Plant Proteomics: An Overview

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Abstract The proteins encoded in a plant have a significant role in its survival and adaptation to external stresses. The cell wall, being the outermost layer, helps in defense against pathogens by production of glycoside hydrolases and proteases that degrade the pathogen external wall. The cell membrane assists in the movement of different molecules into and out of the cell. Different cells communicate with each other with the help of specific signals. Osmotic and salt concentrations are maintained by the embedded ion pumps in the cell membrane. The chloroplast, the only photosynthetic apparatus present in plants, leads to production of energy and also utilizes sunlight for the process of photosynthesis. A number of complex reactions, cycles, and pathways are present in the chloroplast. The mitochondria, also called the powerhouses of the cells, are rich in energy-producing cycles that are required for most of the activities of plants. In the matrix and cristae, a number of enzymes are active continuously. The mitochondrial membrane assists in the survival of the mitochondrion as an independent organelle. The nucleolus, the hub of all the protein-encoding genome, contains many processes. When we begin the analysis of a protein, protein extraction is the first issue. The plant possesses a cell wall that is a critical barrier which should be overcome. Many detergents and other chemicals are applied to break the bonding present in the cell wall, and we then extract our target protein, which is separated using gel electrophoresis. Two-dimensional sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE) facilitates the reaction by separating the proteins with respect to isoelectric point as well as molecular weight. Target proteins are visualized and then digested in gel to process it further for identification of the protein. A mass spectrometer is applied for this purpose to characterize each protein on the basis of charge to mass ratio, leading to unambiguous results. Bioinformatics tools are also used for confirmation of our target protein.

Keywords Cell wall proteome • Lectin • Glycoside hydrolyses • Proteases • Membrane-anchored proteins • Chloroplast proteomics • Lipid metabolism • Carbon metabolism • Oxidative stress and its response • Stroma • Thylakoid membrane • Nucleolus proteomics

1 Introduction

Proteomics is the study of the protein population in a tissue, cell, or subcellular compartment. A proteome is a set of proteins that is expressed in an organism by its genome (Wasinger et al. 1995). Nowadays, the sequence of nucleotides that is obtained from a genome project is transformed to computer files so that better knowledge of those obtained sequences can be retrieved. Thus, from these kinds of projects, functional genes in the genome, the proteins encoded by them, and control of the expression of genes are identified (Lockhart and Winzler 2000). Half the nucleotide sequences do not match in homology with already known proteins, which is coming from genomics research (Maheshwari et al. 2001).

In many of the eukaryotic and prokaryotic organisms whose genome has been completely sequenced many of the protein functions are still unknown. So, the solution to this problem may be the use of "microarray chip" technology (Somerville and Somerville 1999). The functions of a protein not only depends on its nucleotide sequence but are also dependent on posttranslation modification and its interaction with other proteins. If a new open reading frame (ORF) is discovered, then the genomic program tries to find the function of that newly discovered ORF. An ORF results in protein synthesis, and proteins are responsible for the control of biological function. One estimation is that as a result of posttranslational modification, 300,000 human proteins are considered in the PTM product.

Proteomics as shown in Fig. 1 has become an essential tool for understanding the functions of biological processes at the molecular level (Hakeem et al. 2012). The journal *Plant Proteomics* publishes novel and important research articles in the field of proteomics that examine the function and interactions of proteins from plant systems.

2 Cell Wall Proteome

Before the 1980s, the cell wall was considered to be a rigid and static structure but recent research proves the cell wall is dynamic and flexible instead (Cassab 1998). The cell wall is composed of carbohydrates (polysaccharides), lignin (40 %), hemicellulose, pectin, and proteins (10 %). Many of the proteins present in the cell wall are important in the stability of the cell. These cell wall polymers bind covalently and noncovalently to form the functional cell wall.

It has been revealed that the cell wall contains only 5 % to 10 % proteins linked by covalent and noncovalent bonding, forming a network mostly containing proline-rich proteins. Cell wall proteomics as shown in Fig. 2 are divided into nine functional classes by studying the *Arabidopsis* plant. Proteins that act on the cell wall represent 25 % of cell wall proteomics, which include polysaccharide lyases (PLs), carbohydrate esterases (CEs), and many others. The second class represents oxidoreductases, which includes muticopper oxidases, berberine bridge enzymes, and blue copper-binding proteins. The cell wall proteomics has many other functions

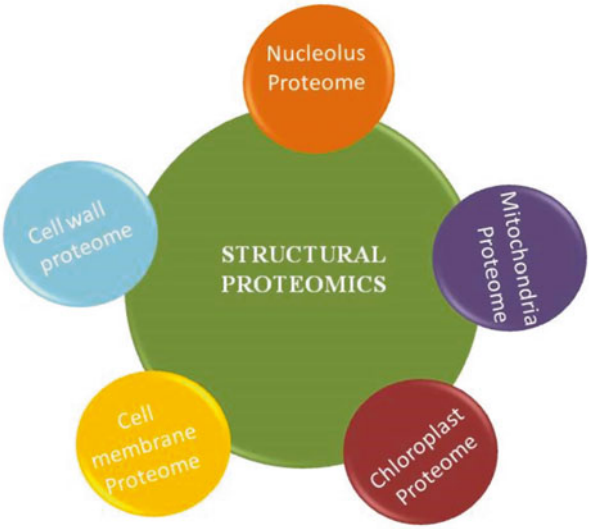

Fig. 1 Structural proteomics

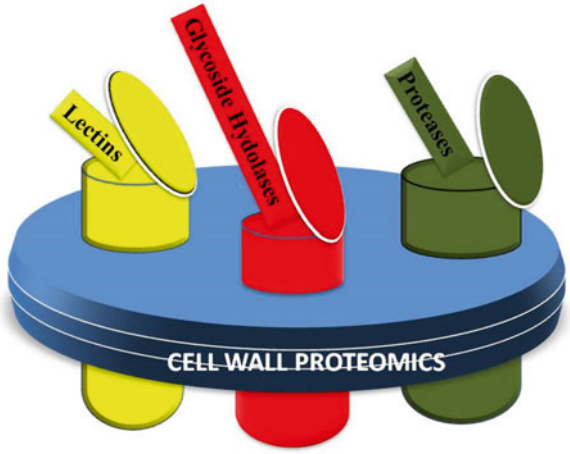

Fig. 2 Cell wall proteomics

such as protein turnover and protein maturation. One of the classes of cell wall proteomics involves signaling, which includes AGP proteins and other proteins such as involved in the transmembrane. Another class of proteins is involved in lipid metabolism and cuticle formation, such as lipid transfer proteins. Leucine-rich repeats (LRR) and glycine-rich proteins (GRPs) have been classified under structural proteins. Other classes include proteases, proteins acting on carbohydrates, miscellaneous proteins, and proteins with unknown functions (Albenne et al. 2013).

The class of cell wall proteomics is concerned with interacting domains, most probably with the carbohydrate-binding domain. Leucine-rich repeats are involved in protein–protein interactions. Those proteins that are not included in any other class have been grouped into miscellaneous proteins such as germins and purple acid phosphatases. There are some puzzling proteins whose functions are still unknown, categorized into the puzzling class of proteins. The function of one eighth of the cell wall proteome is still unknown (Albenne et al. 2013).

One of the structural proteins is hydroxyproline-rich glycoproteins (HRGPs), which are the translator product of the RSH (root-shoot-hypocotyl) gene. In *Arabidopsis thaliana*, the RSH gene is located on chromosome 1. Arabinogalactan proteins (AGPs) are glycoproteins that have been found to be functional in many processes including plant growth, development, embryogenesis, and cell proliferation (Knox 1995). The gene for AGP is on chromosome number 5. AGPs, the largest family, has the highest level of glycosylation of all the HRGPs, and are helpful in different stages of plant growth and plant development (Ellis et al. 2010).

AGPs are also involved in plant response to biotic and abiotic stresses (Gaspar et al. 2004). With the help of bioinformatics, 85 AGP genes were identified in *Arabidopsis thaliana* (Showalter et al. 2010). Another protein, proline-rich cell wall protein (PRP), is vital in specific cell wall structures during the development of the plant and is also involved in defense mechanisms against external damage and infection by pathogens (Ye et al. 1991). The PRP gene is located on chromosome 4 in *Arabidopsis*. Proline-rich proteins are the least glycosylated and thought to insert in the functional mature cell wall. Lectins are the glycoproteins. This carbohydrate protein is important in defense mechanisms against pathogen attack; lectin is important in protection of the cell against pathogens, and in symbiosis and assembly of cell wall polysaccharides. Another function of lectins is to recognize self-endogenous and exogenous ligands (Sharon and Lis 2004).

2.1 Glycoside Hydrolases

This large group of enzymes is involved in the hydrolysis of glycosidic bonds. They are important in plant cell wall metabolism, defense, signaling, and movement of storage reserves and reorganization of carbohydrates. A total of 75 glycoside hydrolases have been identified, subdivided into four groups. The first group of glycoside hydrolases consists of enzymes that are involved in organization of cell wall glycans in the period of growth and development (Minic and Jouanin 2006). Specific substrates for this small group of enzymes have also been identified; most of the substrates are pectin and xylologlucans. These two substrates are soluble in water and thus their enzymes, present outside the cell walls, are called exo-glycoside hydrolase. The multifunctionality of plant glycoside hydrolases makes the cell wall more complex by effectively modifying them even without the involvement of a large number of enzymes.

Some glycoside hydrolases are also involved in defense mechanisms against pathogens. These enzymes include chitinases (GH18, GH19, GH17), which are involved in activity against fungi (Schlumbaum et al. 1986). Plants secrete chitinases and β-1,3-glucanase in spaces within the cell to stop the growth of fungi by destroying their cell wall (Jach et al. 1995). Some glycoside hydrolases are multifunctional, such as β-D glucosidases (GH1), which have many functions such as lignification, signaling, defense, and hydrolysis of secondary metabolites (Xue et al. 1995). Some glycosidase hydrolases are thought to be involved in glycoprotein posttranslational modifications (PTMs) (Kotake et al. 2005).

2.2 Proteases

Proteases are essential in the functioning of enzymes and also protect from different microbes (Schaller 2004). Proteases comprise two sets of enzymes: the endopeptidases, that act upon the internal part of peptide chains, and the exopeptidases, which disrupt peptide bonds present on the termini of peptide chains (Barrett 1994). Research in proteomics has shown that proteases are much diversified in manner; there are multiple enzymes performing diverse functions as in the case of subtilases, carboxy peptidases, aspartases, and a number of other enzymes (Jamet et al. 2006). Enzymes of the subtilases family are especially interesting because they are vital in the production of peptide hormones and other growth factors from earlier peptides. Subtilases are involved in performing three main functions: control of development, protein turnover, and also as components of signaling cascades.

3 Cell Membrane Proteome

The plant cell membrane as shown in Fig. 3 is the outer layer of the protoplasm beneath the cell wall. As in most cell membranes, this is formed by the specific orientation of protein and phospholipid molecules. Cell membranes vary from 7.5 to 10 nm in thickness and consist of about 60% proteins and 40% phospholipids. Different species contain membranes consisting of specific types of polar lipids in specific concentrations that are probably genetically determined. Davson and Danielli (1935) proposed that membranes are made up of a central region consisting of phospholipids and an outer denser region composed of proteins (Danielli and Davson 1935).

The cell membrane is a particular compartment that is involved in structure formation as well as acting as a signaling mediator with extracellular content for the in-and-out movement of signals and materials. External stress results in intracellular reorganization of plants (Buchanan et al. 2000). So, a better knowledge of cell membrane proteins will facilitate logics to improve plant defense. Cell membranes help in controlling a number of critical functions such as metabolites, transport of ions, endocytosis, and cell division and differentiation. All these functions make use

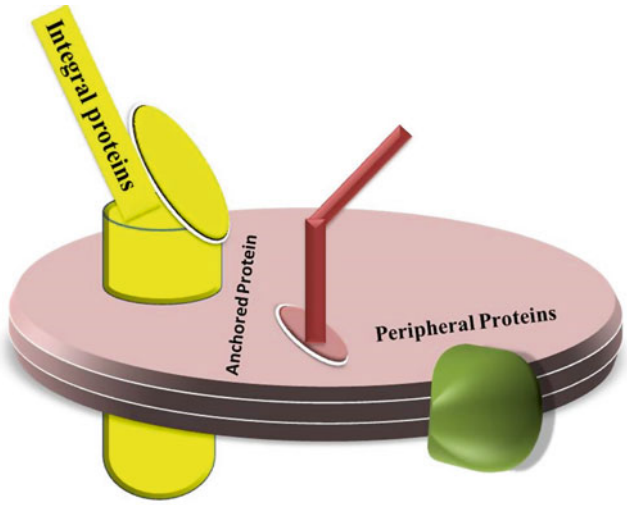

Fig. 3 Cell membrane proteomics

of a number of proteins that have very diverse structure and functions. In *Arabidopsis*, more than 27,000 proteins have been identified (Wortman et al. 2003).

Almost 25 % of proteins were grouped as membrane proteins (Schwacke et al. 2003). The plasma membrane is one of the most complicated systems of the cell that bears proteins and other components which are different with respect to type of cell, developing stage, and external environmental stress.

Cell membrane proteins are of three types:

- Transmembrane proteins
- Membrane-anchored proteins
- Peripheral membrane proteins

3.1 Transmembrane Proteins

Transmembrane proteins are those proteins that pass through the membrane into and out from the cell. The outer domain of the transmembrane protein may be a ligand-binding domain while its inner domain is called the effector domain (Marmagne et al. 2004). Receptors and transporters are the examples of transmembrane proteins, which are 30 % of the total cell membrane proteins (Ward 2001). In *Arabidopsis thaliana*, the total number of transmembrane proteins is considered to be between 4000 (15 %) and 8000 (30 %).

In *Arabidopsis thaliana*, 100 plasma membrane proteins were identified in a cell suspension culture in nonpolar proteins. It was prognosticated that 50 % of the transmembrane protein had domains (Marmagne et al. 2004).

Analysis of purely refined plasma membranes that are taken from leaves and petioles by using the technique named mass spectrometry led to the characterization of proteins as integrated and bordering proteins linked with the plasma membrane. About 238 plasma membrane proteins have been identified, and about 114 are expected to have some membrane domains or be phosphatidylinositol anchored. Of the 238 proteins identified, one third were categorized according to their function. Some families are involved in transport (17 %), signal transduction (16 %), membrane trafficking (9 %), and stress responses (9 %). About 25 % of the proteins that have been characterized in this analysis still lack information about any known function, and it is assumed that half of these somewhat resemble integral membrane proteins. In *Arabidopsis* more than 600 genes encode for protein kinase receptors, which are transmembrane proteins.

3.2 Membrane-Anchored Proteins

Many proteins of plant cells are affixed to membranes with the help of a covalent bond to glycosyl phosphatidylinositol (GPI). These types of proteins do not have a transmembrane domain and intracellular domain and thus are present entirely on the external side of the plasma membrane. GPI-anchored proteins include: enzymes associated to membrane, linkage molecules, initiation antigens, differentiation markers, protozoan coat components, and other miscellaneous glycoproteins. Many kinds of anchored proteins have been discovered in plants. Proteins that are translated in the vicinity of the plasma membrane involve signal transduction molecules such as GTPases and protein kinases. On the other hand, proteins that are anchored at the intracellular membrane include those proteins that help in regulation of vesicular movement (Fu and Yang 2001).

3.3 Peripheral Membrane Proteins

These proteins are lightly bonded to the membrane, most of the time being bonded noncovalently to the extended parts of integral membrane proteins. Peripheral membrane proteins are mostly the various proteins of multicomponent complexes such as photosynthetic proteins and H^+-ATPase in plants.

4 Chloroplast Proteomics

The chloroplast as shown in Fig. 4 is a plant cell organelle that originated from Cyanobacteria. They are involved in essential metabolic and major biosynthetic functions that include photosynthesis and amino acid biosynthesis. Most structural

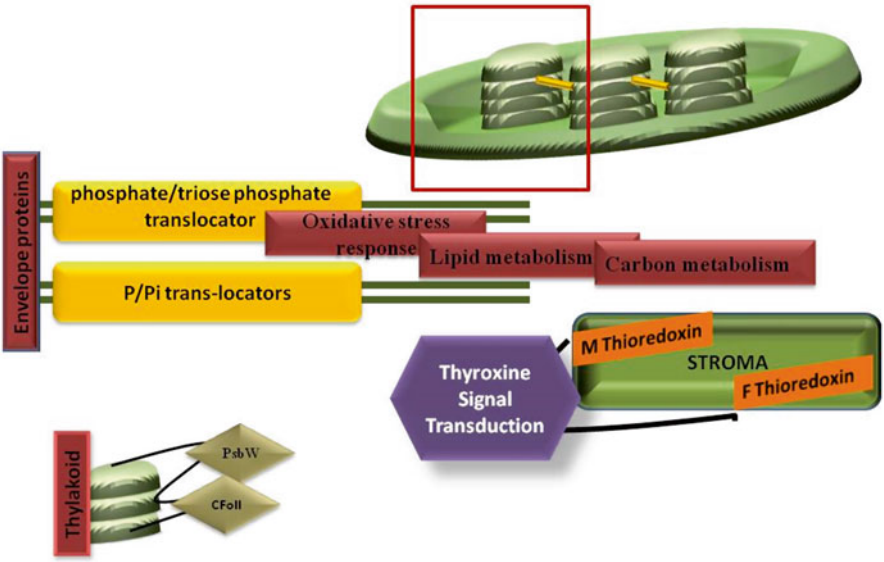

Fig. 4 Chloroplast proteomics

proteins that are involved in the construction of chloroplasts are transcribed by nuclear genes and transported into the chloroplast after being translated in the cytosol (Kleffmann et al. 2004). The three major structural and functional regions of chloroplasts are thylakoids, which are a highly organized membrane network composed of flat, compressed vesicles, the stroma, which is an amorphous matrix mostly rich in hydrophilic proteins and ribosomes, and the chloroplast envelope, a pair of outer membranes with an intermembrane space between them. The chloroplast is the site of carbon dioxide reduction and conversion into carbohydrates, amino acids, and fatty acids. Chloroplasts also bring about nitrite and sulfate reduction, converting them into amino acids (Douc and Joyard 1990; Flugge 2000).

Plastids within the plant cell involve a series of specific envelope proteins such as metabolite transporters, ion channels, pumps, and permease and pore proteins. A major feature of chloroplasts is that they resemble all other plastids and other self-existing organelles, which is why chloroplast formation requires the expression of both nuclear and plastid genes. Envelope membrane proteins take part in controlling the expression of nuclear and plastids genes and also in the specific makeup of chloroplast protein (Joyard et al. 1998).

Varying in size from 120 to 160 kb, the chloroplast genome codes for only 120 proteins; some RNA molecules are also encoded that are involved in transcription and translation of chloroplasts or in the code for small subunits of four complexes which are involved in photosynthesis (Sugita and Sugiura 1996). It is prognosticated that the number of different proteins present in the chloroplast is about 2000 to 5000, so most of the chloroplast proteins are the product of the nuclear genome. Those proteins that are transcribed by nuclear genes are produced as precursors in

the cytosol and are then transported to the chloroplast with the attached N-terminal peptide which is later degraded by proteases as it enters in the vicinity of the chloroplast.

Most of the chloroplast proteins are not actually transcribed by its own genome; rather, they are the product of nuclear genes, and then undergo translation in the cytoplasm and the protein is transported into this organelle. Transportation of these proteins is brought about by the protein import machinery that consists of an outer membrane, the Toc complex, and the inner membrane, the Tic complex (Schnell et al. 1994; Hiltbrunner et al. 2001).

Some polar lipids are also involved in the structural formation of plastid membranes (Douc and Joyard 1990). Some of the lipids are metabolized in the plastid membrane, resulting in many lipid-derived signaling molecules, which take part in the development and defense of the plant (Joyard et al. 1998). There are three membranes and six distinct compartments making up the structure of the chloroplast, which makes protein analysis much more difficult.

4.1 Chloroplast Envelope Proteins

The chloroplast envelope provides a complex network of transport systems that manipulate the in-and-out movement of proteins through the membrane. It also imports many ions and metabolites and also helps in movement out of the chloroplast. The envelope membrane has specific and unique biochemical machinery that depicts the stage of development of the plastid and also the metabolic requirements of different tissues.

It is difficult to identify the proteins especially in the case of plastid envelope membrane where the lipid content is very high (Douc and Joyard 1990). On the other hand, some transporter proteins, which are also embedded in a high content of lipid, cause problems in protein analysis. Because of the problems in the identification and analysis of proteins, very few proteins have been identified and assigned a function.

The first envelope protein to be characterized was phosphate/triose phosphate translocator (Flugge 2000). After that discovery, many substrate-specific outer membrane channels and some inner membrane metabolic translocators such as dicarboxylate and sugar were identified (Flugge 2000). The greatest molecular characterization of genes responsible for production envelope proteins was the characterization of several parts of the envelope proteins import machinery, that is, the Tic/Toc complexes.

To explore new envelope transporters, the cellular proteome analysis strategy was developed to characterize most of the nonpolar envelope proteins. This strategy relies on the utilization of highly refined and categorized membrane fractions, mining of nonpolar proteins with organic compounds, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, and recurrent mass spectrometry analysis. To analyze that large amount of data, a tool based on BLAST

was designed to discover protein, expressed sequence tags (ESTs), and plant genome databases. Of the 54 characterized proteins, only 27 were new envelope proteins that had multiple α-helical transmembrane regions, which may be considered as envelope transporters (Myriam et al. 2002).

The present study of proteins has enabled us to find those with similar attributes among both already known and novel characterized envelope membrane transporters. These properties were utilized to walk through the complete *Arabidopsis* genome and helped us in making a virtual chloroplast envelope protein database. As a result, both protein analysis and in silico approaches helped us in identification of more than 50 candidates that were previously undiscovered as plastid envelope protein transporters. The function of proteins clears up the target of investigation that helps in understanding the chloroplast metabolism in a convenient way (Myriam et al. 2002).

4.2 Function of Envelope Proteins

Some envelope proteins are involved in the transport of ions and metabolites. A series of transport systems takes place in the chloroplast envelope (Seigneurin Berny et al. 1999). Triose P/Pi translocator is an important membrane protein present in the inner membrane of the chloroplast. The triose phosphate translocator that transports inorganic phosphate, 3-phosphoglycerate (3-PGA), and triose phosphate helps in the movement of photoassimilates out of the chloroplasts during the day. During the day, triose phosphates are expelled from the chloroplast stroma in counter exchange with inorganic phosphate (Pi), generated during sucrose synthesis in the aqueous part of the cytoplasm. Involved in photosynthetic acclimation, a light response results in increased tolerance to high-intensity light (Walters et al. 2003).

The transported phosphate is then used for ATP production in the light-dependent reaction. ATP produced as a result of this import is then used for other reactions in the citric acid cycle. The translocator protein exports carbohydrates produced during photosynthesis. The H^+ transporter is a low-affinity H^+/Pi chloroplast transporter that is involved in inorganic phosphate upward movement in the green parts of plants when the plant is Pi sufficient, which is required for Pi repeated translocation during Pi deficiency (Versaw and Harrison 2002). These reactions are dependent upon the tissue, are always expressed in young green tissues, and are also present in both auto- and heterotrophic tissues. It is also expressed in the root stele.

The ADP translocator is important in the import of ADP (Neuhas et al. 1997). The Pi transporter is a high-affinity transporter involved in the movement of external inorganic phosphate. It acts as an H^+ phosphate regulator in low- and high-Pi conditions. It is sensitive to the level of arsenate (Muchhal et al. 1996). In *Arabidopsis thaliana*, this protein is encoded by the PHT1-4 gene present on chromosome 2. It is tissue specific and is mostly expressed in roots, in tissues connecting the lateral roots to the primary root, and is also present in flowers, in senescing anther filaments, and in the abscission zone at the base of siliques. It is expressed in hydathodes and

axillary buds, and in some senescing leaves after Pi starvation, which is localized in all cells of undifferentiated root segments, including root tips and root hairs and in the epidermis, cortex, and stellar regions of mature root segments (Okumura et al. 1998). Some proteins have unpredictable functions: the purposes of these proteins are still unknown, and scientists are trying to determine these functions. Examples are HP45, HP34, and some other transporters as well.

- Amino acid transporters are also present in plants; the specific function of these transporter is still to be confirmed.
- Antiporters (Na^+/H^+) are also found in plants and are vital in the homeostasis of the plant body (Wang et al. 2002).

4.3 Lipid Metabolism

The chloroplast membrane takes part in lipid metabolism, produces lipid derivative growth factors, and forms defense compounds as a biological response to external stimuli. Many enzymes ais in lipid metabolism: acetyl Co-A carboxylase, acyl Co-A synthetase, and desaturases. During proteomic analysis of the chloroplast membrane of *Arabidopsis*, it was proved that chloroplast membranes are involved in lipid metabolism, thus giving more support to proteomics by considering it at the molecular level; for example, 2-lysophosphatidate acyl transferase is involved in glycerolipid biosynthesis.

4.4 Carbon Metabolism

The chloroplast is a region where acetyl Co-A carboxylase enzymes are attached with the inner envelope membrane and are believed to have a fatty acid biosynthetic machinery. The fatty acids that are synthesized in the chloroplast are either used or transported to the cytosol (Rolland et al. 1997). The chloroplast of cotton has enzymes for the Calvin cycle that are found free or membrane bound; those enzymes that are membrane bound have more significant activities compared to the free form. Examples of membrane-bound enzymes include glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, and RuBisCo (Babadzhanova et al. 2002).

4.5 Oxidative Stress and Its Response

Plants experience a wide range of environmental stresses including light, drought, nutrient, and temperature changes that can cause oxidative stress by forming oxygen radicals. These radicals can cause major damage to membrane components

such as lipids and proteins. In such a situation, the large amount of fatty acid hydroperoxide within the membranes can be metabolized in the glutathione cycle. In *Arabidopsis thaliana* many proteins important in the oxidative stress response are present in the envelope membranes, for example, phospholipids, hydroperoxide, glutathione peroxidase (PHGPx), ascorbate peroxidase (APx), and superoxide dismutase (SOD).

The active repair mechanism present in plants effectively tackles the damage caused to a membrane protein under oxidative stress. Two protease families in *Arabidopsis* envelope membranes, the ATP-dependent Clp family and the ATP-dependent FtsH family, are involved in the removal of damaged protein from the envelope membrane. Clp proteins are also involved in the proper functioning of import machinery (Jarvis and Soll 2002).

4.6 Stroma

The chloroplast stroma is not only involved in the citric acid cycle but is also vital in the synthesis of proteins encoded by the organelle. In addition, the stroma also carries out starch and tetrapyrol synthesis. A recent application of proteomics in plants is the study of signal transduction in the chloroplast in which different new pathways were identified for thioredoxin-mediated signaling. In electron transport, the enzymes of the stroma are regulated by the activation of thioredoxin by protein ferredoxin thioredoxin reductase. Until now two chloroplast thioredoxins, m and f, have been identified as involved in signal transduction (Mottohashi et al. 2001). Thioredoxin interacts with its target proteins and forms an intermolecular disulfide bridge between the target proteins and thioredoxin. Then the formed disulfide bridge is reduced, but thioredoxin uses a second cysteine residue nearby, and the reduced target proteins now undergo a conformational change and are activated. If mutation occurs in cysteine being replaced by serine, then the target protein cannot be reduced, resulting in cross-linkage to the thioredoxin (Mottohashi et al. 2001).

4.7 Thylakoid Membrane

A thylakoid membrane is a site for photosynthesis with the help of photosynthetic pigments that are integrated in the membrane, being involved in adaptation to environmental changes. It has alternating dark and light bands of 1 nm. The thylakoid membrane of higher plants basically consists of phospholipids and galactolipids, which are irregularly organized on and across the membranes (Sprague 1987). The lipid synthesis for thylakoid membranes is brought about in the endoplasmic reticulum (ER) and the inner membrane of the plastid envelope, and movement from the inner membrane to the thylakoids is accomplished with the help of vesicles (Benning et al. 2006).

4.8 Thylakoid Lumen

The thylakoid lumen is the aqueous layer that is enclosed by the thylakoid membrane structure. It is involved in phosphorylation, which is an important part of the only photosynthetic process in plants. During daylight, these undergo a light-dependent reaction. For the proper functioning of the lumen, its PH is kept acidic, to pH 4, by the movement of H^+ ions into the lumen. This acidic pH is essential for proper carbon fixation (Lee and Kugrens 1999).

The thylakoid lumen is the least well characterized compartment as compared to other well-characterized parts. It is a continuous space that is present just beneath the thylakoid membrane. With the help of electron microscopy, the thylakoid lumen is seen as densely packed, which makes it even more difficult to characterize. The lumen helps in mediating the electron transport chain and other events related to it. It has also been reported to assist in producing the proton gradient that leads to ATP synthesis and also form currents by the ion channels (Pottosin and Schönknecht 1995).

It was discovered that all the nuclear genome encodes for luminal proteins and that these are produced as precursors in the cytoplasm. The amino-terminal end of these peptides helps in the movement of these proteins into the chloroplast stroma and across the thylakoid membrane into the lumen because they have specific amino-terminal bipartite transit peptides. According to this characteristic, bipartite transit peptides are used as markers for luminal proteins. On the other hand, not all synthesized chloroplast proteins are moved into the luminal space. The bipartite transit-formed peptides PsbW and CFoII are important proteins of the thylakoid membrane (Robinson et al. 1996).

4.9 Chloroplast Biogenesis

The mature chloroplast contains about 3000 proteins (Leister 2003). Metabolism inside the plastids is well organized but the functions of the proteins present are unknown or poorly described. Plastid proteins are encoded by both nuclear and plastid genes (Goldschmidt 1998). The plastids proteins translated from nuclear DNA are encoded in 80S ribosomes and then transported into the organelle. On the other hand, proteins that are encoded from the plastids genome are translated in 70S ribosomes. The chloroplast DNA only codes fewer than 100 proteins in higher plants, whereas the nuclear genome encodes about 95 % of different proteins present in the chloroplast proteome (Martin and Herrman 1998). Chlorobiogenesis is induced by certain environmental and internal signals between plastids and the nuclear genome.

5 Mitochondrial Proteomics

The mitochondria are membrane-bounded organelles responsible for the production of energy, involved in oxidation reactions and the transfer of electrons through the electron transport chain for the production of ATP. A mitochondrion is involved in the metabolism of amino acids and lipids and in the synthesis of nucleotides, vitamins, and cofactors, and it is also involved in the photo-respiratory pathway. Mitochondria are made up of hundreds of different proteins: most of the mitochondrial structural proteins are encoded in the nucleus, and during the formation of mitochondria those proteins are imported through complex enzymes. Another important reaction that occurs in mitochondria is phosphorylation: thus, there are two reactions occurring in mitochondria, oxidation and phosphorylation, and when these two reactions are combined, they are called oxidative phosphorylation, which is a source for the production of ATP in the cell.

5.1 Structure of Mitochondria

Mitochondria have outer and inner membranes as shown in Fig. 5 which basically have two major structural components: phospholipid and proteins (Alberts et al. 1994). The outer and inner mitochondrial membranes have different properties, and this double-membrane organization results in five distinct parts being present in the mitochondrion.

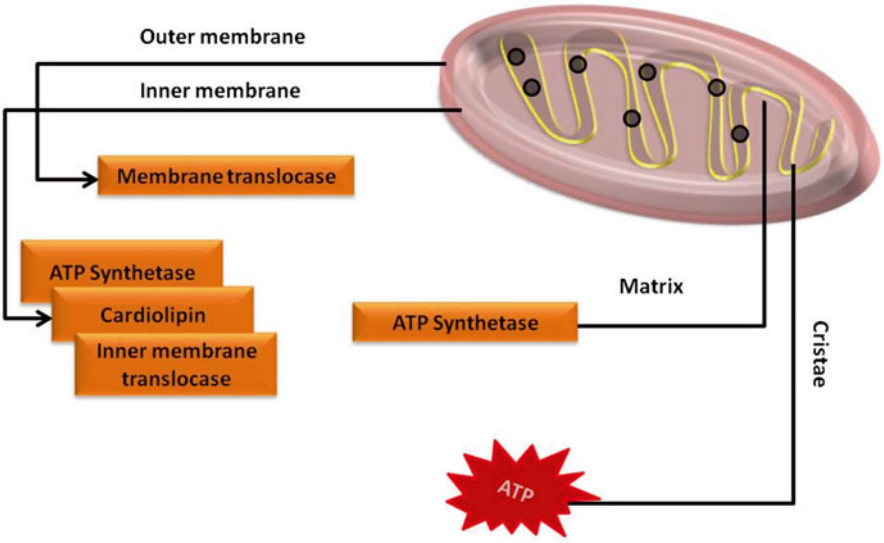

Fig. 5 Mitochondria proteomics

5.2 Outer Membrane

The outer mitochondrial membrane, which is largely made up of channel proteins called porins, surrounds the entire mitochondrion; it has a specific protein: phospholipid ratio similar to that of the eukaryotic plasma membrane (about 1:1 by weight). Porins facilitate molecules of 5 kDa or less in molecular weight to move freely across the membrane (Alberts et al. 1994). Larger proteins can be transported into the mitochondrion only when there is a signal sequence at their N-terminal that attaches to a peptide containing many subunits called membrane translocase, which then effectively moves them through the membrane (Herrmann and Neupert 2000).

Destruction of the outer membrane allows the proteins that are present in the space between the outer and inner membranes to move into the cytosol, causing the immediate death of a cell (Chipuk et al. 2006). The mitochondria-associated ER membrane (MAM) is a complex structure formed as a result of the attachment of the mitochondrial outer membrane to the endoplasmic reticulum membrane. This structure is critical in the mitochondria and with endoplasmic reticulum intracellular calcium signaling, aiding the movement of lipids across the endoplasmic reticulum and mitochondria.

5.3 Inter-Membrane Space

The inter-membrane space is present between the outer membrane and the inner membrane. The concentration gradient of ions and sugars (small molecules) in the space is equal to the concentration gradient of cytosol because the outer membrane is freely permeable to small molecules (Alberts et al. 1994). On the other hand, proteins that are larger in size or have high molecular weight should have a unique signaling sequence to be moved across the outer membrane, so the concentration gradient of the protein content of this space is different from the protein content of the aqueous part of the cytoplasm. Cytochrome *c* is one of the proteins attached to the inter-membrane space (Chipuk et al. 2006).

5.4 Inner Membrane

The inner mitochondrial membrane includes a number of proteins that perform five different kinds of functions. First are the proteins that perform specific reduction–oxidation reactions during oxidative phosphorylation; ATP synthase, which is involved in the production of ATP in the matrix of the mitochondria; specialized movement proteins that control metabolite movement across the matrix; and proteins in the intake machinery, mitochondria fusion, and fission protein.

There are 151 proteins present in the inner membrane, with higher protein content as compared to that of phospholipid. The inner membrane has more than 20 % of the total proteins in mitochondria (Alberts et al. 1994). In addition, the inner membrane is populated with an uncommon phospholipid named cardiolipin. This cardiolipin was first discovered in the heart of cows in 1942. It is mostly present in the mitochondrial as well as the plasma membrane of microbes (McMillin and Dowhan 2002). Cardiolipin consists of four fatty acids instead of two, and these aid in making the inner membrane impermeable to block undesired molecules from penetrating into the mitochondria. The inner membrane lacks specific structures called porins, which is why they are impermeable to all molecules. Most of the ions are transported across the matrix with the help of membrane transporters. Protein movement across the matrix is also carried out with the help of the translocase of the inner membrane (TIM) complex or with the help of Oxa1. After the action of a number of enzymes involved in the electron transport chain on the inner membrane, formation of the membrane potential results.

5.5 Cristae

The mitochondrial membrane has many compartments on its inner side, and each compartment is known as a crista. Cristae increase the surface area of the mitochondrial membrane, resulting in enhanced ability to produce ATP in a very small space. Mitochondria in liver cells have an inner membrane that is greater in size, almost fivefold larger than the outer membrane. The size of the inner membrane depends on the demand of ATP: the greater the demand for ATP, the greater the size of inner membrane will be. Muscle cells have a greater number of these compartments because of their high metabolic rate. These cristae are integrated into the tiny round bodies known as F1 particles, complex and irregularly organized folds attached to the inner membrane that can result in chemical osmotic disturbance (Mannella 2006).

5.6 Matrix

The matrix enclosed by the inner membrane has more than 70 % of the total proteins of the mitochondria (Alberts et al. 1994). The matrix has the majority of enzymes, as already discussed; the matrix thus is the site for many reactions including ATP formation through ATP synthase. The matrix is a complex part that does not contain only a single type of complexes. It has a number of enzymes, some tRNA, and hundreds of copies of the mitochondrial genome. The matrix mostly takes part in pyruvate oxidation and the tricarboxylic acid (TCA) or Krebs cycle. The mitochondrion is an independent compartment that has the ability to make its own RNA and many proteins (Alberts et al. 1994).

5.7 Mitochondria-Associated ER Membrane (Mam)

The mitochondria-associated ER membrane (MAM) is an important factor in the physiology of the cell and also the osmotic balance. Initially, there were some ER vesicles that appeared during cell fractionation, which was considered a problem in fractionating other components, but soon after it was discovered that these vesicles are actually the MAM, an important membranous structure that constitutes one fifth of the total membrane content of the mitochondrial outer membrane. The distance between ER and mitochondrion is about 25 nm, and the binding is maintained with the help of protein-binding complexes (De Brito and Scorrano 2010).

It has also been reported that the MAM are mainly rich in enzymes that help in the exchange of phospholipid and also for channels related to Ca^{2+} signaling (De Brito and Scorrano 2010). It is also involved in the regulation of lipids storage. Evolutionary analysis of MAM gives evidence that it was also involved in controlling calcium signaling (Hayashi et al. 2009).

5.8 Krebs Cycle

The Krebs cycle is an important metabolic process that helps in the production of the energy that is consumed by plants in performing a number of functions. A chain of reactions that make use of oxygen result in the oxidation of acetate, converting it into CO_2 and energy. This cycle is also a source of precursor molecules for other biochemical reactions. One example is ADP, which is a reducing agent. Because of is importance in pathways, it is also inferred that it might be one of the earliest pathways of cellular metabolism and might have been formed abiogenetically (Lane 2009).

5.9 Enzymes

Many enzymes have been are identified that cause the reactions to proceed, including pyruvate dehydrogenase complex, citrate synthase, isocitrate dehydrogenase, 2-oxyglutarate dehydrogenase, succinyl Co-A, succinate dehydrogenase, fumarase, and malate dehydrogenase. These enzymes make the TCA cycle happen.

5.10 Electron Transport Chain

The electron transport chain involves five complexes, namely, complex 1, 2, 3, 4, and 5:

- Complex 1 consists of five subunits (NADH oxidoreductase).
- Complex 2 consists of one subunit (succinate dehydrogenase).

- Complex 3 consists of three subunits (UQ-cytochrome oxidoreductase).
- Complex 4 consists of one subunit (cytochrome *c* oxidase).
- Complex 5 consists of five subunits (ATP synthase complex).

6 Nucleus Proteomics

Nuclear proteins are complex and multifunctional; some are mobile in nature such as transcription factor enzymes for processing of ribosomal RNA and DNA-repair enzymes. Some plant proteins are crucial in protein functions such as zinc finger proteins, glycine dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, transaldolase, actin, and malate dehydrogenase. Transcription regulators are the most dominant class of nuclear proteomics, such as RNA polymerase and TF 2A. Another class of proteins such as MADS box, RCC2, and Heat box are used for transcriptional control (Albenne et al. 2013; Narula et al. 2013). The nucleus matrix proteins as shown in Fig. 6 help in gene localization and expression. DNA methyltransferase and histone deacetylase help in mediating histone acetylation and modulating methylation of DNA by transposable elements silencing. Nuclear proteins assist in regulation of growth and development by transcriptional reprogramming such as ERF, MYB, Whirly, and WRKY factors to cause alteration in transcription factors (Casati 2012; Albenne et al. 2013).

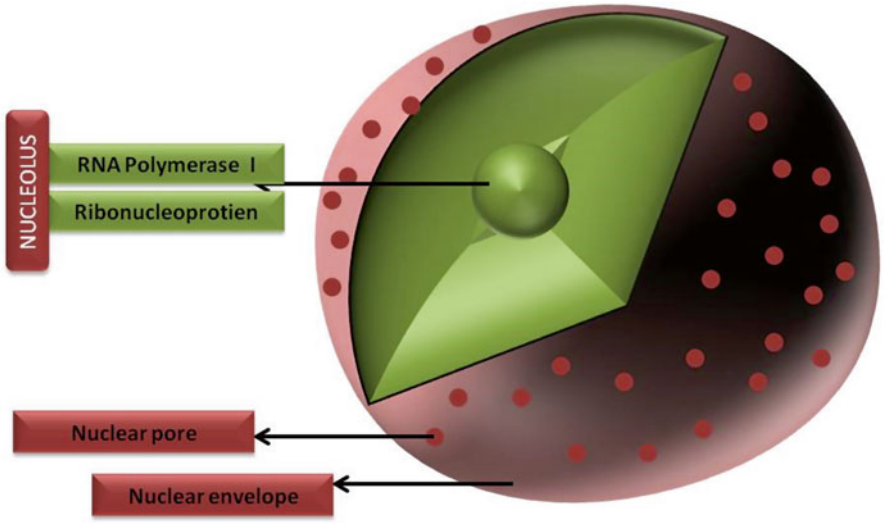

Fig. 6 Nucleus proteomics

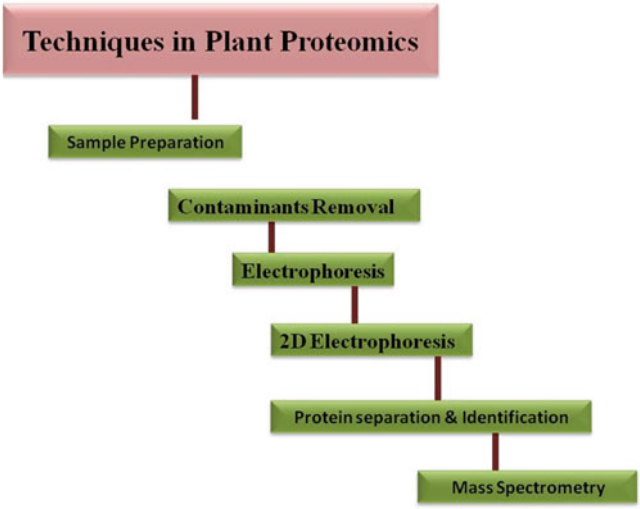

Fig. 7 Techniques in plant proteomics

Monocot nuclear proteins include SBT2 chromatin-binding proteins, RF2B, RING zinc finger proteins, and gypsy-like retroposon (Choudhary et al. 2009; Albenne et al. 2013). Some proteins that have been found in proteome analyses in the Leguminosae family are involved in different cellular functions such as embryogenesis, development, and identification of cells or organs, whereas CHP-rich zinc finger protein, nucleolin, WPP domain proteins, and MYB transcription regulators belong to Solanaceae transcription factors (Abe et al. 2003). Nuclear proteins also involve those involved in protein metabolism such as methyl transferase, ornithine methyl transferase, and homocysteine methyl transferase, which are important in DNA and RNA metabolism (Dahl et al. 2008; Albenne et al. 2013).

Many proteins are involved in cell-cycle regulation; the chromosome box proteins, BUB3 and RCC2 proteins, are important in cell division. CDC5 is an important cell-cycle protein in ciliary motility, trafficking, and mitosis. The Ran cycle is an important component represented by mago nashi proteins, Ran, Ras GTPase, and Ran GTPase. Karyopherins are nuclear proteins important in nuclear trafficking. Besides structural proteins and other proteins, the nucleus contains several enzymes such as polymerase, ligase, RNA processing enzymes, and gyrase. Another important class of nuclear proteins is histone variants that have an important function in maintenance of genome and stability (Ma et al. 2011; Albenne et al. 2013). Another class included mRNA processing proteins such as LSM2, nucleolar RNA processing protein (NRAP), and paraspeckle protein important in protein folding, signal transductors, and in developmental pathways (Caudron-Herger and Rippe 2012). For the maintenance of nuclear proteins and their stability are involved many other nuclear proteins such as different chaperons, proteasome subunit, HSP71, protein sulfide isomerase, DnaJ, and glutathione S-transferase.

Nuclear structural proteins are very diverse in plants. Myosin and actin are the most important, and the oldest proteins have been categorized in structural proteins and are active in DNA-dependent RNA polymerase and arbitrate in exporting RNA from the nucleus (Caudron-Herger and Rippe 2012). Some of the structural proteins are important in nuclear structure and the function of the genome such as tubulin, annexin A1, viscialin, titin, and spectrin. Some of the nuclear proteins are important in disease resistance, mRNA export, and chromosome scaffolding: NUF1, NUP88, NUP82, and nucleoporins.

In the eukaryotic nucleus, many nuclear bodies are involved in DNA replication and gene expression (Lamond and Earnshaw 1998). The nucleolus is a subcompartment of the nucleus and is a site for transcription and processing subunits before exporting to the cytoplasm (Lofantaine and Tollervey 2001). The nucleolus contains RNA polymerase I transcription enzymes and ribonucleoprotien particles, which are required for the modification of nucleotide bases and to process pre-RNA (Filipowicz and Pojacic 2002).

The nucleolus has ribosomal proteins, factors required for rRNA folding and for the export of ribosomal subunits from the nucleopore to the cytoplasm (Fatica and Tollervey 2002). The nucleolus has other functions as well, such as telomerase, cell cycle, cell growth, and aging (Lamond and Earnshaw 1998). The nucleolus is considered to act as a sensor for cellular stresses (Rubbi and Milner 2003). Structure of a plant nucleolus under the transmission electron microscope shows four different regions:

- Fibrillar centers (FC)
- Dense fibrillar components (DFC)
- Granular component (GC)
- Nucleolar cavity (NC)

The fibrillar is a small colorful structure present in the center, which is surrounded by a region of viscous colored material known as dense fibrillar component (DFC); this DFC is further surrounded by a region known as granular components (GC). The nucleolar cavity function is still unknown (Koberna et al. 2002).

Plant nucleoli have an active rDNA transcription unit that is spread throughout the entire nucleolus. DFC makes up 70 % of the nucleolar volume, which is surrounded by GC, whereas FC is not required for transcription.

7 Techniques in Plant Proteomics

The identification of proteins performing a specific function is opening new aspects in the field of molecular biology. Recent discoveries related to the proteins present in the cell wall (Robertson et al. 1997) and the plasma membrane have revolutionized that field (Santoni et al. 1998).

Another property of the plant cell is the presence of a cell wall, which is the outermost layer. Young plant cells are enclosed by a primary cell wall; some plants also do have a secondary wall that lies between cell and primary cell wall. The first step in protein analysis is disruption of the cell wall to access the proteome of that specific cell or tissue part. Many chemical and physical means are applied to disrupt the cell wall, such as lysis buffer, sonification, and high-speed blending (Islam et al. 2004).

Extraction of cell wall proteins (CWPs) is a difficult task; some loosely bound proteins have been reported, and there is no effective procedure for CWP extraction (Jamet et al. 2006). Proteomics involves a number of strategies and methodologies to analyze a specific protein, including protein separation and its identification. Subcellular proteomics and their functions are shown in (Table 1).

7.1 Sample Preparation

Preparing the sample is important for accurate results for protein analysis. Many problems appear; for example, in each cell there are a number of abundant proteins that may be present in the form of complexes, which makes it difficult to target our desired protein. Sample preparation is crucial when we looking for comparative proteomics as we look for even small differences between our controls and experimental results (Freeman and Hemby 2004). In a single cell, it is estimated that there might be ten copies of transcription factors but on the other hand there can be about 1 million copies of an abundant protein. This problem can be overcome by removing the most abundant protein, which will reduce the complexity of the whole sample.

Homogenization helps in making our sample uniform in terms of both composition and structure. It can be done by five different methods: mechanical, or by using ultrasonics, applying pressure, performing freeze-thaw, and osmotic and detergent lysis. Protein solubilization has special importance as it affects the efficiency of our expected final results and also affects the success of the whole experiment. Isolated proteins are often in insoluble form so we need to break the interactions present between them to make it soluble. It usually involves the breakage of interactions such as disulfide bonds, Van der Waals forces, and ionic and hydrophobic interactions (Rabilloud et al. 1997). An important point to be kept in mind while breaking these interactions is that there should be no modification or aggregation while solubilizing proteins as this can result in protein loss. Thus, we mostly use chaotropes such as urea/thiourea, detergents, reducing agents, tributyl phosphine, and protease inhibitors as sample buffer (Gorg et al. 2004). Chaotropes break hydrogen bonds and also disrupt hydrophilic interactions, which enables proteins to unfold. Reagents used mostly include urea, a neutral chaotropic agent used at high concentrations (5–9 M) to disrupt the secondary structure of proteins (Rabilloud et al. 1997).

Table 1 Subcellular proteomics and their functions

Subcellular organs	Proteins	References
Cell wall proteins	Structural proteins, oxidoreductases, protein related to lipid metabolism, miscellaneous proteins, proteins with unknown functions, proteases, proteins acting on carbohydrates, proteins with interacting domains	Albenne et al. (2013)
Cell membrane proteins	Signaling mediator, metabolite, transport of ions, endocytosis, and cell division and differentiation. Transmembrane protein as membrane trafficking and stress responses. Membrane-anchored protein as membrane trafficking, and stress responses. Peripheral protein mostly photosynthetic proteins and H^+-ATPase	Marmagne et al. (2004) Fu and Yang (2001)
Chloroplast proteins	Metabolite transporters, ion channels, pumps, and permease and pore proteins, thioredoxin proteins, chloroplast envelope proteins, stroma proteins, thylakoid proteins	Benning et al. (2006) Mottohashi et al. (2001)
Mitochondrial proteins	Translocase, mitochondria-associated ER membrane (MAM), translocase of inner membrane (TIM), succinyl Co-A, succinate dehydrogenase, fumarase	De Brito and Scorrano (2010) Hayashi et al. (2009)
Nucleolus proteins	Glycine dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, transaldolase, actin and malate, MADS box, RCC2	Filipowicz and Pojacic (2002)

Detergents break hydrophobic interactions, enabling the protein extraction. Detergents have been classified on the base of hydrophilic group, ionic, nonionic, and zwitterionic. Ionic includes anionic sodium dodecyl sulfate (SDS). Nonionic are uncharged, which includes octyl glucoside (Triton X-100). Zwitterionic are both positively and negatively charged groups with a net charge of zero: this includes CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO). Reductants break the disulfide bonds between cysteine residues, so they promote unfolding of proteins. Mostly, sulfhydryl reducing agents such as dithothreitol (DDT) and dithioerythritol (DTE) are used.

7.2 Contaminants Removal

pH and ionic strength affect the solubility of proteins. To maintain pH at a suitable level, buffers and salts are added to the sample. These buffer and salts often affect the further procedures in protein separation and spectrometric analysis, so they need to be removed before going further in our experiments (Visser et al. 2005). Salt added for preventing proteins from being precipitated interfere in 2-D electrophoresis. Salts are mostly removed by ultracentrifugation, gel filtration, and precipitation with TCA (Simpson 2004).

Detergents can be removed by using dialysis, that is, gel filtration chromatography. Lipids are also present in samples as some proteins are attached to lipids to form complex structures. This interaction with lipids reduces the solubility of proteins. A centrifugal filter device is used with the CHAPS, which allows effective lipid salt removal.

7.3 Protein Separation and Identification

Proteins in a cell vary from each other in their size and sequence of amino acids. There is not even one single method that can separate protein alone. Mostly procedures are optimized for a specific sample. Separation of protein is based upon the charge, size, and confirmation.

7.4 Electrophoresis

Polyacrylamide gel electrophoresis in SDS was first explored in 1949 (Rosenfeld et al. 1992). Separation is done under an electric field. As proteins carry a negative charge, the electric field forces its movement toward the other end. Protein migration depends upon its charge, size, and shape (Hale et al. 2004).

7.5 2D Gel Electrophoresis

The most widely used electrophoresis for separation of protein is by 2D PAGE (Gorg et al. 1999). For the past 20 years, it has also been used for the separation of a mixture of complex proteins with large molecular weight and sequence (Ong and Pandey 2001). In this approach, the protein is fractionated by isoelectric focusing in the first dimension in which separation is done based on the charge using a wide range of pI. In the second dimension, proteins are separated on SDS-PAGE based on their molecular weight (Gorg et al. 2000). A rapid improvement in resolution and reproducibility in the past 10 years has enhanced the popularity of 2DE (Rabilloud 2002). These improvements have resulted in the introduction of commercially available IPG systems that separate proteins within the narrow range of pI (Gorg et al. 2000), and also the availability of the latest fluorescent stains such as Sypro Ruby, which is highly sensitive and able to identify even proteins in very low abundance (Berggren et al. 2000). As a result, the 2DE is more applicable and reproducible with a higher capacity of loading and rapid identification of spot by utilizing the software (Wilkins et al. 1999). 2DE is the backbone in many protein analyses. After the protein has been separated on 2DE, it is visualized. After our target peptide is identified, it is excised from the gel with the help of the enzyme trypsin, and now mass spectrometric analysis can be done.

7.6 Identification by Mass Spectrometry

Developments made during the past few years in mass spectrometry have resulted in the assurance that it is the only method by which we can easily identify proteins; posttranslation modification can also be characterized (Packer and Harrison 1998). The latest ionization methods and other mass analyzer strategies have led to some astonishing improvements in this technology that have made it more accurate, higher in resolution, and also applicable to characterizing very large protein molecules (Costello 1999). Another major advancement in this technology is the introduction of the desorption method, allowing it to analyze large molecules without any complex fragmentation. The two most common methodologies used for protein identification and characterizations are matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) and electrospray ionization (Liang et al. 1996). Selection of the type of mass spectrometry is based upon such factors as the nature and source of the protein. In many of the proteome analyses, we always look to MALDI-TOF first. It is based on the mass-to-charge ratio of the peptide fragments that are produced by enzymatic cleavage of the parent protein using the trypsin enzyme. That fragmentation leads to unique peptides known as the peptide mass fingerprint (PMF) (Griffin et al. 2001). Bioinformatics strategies are also involved when a PMF is obtained. PMF along with the values of pI and molecular mass is compared to the theoretical values already present in the database, which helps in

removal of redundant data. These experimentally obtained values are compared to that reported in the database. After comparison by means of some algorithm, it gives results and computes scores.

In some cases, the PMF leads to some doubts in scores that can give a clear result. It happens when comparison of experimental results is done with the one already reported, and it gives more than two possible results on the basis of scores. In that situation we look for only one approach, that is, de novo sequencing, by a tandem mass spectrometer. A number of spectrometers can help in the analysis of proteins (Aebersold and Goodlett 2001). Data that are obtained by one or multiple MS in analysis of protein leads to characterization with a very high level of accuracy (Blackstock and Weir 1999). This methodology also gives information related to the posttranslation modifications in addition to the amino acid sequence (Bardor et al. 1999).

8 Future Perspectives

Plant proteomics is progressing continuously at a great pace in many aspects, and is also involved in developments in model systems, as in the case of yeast and *Escherichia coli* (Mann et al. 2001). With the advances of the technological era, revolutionary improvements in protein analysis and methodologies have made progress even more rapid. New analytical techniques are being introduced or are about to be applied in this field, which will make it easier to analyze even small samples with much greater accuracy and specificity. The future is expected to bring a lab-on-chip that will be able to able to analyze nano quantities (Nelson et al. 2000) of sample involving nano-separation with high resolution. Within a few years, developments will lead to the characterization of posttranslational modification and other protein complexes in an easy and specific way (Gavin et al. 2002). The development of a number of bioinformatics tools that are related to proteins will make data comparison and retrieval easier, hence making our proteomic approach rapid, and also allowing storage of vast amounts of data (Reiser et al. 2002).

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