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Proteomic approach for analyzing cellular responses to abiotic stresses in crop plants

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Introduction

With tremendous advances in new technologies in the field of biological sciences, it is possible to get more precise and broad information on plants. This is mainly due to the flood of information on the decoded genomes of two plants. *Arabidopsis thaliana*, was the first plant, whose genome was fully decoded and almost perfectly annotated (Bernal et al., 2001). In 2005, the complete genome sequence of rice which provides both the linear order of the 37,544 genes and their positions on the 12 rice chromosomes became finally available to the public in 2005 (International rice genome sequencing project, 2005). The vast genomic data of these two flowering plants represents a significant landmark in the history of plant biology. However, which parts of genome sequence are valuable as genes encoding protein, and the function of protein and its abundance in plant cells can not be predicted from the genome sequence.

Advanced genomic technologies like DNA-based microarray and serial analysis of gene expression have become available to profile genome-wide expression of unprecedented numbers of genes at the mRNA level (Kawasaki et al., 2001). However, it is not genes but proteins that are directly responsible for the function of cells. Moreover, the various biological processes of living cells in plants are

mediated by mainly protein, which interact with metabolites, phospholipids, carbohydrates and nucleic acids. In this respect, such a genomic tool does not provide any direct information on protein levels and their modification (Anderson & Anderson, 1998) mainly due to post-translational regulation like phosphorylation, which results in a lack of correlation between mRNA and protein abundance (Gygi et al., 1999).

Although the idea of measuring all the proteins produced in an organism was suggested in the early 1980s (Anderson et al. 1982), this idea has been merely a possibility for years. With technological advances in protein separation and protein identification, the term proteome, the total PROTEin complement of a genOME, was first heralded at a scientific conference in 1994. Since then, the field of proteomics has evolved, which involves studying the proteome and a systematic analysis of the protein population in a cell, subcellular compartment, tissue, and whole organisms (Huber, 2003).The fundamental technique for proteomics is two-dimensional gel electrophoresis (2-DGE) which separates simultaneously thousand of proteins (O'Farrell, 1975). In high-resolution 2-DGE, proteins are separated by isoelectric point [pI(s)] in the first dimension (isoelectric focusing; IEF) and molecular size [M_r(s)] in the second dimension (sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SDS-PAGE). This technique enables subsequent characterization either in gel or after blotting on to membrane at a low cost (Herbert et al., 2001), and is capable of generating high-quality 2-D gel reference map in a short period of time. Along with the mass spectrometry-based protein identification methods, proteomics became a high-throughput approach that is being used to address biological function of plants by studying globally expressed proteins in a cell, given tissue and whole organisms (Rakwal & Agrawal, 2003; Agrawal & Rakwal, 2006). An overall view that encompasses the steps from protein samples to database construction has been schematically depicted in Fig. 1. Plant proteomics is still in its infancy compared to the proteomic analyses of other organism. This is partly due to the lack of availability of genomic database from plants. However, plant proteomics is becoming a major area of study in plant biology, including on the hot research area of environmental

stress biology. This is due to the fact that responses to both biotic and abiotic stresses are a matter of great concern of crop scientists as these crops form the basis of our food and nutritional resources.

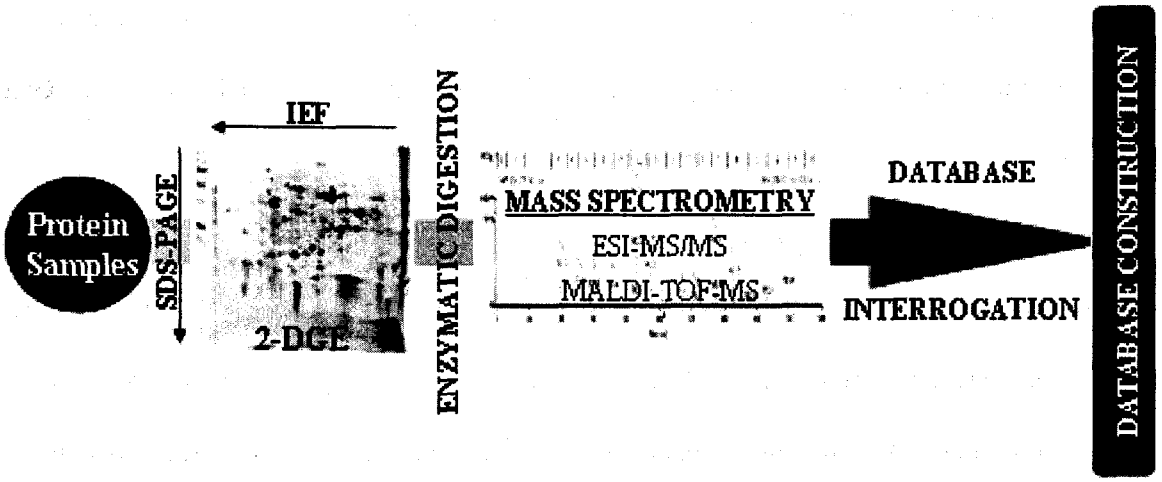

Fig. 1. The proteomics workflow.

A number of unfavorable abiotic stress factors strongly reduce crop growth and yield; these include water deficit, soil salinity, low temperature, flooding and inavailability of nutrients. Each of abiotic stress factors affects crop plants either specifically or in a concerted manner together with other stress factors. In a published report, it has been calculated that maximum 80% of the record yield of major crop plants was reduced by abiotic stress factors, depending on the species (Bray et al., 2000). Therefore, there have been a numerous physiobiochemical and molecular approaches directed towards understanding the plant's responsiveness and tolerance mechanisms to different abiotic stress factors (Ashraf & Harris, 2005). Proteomic approaches have been rapidly applied in this field of plant biology, as shown in table 1. It can be expected that proteome studies will contribute to gaining further insights into the cellular events of plant in response to abiotic stresses, and their results will be used for future breeding of stress-tolerant crop plants.

This presentation is intended to provide for either established or up-coming researchers a primal technique in proteomic approach and examples of its application to analyzing the responses of crop plants to abiotic stresses. For this purpose, firstly, we will describes techniques for protein sample

preparation which is the first and most critical step for subsequent protein separation and identification in proteomics. Secondly, we will summarize the achievements of proteomic analysis made on responses of crop plants exposed to different abiotic stress factors, including salt stress. Finally, we will propose what should be the future directions for the proteomic study on the response of crop plants to the abiotic stress factors.

Table 1. Reports on the responses to different abiotic stress factors in crop plants.

Stress factors	Crop species	References
Drought	Maize	[Riccardi et al., 1998; Riccardi et al., 2004; Vincent et al., 2005]
	Sugar beet	[Hajheidari et al., 2005]
	Rice	[Salekdeh et al., 2002; Rakwal & Komatsu, 2004; Ali & Komatsu, 2006]
Salinity	Rice	[Abbasi & Komatsu, 2004; Yan et al., 2005; Kim et al., 2005; Parker et al., 2006]
	Wheat	[Majoul et al., 2000; Ouerghi et al., 2000]
	Maize	[Zörb et al., 2004]
Low temperature	Rice	[Imin et al., 2004; Cui et al., 2005; Yan et al., 2006; Yang et al., 2006]
Flooding	Rice	[Dubey et al., 2003]
	Maize	[Chang et al., 2000]
Low nitrogen application	Wheat	[Bahrman et al., 2004]

Protein Sample Preparation

The ideal sample preparation for plant proteome study should be the extraction of a maximum number of proteins from a given plant material. A good extraction buffer should extract all proteins in a

quantitative manner and should protect proteins from proteolytic degradation. Selection of a suitable extraction buffer is the key for good sample preparation.

Urea-based O'Farrell buffer (Lysis buffer; LB) is a commonly used and well-established buffer for protein extraction (O`Farrell, 1975). From our experiences, it has been confirmed that LB is easy to use and effectively extracts proteins from rice leaves regardless of the growth stage of rice (Rakwal & Agrawal, 2003; Agrawal & Rakwal, 2006). Besides, it was found that a modified lysis buffer supplemented with thiourea plus Tris-HCl and Trizma-base (LB-TT) increases considerably the solubilization of proteins as well as the number of spots on 2-D gels (Fig. 2).

Depending on the plant materials, such as soybean seeds, proteins are not easily extracted by common extraction methods due to high levels of interfering compounds. For this reason, a couple of methods have been developed and used together with LB-based protein extraction method. Among the most popular techniques is the use of tricholoroacetic acid (TCA) and acetone (TCA/acetone) for the direct precipitation of proteins from a given cell material (Cho et al., 2006). Another one is the solubilization of proteins in phenol followed by precipitation with methanol and ammonium acetate (Hajduch et al., 2005). By an extensive washing crude samples with organic solvent and aqueous TCA, these techniques are effective on removing salt, pigments, lipids and other water soluble contaminants.

Basically, proteome can be simply defined as a large number of proteins with varying levels of abundance, hydrophobicities, and diverse pI(s) and M_r(s) in a cell or organism. Therefore, along with suitable protein extraction buffers, protein simplification methods to reduce the heterogeneity of protein sample are also necessary for a good protein sample preparation. For an example, when one's target proteins are located in a particular range of molecular size, SD-SPAGE can be used for selective partitioning complex protein mixtures on the basis of their molecular size. Under this aspect, Kim et al. (2003) used SDS-PAGE to enrich high molecular weight or low abundance proteins and simultaneously increase the number of protein spots. In this technique, rice (cv. Jinheung) leaf proteins were separated by SDS-PAGE, and the gel segment of desired molecular size was cut and extracted.

After acetone precipitation and resolubilization of the protein extracts, 2-DGE was performed. This technique resulted in 27% increase in protein spots over un-fractionated samples, and 17% were enhanced. In addition, it should be known that a particular protein extraction method does not cover enough information on the proteomic nature of a plant material (Agrawal and Rakwal, 2006). Therefore, it would be necessary to use at least two different extraction buffers depending on the type of plant materials.

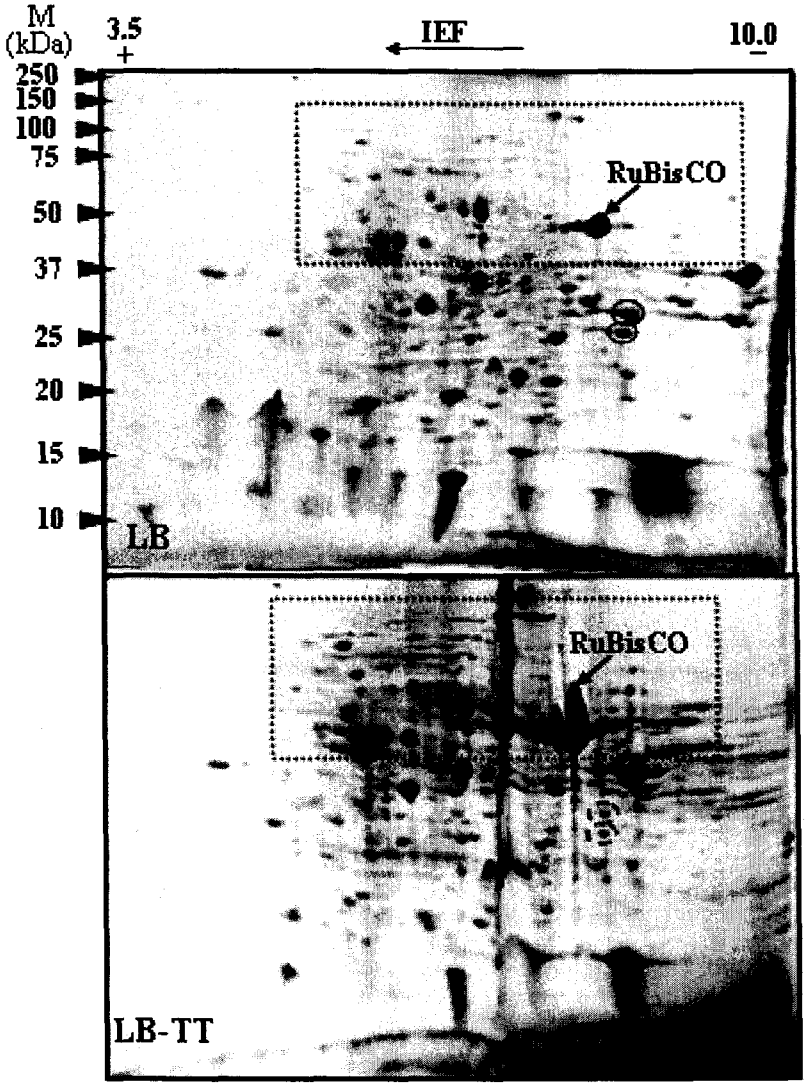

Fig. 2. Effect of a slight modification of protein extraction buffer on the 2-DE protein profiles. Proteins were extracted from rice leaves using two different LBs (LB and LB-TT).

Summary of Proteome Studies on Abiotic Stresses in Crop plants

In nature, crop plants are often exposed to a variety of abiotic stress during their growth duration. Adverse effects of abiotic stresses can reduce considerably the growth and yield of crop plants. To cope with these stresses, crop plants have evolved a number of highly sophisticated strategies associated with stress signal recognition, transduction, and subsequent physiobiochemical responses in a complex network. In this section, we will summarize a number of proteomic approaches, which have been applied to investigate on alterations of global protein expressions in crop plants under various abiotic stress factors.

Drought

Two unrelated lines of maize and their hybrid were used to analyze protein expression profiles changing in maize leaves after drought stress (Riccardi et al., 1998). Among a total of 78 protein spots on 2-D gels showing significant quantitative differences, a majority of protein spots are identified as well-known water stress-responsive proteins (e.g. RAB17, ABA-stress-ripening protein, and ferritin) as well as enzymes involved in basic metabolic pathways such as glycolysis and the Krebs cycle. Based on the drought stress-induced up-regulation of proteins involved in phenylpropanoid metabolism, such as caffeate *O*-methyltransferase, β-glucosidase, cysteine synthase and glutamate semialdehyde aminotransferase, the authors suggested that the lignin-biosynthesis pathway in maize leaves could be activated under drought stress. This idea was supported in a paper by Vincent et al. (2005). In this study, protein expression profiles with respect to candidate proteins were consistent with the physiobiochemical alterations such as a reduction of lignin content and a migration of the lignified area on the maize leaves.

In a proteome analysis of sugar beet leaves under drought stress (Hajheidari et al., 2005), the expression of 79 proteins were differentially regulated by the stress; of these, 8 spots were not detected

in the non-stressed controls after 2-DGE. In this study, identified proteins were previously known to be stress-responsive proteins, including the small heat shock protein (HSP), cytosolic Cu-Zn super oxide dismutase (Cu-Zn SOD), 2-cysteine peroxidase, cyclophilin, nucleoside-diphosphate kinase, a nascent polypeptide-associated complex α-chain, and the large subunit of RuBisCO. Proteomic studies using a variety of plant species have shown that the expression of these stress-responsive proteins was genetically variable. Moreover, these proteins are certainly involved in protection mechanisms in plants. Therefore, these proteins can be used as potential biomarker for screening the drought tolerance of crop plants.

Using two different lines of rice, Salekdeh et al. (2002) demonstrated cultivar specific differences in protein expression pattern of rice during drought stress. The drought stress-responsive proteins identified in these studies were proteins belonging to photosynthesis, cell elongation, anti-oxidant, metabolism and lignification. Fifteen proteins showed similar expression patterns in the two cultivars, whereas 27 proteins showed up- or down-regulated expression in one line and failed to respond in the other. More recently, Ali et al. (2006) reported a comparative proteomic study of rice leaf sheath under drought stress using Nipponbare and the drought-tolerant cultivar, Zhonghua 8. Compared with Nipponbare, the drought-tolerant cultivar Zhonghua 8 showed relatively higher expression level of proteins, which were identified as actin polymerization factor, photosystem II (PSII) oxygen evolving complex protein, oxygen evolving enhancer protein 2 (OEE2), and light harvesting complex chain II. Besides, they also examined the changes of protein profiles under different stresses. The expression of proteins such as actin depolymerising factor, light harvesting complex chain II and SOD were regulated under drought and osmotic stress, but remained unchanged under salt stress, cold stress. It was also found that actin depolymerizing factor was up-regulated by drought stress in all organs of rice seedlings. These results suggest that actin depolymerizing factor may be one of the specific proteins in rice conferring tolerance to drought or osmotic stress.

Salinity

Abbasi & Komatsu (2004) studied salt-responsive proteins of rice seedlings exposed to 50, 100 and 150 mM NaCl for 6 to 48 hours. In rice seedlings exposed to low salinity (50 mM NaCl), accumulation of eight proteins was enhanced with a maximum expression level at 24 hours. On the other hand, longer exposure and higher salinity level induced down-regulation of proteins which were initially responsive. Tissue-specificity was revealed for LSY262 (leaf sheath and root), and fructose bisphosphate aldolases, PSII oxygen evolving complex protein, and OEE2 (leaf sheath and blade) proteins. As a result, it was suggested that the induced expression levels of those proteins are part of the coordinated responses of rice to protect against low doses and short term NaCl stress.

In 2005, we investigated the effect of salt stress on the leaf proteome of rice seedlings by using a hydroponically cultured rice seedling (Kim et al., 2005). In our study, 18-day-old rice seedlings were treated with or without 130 mM NaCl for 4 days. Prior to the proteome analysis, we also examined salt stress-induced physiobiochemical responses of rice. From the results, we observed that the morphological damage symptoms (wilting and browning of the 3rd leaf and inhibition in overall seedling growth) in the 3rd leaves were associated with decrease in photosynthetic activity, and increase in lipid peroxidation (see figures presented in Kim et al., 2005). A comparison of protein profiles between the untreated control and salt-stressed 3rd leaves revealed 55 differentially expressed protein spots, where 47 spots were increased over the control. Of these changed spots, 33 protein spots (27 increased and 5 decreased) were identified by electrospray ionization mass spectrometry (Fig. 3). Most of these identified proteins belonged to major metabolic processes like photosynthetic carbon dioxide assimilation and photorespiration. In addition, using 2-DGE immunoblot and enzyme activity analyses of rice leaves, we also found considerable changes in the key marker enzymes associated with oxidative damage to salt stress; ascorbate peroxidase was induced, and catalase was suppressed.

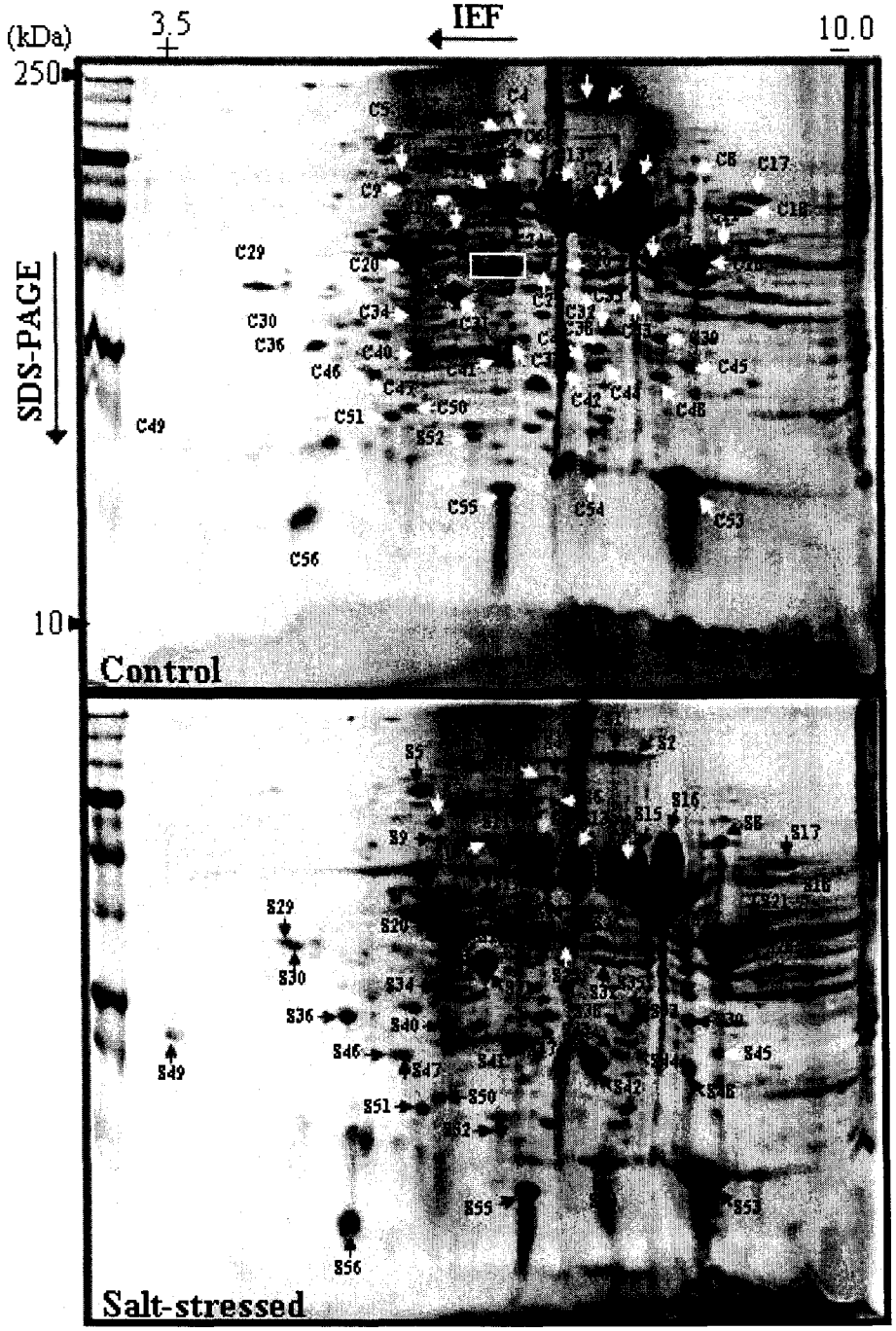

Fig. 3. Effect of salt stress on 2-DGE protein profiles in rice leaves. Numbered arrows and box show changes in proteins at 4 days after salt stress treatment (130mM NaCl).

In wheat, Majoul et al. (2000) analyzed protein patterns in root using two wheat cultivars, differing in their sensitivity to NaCl. In their results, protein profiles for control and salt-stressed seedlings were qualitatively similar, but a 26 kDa protein was found to be significantly up-regulated in the tolerant cultivar. Edman sequencing revealed strong similarities with two proteins, Rab 24 from rice and peroxiredoxin B15C from barley. Another research group has examined leaf protein profiles of two cultivars having different sensitivity to salt stress (Ouerghi et al., 2000). Out of 500 leaf protein spots detected from both what cultivars, 12 protein spots were differentially changed by salt stress. In the salt-sensitive cultivar 12 protein spots was down-regulated. But, in the tolerant cultivar, only 5 protein spots were down-regulated, 2 protein spots were up-regulated, and 5 protein spots remained unchanged.

In maize, using a Na^+-excluding inbred line protein patterns in response to salt stress were analyzed for roots and shoots (Zörb et al., 2004). In this study, even a lower level of salt stress (25 mM NaCl) altered the expression of 31% of shoot proteins and 45% of root proteins. In this level of salt stress, maize plants did not showed physiological changes in growth and toxic ion concentrations. Under a higher salt stress (100 mM), more than 80% of detected proteins were differentially expressed. Based on the protein identification, it was revealed that salt stress-responsive proteins are involved in protein biosynthesis and its modifications, carbon metabolism and nitrogen metabolism.

Low Temperature

Imin et al. (2004) analyzed the effect of low temperature on the proteome profiles of rice anther at the young microspore stage. Out of 70 anther proteins whose expression was differentially regulated in response to low temperature, 65 low temperature-responsive protein spots were analyzed by MS. The majority of 18 identified proteins were matched to ascorbate peroxidase, a major antioxidant enzyme involved in scavenging hydrogen peroxide. Profiling of other identified proteins indicated that low temperature at the young microspore stage induces partial degradation of proteins in rice anthers. Later

on, Cui et al. (2005) studied on the low temperature-responsive proteins of rice leaves. Results from this study implicated that protein quality control mediated by chaperones and proteases as well as augmentation of cell wall components play important roles in tolerance of rice to low temperature. Moreover, among the stress-responsive proteins, 43% were predicted to be located in the chloroplasts, implying that proteins in the chloroplasts are affected by cold stress.

In 2006, a comparative proteome approach for analyzing rice's responses to low temperature (Yan et al., 2006). By MS analysis, 85 differentially expressed proteins were identified, mainly involved in photosynthesis (35%), energy metabolism (8.2%), photorespiration (7.1%), carbon metabolism (7.1%), redox homeostasis (5.9%), translation (5.9%), signal transduction (4.7%), protein processing (4.7%), sulphur (2.4%) and nitrogen metabolism (2.4%) as well as RNA processing (1.2%). It was also found that low temperature enhanced degradation of many identified proteins, especially proteins involved in photosynthesis. Interestingly, gene expression analysis for 44 different proteins by quantitative real time PCR showed that the mRNA levels were not well correlated to the protein levels.

Flooding

Chang et al. (2000) applied a proteomic approach to examine the patterns of protein synthesis in maize root tips under low-oxygen condition. In their study, maize seedlings were grown under hypoxia for 4 h prior to 13 h of anoxia and 26 h of normal condition. It was found that the tolerance to maize to anoxia was significantly enhanced by 2-4 h of hypoxia pretreatment. Hypoxia acclimation enhanced selectively the synthesis of a few proteins in root tips, but subsequent anoxia treatment inhibited protein synthesis. Based on these results, it was suggested that protein synthesis during hypoxia, but not during subsequent anoxia, is crucial for acclimation. Among 46 root tip proteins identified by MS, some proteins are important enzymes involved in cytoplasmic and organellar translation (eIF-4A, eEF-2, and mitochondrial elongation factor), oxidative phosphorylation (subunits of the F1-ATPase), protein folding (mitochondrial chaperonin 60), and intracellular trafficking (Golgi-associated protein

se-wap41). Hypoxia-induced expression of anaerobic proteins (alcohol dehydronase 1, enolase 1, and GAPDH) is also known as preferentially synthesized proteins during hypoxic acclimation in previous studies (Drew, 1997; Lal et al., 1998). In rice, Dubey et al. (2003) has constructed proteome maps of flood-tolerant FR13A and flood-sensitive IR54 rice cultivars. Based on the identification of protein spots, they also found that sucrose synthase, glyceraldehyde 3-phosphate dehydrogenase, UDP-glucose-6-dehydrogenase, and asparagine synthetase are associated with flooding stress-response of rice. Besides, it was considered that carbohydrate may function as controlling the responses of rice to low oxygen stress caused by flooding stress.

Low Nitrogen Application

To evaluate differences in nitrogen utilization of wheat, Bahrman et al. (2004) applied proteomic approach using two wheat varieties (Arche and Récital) grown under four nitrogen level. Out of 532 and 533 leaf protein spots detected in Arche and Recital, they found that 55 spots showed a significant genotypic variation, and the expression of 76 protein spots was considerably regulated by different nitrogen application level. Most of these identified proteins were mainly enzymes involved in metabolic pathway, including carbon fixation and energy production (2,3-bisphosphoglycerate-independent phosphoglycerate mutase, enolase 2, phosphoglycerate kinase, fructose 1,6-bisphosphate aldolase, ribose 5-phosphate isomerase precursor, malate dehydrogenase, RCAA, OEE1, and ATP synthase beta subunit).

In rice, we also analyzed the effect of low nitrogen (low-N) application on the protein profile of rice leaf (Fig. 4). The leaves of a representative low-N-responsive rice cultivar (BG90-2) were used for proteomic analysis. Out of 50 protein spots whose expression was differentially regulated by low-N application, 41 proteins were identified by MS. Assignment of proteins into major (energy metabolism, photosynthesis and oxidative stress) and minor functional categories revealed a number of novel low-N-responsive proteins, including those having energy/photosynthesis- and defense/stress and iron

homeostasis-related functions. Our results suggest that these proteins may be useful as potential biomarkers for rice response to low-N.

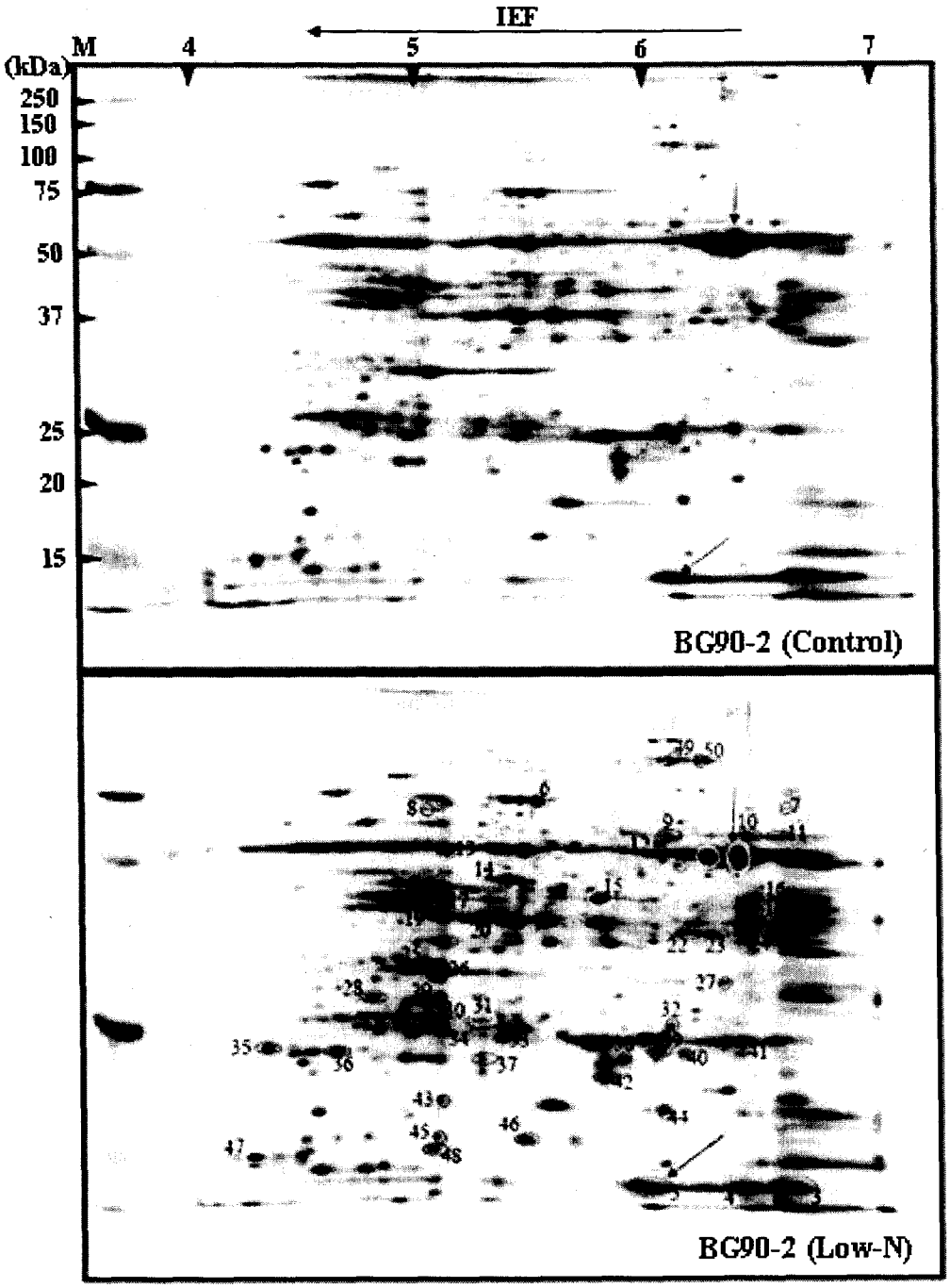

Fig. 4. The 2-DGE protein profiles of leaf protein extracted from rice seedlings treated with low-N. Numbered spots (1-50) show proteins selected based on their induction/suppression. The arrows indicate the RuBisCO large subunit (LSU, spot no. 1) and small subunit (SSU, spot no. 5).

Conclusions and perspectives

As described in this presentation, proteome studies have provided us to have deeper insights into the global expression of proteins differentially regulated in crop plants under various abiotic stress conditions. Some of studies have also revealed a few proteins which can be used as potential biomarkers for characterizing the stress-specific responses of crop plants. However, a substantial number of proteins affected by abiotic stresses remains annotated as hypothetical proteins, due to a lack of any sequence similarity to any proteins of known function. The identity and physiological role of the responsive proteins remain to be elucidated. Besides, most of studies reflect the final down-stream responses of proteins to stress factors. Therefore, analysis of early events at the protein level is needed for future proteomic analysis of abiotic stress responses in plants.

To cover a wider fraction of proteome in crop plants exposed to various abiotic stresses, different strategies are needed for the substantial improvement of proteome analysis. Firstly, a better protein sample preparation enables us to separate individual proteins clearly on 2-D gels, and subsequently this lead to a better identification of each protein by MS-based methods. Therefore, researchers should always be aware of preparing good protein samples which is the most suitable for their biological questions. In this aspect, the proteome analysis of separated organelles from stressed plants as well as using pre-fractionated protein extracts will result in a higher resolution of the proteome analysis. Secondly, 2-DGE is the most widely used in proteome studies as a powerful and stable technique, but still there is a room for refining the technique. Use of pre-cast IPG technology along with large-format pre-cast 2-D gels will provide a higher resolution of protein spots. Use of fluorescence-based protein detection can also help in improving the sensitivity of 2-DGE. The utilization of 2-DGE can be broadened by coupling with other techniques like immunoblotting for characterization of antibody specificity. Finally, more sophisticated protein identification methods such label-free quantitative LC-based techniques will also contribute to higher analytical depth.

Construction of high-quality 2-D gel reference maps will be essential to provide the most visible and useful datasets for the plant proteomic researcher. Image analysis is the first step towards creating a 2-D gel reference map, and for this a number of software's are available. In spite of the reliability of software-based image analyses, optical confirmation by researcher is still needed to analyze the gels. Although there are 2-D gel reference maps providing information on rice proteome (htpp://gene64.dna.affrc.go.jp/RPD/), it should be cautioned that the reference maps do not meet the criteria, reproducibility, required for high-quality reference maps (Agrawal et al., 2006). Comparative studies on proteomes of crop plants with different level of stress tolerance will provide us with more comprehensive proteomic information under diverse abiotic stresses, which will help in developing protein biomarkers involved therein. Both 2-DGE and MS-based proteomic technologies are suitable for the discovery of protein biomarkers. Because the generation of high-quality biomarkers is not an easy task, the proteomics approach needs to be validated under extensive experimentation using diverse abiotic stress factors.

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