Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 815 (2005) 137–145
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

System, trends and perspectives of proteomics in dicot plants Part III: Unraveling the proteomes influenced by the environment, and at the levels of function and genetic relationships

Ganesh Kumar Agrawal[a,1], Masami Yonekura[b], Yumiko Iwahashi[c], Hitoshi Iwahashi[d], Randeep Rakwal[a,d,*]

[a] *Research Laboratory for Agricultural Biotechnology and Biochemistry (RLABB), GPO Box 8207, Kathmandu, Nepal*
[b] *Food Function Laboratory, School of Agriculture, Ibaraki University, Ami, Ibaraki 300-0393, Japan*
[c] *National Food Research Institute (NFRI), Kannondai 2-1-12, Tsukuba, Ibaraki 305-8642, Japan*
[d] *Human Stress Signal Research Center (HSS), National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan*

Received 26 April 2004; accepted 2 November 2004
Available online 15 December 2004

Abstract

This review is devoted to the proteomics studies in dicotyledoneous (dicot) plants, such as *Arabidopsis*, *Medicago*, potato, soybean, and tomato, under the influence of the environment and at the functional and genetic relationship levels, where the two core technologies, two-dimensional gel electrophoresis (2-DGE) and mass spectrometry (MS) have been instrumental in unraveling the proteomes affected therein. Abiotic and biotic stress responses, including the affect of allergens, the symbiotic interaction between the members of the Leguminoseae family and genera of nitrogen fixing bacteria, phosphoproteomics, and proteomics in revealing the genetic relationships between species and genera have been the subject of many proteomics studies, and these are discussed in this review. In all, these studies have complemented and extended the studies of developmental proteomics [G.K. Agrawal, M. Yonekura, Y. Iwahashi, H. Iwahashi, R. Rakwal, J. Chromatogr. B (2004)].

Keywords: Dicotyledoneous plants; Proteomic studies; 2-DGE; MS; Protein identification

Contents

1. Introduction 138
2. Environmental proteomics 138
   2.1. Abiotic stress 138
      2.1.1. Cold 138
      2.1.2. Drought 139
      2.1.3. Ethylene 139
      2.1.4. Heat 139
      2.1.5. Oxidative stress 139

* Corresponding author. Fax: +81 29 861 6066.
E-mail addresses: gkagrawal@onebox.com (G.K. Agrawal), rakwal-68@aist.go.jp (R. Rakwal).
[1] Co-corresponding author.

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.11.022

2.2. Biotic stress 140
2.2.1. Fungal infection 140
2.2.2. Viral infection 140
2.3. Allergens 140
3. Symbiosome proteomics 140
3.1. Nodulation 140
3.2. Symbiosome membrane proteins 141
3.3. Peribacteriod membrane and space proteins 141
4. Phosphoproteomics 142
4.1. Elicitors responsive phosphoproteins 142
4.2. Mitochondrial phosphoproteins 142
5. Genetic relationships by proteomics 142
5.1. Mutant analysis 142
5.2. Taxonomical analysis of plant family 143
5.3. Assessment of genetically modified (GM) plants 143
6. Conclusions and perspectives 143
7. Nomenclature 144
Acknowledgements 145
References 145

1. Introduction

With the proteomes investigated/established for the complex developmental stages [1], the next stage involves for extended proteomic studies in the dicotyledoneous (dicot) plants, mainly due to unfavorable conditions for plant in their environment. A unique symbiotic interaction between bacteria and dicot plants in the environment gives rise to the symbiosome within infected cells of root nodules. The study of the symbiosome and associated processes at the level of the proteome is a hot topic among the symbiotic research community, and can be referred to as symbiosome proteomics. Two other disciplines are gaining prominence in plant proteomics, including dicot plants: phosphoproteomics and proteomics in genetic relationships. Phosphorylation of proteins is an important post-translational modification (PTM), and the identification of phosphoproteins (phosphoproteomics) is needed to characterize entire phosphorylation cascades involved in a broad range of biological function(s). Proteomics has also invaded the classical field of taxonomy, with genetic relationships between species and genera that are being established based on the comparison of protein patterns. As in developmental proteomics [1], both two-dimensional gel electrophoresis (2-DGE [2–4]) and mass spectrometry (MS [5–7]) have been instrumental in the investigation of their proteomes (Fig. 1); these core technologies have been discussed in detail in part I of the review [8]. Undoubtedly, the growing disciplines of proteomics are highly relevant in our quest to understand the cause/effect relationships of the environment and evolution on plant growth and development. In the following sections, these disciplines have been summarized/discussed for the dicot plants, *Arabidopsis*, *Medicago*, potato, soybean, tomato, etc.

2. Environmental proteomics

Plants, which are sessile in nature and must adapt to the ever-changing environmental conditions, have fairly complex and well-developed mechanisms to cope with a variety of biotic and abiotic stress. How the environment brings changes in protein profiles under a particular factor/stimuli is the subject of environmental proteomics. Dicot plant materials, which have been investigated for their proteomes under unfavorable environmental conditions, are described below.

2.1. Abiotic stress

2.1.1. Cold

Two proteome studies were conducted; one involves total protein from flax (*Linum usitatissimum* L.) hypocotyls [9] and another involves nuclear protein fraction from *Arabidopsis* seedlings [10]. The hypocotyls of flax cultivar (cv.)

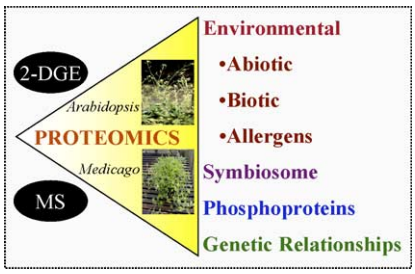

Fig. 1. Proteomes influenced by the environment, and at the levels of function and genetic relationships. 2-DGE and MS are instrumental in the separation and identification of proteins. *Arabidopsis thaliana* (ecotype Columbia) and *Medicago truncatula* genotype Jemalong A17 are model dicot plants, whose photographs were kindly provided by Dr. Akihiro Kubo (NIES) and Prof. Richard Oliver (Murdoch University), respectively.

Ariane seedlings grown for 6 days were subjected to cold stress (4 °C) in time variations of 1–120 min [9]. Selection of this time window was based on the observed rapid increase (within a minute) in cytosolic calcium level due to cold stress. It was also reasoned that early time profiles might reveal the proteins responsible for sensing low temperature. 2-DGE followed by silver staining revealed a set of seven proteins (CS (cold shock) A–G) responsive to cold shock. Three proteins (CSD, CSE and CSG) were induced within a minute of cold shock, and were absent from untreated hypocotyls. Among these, CSD and CSG showed a transient response, being present until 60 min but absent at 120 min.

Nuclear proteins were isolated from *Arabidopsis* seedlings exposed to 4 °C for 6 h [10]. One hundred and eighty four protein spots were identified, of which 40 were induced, and 14 were repressed by more than a factor of 2, due to cold stress. Among the identified proteins were heat shock proteins (HSPs) 70/90, transcription factors (AtMYB2 and OBF4), DNA-binding proteins (DRT102 and Dr1), catalytic enzymes (phosphoglycerate kinase, serine acetyltransferase, and glyceraldehyde-3-phosphate dehydrogenase), syntaxin, calmodulin, and germin-like proteins.

2.1.2. Drought

Two auxin-insensitive *Arabidopsis* (ecotype Columbia) mutants (*axr1*, *axr2*) differentially affected in specific drought responses, and the wild type, were used to create the 2-DGE profiles under the well-watered and drought-stressed conditions [11]. Subtle changes in protein patterns induced by progressive drought stress and/or mutations affecting the auxin response pathway were observed. Around 600 hundred reproducible protein spots were detected on leaf or on root 2-DGE gels within a p*I* range of 5–7 and Mr range of 20–100 kDa (10^2–10^5 molecular masses) by silver staining. Thirty protein spots were affected by drought and/or at least one mutation specifically in leaves, 15 in roots and 8 others in both organs. Although these changed proteins were not identified, it was suggested that these proteins could play a role both in the auxin and the drought response pathways.

2.1.3. Ethylene

2-DGE silver stained gels in conjunction with MALDI-TOF-MS were used to identify the effect of ethylene on the changes in protein profiles during early root (from 4-day-old seedlings) epidermal development in *Arabidopsis* (ecotype Columbia) seedlings [12]. Most of the young root proteins identified were soluble metabolic enzymes of known biological functions, while many others were unknown. Three distinct glutathione *S*-transferase (GST) isoforms, AtGSTF2, AtGSTF8, and AtSTU19, were expressed early in root epidermal establishment. AtGSTF2 was specifically up-regulated by ethylene at both protein and mRNA levels.

2.1.4. Heat

Two plant species mung bean (*Phaseolus aureus*) and tomato (*Lycopersicum esculentum*) were studied with respect to heat stress [13,14]. In mung bean (cv. Roxborough), a preparative continuous elution sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) combined with 2-DGE immunoblotting was used to study the HSPs from heat shocked (42 °C) radiolabeled hypocotyls derived from seeds germinated for 2 days in the dark [13]. Ten HSPs with molecular masses of 20, 21.5, 23, 29, 34, 38, 55, 62, 70, 85 kDa were separated and visualized on SDS–PAGE. Among these, HSP55 was the most strongly radiolabeled. Furthermore, using 2-DGE and immunochemical analysis, the HSP29 isolate revealed the presence of seven radiolabeled peptides, where six cross-reacted with a monoclonal HSP29 antibody.

In tomato, a preliminary study of heat stress on tomato fruits led to the observation that the water of the pericarp tissues did not change [15]. Based on this observation, it was assumed that newly synthesized proteins might have protected the pericarp tissue from heat. To prove this assumption, changes in tomato pericarp proteins under heat stress (37 °C for 1 day) were analyzed by 2-DGE [14]. A total of 1200 proteins were resolved and detected by Coomassie brilliant blue (CBB) staining under control (minus heat stress) conditions. About 23.7% (293) of the proteins that are present in the control decreased by heat stress. The expression of 24.9% (315) of the proteins did not change by heat stress. In addition, the expression level of 26.9% (341) of the proteins increased by heat stress, and 14 proteins appeared only under the heat stress condition. Among the identified changed proteins were 23 kDa subunit of the oxygen evolving enhancer-2 protein precursor, 22 kDa mitochondrial HSP, invertases, polygalacturonase, and isoforms of ascorbate peroxidase (APX).

2.1.5. Oxidative stress

Arabidopsis (cv. Landsberg *erecta*) stem explant suspension-cultured cells (maintained for over 9 years) were treated with hydrogen peroxide (H_2O_2, 88 mM), menadione (400 μM, a redox active quinone that generates intracellular superoxide), antimycin A (25 μM, an inhibitor of complex III of the mitochondrial respiratory chain) for 16 h to investigate the oxidative stress [16]. It was mentioned that these treatments decreased cell growth, but there was no appreciable loss of cell viability. Highly purified mitochondria, extracted using a double Percoll gradient method, were subjected to protein isolation. Proteins were separated by 2-DGE, and analyzed by Q-TOF-MS/MS. Twenty five protein spots showed more than three-fold induction after H_2O_2/menadione treatment; a subset of these proteins also increased in antimycin A treated samples. A set of 10 protein spots decreased significantly during these stress treatments. Furthermore, a specific set of mitochondrial proteins (subunits of ATP synthase, complex I, succinyl CoA ligase, aconitase, and pyruvate and 2-oxoglutarate dehydrogenase complexes) were degraded by these stresses. Nine increased proteins (thioredoxin-dependent peroxidase and thioredoxin reductase-dependent protein disulphide isomerase) in stressed mitochondria were

not present in the control, indicating that they are either inducible or extramitochondrial.

Tomato (*L. esculentum* Mill, cv. Sweet 100, Vilmorin and France) suspension-cultured cells were also used to examine the effect of oxidative stress-inducing chemical agents (methyl viologen (MV), digitonin, gamma irradiation, H_2O_2) and heat shock (40 °C) by [^{35}S]-labeling of proteins, 2-DGE and immunoblotting [17]. Compared to control experiments, heat shock and gamma irradiation induced various stress proteins. The three main gamma-induced proteins corresponded to HSP15, HSP17 and HSP22. H_2O_2 treatment induced nine proteins, out of which seven were the HSPs including the major induced protein HSP22. HSP17 was weakly induced by MV or digitonin treatments, while HSP15 was induced by MV application only.

2.2. Biotic stress

2.2.1. Fungal infection

In the xylem sap of fungal (*Fusarium oxysporum* f. sp. *lycopersici* (Fol) isolates Fol004, Fol007, and Fol029) infected tomato plants (5- to 8-week old *L. esculentum* line GCR161, resistant to Fol004, and C32, susceptible to all isolates), proteins associated with compatible and incompatible interactions were surveyed [18]. Proteins were separated by SDS–PAGE, and bands of interest were excised and analyzed by MALDI-TOF-MS and Q-TOF-MS/MS. Pathogenesis-related (PR) proteins [PR-1 isoforms, and acidic and basic β-1,3-glucanases (PR-2)] were detected in compatible interactions only, concomitantly with disease development. A new member of the PR-5 (22 kDa) family was identified in both types of infection.

2.2.2. Viral infection

The intracellular fluid of soybean (*Glycine max* cv. Hodgson) leaves, locally infected with tobacco necrosis virus and showing necrotic local lesions, was used to study the acidic and basic PR proteins, using native-PAGE and 2-DGE followed by silver staining [19]. This resulted in the detection of 20 PR proteins (10 acidic- and 10 basic-PR proteins).

2.3. Allergens

Allergy to food is a serious problem world wide, and allergy to two important foods, sesame (*Sesamum indicum*) seed and oilseed rape (*Brassica napus*) pollen, is becoming increasingly prevalent. A study was conducted to find allergenic proteins in sesame seeds [20]. Seed proteins were separated by SDS–PAGE and 2-DGE followed by immunoblotting analysis and N-terminal amino acid sequencing. A total of 10 (7–78 kDa) IgE-binding proteins were detected, and 4 were positively identified. The major cross-reacting proteins were 45 and 7 kDa proteins, identified as 7S vicilin-type globulin (named Ses I 3) and 2S albumin (Ses I 2), respectively. In addition, a 78 and 34 kDa protein showed homology to the embryonic abundant protein and the seed maturation protein of soybeans, respectively. Furthermore, the detection of conserved IgE-binding epitopes in common food allergens might be a useful tool for predicting cross-reactivity to certain foods. For identifying oilseed rape pollen allergens, water extractable pollen proteins were separated by isoelectric focusing and analyzed by 2-DGE, immunoblotting and N-terminal amino acid sequencing [21]. Three proteins (40, 70 and 80 kDa) displayed identities with the berberine bridge protein (a reticulin oxidase), a receptor-like protein kinase and the cobalamin-independent methionine synthetase from *Arabidopsis*, respectively. Identification of a receptor-like protein kinase may represent a new class of allergens.

3. Symbiosome proteomics

The formation of nitrogen fixing root nodules in legume plants results from the symbiotic interaction between the members of the Leguminoseae family and genera of nitrogen fixing bacteria, *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium*. During this event, genes from both the plant and these bacteria play a role in the establishment and maintenance of this interaction [22,23], in which the plant supplies reduced carbon to the bacteriod (the nodule-residing state of the bacteria) in exchange for fixed nitrogen. Among the legumes, *M. truncatula* has been accepted as the nodal species for comparative and functional legume genomics, and *Sinorhizobium meliloti* strain 1021 has been proposed as a model microsymbiont [24]. Formation of the symbiosome occurs within the infected cells of root nodules. The study of the symbiosome and associated processes at the level of the proteome is referred to as symbiosome proteomics.

3.1. Nodulation

A proteome map of *Melilotus alba* infected with *S. meliloti* 1021 was established to provide insight into the symbiotic processes [25]. The reason for selecting *M. alba* (small-seeded, autogamous, and diploid legume) is that this genus is known to have almost same gene expression characteristics as alfalfa, *M. sativa* [26], and has a series of nodulation mutants blocked at various stages of bacterial infection and nitrogen fixation [27]. The proteome maps were generated from control *M. alba* roots, wild-type nodules, and cultured *S. meliloti* and *S. meliloti* bacteroids, and compared. Root or root nodules were harvested 12 days after mock or *S. meliloti* inoculation. Over 1700 individual abundant proteins were resolved in silver stained proteome maps of mock-inoculated roots and root nodule tissue of *M. alba*, respectively. Out of these, over 250 proteins were induced or up-regulated in the nodule, among which over 180 were from bacteroid, while 70 appeared to be novel to nodule tissues and are potentially new nodulins. Moreover, 20 root proteins were down-regulated in the nodule tissue. A comparison of bacteroid and cultured bacteria also revealed over 1700 protein spots. Around 350

proteins were down-regulated in the bacteroid form of the rhizobia, compared with cultured cells, whereas 130 proteins up-regulated in the bacteroid. The putative identity of nearly 100 nodule, bacterial, and bacteroid proteins, were assigned by using N-terminal amino acid sequencing and MALDI-TOF-MS. These identified proteins included the previously identified nodule proteins leghemoglobin and NifH as well as proteins involved in carbon and nitrogen metabolism in *S. meliloti*. Bacteroid cells showed down-regulation of several proteins involved in nitrogen acquisition, including glutamine synthetase, urease, a urea–amide binding protein, and a photosystem II isoform, indicating that the bacteroids were nitrogen proficient. The down-regulation of several enzymes involved in polyhydroxybutyrate synthesis and a cell division protein was also observed.

The first proteome reference maps of *M. truncatula* cv. Jemalong A17 were also established using 2-DGE in conjunction with peptide mass fingerprinting (PMF) to dissect the nodulation and root developmental pathways [28]. Over 2500 and 450 root (of 5-day-old seedlings) protein spots were displayed reproducibly across a p*I* range of 4–7 and 6–11, respectively, by silver staining. CBB staining revealed 1500 and 50 protein spots, respectively, in the same p*I* range. A total of 485 most abundant proteins were analyzed by PMF. By matching against the expressed sequence tag database (*M. truncatula*), 179 proteins could be identified. The majority of identified proteins were metabolic enzymes and stress response proteins, and 44% of proteins occurred as isoforms, a result that could not have been predicted from sequencing data alone. This result adds to the growing evidence that many so-called nodulins are not specifically expressed during nodulation. The identified proteins were classified into 12 categories; plant metabolism (40%), flavanoid metabolism (5%), defense and stress response (18%), protein synthesis and processing (14.5%), hormone or developmental regulated (7%), membrane transport (5%), cytoskeleton (4%), transcription and its regulation (3%), cell wall structure and synthesis (1.25%), nodulins (1.25%), signaling (0.5%), and cell cycle (0.5%). PR proteins (PR10 class) were the most abundant proteins, detected on the gels (for further details see [28]). PR10-1 expression has been found to be constitutive in roots [29], implying its importance for normal plant development. Moreover, a number of isoforms of enzymes of the flavanoid pathway were detected, reflecting the importance of flavanoid metabolism in legumes [30]. The 2-DGE protein gels of *M. truncatula* were surprisingly similar to protein patterns on gels of nodulated roots of white sweet clover (*M. alba*) [25]. Moreover, striking similarity to the gels of subterranean clover (*Trifolium subteraneum*) root proteins was seen, where PR10-1, APX, α-tubulin, chaperonin 10, a-fucosidase, peroxiredoxin and ripening-induced proteins were the common proteins [31]. The root proteome reference map along with predicted identities can be found on the web (http://semele.anu.edu.au/2d/2d.html).

Another study examined the root protein profiles of *M. truncatula*, inoculated either with the arbuscular mycorrhizal fungus *Glomus mosseae* or with the nitrogen fixing bacterium *S. meliloti*, by 2-DGE and silver staining [32]. Protein identification was done on a MALDI-TOF-MS (PMFs) and by Q-TOF-MS/MS, which allowed for de novo sequencing of tryptic-digested proteins. One protein induced in nodulated roots was identified as a *M. truncatula* leghemoglobin, whereas another protein was identified as an elongation factor Tu from *S. meliloti*. In the inoculated (mycorrhizal) roots, the identified proteins included GST, fucosidase, myosin-like protein, serine hydroxymethyltransferase, cytochrome-c-oxidase, and a protein of unknown function.

3.2. Symbiosome membrane proteins

The symbiosome membrane serves both as a physical interface and as a mediator of metabolite exchange between the symbionts, both functions being essential for nodule function. In mature root nodule cells, the symbiosome membrane represents a mixture of proteins that resembles most closely the protein constituents of the plasma membrane and the tonoplast [33,34]. In order to understand the biogenesis and function of the symbiosome membrane, 2-DGE coupled with LC-MS/MS was used to identify proteins present in the symbiosome membrane of the *M. truncatula* genotype A17 (Jemalong) [35]. Root nodules, harvested 2–3 weeks post inoculation of *S. meliloti* strain 2011, were used for fractionation into the symbiosome membrane (110 spots), symbiosome space (200 spots) and bacteriod (220 spots) proteins. A total of 51 proteins were identified; 28 were the symbiosome membrane proteins (classified into four functional categories: protein destination (56%), energy and transport (24%), nodule-specific (10%), and unclassified (10%), and 23 were the bacterial proteins (classified into two functional categories: protein destination/storage (35%), and others). Symbiosome membrane proteins included H^+-ATPase, ENOD16, ENOD8, nodulin-25, BiP, HSP70, PDI, multifunctional aquaporin, a putative syntaxin, etc. These results provide a basis to hypothesize mechanism of symbiosome membrane formation and function.

3.3. Peribacteriod membrane and space proteins

The compartment that harbors the bacteroids is surrounded by a peribacteroid membrane (PBM), which originates from the plant plasma membrane. The PBM and the space between the PBM and the bacteroid membrane, called peribacteroid space (PS), mediate the exchange of metabolites between the symbionts. The PBM and the PS were isolated by a standard differential centrifugation procedure, including a Percoll gradient from pea root nodules, and used for proteome analyses [36]. Proteins in the PBM and PS fractions were separated by 2-DGE, and 89 protein spots were analyzed by tandem MS, resulting in the identification of 46 protein spots. It was found that PS and even PBM preparations from pea symbiosome always contain abundant amounts of bacteroid proteins as a contaminant. Interestingly, in addition to

a few PS/PBM proteins, a number of endomembrane proteins (less likely representing a contaminate), including V-ATPase, BIP, and an integral membrane protein known from COPI-coated vesicles, were found in the PBM fraction, supporting the role of the endomembrane system in PBM biogenesis.

4. Phosphoproteomics

Phosphorylation, an important factor in the integration of signals within the cell, of a protein can alter its behavior in almost every conceivable way, including its intrinsic biological activity, subcellular location, half-life and interaction with other proteins. It is also the most common PTM of proteins, and is among the best-studied processes involved in the regulation of cellular metabolism [37]. Protein phosphorylation is an important event in defense signaling to initiate responses to diverse environmental stresses, and is involved in both positive and negative regulation of the defense pathways. For example, mutations in the FLS2 LRR-kinase in *Arabidopsis* render the plant insensitive to the bacterial elicitor, flagellin [38]. Mutations in EDR1, a mitogen-activated protein (MAP) kinase kinase kinase [39], and in MAP kinase 4 [40], result in *Arabidopsis* plants that are more resistant to virulent pathogens. Although rapid changes in pattern of phosphorylated proteins in elicitor treated suspension-cultured cells [41,42] and in ozone (O_3)-fumigated plants [43], have been noted, relatively few of them have been identified. Preliminary studies in *Arabidopsis* identified chloroplast thylakoid membrane phosphoproteins and their phosphorylation sites using MALDI-TOF-MS and ESI-MS [44]. Moreover, a bioinformatic screening and in silico analyses together with immunological studies indicated that *Arabidopsis* proteins specifically phosphorylated on tyrosine residues are much higher than in yeast [45]. The study of phosphorylated proteins constitutes phosphoproteomics.

4.1. Elicitors responsive phosphoproteins

A "directed proteomics" approach was employed to identify phosphorylated proteins in *Arabidopsis* suspension-cultured cells in response to microbial elicitors [46]. Radioactive [^{32}P] orthophosphate was used to pulse-label suspension-cultured cells, followed by separation and identification of isolated proteins by 2-DGE and MS, respectively. A number of proteins showed increased or decreased incorporation of radioactive phosphate within 4 min of flagellin 22 (flg22), a peptide corresponding to the most conserved domain of bacterial flagellin, elicitor treatment. A protein termed AtPhos43, having an acidic p*I* in a region of low protein complexity, was analyzed by ESI-MS/MS, and predicted to be a cytosolic protein with two-ankyrin motifs at the C-terminus. Immunoblotting revealed that AtPhos43 is differentially phosphorylated in response to flg22 (bacterial) and chitin (fungal) elicitors. A Phos43 protein, related to AtPhos43 was also found in tomato suspension-cultured cells treated with flg22, suggesting conservation of some pathways between *Arabidopsis* and tomato. However, as the phosphoprotein patterns after flg22 treatment in these two species were quite different, one should keep in mind that the identities of related phosphoproteins in plants showing similar responses should not be assigned by simple comparison of phosphoprotein proteome maps. A continuous effort in this direction led to the identification of another protein phosphorylated rapidly in response to flg22, namely syntaxin (AtSyp122) [47]. AtSyp122 was phosphorylated prior to elicitation, as determined by 2-DGE-immunoblotting analyses, and shown to be calcium-dependent by an in vitro analysis.

4.2. Mitochondrial phosphoproteins

Phosphorylated proteins of potato (*Solanum tuberosum* L.) tuber mitochondria were studied by 2-DGE in conjunction with nano-ESI-MS/MS and MALDI-TOF-MS, resulting in the identification of 14 phosphoproteins [48]. Out of the 14, 7 phosphoproteins were found to be involved in the tricarboxylic acid cycle or associated reactions, whereas four were the subunits of respiratory complexes and involved in electron transport, ATP synthesis and protein processing, two are the HSPs, and one was involved in defense against oxidative stress. Phosphoprotein pattern of potato tuber mitochondria was reported to be very similar to that of the *Arabidopsis*, suggesting conserved function of these phosphorylated mitochondrial proteins between these two species [48]. Therefore, protein phosphorylation is likely to be one of the fundamental mechanisms for signal integration of the complex network of mitochondrial processes.

5. Genetic relationships by proteomics

The genetic relationships between species and genera have been usually based on molecular studies. In the late 1980s, it was shown that such relationships could be also established based on the comparison of protein patterns. The usefulness of qualitative and quantitative variation of protein spots, separated by 2-DGE for distinguishing between very closely related genotypes as part of a taxonomic study, has been demonstrated [49,50]. Comparative study of protein profiles among plants can lead to the establishment of genetic relationships by proteomics.

5.1. Mutant analysis

In order to estimate the biochemical distance indices between developmental mutants of *Arabidopsis* and wild type plants, cultivated in the presence of various hormones, total proteins were analyzed using 2-DGE [51]. Computer-based analysis of 2-DGE profiles followed by statistical treatment of data was used for creating a phenogram that described the biochemical distances between the different genotypes. It was shown that phenotypical and physiological

analysis of various genotypes were in excellent agreement with the 2-DGE analysis, and therefore may be helpful for physiological analysis of mutants, as exemplified for *crystal* mutants.

5.2. Taxonomical analysis of plant family

A comparative proteomic approach was undertaken to establish the genetic relationships in the Brassicaceae family, which comprises numerous agronomically and economically important crop plants, such as cabbages, mustards, rapes, radishes, and *Arabidopsis* [52]. Various varieties of *Brassica*, cabbage and radish, along with two ecotypes of *Arabidopsis*, L. *erecta* and Columbia were used. For this purpose, the aerial part of the seedlings, grown on water-imbibed filter paper in large Petri dishes for 7 days in the dark at circa 20 °C, was used. Selection of this developmental stage is based on the work in Triticeae, where variations in gene expression, i.e. the appearance or disappearance of spots, are scarce between the fifth and the ninth day. Proteome comparisons were done using a global gel with 2273 reproducible spots, which were obtained from eight synthetic mixed gels from all samples using the *B. napus* protein profile as a reference (for further details on distance indices calculation, phenetic trees and factorial correspondence analysis see [52]). Genetic distances were calculated on the basis of common and distinct spots. Results obtained from the study were in good agreement with the established taxonomy of the Brassicaceae.

5.3. Assessment of genetically modified (GM) plants

Proteomics was also applied to study the safety of genetically (GM) modified plants. For this, a transgenic tomato plant resistant to virus (tomato spotted wilt virus) and its corresponding wild type (unmodified hybrid) was studied by comparing their 2-DGE protein profiles [53]. No significant differences, either qualitative or quantitative, could be detected; indicating that expression of major proteins was unmodified by the genetic manipulation.

6. Conclusions and perspectives

It is not surprising to see that proteomics has evolved into more focused and specialized areas—related to the environment, function, and genetic relationships (Fig. 1), and therefore, it is the perfect time to start placing the proteomes to their defined areas (disciplines) to lay a strong foundation, and provide systems and trends for the current and up-coming proteomic researcher(s). Proteomics studies in these disciplines have also played an important role in defining the proteomes in relation to the environment, symbiosome, PTMs, inter-species interactions, intra- and inter-cellular signaling, inter-species variations and food safety, and at the same time linking them to developmental proteomics.

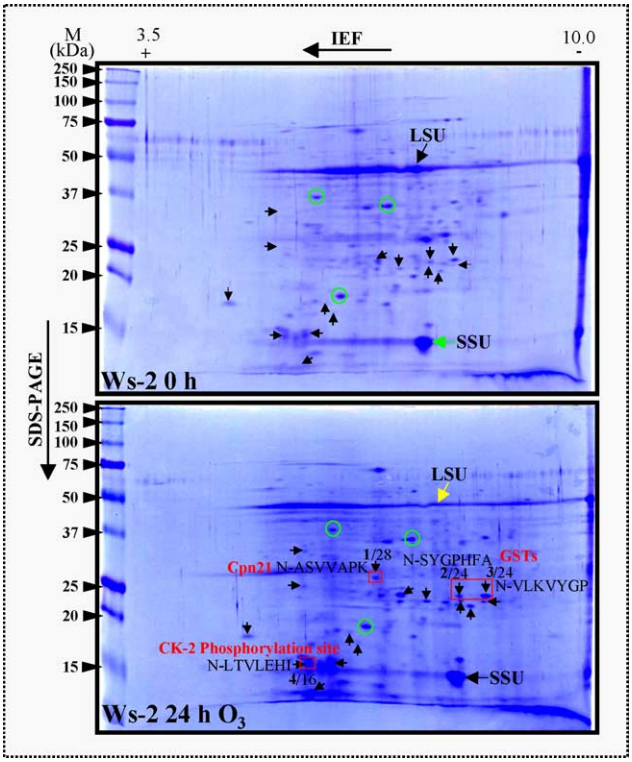

Fig. 2. Effect of ozone (O_3) on protein profiles in *Arabidopsis*. The 16-day-old *Arabidopsis* (ecotype Wassilewskija-2) plants were placed in an O_3 chamber and exposed to 0.2 ppm O_3 for 24 h. Plants before the start of the experiment served as the 0 h control. All the leaves were collected, and total proteins were extracted in homogenization buffer (Tris–HCl, pH 7.5). Approximately 200 μg total protein extract was separated by 2-DGE (for details on methods, see [57]), and the separated proteins were visualized using CBB stain. The proteins marked by black arrows show induction at 24 h; the positions of the same proteins are also marked in the control. Protein spots numbers 1–4 (red boxes) were N-terminally sequenced, and the amino acid sequences are mentioned on the gel. RuBisCO LSU and SSU are marked for reference, and the LSU and SSU levels are decreased (yellow arrow) and increased (black arrow) after O_3 treatment, respectively. M, molecular mass standards (Precision Plus Protein Standards, Bio-Rad).

The environmental proteomics studies highlight the cataloguing of proteins induced or suppressed under specific stress conditions. Although the effect of viral and fungal pathogens on protein changes has been investigated to only some degree, it is notable that the abiotic stress responses have provided more insights into the protein changes therein. One important environmental stressor, whose study is lacking in dicot plants, is the effect of the gases, O_3 and sulfur dioxide (SO_2). Both O_3 and SO_2 are well-known environmental gaseous pollutants, causing serious health hazards in humans and animals, and damage to plant growth, development and productivity [54–56]. Proteomics would be a valuable tool to examine their effects, as has been recently shown for the monocot model plant, rice [57,58]. We present an example of O_3-induced changes in protein profiles using *Arabidopsis* (ecotype Wassilewskija-2) as a representative dicot model (Fig. 2). Using 2-DGE and CBB staining, followed

by protein identification upon Edman sequencing, a number of proteins (spots marked by black arrows) were induced by O_3 in the leaves of 16-day-old *Arabidopsis* plants, within 24 h over the control (0 h). The N-terminal amino acid sequences of the four protein spots (boxed in red) clearly show that they are all stress-related proteins: chaperonin (Cpn) 21 (spot 1), GSTs (spots 2 and 3), and a protein kinase with a casein kinase (CK)-2 phosphorylation site (spot 4). Interestingly, the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit (LSU) was drastically reduced, whereas the small subunit (SSU) was increased, suggesting that photosynthesis is affected by O_3. These findings demonstrate the power of 2-DGE coupled with Edman sequencing in investigating the leaf proteome in response to O_3.

Symbiosome proteomics has resulted in the identification of molecular events occurring in plant root symbioses and associated processes. It seems that nodulins are not only involved in the symbiotic events, but also play a role in normal plant development, as evidenced from the identification of two nodulins (ENOD18 and a homologue of narbonin) in uninfected root tissues. Phosphoproteomics has provided a good start towards the identification of phosphorylated proteins involved in signal perception and transduction pathways in plants, which will subsequently lead to the dissection of signaling network(s). We again provide an example on the use of the phosphoprotein fluorescent gel stain, Pro Q Diamond ([59], and see conclusions and perspectives in reference [8]), in rapidly identifying phosphoproteins and changes therein upon stress, in this case treatment with O_3, in 16-day-old bean (*Phaseolus vulgaris* L. cv. IDIAP R-3) plants (Fig. 3). The fluorescent protein bands within the boxes indicate the phosphoproteins, and the total protein, representing equal loading, is stained with SYPRO Ruby. Finally, proteomics in genetic relationship studies has revealed that this "omic" technology can be used to reach a definite conclusion and/or support the conclusions drawn based on classical taxonomical and/or molecular approaches. Moreover, it also seems to be quite suitable for the evaluation of food safety in GM plants.

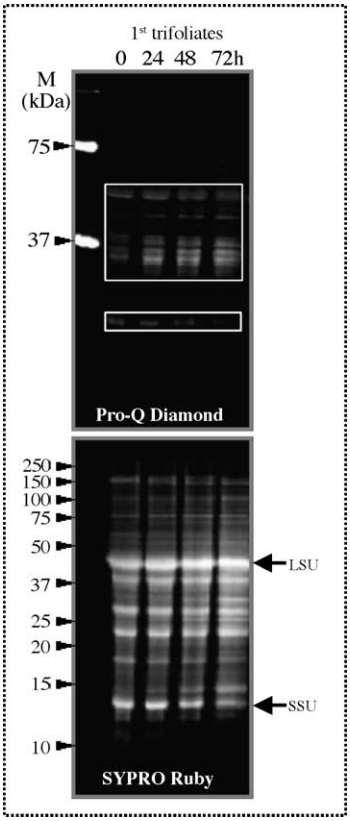

Fig. 3. Visualization of phosphoproteins by Pro Q Diamond dye. The 16-day-old bean (cv. IDIAP R-3) plants were placed in an ozone (O_3) chamber and exposed to 0.2 ppm O_3 for 72 h. First trifoliate leaves were collected at appropriate time periods, and total proteins extracted in homogenization buffer (Tris–HCl, pH 7.5). Approximately 50 μg of the total protein extract was separated by SDS–PAGE (for details on methods, see [57]), and the separated proteins were visualized with fluorescent stains, Pro Q Diamond (for phosphoproteins) and SYPRO Ruby (for total protein) using a UV-transilluminator (ATTO, Tokyo Japan). The boxed regions indicate the phosphoproteins. The RuBisCO LSU and SSU are marked for reference. M, molecular mass standards (Precision Plus Protein Standards, Bio-Rad).

7. Nomenclature

APX	ascorbate peroxidase
CBB	Coomassie brilliant blue
CS	cold shock
ESI-MS/MS	electrospray ionization tandem-mass spectrometry
nESI-MS/MS	nanoesi-tandem MS
ESI-Q-TOF-MS	ESI-quadrupole time of flight tandem MS
Flg22	Flagellin 22
GM	genetically modified
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HPLC	high-performance liquid chromatography
HSP	heat shock protein
kDa	kilo Dalton
LC-MS/MS	LC-tandem MS
MALDI	matrix-assisted laser desorption/ionization
MAPK	mitogen-activated protein kinase
M_r	molecular mass
MS	mass spectrometry
PAGE	polyacrylamide gel electrophoresis
PBM	peribacteroid membrane
p*I*	isoelectric point
PMF	peptide mass fingerprinting
PR	pathogenesis-related
PS	peribacteroid space
PTM	post-translational modification
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulfate
2-DGE	two-dimensional gel electrophoresis

Acknowledgements

We are grateful to Ms. Junko Shibato (HSS, AIST) for her help during the preparation of this manuscript. Authors highly appreciate the vision and collaboration established with Drs. Vishwanath Prasad Agrawal (RLABB, a private non-profit research organization, Kathmandu, Nepal), Masami Yonekura (School of Agriculture, Ibaraki University, Ami, Japan), Shigeru Tamogami (Akita Prefectural University, Akita, Japan), Akihiro Kubo (National Institute for Environmental Studies, Tsukuba, Japan), and Nam-Soo Jwa (Sejong University, Seoul, Korea), in proteomics of plant materials, including the dicot model *Arabidopsis*. The seeds of *Phaseolus vulgaris*, were kindly provided by Ms. Nilka Lineth Torres Stanziola (University of Panama, Panama), JICA trainee at HSS, AIST.

References

[1] G.K. Agrawal, M. Yonekura, Y. Iwahashi, H. Iwahashi, R. Rakwal, Proteomics Topical, in press.
[2] P.F. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[3] J. Klose, Humangenetik 26 (1975) 231.
[4] G.A. Scheele, J. Biol. Chem. 250 (1975) 5375.
[5] J. Li, S.M. Assmann, Plant Physiol. 123 (2000) 807.
[6] B.R. Herbert, J.L. Harry, N.H. Packer, A.A. Gooley, S.K. Pedersen, K.L. Williams, Trends Biotechnol. 19 (2001) S3.
[7] R. Aebersold, M. Mann, Nature 422 (2003) 198.
[8] G.K. Agrawal, M. Yonekura, Y. Iwahashi, H. Iwahashi, R. Rakwal, Proteomics Topical, J. Chromatogr. B 815 (2005) 125.
[9] M. Tafforeau, M.C. Verdus, R. Charlionet, A. Cabin-Flaman, C. Ripoll, Electrophoresis 23 (2002) 2534.
[10] M.S. Bae, E.J. Cho, E.Y. Choi, O.K. Park, Plant J. 36 (2003) 652.
[11] J. Leymarie, C. Damerval, L. Marcotte, V. Combes, N. Vartanian, Plant Cell Physiol. 37 (1996) 966.
[12] H.G. Mang, E.O. Kang, J.H. Shim, S.Y. Kim, K.Y. Park, Y.S. Kim, Y.Y. Bahk, W.T. Kim, Biochim. Biophys. Acta 1676 (2004) 231.
[13] D.H. Wu, D.L. Laidman, Phytochemistry 44 (1997) 985.
[14] Y. Iwahashi, H. Hosoda, Electrophoresis 21 (2000) 1766.
[15] Y. Iwahashi, A.K. Horigane, K. Yoza, T. Nagata, H. Hosoda, Mag. Reson. Imaging 177 (1999) 767.
[16] L.J. Sweetlove, J.L. Heazlewood, V. Herald, R. Holtzapffel, D.A. Day, C.J. Leaver, A.H. Millar, Plant J. 32 (2002) 891.
[17] N. Banzet, C. Richaud, Y. Deveaux, M. Kazmaier, J. Gagnon, C. Triantaphylides, Plant J. 13 (1998) 519.
[18] M. Rep, H.L. Dekker, J.H. Vossen, A.D. de Boer, P.M. Houterman, D. Speijer, J.W. Back, C.G. de Koster, B.J.C. Cornelissen, Plant Physiol. 130 (2002) 904.
[19] P. Roggero, S. Pennazio, Electrophoresis 11 (1990) 86.
[20] K. Beyer, L. Bardina, G. Grishina, H.A. Sampson, J. Allergy Clin. Immunol. 110 (2002) 154.
[21] H. Chardin, C. Mayer, H. Senechal, M. Tepfer, F.X. Desvaux, G. Peltre, Int. Arch. Allergy Immunol. 125 (2001) 128.
[22] M.R. Bladergroen, H.P. Spaink, Curr. Opin. Plant Biol. 1 (1998) 353.
[23] M. Schultze, A. Kondorosi, Annu. Rev. Genet. 32 (1998) 33.
[24] D.R. Cook, Curr. Opin. Plant Biol. 2 (1999) 301.
[25] S.H.A. Natera, N. Guerreiro, M.A. Djordjevic, Mol. Plant-Microbe Interact. 13 (2000) 995.
[26] B.E. Kneen, T.A. LaRue, Plant Sci. 58 (1988) 177.
[27] L.J. Utrup, A.J. Cary, J.H. Norris, Plant Physiol. 103 (1993) 925.
[28] U. Mathesius, G. Keijzers, S.H.A. Natera, J.J. Weinman, M.A. Djordjevic, B.G. Rolfe, Proteomics 1 (2001) 1424.
[29] P. Gamas, F. de Billy, G. Truchet, Mol. Plant-Microbe Interact. 11 (1998) 393.
[30] H.A. Stafford, Bot. Rev. 63 (1997) 27.
[31] A.C. Morris, M.A. Djordjevic, Electrophoresis 22 (2001) 586.
[32] G. Bestel-Corre, E. Dumas-Gaudot, V. Poinsot, M. Dieu, J.F. Dierick, T.D. van, J. Remacle, V. Gianinazzi-Pearson, S. Gianinazzi, Electrophoresis 23 (2002) 122.
[33] N.J. Brewin, Annu. Rev. Cell Biol. 7 (1991) 191.
[34] P. Mylona, K. Pawlowski, T. Bisseling, Plant Cell. 7 (1995) 869.
[35] C.M. Catalano, W.S. Lane, D.J. Sherrier, Electrophoresis 25 (2004) 519.
[36] G. Saalbach, P. Erik, S. Wienkoop, Proteomics 2 (2002) 325.
[37] T. Xing, T. Ouellet, B. Miki, Trends Plant Sci. 7 (2002) 224.
[38] L. Gomez-Gomez, T. Boller, FLS2: Mol. Cell 5 (2000) 1.
[39] C.A. Frye, D. Tang, R.W. Innes, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 373.
[40] M. Petersen, P. Brodersen, H. Naested, E. Andreasson, U. Lindhart, B. Johansen, H.B. Nielsen, M. Lacy, M.J. Austin, J.E. Parker, S.B. Sharma, D.F. Klessig, R. Martienssen, O. Mattsson, A.B. Jensen, J. Mundy, Cell 103 (2000) 1111.
[41] A. Dietrich, J.E. Mayer, K. Hahlbrock, J. Biol. Chem. 265 (1990) 6360.
[42] F. Lecourieux-Ouaked, A. Pugin, A. Lebrun-Garcia, Mol. Plant-Microbe Interact. 13 (2000) 821.
[43] G.K. Agrawal, R. Rakwal, M. Yonekura, A. Kubo, H. Saji, J. Plant Physiol. 159 (2002) 361.
[44] A.V. Vener, A. Harms, M.R. Sussman, R.D. Vierstra, J. Biol. Chem. 276 (2001) 6959.
[45] A. Carpi, G. Di Maira, M. Vedovato, V. Rossi, T. Naccari, M. Floriduz, M. Terzi, F. Filippini, Proteomics 2 (2002) 1494.
[46] S.C. Peck, T.S. Nuhse, D. Hess, A. Iglesias, F. Meins, T. Boller, Plant Cell 13 (2001) 1467.
[47] T.S. Nuhse, T. Boller, S.C. Peck, J. Biol. Chem. 278 (2003) 45248.
[48] N.V. Bykova, H. Egsgaard, I.M. Moller, FEBS Lett. 540 (2003) 141.
[49] A. Gorg, W. Postel, M. Baumer, W. Weiss, Electrophoresis 13 (1992) 192.
[50] M. Zivy, S. El Madidi, H. Thiellement, Electrophoresis 16 (1995) 1295.
[51] V. Santoni, M. Delarue, M. Caboche, C. Bellini, Planta 202 (1997) 62.
[52] K. Marques, B. Sarazin, L. Chane-Favre, M. Zivy, H. Thiellement, Proteomics 1 (2001) 1457.
[53] D. Corpillo, G. Gardini, A.M. Vaira, M. Basso, S. Aime, G.P. Accotto, M. Fasano, Proteomics 4 (2004) 193.
[54] W.A. Chameides, P.S. Kasibhatla, J. Yienger, H.I.I. Levy, Science 264 (1994) 74.
[55] J.A. Laurence, C.P. Andersen, Environ. Int. 29 (2003) 155.
[56] J.N. Cape, D. Fowler, A. Davison, Environ. Int. 29 (2003) 201.
[57] G.K. Agrawal, R. Rakwal, M. Yonekura, A. Kubo, H. Saji, Proteomics 2 (2002) 947.
[58] R. Rakwal, G.K. Agrawal, Electrophoresis 24 (2003) 3378.
[59] T.H. Steinberg, B.J. Agnew, K.R. Gee, W.Y. Leung, T. Goodman, B. Schulenberg, J. Hendrickson, J.M. Beechem, R.P. Haugland, W.F. Patton, Proteomics 3 (2003) 1128.