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Review

Proteomic Studies in Plants

Ohkmae K. Park*
Kumho Life and Environmental Science Laboratory, Gwangju 500-712, Korea

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Proteomics is a leading technology for the high-throughput analysis of proteins on a genome-wide scale. With the completion of genome sequencing projects and the development of analytical methods for protein characterization, proteomics has become a major field of functional genomics. The initial objective of proteomics was the large-scale identification of all protein species in a cell or tissue. The applications are currently being extended to analyze various functional aspects of proteins such as post-translational modifications, protein-protein interactions, activities and structures. Whereas the proteomics research is quite advanced in animals and yeast as well as *Escherichia coli*, plant proteomics is only at the initial phase. Major studies of plant proteomics have been reported on subcellular proteomes and protein complexes (*e.g.* proteins in the plasma membranes, chloroplasts, mitochondria and nuclei). Here several plant proteomics studies will be presented, followed by a recent work using multidimensional protein identification technology (MudPIT).

Keywords: Functional genomics, Mass spectrometry, Proteomics, Plant, Two-dimensional gel electrophoresis

Introduction

Proteomics can be defined as the systematic analysis of proteome, the protein complement of genome (Pandey and Mann, 2000; Patterson and Aebersold, 2003; Phizicky *et al.*, 2003). This technology allows the global analysis of gene products in various tissues and physiological states of cells. With the completion of genome sequencing projects and the development of analytical methods for protein characterization, proteomics has become a major field of functional genomics. The initial objective of proteomics was the large-scale identification of all protein species in a cell or tissue. The applications are currently diversified to analyze various functional aspects of proteins such as post-translational modifications, protein-protein interactions, activities and structures. Here, general technologies of proteomics will be briefly introduced, followed by the overall progress in plant proteomics.

With the aim of profiling proteins in biological samples, proteomics has long been associated with the techniques of two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS) (Shevchenko *et al.*, 1996; Wilkins *et al.*, 1996). Classical 2DE had limitations in resolution and reproducibility of gels, which were mostly overcome by using immobilized pH gradient (IPG) strips (Gorg, 1991). The improved techniques in 2DE allowed comprehensive protein visualization on 2D gels. Proteomics was further advanced by the development of biological MS and the growth of searchable sequence databases. The MS techniques that were developed for the ionization of proteins and peptides include matrix-associated laser desorption ionization (MALDI) and electrospray ionization (ESI) (Karas and Hillenkamp, 1988; Fenn *et al.*, 1989). Combined with time of flight (TOF), ion trap and triple-quadrupole tandem MS (MS/MS) spectrometers, these offer high sensitivity and mass accuracy (Aebersold and Mann, 2003).

Although 2DE-based proteomics has proven powerful for the global analysis of proteins, it still retains technical problems that need to be solved (Corthals *et al.*, 2000; Gygi *et al.*, 2000). It is costly and a labor- and time-consuming process, limiting high-throughput analysis of protein expression. In addition, the entire protein profiling and quantification are not possible due to the limited loading capacity and incomplete staining methods. An alternative method to analyze proteins directly by MS, without gel separation, has been developed to overcome these 2DE-associated limitations. It is referred to as multidimensional protein identification technology (MudPIT) or liquid chromatography (LC)-MS/MS that couples capillary, high-performance liquid chromatography (HPLC) to MS/MS and allows automated analyses of peptide mixtures that are generated from complex protein samples (Appella *et al.*, 1995; Washburn *et al.*, 2001; Wolters *et al.*, 2001).

*To whom correspondence should be addressed.
Tel: 82-62-970-2625; Fax: 82-62-972-5085
E-mail: omkim@kkpc.com

Furthermore, quantitative proteomics became feasible using an innovative reagent, termed isotope-coded affinity tag (ICAT), in the LC-MS/MS system (Han *et al.*, 2001).

Several intriguing strategies have been developed toward large-scale studies of protein functions. Following the successful applications of DNA chip methods, protein microarray technology has emerged (Macbeath, 2002; Mitchell, 2002). The complicated features of protein microarray are that proteins have different biochemical properties and need high-throughput methods for expression and purification. There are some reports of comprehensive protein microarray screening (Houseman and Mrksich, 2002; Schweitzer *et al.*, 2002). In addition, studies of protein-protein interactions lead into cellular protein functions. There have been large-scale analyses of protein complexes in yeast, using the tandem-affinity purification (TAP) approach combined with MS (Gavin *et al.*, 2002; Ho *et al.*, 2002). Researchers isolated and identified more than 200 protein complexes, providing clues how an entire proteome is organized into functional units.

While proteomics research is advanced in animals and yeast, plant proteomics is still at the initial phase (Zivy and de Vienne, 2000; van Wijk, 2001; Kersten *et al.*, 2002). The progress in plant proteomics has been largely made by 2DE-based proteomic approaches. Since the resolution of protein spots on a 2D gel is limited by factors such as abundance, size, and other electrophoretic properties, the complete proteome has been fractionated into sub-proteomes such as subcellular compartments, organelles and multiprotein complexes to improve sensitivity and resolution and to reduce the overall complexity (Jung *et al.*, 2000). Several proteomics groups have studied subcellular proteomes and protein complexes in plants, *e.g.* proteins in the plasma membranes, chloroplasts, mitochondria and nuclei (Rouquie *et al.*, 1997; Peltier *et al.*, 2000; Prime *et al.*, 2000; Peltier *et al.*, 2001; Kruft *et al.*, 2001; Millar *et al.*, 2001; Bae *et al.*, 2003). In a recent paper, MudPIT was used to analyze rice proteome, in which the combined 2DE and MudPIT approaches identified 2,528 unique proteins (Koller *et al.*, 2002; Whitelegge, 2002). In this article, several plant proteomics studies will be presented that focused on subcellular proteomes and protein complexes (Table 1). A recent work using MudPIT will also be briefly described.

Chloroplast Proteome

It is estimated that the chloroplast contains 2,500 to 5,000

Table 1. Proteomic studies in different subcellular compartments in plants

Subcellular compartments	Summary of studies	References
Chloroplast	61 lumenal and peripheral thylakoid proteins in pea identified by MALDI-TOF MS, ESI MS/MS and Edman sequencing;	Peltier *et al.*, 2000
	more than 100 envelope membrane proteins in *Arabidopsis* identified by LC-MS/MS;	Ferro *et al.*, 2003
	350-kDa ClpP protease complex with 10 different isoforms in *Arabidopsis* identified by blue-native gel electrophoresis, MALDI-TOF MS and ESI MS/MS;	Peltier *et al.*, 2001
	light-harvesting proteins of photosystems I & II in 14 and 9 different plant species, respectively, identified by reverse phase HPLC-ESI-MS	Zolla *et al.*, 2002, 2003
Mitochondria	52 mitochondrial proteins in *Arabidopsis* identified by immunoblotting, Edman sequencing, MALDI-TOF MS and ESI MS/MS;	Kruft *et al.*, 2001
	91 mitochondrial proteins in *Arabidopsis* identified by MALDI-TOF MS;	Millar *et al.*, 2001
	protein complexes, ATP synthase complex, cytochrome *c* reductase complex and TOM complex, in *Arabidopsis* resolved by 3DE;	Werhahn and Braun, 2002
	subunits of TOM complex in *Arabidopsis* identified by immunoblotting and direct sequencing	Werhahn *et al.*, 2001
Nucleus	184 nuclear proteins in *Arabidopsis* identified by MALDI-TOF MS	Bae *et al.*, 2003
Plasma membrane	82 proteins in plasma membrane and soluble fractions in *Arabidopsis* identified by direct sequencing;	Santoni *et al.*, 1998
	38 plasma membrane proteins associated with cold acclimation in *Arabidopsis* identified by MALDI-TOF MS	Kawamura and Uemura, 2003
Cell wall	111 cell wall proteins in *Arabidopsis* identified by MALDI-TOF MS;	Chivasa *et al.*, 2002
	extracellular proteins responding to fungal elicitors in *Arabidopsis* suspension cultures identified by immunoblotting and MALDI-TOF MS	Ndimba *et al.*, 2003
Post-translational modification	thylakoid proteins reversibly phosphorylated in *Arabidopsis* identified by MALDI-TOF MS and ESI MS/MS;	Vener *et al.*, 2001
	30 GAPs identified by LC-MS/MS and 248 GAPs predicted by a bioinformatic analysis in *Arabidopsis*	Borner *et al.*, 2003

proteins. Since the chloroplast genome encodes approximately 120 proteins, most proteins are encoded by the nuclear genome and transported into the chloroplast (Keegstra and Cline, 1999). To have a complete picture of the chloroplast proteome, further fractionation into smaller sub-proteomes is required.

The soluble and peripheral proteins in the thylakoids of pea were systematically analyzed by using 2DE, MS and N-terminal Edman sequencing, followed by database searching (Peltier *et al.*, 2000). Among the estimated 200 to 230 lumenal and peripheral proteins, 61 proteins were identified. For 18 of those, no corresponding full-length genes could be found in the databases. The predictions for chloroplast localization and transit peptides were made with three on-line programs, ChloroP, PSORT and SignalP, showing that a combination of these three programs provide a useful tool for evaluating localization and targeting.

The chloroplast envelope membranes from *Arabidopsis* were subjected to an extensive proteomic analysis (Ferro *et al.*, 2003). Purification and extraction methods were developed to prepare highly-purified envelope membranes and as many proteins as possible. LC-MS/MS analyses were then performed, leading to the identification of more than 100 proteins. Regarding functions, approximately 50% of the identified proteins have functions known, or very likely to be associated, with the chloroplast envelope. Proteins implicated in proteolysis, carbon metabolism and oxidative stress response were also found.

A 350-kDa ClpP protease complex with 10 different subunits was identified in the chloroplasts of *Arabidopsis*, using blue-native gel electrophoresis, followed by MALDI-TOF MS and nano-ESI MS/MS (Peltier *et al.*, 2001). It includes pClpP, 5 nuclear-encoded ClpP proteins, 3 nuclear-encoded ClpR proteins, as well as 1 novel Clp protein, ClpS1. Several truncations and errors in intron and exon prediction of the annotated Clp genes were corrected using MS data and by matching genomic sequences with cDNA sequences.

The light-harvesting proteins of photosystems I & II from different plant species were extracted and identified on the basis of their intact molecular masses by reversed phase HPLC-ESI-MS (Zolla *et al.*, 2002, 2003). During the last several years, reversed phase HPLC-ESI-MS has evolved into a highly powerful tool for accurate mass measurements of proteins (Premstaller *et al.*, 2001) and has been applied to the analysis of plant proteins (Whitelegge *et al.*, 1998; Huber *et al.*, 2001; Gómez *et al.*, 2002).

Mitochondrial Proteome

The *Arabidopsis* mitochondrial proteome was systematically analyzed (Kruft *et al.*, 2001; Millar *et al.*, 2001). Using different conditions that were combined for protein solubilization and isoelectric focusing, up to 800 proteins were resolved on 2D gels. About 100 different mitochondrial proteins were identified by MS, direct protein sequencing or immunoblotting analyses. The identified proteins participate in various processes, such as respiration, citric acid cycle, amino acid and nucleotide metabolism, protein import, processing, assembly, transcription, membrane transport and antioxidant defense. More than 20% of the identified proteins were not previously described for plant mitochondria, indicating novel mitochondrial functions.

The *Arabidopsis* mitochondrial proteome was further subdivided into sub-proteomes on the basis of protein complexes (Werhahn and Braun, 2002). The mitochondrial protein complexes that are separated by blue-native gel electrophoresis were electroeluted and analyzed by 2DE. This method allowed the resolution of the ATP synthase complex, the cytochrome *c* reductase complex and the preprotein translocase of the outer mitochondrial membrane (TOM complex). The TOM complex from *Arabidopsis* was purified and characterized (Werhahn *et al.*, 2001). On blue-native gels, the TOM complex run at 230 kDa and could be dissected into different subunits. A 2DE analysis led to the identification of multiple forms of TOM20.

Nuclear Proteome

The *Arabidopsis* nuclear proteome was comprehensively characterized (Bae *et al.*, 2003). Nuclear proteins were isolated and analyzed using 2DE and MALDI-TOF MS. Proteomic analyses led to the identification of proteins that are implicated in a variety of cellular functions such as signaling, gene regulation, structure, translation, proteolysis and various RNA-associated functions. Furthermore, the changes in the nuclear proteome were analyzed in response to cold stress. Of the identified 184 proteins, 54 were up- or downregulated in response to cold treatment.

Plasma Membrane Proteome

The proteome of the plasma membrane is a difficult one for reproducible and complete mapping due to its hydrophobic nature. Specific strategies have been developed to recover the hydrophobic membrane proteins on 2D gels (Santoni *et al.*, 2000). Efforts have been employed to construct 2DE reference maps of the plasma membrane under optimized solubilizing conditions (Rouquie *et al.*, 1997; Santoni *et al.*, 1998).

In order to identify *Arabidopsis* plasma membrane proteins that are associated with the early stage of cold acclimation, highly-purified plasma membrane fractions from non-acclimated and cold-acclimated *Arabidopsis* leaves were analyzed using MALDI-TOF MS (Kawamura and Uemura, 2003). The proteins that changed in quantity during cold acclimation included those that are associated with membrane repair by membrane fusion, protection of the membrane against osmotic stress, enhancement of CO_2 fixation and proteolysis.

Secreted Proteome

Proteins were sequentially extracted from enriched *Arabidopsis* cell wall fractions using $CaCl_2$ and a urea buffer, separated by 2DE, and identified by MALDI-TOF MS and genomic database searches (Chivasa *et al.*, 2002). Among the identified proteins were classical cell wall proteins of known functions. The results also demonstrated the unusual cell wall localization of proteins that are normally associated with other organelles and the existence of extracellular phosphorylation in plants. In addition, *Arabidopsis* cell suspension cultures were investigated for the proteomic changes that are induced by fungal elicitors (Ndimba *et al.*, 2003). There were extracellular proteins that changed in abundance or in phosphorylation following elicitation.

Post-translational Modifications

Proteins undergo post-translational modifications such as phosphorylation, glycosylation, sulfation, prenylation, acetylation and ubiquitination. Post-translational modifications modulate protein functions including activity, stability and localization. Proteomic strategies have been developed to characterize protein modifications that cannot be predicted from a genomic sequence (Mann and Jensen, 2003).

The reversible phosphorylation of chloroplast thylakoid proteins in *Arabidopsis* was analyzed using MALDI-TOF MS and ESI MS/MS (Vener *et al.*, 2001). By comparing the levels of phospho- and nonphosphopeptides, the *in vivo* phosphorylation states of these proteins were determined under different physiological conditions.

The combination of 2DE and 1DE with Triton X-114 phase partitioning showed that glycosylphosphatidylinositol (GPI)-anchored proteins (GAPs) are an abundant class of proteins in plants. They are present at the plasma membrane and also released into the extracellular matrix (Boner *et al.*, 2003). Using LC-MS/MS, 30 GAPs were identified. Furthermore, a bioinformatic analysis was performed using the improved search algorithm, which raised the total to 248 predicted GAPs in *Arabidopsis* (Borner *et al.*, 2002, 2003).

Rice Proteomics

Rice is the first cereal crop genome to be decoded. The rice proteome has been systematically analyzed using 2DE, N-terminal sequencing and MS methods (Komatsu *et al.*, 2003; Rakwal and Agrawal, 2003).

Recently, a systematic proteomic analysis of rice leaf, root and seed tissue using two independent technologies, 2DE followed by LC-MS/MS and MudPIT, allowed the detection and identification of 2,528 unique proteins (Koller *et al.*, 2002). Among them, 189 proteins (corresponding to 7.5%) that were detected in all of the tissues were found to be involved in the central metabolic pathways, transcription control and mRNA biosynthesis, and protein biosynthesis. The majority of proteins showed a tissues-specific expression pattern. The expression patterns of proteins, which were identified and classified as being involved in metabolic pathways were visualized on a metabolic map to illustrate the contribution of proteins to tissue-specific metabolic pathways.

Conclusions

In the post-genomic era, proteomics is positioned at the center of the functional genomics to study gene function on a genome-wide scale. The unique feature of proteomics is its feasibility to analyze the changes occurring at the protein level that cannot be predicted from genomic sequence. Proteins undergo post-translational modifications, proteolysis, recycling, multicomplex formation and subcellular translocation that are key events to regulate protein functions in cellular processes. Proteomics can eventually reveal all proteins in a cell or tissue at any given time, including those with post-translational changes. However, it also has technical limitations that are continually being researched. With the improved strategies, proteomics will surely provide powerful tools to disclose gene function in plant biology.

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