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RESEARCH ARTICLE

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Evaluation of two-dimensional gel electrophoresis-based technology on plant calmodulin isoforms

Abstract The two-dimensional gel electrophoresis-based technique is commonly used in protein analysis and identification. Though this technique is powerful, mature and sensitive there still remain some questions regarding its ability to characterize different types of proteomes. Different calmodulin isoforms play different roles in response to varying external stimulant signals during plant development, but their advanced structures are too small to be distinguished and purified at protein level. In this paper, a two-dimensional gel electrophoresis-based technology was investigated to identify different calmodulin isoforms, which will lay a foundation for subsequent researches.

Keywords 2D gel electrophoresis, plant calmodulin, isoform

1 Introduction

Ca^{2+} ion flux, as an important second messenger, participates in many signal transduction processes induced by biotic- or abiotic stress (Hetherington and Brownlee, 2004). The Ca^{2+} ion flux signaling is transduced via Ca^{2+} receptors such as calmodulin (CaM), which is a major Ca^{2+} receptor in both animals and plants. When CaM combines with Ca^{2+}, its structure will be changed correspondingly to regulate or activate the target protein downstream to control different physiological procedures (Sun, 2001).

The basic structures of CaM proteins are highly conservative in eukaryotes based on previous research, but many CaM genes which encode the proteins with the same or similar structure have been found in single plants by gene expression technology in recent years, and named CaM isoforms. Takezawa et al. (1995) isolated 8 independent CaM genes (*PCaM1–8*) from potato and grouped them into 5 types, of which *PCaM5–8* encoded the same CaM isoform. From wheat, Yang et al. (1996) indentified 10 CaM genes and distinguished them into 4 CaM subfamilies (SF1–4) and 3 different isoforms. The cDNAs of the SF-1, SF-3, SF-4 subfamilies were grouped into one isoform, but SF-2 encoded 2 isoforms at least. In addition, it was reported that different CaM multigenes were isolated in soybean and pea, respectively (Lee et al., 1995; Duval et al., 2002).

Eriko Ishigaki (2004, 2005) and Reona Takabatake (2007) revealed that the CaM multigene families of plants have many different aspects, such as tissue distribution, regulation of gene expression and physiological functions. According to the results of Heo et al. (1999) regarding different CaM isoforms in soybean, the expression of *SCaM4* and *5* remained low but increased quickly when it was induced by pathogens, but the expression level of the other 3 types remained unchanged. After *SCaM4* and *5* were transformed, the tobacco showed more resistance to the fungal pathogen and tobacco mosaic virus (TMV). All the researches suggested that these two isoforms of *SCaM4* and *5* took part in plant signal transduction. Thus, further research on investigating the function of each isoform will be very helpful to understand the specificity of the Ca^{2+}-CaM system.

Specific antibodies are necessary to investigate the location, quantity and physiologic function of CaM isoforms, but they are difficult to be prepared, as different CaM isoforms are different from each other in only a couple of amino acid residues. However, the 2D gel electrophoresis technique which combined ampholine electrophoresis with SDS-polyacrylamide gel electrophoresis and became widely used in the recent past, provides a new method for studying each isoform expression. It groups proteins according to their isoelectric points and molecular weights. In this paper, 2D electrophoresis was used to identify different CaM isoforms of wheat, which lays a foundation for subsequent researches.

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2 Materials and methods

2.1 Plant materials

The seeds of wheat cultivar L10 (*Triticum aestivum* L.) were planted into organic soil in pots of 10 cm diameter in a greenhouse at 25/20°C, with a 16-h-day and 8-h-night photoperiodic regime; the intensity of illumination was 400 $W \cdot m^{-2}$. When the first euphylla fully developed, wheat leaf rust race 260 was inoculated to form an incompatible combination with L10.

2.2 Protein extraction

2.2.1 Extraction of dissolvable protein

After plants were inoculated for 8 h, the first leaves were sampled and stored in sealed plastic bags at −80°C. 0.5 g leaves were ground to fine powder in liquid nitrogen. The extraction procedure was as follows: 1 mL extraction buffer (50 $mmol \cdot L^{-1}$ Tris-HCl (pH 8.0), 2 $mmol \cdot L^{-1}$ EGTA, 1 $mmol \cdot L^{-1}$ PMSF, 1 $mmol \cdot L^{-1}$ DTT) was added and the reagent was spun at 20000×g for 30 min. Then 1∶5 (V/V) pre-cold 50% trichloracetic acid was added, the tubes were placed on ice for 1 h, and the sample was centrifuged at 20000×g for 15 min; it was then washed with cold acetone (including 0.3% DTT) and centrifuged 3 times, and the precipitate was then placed on ice for 5 min and was dried out.

0.5 mg dried protein was weighed and transferred into a tube, adding 400 μL lysis buffer (8 $mol \cdot L^{-1}$ urea, 4% ($m \cdot V^{-1}$) CHAPS, 65 $mmol \cdot L^{-1}$ DTT, 0.2% (V/V) Boi-Lyte and ampholining buffer (pH 3–5.5, 0.001% bromchlorphenol blue)), and schizolysed in room temperature for 1 h. Subsequently, the sample was centrifuged at 20000×g at 17°C for 30 min. The concentration of supernatant was detected according to the description of Bradford, and storing of the extraction was done at −80°C.

2.2.2 Extraction of dissolvable thermostable proteins

0.5 g of 7-day-old plant leaves was weighted and ground into powder in liquid nitrogen then transferred into 2 mL Eppendorf tubes. Premixed 1 mL extraction buffer (50 $mmol \cdot L^{-1}$ Tris-HCl (pH 8.0), 2 $mmol \cdot L^{-1}$ EGTA, 1 $mmol \cdot L^{-1}$ PMSF, 1 $mmol \cdot L^{-1}$ DTT) was added and the tubes were incubated at 92°C for 3 min; the reagent was cooled as quickly as possible, and then centrifuged at 20000×g at 4°C for 30 min. There were two methods for precipitating protein A and protein B. Protein A was a trichloroacetic acid precipitation method which is the same as that described in 2.2.1, and protein B was an acetone precipitation method where 300 μL of supernatant with cold acetone 900 μL was suspended in a 2 mL Eppendorf tube at −20°C for 2 h.

2.3 Isoelectric focusing electrophoresis and SDS-PAGE electrophoresis

2.3.1 Isoelectric focusing electrophoresis

After the plastic cover sheet of a pH 3–6, 17 cm stripe was removed, the immobilized pH gradient (IPG) stripes with gel-side down were laid onto the sample solution, distributed along the bottom of the rehydration tray, then covered with mineral oil and incubated at room temperature for 4 h, followed by initially rehydrating for 8 h so that the IPG was rehydrated completely. The IPG was then placed into an IEF tray transferred into a Protean IEF Cell. The program was set as the following 4 steps: 250 V for 1 h, 500 V for 1 h, 1000 V for 4 h, and 10000 V till reaching 60000 Vh. The maximum electric current intensity was kept at less than 50 V. A per stripe during each process of running.

2.3.2 Equilibration

Once isoelectric focusing was finished, the IPG stripes were covered with 10 mL equilibration buffer (0.375 $mol \cdot L^{-1}$ Tris-HCl (pH 8.8), 6 $mol \cdot L^{-1}$ urea, 20% (V/V) glycerin, 2% SDS, trace amount of Bromophenol blue, 0.13 $mol \cdot L^{-1}$ DTT) in a new plate. Fifteen minutes later, the stripes were removed and incubated in the new equilibration buffer (0.375 $mol \cdot L^{-1}$ Tris-HCl (pH 8.8), 6 $mol \cdot L^{-1}$ urea, 20% (V/V) glycerin, 2% SDS, trace amount of Bromophenol blue, 0.135 $mol \cdot L^{-1}$ indoacetamide) for 15 min .

2.3.3 SDS-PAGE

The IPG stripes were placed on top of the second dimension gels (12% SDS-PAGE) with great care and sealed with low melting-point agarose. The process was run according to the following steps: the first 20 min at 100 V followed by 200 V until the Bromophenol blue dye reached 0.5 cm from the bottom of the gels. Middle molecular weight protein standard was used as protein marker.

2.3.4 Coomassie staining

The gels were peeled off the plastic plate carefully and fixed in new trays with 40% alcohol and 10% acetic acid for 30 min. This step could precipitate and fix the protein into the gels. The gels were then incubated in the staining solution (0.116% Coomassie blue R-250, 25% alcohol and 8% acetic acid) over night and shaken slowly in the destaining solution (25% alcohol and 8% acetic acid) until the protein points appeared clearly.

2.4 Western blotting of CaM isoforms

After two-dimensional gel electrophoresis, the gel (8.5 cm

× 4.0 cm) was cut down with the center around the point of the molecular mass of 17 kDa and pI of 4.0. A piece of PVDF membrane (Millipore Immobion-P) was cut and wetted for about 5 min in methanol on a rocker at room temperature. The methanol was then removed and 1×Blotting buffer was added before its use. The gel was transferred onto the PVDF membrane for 1 h at 100 V in 25 mmol·L^{-1} Tris, 192 mmol·L^{-1} glycine and 20% (V/V) methanol at pH 8.3 using a mini-transblot system (Bio-Rad). After electroblotting, proteins were fixed with 0.2% (V/V) glutaraldehyde in TBS (10 mmol·L^{-1} Tris–HCl pH 7.5, NaCl 150 mmol·L^{-1}) for 50 min under constant agitation. The membrane was blocked with TBS containing 5% (V/V) BSA for 60 min, and incubated with primary antibody diluted in blocking buffer for 60 min at room temp. After washing in TBST (0.05% Tween-20 in TBS), the membrane was incubated for 1 h at room temperature with the secondary antibody diluted in Blocking buffer for 60 min at room temperature. After several washes in TBST and a final wash in TBS, immunodetection was performed with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

3 Results and analysis

CaM protein with 148 amino acids is a kind of small heat-resistant molecular acid protein with a molecular weight of 16.670 kDa and pI of 3.9 to 4.3. In order to separate the isoforms of CaM protein, 17 cm (pH 3–6) IPG stripes were selected for isoelectric focusing. The results showed that points gathered at pH 5–6. Few, if any, were at pH 3.5–4.5, according to the extraction method of dissolvable proteins with no heat step (Fig. 1). Considering the heat stability of CaM, we added the extraction solution into the tube, ground the samples and heated the supernatant at 92°C for 3 min to remove foreign matters and impure proteins. As shown in Fig. 2, some protein points appeared at pH 3–4. Furthermore, it was shown, by replacing trichloroacetic acid with acetone to precipitate proteins, that there existed more proteins at pH 3–4 (Fig. 3). The results above revealed that the two improved steps were necessary for 2D gel electrophoresis to study CaM. On the basis of confirming the ideal 2D gel electrophoresis system and western blotting, two protein spots (Fig. 4) had an apparent molecular mass of 16 kDa and pI of 4.0, which means two wheat calmodulin isoforms were detected.

4 Discussion

At present, the study of CaM isoforms is generally conducted using the specific antibody of CaM isoforms. In this paper, the technology of two-dimensional gel electrophoresis was used to separate wheat calmodulin isoforms, which is useful for further studies.

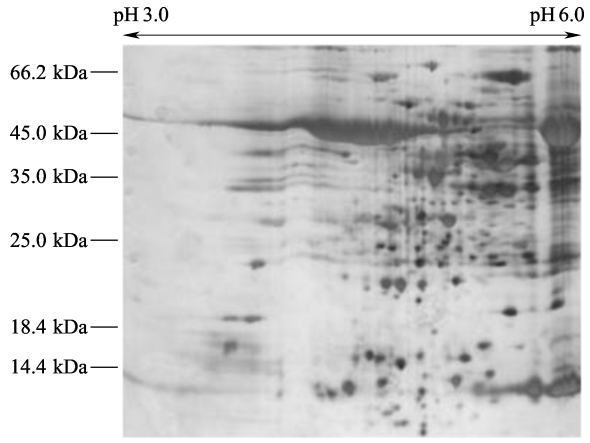

Fig. 1 2-DE result of no heat and precipitate with trichloroacetic acid

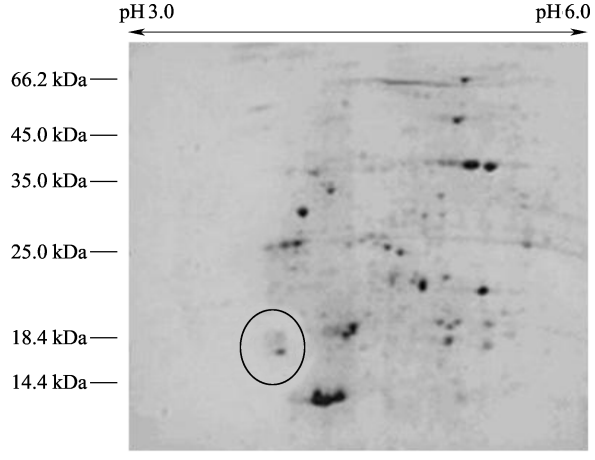

Fig. 2 2-DE result of heat and precipitate with trichloroacetic acid

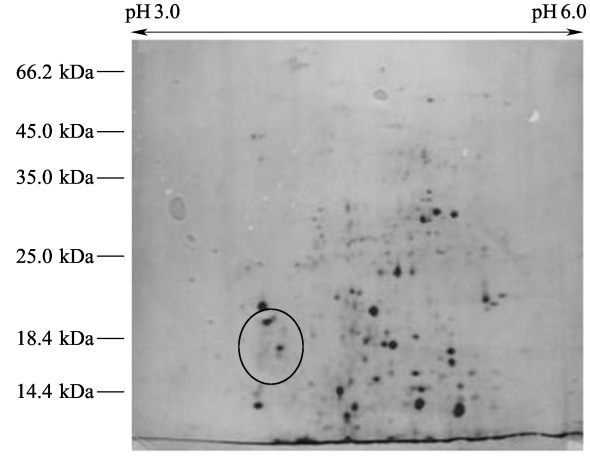

Fig. 3 2-DE result of heat and precipitate with acetone

The procedure of 2D gel electrophoresis is extremely complicated, as the result will be affected by many factors such as the protein extraction process, sample dissolution

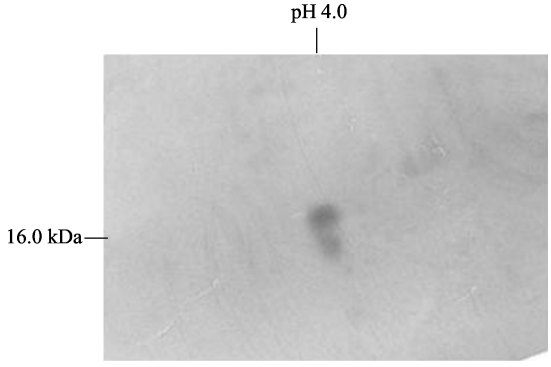

Fig. 4 Western blotting of CaM isoforms

and equilibration, and IPG selection. We approached a 2DE method to identify each isoform of plant CaM. As suggested above, the precipitation method could affect the acid protein distribution significantly. Compared with trichloroacetic acid, the acetone was more efficient in protein precipitation because there were more pH 3.5–4.5 points detected on the gel, whose molecular weight was diverse, from 14 to 20 kDa. The key might be redundant salt and acid radical ions. After being precipitated by trichloroacetic acid, salt ions might not be removed completely by acetone, and on the other side, many acid radical ions would maintain a lower pH value in extracted proteins that would not be dissolved because the pH value of the solution was near the pI. Based on these two reasons, the aim protein would not be identified in electrophoresis.

With the high thermotolerance of CaM considered, the protein was heated at 90–95°C for 3–5 min. Some foreign matter and impure proteins were denatured and precipitated from the solution. This measurement could facilitate the detection by not only elevating the resolution of the 2D electrophoresis, but also by enriching the CaM protein amount.

As reported, there are three types of calmodulin isoforms in wheat (Yang et al., 1996). However, only two isoforms were identified in the western blot analysis after 2D gel electrophoresis. Perhaps the calmodulin isoforms encoded by the cDNA of SF-1, SF-3 and SF-4 were similar in isoelectric point and molecular weight with one of the calmodulin isoforms encoded by the cDNA of SF-2, which caused the difficulty in separating the two isoforms. With narrow-ranged strips like pH 3.5–4.5 strip, the resolution can be increased.

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