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A Review on Proteomics: Tools and Techniques in Analysis of Plant Metabolites

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Abstract

Worldwide, crop yields are lost due to abiotic and biotic stresses. A wide range of research is conducted on the genome, transcriptome, proteome, and metabolome levels to reveal the mechanisms of plant reactions in response to their interactions with the environment. These studies will provide data to define the biochemical and physiological mechanisms behind plant resistance or susceptibility to affecting factors/stresses. The field of plant proteomics focuses on large-scale functional analysis of plant proteins. Researchers use proteomics-based mass spectrometric techniques to breed new varieties of crops that are tolerant to affecting stresses and have good agronomic properties. Molecular and cellular pathways are better understood through the large-scale analysis of proteins that provides a more comprehensive picture of how living systems function. Despite the high dynamic range and difficulties in assessing low abundance proteins, proteome analysis is an extremely challenging task. Various methods have been employed to study proteins, including gel-based techniques, protein microarrays, mass spectrometry-based approaches like MALDI and SELDI, liquid chromatography with high and ultra-high resolution, and fourier transform ion cyclotron resonance mass spectrometry. This review will give a brief overview of some of these techniques and their most recent developments.

Keywords: Proteomics, Techniques, Abiotic and Biotic Stresses, Plant Metabolites

Proteomics: The term “proteomics” was first coined by Marc Wilkins during the Siena meeting in 1994 to denote the “PROTein complement of a genOME” (Wilkins *et al*. 1996). The proteome identifies the majority of a gene's functional information. The total amount of proteins in a cell at any given time, as determined by their location, interactions, post-translational modifications, and turnover, is known as the "proteome". Proteomics is the study and characterization of a complete set of proteins expressed by a genome, a cell or a tissue at a given time (Wilkins *et al*. 1995). It involves the characterization of proteome, which includes expression, structure, functions, interactions and modifications of proteins at any stage (Domon and Aebersold, 2006). It complements other "omics" technologies like genomics and transcriptomics to clarify the identity of an organism's proteins and to understand the makeup and purposes of a specific protein. Technologies based on proteomics are used in a variety of ways for various research settings, including the identification of various diagnostic markers, the development of vaccine candidates, the comprehension of pathogenicity mechanisms, the alteration of expression patterns in response to various signals, and the interpretation of functional protein pathways in various diseases (Bilal *et al*. 2017). Additionally, the proteome changes from time to time, from cell to cell, and in reaction to outside stimuli.

Although it is far more complicated than genomics, proteomics is one of the most important methodologies for understanding how genes work (Lander *et al*. 2001). Analysis of the transcriptome or proteome can help distinguish between two biological states of the cell, allowing for the determination of variations in gene expression levels. For comprehensive transcriptome study on a wide scale, microarray chips have been developed. Microarrays, however, cannot be used to directly assess an increase in mRNA synthesis (Canales *et al*. 2006). Proteins are biological effectors, and their quantities depend on host translational control and regulation in addition to corresponding mRNA levels. Proteomics would therefore be viewed as the most pertinent data collection for describing a biological system. (Cox and Mann, 2007).

In general, proteomic approaches can be used (a) for proteome profiling, (b) for comparative expression analysis of two or more protein samples, (c) for the localization and identification of post translational modifications, and (d) for the study of protein–protein interactions (Baltimore, 2001; Chandramouli and Qian, 2009). Proteomics is used in diverse fields such as medicine, oncology, food microbiology, and agriculture as well (Chandramouli and Qian, 2009).

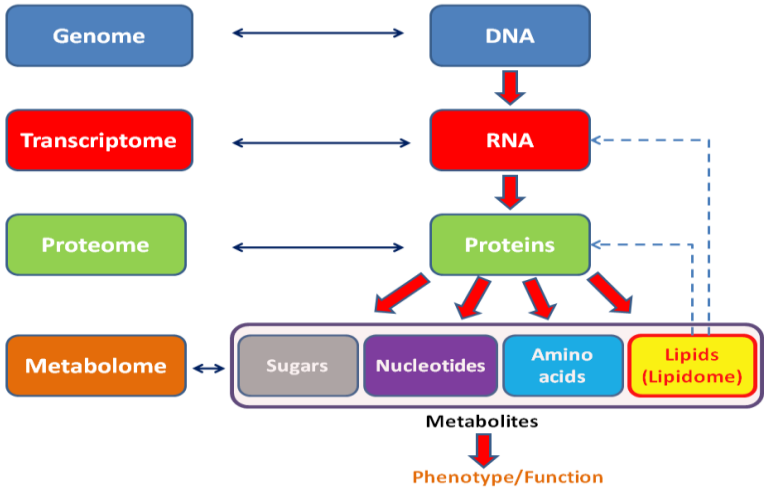

Figure. 1. Progression from Genome to Proteome

Source: Wikipedia

Types of proteomics: Expression proteomics, functional proteomics, and structural proteomics are the three primary subtypes of proteomics.

1. Expression proteomics or Protein expression proteomics: Expression proteomics is the quantitative analysis of protein expression in samples that differ in some way. With this method, it is possible to compare the protein expression of different samples' sub-proteomes or the complete proteome (Banks *et al.* 2000). Additionally, it can be used to find novel proteins involved in signal transduction or proteins associated with particular diseases (Graves and Haystead, 2002). Studies of the patterns of protein expression in various cells typically use expression proteomics techniques. To find changes in protein levels, for instance, a tumour tissue sample is contrasted with a normal tissue sample. Using 2- DE and MS methods, differences in protein expression that are present or absent in tumour tissue relative to normal tissue are found (Hinsby *et al*. 2003).

2. Structural proteomics: To determine the three-dimensional structure and structural complexities of functional proteins, nuclear magnetic resonance spectroscopy and X-ray crystallography are used in structural proteomics. It details every protein interaction such as membranes, cell organelles, and ribosomes in the mixture (Jungbauer and Hahn, 2009). An example of structural proteomic is the study of the nuclear pore complex (Rout *et al*. 2000).

3. Functional proteomics: This kind of proteomics examines the relationships between proteins in the cell as well as their function and molecular mechanisms. Specifically, it examines how members of a particular protein complex engaged in a given process interact with partners from an unidentified protein. This may specify the protein's biological function (Gavin *et al.* 2002). Additionally, detailed descriptions of cellular signalling cascades can result from the understanding of protein-protein interactions in vivo (Monti *et al.* 2007).

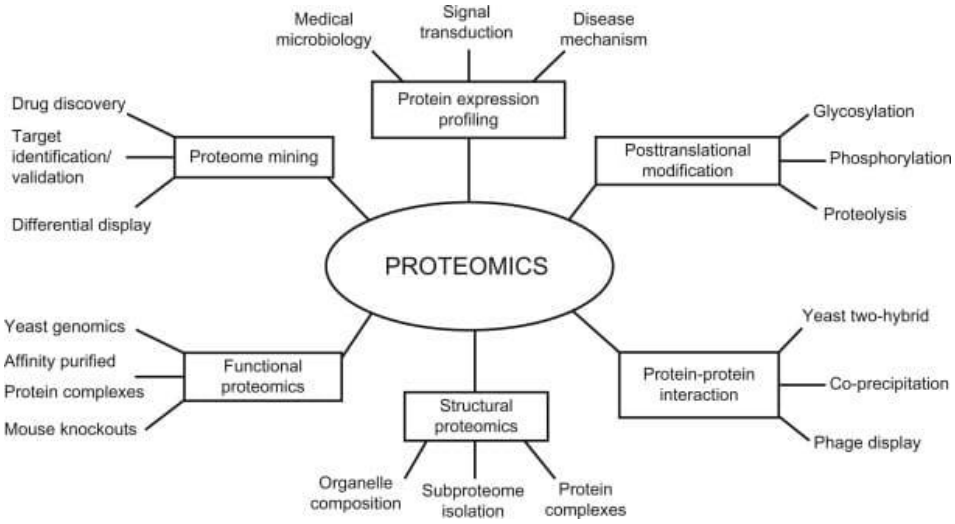

Figure. 2. Types of Proteomics and their applications to biology (Graves and Haystead, 2002)

Proteomics and plant metabolites: In the natural and agricultural environments, plants are subjected to a variety of biotic and abiotic stressors (Zipfel and Oldroyd, 2017), which threaten their survival and growth. Unfavourable environmental conditions like drought, too much salt, floods, extremely high and low temperatures, heavy metals, and radiation are examples of abiotic stressors (McDowell and Dangl, 2000; Sarwat *et al.* 2013). Pathogenic bacteria, fungi, viruses, and nematodes, as well as pest attacks and the invasion of parasitic plants, all cause biotic stress. It is estimated that pathogens and pests are responsible for 30.0% of rice yield losses globally in 2019 (Savary *et al*. 2019); therefore, these stresses pose a threat to food availability. Plants have evolved a variety of dynamic constitutive and inducible defensive mechanisms in response to biotic stimuli to shield themselves from the harm brought on by invasive diseases. In addition to structural rigidity, inherent defence mechanisms such as cell walls, waxy epidermal cuticles, and barks serve as the first line of defence. Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) are inducible defence mechanisms that have been clarified using a "zigzag" paradigm (Bigeard *et al*. 2015) (Jones and Dangl, 2006). The activation of complex networks of signalling cascades linked to the production of reactive oxygen species (ROS) and the activation of hormones is important. Furthermore, these cascades regulate kinase signalling to activate transcription factors (TFs). Because of this, several secondary metabolites (SMs) and antibacterial substances including phytoalexins and phenolics are produced (Jain *et al*. 2019).

The final genomic products, proteins and metabolites, play a crucial role in essential life processes. Plants use a variety of protein classes to combat biotic stress, such as: (1) catalytic enzymes involved in cell wall modifications, phytohormones, ROS, and pathogenesis-related (PR) proteins; (2) TFs and posttranslational factors; and (3) receptors and receptor-like kinases, (Wu *et al*. 2016; Wu *et al*. 2017; Meng *et al*. 2019). Plant metabolites, however, serve different purposes. According to Kang *et al*. (2019), there are more than 200,000 plant metabolites, which have been divided into three major categories: primary metabolites, secondary (or specialized) metabolites, and hormones, all of which serve overlapping purposes (Erb and Kliebenstein, 2020). These secondary metabolites act as antimicrobial compounds, damage-associated molecular patterns (DAMPs), and pathogen virulence factors, pollinator attractors in addition to regulating callose deposition and programmed cell death (Piasecka *et al*. 2015; Zaynab *et al*. 2018). Because they can be employed as medicines, colours, scents, nutritional supplements, and flavours, secondary metabolites have long piqued human curiosity. Unfortunately, a lot of the most advantageous secondary metabolites are produced in so small amounts that it is impossible to extract them for use in commerce. In an effort to manufacture more of these desirable molecules, researchers have investigated secondary metabolic pathways. The complexity of secondary metabolic pathways, which contain several enzymatic steps, considerable branching and crosstalk, and compartmentalization of various processes in various cell types or organelles, is another obstacle. Using feeding experiments and labelled intermediates, traditional methods for studying secondary metabolism require the step-by-step characterisation of individual processes. Since proteomics may detect not only enzymes but also regulatory proteins and proteins involved in the movement of intermediates between compartments, it can speed up the discovery process in plant secondary metabolism (Twyman, 2004).

Also, studying proteins and metabolites is critical to understanding plants' responses to different biotic stressors. It is also crucial to understand how proteins behave under stressed conditions to clarify how stress tolerance and crop injury are caused. The proteomics approach is used to detect and analyse proteins. A wide range of proteins can be identified using this tool, including changes in protein levels during specific developmental stages or under stress (Tan *et al*. 2017; Liu *et al*. 2019). Additionally, proteomics can reveal metabolic processes and their possible interactions with regulatory pathways. Proteomic techniques are ideal for analysing translated regions. Additionally, several proteins undergo post-translational modifications, such as removing signal peptides, phosphorylating, and glycosylating, which are crucial to their function. Thus, proteomics, combined with genome-sequence data and modern bioinformatics, provides a powerful tool for identifying and characterizing novel proteins as well as illustrating changes in relative protein abundances over time (Hossain and Komatsu, 2014a,b). A metabolomics approach provides a more efficient mechanism for detecting metabolites that are end products of different regulatory processes and elucidating molecular mechanisms responsible for variations in plants (Arbona *et al*. 2013).

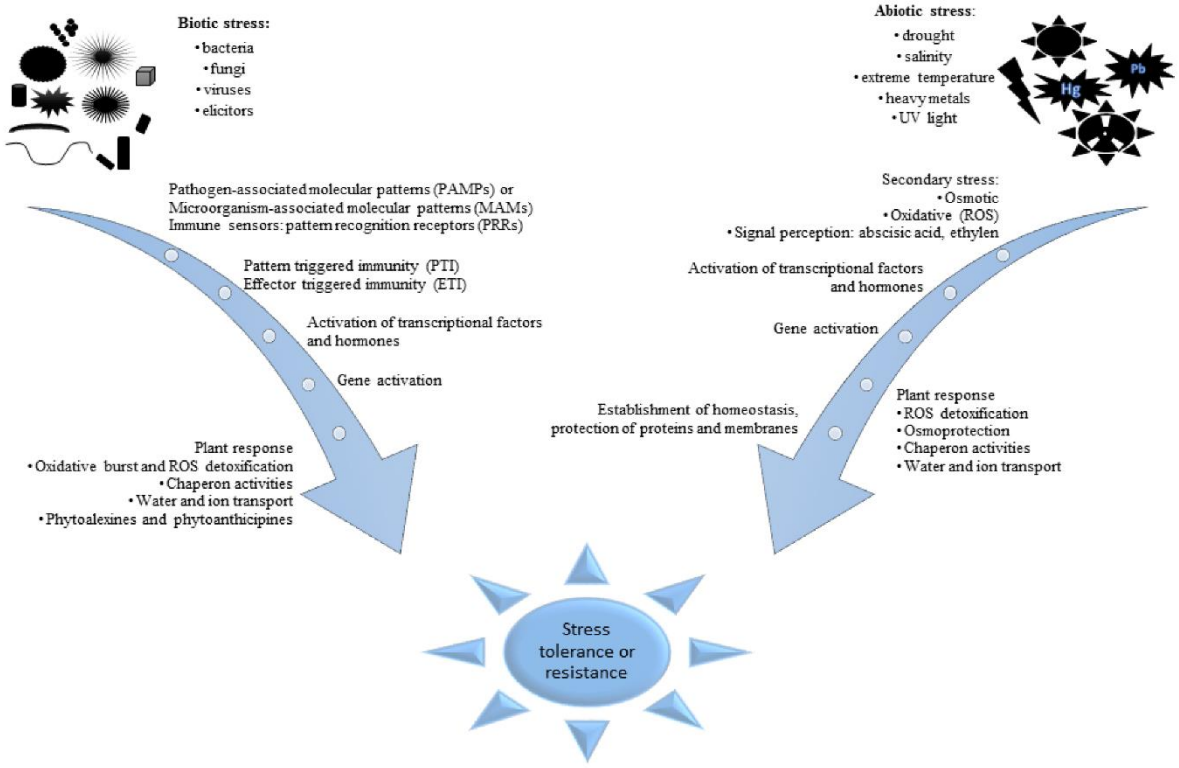

Figure. 3. Stages of a plant's response to biotic and abiotic stressors. Specific mechanisms may respond differently to different environmental inputs. Each one, however, results in a better plant's ability to respond to stress and a future response that is more effective. Source: Piasecka *et al*. 2019

Techniques used to study plant proteomics and metabolomics: Analyses of proteomics and metabolomics use a range of methods. For instance, gel-based or gel-free methodologies can be used in proteomics studies. For global protein studies, gel-based techniques, such as two-dimensional gel electrophoresis (2-DE) and difference gel electrophoresis, are most frequently used (DIGE). Numerous proteins can be found in a single polyacrylamide gel using sophisticated mass spectrometry (MS) methods, enabling investigations of their mass-to-charge ratio and post-translational changes (Vanderschuren *et al*. 2013; Tan *et al*. 2017).

Gel-free methods were created to overcome the shortcomings of gel-based methods, such as repeatability, bias, the requirement for technical skill, and challenges in detecting proteins that are scarce or have high acidity or basicity (Tan *et al*. 2017). Three labelling techniques are used in this method: metabolic labelling using stable isotope labelling by amino acids in cell culture (SILAC) and 15N labelling; tag-based labelling using isotope-coded affinity tag (ICAT), isobaric tags relative and absolute quantification (iTRAQ), tandem mass tag (TMT), and dimethyl and 18O labelling; and label-free techniques using multidimensional capillary liquid chromatography (LC) coupled to nano-electrosp (Tan *et al*. 2017; Ludwig *et al*. 2018).

A variety of metabolomics techniques, including gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) combined with mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, have been developed as a result of advances in analytical chemistry (Fukusaki and Kobayashi, 2005; Piasecka *et al*. 2019). A common technique for measuring the amounts

of volatile and semi-volatile organic chemicals in various samples is gas chromatography-mass spectrometry (GC-MS). On the other hand, LC-MS is a more thorough approach, and crude extracts can be used to measure a range of metabolites. By combining high-resolution mass analysis techniques like time-of-flight, Fourier analysis, and Orbitrap-based MS with ultra-performance liquid chromatography over time, LCMS has been improved, enabling the collection of more effective metabolomics data (Salem *et al*. 2020).

Another effective method is CE-MS, however, due to the labour-intensive and varied extraction requirements, it is rarely utilised to evaluate plant metabolites. However, this extremely sensitive technique, in particular for highly charged metabolites, can categorise metabolites into classes that other techniques cannot (Fukusaki and Kobayashi, 2005; Salem *et al*. 2020). NMR spectroscopy is thought to be less biased because it is ionization independent. Additionally, this technique may detect novel chemicals, requires little sample preparation, and is extremely reproducible (Valentino *et al*. 2020). NMR is hardly ever utilised to examine the metabolomics of rice under biotic stress.

In order to effectively identify and categorise proteins and metabolites based on their unique activities and associated pathways, proteomics and metabolomics require the use of numerous analysis techniques and databases. The specifics of various analysis tools have also been well-documented (Piasecka *et al*. 2019; Sarim *et al*. 2020). Gene Ontology Knowledgebase (http://geneontology.org/), the Protein Database of the National Center for Biotechnology Information, RiceCyc (http://pathway.gramene.org/gramene/ricecyc.shtml), OryzaCyc in the Plant Metabolic Network database (www.plantcyc.org), and Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) are some of the frequently used databases.

A. Conventional techniques

1. Chromatography-based techniques

1.1 Ion exchange chromatography: The IEC is a versatile tool for protein purification because of the charged groups on its surface. Different proteins have different amino acid sequences; some amino acids are anionic and others are cationic. The net charged content of a protein at physiological pH is calculated using equilibrium between these charges. The protein is divided first by the type of charge (anionic or cationic), and then by the relative strength of the charge. The IEC is very valuable because it is cheap and has buffer-condition survival capabilities (Jungbauer and Hahn, 2009).

Four peaks were obtained in the full separation of *Nigella sativa* proteins that preserve immune modulatory activity after IEC fractionation (Haq *et al*. 1999). Pharmaceutical products economically value proteins produced in transgenic plants. Aprotinin is one such; it is a serine protease inhibitor produced in maize seed and refined via IEC (Azzoni *et al*. 2005).

1.2 Size exclusion chromatography: Proteins are separated by SEC on the basis of molecular size by passing through a porous carrier matrix with distinct pore sizes based on permeation. The SEC is a reliable method that can handle proteins under a variety of physiological circumstances, including those involving the presence of detergents, ions, co-factors, or different temperatures. The SEC is an effective method for the purification of non-covalent multi-meric protein complexes under biological circumstances and is used to separate low molecular weight proteins (Voedisch and Thie, 2010).

Through SEC, *A. thaliana*'s intrinsically disordered proteins were also purified. These are expressed at a later stage of seed development and play a big part in signal transduction and transcription control (Yoo *et al.* 2014).

1.3 Affinity chromatography: A significant advance in protein purification was made using affinity chromatography, which allows scientists to study protein breakdown, post-translational changes, and protein-protein interactions. The reversible interaction between the affinity ligand of the chromatographic matrix and the proteins to be purified is the fundamental tenet of affinity chromatography. There are many uses for affinity chromatography in identifying microbial enzymes that are primarily responsible for disease (Hage *et al*. 2012).

By using metal chelate affinity chromatography, the HIV-I reverse transcriptase homodimer and heterodimer were quickly isolated (Grice *et al.* 1990). The practical uses of bacteriophages in biotechnology and medicine convince people that phage purification is overly necessary. Away from bacterial waste and other contaminating bacteriophages, the T4 bacteriophages have been purified. Both fusion and wild-type proteins were generated by the bacteria in the "competitive phage display." The fusion proteins were incorporated into the phage capsid and made it possible to successfully purify T4 bacteriophages (Ceglarek *et al.* 2013).

1.4 Enzyme-linked immunosorbent assay: The ELISA is a popular immunoassay for diagnostic application and is extremely sensitive. The assay makes use of the presence of an antigen or antibodies on a solid surface, the addition of enzyme-conjugated antibodies, and the measurement of changes in enzyme activities that are inversely correlated with the levels of an antigen and antibodies in a biological material (Lequin, 2005).

In order to safeguard those who are sensitive to wheat, wheat proteins have been identified in meals that trigger allergic reactions in vulnerable individuals using ELISA kit (Sharma, 2012). *Bacillus thuringiensis* Cry1Ac protein was detected using a sandwich ELISA from transgenic BT cotton since its release has a negative impact on the environment (Wang *et al*. 2007). To find *Botrytis cinerea* in fruit tissue, an indirect competitive ELISA was created. The phytopathogenic fungus *B. cinerea*, which causes grey mould and frequently manifests as a latent infection, deteriorates good fruits (Fernández *et al*. 2011).

1.5 Western blotting: In Western blotting, a low abundance protein is precisely detected by enzyme conjugated antibodies after being separated from other proteins using electrophoresis, transferred to nitrocellulose membrane, and other steps (Kurien and Scofield, 2006). Western blotting is a popular method for identifying antigens from a variety of microorganisms and is useful for identifying infectious illnesses.

In order to identify and validate 10 rice reference proteins, Li *et al*. used Western blotting. The two most prevalently expressed proteins in rice were elongation factor 1 and heat shock proteins (Li *et al*. 2011). The Plum Pox Virus (PPV) capsid proteins from infected *Nicotiana benthamiana* were discovered by Kollerova *et al*. (2008). Western blot analysis was used to confirm that the *Plasmodium falciparum* PfCP-2.9 gene was expressed in tomatoes (Kantor *et al*. 2013). Western blotting was used to identify the specific IgE against Ara h1, Ara h2, and Ara h3 in patients with peanut allergies (Koppelman, *et al*. 2004).

1.6 Edman sequencing: The procedure includes chemical reactions to remove and identify residues of amino acids that are found at the N-terminus of polypeptide chains. The development of therapeutic proteins and the quality control of biopharmaceuticals were significantly aided by Edman sequencing (Smith, 2001).

Due to the consumption of bakery goods and fast meals, the prevalence of sesame seed allergy has been rising. Through the use of 2D-PAGE and SDS-PAGE, the main Sesamum indicum allergy proteins from allergic patients have been identified for further analysis using Edman sequencing. These proteins' IgE-binding epitopes were discovered, and they may be useful in immunotherapeutic methods (Beyer *et al.* 2002). To ascertain their function, rice leaf sheath proteins were isolated and subjected to MS and Edman sequencing analysis. Since the majority of proteins tested by both approaches have a similar amino-acid sequence, it is suggested that these two methods be used to identify plant proteins.

2. Advanced techniques

2.1 Protein microarray: The growing family of proteomics techniques that can perform high-throughput detection from a tiny amount of sample are protein microarrays, commonly referred to as protein chips. Analytical protein microarrays, functional protein microarrays, and reverse phase protein microarrays are the three categories into which protein microarrays can be divided (Sutandy *et al*. 2001).

2.2 Analytical protein microarray: The most representative category of analytical protein microarrays is the antibody microarray. Direct protein labelling is used to find proteins that have been captured by antibodies. These are often employed to assess the degree of protein expression and binding affinities (Sutandy *et al*. 2001; Ebhardt *et al*. 2015; Rosenberg and Utz, 2015).

In order to characterize plant kinases using protein microarrays and identify cellular signalling networks, analytical and experimental methods have been established (Brauer *et al.* 2014). *Arabidopsis*'s mitogen-activated protein kinases (MAPKs) have been described. Plants that respond to a variety of external stimuli use molecules called MAPKs, which are highly conserved single transduction molecules (Feilner *et al*. 2005).

2.3 Functional protein microarray: Purified proteins are used to create functional protein microarrays, which allow for the investigation of a variety of interactions, including those involving protein-DNA, protein-RNA, protein-protein, protein-drug, protein-lipid, and enzyme-substrate relationships (Sutandy *et al.*2001). Analysis of the substrate selectivity of protein kinases in yeast was the first application of functional protein microarray (Zhu *et al*. 2001). The activities of thousands of proteins were identified via functional protein microarray. *A. thaliana*'s protein-protein interactions were investigated, and Calmodulin-like proteins (CML) and Calmodulin substrates (CaM) were discovered (Popescu *et al*. 2007).

2.4 Reverse-phase protein microarray: Arrayed on nitrocellulose slides that are probed with antibodies against target proteins are cell lysates collected from various cell states. Then, using fluorescence, chemiluminescent, and colorimetric assays, antibodies are found. Reference peptides are printed on slides to quantify proteins. To identify the changed or dysfunctional protein characteristic of a particular disease, these microarrays are used (Sutandy *et al*. 2001).

The phosphorylation status and protein expression in human stem cells and acute myelogenous leukaemia cells were examined on a wide scale using reverse-phase protein microarray analysis of hematopoietic stem cell and primary leukaemia samples (Tibes *et al*. 2006). By observing the apoptosis, DNA damage, cell-cycle control, and signalling pathways, the reverse-phase protein microarray technique was tested for quantitative analysis of phosphoproteins and other cancer-related proteins in non-small cell lung cancer (NSCLC) cell lines (Ummanni *et al*. 2014).

B. Gel-based approaches

1. Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis: SDS-PAGE is a high-resolution method for sorting proteins based on their size, which makes it easier to approximate molecular weight. In a liquid with a pH different from their isoelectric point, proteins are capable of moving with an electric field. Depending on the ratio of charge to mass, various proteins in a mixture move at varying speeds. Proteins are denatured by the addition of sodium dodecyl sulfate, thus they must be separated completely by molecular weight (Dunn, 1986).

The *cleome* spp. is particularly beneficial for the treatment of cough, fever, asthma, rheumatism, and many other ailments. They are consumed as green vegetables in African nations. SDS-PAGE was used to compare the proteins in the leaves and seeds of various cleome species (Aparadh *et al*. 2012). Under drought stress and non-stress circumstances, the chickpea (*Cicer arientinum*) seed and leaf storage proteins were profiled (Kakaei *et al*. 2012). *Brassica* species' seed storage proteins are also identified in order to assess the genetic diversity among various genotypes (Sadia *et al*. 2009).

2. Two-dimensional gel electrophoresis: Proteins can be separated based on their mass and charge using the effective and dependable two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Depending on the size of the gel, 2D-PAGE can resolve 5,000 distinct proteins in succession. In the first dimension, the proteins are divided based on charge, while in the second, they are divided based on variations in mass. The 2-DE is successfully used for the study of metabolic pathways, post-translational modifications, mutant proteins, and post-translational modifications. 2-DE, a highly sensitive method, was brought into the study of bacterial physiology by Neidhardt and van Bogelen (Issaq *et al*. 2008).

Due to the low protein concentration, high protease activity, and high levels of interfering substances like polyphenols, flavonoids, terpenes, lignans, and tannins, protein extraction from grapes is difficult; however, Marsoni *et al*. (2015) were able to successfully extract the proteins from grape tissue using 2-DE. The mature rice leaves' proteins were likewise isolated by Islam *et al*. (2004) and used in the proteome study.

3. Two-dimensional differential gel electrophoresis: By activating the dye at a certain wavelength, proteins that have been tagged with Cy Dye can be easily seen in 2D-DIGE. Because the plasma membrane reacts to both biotic and abiotic stress in plants, the characterization of plasma proteins offers new insight into the biological processes that are unique to plants. Rice and *A. thaliana*'s plasma membrane proteomes were described by Komatsu (Marouga *et al*. 2005). We looked into how rice plants that were 10 days old responded to salt stress using their apoplastic proteins. Soluble apoplastic proteins from the rice shoot stem were isolated for differential analysis and compared to untreated samples; it was discovered that these proteins were engaged in oxidation-reduction reactions, glucose metabolism, and protein processing and degradation (Song *et al*. 2011).

Due to challenges with protein extraction, the quantity of proteins in various plant tissues and the absence of well-defined genome sequences, quantitative plant proteomics is more difficult. For MS to be used successfully in proteomics, it must have higher resolution power, exact mass measurements, higher scanning rates, and precise chromatogram alignment (Abdallah *et al*. 2012).

C. Quantitative techniques

1. ICAT labelling: Chemical labelling chemicals are employed in the ICAT, an isotopic labelling technique, to quantify proteins. The ICAT has also increased the variety of proteins that may be studied and made it possible to precisely quantify and identify protein sequences from intricate combinations. The ICAT reagents include reactive group, isotopically coded linker, and affinity tag for isolating labelled peptides (Shiio and Aebersold, 2006).

With almost 4,000 genes, Mycobacterium TB is regarded as one of the most significant human pathogens. Combining Liquid Chromatography (LC), Tandem Mass Spectrometry (MS/MS), and ICAT, the proteome analysis was completed (Schmidt *et al.* 2004). Additionally, the combination of approaches enables a thorough understanding of the biological system.

2. Stable isotopic labelling with amino acids in cell culture: SILAC is a quantitative proteomics method based on MS that relies on metabolic labelling of the entire cellular proteome. Through the use of "light" or "heavy" amino acid labels, the proteomes of various cells raised in cell culture are distinguished through MS. The SILAC has been created as a quick method to research post-translational changes, cell signalling, and the regulation of gene expression. SILAC is a crucial method for secretory pathways and proteins in cell culture as well (Ong and Mann, 2006).

In order to determine the proteome turnover rate and changes in metabolism under salt stress conditions, the salt stress response and protein dynamics in the photosynthetic bacterium *Chlamydomonas reinhardtii* have been examined using SILAC. The most prevalent protein in *C. reinhardtii* was shown to be RuBisCO (Mastrobuoni *et al*. 2012).

3. X-ray crystallography: The method of choice for determining the three-dimensional structure of proteins is X-ray crystallography. The size of the repeating unit that produces the crystal and the symmetry of the crystal packing are revealed by subjecting the highly pure crystallised samples to X-rays and processing the resulting diffraction patterns. Numerous topics like the viral system, protein-nucleic acid complexes, and immunological complexes can be studied using X-ray crystallography. Additionally, the precise information regarding the clarification of enzyme mechanism, drug design, site-directed mutagenesis, and protein-ligand interaction is provided by the three-dimensional protein structure (Smyth and Martin, 2000).

Non-specific lipid transfer proteins allow phospholipids, glycolipids, steroids, and fatty acids to flow between membranes (nsLTPs). The volume of the hydrophobic cavity varies depending on the size of the bound ligands, according to the comparative structure of maize nsLTP in complex with many ligands (Han *et al*. 2001).

D. High-throughout techniques

1. Mass spectrometry: The mass to charge ratio (m/z), which is measured by MS and is useful for figuring out the molecular weight of proteins. Three steps make up the entire process. In the first stage, the molecules must be converted to gas-phase ions, which is difficult for biomolecules in a liquid or solid phase. In the second phase, ions are separated according on their m/z values in a space known as a mass analyzer while being subjected to electric or magnetic fields. The separated ions are then measured, together with the quantity of each species with a certain m/z value. The most popular ionisation techniques include electrospray ionisation (ESI), surface enhanced laser desorption/ionization (SELDI), and matrix-assisted laser desorption ionisation (MALDI) (Yates, 2011).

Through the use of MS, post-translational modifications in plants, such as protein phosphorylation, have been identified. After *Fusarium oxysporum* infection, the most prevalent proteins in tomato (*Lycopersicon esculentum*) xylem sap were found using mass spectrometric sequencing and peptide mass fingerprinting (Novakova *et al*. 2011).

2. NMR spectroscopy: The NMR is a top technique for examining the molecular makeup, protein folding, and behaviour. NMR spectroscopy often comprises several steps, each utilising a distinct set of extremely specialised procedures. To verify the structure, samples are prepared, measurements are taken, and then interpretive procedures are used. In numerous fields of research, including structure-based drug design, homology modelling, and functional genomics, the protein structure is crucial (Wiese *et al*. 2007).

For the soil to receive the necessary nutrients and for the atmosphere to produce CO2, plant litter decomposition is crucial to the nitrogen and carbon cycles. We employed HR-MAS NMR spectroscopy to track the environmental degradation of wheatgrass and pine residues using 15N- and 13C-labeled plant components. All plant tissues lost condensed and hydrolysable tannin, while aliphatic components (cuticles, waxes), aromatic (partly lignin), and a tiny amount of carbohydrate persisted, according to the spectra (Kelleher *et al*. 2006).

E. Bioinformatics analysis: Proteomics requires the use of bioinformatics, therefore its implications have been growing along with the development of high-throughput techniques that rely on robust data processing. Novel techniques are being offered by this young and developing discipline to handle enormous and diverse proteome data and advance the discovery process (Vihinen, 2001).

Over the past few years, the use of bioinformatics for proteomics has become much more popular. The creation of a new algorithm that allows for the analysis of larger amounts of data with greater specificity and accuracy aids in the identification and quantification of proteins, making it possible to obtain detailed information about the expression of proteins.

An overview of common proteomic technologies, applications and their limitations are briefed in table no. 1.

Method used to study plant proteomics and metabolomics:

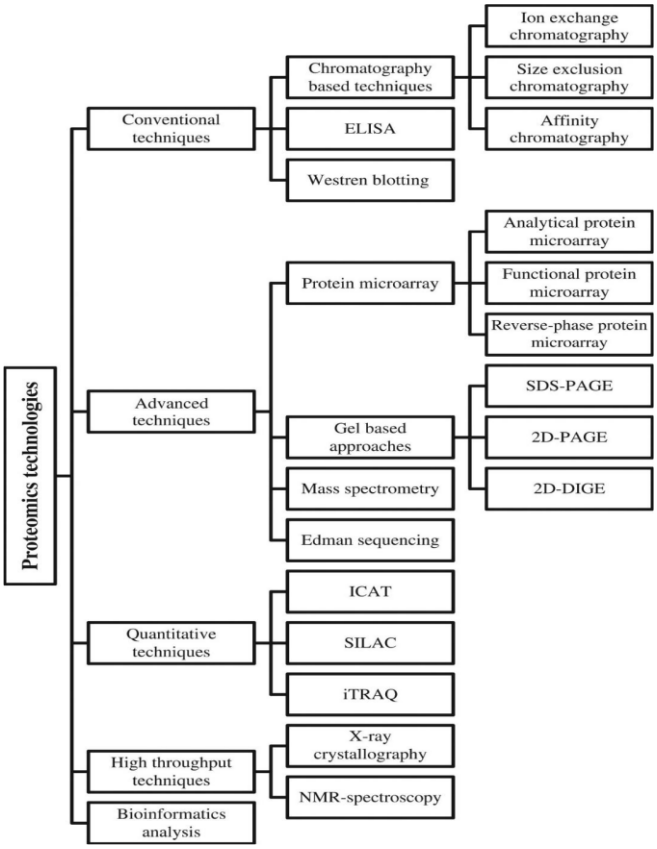

Figure. 4. An overview of proteomics techniques (Aslam *et al.* 2016)

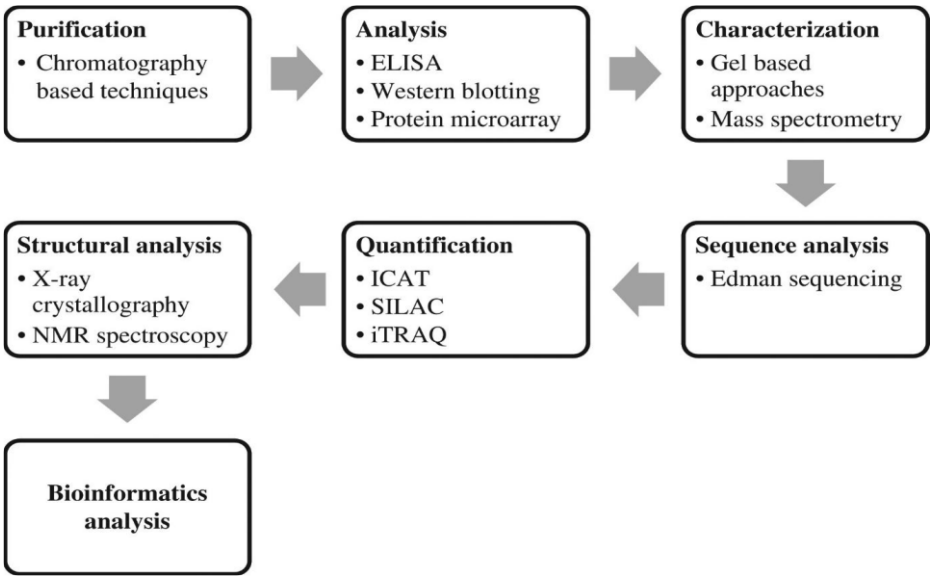

Figure. 5. Applications of proteomics techniques (Aslam *et al.* 2016)

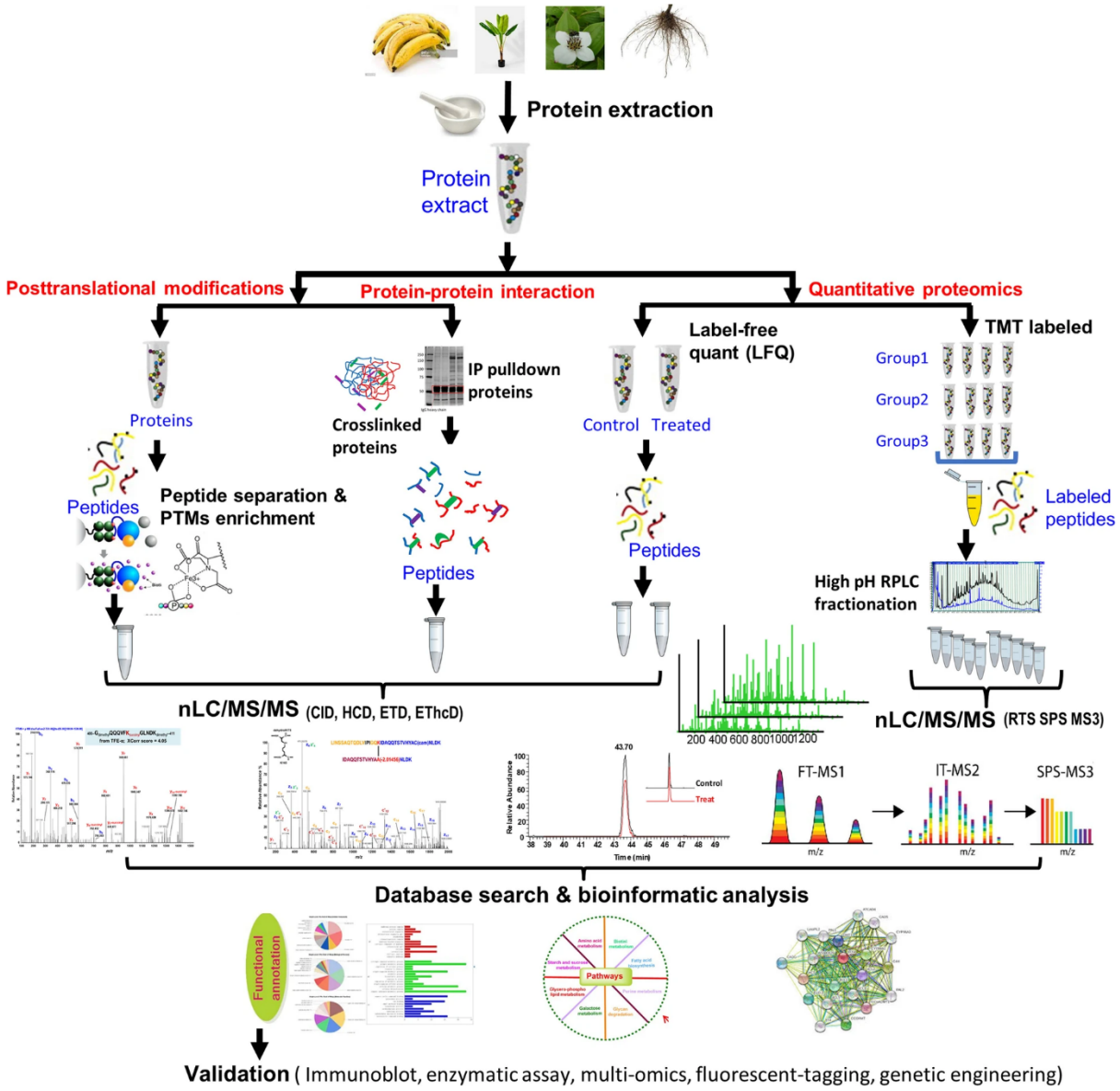

Figure. 6. Workflows for broad plant proteomics are shown schematically. Using either gelbased or gel-free procedures, proteins that have been isolated from plant materials are fractionated. For the identification and measurement of posttranslational modifications, protein-protein interactions, and quantitative proteomics, three main techniques of proteomics analysis were presented. Database searches, statistical analysis, and bioinformatics analysis are used to process the MS raw data produced by each workflow utilising various mass spectrometric techniques. Last but not least, the results need to be further verified in order to either generate biological hypotheses or confirm the earlier mechanistic hypotheses. Source: Yan *et al.* 2022

Table 1. Common proteomic technologies, applications and their limitations

Technology	Application	Strengths	Limitations
2DE	Protein separation Quantitative expression profiling	Relative Quantitative PTM information.	Poor separation of acidic, basis, hydrophobic and low abundant proteins.
DIGE	Protein separation Quantitative expression profiling	Relative Quantitative PTM information High sensitivity Reduction of intergel variability	Proteins without lysine cannot be labeled Requires special equipment for visualization and fluorophores are very expensive
ICAT	Chemical isotope labeling for quantitative proteomics	Sensitive and Reproducible Detect peptides with low expression levels.	Proteins without cysteine residues and acidic proteins are not detected
SILAC	Direct isotope labeling of cells Differential expression pattern	Degree of labelling is very high Quantitation is straightforward	SILAC labeling of tissue samples is not possible
iTRAQ	Isobaric tagging of peptides	Multiplex several Samples Relative quantification High-throughput	Increases sample complexity Require fractionation of peptides before MS.
MUDPIT	Identification of protein-protein interactions Deconvolve complex sets of proteins	High separation Large protein complexes identification	Not quantitative Difficulty in analyzing the huge data set Difficult to identify isoforms
Protein array	Quantitate specific proteins used in diagnostics (biomarkers or antibody detection)	High-throughput Highly sensitive Low sample consumption	Limited protein production Poor expression methods Availability of the

	and discovery research		antibodies Accessing very large numbers of affinity reagents.

Source: Chandramouli and Qian, 2009

Protein Quantification				
Relative Quantification	*procedure*	*Rationale*	*application in plants*	*References*
2-dimensional gel electrophoresis (2D-PAGE)	The protein complement of 2 or more differentially treated samples is compared, either between different gels or on the same gel after differential labeling (DIGE)	Proteins that are affected by the treatment decrease or increase in abundance (determined by staining) and modified proteins will be shifted in molecular mass and/or isoelectric point	• Proteome response to different types of biotic and abiotic stresses (cold, salt, osmotic stress, elevated CO_2 levels, pathogen attack) • Light/dark shift of etiolated rice seedlings (plastid proteome analysis) • Red/far-red/blue light response of entire plants • Effect of auxin application on roots • Soluble chloroplast proteome in different differentiated cell types • Effect of anoxia on the mitochondrial proteome • Pollen development following pollination • Cotyledon dedifferentiation after hormone treatment • Seedling development/combined with phosphoprotein staining	Riccardi et al., 1998, Salekdeh et al., 2002, Bae et al 2003, Abbasi et al., 2004, Millar et al., 2004, Hajheidari et al., 2005, Cui et al., 2005, Majeran et al., 2005, Taylor et al., 2005, Kim et al., 2006, Sorin et al., 2006, Dai et al., 2007, Kleffmann et al., 2007
ICAT	Isotope labeling at Cys, mixing of two samples	Intensity read-back (TIC) comparison of isotopically labeled and unlabeled peptide	• Localization of isotopically labeled peptides in a density gradient (LOPIT), inference of protein localization by pairwise comparison of abundance profiles • Comparison of chloroplast proteome in different differentiated cell types	Majeran et al., 2005, Dunkley et al., 2006
ITRAQ	Isotope labeling at primary amine groups, mixing of up to eight samples	Intensity read-back (TIC) comparison of reporter ions dissociated from differentially labeled peptides	• Quantitative comparison of CLP mutants • Dissection of the phosphoproteome response to *Pseudomas syringae* attack • Phosphorylation dynamics at the plasma membrane in response to flagellin elicitation	Jones et al., 2006, Rudella et al., 2006, Nuhse et al., 2007
TIC comparison	Peptide's TIC read-back in two different samples	Identical ionization properties of identical peptides allow TIC comparison between different LC runs (neglects ion suppression effects)	• Proof-of-concept comparison of etiolated and illuminated Arabidopsis cell cultures • Comparison of chloroplast proteomes in different differentiated cell types	Majeran et al., 2005, Fischer et al., 2006
Metabolic labeling	Labeling of *de novo* synthesized proteins by growing cells on ^{15}N containing media as the only nitrogen source	Proteins from cells grown with ^{15}N as the only nitrogen source can be distinguished from those of other cells by a mass shift in MS, quantification by intensity read-back ratio (TIC)	• Proof-of-concept comparison of etiolated and illuminated Arabidopsis plants (partial metabolic labeling) • Proof-of-concept study for the labeling of entire plants • Quantitative proteomics of leaf senescence • Quantitative analysis of the heat shock response	Engelsberger et al., 2006, Huttlin et al., 2007, Hebeler et al., 2008, Palmblad et al., 2008
SILAC	Similar to metabolic labeling, but instead of inorganic ^{15}N, labeled amino acids are provided	Proteins from cells grown with labeled amino acids can be distinguished from those of other cells by a mass shift in MS, quantification by intensity read-back ratio (TIC)	• Proof-of-concept study to analyze the effect of salicylic acid treatment on the accumulation of glutathione S-transferases	Gruhler et al., 2005
Absolute Quantification				
Spiking isotopically labeled peptide into sample (AQUA)	Isotope labeling of selected peptides, spiking into sample	Comparison of native peptide to TIC of labeled peptide with known concentration	• Proof-of-concept quantification of sucrose synthase from complex protein mixtures	Wienkopp and Weckwerth, 2006
Peptide count (APEX)	Counting identified peptides per protein, normalization to expected peptides or MW and overall sampling depth	Number of detected peptides is proportional to protein abundance	• Assessment of protein abundance in a chloroplast proteome analysis • Analysis of the correlation between transcript and protein accumulation in different plant organs	Kleffmann et al., 2004 Baerenfaller et al., 2008
Staining methods combined with gel electrophoresis	Intensity, combined with MS-based protein identification	Staining intensity is proportional to protein abundance	• Analysis of the native stroma proteome of chloroplasts, colorless native gels were stained and the staining intensity used for protein complex quantification	Peltier et al., 2006

Figure. 7. Protein quantification methods and their application to plant proteomics (Baginsky, 2008)

Top-down proteomics and bottom-up proteomics approaches

1. Top-down proteomics: This method separates the proteins in a sample of interest before characterising each one separately. Protein separation is carried out using techniques like 2DE, DIGE, or MS based on mass and charge. The proteins are initially separated on the gel when employing 2D electrophoresis techniques, and then each one is individually digested into peptides that are then examined by a mass spectrometer. When employing a mass spectrometer (MS) directly, the undigested sample including the full proteins is introduced into the instrument, the proteins are sorted, and then specific proteins are chosen for digestion and a further round of MS to analyse the peptides (Beeton-Kempen, 2020).

Top-down Research on various PTMs (Post translational modifications) and protein isoforms is more suited for MS. However, it is constrained by the challenges associated with purifying complex protein mixtures and the declining sensitivity of MS toward larger proteins (namely > 50 to 70 kDa) (Beeton-Kempen, 2020).

2. Bottom-up proteomics or "shotgun" proteomics: To determine which proteins were present in the sample, all of the proteins are first digested into a complicated mixture of peptides, and then these peptides are examined. Initially, proteins are digested, and the resulting peptide mixture is fractionated before being submitted to MS, often in an LC-MS/MS setup. Automated search techniques are used to compare the obtained peptide sequences to databases that already exist. These search engines compare the anticipated spectra of proteins produced by in silico digestion to the experimentally acquired peptide spectra (this is known as "peptide-spectrum matching") (Beeton-Kempen, 2020).

Bottom-up workflows can take a variety of forms, including data-dependent, data-independent, and hybrid approaches. Bottom-up MS depends primarily on inference and uses peptides (between 5 and 20 amino acids in length) because they are simpler to fractionate, ionise, and fragment. This method gives an indirect indication of the proteins that were initially present in samples (Beeton-Kempen, 2020).

Applications of proteomics

Personalized Medicine: To increase efficacy and minimise side effects, disease treatments should be personalised for each patient based on their genetic and epigenetic composition. Proteomics data will probably contribute another dimension for patient-specific therapy, even if genomes and transcriptomics have been the main focus of such studies up until now (Beeton-Kempen, 2020).

Biomarker discovery: Identification of protein indicators for e.g., glioblastoma diagnosis and prognosis, and assessing patients' responses to treatment therapies such as stem cell transplants (Beeton-Kempen, 2020).

Drug discovery and development: Selecting suitable protein targets for drug development, determining their drug ability, and creating medicines that are directed at these prospective therapeutic protein targets (*e.g.*, for hepatocellular carcinoma) (Beeton-Kempen, 2020).

Systems biology: Systematic studies of medication action, toxicity, resistance, and efficacy; systemic studies of disease pathways and host-pathogen interactions to identify prospective biomarkers and therapeutic targets (Beeton-Kempen, 2020).

Agriculture: Studies into the connections between plants and pathogens, as well as crop engineering for improved resistance to environmental challenges like flooding and drought (Beeton-Kempen, 2020).

Food science: The improvement of food nutrition, allergen identification, and food safety and quality management (Beeton-Kempen, 2020).

Paleoproteomics: The investigation of prehistoric proteins to deepen our knowledge of evolution and archaeology (Beeton-Kempen, 2020).

Astrobiology: Studies on the primordial organic materials discovered on meteorites and how mammals' immune systems may react to exo-microbes found in space (Beeton-Kempen, 2020).

Challenges: The inability to measure the full proteome presents a fundamental difficulty in proteomics when researching plants or any other complex biological system (Ahn *et al*. 2007). The ability to perform large-scale protein quantification and allow for comparative proteomics research is a key barrier to proteomics (Schulze and Usadel, 2010). This would give the technology a strong biological application and make it possible to examine overall protein changes throughout processes like plant development or stress reactions. The inability to quantify the whole protein complement as well as technological restrictions in quantification methods are currently the limits of comparative proteomics. Early comparative studies compared samples of interest using 2D-PAGE arrays, but as technology advanced, multiplexing and enhanced sensitivity, fluorescent dyes were added. Although the method still has problems with the 2Dinherent PAGE's drawbacks (Taylor *et al*. 2011). As sample analysis has moved away from gel arraying methods, methods utilizing mass spectrometry quantification have become increasingly popular.

Future prospects: Agricultural crops are increasingly at risk of having their yields reduced by biotic stress, thus it is crucial to understand how plants react to it. The fundamental knowledge of how plants react to biotic stress needs to be further developed. Precise control targets for plant immunity will be revealed by an understanding of proteomics at the cellular and subcellular levels. Recent research has shown that complex molecular pathways play a role in biotic stress, as was previously discussed. Measurement of proteins with important roles and contributions to biotic stress processes is possible using MS-based proteomic approaches. However, there are still a lot of unanswered problems surrounding plant proteomics and its use in biotic stress (Kieu *et al*. 2021).

Proteomics has been shown to be a useful approach for identifying proteins involved in plants' reactions to abiotic stress, enabling functional genome analysis. Proteins, such as enzymes involved in the removal of ROS and protein heat shock (HSP), have been identified as common mechanisms of response to various abiotic stress factors (water and salt), which are expressed in different parts of the cell. HSP70 is particularly prevalent in water and salt stress. The production of proteins involved in the manufacture of osmolytes, aquaporins, and LEA proteins linked to water stress are only a few examples of the specific response mechanisms to stress that have been discovered (Kieu *et al*. 2021).

Proteomics enables us to create cutting-edge techniques to improve the tolerance of various plants and crops to biotic and abiotic stress conditions and so helps to our understanding of the intricate mechanisms of plant response to environmental influences (abiotic and biotic stress). Utilizing various biological models (mutant or transgenic plants), comparative proteomic studies on various tissues,

organs, organelles, and membranes allow for the monitoring of protein expression at various times, greatly aiding in the understanding of the mechanisms of adaptation of plants under various stress conditions. Networks of interactions between the genes, proteins, and metabolites involved in stress response mechanisms will be established with the aid of additional research on protein-protein and protein ligand interactions as well as advancements in techniques like genomics, transcriptomics, and metabolomics (Kieu *et al*. 2021).

Resolve proteins with pI (s) ends and lessen the impact of abundant proteins like Rubisco, which obstruct the display of other proteins of interest, in order to improve protein extraction processes in plant tissues, which are thought to be refractory. With the development of new methodologies, known as second generation proteomics, which address some issues related to the analytical variability of the technique, quantitative studies of proteins may become more common. These methodologies enable the achievement of results with greater reproducibility, in stages of development and protein comparison of organs and between genotypes. The development of new computing platforms will facilitate the information sharing and quick advancement of proteomics research globally (Kieu *et al*. 2021).

Conclusion: Cells rely on proteins to perform their functions. In the cell, they perform almost all biochemical functions and interact with a wide range of molecules. Thus, proteins are functionally the most important components of biological systems, and a true understanding of these systems can only be acquired through direct analysis of proteins. Over the past few years, the study of proteins has gained much attention as a growing field in biology. Novel techniques for the separation and identification of proteins play an important role in the field's success. Gel-based methods, such as 2D-PAGE and 2D-DIGE, and non gel-based methods exist. The most commonly used methods for identifying proteins are mass-based techniques such as MALDI. Proteins at the proteome level are accurately quantified using stable isotope labelling methods, such as SILAC and iTRAQ, and label-free methods. X-ray crystallography and NMR spectrometry are used in structural proteomics. Abiotic stress responses in plants can be identified through proteomics, allowing functional genome analysis. Many biotic and abiotic stress factors can be responded to by proteins, which are expressed in different parts of the cell. Thus, this omic approach contributes to the understanding of plant responses to environmental factors. Identification of stress-responsive target proteins and their post-translational modifications. Proteome-level research is needed to better understand the minute changes in a cell's protein signature in response to flooding and drought stress. It is possible to monitor protein expression in different tissues, organs, organelles, and membranes by using different biological models (mutant or transgenic plants), which contribute greatly to understanding the mechanisms of plant adaptation under stress. Using comparative organelle proteomes could provide valuable insight into stress signalling pathways. Further improvements in sample preparation and fractionation strategies are needed to further understand plant stress response mechanisms using convergent MS techniques and bioinformatics.

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