PROTEOME ANALYSIS TO DECODE PLANT-MICROBE INTERACTIONS IN THE RHIZOSPHERE

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Abstract

Understanding the complex interactions between plants and microbes in the rhizosphere is vital for improving agricultural productivity and promoting sustainable crop production. To unravel theseinteractions, a comprehensive approach incorporating OMICS techniques such as proteome and secretome analysis is employed. Proteome analysis, which examines the complete set of proteins expressed by an organism, provides valuable insights into the molecular mechanisms underlying plant- microbe interactions in the rhizosphere. This environment serves as a critical habitat where plants and microorganisms interact, influencing each other's growth and functionality. Advanced techniques like one and two-dimensional electrophoresis, difference gel electrophoresis (DIGE), highly sensitive mass spectrometry (MS), and isotope labeling methods such as isobaric tags for relative and absolute quantification (iTRAQ) are utilized to analyze and compare protein expression profiles. Another significant aspect of proteome analysis in plant-microbe interactions is the study of the secretome. The secretome refers to the proteins released by microorganisms into the extracellular environment. These secreted proteins play essential roles in host recognition, colonization, and pathogenicity. By examining the secretome, researchers can gain insights into the virulence factors and mechanisms of pathogenicity employed by microorganisms. Techniques like vacuum infiltration centrifugation (VIC) and gravity- extraction method (GEM) are employed to isolate secreted proteins for analysis.

Keywords: Genomics, Metabolomics, Omics, Plant–Microbe Interaction, Proteomics

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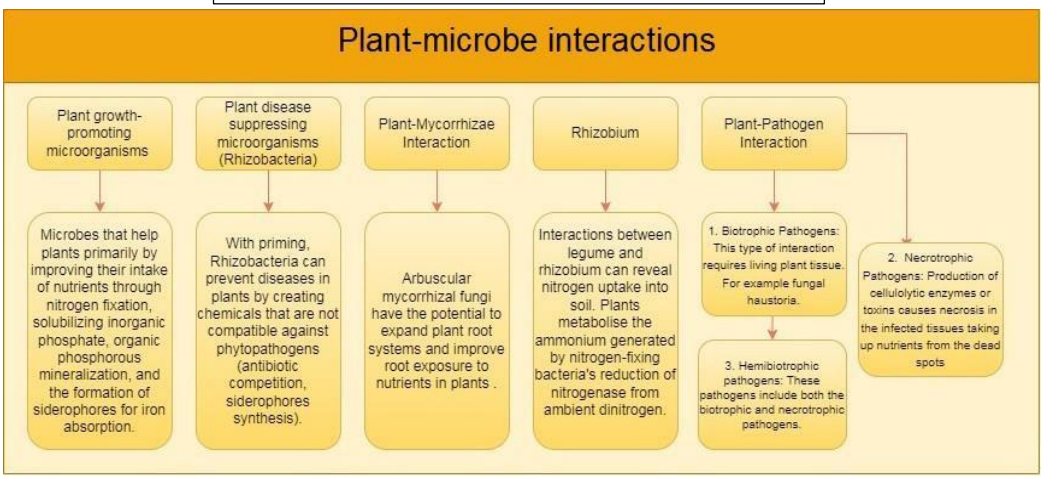
1. Introduction

In nature, since billions of different microorganisms inhabit and populate the plant's parts, including the rhizosphere, rhizoplane, endosphere, and phyllosphere, these are considered to be equally important in the growth and regulation of the plant. Consequently, it has been suggested that plants and their microflora act as metaorganisms [1]. The constrained region of a plant's root and soil surface is known as the rhizosphere, which is crucial for a variety of ecological processes like nitrogen fixation, decomposition of organic matter, plant, stress, tolerance, carbon absorption, and even more. Plants' roots build an interaction between the plant and the soil ecosystem, resulting in a vast store of microbial life. It is recognized that microorganisms get chemo attracted to exudates and migrate towards them, which enables the microbes to populate and grow in root environment [2]. Numerous variables, including biotic and abiotic aspects, such as diseases and insect pests, types of plants, genes, root morphologies, and plant developmental phases, have an impact on the microbiological communities that reside in the plant's rhizosphere [3, 4]. By supplying accessible mineral nutrients and producing phytohormones, rhizosphere microbiota, also known as plant growth promoting rhizosphere microorganisms (PGPM), regulate plant development and disease resistance [5].Both plant species and soil characteristics have an impact on the variety and makeup of the rhizosphere microbial population. All plants have limited access to mineral resources and are frequently harmed by diseases. Plants establish these rhizosphere interactions independent of their host species to get around these restrictions. Rhizobial communities in the bulk soil pool are subject to selection pressure from plants in order to acquire certain functional features required or plant fitness. These also help employ a variety of processes such as metabolic changes generation of exopolysaccharides, root colonization, and improved plant nutrition. As a result, the plant's functions are considerably expanded by the rhizosphere microbiome [6]. They do this by producing antibiotics, inducing systemic resistance, enhancing rhizosphere competence, and producing antagonistic substances for biocontrol. Besides enhancing the plant's indirect growth, these microbes help increase resilience to several of biological and abiological stressors, including invading pathogens or heavy metal contamination [7-9].Numerous microbial processes, primarily bacterial ones, such as the synthesis of antibiotics and the fixation of nitrogen, are known to be regulated by cell density-dependent quorum sensing. The survivability of type of microbial strain is impacted by quorum sensing. For example, by releasing some toxic compounds), which additionally impacts colonization of other species in that rhizosphere within the natural circumstances [10]. Furthermore, it has been shown that PGP microbes' capacity to provide amino acids, some vitamins, NADH enzymes, lipopolysaccharides (LPS), or fimbriae to root colonization [2, 11, 12].The PGPR are primarily categorized as biopesticides, phytostimulators, and fertilizers.By providing nutrients to the host, soil microorganisms help plants flourish. *Rhizobium* spp., *Allorhizobium* spp., *Trichoderma hamatum Trichoderma asperellum, Pseudomonas fluorescens*, and *Trichoderma* spp. are a examples of biofertilizers [13-15]. Understanding these reactions, how they are regulated, and how they are connected is the goal of research on plant-microbe interactions (Figure 1). These studies can provide appropriate information that may be used to develop plants with enhanced disease resistance and innovative symbiotic relationships. Proteomics, a technique that involves characterizing a group of proteins under particular circumstances, is an important technique for developing plant biology and even plant-microbe interaction [16]. Proteomics approaches are substantial and pertinent in many research findings, which demonstrate that relying solely on genomics approaches are insufficient as they lose a lot of significant information about the final products, The proteins, rather than the genes that code for them, are what cause the identified trait. The study of the many characteristics of protein layouts and configurations as well as their activities is made possible by new methodologies in proteomics that are constantly being developed. Large-scale technology of proteomics is used in a complicated sample to examine form and composition of proteins associated with plant-microbe interactions [17]. Various proteomics methods, involving 2-D gel electrophoresis and highly sensitized mass spectrometry (MS), isotope coding with an affinity tag (ICAT), and iTRAQ, are being utilised to decode these plant microbe interactions. A smaller number of documented research studies have addressed plant-microbial interactions, with the majority of them concentrating on the identification of alterations in protein expression as a result of a stimulus to toxins, nutritional requirements, genetic changes, or unbalanced expression of certain genes. However, in further investigation, a range of genetic and biochemical methods will need to be used in order to demonstrate a link between the activities of the relevant proteins and specific stimuli [18]. Secretome analysis essentially explains the comprehensive study of proteins

produced in the extracellular environment of microbial cells at any given moment and under certain circumstances by a variety of secretory systems involving constitutive and regulated organelles. The extracellular environment or extracellular space (ECS) is controlled by these secreted proteins, which maintain cell shape, participate in signaling and defense responses, and are also essential for stress responses [19]. The complete process of host identification, colonization, and penetration in plant tissues is guided by the secretome, a catalog of data on virulence factors and pathogenicity. Assuming that these several secretomic proteins are engaged in pathogen control or plant-microbe interaction, secretomic proteins are also essential for maintaining cell shape, cell communication, and, most significantly, the plant defense system. In one way or another, bacteria, fungus, and other creatures are either symbiotic or biotrophic with plants, or they are saprophytes of plants. Studying what is happening in this specific region may greatly help to build a notion of plant- microbe interaction since every contact between two organisms, whether it be pathogenic or symbiotic, happens in a nearby region of plant components. The mechanism of several interactions between plants and these microbes may be uncovered with the use of secretome analysis. Studies of secreted proteins that have been developed in natural, biotic, and abiotic environments have identified a number of unique secreted protein types, together with that of the leaderless secretory proteins (LSPs). These secreted proteins are being analyzed with two methods – vacuum infiltration centrifugation (VIC) method and gravity-extraction method (GEM) [19].

Figure1: Types of plant microbe interaction



2. Literature Review-

2.1. Omics to Understand Plant-Microbes Interaction

Understanding the beneficial and detrimental effects of microorganisms on plants requires a thorough understanding of plant-microbe interactions. Microbes may also have an indirect impact on plants by altering the surroundings of plants. The development of omics tools and sequencing technology in the genomics era has expedited biological science [20]. As an ecosystem management factor in holobiont theory, important or "fundamental" microbiome members that constantly interact with plants directly are being sought using next-generation sequencing technology [20]. High-throughput sequencing is being done at remarkable geographical and temporal levels utilizing marker genes, such as the 16S rRNA, ITS, or 18S rRNA genes, although these techniques lack insight information. In order to complement taxonomic methodologies, it is essential to incorporate or, at minimum, follow up with available data (proteomics and secretomics) (Figure2). Comparative functional genomics, in conjunction with other downstream omics techniques, is anticipated to be a major step in finding of plant and microbial interaction that underlie consistently identified core microbiome taxa or functions [21,22].

2.2. Proteomics

All the proteins that a genome expresses together are referred to as the "proteome." The proteins in a single cell vary significantly according to the working of the genes being in switched on or switched off in reaction to the external environment, whereas the genome mostly remains intact. Inclusion of structural, functional, and dynamic data is necessary to answer the issue of how one protein controls the functioning of

another by binding to it. A more comprehensive knowledge of the relationship between plants and microbes may be obtained by focusing only on the proteins, as they are either directly or indirectly engaged in all biochemical activities (Table 1). Some researchers refer to the total number of proteins made by a particular cell in a limited amount of time as that of the "functional proteome," which reflects the distinct identity of the proteome [23-25]. Because proteins preserve cellular homeostasis, their significance in the plant-microbe interaction becomes crucial. They play a major role in controlling cellular functions and signaling networks [26]. It is somewhat important to understand that proteins are not only simple translations of genes but some essential links between genetic and physical traits [27]. For instance, efforts to chemically delineate proteins and their functions are often accomplished using mass spectrometry-based methodologies [28]. Proteomics research has advanced over the last several decades, providing new comprehensions into how any plant detects the microorganisms they harbor and control their establishment, persistence, and function Proteomics investigation is recognized as an important way to understand how organisms exchange signals with each other. This goes beyond just mapping and analyzing the composition and expression of proteins on a large scale [29].

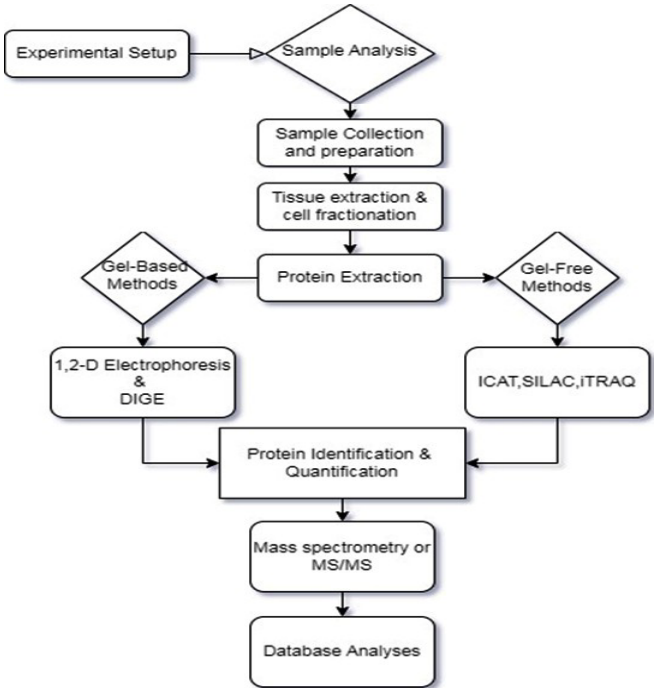


Figure2: Process depiction of proteomics

Evaluating the proteomes of cells and subcellular components is necessary to observe the signaling processes that take place during the initial stages of the interaction between plants and microbes. This also gives insight into what happens within a specific compartment of the host cell during this relationship [30]. Proteome assessment involves protein extraction as well as separation, breakdown to peptides, and then recognition using different methods [30]. New methods and processes are constantly being developed to improve High-throughput Proteomics at every stage of the workflow. This starts in the laboratory, with the techniques used for tissue and cell fractionation, protein extraction, depletion, purification, separation, and MS analysis. The process ends at the computer level, with the development of algorithms for protein identification and bioinformatics tools for data analysis, as well as the creation of databases and repositories to store and share the data obtained. As one technique cannot fully solve the complexities of live beings, experimental analysis must be confirmed [31-33]. Proteomic approaches have several drawbacks, including sensitivity, resolution, and data acquisition speed. There are several challenges in proteomics that require further attention and advancement, such as achieving deeper coverage of the proteome, conducting proteomic studies on unsequenced or "orphan" species, utilizing top-down proteomics approaches, improving protein quantification techniques, and exploring interatomics. The majority of these constraints and problems stem from the troubles of engagement with proteins' varieties and different types of physicochemical qualities [34, 35]. Each

proteomic experiment requires a solid experimental design to be successful. In plant proteomics experiments, the harvesting protocol is particularly important due to several factors. Firstly, plant tissues tend to have a lower protein content compared to other systems. Additionally, the presence of the cell wall and vacuoles can make it more challenging to extract proteins. Proteases and oxidative enzymes can also be present, which can degrade proteins. Finally, plant tissues tend to accumulate large amounts of polysaccharides, lipids, phenolics, and other secondary metabolites, which can interfere with protein extraction and downstream analysis. The procedures taken on must be tailored for the specific tissue and the investigation goal. The approach should be highly consistent and able to draw out as many protein species as possible while limiting impurities and avoiding anomalous protein breakdown and alteration [36]. Various research approaches have been considered to be ideal for the analysis.Proteomics can be divided into different sub-areas depending on the specific goals and objectives. These sub-areas include descriptive proteomics, which encompasses subcellular proteomics, comparative proteomics, post-translational modifications, interactomics, and proteinomics. another sub-area that can be identified is translational proteomics [37].

Table 1 : Proteome analysis for plant-microbe interactions

S.No.	Name of the plant species	Microbe associated	Interaction Results	Reference
1.	*Vitis vinifera*	*Candidatus Phytoplasma vitis*	Forty eight proteins were found to have varying levels of abundance, phosphorylation, or both in response to Flavescence dorée phytoplasma infection.	[37]
2.	*Vigna mungo*	*Mung bean yellow mosaic virus*	In both compatible and incompatible interactions, biochemical examination demonstrated a rise in phenolics, hydrogen peroxide, and carbohydrate levels.	[53]
3.	*Actinidia chinensis*	*Pseudomonas syringae pv. actinidiae*	Pathogenesis-related (PR) polypeptides or components involved in basal protection, oxidative stress, heat shock, and associated transport and signalling mechanisms were among the protein species that were more prevalent in infection.	[54]
4.	*Apple rootstocks*	*Erwinia amylovora*	In planta study, numerous proteins associated to RNA processing were shown to be more prevalent in the more virulent strain than in the less virulent strain.Plants resist infection by relying on innate immunity of each cell and systemic signals generated at infection sites. Following infection, the host undergoes an oxidative stress response that includes superoxide buildup, lipid peroxidation, electrolyte leakage, and enzyme stimulation, suggesting an incompatible relationship.	[55]
5.	*Oryza sativa*	*Magnaporthe oryzae*	The quantity of proteins involved in cell signalling, including pathogerecognition and signal transduction, is changed in plants reacting to pathogen assault from the start.Plant defence responses include cell wall strengthening, phytoalexin production, and the creation of defense-related proteins.	[59]
6.	*Populus*	*Botryosphaeria dothidea*	Infected species had a larger percentage of genes 365 encoding proteins associated to cell, extracellular area,	[60]

			macromolecular complex, and 366 antioxidant cellular component subcategories. Only 367 genes encoding organelle-related proteins were less numerous.	
7.	Tomato Plants	*Verticillium dahliae*	Some homologs of sixty-two proteins found in the incompatible interaction have been annotated for defence response and cell wall strengthening in other pathosystems, including the tomato-Fusarium system. Other defense-related proteins, such as pathogenesis-related (PR) proteins, were found in both resistant and susceptible relationships. Furthermore, homologs of a defense-associated mitogen-activated protein kinase generated in the incompatible contact studied here are known to drive hypersensitive response induction.	[61]
8.	*Theobromacacao* L.	*Moniliophthora perniciosa*	A combined total of 554 proteins were found, with 246 found in the prone Catongo genotype and 308 found in the resistant TSH1188 genotype. The proteins discovered were mostly involved in metabolism, energy, defence, and oxidative stress. The resistant genotype had more expressed proteins with more diversity related to stress and defence, whereas the susceptible genotype had more suppressed proteins.	[62]

2.3. One and two-dimensional electrophoresis

A simple and dependable way to analyze crude plant extracts is to use one-dimensional electrophoresis and SDS-PAGE, along with appropriate software, for fingerprinting. This technique is especially useful when working with hydrophobic or low molecular weight proteins. In-gel stable isotope labeling can be utilized to quantify changes in protein concentration or modifications within a protein. SDS-PAGE, band cutting, trypsin digestion, and LC separation of the resulting peptides are currently the most effective proteomic techniques for achieving high protein coverage [38, 39]. Quantitative Proteomics techniques like iTRAQ [40] can be facilitated by Electrophoretic methods that are compatible with chemical labeling [41]. The 2-DE method is the most prevalent separation technique and is continually being improved in various areas such as separating hydrophobic proteins, staining gels, capturing and analyzing images, and automating the process [42-44]. 2-DE is indeed a large-scale protein separation process that was created in the 1970s [45]. This method starts with separating proteins depending on their isoelectric points (in the first step). Isoelectric focusing (IEF) was used first, then by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second place (SDS-PAGE).Combining the approaches above in perpendicular orientations allows for the separation of hundreds of proteins in the same gel. Upon staining, proteins show as round or elliptical spots on the final two-dimensional gel rather than the rectangular bands found on one-dimensional gels. Although the overall separating capability of large-format two-dimensional gels is predicted to be 5000 spots per gel, when a sensitive detection method is utilized, a single two-dimensional separation of a complicated combination such as a whole-cell or tissue extract may generate 1000 to 2000 well-resolved spots [46]. Electrophoretic methods that work with chemical labeling can aid in Quantitative Proteomics techniques like iTRAQ. The most commonly used separation technique is 2-DE, which is constantly being improved in different areas such as the separation of hydrophobic proteins, gel staining, image capture and analysis, and automation. Separated protein spots can be analyzed using Western blotting, pre-electrophoresis fluorescence labeling, post-electrophoresis staining with coomassie blue dye, silver staining, or SYPRO dyes, differential expression analysis, and identification through mass spectrometry. [47] This method has some

technical limitations, such as inadequate separation of proteins and difficulty resolving less abundant, basic, or hydrophobic proteins. However, the reproducibility of 2-D gels has improved with the availability of equipment and reagents that utilize immobilized pH gradients [48]. The 2-D gel method has some technical limitations, including difficulty separating certain types of proteins. However, the use of immobilized pH gradients has improved the reproducibility of the method. To address the challenge of sorting low abundance and basic proteins, nonequilibrium pH gradient electrophoresis (NEPHGE) is being employed. This approach prevents protein precipitation by preventing accumulation at their isoelectric points [49].

2.4. DIGE~ Difference gel electrophoresis

The 2-D gel method has limitations in separating certain types of proteins, but the use of immobilized pH gradients has improved its reproducibility. To address sorting low abundance and basic proteins, nonequilibrium pH gradient electrophoresis (NEPHGE) is being used. DIGE is a valuable approach for comparative proteome research as it is highly repeatable, precise, and sensitive. It was created to increase repeatability when comparing samples by separating them on the same gel, allowing for the detection of genuine biological changes. Test and control samples are labeled with two fluorescent dyes (Cy3 and Cy5) using minimum approaches [50]. These dyes have a high sensitivity as well as a broad range of sample detection. The protein samples labeled differently are mixed in equal amounts and run on the same gel through 2D gel electrophoresis. This ensures that proteins with similar properties from different samples move together on the gel, and their fluorescence signals are overlaid, facilitating precise analysis of differences in their expression levels [51]. By directly comparing samples under identical electrophoretic conditions, the DIGE approach demonstrated greater sensitivity and linearity, avoided post-electrophoretic processing (fixing and destaining), and improved repeatability. The generated pictures are then processed using software created particularly for 2-D DIGE analysis, such as De Cyder. The DIGE approach has certain limitations; proteins lacking lysine cannot be tagged, specific equipment is required for viewing, and fluorophores are highly costly but still preferred over simple 2-D gel electrophoresis [52].

2.5. Other techniques for Proteomics

As mentioned earlier, there are several proteomic techniques that do not involve gel-based methods, such as iTRAQ, ICAT, SILAC, protein microarrays, and label-free comparative LC-MS. These techniques can also be used to investigate plant-microbe interactions. Additionally, they can be useful for identifying protein phosphorylation. In plant systems, such technologies have two key drawbacks: it is difficult to obtain repeatable labeling, and is expensive [37]. The isotope-coded affinity tag (ICAT) approach was employed to detect pairwise modifications in protein expression by differentially labeling proteins or peptides with stable isotopes, then by identification and quantification with a mass spectrometer. If a peptide with the same sequence is identified in two different biological samples and there is a variation in the measurements when comparing the peptide labeled with a heavy isotope to the one with a regular isotopic distribution, it indicates alterations in protein expression. This method allows for the evaluation of the expression of several proteins between two distinct biological states at the same time [53]. SILAC stands for Stable Isotope Labeling by Amino acids in Cell culture which is a powerful technique that utilizes mass spectrometry and metabolic integration of amino acids labeled with stable isotopes to measure the changes in the relative proteome under different treatments. It involves introducing a specific variant of the amino acid labeled as 'light' or 'heavy' into two separate samples. In SILAC, two cell populations are grown in the same culture medium, except that one contains a 'light' version of a specific amino acid, while the other includes a 'heavy' variant. Since these labeled amino acids are chemically alike to their natural counterparts, they do not interfere with the cell's normal growth while producing proteins and peptides that can be differentiated based on mass, making them suitable for mass spectrometry analysis. SILAC is an excellent technique for monitoring changes in post-translational modifications [54]. Several proteomics investigations rely heavily on protein relative quantitation. iTRAQ stands for Isobaric Tags for Relative and Absolute Quantification is a chemical tagging method that attaches to all peptides in a protein digest through free amines at the peptide N-terminus and lysine side chains. After labeling, the samples are combined and analyzed together. Since the tags used are isobaric, the mass of labeled peptides remains unchanged in mass spectrometry. Rather than measuring individual signals, the signal from the same peptide in all samples is averaged, resulting in a slight increase in sensitivity. This cumulative intensity of sequence ions after peptide fragmentation assists in boosting sensitivity. Despite the fact that iTRAQ labels have the same mass, each tag has a unique

isotope distribution that results in the generation of a tag-specific reporter ion upon fragmentation. The relative amounts of the labeled peptide in different samples can be determined by comparing the signal intensities of these tags [55].

2.6. Protein identification by mass spectrometry

Mass spectrometric (MS) studies have two advantages: sensitivity and selectivity with quick speed. The incorporation of desorption methods like electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) was a significant development that enabled large molecules to be transformed into ions in the gas phase for mass spectrometric examination without altering their structure or shape [56]. This breakthrough turned MS into a powerful tool for large-scale proteomic analysis. To quantify proteins, peptide mixtures produced by proteolytic digestion are analyzed using mass spectrometry, resulting in either peptide mass fingerprinting (PMF) or tandem mass spectrometric (MS/MS) data [57]. These data are searched against protein databases using various methods. The PMF method generates a protein's unique "peptide mass fingerprint," which is a list of experimentally determined peptide masses that is compared to the theoretically derived PMFs of each item in the database. When the PMF of the target protein matches a particular protein candidate in the database, identification occurs. The MS/MS technique, in addition to PMF data, provides structural information related to the peptide sequence, which improves the specificity of the target protein identification [58]. The peptide mixture is initially analyzed in the standard MS mode to obtain typical PMF results. After selecting a specific peptide ion as the parent ion, it undergoes fragmentation in the MS/MS mode through collision with an inert gas in a collision cell. The resulting fragments, called daughter ions, are then separated and analyzed in the second half of the tandem mass analyzer, which generates an MS/MS spectrum. Sometimes, further fragmentation of daughter ions is performed to obtain more sequence information (MS/MS). To identify the peptide, the acquired MS/MS spectra of different regions of the target protein are compared to the calculated spectra of all peptides in the database. Mass spectrometry-based proteomics has made significant contributions to various fields of biology by enabling the detection of protein post-translational modifications (PTMs) and the identification of new biomarker proteins for different diseases. PTMs result in a diverse population of proteins that perform crucial cellular functions, including plant defense against pathogens during plant-pathogen interactions [49, 59, 60, 61].

3. Conclusion

Despite the advances in proteomics technology, only a small fraction of the cell proteome has been characterized so far, and the functions of many proteins remain unknown even in well-studied organisms. Therefore, in the current era of plant proteomics, the next step is expected to involve the integration of data from multiple "omics" levels using a Systems Biological approach. This approach involves combining experimental data with genome-scale metabolic network modeling and mathematical modeling to predict the molecular behavior of plant species in their natural environment. The different omics levels serve both as an output of the system and as input to the models.

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