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Full Length Research Paper

A two-dimensional electrophoresis protocol suitable for *Medicago truncatula* leaf proteome

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Medicago truncatula **leaves were used as the experimental materials. Total proteins of leaves were extracted by trichloracetic acid (TCA)-acetone method and proteins had a better separation using gel strips, forming an immobilized non-linear 3 to 10 pH gradient focusing 123,000 vhr combined with 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie Brilliant Blue G-250 and digitalized gels were analyzed using the PDquest 8.0.1 software. The results indicated that 931 protein dots were detected in the gel. A technology suitable for the *M. truncatula* leaves protein extraction by TCA/acetone and a protocol for two-dimensional electrophoresis (2-DE) was established, which provides technical support for *M. truncatula* leaf proteome research.**

Key words: *Medicago truncatula*, proteome, two-dimensional polyacrylamide gel electrophoresis (2-DE), isoelectrofocusing (IEF).

INTRODUCTION

Proteomics is defined as the systematic analysis of the proteome and the protein complement of genome (Pandey and Mann, 2000). Proteome analysis has become a powerful tool in the functional characterization of plants. Due to the availability of vast nucleotide sequence information and based on the progress achieved in sensitive and rapid protein identification by mass spectrometry, proteome approaches open up new perspectives to analyze the complex functions of model plants and crop species at different levels. Two-dimensional polyacrylamide gel electrophoresis (2-DE) is one of the most efficient and powerful methods to study complex patterns of gene expression at the level of proteins (Celis et al., 1998). Then, the primary task of proteome research is to establish high resolution, reproducible 2-DE technology system.

Medicago truncatula, with its small diploid genome, self-fertile nature, prolific seed production, rapid generation time and ease of transformation, has made it a good legume model (Frugoli and Harris, 2001; Cook, 1999). As a model, the impressive achievements in genome and expressed sequence tag (EST) sequencing have provided a wealth of information. However, the information is insufficient in answering questions concerning gene function, developmental/regulatory biology, and the biochemical kinetics of life. Gene expression at the transcriptional level provides important information regarding early stage transmission from genome to cellular machinery, but mRNA levels are not always consistent with the abundance of cognate proteins (Ideker et al., 2001; Gygi et al., 1999). Protein is not only the final executant of life functions, but also the key to understanding physiological, pathological and

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Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **EST,** expressed sequence tag; **IEF,** isoelectrofocusing.

pharmacological functions. Proteomics provides a more direct assessment of biochemical processes by monitoring the actual proteins performing the enzymatic, regulatory and structural functions encoded by the genome and transcriptome.

The abundance of proteases (Tsugita et al., 1994) and the presence of phenols (Cremer and Van de Walle, 1985) in green tissues extract result in protein degradation and give rise to horizontal streaks (Gorg et al. 2004), abundance of the photosynthetic enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) that migrates as several large irregularly shaped spots (des Francs et al., 1985). These facts had a serious effect on the separation result of 2-DE. Some reference maps of different organs, including root (Mathesius et al., 2001), stem, leaf, flower, seed pots, cell suspension cultures (Lei et al., 2005; Watson et al., 2003) and embryogenic tissue culture cells have been established (Imin et al., 2004). Asirvatham et al. (2002) quantified the coefficient of variance for both analytical and biological sources with 2-DE. But all of them used the 11 cm IPG strips and got a low resolution maps, without respect to the influencing factors. The programs could not suit the 17 cm IPG strips. In this paper, leaves of *M. truncatula* were used as the experimental material to study the influencing factors on 2-DE. A technology study for the *M. truncatula* leaves protein 2-DE was established, which provides technical support for *M. truncatula* leaf proteome research.

MATERIALS AND METHODS

Plant material and growth conditions

M. truncatula Gaertn. cv. Jemalong seeds were scarified in concentrated anhydrous sulphuric acid and sterilized in 50%(v/v) sodium hypochloride. Then they were immersed in 70% (v/v) ethanol for 2 min, rinsed with distilled water and placed in Petri dishes with soaked filter paper. After 3 days at 4°C in dark conditions, Petri dishes were transferred to the growth chamber (thermoperiod of 25/18°C, day/night, and relative humidity of approximately 40%) for 2 to 3 days still in dark conditions. Germinated seeds were maintained another week in Petri dishes but under light conditions (photoperiod of 16/8 h, day/night). Afterwards, seedlings were individually transferred to 30 × 30 cm pots with vermiculite. The plants were maintained in the growth chamber under the same conditions. Eight-week-old plants were used for leaf tissue. The top two apical unfolded trifoliates were sampled for leaf tissue.

Total protein extracts

Total proteins of leaves were extracted by TCA-acetone method (Shultz et al., 2005; Saravanan and Rose, 2004). 0.5 g leaf was ground in liquid N_2 using a mortar and pestle. The powdered tissue was placed in 50 ml tubes and then precipitated at -20°C with 10 % (w/v) TCA in acetone containing 0.07 % (w/v) 2-mercaptoethanol. Later, vortexed samples were incubated at -20°C for 45 min. Proteins were recovered by centrifuging at 14,000 g for 15 min. The protein pellets were washed with a cold solution of 90% acetone and 10% water containing 0.07% 2-mercaptoethanol twice to remove residual TCA (One sample was washed with 100% acetone instead of 90% acetone). Then the pellets were washed again with 0.2% DTT instead of 2-mercaptoethanol, air-dried and resuspended in 2-DE solubilization buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholytes. The protein concentration was then quantified by Bradford method (Bradford, 1976) using a commercial dye reagent (Bio-Rad) and bovine serum albumin as the standard.

2-D Electrophoresis

Proteins were first separated by isoelectrofocusing (IEF). Proteins (900 µg per sample) were separated using gel strips, forming an immobilized nonlinear 3 to 10 pH gradient (Immobiline DryStrip, 17 cm; Bio-Rad). Strips were rehydrated in the protein IEF Cell (Bio-Rad); passive rehydration was done for 4 h and active rehydration, 12 h at 20°C. IEF was performed at 20°C in the protein IEF Cell system for 1 h at 250 V; 1.5 h at 1,000 V; volt gradient to 9,000 V in 5 h; and 9,000 V for 60,000 volt-hours (vhr), 90,000, 105,000, 115,000, 123,000 and 125,000 vhr, respectively. Before the second dimension, each gel strip was incubated at room temperature for 2 × 15 min, in 2 × 5 ml equilibration solution as described by Gallardo et al. (2002). Proteins were then separated in vertical polyacrylamide gels (12% (w/v) acrylamide) in a PAC 3000 Electrophoresis system (Bio-Rad). Electrophoresis was run at 12°C for 1.5 h at 70 V, then at 4°C, 200 V until the dye front reached the bottom of the gel.

Protein staining and analysis of 2-D gels

Gels were stained with either Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA) according to Candiano et al. (2004). Image acquisition was done using a Sharp JX-330 scanner (Amersham Biosciences) with a resolution of 300 ppi and an optical density which ranged from 0.05 to 3.05. Image analysis was carried out on Coomassie Blue gels with the PDQuest 8.0.1(Bio-Rad).

RESULTS

Protein extraction methods

Compared with 100% acetone, 90% acetone washed sample can attain set volt (9,000 V), and get a stable electrophoresis process (Figure 1). In 100% acetone washed sample, the volts were limited to 6,000 to 7,000 V. Adding desalt procedures and prolonged desalt time could not reduce the current. The incompletely focused proteins show a lot of horizontal streaking in the gel.

2D-PAGE analysis

We further compare the focusing result of *M. truncatula* leaf proteins under different focusing volt-hours (vhr) (Figure 2). They all have different focusing states. The 60,000 vhr focusing gel, due to incomplete focusing shows a lot of proteins streaking in gel. Some abundance proteins streaking such as Rubisco covered other proteins, with their effects detected. Along with the

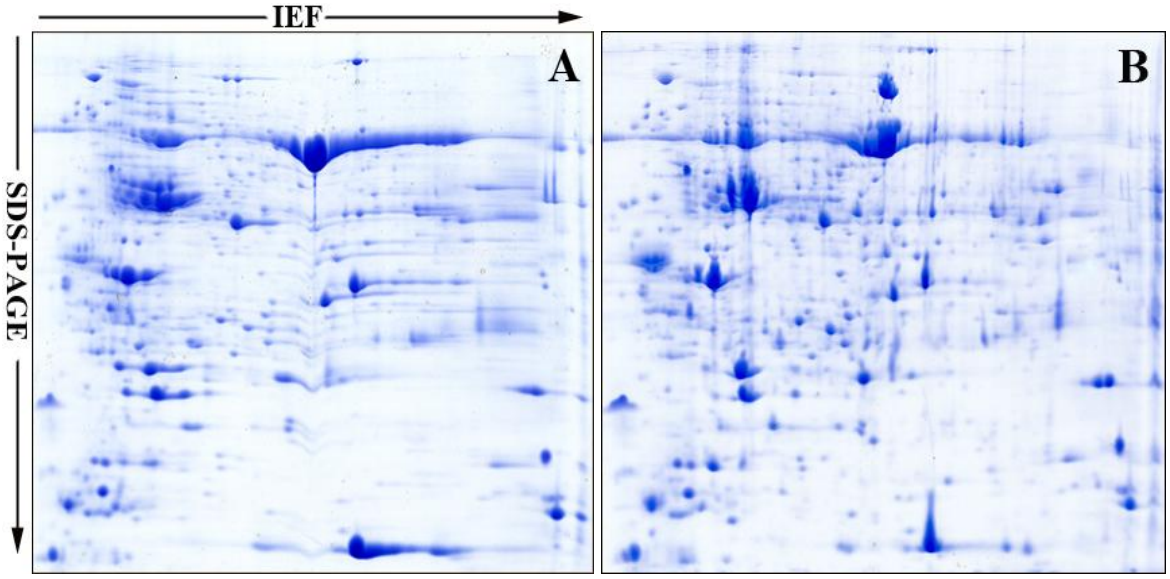

Figure 1. 2-D gels with different washing methods. A, Washed with pure acetone. B, washed with 90% acetone.

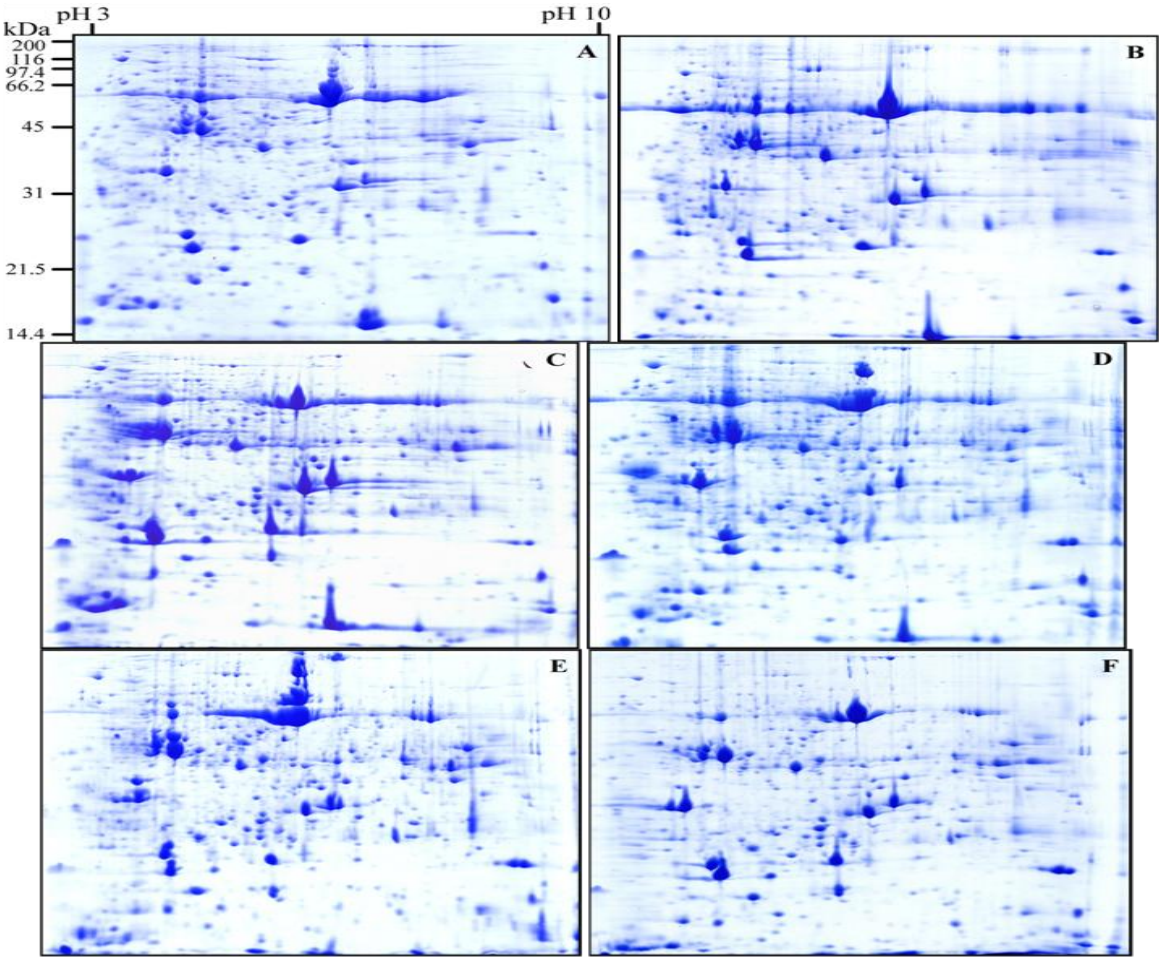

Figure 2. 2-D gels proteome map of *M. truncatula* leaves with different focusing state. A, 60 Kvhr; B, 90 Kvhr; C, 105 Kvhr; D, 115 Kvhr; E, 123 Kvhr; F, 125 Kvhr.

focusing time extension, horizontal streaking was gradually reduced. This was obviously seen in the Bubisco protein. When focusing up to 123,000 vhr, proteins are focused into discrete spots, and also more proteins can be detected. When focusing reaches 125,000 vhr, horizontal streaking happens again. Qualitative analysis revealed that 638, 859, 930, 822, 931 and 602 protein spots were detected in 60,000, 90,000, 105,000, 115,000, 123,000 and 125,000 vhr focusing gel, respectively. Along with the focusing time extension, we detected more protein spots in the gel. The most protein spots detected in the gel was focusing 123,000 vhr, and focusing 105,000 vhr gel showed the second highest quantity of protein spots. When focusing 125,000 vhr, the quantity of protein spots has a sudden decrease.

DISCUSSION

Sample extraction was one of the most crucial steps of 2-DE. Most problems can be traced to co-extraction of non-protein cellular components that can affect protein migrations (Gorg et al., 2000). TCA/acetone precipitation is one of the most commonly methods used for plant protein sample extraction (Wang et al., 2006), which can consume shorter times in whole process and enrich alkaline proteins. Ice-cold TCA on the low pH could induce proteases inactivated and prevent protein degradation, which is also very useful for removing interfering compounds, such as salt (Shaw and Riederer, 2003) or polyphenols. Lower molecular weight (Mr) polysaccharides may be removed by precipitation in TCA (Shaw and Riederer, 2003). However, there were many protein losses due to incomplete precipitation or resolubilization of proteins. Moreover, a low pH can induce protein degradation, and shorten the precipitation time to 45 min, with both ensuring the precipitation of proteins and reducing protein loss (Tsugita et al., 1996). Compared with 100% acetone, 90% acetone has a better capacity to remove water solubility impurity. And the samples washed with 90% acetone could attain setting volt in IEF, which plays an important roles in improving samples' quality and stability of 2-DE.

IEF is an important link in 2-DE, which directly influences the quality of 2-DE gel; and both incomplete focusing and over-focusing can lead to horizontal streaking. All protein samples are different and require different isoelectric focusing parameter. Horizontal streaking can lead to decrease of the quantity of protein spots. There was a lot of horizontal streaking on 60,000, 90,000, 105,000, 115,000 and 125,000 vhr focused gel; with the 123,000 vhr focusing gel showing a better result. When focusing over 123,000 vhr, the decrease in the quantity of protein spots is shown in the gel. The horizontal streaking observed in Figures 2A, B, C and D appears to be as a result of incomplete focusing, prolonged focusing times that decreased horizontal streaking on the subsequent second-dimension gel. The horizontal streaking in Figure 2C, D, E and F may be the result of water and protein removal. Stochaj et al. (2006) and Wu et al (2010) think overextending the Vhr in IEF may cause over-focusing; and over-focusing must be avoided. Focusing 123,000 vhr was a suitable parameter for this protocol. The parameters of IEF were optimized in this study. Passive and active rehydrations were adopted to make sure the sample is adsorbed. A 50 V initial voltage was convenient sample adsorbed to gel. Gorg (2000) indicated that a 30 to 50 V initial voltage can drive the sample into the gel. The samples extracted by the process of our research have two standard desalt steps that ensure IEF process works well, and do not need to add extra desalt steps and prolonged focusing times. A technology suitable for the *M. truncatula* leaves protein 2-DE was established, which provides technical support for *M. truncatula* leaf proteomics research.

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