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Protein Extraction/Solubilization Protocol for Monocot and Dicot Plant Gel-Based Proteomics

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Sample preparation in plant proteomics is tedious, requiring modifications depending on the type of tissue involved. Here, we describe a protein extraction protocol for both monocotyledonous (monocot) and dicotyledonous (dicot) species, which significantly improves the solubilization of total proteins. For example, we used the primary leaf tissue and seeds from rice, a cereal crop and genome model system. Total protein was first precipitated with trichloroacetic acid/acetone extraction buffer (TCAAEB) and subsequently solubilized with a modified O'Farrell lysis buffer (LB) containing thiourea and tris (LB-TT). Separation of total leaf proteins by two-dimensional gel electrophoresis (2-DGE) revealed improved solubilization, as determined by an increased number of spots detected with Coomassie brilliant blue (CBB) staining. In addition, the resolution was better than when LB-TT was used alone for protein extraction. Seed proteins could be extracted in LB-TT itself without the need for TCAAEB, which resulted in a highly insoluble precipitate. Our CBB-stained 2-D gel protein profiles also demonstrated the efficacy of this protocol for total protein extraction/solubilization from the dicot genome model (*Arabidopsis*), a dicot disease model (cucumber), and two other important monocot cereal crop models (maize and wheat). Moreover, this is the first report on generating a 2-D gel proteome profile for wheat crown and cucumber leaf tissues. Finally, as examples of proteome reference maps, we obtained silver nitrate-stained, large-format 2-D gels for rice leaf and wheat crown LB-TT solubilized proteins.

Keywords: *Arabidopsis*, cucumber, lysis buffer, maize, rice, wheat

Proteomics, a rapidly progressing discipline in this functional genomics era, is essential not only for determining the functional components of genes and proteins, but also, and more importantly, for addressing the question of post-translational modifications because the one-gene/one-protein theory no longer holds true (Pandey and Mann, 2000; Hancock, 2004a, b; Agrawal et al., 2005a, b, c; Agrawal and Rakwal, 2006). Two major methodologies have now been identified in proteomics -- two-dimensional gel electrophoresis (2-DGE) and mass spectrometry (MS) - also referred to as gel- and non gel-based technologies, respectively (O`Farrell, 1975; Rabilloud, 2002; Yates, 2004; Agrawal and Rakwal, 2006). These two are the basis for current proteomics studies, and are used for separating and identifying proteins at the proteome level. Compared with yeast and animal proteomics, that of plants species lags in the amount of its research and publications, not because the technology is lacking, but mostly because the plant genome sequence database is small and sampling complex (Agrawal and Rakwal, 2006). Although *Arabidopsis thaliana* is an excellent genome model, rice (*Oryza sativa* L) and other cereal crops are the most important food sources for the world's population, and thus are demanding increased research attention in the field of plant sciences. Moreover, because only the rice genome has been almost completely sequenced (International Rice Genome Sequencing Project, 2005), this plant has become a crucial reference for monocots, and serves as a cornerstone for functional genomics in cereal crops (Agrawal and Rakwal, 2006).

Our current study focuses on protein extraction from various model plants: rice, *Arabidopsis* (Kim et al., 2005), maize, wheat, and cucumber. One of our objectives has been to develop an extraction protocol that can accommodate at least all the model plants, and be coupled with the improved extraction and solubilization of proteins for downstream 2-DGE. Here, we briefly describe the rationale behind investigating these particular species and stresses at the proteome level. As with any other "omic technology", one of the most critical steps in initial experimental design for proteomics is good sample preparation. Because plant tissues are complex and diverse, protein extraction becomes a first and limiting step for subsequent separation and identification by 2-DGE or MS. Despite inherent drawbacks, such as the non-separation of basic and membrane proteins, we must emphasize that 2-DGE is still one of the most efficient proteomics techniques, providing a visual method for identifying relative quantitative differences between proteins

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expressed in a given sample under normal and stress conditions. These proteins can be unambiguously identified using either the classic N-terminal amino acid sequencing approach or high-throughput MS via matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-MS (MALDI-TOF-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Here, our focus is on trichloroacetic acid (TCA)/acetone extraction buffer (TCAAEB), coupled to a modified lysis buffer (LB) for solubilization of precipitated plant proteins in order to have good resolution on 2-DGE.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Rice: Seedlings of *O. sativa* L. japonica-type cv. Nakdong were grown for 14 d under white fluorescent light (wavelength 390 to 500 nm, 150 mol m^{-2} s^{-1}, 12-h photoperiod) at 25°C and 70% relative humidity (RH) (Agrawal and Rakwal, 2006). Leaf segments (about 2 cm long) were cut, with clean scissors, from the third and fourth leaves of randomly selected seedlings, divided into 100-mg samples, and immediately ground in liquid nitrogen or kept frozen at -80°C until use. For the seed, 150-mg samples of freshly dehusked, dry, mature seeds from field-grown cv. 'Nipponbare' were used as experimental material.

Arabidopsis: Seeds of Col-0 ('Columbia') were sown on 2 × 2.3 cm^2 blocks of glass wool (Minipot; Nittobo, Japan), where they were kept for 2 d at 4°C for vernalization. Afterward, the seedlings were reared in a growth chamber at 22°C, under 14 h of light at 100 nmol m^{-2} s^{-1} from white fluorescent lamps, and at 50 to 60% RH. Sixteen-day-old seedlings were removed with forceps from the glass wool blocks, then weighed and immediately frozen in liquid nitrogen before storage at -80°C.

Maize: Maize (*Zea mays* L. cv. Guarare 8128) plants were grown for 16 d in the greenhouse under natural light, at a controlled temperature of 25°C and 70% RH. Leaf segments (about 2 cm long) were cut, with clean scissors, from the third and fourth leaves of randomly selected seedlings, divided into 100-mg samples, then immediately ground in liquid nitrogen or kept frozen at -80°C until use.

Wheat: Wheat (*Triticum aestivum* L. em Thell., line 'Iris') seedlings were grown in 3 × 21 cm Ray Leach Containers (USA) that were filled by two-thirds with Turface MVP soil conditioner (Profile Products, USA) for easy root removal. These were then topped with soil as described previously (Subramanyam et al., 2006). Throughout the experimental period, the growth chamber was maintained at 18°C and under a 24-h photoperiod from light illumination at 250 to 300 mol m^{-2} s^{-1}. The plants were watered as required and fertilized with Peter's fertilizer (WR Grace, USA). Both the plant crown (extending from the junction of the root and aerial portion to about 1 cm below the ligule of the first leaf) and the leaf blade tissue were harvested into liquid nitrogen and stored at -80°C. The embryos (50 mg) of wheat (cv. 'Norin 60') were carefully excised from dry, mature seeds using a carbide bur (1/4 size; Emesco, Germany) and scalpel under a light microscope.

Cucumber: Cucumber (*Cucumis sativus* L., cv. 'Shin Suyo Tsukemidori') seedlings were raised in a seeding box containing cultivation soil (Kureha Chemical, Japan) for easy removal of their roots. After approximately 10 d, the seedlings were transplanted to 10 × 10 cm plastic pots containing ¾-capacity pre-made soil and fertilizer mix (Nihon Hiryo, Japan). They were then reared in a phytotron under natural light conditions at 25°C, with daily watering. The third leaves were harvested from ca. 25-day-old plants and

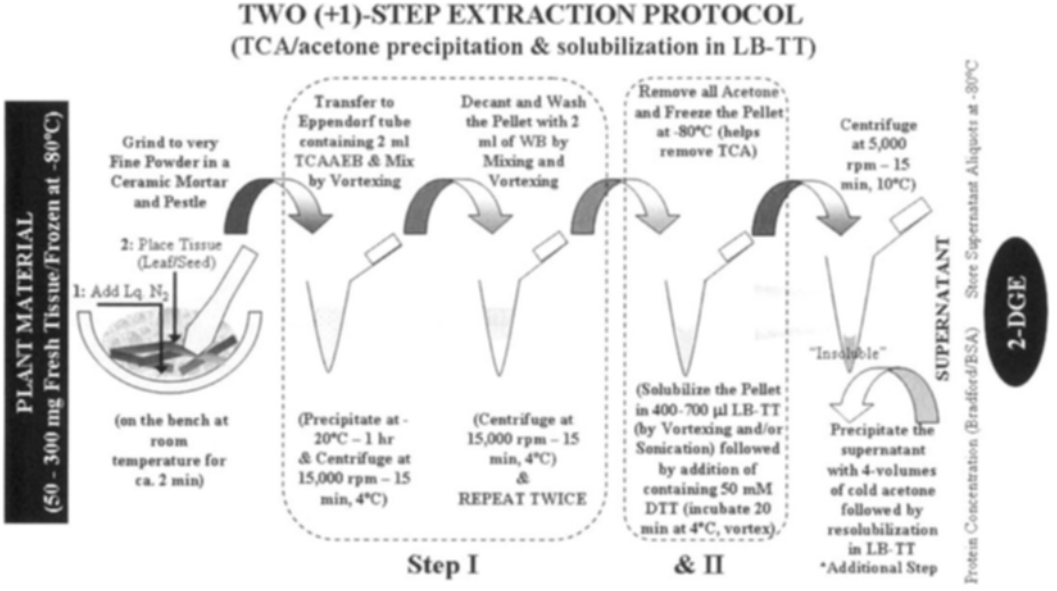

Figure 1. Two (+1)-step protein extraction protocol. Schematic description of steps involved in protein extraction, starting from grinding of leaves to very fine powder in liquid nitrogen, followed by precipitation of proteins in TCA/acetone extraction buffer (TCAAEB), treatment with wash buffer (WB), and solubilization of precipitated protein pellet in lysis buffer supplemented with thiourea and Tris (LB-TT), then protein determination before final 2-DGE.

stored at -80°C.

Extraction of Total Protein

Extraction of total protein was performed through a two (+1)-step protein extraction protocol (Fig. 1). Leaf samples pooled from individual seedlings were placed in liquid nitrogen, and ground thoroughly to a fine powder with a mortar and pestle (pre-cooled). The tissue powder (ca. 100 mg) was transferred to sterile tubes containing TCAAEB [acetone with 10% (w/v) TCA and 0.07% 2-mercaptoethanol (2-ME)]. Proteins were precipitated for 1 h at -20°C, followed by centrifugation at 15000 rpm for 15 min at 4°C. The supernatant was decanted, and the pellet was cleansed twice with wash buffer [acetone containing 0.07% 2-ME, 2 mM EDTA, and EDTA-free protease inhibitor cocktail tablets (Roche) in a final volume of 100 mL buffer]. Afterward, all the acetone was removed by air-drying the pellet at ambient room temperature (RT). The pellet was then kept at -80°C for at least 24 h before being solubilized in lysis buffer (LB)-TT [7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 18 mM Tris-HCl (pH 8.0), 14 mM trizma base, two EDTA-free protease inhibitor cocktail tablets, 0.2% (v/v) Triton X-100 (R), and 50 mM dithiothreitol (DTT), to a final volume of 100 mL]. This mixture was incubated for 20 min at 4°C with occasional vortexing and sonication, then centrifuged at 5,000 rpm for 15 min at 10°C. If further purification/clean-up of the solubilized protein samples was necessary, the supernatant from the last step was precipitated in 4 volumes of cold (-20°C) acetone and solubilized in LB-TT as above. For the seeds (ca. 150 mg), LB-TT was directly added to broken seed pieces and ground rapidly and thoroughly in a cold mortar with pestle, followed by incubation and centrifugation of the homogenate as above. The supernatant was used for protein determination with a Coomassie Plus™ (PIERCE, USA) protein assay kit, and was stored in aliquots at -80°C until analyzed by 2-DGE. When the sample was prepared for hand-cast tube gels in the first dimension, 1% (v/v) ampholyte (pH 3 to 10) was added in the LB-TT.

Hand-Cast Two-Dimensional Gel Electrophoresis

2-DGE was performed in hand-cast IEF tube gels on a Nihon Eido vertical IEF electrophoresis unit (Nihon Eido, Japan). This was followed by the second dimension using hand-cast polyacrylamide gels (15%) on a Nihon Eido SDS-PAGE vertical electrophoresis unit (Agrawal and Rakwal, 2006). Briefly, IEF gels were made in glass tubes (3 × 5 × 14/16 (H) mm) sealed at the bottom with parafilm. IEF gel solution [28.38% (w/v) acrylamide and 1.62% (w/v) bisacrylamide] was prepared, and loaded into the gel tubes. After polymerization, total soluble protein (ca. 350 µg) in LB-TT (maximum volume of 200 µL) was loaded onto the surface of the gel and overlaid with 20 µL of 1/3 LB-TT. Basic (1% v/v *N,N,N,N'*-tetramethylethylenediamine) and acidic (0.02 N H_3PO_4) reservoir buffers were added, and IEF was carried out according to the following voltage-time program: 200 V for 30 min, 400 V for 16 h, and 600 V for 1 h; constant voltage was used. The tube gels were either used immediately after equilibration for the second-dimension separation or stored at -80°C. Equilibration of the tube gels was done twice with gentle agitation (15 min each) in SDS-sample buffer [62 mM Tris (pH 6.8) containing 10% (v/v) glycerol, 2.5% (w/v) SDS, and 5% (v/v) 2-ME]. Molecular masses were determined by running standard protein markers (DualColor PrecisionPlus Protein™ Standard; Bio-Rad, USA).

Pre-Cast IPG and DALTtwelve

IEF on pre-cast IPG strip gels was conducted on an IPGphor unit as prescribed by the manufacturer (GE Healthcare Bio-Sciences AB, Sweden), with some modifications to the rehydration and IEF protocols. The volume carrying 150 µg total soluble protein was mixed with LB-TT containing 0.5% (v/v) pH 4 to 7 IPG buffer to bring it to a final volume of 450 µL. A trace of bromophenol blue (BPB) was added and the entire mixture was kept at RT for 5 min, then vortexed and centrifuged at 15000 rpm for 15 min at 10°C, followed by pipetting into a 24-cm strip holder tray. IPG strips (pH 4 to 7; 24 cm) were carefully placed onto the protein samples, covered with a lid, and put into the IPGphor unit. These strips were positioned gel-face down on the protein samples to avoid air bubbles and allow for passive rehydration with the protein samples for 1.5 h. This was followed by overlaying the IPG strips with 1.5 mL of cover fluid, a process directly linked to a five-step active rehydration and focusing protocol (Step 1: active rehydration, step-n-hold, 50 V for 12 h; Step 2: step-n-hold, 100 V for 1 h; Step 3: gradient, 500 V for 4 h; Step 4: gradient, 8000 V for 12 h; and Step 5: step-n-hold, 8000 V for 3 h). A total of 77000 V·h was used for the 24-cm strip, and the entire procedure was carried out at 20°C. Following IEF, the IPG strips were removed from the strip holder and blotted on a Kimwipe to remove the cover fluid. They were then immediately used for the second dimension or stored at -20°C. The strip gels were incubated in 20 mL equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS] containing 2% (w/v) DTT for 20 min with gentle agitation, followed by incubation in the same (newly prepared 20 mL) equilibration buffer supplemented with 2.5% (w/v) iodoacetamide at RT for the same time period as above. Preceding the second dimension separation, the IPG strips were rinsed with cathode running buffer [0.025 M Tris, 0.192 M glycine, and 0.2% (w/v) SDS], placed on polyacrylamide gels (DALT Gel, 12.5% of 255 × 196 × 1 mm size), and overlaid with an overlay agarose solution [60 mM Tris-HCl (pH 6.8), 60 mM SDS, 0.5% (w/v) agarose, and 0.01% (w/v) BPB]. The lower anode buffer contained 0.05 M diethanolamine and 0.05 M acetic acid. SDS-PAGE as the second dimension was performed at a constant current of 2 W overnight (ca. 14 h) on an Ettan DALT*twelve* System (GE Healthcare Bio-Sciences AB) as per the manufacturer's instructions. Molecular masses were determined by running standard protein markers (Bio-Rad).

Protein Visualization and Image Analysis

To visualize the protein spots, the gels were stained with either CBB R-250 (Fluka Chemie GmbH, Switzerland) or silver nitrate. With the CBB, they were first stained for ca. 30

min before the excess dye was washed from the gels with a destaining solution to clear the background. This protocol had been recently reviewed (Agrawal and Rakwal, 2006). For the silver nitrate, staining was carried out exactly according to the manufacturer's instructions for the PlusOne Silver Staining Kit (GE Healthcare Bio-Sciences AB). Protein patterns in the gels were recorded as digitalized images using a digital scanner (resolution 300 dpi, greyscale, CanoScan 8000F; Canon, Japan), and saved as TIFF files. Protein spots on the gel were quantitated in profile mode according to the operating manual for the ImageMaster 2D Platinum software (GE Healthcare Bio-Sciences AB).

RESULTS AND DISCUSSION

The selection of a suitable extraction buffer is the key to good sample preparation; depending on the plant materials and their origin, that buffer may change for each evaluation. Moreover, a good buffer should help one achieve two important goals: the extraction of all proteins in a quantitative manner, and the protection of proteins from proteolytic degradation. Agrawal and Rakwal (2006) have now proposed two modifications that improve on this protein extraction and solubilization using the original O'Farrell lysis buffer (LB; O'Farrell, 1975). Their long experience in rice proteomics has led them to determine that an LB supplemented with thiourea plus tris (LB-TT) dramatically increases the solubilization of proteins from the leaves of mature (two-month-old, just before heading) rice (cv. Nipponbare) plants. This is evidenced by a rise in the abundance of large subunit (LSU) and other medium- and high-molecular-weight proteins, as well as the prevention of fragmentation of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) compared with the performance of either LB alone or LB supplemented with thiourea (LB-T). Their results have revealed that even a slight modification in LB buffer composition can bring stability by reducing protein degradation, along with simultaneously increasing the number of spots on 2-D gels. This development has now prompted us to apply the LB-TT buffer to other model systems, such as *Arabidopsis*, maize, wheat (Williams et al., 2003; Puthoff et al., 2005; Sardesai et al., 2005a, b; Subramanyam et al., 2006), and cucumber (Kuc, 1982; Narusaka et al., 2001; Cools and Ishii, 2002). In these model plants, comparative proteomics will be utilized to investigate plant responses to abiotic and

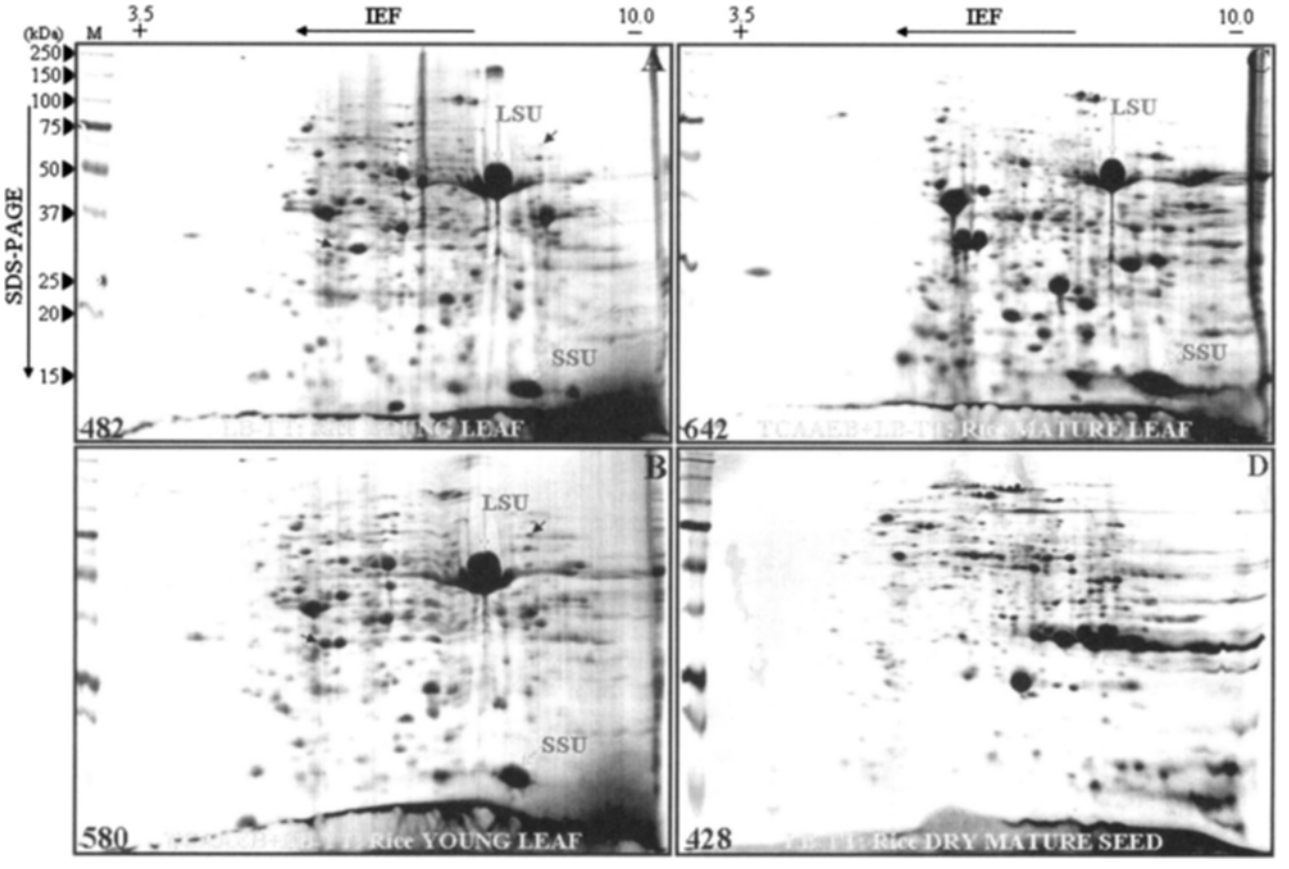

Figure 2. Representative 2-D gel images of rice leaf and seed proteins separated by hand-cast IEF tube gels in first dimension. Images at left are 2-D gel protein profiles from young rice (cv. Nakdong) leaves extracted by directly grinding with LB-TT (**A**), or according to extraction protocol presented in Figure 1 (**B**). Images to right are of 2-D gel protein profiles from mature rice (cv. Nipponbare) leaves (**C**) and dry mature seed (**D**). For each sample, ca. 350 μg of total soluble protein was loaded at cathodic end of 11-cm-long tube gel. IEF (pH 3-10) and SDS-PAGE (15%; 12 × 14 cm) were carried out in second dimension. Proteins were visualized by staining with CBB, and spot numbers in each gel are indicated at bottom left-hand corners. LSU and SSU are RuBisCO large and small subunits. Black arrows mark representative protein spots increased in B (TCAAEB/LB-TT) over A (LB-TT).

biotic stresses.

Using TCAAEB and LB-TT in our two (+1) protein extraction and solubilization protocol schematically presented here (Fig. 1), we have demonstrated its (protocol) suitability in at least five plant species (rice, *Arabidopsis*, maize, wheat, and cucumber) and three tissue types -- leaf, seed, and crown (Fig. 2-4). This method is relatively simple, requires only a small amount of tissue (50 to 150 mg), and involves quickly grinding the sample to a very fine powder in liquid nitrogen, followed by precipitation in TCAAEB in 2 mL Eppendorf tubes. It should be noted that improper or weak grinding can cause problems with complete extraction of proteins and subsequent solubilization in the LB of interest. The next critical step is the washing of the resultant precipitates; a thorough and complete cleansing will remove any impurities (including interfering pigments bound to the protein), and help in subsequent protein solubilization. Solubilization in LB-TT with occasional vortexing at 4°C for ca. 20 min, followed by centrifugation, produces the total soluble protein extract. An additional precipitation step using the solubilized protein and cold acetone can help in further clean-up of the protein sample, which provides good resolution in the first dimension IEF.

In testing this protocol, equal amounts of protein were loaded for each extraction procedure in order to compare the results by 2-DGE (Fig. 2-4). In young rice leaves, proteins were resolved over a pH range of 3.5 to 10.0; using TCAAEB prior to solubilization with LB-TT, ca. 580 CBB-stained spots were detected by computerized image analysis software versus the ca. 482 spots seen with LB-TT alone. Mature rice leaf proteins could be nicely separated, revealing ca. 642 CBB-stained spots; this method provided a significant improvement in gel profiles (Fig. 2, 5) over the previous procedure of Islam et al. (2004), which also used TCA but recommended longer vortexing times (1 h) in the presence of glass beads. For seed samples, although TCAAEB precipitation resulted in an insoluble precipitate after the proteins were extracted from the powder, we found that grinding directly in LB-TT resulted in well-separated protein patterns on 2-D gels, as evidenced by the 428 protein spots detected by CBB staining (Fig. 2). However, it was also possible to precipitate the proteins extracted in LB-TT by acetone (refer to additional step III; Fig. 1), thereby obtaining a more concentrated protein amount for 2-DGE (data not shown). With *Arabidopsis*, we could observe even more clearly the effect of TCAAEB, in that significantly increased protein amounts were extracted from leaf samples after solubilization in LB-TT (589 spots), versus LB-TT (441

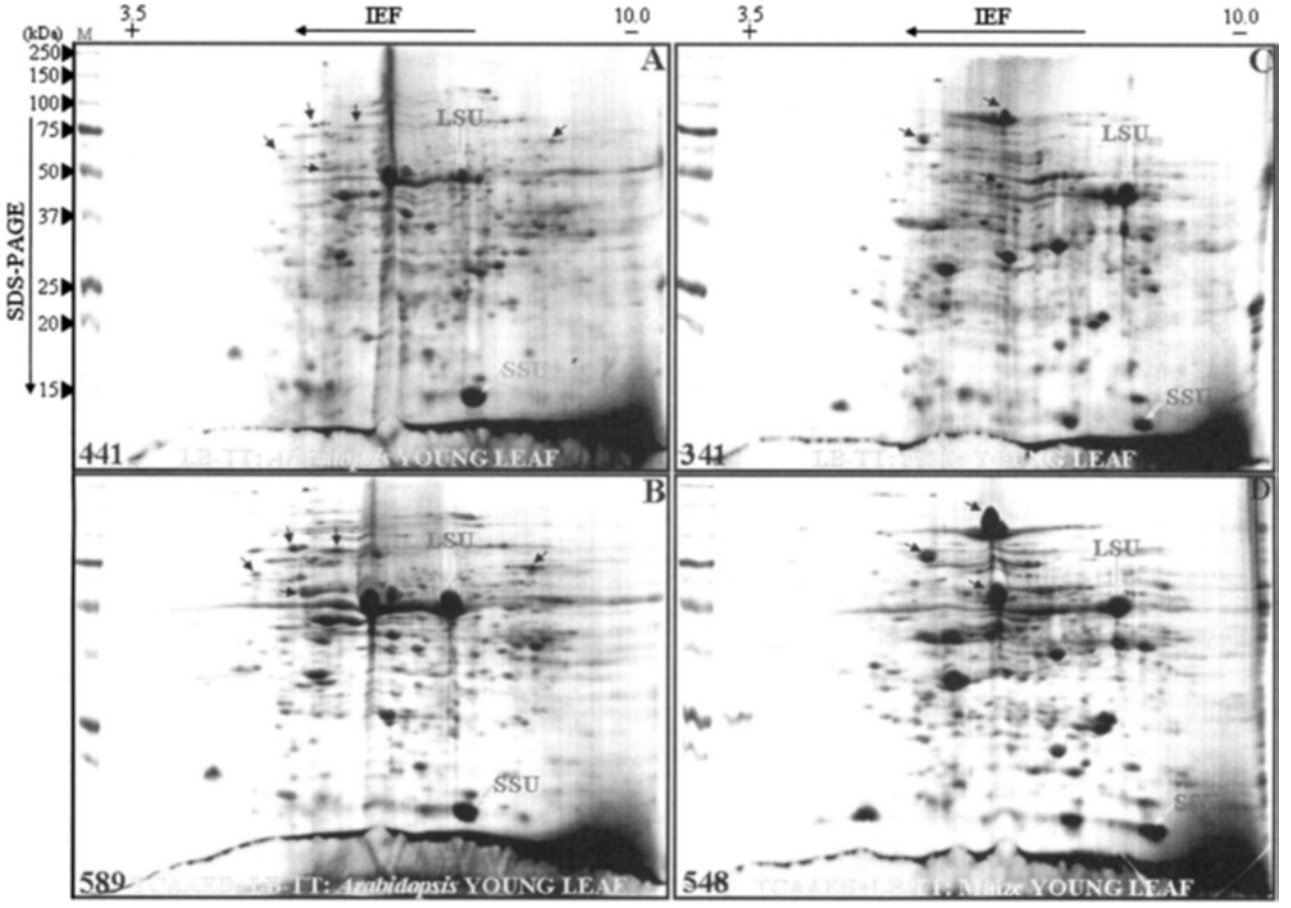

Figure 3. Representative 2-D gel images of *Arabidopsis* and maize leaf proteins separated by hand-cast IEF tube gels in first dimension (pH 3-10). Respective images at left and right are 2-D gel protein profiles from young *Arabidopsis* (cv. Col-0) leaves and young maize (Panama cv. Guarare 8128) leaves extracted by directly grinding with LB-TT (**A**, **C**) or by following extraction protocol presented in Figure 1 (**B**, **D**). Protein measurements, 2-DGE method, visualization, and image analysis were done as described in Figure 2. Black arrows mark representative protein spots increased in B and D (TCAAEB/LB-TT) over A and C (LB-TT).

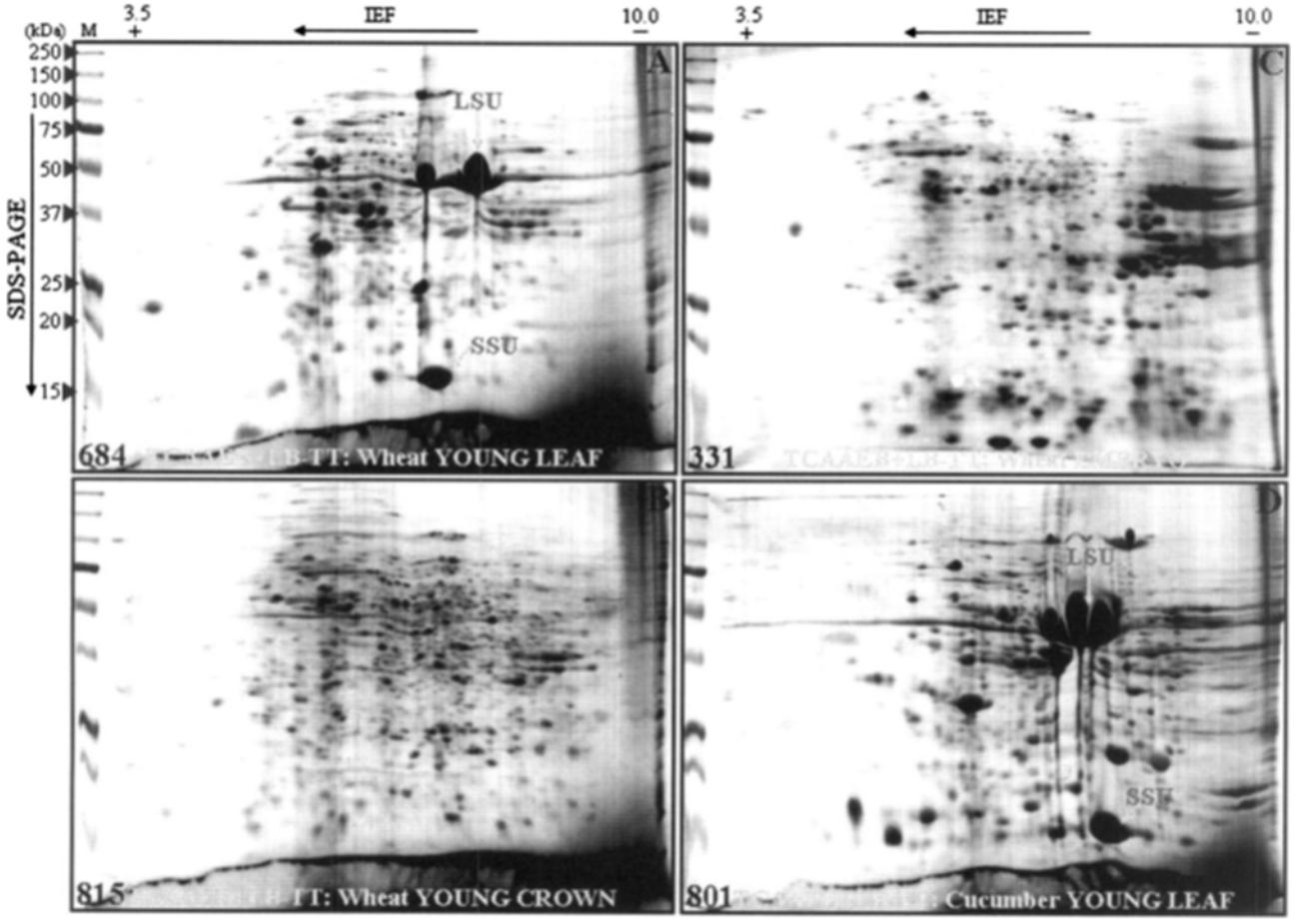

Figure 4. Representative 2-D gel images of wheat leaf, crown, and embryo, and cucumber young-leaf proteins separated by hand-cast IEF tube gels in first dimension (pH 3-10). First three 2-D gel protein profile images are of wheat young leaf (**A**), crown (**B**), and embryo (**C**), while image (**D**) is of cucumber proteins following extraction protocol presented in Figure 1. 2-DGE was carried out as in Figure 2.

spots) alone (Fig. 3). A similar result was obtained with maize leaves, where 548 spots were detected by using the TCAAEB plus LB-TT combination, compared with only 341 spots seen from LB-TT alone (Fig. 3). Moreover, as shown by the individual gel images in Figure 2 and 3, the presence of large new protein spots could be easily distinguished with the TCAAEB plus LB-TT protocol. In Figure 4, we further demonstrated that this protocol worked extremely well for extracting and separating proteins from the leaves (684 spots), crowns (815 spots), and embryos (331 spots) of wheat, as well as from cucumber leaves (801 spots).

Although the hand-cast IEF tube gels could separate total soluble protein (Fig. 2-4), it became clear that the one spot-one protein idea was redundant; significant overlaps occurred in protein spots when using the hand-cast small size IEF tube gels (11 cm) and a non-linear pH gradient. Therefore, as Agrawal and Rakwal (2006) have discussed, it was imperative to shift toward using the pre-cast linear IPG strips and large-format gels for 2-DGE. Here, we demonstrated a significant increase in the number of proteins separated and detected in rice leaves (910 protein spots) and wheat crowns (907 protein spots) using large-format, pre-cast 24 cm (pH 4-7) IPG strips and 26 × 20 × 1 mm polyacrylamide gels, for the first and second dimensions, respectively (Fig. 5). It should be noted that these greater numbers resulted from a very low amount of protein loading (150 μg) compared with fewer protein spots when larger amounts of protein (350 μg) were loaded. Moreover, the individual spots were highly defined and showed almost no streaking, indicating good resolution in the first dimension, which reflected the qualitative aspect of the LB-TT for protein solubilization prior to IPG. Therefore, we recommend IPG not only for the better resolution of protein spots (and increased amounts), but also for greater reproducibility and generation of 2-DGE reference maps (Zhan and Desiderio, 2003). Moreover, a reproducible system (large-format 2-DGE) can be applied from lab to lab in developing 2-D maps for comparative proteomics.

Our study results demonstrate a simple and efficient proteomics methodology for protein extraction and 2-DGE to establish reference maps of monocot and dicot sample tissues for applications in comparative proteomics. Using TCAEEB/LB-TT, we have achieved not only significant improvement in the solubilization of proteins from rice tissues, but also a dramatic alteration in protein spot numbers, quality and quantity from *Arabidopsis*, maize, wheat, and cucumber samples processed by 2-DGE. We are currently working toward the proteomics of those five species, with the goal of identifying differentially expressed proteins under the variety of environmental stress conditions that plague commercial crop

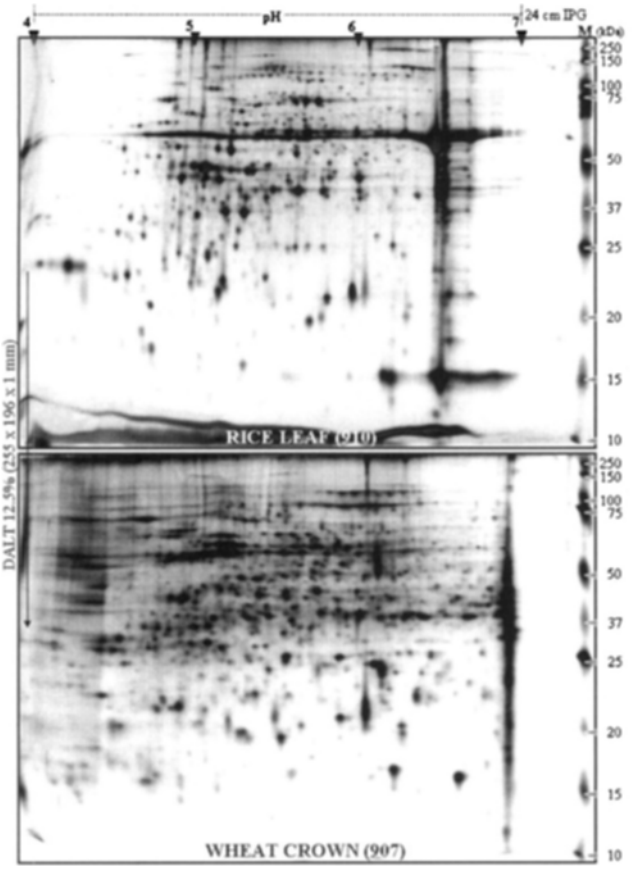

Figure 5. Representative 2-D gel images of rice leaf and wheat crown proteins (150 μg) separated on 24-cm-long pre-cast IPG strip gels (pH 4-7) and large-format, pre-cast 12.5% polyacrylamide gels (255 × 196 × 1 mm). Proteins were visualized by staining gels with silver nitrate; spot numbers in each gel are indicated at bottom-center of image.

production. This in turn will lead to assigning potential protein markers ("biomarkers"; Hancock, 2004a, b), for certain stress factors and in crop breeding programs worldwide. Such a challenging task, however, requires deep proteome measurements that will provide greater insight into the effect of ozone (Agrawal et al., 2002), mutations, varietal differences, and biotic stresses. The importance of IPG and large SDS-PAGE gels in proteomics is beyond doubt, and we now must set a standard for gel-based proteomics in order not to discount the value of 2-DGE in plant research.

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