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Phytochemistry 65 (2004) 1487–1498
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Review

Technical aspects of functional proteomics in plants

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Received 10 January 2004; received in revised form 14 April 2004

Abstract

Since the completion of genome sequences of several organisms, attention has been focused to determine the function and functional network of proteins by proteome analysis. This analysis is achieved by separation and identification of proteins, determination of their function and functional network, and construction of an appropriate database. Many improvements in separation and identification of proteins, such as two-dimensional electrophoresis, nano-liquid chromatography and mass spectrometry, have rapidly been achieved. Some new techniques which include top-down mass spectrometry and tandem affinity purification have emerged. These techniques have provided the possibility of high-throughput analysis of function and functional network of proteins in plants. However, to cope with the huge information emerging from proteome analyses, more sophisticated techniques and software are essential. The development and adaptation of such techniques will ease analyses of protein profiling, identification of post-translational modifications and protein–protein interaction, which are vital for elucidation of the protein functions.

Keywords: Proteome; Porteomics; Mass spectrometry; Protein profiling; Post-translational modification; Protein–protein interaction; Plant

Abbreviations: DIGE, differential in-gel electrophoresis; ESI, electrospray ionization; FT MS, Fourier transform ion cyclotron resonance mass spectrometry; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; ICAT, isotope-coded affinity tag; IT MS, ion trap mass spectrometer; LC, liquid chromatography; MALDI, matrix assisted laser desorption ionization; MS, mass spectrometry; Q MS, quadrupole mass spectrometer; SELDI, surface enhanced laser desorption ionization; SPR, surface plasmon resonance; TAP, tandem affinity purification; TOF, time-of-flight; 2-DE, two-dimensional electrophoresis.

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doi:10.1016/j.phytochem.2004.05.019

1. Introduction

Sequence analysis of genomic DNA, which started in 1990s on a full scale, developed rapidly during the last decade. The entire sequence of genomic DNA is now available for many organisms including higher plants, animals and human. Based on this analysis, the number of genes in the genomes have been estimated. For example, about 32,000 genes encoding proteins are considered to be present in the human genome, suggesting that the human proteome consists of at least 32,000 proteins. Functions of only 50% of the proteins have been assigned by retrieval of the available databases. Similar situations can also be found in other organisms, suggesting a prime need to determine the functions of the unknown proteins and their functional network by proteome analysis.

Recently, large interest has been focused into transcriptome analysis, which is the comprehensive analysis at the transcriptional level of genes. Although gene expression can be analyzed at the transcriptional level, protein expression cannot always be analyzed from gene expression because of relatively low correlation (correlation coefficient about 0.5) in quantity between mRNA and protein (Anderson and Seilhamer, 1997). In addition, the DNA sequence and the expression of mRNA do not provide any information of protein post-translational modification, structure and protein–protein interaction. Almost all proteins are post-translationally modified, and then form specific structure and function through protein–protein (ligand) interaction. Therefore, it is important to analyze the protein itself.

Although proteome research started after the end of the genome sequence analysis, the developments over the last few years have been remarkable, and the new field of science, "proteomics", was introduced. In proteome analysis, a large number of proteins, not a few proteins, are analyzed by high-throughput methods such as two-dimensional electrophoresis (2-DE) and mass spectrometry (MS). In this aspect, proteome analysis is very different from conventional protein analysis.

Although a number of reviews on proteome research in plants are available (Thiellement et al., 1999; Zivy and de Vienne, 2000; Rossignol, 2001; van Wijk, 2001; Holtorf et al., 2002; Kersten et al., 2002; Roberts, 2002; Thiellement et al., 2002; Islam and Hirano, 2003; Rakwal and Agrawal, 2003), comprehensive information on proteome research encompassing most of the plants for which genome sequence are available are scant. In this review, we attempt to combine most of the information on proteome research accomplished in plant science, and discuss the suitability and pitfalls of currently available techniques related to proteome analysis.

2. Procedure of proteome analysis

In proteome analysis, first a number of proteins are separated. 2-DE (O'Farrel, 1975; Klose, 1975) is often used for the separation of proteins. A peptide map called "peptide mass fingerprint" is constructed by MS in order to identify the protein. Otherwise, the partial amino acid sequence is determined by MS ("de novo sequencing") and occasionally by a gas-phase protein sequencer. Then, the database constructed by the genome analysis is retrieved, and the corresponding gene is identified. The entry of the protein sequence database shows which proteins have been ascribed particular functions. If the sequence of protein is similar to that of some other proteins with known function, the function

of the target protein can be implied. When the protein function is not identified by the homology search, the post-translational modification, expression time, subcellular localization, expression level, protein–protein interaction, tertiary structure, enzymatic activity, physiological activity, etc. are analyzed in order to determine the function of protein. Finally, the information on 2-DE pattern, amino acid sequence, structure and function of each protein are compiled into the database.

3. Mass spectrometry and proteome analysis

MS for proteins and peptides has rapidly developed since 1980s. Nowadays, it is possible to measure the mass of proteins and peptides at the fmol level using MS with high accuracy, and to identify efficiently a number of proteins using software developed for proteome research. MS is also used frequently in the analysis of the protein expression, post-translational modification and protein–protein interaction.

An MS instrument is composed of ion source, mass spectrometer and ion detector. Although there are several methods in ionization of proteins and peptides in the ion source, two "soft" ionization methods, matrix-assisted laser desorption ionization (MALDI) and eletrospray ionization (ESI), are usually used. Generally, time-of-flight (TOF) MS is combined with MALDI, while quadrupole MS (Q MS) and ion trap MS (IT MS) are combined with ESI. Recently, Q-TOF MS in which TOF is appended to quadrupole MS has been developed. MALDI-TOF/TOF MS and MALDI Q-TOF were also developed. The characteristic of each MS is different depending on the type of ionization and mass spectrometer. There is no MS that can be useful for all fields of proteome analysis. It is necessary to choose the instrument suitable for the purpose of the analysis. MALDI-TOF MS is often used for high-throughput identification of the protein by peptide mass fingerprinting. In the analysis of amino acid sequence and post-translational modification, MS/MS such as ESI IT and ESI Q-TOF MS are used. Recently, Fourier transform ion cyclotron resonance MS (FT MS) instrument have been refined. This instrument using the phenomenon of ion cyclotron resonance can fragment the protein ionized by ESI or MALDI to determine the amino acid sequence and post-translational modification. FT MS allows analysis with high sensitivity and extremely high accuracy.

In most MS analyses such as MALDI-TOF MS, ESI Q-TOF MS and ESI IT MS, the proteins are analyzed after digestion with protease into the peptides, because large molecular weight of proteins cannot be analyzed directly. This approach is called "bottom-up proteomics". In contrast, in FT MS, the whole protein can directly be applied to obtain information. FT MS allows analyzing the sequence information and post-translational modification of proteins automatically without preparation by 2-DE and liquid chromatography (LC). This approach is called "top-down proteomics" or top-down mass spectrometry (Mortz et al., 1996; Forbes et al., 2001; Ge et al., 2002; Sze et al., 2002; Ver-Berkmoes et al., 2003).

4. Protein expression and its detection

4.1. Protein profiling

A common goal of proteome analysis is to determine the protein functions and to explore the functional network of proteins. This is accomplished by elucidation of the protein expression profile, post-translational modifications and protein–protein interactions. The profiling of a large number of proteins is done to determine the correspondence of protein with the gene. Matsudaira (1987) first reported a method in which proteins separated by 2-DE were electroblotted onto the polyvinylidene difluoride membranes, and the partial amino acid sequence determined by a gas-phase protein sequencer. With this method, the partial amino acid sequences of a number of proteins separated by 2-DE in plants (Hirano, 1989) were determined for protein identification. Afterward, this technique was widely used to identify proteins in rice (Komatsu et al., 1994; Tsugita et al., 1994; Zhong et al., 1997; Hirano, 1997; Woo et al., 2002), *Arabidopsis* (Kamo et al., 1995; Santoni et al., 1998), wheat (Skylas et al., 2000), barley (Flengsrud, 1993) and tobacco (Rouquie et al., 1997).

Recently, protein identification has been accomplished by comparing the experimentally obtained peptide mass fingerprints with the theoretical ones in the databases. Alternatively, the partial amino acid sequences of proteins separated by 2-DE have been determined by ESI Q-TOF MS and ESI IT MS to identify the proteins.

Although 2-DE allows simple, rapid and reproducible separation of a number of proteins, it is difficult to separate high molecular weight and basic proteins. To overcome this problem, "shotgun" method has been developed (Link et al., 1999; Smith et al., 2002). In this method, the proteins extracted from the cells are digested with protease such as trypsin and lysylendopeptidase, and the resultant peptides are analyzed by multidimensional LC, followed by MS/MS to determine the sequences. Using the shotgun method, a comprehensive proteome analysis has been performed in many organisms including yeast and human.

In plants, the identification of proteins by MS was performed in rice (Koller et al., 2002; Fukuda et al., 2003), *Arabidopsis* (Santoni et al., 1998; Gallardo et al., 2002), maize (Touzet et al., 1996), barrel medic (*Medicago truncatula*) (Watson et al., 2003), etc. The most

comprehensive studies were performed in rice (Koller et al., 2002) and barrel medic (Watson et al., 2003), which are model plants in cereals and legumes, respectively. Koller et al. (2002) identified a total of 2528 unique proteins including 1022 different proteins from leaves, 1350 different proteins from roots and 877 different proteins from seeds. Most of the proteins (67.2%) have known functions or homologous sequences to other proteins with known functions in the database. The identified proteins were classified into 16 functional categories (Fig. 1). The most abundant class of proteins were involved in metabolic processes (20.8%). There were many proteins involving in protein synthesis, protein degradation and signal transduction. Of the 2528, 189 proteins were expressed in all three organs, but 622, 862 and 512 proteins were expressed only in leaves, roots and seeds, respectively. Enzymes involved in central metabolic pathways are present in all tissues, while many proteins showed a tissue-specific expression pattern. Similarly, Watson et al. (2003) identified a total of 304 proteins from leaves, stems, roots, flowers, seed pods and cell suspension cultures of barrel medic by MS. The functions of 55% of proteins were assigned by homology to known sequences. An average 61% of proteins were detected in one or more tissues, while 39% were found in only one tissue and related to the function specific to the tissue.

Protein expression varies depending on particular species, variety, growth stage, organ and cell organelle in particular environment. The expression profile is closely related to the function of proteins. When proteins are extracted from the cells under different conditions and are compared, it is called "protein differential display" analysis. With this analysis, the interspecies and varietal differences of plant proteins (Hirano, 1982) have been studied, and many proteins specific to the organ and tissue (Kehr et al., 2001; Mayfield et al., 2001; Porubleva et al., 2001; Koller et al., 2002; Shen et al., 2002; Watson et al., 2003), and growth stage (Bardel et al., 2002; Finnie et al., 2002; Gallardo et al., 2002; Maltman et al., 2002; Wilson et al., 2002) of plants have been detected. The proteins which were up- or down-regulated by hormone treatment (Konishi and Komatsu, 2003; Shen et al., 2003; Tanaka et al., 2004), disease (Konishi et al., 2001) and stress such as low temperature (Tafforeau et al., 2002), heat (Majoul et al., 2003), drought (Riccardi et al., 1998; Rey et al., 1998; Salekdeh et al., 2002), salt (Ramani and Apte, 1997) and ozone (Agrawal et al., 2002) were also investigated by 2-DE. The proteins, which expressed in the near-isogenic lines with phenotypes such as semi-dwarfism (Hirano et al., 1991) and various mutants (Damerval and Le Guilloux, 1998; see Thiellement et al., 1999), were detected by 2-DE. These analyses are essential to identify the proteins involved in growth, differentiation, disease resistance and stress tolerance of plants. On the other hand, allergen proteins (Weiss et al., 1997), wheat germ quality-related proteins for beer (Gorg et al., 1992), wheat quality-related proteins (Gottlieb et al., 2002), etc. were identified by 2-DE. This is important to determine the function of the plant proteins in foods.

There are many reports on the proteins which appear in organelles such as plasma membrane (Santoni et al., 1998, 1999), mitochondria (Heazlewood et al., 2003; Kruft et al., 2001; Millar and Heazlewood, 2003; Millar et al., 2001), chloroplast (Peltier et al., 2000; Ferro et al., 2002; Gomez et al., 2002; Schubert et al., 2002), peroxisome (Fukao et al., 2002), amyloplast (Andon et al., 2002; Islam et al., 2003a) and ribosome (Yamaguchi et al., 2003; Yamaguchi and Subramanian, 2003) of various plant species including *Arabidopsis*, rice and wheat. These proteins were detected by 2-DE and identified by mainly MS. On the other hand, Gomez et al. (2002) analyzed comprehensively proteins from thylakoid membrane subdomains (grana) of pea and spinach. In this analysis, they identified the thylakoid membrane proteins by the shotgun method. Recently, Zabrouskov et al. (2003) applied the chloroplast proteins from *Ara-*

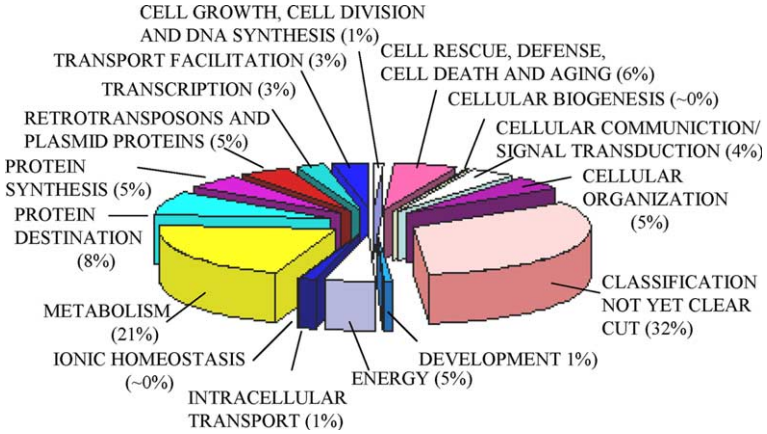

Fig. 1. Functional classification of a total of 2528 proteins identified in the rice leaf, root and seed (cited from Koller et al., 2002).

bidopsis to FT MS to identify them by top-down proteomics. This is the first application of the top-down mass spectrometry to plant proteome analysis.

4.2. Analysis of subcellular localization

Recently, a high-throughput method using epitope tag or green fluorescent protein (GFP), which can analyze the subcellular localization of proteins, has been developed. In the epitope tagging, the chimera DNA, which encodes a target protein and a specific epitope peptide, is introduced into a vector to transfect to the host cell. The expression site of the fusion protein can be detected using the antibody by microscopy. Instead of the epitope tag, GFP is also used, and in this case, the fusion protein is detected as a fluorescent protein. In yeast, the localization of almost all proteins was identified by these methods (Kumar et al., 2002; Huh et al., 2003). In plants, Cutler et al. (2000) constructed GFP::cDNA fusion library and introduced it to *Arabidopsis* by *Agrobacterium* transfomation system. The subcellular localization of proteins was identified by the fluorescence of GFP.

4.3. Differential in-gel electrophoresis

Differential in-gel electrophoresis or difference gel electrophoresis (DIGE), a recently developed 2-DE technique with high reproducibility (Unlu et al., 1997), is gaining popularity. In this technique, the proteins are extracted from different types of cells, labeled with different fluorescent reagents, mixed and then separated by 2-DE using a single gel. The proteins are detected separately at the excitation wavelength specific to the different fluorescent reagents. Two patterns are analyzed by an image analyzer, and the difference of the protein expression is easily detected. This technique is useful to reduce the gel to gel variation.

4.4. Surface enhanced laser desorption ionization-mass spectrometry and isotope-coded affinity tagging

The surface enhanced laser desorption ionization (SELDI)-MS is used in protein differential display analysis without using 2-DE. In SELDI MS, the protein is immobilized in 2 mm diameter of holes on the surface of metal chip plate by static electricity, hydrophobicity or metal affinity, antibody affinity, receptor ligand interaction, etc (Issaq et al., 2002). The immobilized protein is directly analyzed by MALDI-TOF MS. In this method, the sample is easily concentrated, and the contaminant is removed from the sample on the chip. Therefore, the differential display of low abundant proteins can be performed. There are many reports of SELDI-MS analysis in mammals, but not many in plants. El-Gendy et al. (2001) immobilized two cell wall proteins of spring wheat, which may be involved in defensive reactions to pathogenic attack, onto reverse-phase protein chip surface, and identified their target proteins using MS.

Isotope-coded affinity tag (ICAT) labeling is also used in protein differential display analysis without 2-DE. In ICAT labeling, the proteins from different cells are labeled by ICAT with different molecular masses (Gygi et al., 1999). The proteins of two samples are mixed, and digested with protease such as trypsin and analyzed by LC-MS/MS. The difference in protein quantity is determined by measuring the peptides labeled with heavy and light ICAT reagent. This technique was successfully used for the first time in plants by Islam et al. (2003b) to analyze the difference of protein composition between wheat cv. Chinese Spring and its chromosome deletion lines. Although as an initial step to investigate the feasibility of quick protein identification using ICAT-ESI MS/MS has been reported, several challenges become apparent. For example, it was often difficult to identify C-terminal fragments due to signals resulted from the fragmentation of ICAT reagents (Islam et al., 2003b). In addition, peptides labeled with ^{2}H and ^{1}H affect the retention time in HPLC due to their differences in polarity (Williams et al., 2002). Difficulties in peptide quantification caused by ^{2}H and ^{1}H are also apparent from other reports (Zhang and Regnier, 2002). In recent studies, $^{2}H/^{1}H$ have been replaced by $^{12}C/^{13}C$, and found that the incorporation of $^{12}C/^{13}C$ has no measurable effect on peptide elution time (Hansen et al., 2003). Another problem in ICAT system is that the cleaning and purification of samples from salts and detergent by columns limits the use of wide range of urea-based solvents, considered to be ideal for plant protein extraction. Therefore, sample extraction procedure of ICAT has to be improved to make a valid comparison.

5. Post-translational modification

5.1. Detection of post-translational modification

In order to perform the intrinsic functions, most proteins need to be post-translationally modified. Therefore, the analysis of post-translational modification is important to determine the in vivo state of proteins. The area of proteomics which deals with post-translational modification is called ''modificomics''. Various post-translational modifications such as removal of signal peptide, processing of precursor polypeptide, and modifications of amino acids have been reported. Of them, the modifications of amino acids are frequently detected by MS. In this case, the protein extract is digested with protease such as trypsin. The molecular mass of the resultant peptides is analyzed by MS, and the modified amino acid can be identified from the

differences (http://www.abrf.org/index.cfm/dm.home) between actual and theoretical molecular masses. Database and software for identifying the modified amino acids from the mass difference are available elsewhere.

Among various post-translational modifications, phosphorylation and glycosylation have been widely studied. Protein phosphorylation is closely related to signal transduction. The research field of the phosphoprotein in proteomics is called "phosphoproteomics". Phosphopeptides cannot be often detected by usual MS analysis. Various methods have been developed in order to detect the phosphopeptides more effectively. For example, Zhou et al. (2001) selectively isolated the phosphopeptide by modifying phosphate group of phosphoserine and phosphothreonine of the peptide with sulhydryl group, followed by purifying with amino beads. Oda et al. (2001) substituted the phosphate group of phosphoserine with biotin, and identified the biotinylated peptide by MS. The steps of derivatization and purification of phosphopeptide are very common in these methods. This technique needs to be improved for effective isolation of phosphopeptide.

Glycosylation of proteins is related to the function such as cell recognition, membrane binding, enzymatic activity, interaction between proteins, etc. Recently, Kaji et al. (2003) have developed a novel method for high-throughput detection of glycoprotein. In their method, proteins were purified by lectin column, and ^{18}O was introduced into the Asn-linked oligosaccharide-binding site using glycopeptidase and the ^{18}O-labeled peptides were identified by LC-MS/MS. They detected 250 proteins with Asn-linked oligosaccharide in nematode by this method. This method can be applied to analyze plant glycoproteins.

Glycosylphosphatidylinositol (GPI)-anchored proteins are targeted to the plant cell surface and are likely to be related to extracellular matrix remodeling and signaling. Borner et al. (2003) identified GPI anchored proteins using Triton X-114 phase partitioning and phosphatidylinositol-specific phospholipase C in *Arabidopsis*. In this study, a number of phospholipase C sensitive proteins were detected by 2-DE and SDS-gel electrophoresis. They identified 30 GPI-anchored proteins including β-1,3 glucanase, phytocyanine, receptor-like protein by LC-MS/MS.

5.2. Functional analysis of post-translational modification

As discussed above, MS analysis has a great potential to identify post-translational modifications of a large number of proteins. However, even if post-translational modification is detected, it is not easy to estimate the function of protein based on the modification. Because there is not enough information on the role of post-translational modification are available to estimate the function, and also in our knowledge, there is no database and software which can predict the function from the information of post-translational modification detected by MS analysis. Therefore, this can be an area of interest which might play a significant contribution to the proteome analysis.

Kimura et al. (2000, 2003) focused into the yeast 26S proteasome, which is a multifunctional protein complex with proteolytic activities and exists in many organisms from archaebacteria to higher plans, animals and human. The post-translational modification of the 26S proteasome was investigated comprehensively. The 26S proteasome consists of two units, a 20S proteasome and a pair of 19S regulatory particles, containing 28 and 36 different subunits, respectively, in yeast. Many subunits were found to be N-acetylated. In yeast, there are three *N*-acetyltransferases, Nat A, Nat B, and Nat C, containing NAT1, MAK3, and NAT3 subunits, respectively (Polevoda and Sherman, 2000). Analysis of the 20S proteasome subunits purified from the normal strain and each of the NAT1, MAK3 and NAT3 deletion mutants indicated that six subunits were N-acetylated with NAT1, two subunits with MAK3, and a subunit with NAT3 (Kimura et al., 2000). The latent 20S proteasome has three proteolytic activities corresponding to trypsin-, chymotrypsin- and caspase-like activities. It was found that the chymotrypsin-like activity was higher in the NAT1 deletion mutant than the normal, suggesting that N-acetylation may be related to the proteolytic activity of the 20S proteasome. It is likely that lack of N-acetylation of the α-subunits in the NAT1 deletion mutant possibly results in change of higher order structure of the 20S proteasome, leading opening of the channel of 20S proteasome catalytic cavity and subsequent activation of the chymotrypsin-like activity of the 20S proteasome (Kimura et al., 2000). The 19S regulatory particle was also purified in yeast, and 36 subunits were separated by 2-DE and identified by MALDI-TOF MS. At least 24 of the 19S regulatory particle subunits were N-acetylated (Kimura et al., 2003). No information on the function of the N-acetylation of the 19S regulatory particle subunits is available at present. About 50% of one of the 19S regulatory subunits was N-myristoylated, suggesting that some part of the 26S proteasome works on the membrane (Kimura et al., 2003). On the other hand, three α-subunits to be phosphorylated were identified in the yeast 20S proteasome, and that the chymotrypsin-like activity was found to be affected by dephosphorylation of these subunits with alkaline phosphatase (Iwafune et al., 2002). Phosphorylated subunits in the 19S regulatory particle were also identified. It was found that the dephosphorylation of the proteasome with alkaline phosphatase affects chymotrypsin-like activity of the 20S proteasome and ATPase activity of the 19S regulatory particle (Nishimura et al., 2002). To analyze the differ-

ence of post-translational modification between rice and yeast, all of the rice DNAs that encode the 26S proteasome subunits were cloned (Sassa et al., 2000; Shibahara et al., 2002). The DNA sequences were determined and the amino acid sequences were deduced. From the results of protein and DNA analyses, a difference was found in the N-acetylation of the 20S proteasome subunit, that is, the rice β6 subunit has been N-acetylated, suggesting that the functional difference may exist in the β6 subunit between rice and yeast. The data obtained in these analyses were compiled into the database for performing the high-throughput proteome analysis. Similar type of information are necessary to understand protein functions under the condition for post-translational modification.

6. Protein–protein interaction

6.1. Immunoaffinity purification

Proteins function by interacting with other proteins and ligands. Analysis of protein–protein (ligand) interaction is important to determine the function of proteins. The analysis of protein–protein (ligand) interaction is called ''interactome'' analysis, and the scientific field is called ''interaction proteomics''. In proteome analysis, the interactions are often analyzed by immunoaffinity purification, surface plasmon resonance analysis (SPR), protein chip and MS.

In immunoaffinity purification, epitope tagging and tandem affinity purification (TAP) are the two techniques used to detect protein–protein interaction. In the epitope tagging, DNA which encodes the fusion protein of a target protein and epitope is overexpressed in the cell. The protein interacted with the target protein is purified by immunoprecipitation using antibody, separated by electrophoresis and identified by MS. In the TAP method, the fusion protein where two epitopes (IgG binding domain of protein A and calmodulin binding peptide) are conjugated to the target protein is overexpressed, and the protein complex consisting of the target protein and the interacted proteins is purified by two steps of purification using IgG- and calmodulin-beads (Rigaut et al., 1999). This method is frequently used to detect protein–protein interaction in many organisms. Rivas et al. (2002) used this method to purify the 420-kDa heteromultimeric membrane-associated protein complex interacted with the tomato Cf-9 protein. The *Cf-9* gene confers race-specific resistance to the fungal pathogen expressing the avirulence gene *Arv-9*.

6.2. Surface plasmon resonance spectrometry

In SPR, the interaction is analyzed using the phenomenon called surface plasmon resonance. Ligands such as proteins are immobilized on the sensor chip surface, and the sensor chip is installed in SPR spectrometer. The protein solution is applied into the flow cell. When the specific protein (analyte) binds to the ligand, the surface plasmon resonance signal changes. This change is measured by the photodiode array detector to detect the protein–protein (ligand) interaction. In SPR, it is also possible to analyze the binding kinetics. The binding protein is digested in the flow cell with protease, and the digests are analyzed by MS to identify the protein. Laukens et al. (2001) found the interaction of cytosolic glyceraldehydes 3-phosphate dehydrogenase and two nucleoside diphosphate kinase isoforms with cAMP in tobacco bright yellow 2 cells by SPR and ESI Q-TOF MS. The problem is that the throughput of this method.

6.3. Genome manipulation by chromosome deletion

Recently, Islam et al. (2003) studied protein–protein interaction in wheat seed proteome by using chromosome deletion lines. The changes in protein composition of wheat seed proteome were investigated by using 39 ditelocentric lines (Islam et al., 2002), which carry all the normal chromosome (euploid) complement of wheat chromosome for which one arm is missing, and also by using fine deletion lines of chromosomes 1B (Islam et al., 2003b). Proteins were separated by 2-DE and visualized by staining with Commassie brilliant blue. Quantitative analysis of protein-spots was performed by PDQuest software. Variations in protein-spots between the euploid and the 39 ditelocentric lines were evaluated. Out of the 1755 major spots detected in 39 ditelocentric lines, 147 (11%) were disappeared, 978 (71%) were up-regulated and 247 (18%) were down-regulated. Correlation studies in changes in protein intensities among 24 protein spots across the ditelocentric lines were performed. High correlations in changes of protein intensities were observed among the proteins encoded by genes located in the homoeologous arms. The feasibility of a new analytical approach to identify protein–protein interactions based on ICAT labeling of peptides in tryptic digest followed by ESI Q-TOF MS has been investigated. The down- and up-regulated proteins were detected in the fine chromosome deletion lines. This approach shows a possibility to identify wheat seed proteins and to understand their interactions, which are reported to be difficult by 2-DE due to co-synthesis of proteins by genes from three genomes, A, B and D.

Although the genome manipulation technique was successfully used by Islam et al. (2003a,b,c) to predict protein–protein interaction in wheat seed proteome, this technique is not feasible for diploid species where genome manipulation is not as easy as the hexaploid wheat.

6.4. Protein microarray analysis

The DNA microarray which rapidly developed in 1990s has become an epoch-making technique for gene expression analysis. Using a similar idea, the research area of protein microarray has started in order to analyze the expression profile of proteins and their function (MacBeath, 2002). However, this technique has been facing a number of problems since it came to existence. For example, some proteins change their conformation when it comes in contact with the chip and thus lose its activity. In many cases, the surface of plate is chemically modified, and the proteins are immobilized covalently with the modification group. In order to make this technology more popular, it is necessary to improve the material of the chip, to refine the methods for the surface treatment and protein immobilization, and also the methods for detection need to be improved.

To produce the protein chip, native proteins or proteins which are expressed by genetic manipulation are purified and immobilized on the plate. However, the purification of many protein speceies is not easy, and this has become a bottleneck to produce the high-density protein chip.

The high-density protein chip can be produced, if proteins separated by 2-DE are electroblotted onto the chip plate. High-throughput analysis of the protein–protein interaction can be performed when the proteins interacted with the target proteins on the chip are identified by MS. However, such chip-plates on which the target proteins are directly transferred from the gels, were not available.

Recently, a diamond-like carbon coated stainless steel plates modified with *N*-hydroxysuccinimide ester has been developed. The target proteins in the gels are immobilized on the diamond-like carbon coated plate with high blotting efficiency (30–70%). Proteins extracted from the cells are probed with the proteins on the diamond-like carbon coated plate, and the interacted proteins are detected by MALDI-TOF MS (Hirano et al., unpublished). This technique has a great potential of high-throughput analysis of proteins interacted with thousands of plant proteins separated by 2-DE.

7. Proteome bioinformatics

Since the development of proteomics, enormous information on proteome analysis has been produced. Unfortunately, unlike the DNA sequence database, no unified proteome database is available for proteomic study. However, some organizations are coming forward to make protein databases. For example, the note (annotation) on the characteristics of the proteins and the sequence data of a number of proteins are also available in the Web site: (http://www.expasy.ch/alinks.html). The databases are linked with other databases such as DNA sequence database and protein structure database. In addition, the following databases contain satisfactory information for specific plants.

- *Arabidopsis* http://sphinx.rug.ac.be:8080/ppmdb/index.html
- Maize http://moulon.inra.fr/imgd
- *Pinus pinaste* http://www.pierroton.inra.fr/genetics/2D/

In order to analyze the function of a number of proteins, it is important to develop the software for proteome analysis. Various software, for example, 2-DE pattern image analysis, MS analysis, identification of the protein based on the peptide mass fingerprint and amino acid sequence, detection of the post-translational modification is available. However, there is no sophisticated software which, for example, can predict the function of proteins from the data of amino acid sequence, post-translational modification, protein–protein interaction and higher order structure.

8. Technical perspective

The technology for separation and identification of a number of proteins has been rapidly developed in proteomics. The techniques such as 2-DE, DIGE, ICAT, shotgun analysis and MS are considered to be useful for separation and identification of proteins in plants. Recently, some new techniques such as FT MS for top-down proteomics and TAP for analysis of protein complex have developed for analysis of function and functional network of proteins. However, further development of the technology is required to cope up with current demand of high throughput proteomics. Although a number of software packages are available to identify proteins and to predict their functions, no software is available to analyze the post-translational modification and protein–protein interaction, which are particularly important for elucidation of the protein function.

Efficiency of the protein functional analysis in proteomics is strongly "database-dependent". In many cases, the function and functional network are determined by retrieval of the database based on the data obtained by actual analysis of proteins. Therefore, the improvement of quality and quantity of the proteome database and construction of the software, which can analyze the protein function, are essential for proteome research.

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