaction monitoring, *SWATH/DIA* sequential window acquisition of all theoretical spectra–data-independent acquisition, *AQUA* absolute quantification, *Qcon-CAT* quantification concatamers, *PSAQ* protein standard absolute quantification

comparative samples. Nevertheless, the issue of less accuracy in protein quantification still remains a major limiting factor of 2DGE, since the abundance of individual proteins is being indirectly quantified by protein spot intensity-based densitometry analysis (Brewis and Brennan 2010). Among the various proteomics approaches, 2DGE is the most widely used tool, mainly, due to its affordability and acquaintance. More than 50% of the research publications in phyto-pathoproteomics, have employed 2DGE for proteomic analyses. Therefore, this technique is considered as the workhorse of proteomics.

Three-dimensional gel electrophoresis (3DGE)

3DGE was developed to alleviate some of the limitations of 2DGE such as lack of accuracy in protein identification and discrepancies in relative quantification of spots that occur mainly due to co-migration of proteins. In this method, the co-migrated spots are further separated (for the third time) in-gel in alternative buffer systems involving different ion carriers (Colignon et al. 2013). Apart from enhancing the accuracy of protein identification, this method aids in unambiguous identification of post-translational modification (PTM) of proteins (Rabilloud 2013). Despite its

potential in resolving co-migrated spots that are often encountered during complex plant proteome analysis, this principle is yet to be employed in phyto-pathoproteomics associated studies.

Stable isotope labeling by amino acids in cell culture (SILAC)

SILAC is a shotgun proteomics tool developed using in vivo labelling strategy for MS-based relative quantification of protein samples. It works on the principle of incorporation of non-radioactive heavy isotopes in either amino acids or metabolites in the culture medium, which can be easily identified by tandem MS (Geiger et al. 2011). However, SILAC can only be employed for metabolically active cell culture samples like suspension cultures or in vitro grown pathogen cultures and tissue-cultured plants (Harsha et al. 2008). Besides, the approach is relatively more expensive and cumbersome, when compared to other shotgun proteomic approaches. Because of these limitations, thus far, only two phyto-pathoproteomics studies have employed this technique for proteome analysis (Phillips et al. 2011; Rowland et al. 2015).

Isotope-coded affinity tag (ICAT)

ICAT is an in vitro labelling technique for MS-based relative quantification of complex protein mixtures. ICAT reagents comprise of three structures viz., an affinity tag like biotin, linker containing stable isotope, and a reactive group to bind with the thiol groups (cysteines) of proteins. Besides, heavy and light isotopic forms are used for labelling different samples. Thus, the technique adopts the strategy of chromatographic fractionation of ICAT labelled tryptic peptides, followed by identification and quantification of proteins using tandem MS (Shiio and Aebersold 2006). Similar to SILAC, the cumbersome procedures and expensive tags have made ICAT approach inept for use and paved way for the development of a new tagging and quantification technique called iTRAQ.

Isobaric tag for relative and absolute quantification (iTRAQ)

iTRAQ, a MS-based relative quantification approach employs isobaric tags, thus enabling multiplex analysis of protein samples. In this method, the digested peptides are labelled at lysine residues and at N-terminal end before proceeding with liquid chromatography–mass spectrometry (LC–MS/MS). Since the labels are isobaric and uniform to all peptides, the sensitivity of detecting peptides is higher than other shotgun approaches (Evans et al. 2012). Unlike SILAC and ICAT, iTRAQ tags are labelled directly to protein/peptide mixture and so, this approach has been employed in more than 15 phyto-pathoproteomic studies, with a linear increment in the recent years (Online resource Supp. ESM_1a). Among the shotgun approaches available for labelling and quantification, iTRAQ is the most preferred and relatively less sophisticated method for phyto-pathoproteomic studies.

Multidimensional protein identification technology (MudPIT)

MudPIT is the first gel-free approach developed for complex proteome analysis (Washburn et al. 2001). In this method, the complex peptide mixture of proteins are separated by a biphasic or triphasic microcapillary column packed with strong cation exchange and reverse phase matrices and then subjected to tandem MS. The multidimensional separation of peptides enhances the sensitivity and identification of low abundant peptides (LAPs) and hence generates an exhaustive list of proteins present in a sample (Florens and Washburn 2006). In addition, spectral counting-based quantification of peptides is possible with this approach. MudPIT is less complex and cheaper than iTRAQ, however, the quantitation accuracy is relatively less and largely dependent on sample preparation. As far as plant–pathogen interactions are concerned, this approach did not find much applications in the recent years.

Deep proteome analysis

Deep proteome analysis or deep proteomics is one of the shotgun proteomics approaches developed for obtaining maximum coverage of proteins present in a complex protein mixture. The approach relies on a three dimensional workflow (not 3DGE), which involves three protein separation techniques viz., 1D PAGE, in-gel IEF and reverse phase LC–MS. Besides, gel fractionation and trypsin digestion steps are employed after 1D-PAGE for further enhancement of the resolving power (Atanassov and Urlaub 2013). Sometimes, MudPIT approach is followed after trypsin digestion of fractionated gel slices to obtain the maximum and deeper coverage of peptides. These kind of combined approaches coupled with Orbitrap MS enhance the sensitivity and peptide coverage by many folds, which would improve the identification of LAPs and post-translationally modified proteins. Due to this enhanced sensitivity and peptide coverage, this is the most often used approach in the recent years for understanding plant–pathogen interactions, after 2DGE–MS. With stringent identification parameters for database search, this approach is being increasingly employed for non-model plants.

Comprehensively, among the global proteome profiling approaches, iTRAQ and deep proteomics approaches have potential applications and enormous scope in bailing out phyto-pathoproteomics field from the conventional and basic 2DGE–MS approach.

Targeted proteome analysis

Targeted (selective) proteome analysis is the next major class of proteomics that was grouped based on the coverage of proteins. Targeted proteome analysis is preferred mainly for two reasons, identification of interacting proteins or proteins of interest (immunoprecipitation) and identification of post-translationally modified proteins. Generally, targeted proteome analysis focuses on profiling or identification of a specific protein or selective group of proteins through motif or PTM-specific stains, antibodies (immunoassay) or targeted MS assays (Fig. 1). Among the available methods for screening and identification of post-translationally modified proteins, MS-based approaches are gaining more attention and utility over others. It is because of their ability to systematically configure any protein or protein modifications of interest and the advantage of multiplexing many target peptides with quantitative information in a single experiment (Liebler and Zimmerman 2013).

Gel-based targeted proteomics

For gel-based targeted analysis of proteins, protein-specific or PTM-specific staining procedures are followed after global profiling of proteins using 2DGE. In this approach, the proteins of interest can be visualized after staining with PTM-specific stains like phosphoprotein specific gel stain (Pro-Q® Diamond, etc.), glycoprotein gel stain (Lissamine rhodamine B sulfonyl hydrazine), or motif/PTM specific immunostains (with antibodies). Alternatively, PTMs such as phosphorylation can also be identified by comparing the phosphatase treated proteome profiles with the untreated profiles, which would aid in mapping differential migration of proteins (Yamagata et al. 2002). Besides, there are a few publicly available web-based tools like JVirGel (Hiller et al. 2003) and ProMoST (Halligan et al. 2004), that aid in putative identification of modified proteins in *in silico* gels. However, the probability of identifying less abundant, immunospecific and post-translationally modified proteins are very low with these approaches. Besides, these methods are limited to only certain PTMs (phosphorylation, glycosylation, acetylation, and ubiquitination) and can be identified only after MS analysis (Mann and Jensen 2003). Due to the advances in chromatography and MS-based targeted proteomic approaches, gel-based targeted proteomic approaches are not followed in the recent years. For instance, pertaining to plant pathology, only a few publications have used this approach since 2001, but not beyond 2014 (Online resource Supp. ESM_1a).

Affinity and reactive chemistry-based proteomics

Before profiling the proteome, proteins/peptides of interest can be specifically enriched or purified using affinity and reactive chemistry-based techniques such as immunoprecipitation (IP), strong anion exchange (SAX), strong cation exchange (SCX) and immobilized metal affinity chromatography (IMAC) techniques. Generally, IP method is used for isolation of specific proteins with or without the interacting proteins and enrichment of specific post-translationally modified proteins (phosphorylation, acetylation, ubiquitination) prior to profiling with LC–MS/MS (Kabo-ord and Perr 2008). Further, chemical derivatization strategies such as β-elimination and Michael addition reaction of phosphate groups would be employed to tag biotin for direct affinity purification of phosphopeptides (McLachlin and Chait 2003). However, these chemical modification strategies have many limitations and hence, currently, inept for application.

On the other hand, chromatography techniques such as SAX, SCX and IMAC are used only to enrich specific post-translationally modified peptides. For instance, to enrich phosphor peptides SAX chromatography employs titanium dioxide (TiO_2) and dihydroxybenzoic acid (DHB) (Macek et al. 2009), SCX chromatography utilizes charged peptide-centric approach (Mohammed and Heck 2011) and IMAC uses Fe^{3+}, Ga^{3+}, Al^{3+}, Zr^{3+} and Co^{2+} ions (Ficarro et al. 2002). Sometimes, the combination strategies like SCX coupled with SAX are used to enhance the enrichment of phosphopeptides before MS analysis (Villen et al. 2007). Similarly, for the enrichment of both mono- and multi-phosphorylated proteins, one of the combination strategies called sequential elution from IMAC (SIMAC), that involves IMAC followed by SAX with TiO_2 is employed (Thingholm et al. 2009).

Mass spectrometry-based targeted proteomics

MS-based methods are transforming rapidly in both instrumentation per se and post data acquisition analysis for targeted identification and quantification. While any tandem MS can be used for targeted quantification, only a few tools like triple quadrupole, quadrupole Trap (Q-Trap) and Linear Trap Quadrupole-Orbitrap (LTQ-Orbitrap) are widely employed. The principle behind the quantification of PTM peptides is the detection of multiple occurrences of modification-specific transitions in b- and y-ions during

fragmentation in a MS instrument. The process of detecting specific signals or modifications that are created from these transitions in triple quadrupole is called as selected reaction monitoring (SRM). Similarly, the process of detecting multiple modifications is called multiple reaction monitoring (MRM) (Liebler and Zimmerman 2013). In other words, SRM is a non-scanning approach, in which collision-induced dissociation is used to increase selectivity, whereas, MRM can be measured within the same experiment on a chromatographic time scale by rapidly toggling between different precursor/fragment pairs and records the signal of each transition as a function of the elution time. The data of the peak areas of SRM/MRM transitions of different samples are used for relative quantification or abundance of targeted peptide.

Parallel reaction monitoring (PRM), a method similar to MRM is developed using hybrid instruments like LTQ-Orbitrap, provides increased speed, sensitivity and selectivity (Gallien et al. 2014). Since PRM does not require prior information about target transitions, it is relatively easier to build data acquisition steps than SRM/MRM methods (Rauniyar 2015). Sequential window acquisition of all theoretical spectra (SWATH), a high throughput targeted label-free relative quantification method relies on data-independent acquisition (DIA) mode, wherein the targeted data can be extracted from the globally acquired data. However, this high-throughput and flexibility in targeting from post data acquisition reduces its quantification accuracy as compared to SRM/MRM (Kockmann et al. 2016).

All the aforementioned MS-based label-free quantification methods are proportional (relative) and suffer from slight precision errors between samples, despite many advances in detection and processing algorithms. Therefore, for high precision and accurate quantification of biologically important samples, the strategy of isotope dilution is implemented with SRM/MRM/PRM techniques, wherein the samples are spiked with defined amounts of isotope-labelled analogues (Jorrin-Novo et al. 2015). After isotope labelling, the true and precise abundance of each protein or peptide is determined by standards such as Absolute Quantification (AQUA), Quantification Concatamers (QconCAT) and Protein Standard Absolute Quantification (PSAQ). While AQUA standards are synthetic peptides that are spiked into the samples after proteolysis step, QconCAT standards are chimerical proteins of different peptides that are spiked into the samples before proteolysis step. PSAQ standards are full-length proteins with similar biochemical properties of the target protein (Brun et al. 2009). QconCAT, PSAQ and AQUA methods are highly specific and sophisticated, and finds a better scope in clinical applications than in understanding plant–pathogen interactions.

Comprehensively, to profile the post-translationally modified proteins which play a vital role in regulating plant–pathogen interactions, affinity and reactive chemistry-based approaches are mostly employed, as evidenced by more than 20 research publications till date. On the other hand, the MS-based targeted proteomics, especially MRM approach that has huge scope is just emerging at its budding stage in the field of phyto-pathoproteomics with just four publications in the past 3 years.

Scope of proteomic applications in understanding plant–pathogen interactions

The second major focus of this review is to provide an overview on the scope of applications of proteomics in understanding plant–pathogen interactions, for which empathizing fundamental concepts of plant–pathogen interactions is essential. To invade a plant, pathogen has to overcome various lines of defense established by the host ranging from passive barriers to biomolecules of active defense. Therefore, most of the pathogens secrete an array of effectors against the multitude of defense orchestrated by the host (Jones and Dangl 2006; Gohre and Robatzek 2008; Hogenhout et al. 2009). As part of the first line of active defense, plants recognize the signatures of the pathogens, which are referred to as pathogen associated molecular patterns (PAMPs) through cell surface receptors or pattern recognition receptors (PRRs) that are present in the plant cell membrane surfaces and induce PAMP-triggered immunity (PTI) (Boller and Felix 2009; Zipfel 2009; Bohm et al. 2014). While, PAMPs can be of any pathogen-derived biomolecules such as proteins, polysaccharides, glycoproteins and lipopolysaccharides, PRRs are the host proteins, often conserved with leucine-rich repeat (LRR), transmembrane and kinase domains (Monaghan and Zipfel 2012). Proteomics as a strategy has a great scope in exploring these PAMPs and its interacting partners—PRRs in membrane/cell surface to delineate initial perception events and induction of PTI during plant–pathogen interactions (Fig. 2).

Generally, effectors are evolved to enable parasitism by suppressing plant immunity, especially, PTI and modifying host physiology to support growth of the invading pathogen, which leads to effector-triggered susceptibility (ETS) (Gohre and Robatzek 2008; Lapin and Van den Ackerveken 2013). However, effectors can also elicit host defense, if it is recognized by certain intracellular receptors, either directly or indirectly and trigger a second line of active defense. The immunity, thus activated is rapid, enhanced and robust, and referred to as effector-triggered Immunity (ETI) (Zhang and Zhou 2010; Wu et al. 2014). Since, the effector proteins pose/confer either positive or

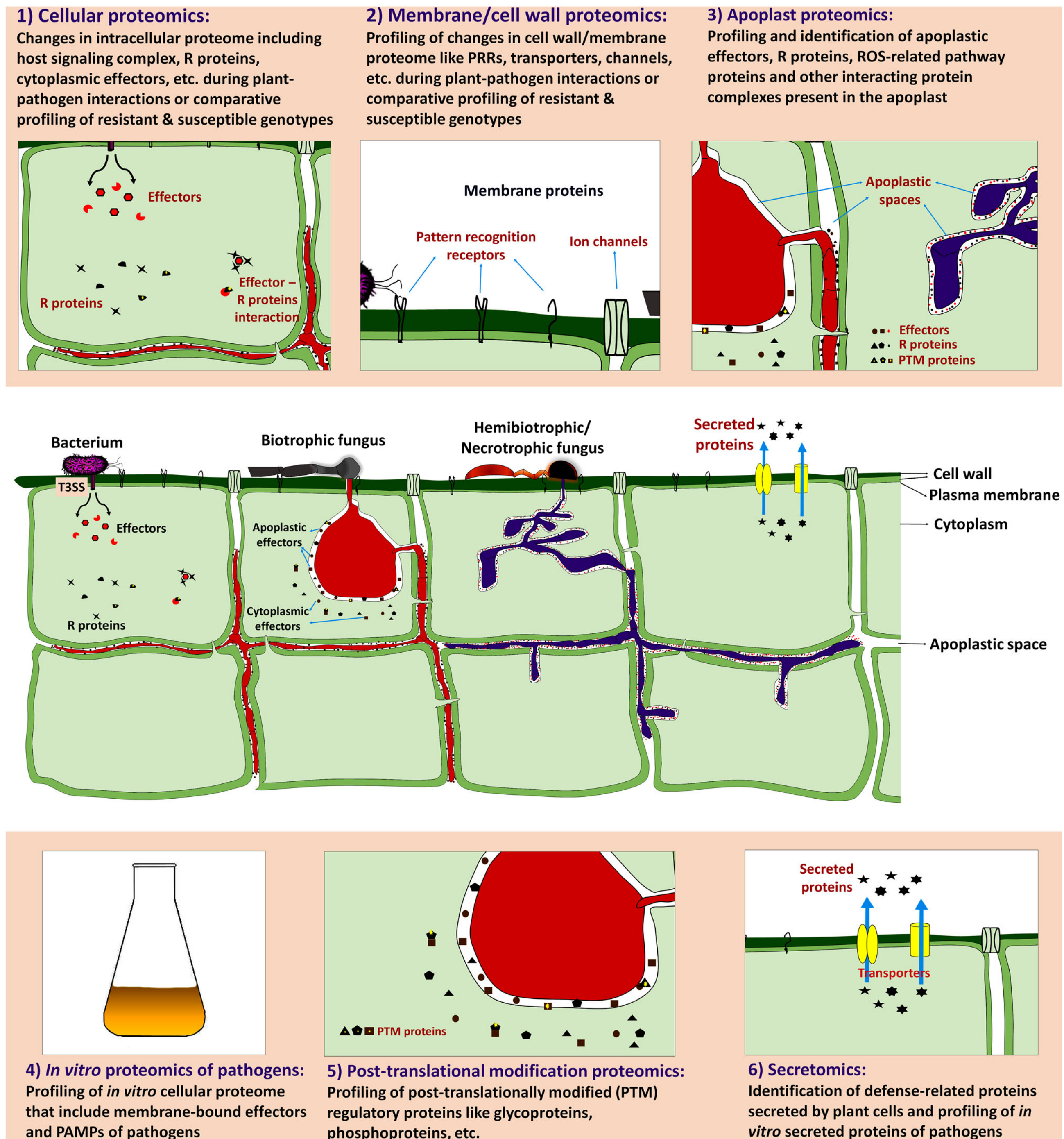

Fig. 2 Various facets of phyto-pathoproteomics applications in elucidating the molecular interface of plant–pathogen interactions

negative fitness to the pathogen based on its host specificity, they are found to be the most rapidly evolving genes (Win et al. 2012, Dou and Zhou 2012). Intracellular receptors, which are often referred to as nucleotide binding-leucine rich repeat (NB-LRR) domain containing proteins and the PRR proteins are collectively called as resistance proteins (R proteins) (Takken et al. 2006; Eitas and Dangl 2010). To avert the recognition by these R proteins, pathogens often modify its effectors evolutionarily by InDel (insertional/deletional) mutation, PTM and alternative splicing without compromising on their activity. Sometimes, pathogens even lose their effector(s), completely, if it renders negative fitness. These alteration events are reported to be one of the major mechanisms for gain of virulence and emergence of new virulent races against the same genotype of plants over a period of time

(Stergiopoulos et al. 2007; Howden and Huitema 2012). PTMs such as phosphorylation, glycosylation, methylation, acetylation, sumoylation and ubiquitination play a major role in spatial and temporal regulation of signaling and other cellular processes by controlling the functions of specific proteins in both the host as well as pathogens (Walsh et al. 2005). All these PTM processes and their functions can only be probed with advanced proteomic technologies and hence, investigation of PTMs in different cellular processes is emerging as one of the major frontier areas of proteomics research (Fig. 2).

Effectors that are secreted into and function in the intercellular spaces are referred to as apoplastic or extracellular effectors, whereas the effectors that are secreted into or translocated into the host cytoplasm via plasma membrane are referred to as cytoplasmic effectors (Stergiopoulos and De wit 2009). Generally, apoplastic spaces are filled with a circulating fluid which contains a myriad of host defense proteins that act against the cell wall of colonizing pathogen and their secreted proteins especially, apoplastic effectors. Since, the intercellular apoplastic space is one of the major battle zones, wherein the molecular warriors of both host and pathogen dynamically interact, it is regarded as one of the potential areas for probing with proteomic approaches (Delaunois et al. 2014). When cytoplasmic effectors are recognized by plants that express direct ligand-receptor or corresponding resistance (R) protein, it triggers hypersensitive response (HR) or systemic acquired resistance (SAR) as a consequence of ETI (Giraldo and Valent 2013). Often, pathogens do secrete effectors or other pathogenicity determinants under in vitro culture conditions either with the perception of some host-related signatures or even without the perception of any host-signals. Similarly, plants also secrete a myriad of defense-related proteins in their root exudates, external cell surface and in cell suspension cultures, either actively or passively. Hence, exploration of secreted proteins of both plants and pathogens separately, and during their interactions using proteomic tools have immense prospects in yielding novel insights on plant–pathogen interactions (Fig. 2).

Plant's active defense system is orchestrated by a complex signaling network of defense mechanisms, primarily involving salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET)-mediated pathways. Generally, PTI and ETI activations are mediated by both MAP kinase and ROS-related pathways. However, the defense triggered by PTI is transient, when compared to the robust ETI, which is often associated with hypersensitive response (HR) and systemic acquired resistance (SAR) (Tsuda et al. 2009). But, HR is associated with JA/ET-mediated pathways, SAR is associated with SA-mediated pathway. SA-mediated pathway activation, in turn, leads to the production of pathogenesis-related (PR) proteins. These crosstalks between ETI and PTI, and SA and JA/ET-mediated pathways suggested extensive sharing of signaling machineries, but they are utilized differently for induction of PTI or ETI responses (Katagiri and Tsuda 2010). Collectively, the host proteins that are involved or regulated in these processes are referred to as stress-induced proteins. Proteomic investigations on changes or differences in all these complex cellular processes in a plant or pathogen (in vitro) or during interactions would yield a greater deal of information from a single experiment. For instance, comparative proteome profiling of two host genotypes differing in disease resistance, two pathotypes differing in virulence, host–pathogen interaction versus mock control, resistant and susceptible interactions and resistance–inducer interactions with host versus control.

Following are the common molecular events like infection strategies and the consequent defense mechanisms of few major plant–pathogen interactions viz., plant–fungus/oomycete interactions, plant–bacterial interactions and plant–virus interactions to highlight the potential scope for proteomics application. Among the thousands of plant diseases that cause serious crop losses worldwide, a major proportion of the diseases are caused by filamentous pathogens namely, fungi and oomycete (Fisher et al. 2012). The interaction of fungus/oomycete with a host is different from the rest of pathogens mostly because of their mode of infection or nutritional lifestyle. The three distinct life styles are biotrophic, hemibiotrophic and necrotrophic. While the biotrophic pathogens employ haustorial structures for feeding in cytoplasm and colonize without killing the host, the necrotrophic pathogens use invasive hyphal structures for penetration and colonization, and kill the host cell. Hemibiotrophic pathogens follow biotrophic lifestyle at initial stages of colonization using primary hyphae, but subsequently transit to a necrotrophic phase involving secondary hyphal structures (Giraldo and Valent 2013). Further, the hyphal structures of both biotrophic and hemibiotrophic pathogens extensively invade the apoplastic spaces. These haustorial and hyphal structures are the actual sites for secretion of a plethora of effectors and plant cell wall degrading enzymes, and interaction with defense-related proteins of host, often with protease activities. To evade the host's perception of its PAMPs during interaction, filamentous pathogens employ many mechanisms, which include scavenging or masking of PAMPs, blocking the signaling cascade that induces PTI with a specific set of effectors. On the other hand, plant's response includes cell wall strengthening by lignification, callose deposition, suberization, etc., HR/necrosis around the site of infection (in incompatible interactions), and production of reactive oxygen species (ROS), phytoalexins and PR proteins (Faulkner and Robatzek 2012). Generally, SA-mediated

defense pathway plays a major role in conferring disease resistance during biotrophic interactions, whereas it is JA/ET-mediated pathway, in case of necrotrophic interactions. However, often these disease resistance pathways are reported to be antagonistic and synergistically active (Tsuda et al. 2009).

A cascade of events that occur during perception of bacterial PAMPs, secretion of effectors [through type three secretion system (T3SS)] and subsequent effector–host target interactions were well established at molecular level in plant–bacteria interactions than plant–fungal/oomycete interactions. For instance, this kind of interaction affects the host defense machinery at multiple levels as follows: inhibition of enzymatic degradation in apoplast, elicitor masking/camouflage, inhibition of receptor activation, downregulation of mitogen-activated protein kinases (MAPK) signaling, modification of defense transcriptome, degradation of host defense components, secretion of plant cell wall degrading enzymes through T2SS, exploiting host proteasomal degradation machinery and interference in vesicular trafficking, etc. (Gohre and Robatzek 2008; Win et al. 2012). On the other side, the basic defense responses of plant's active immune system to resist the bacterial attack is akin to plant–fungus interactions, which includes induction of HR/necrosis, cell wall strengthening, synthesis/production of ROS, phytoalexins, PR proteins and other antimicrobial proteins.

When compared to fungi/oomycete and bacteria, most of the plant viruses may contain only an insignificant number of proteins ranging from at least 3–12. But these miniscule number of proteins can significantly harbor and manipulate host's molecular machinery systems, especially the protein biosynthesis processes (Hull 2013). Generally, plant virus transmission takes place through injured sites of the host or mediated by vectors. During compatible interactions, viruses utilize host's molecular machinery systems for replication and protein synthesis, and spread through plasmodesmata, symplast and vascular bundles. During these replication and movement events, it alters host proteome by affecting endomembrane and cytoskeleton systems, photosynthetic processes, amino acid metabolism, cell wall biogenesis, etc. (Alexander and Cilia 2016). On the other hand, incompatible interactions result in HR, cell wall strengthening, production of ROS, PR proteins, etc. Apart from these, a unique defense mechanism that plants employ against viruses during interactions is RNA silencing. However, many viruses use silencing suppressor proteins to counterattack this mechanism (Burgyan 2008). Though the direct interaction between viral proteins and host proteins are very less, their impact on cellular proteome is huge (Sergeant and Renaut 2010). Certain plant–virus interactions may be asymptomatic, but would impact moderate proteome level changes because of viral replications.

Decisively, proteomics has immense scope and potential applications in identifying these molecular warriors and present a picturesque knowledge of this complex phenomenon, which includes infection strategies, pathogenesis and plant defense mechanism. Therefore, based on the targets of cellular components, the applications of phyto-pathoproteomics can be classified into cellular proteomics, membrane/cell wall proteomics, apoplast proteomics, secretomics, PTM proteomics (phosphorylation, addition/removal of glycosyl group, etc.) and in vitro proteomics of pathogens (Fig. 2). On the other hand, based on broad objectives/interactions involved in a study, proteomics applications can be classified into descriptive proteomics or reference mapping (cataloguing the whole proteome), plant–fungus interactions, plant–oomycete interactions, plant–bacteria interactions, plant–virus interactions, plant–inducer interactions, etc. Besides these categories, the terms such as proteomics and translational proteomics are used to refer studies on protein structure and those that have immediate application to society, respectively (Jorrin-Novo et al. 2015). Sometimes, certain proteomic studies are referred with specific tissues/organs from which it was analyzed. E.g. seed proteomics, root proteomics, leaf proteomics, etc. However, these categorizations are context based and not stringent. There may be overlaps between these categories for different applications.

Present status of proteomics applications in understanding plant–pathogen interactions

The field of 'proteomics' have completed more than one and a half decades, ever since, 2DGE–MS was applied for successful profiling and large scale identification of proteins. Particularly, phyto-pathoproteomics started to flourish with various applications of core proteomic technologies, as evidenced by the rising number of research publications, from 2000 onwards to till date. The status and applications of phyto-pathoproteomics can be easily comprehended from the publications' perspective. Hence, in this review, for the first time, all the research articles published in peer-reviewed journals on phyto-pathoproteomics during the period 2000–2016, were compiled (Online resource Supp. ESM_1a) and various factors like type of interactions, crop, techniques, etc. were analyzed. Thus, this review uniquely presents the status of research publications in different categories of plant–pathogen interactions, crops and non-crops, and utilization of different proteomic approaches in phyto-pathoproteomics.

Publications relevant to plant–pathogen interactions were screened from 'PubMed' (http://www.ncbi.nlm.nih.gov/pubmed), using advanced search options. The listed publications were manually screened further to exclude

literature on proteome of non-pathogenic microbes, beneficial pathogens with economic potentials, symbiotic plant–microbe interactions, nematode and plant–nematode interactions, and studies associated with biocontrol agents. So, the final number of publications that were published on plant–pathogen interactions using core proteomics strategies were found to be 486 (comprising both review and research papers) from the year 2000 to August, 2016 (Online resource Supp. ESM_1a).

An overview of publications during this period showed that proteomic studies on plant–pathogen interactions have gained momentum, since 2002, with the availability of genome information of model pathosystems (Fig. 3). Remarkably, a phenomenal increase in the number of publications was observed since 2007, which marks the transition point in application of proteomics from model plants (*Arabidopsis*, rice) to other crops as well as in profiling of in vitro proteome of pathogens (Online resource Supp. ESM_1b). It can also be observed that the increased availability of many pathogenomes and development of simple protein extraction procedures attracted more researchers to preliminarily start with profiling of pathogens, more than the host or host–pathogen interactions, throughout the period. Research publications on cellular proteomics began to dominate over other facets of proteomics from 2003 to till date with a maximum proportion of 39%. The reasons behind this growth are a comprehensive representation of proteome by cellular proteomics and lack of standard methodologies to extract proteins from sub-cellular components (Fig. 4a). While, in vitro proteomics of pathogens and secretomics share 15 and 8% of total publications, respectively, other emerging important categories namely, apoplast proteomics, membrane/cell wall proteomics, PTM proteomics and organelles proteomics, collectively shared just 17% of publications. Nevertheless, the rising number of publications in apoplast proteomics, membrane/cell wall proteomics and PTM proteomics are encouraging in recent years, although most of them were investigated only on model pathosystems (Online resource Supp. ESM_1b). Interestingly, the proportion of 21% of review articles on core phyto-pathoproteomics, highlights their significance and contribution in providing impetus to the growth of phyto-pathoproteomics.

Based on broad objective or category of host–pathogen interactions, the publications were grouped into ten different sections (Fig. 4b). This grouping revealed that maximum number of proteomic publications (95) were published on plant–fungus interactions, followed by plant–inducer interaction studies (63) with an objective of mapping defense-related proteins. While the group 'pathogen virulence' representing the profiling of pathogenicity-related proteins (in vitro) accounts for 58 publications, the group 'reference proteome', which presents the catalogue of proteins present in a proteome accounts for 29 publications. The groups namely, plant disease resistance and plant disease resistance-transgenic that involved only host plants to identify disease resistance-related proteins recorded the least number of publications, 18 and 19, respectively.

The third categorization of publications on plant–pathogen interactions is based on the family of host plants. Conventionally, many techniques or strategies are usually applied first on model systems, a system which is well studied. Similarly, in the field of phyto-pathoproteomics, most of the early investigations employed rice, tomato, *Arabidopsis* and tobacco until 2004 (Online resource Supp. ESM_1b). Later many other crop pathosystems namely maize, wheat and potato were investigated in detail. As far as phyto-pathoproteomics is concerned, all the above seven

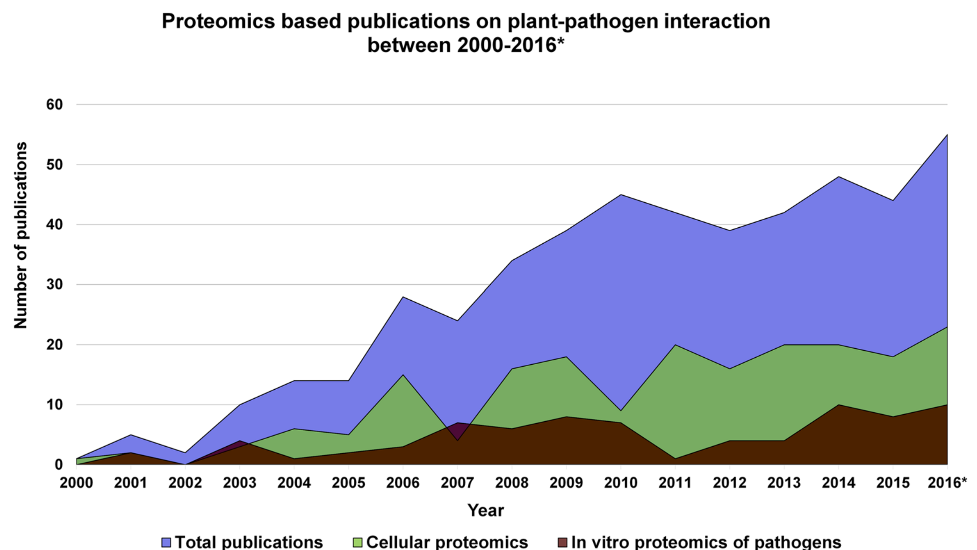

Fig. 3 Area graph representing the number of proteomics-based research publications on plant–pathogen interactions during the period 2000–2016*. *Only publications until August, 2016 were considered for analysis

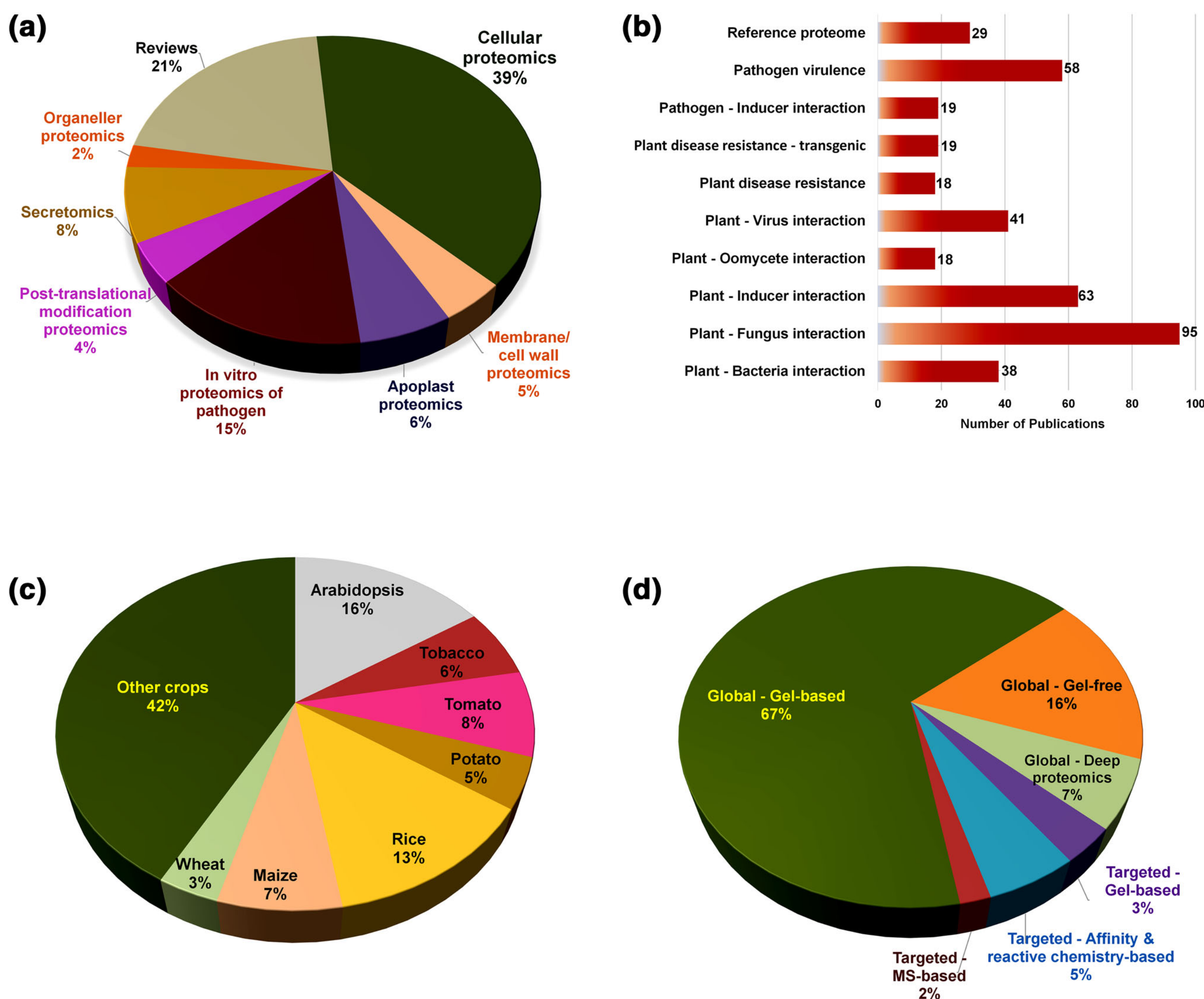

Fig. 4 An overview on the status of application of proteomics in understanding plant–pathogen interactions from the perspective of number of publications published in peer-reviewed journals between 2000 and 2016* (*Only publications until August, 2016 were considered for analysis). **a** Categorization based on cellular components/function; **b** grouping based on major objective/host–pathogen interactions; **c** categorization based on different crops and non-crops; **d** categorization based on the utilization of proteomic strategies

plants representing diverse plant families became model systems for all other crops with regard to protein extraction protocols from different cellular components, reference protein databases, etc. Together, these seven plants account for 58% of total phyto-pathoproteomics related publications, wherein *Arabidopsis* shares 16% and rice shares 13% of publications (Fig. 4c). Only the remaining 42% of publications represented other crops, which clearly indicates that there are still a lot of crops left without realizing the potential applications of proteomics. On the other side, proteomic research in many crops has not even proceeded beyond the basic cellular proteomics strategy. In other words, application of proteomics is still at its infancy in many crops with just one or two publications. Besides, these analyses have also indicated that the proteomic tools yet to be utilized to its true potential in several crops, when compared to the exploitation of high throughput genomics and transcriptomics tools.

On the status of application and utilization of proteomic technologies front, more than two-thirds of the publications on plant–pathogen interactions have adopted gel-based proteomic approaches for global analysis of proteome (67%), the reasons for which has been discussed already in advances in proteomic technologies section (Fig. 4d). Since 2006, the application of global-gel free and targeted approaches like shotgun and modification specific enrichment/separation and identification have started and altogether witnessed only 33% of publications. Though the recently developed targeted approaches like MS-based targeted proteomics possess huge merits and witnessed

revolutionary research in human pathology, the adoption of these advanced proteomic technologies to study plant–pathogen interactions is very slow and limited.

Major findings and contributions of phyto-pathoproteomics

There are nearly four hundred publications on proteomics which substantially contributed, complemented and supplemented to the fundamental understanding of different plant–pathogen interactions (Online resource Supp. ESM_1b). As this review is not focused on the contributions, significance and important findings of individual research publications in phyto-pathoproteomics, the readers are advised to refer to few critical reviews, which are focused on specific categories of phyto-pathoproteomics (Online resource Supp. ESM_2).

Challenges of phyto-pathoproteomics

Though a steady growth in proteomics application was witnessed in many crops, the non-availability of whole genome information of few crops has either restricted the exploitation of proteomics tools or suffered from far lesser or no protein/peptide coverage. This has resulted in inaccuracy in identification of proteins during post-data acquisition (*in silico*) analysis (Barnabas et al. 2015). Similarly, lack of standard protocol for protein extraction from hardy tissues, membranes/cell wall and apoplastic spaces from major crops remains a serious hurdle for realizing the full potential of proteomics in such systems. One of the best examples for the above mentioned challenges is sugarcane, a tough and hardy polyploidy crop. Despite the huge economic importance, the absence of genome information and toughness of cane stalks prevented the application of proteomics to study stalk diseases until the establishment of a standard protocol by Amalraj et al. (2010). Even after the establishment of standard extraction protocol, the crop suffers from less protein coverage, despite using reference databases of closely-related species and de novo sequencing methods (Barnabas et al. 2016).

Identification of LAPs remains to be one of the major impediment for proteomics, especially phyto-pathoproteomics, wherein the indispensable and crucial proteins present in the cell wall and apoplastic spaces are less abundant and often modified by PTM processes (Gupta et al. 2015). These limitations can be overhauled by subjecting the protein mixture to specific LAP enrichment procedures and subsequent analysis by advanced proteomic approaches like isotope-based or MS-based targeted analysis, deep proteome analysis and peptide ligand-based approaches (Delaunois et al. 2014; Righetti and Boschetti 2016).

Conclusion

The science of proteomics is evolving tremendously in both technology and application fronts with the advent of robust and sophisticated tools. However, the perception and adoption of this technology for understanding plant–pathogen interactions is not much encouraging, which is mainly due to lack of expertise, acquaintance and cognizance of scope and potential applications. Hence, a huge impetus is required with path-breaking findings in the form of high impact proteomic publications in challenging crops or cellular components to boost the adoption of emerging advanced technologies. The integration of advanced technologies like MS-based PTM identification using MRM and approaches like enrichment of LAPs for the exploration of apoplast proteomics and membrane proteomics would supercharge the ongoing pace of applications of proteomics in all fronts of phyto-pathoproteomics, which are otherwise tedious and cumbersome to be addressed by resorting to other tools of 'omics'.

Acknowledgements The authors would like to express their gratitude to Indian Council of Agricultural Research (ICAR), New Delhi. The authors thank Director, ICAR-Sugarcane Breeding Institute for providing facilities and continuous encouragement. We also place on record our sincere thanks to Department of Science and Technology, Govt. of India for the support. The authors are indebted to INPPO (http://www.inppo.com/) for their support and encouragement.

Compliance with ethical standards

Conflict of interest The authors declare that they do not have any conflict of interest.

References

Agrawal GK, Bourguignon J, Rolland N, Ephritikhine G, Myriam F, Jaquinod M, Alexiou KG, Chardot T, Chakraborty N, Jolivet P, Doonan JH, Rakwal R (2011) Plant organelle proteomics: collaborating for optimal cell function. Mass Spectrom Rev 30:772–853. doi:10.1002/mas.20301

Agrawal GK, Sarkar A, Righetti PG, Pedreschi R, Carpentier S, Wang T, Barkla BJ, Kohli A, Ndimba BK, Bykova NV, Rampitch C, Zolla L, Rafudeen MS, Cramer R, Bindschedler LV, Tsakirpaloglou N, Ndimba RJ, Farrant JM, Renaut J, Job D, Kikuchi S, Rakwal R (2013) A decade of plant proteomics and mass spectrometry: translation of technical advancements to food security and safety issues. Mass Spectrom Rev 32:335–365. doi:10.1002/mas.21365

Agrios GN (2005) Plant pathology. Elsevier Academic Press, New York

Alexander MM, Cilia M (2016) A molecular tug-of-war: global plant proteome changes during viral infection. Curr Plant Biol 5:13–24. doi:10.1016/j.cpb.2015.10.003

Altenbach D, Robatzek S (2007) Pattern recognition receptors: from the cell surface to intracellular dynamics. Mol Plant Microbe Interact 20:1031–1039. doi:10.1094/MPMI-20-9-1031

Amalraj RS, Selvaraj N, Veluswamy GK, Ramanujan RP, Muthurajan R, Palaniyandi M, Agrawal GK, Rakwal R, Viswanathan R (2010) Sugarcane proteomics: establishment of a protein extraction method for 2-DE in stalk tissues and initiation of sugarcane proteome reference map. Electrophoresis 31:1959–1974. doi:10.1002/elps.200900779

Atanassov I, Urlaub H (2013) Increased proteome coverage by combining PAGE and peptide isoelectric focusing: comparative study of gel-based separation approaches. Proteomics 13:2947–2955. doi:10.1002/pmic.201300035

Barnabas L, Ramadass A, Amalraj RS, Palaniyandi M, Rasappa V (2015) Sugarcane proteomics: an update on current status, challenges, and future prospects. Proteomics 15:1658–1670. doi:10.1002/pmic.201400463

Barnabas L, Ashwin NMR, Kaverinathan K, Trentin AR, Pivato M, Sundar AR, Malathi P, Viswanathan R, Rosana OB, Neethukrishna K, Carletti P (2016) Proteomic analysis of a compatible interaction between sugarcane and *Sporisorium scitamineum*. Proteomics 16:1111–1122. doi:10.1002/pmic.201500245

Bodzon-Kulakowska A, Bierczynska-Krzysik A, Dylag T, Drabik A, Suder P, Noga M, Jarzebinska J, Silberring J (2007) Methods for samples preparation in proteomic research. J Chromatogr B 849:1–31. doi:10.1016/j.jchromb.2006.10.040

Bohm H, Albert I, Fan L, Reinhard A, Nürnberger T (2014) Immune receptor complexes at the plant cell surface. Curr Opin Plant Biol 20C:47–54. doi:10.1016/j.pbi.2014.04.007

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60:379–406. doi:10.1146/annurev.arplant.57.032905.105346

Brewis IA, Brennan P (2010) Proteomics technologies for the global identification and quantification of proteins. Adv Protein Chem Struct Biol 80:1–44. doi:10.1016/B978-0-12-381264-3.00001-1

Brun V, Masselon C, Garin J, Dupuis A (2009) Isotope dilution strategies for absolute quantitative proteomics. J Proteomics 72:740–749. doi:10.1016/j.jprot.2009.03.007

Burgyan J (2008) Role of silencing suppressor proteins. Methods Mol Biol 451:69–79

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host–microbe interactions: shaping the evolution of the plant immune response. Cell 124:803–814. doi:10.1016/j.cell.2006.02.008

Colignon B, Raes M, Dieu M, Delaive E, Mauro S (2013) Evaluation of three-dimensional gel electrophoresis to improve quantitative profiling of complex proteomes. Proteomics 13:2077–2082. doi:10.1002/pmic.201200494

Da Silva ACR, Ferro JA, Reinach FC, Farah CS, Furlan LR, Quaggio RB, Monteiro-Vitorello CB, Van Sluys MA, Almeida NA, Alves LM (2002) Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature 417:459–463. doi:10.1038/417459a

Delaunois B, Jeandet P, Clement C, Baillieul F, Dorey S, Cordelier S (2014) Uncovering plant-pathogen crosstalk through apoplastic proteomic studies. Front Plant Sci 5:249. doi:10.3389/fpls.2014.00249

Dou D, Zhou J-M (2012) Phytopathogen effectors subverting host immunity: different foes, similar battleground. Cell Host Microbe 12:484–495. doi:10.1016/j.chom.2012.09.003

Eitas TK, Dangl JL (2010) NB-LRR proteins: pairs, pieces, perception, partners, and pathways. Curr Opin Plant Biol 13:472–477. doi:10.1016/j.pbi.2010.04.007

Evans C, Noirel J, Ow SY, Salim M, Pereira-Medrano AG, Couto N, Pandhal J, Smith D, Pham TK, Karunakaran E, Zou X (2012) An insight into iTRAQ: where do we stand now? Anal Bioanal Chem 404:1011–1027. doi:10.1007/s00216-012-5918-6

Faulkner C, Robatzek S (2012) Plants and pathogens: putting infection strategies and defence mechanisms on the map. Curr Opin Plant Biol 15:699–707. doi:10.1016/j.pbi.2012.08.009

Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. Nat Biotechnol 20:301–305

Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186–194. doi:10.1038/nature10947

Florens L, Washburn MP (2006) Proteomic analysis by multidimensional protein identification technology. Methods Mol Biol 328:159–175

Gallien S, Bourmaud A, Kim SY, Domon B (2014) Technical considerations for large-scale parallel reaction monitoring analysis. J Proteom 100:147–159. doi:10.1016/j.jprot.2013.10.029

Geiger T, Wisniewski JR, Cox J, Zanivan S, Kruger M, Ishihama Y, Mann M (2011) Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. Nat Protoc 6:147–157. doi:10.1038/nprot.2010.192

Giraldo MC, Valent B (2013) Filamentous plant pathogen effectors in action. Nat Rev Microbiol 11:800–814. doi:10.1038/nrmicro3119

Goff SA, Ricke D, Lan T-H, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). Science 296:79–92. doi:10.1126/science.1068275

Gohre V, Robatzek S (2008) Breaking the barriers: microbial effector molecules subvert plant immunity. Annu Rev Phytopathol 46:189–215. doi:10.1146/annurev.phyto.46.120407.110050

Gonzalez-Fernandez R, Prats E, Jorrín-Novo JV (2010) Proteomics of plant pathogenic fungi. J Biomed Biotechnol 2010:932527. doi:10.1155/2010/932527

Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B, Goldman BS, Cao Y, Askenazi M, Halling C, Mullin L (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science 294:2323–2328. doi:10.1126/science.1066803

Gupta R, Wang Y, Agrawal GK, Rakwal R, Jo IH, Bang KH, Kim ST (2015) Time to dig deep into the plant proteome: a hunt for low-abundance proteins. Front Plant Sci 6:1–3. doi:10.3389/fpls.2015.00022

Halligan BD, Ruotti V, Jin W, Laffoon S, Twigger SN, Dratz EA (2004) ProMoST (Protein Modification Screening Tool): a web-based tool for mapping protein modifications on two-dimensional gels. Nucleic Acids Res 32:638–644. doi:10.1093/nar/gkh356

Harsha HC, Molina H, Pandey A (2008) Quantitative proteomics using stable isotope labeling with amino acids in cell culture. Nat Protoc 3:505–516. doi:10.1038/nprot.2008.2

Hiller K, Schobert M, Hundertmark C, Jahn D, Münch R (2003) JVirGel: calculation of virtual two-dimensional protein gels. Nucleic Acids Res 31:3862–3865. doi:10.1093/nar/gkg536

Hogenhout SA, Van der Hoorn RAL, Terauchi R, Kamoun S (2009) Emerging concepts in effector biology of plant-associated organisms. Mol Plant Microbe Interact 22:115–122. doi:10.1094/MPMI-22-2-0115

Howden AJM, Huitema E (2012) Effector-triggered post-translational modifications and their role in suppression of plant immunity. Front Plant Sci 3:160. doi:10.3389/fpls.2012.00160

Hull R (2013) Plant virology. Elsevier Academic Press, New York

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329. doi:10.1038/nature05286

Jorrin-Novo JV, Pascual J, Lucas RS, Romero-Rodríguez MC, Rodríguez-Ortega MJ, Lenz C, Valledor L (2015) Fourteen years of plant proteomics reflected in "Proteomics": moving from model species and 2-DE based approaches to orphan species and gel-free platforms. Proteomics 15:1–45. doi:10.1002/pmic.201400349

Kaboord B, Perr M (2008) Isolation of proteins and protein complexes by immunoprecipitation. In: Posch A (ed) Methods in molecular biology—2D PAGE: sample preparation and fractionation, vol 424. Humana Pre, Clifton, pp 349–364. doi:10.1007/978-1-60327-064-9_27

Katagiri F, Tsuda K (2010) Understanding the plant immune system. Mol Plant Microbe Interact 23:1531–1536. doi:10.1094/MPMI-04-10-0099

Klose J (1975) Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 26:231–243

Kockmann T, Trachsel C, Panse C, Wahlander A, Selevsek N, Grossmann J, Wolski WE, Schlapbach R (2016) Targeted proteomics coming of age—SRM, PRM and DIA performance evaluated from a core facility perspective. Proteomics. doi:10.1002/pmic.201500502

Lapin D, Van den Ackerveken G (2013) Susceptibility to plant disease: more than a failure of host immunity. Trends Plant Sci 18:546–554. doi:10.1016/j.tplants.2013.05.005

Liebler DC, Zimmerman LJ (2013) Targeted quantitation of proteins by mass spectrometry. Biochemistry 52:3797–3806. doi:10.1021/bi400110b

Macek B, Mann M, Olsen JV (2009) Global and site-specific quantitative phosphoproteomics: principles and applications. Annu Rev Pharmacol Toxicol 49:199–221. doi:10.1146/annurev.pharmtox.011008.145606

Mann M, Jensen ON (2003) Proteomic analysis of post-translational modifications. Nat Biotechnol 21:255–261. doi:10.1038/nbt0303-255

McLachlin DT, Chait BT (2003) Improved β-elimination-based affinity purification strategy for enrichment of phosphopeptides. Anal Chem 75:6826–6836. doi:10.1021/ac034989u

Mohammed S, Heck AJR (2011) Strong cation exchange (SCX) based analytical methods for the targeted analysis of protein post-translational modifications. Curr Opin Biotechnol 22:9–16. doi:10.1016/j.copbio.2010.09.005

Monaghan J, Zipfel C (2012) Plant pattern recognition receptor complexes at the plasma membrane. Curr Opin Plant Biol 15:349–357. doi:10.1016/j.pbi.2012.05.006

O'Farrell PF (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021

Patterson SD, Aebersold RH (2003) Proteomics: the first decade and beyond. Nat Genet 33:311–323. doi:10.1038/ng1106

Phillips CM, Iavarone AT, Marletta MA (2011) Quantitative proteomic approach for cellulose degradation by *Neurospora crassa*. J Proteome Res 10:4177–4185. doi:10.1021/pr200329b

Rabilloud T (2013) When 2D is not enough, go for an extra dimension. Proteomics 13:2065–2068. doi:10.1002/pmic.201300215

Rauniyar N (2015) Parallel reaction monitoring: a targeted experiment performed using high resolution and high mass accuracy mass spectrometry. Int J Mol Sci 16:28566–28581. doi:10.3390/ijms161226120

Righetti PG, Boschetti E (2016) Global proteome analysis in plants by means of peptide libraries and applications. J Proteom 143:3–14. doi:10.1016/j.jprot.2016.02.033

Rowland E, Kim J, Bhuiyan NH, van Wijk KJ (2015) The *Arabidopsis* chloroplast stromal N-terminome: complexities of amino-terminal protein maturation and stability. Plant Physiol 169:1881–1896. doi:10.1104/pp.15.01214

Salanoubat M, Genin S, Artiguenave F, Gouzy J, Mangenot S, Arlat M, Billault A, Brottier P, Camus JC, Cattolico L, Chandler M, Choisne N, Claudel-Renard C, Cunnac S, Demange N, Gaspin C, Lavie M, Moisan A, Robert C, Saurin W, Schiex T, Siguier P, Thebault P, Whalen M, Wincker P, Levy M, Weissenbach J, Boucher CA (2002) Genome sequence of the plant pathogen Ralstonia solanacearum. Nature 415:497. doi:10.1038/415497a

Scheele GA (1975) Two-dimensional gel analysis of soluble proteins. Characterization of guinea pig exocrine pancreatic proteins. J Biol Chem 250:5375–5385

Sergeant K, Renaut J (2010) Plant biotic stress and proteomics. Curr Proteom 7:275–297. doi:10.2174/157016410793611765

Shiio Y, Aebersold R (2006) Quantitative proteome analysis using isotope-coded affinity tags and mass spectrometry. Nat Protoc 1:139–145. doi:10.1038/nprot.2006.22

Simpson AJ, Reinach FC, Arruda P, Abreu FA, Acencio M, Alvarenga R, Alves LC, Araya JE, Baia GS, Baptista CS, Barros MH (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*. The *Xylella fastidiosa* consortium of the organization for nucleotide sequencing and analysis. Nature 406:151–159. doi:10.1038/35018003

Song J, Sun R, Li D, Tan F, Li X, Jiang P, Huang X, Lin L, Deng Z, Zhang Y (2012) An improvement of shotgun proteomics analysis by adding next-generation sequencing transcriptome data in orange. PLoS ONE 7:5–10. doi:10.1371/journal.pone.0039494

Stergiopoulos I, de Wit PJGM (2009) Fungal effector proteins. Annu Rev Phytopathol 47:233–263. doi:10.1146/annurev.phyto.112408.132637

Stergiopoulos I, De Kock MJD, Lindhout P, De Wit PJGM (2007) Allelic variation in the effector genes of the tomato pathogen *Cladosporium fulvum* reveals different modes of adaptive evolution. Mol Plant Microbe Interact 20:1271–1283. doi:10.1094/MPMI-20-10-1271

Takken FL, Albrecht M, Tameling WI (2006) Resistance proteins: molecular switches of plant defence. Curr Opin Plant Biol 9:383–390. doi:10.1016/j.pbi.2006.05.009

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796–815. doi:10.1038/35048692

Thelen JJ (2007) Ch 1: introduction to proteomics: a brief historical perspective on contemporary approaches. In: Samaj J, Thelen JJ (eds) Plant proteomics. Springer, Berlin, Heidelberg, pp 1–13. doi:10.1007/978-3-540-72617-3_1

Thingholm TE, Jensen ON, Larsen MR (2009) Analytical strategies for phosphoproteomics. Proteomics 9:1451–1468. doi:10.1002/pmic.200800454

Tsuda K, Sato M, Stoddard T, Glazebrook J, Katagiri F (2009) Network properties of robust immunity in plants. PLoS Genet. doi:10.1371/journal.pgen.1000772

Unlu M, Morgan ME, Minden JS (1997) Difference gel electrophoresis. A single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077

Villen J, Beausoleil SA, Gerber SA, Gygi SP (2007) Large-scale phosphorylation analysis of mouse liver. Proc Natl Acad Sci USA 104:1488–1493. doi:10.1073/pnas.0609836104

Walsh CT, Garneau-Tsodikova S, Gatto GJ (2005) Protein posttranslational modifications: the chemistry of proteome diversifications. Angew Chem Int Ed Engl 44:7342–7372. doi:10.1002/anie.200501023

Washburn MP, Wolters D, Yates JR (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol 19:242–247

Wilkins MR, Sanchez JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, Williams KL (1995) Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Eng Rev 13:19–50

Win J, Chaparro-Garcia A, Belhaj K, Saunders DG, Yoshida K, Dong S, Schornack S, Zipfel C, Robatzek S, Hogenhout SA, Kamoun S (2012) Effector biology of plant-associated organisms: concepts and perspectives. Cold Spring Harb Symp Quant Biol 77:235–247. doi:10.1101/sqb.2012.77.015933

Wood DW, Setubal JC, Kaul R, Monks DE, Kitajima JP, Okura VK, Zhou Y, Chen L, Wood GE, Almeida NF, Woo L (2001) The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science 294:2317–2323. doi:10.1126/science.1066804

Wu L, Chen H, Curtis C, Fu ZQ (2014) How plants deploy effector-triggered immunity to combat pathogens. Virulence 5:710–721. doi:10.4161/viru.29755

Yamagata A, Kristensen DB, Takeda Y, Miyamoto Y, Okada K, Inamatsu M, Yoshizato K (2002) Mapping of phosphorylated proteins on two-dimensional polyacrylamide gels using protein phosphatase. Proteomics 2:1267–1276. doi:10.1002/1615-9861(200209)2:9<1267:AID-PROT1267>3.0.CO;2-R

Yu J, Hu S, Wang J, Wong GK, Li S, Liu B, Deng Y, Dai L, Zhou Y, Zhang X, Cao M (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). Science 296:79–92. doi:10.1126/science.1068037

Zhang J, Zhou J-M (2010) Plant immunity triggered by microbial molecular signatures. Mol Plant 3:783–793. doi:10.1093/mp/ssq035

Zipfel C (2009) Early molecular events in PAMP-triggered immunity. Curr Opin Plant Biol 12:414–420. doi:10.1016/j.pbi.2009.06.003