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The use of two-dimensional gel electrophoresis in the analysis of organ-specific maize proteins

Two-dimensional gel electrophoresis with non-equilibrium pH gradient electrophoresis in the first dimension and sodium dodecyl sulfate-polyacrylamide gels in the second dimension has been used for the analysis of organ-specific proteins in maize. The method has been used to study the whole protein pattern of developing organs and adult leaves as well as protein patterns of *in vitro* translation. Examples of two-dimensional immunoblotting and *in vitro* translation of endosperm-specific proteins are also shown. Two-dimensional gel electrophoresis appears as an essential analytical step in the identification of organ-specific proteins and for the detection of the protein products related to organ-specific genes identified by other means.

1 Introduction

The detection of genes having an organ-specific expression is an interesting approach to the study of gene regulation. Promotor elements responsible for the organ-specificity of genes have been shown in plants [1] and they may be important to design experiments for plant transformation. Several approaches are possible to achieve the cloning of such genes. One is to employ the available cloning procedures to proteins that are known to have an organ-specific expression. This is the case, for instance, of storage proteins, exclusively present in seed tissues of the plant. A second approach is to use a differential screening approach on cDNA or genomic libraries by means of labelled cDNAs synthesized from mRNA extracted from different organs. A third approach is to study the protein pattern of specific organs and to attempt the cloning of polypeptides appearing in specific organs or developmental stages of the plant. The three approaches described have been employed in our laboratory and the use of two-dimensional (2-D) gel electrophoresis is an essential analytical tool in these experiments [2, 3].

In the first steps of the detection of organ- or stage-specific proteins the 2-D gel electrophoresis is the method having a resolution that may allow a large population of polypeptides to be separated and the appearance of specific spots to be observed. The use of *in vitro* translation and *in vivo* labelling is a necessary approach for cDNA cloning as the protein pattern obtained in these cases is more directly correlated with the population of mRNAs. Finally if antibodies are available, 2-D gel electrophoresis may be used to follow specific steps in the biosynthesis and processing of the protein. The method used in our laboratory, non-equilibrium pH gradient electrophoresis (NEPHGE), is based on the method of O'Farrell [4] as modified by Meyer and Chartier [5]. One disadvantage of the original isoelectric focusing (IEF) is the severe cathodic drift and consequent loss of basic proteins. Although in the NEPHGE system proteins are not strictly separated according to their isoelectric points, resolution of cathodic proteins is greatly improved. It is important to note that this method needs well-defined experimental conditions to have reproducible results, in particular when protein patterns from different organs are to be compared.

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Abbreviations: **2-D**, two-dimensional; **DTT**, dithiothreitol; **IEF**, isoelectric focusing; **NEPHGE**, non-equilibrium pH gradient electrophoresis; **SDS**, sodium dodecyl sulfate; **TCA**, trichloroacetic acid

2 Materials and methods

2.1 Plant material

Seedlings of *Zea mays* L. pure inbred line W64A were grown on soaked filter papers at 28 °C in darkness for 7 days. Roots and coleoptiles were used at this stage. Adult green leaves were collected from seedlings grown in the greenhouse for 30 days. Kernels were harvested 20 days after pollination or at maturity, and endosperms were isolated after removing the pericarp and embryo by hand. Protein bodies from endosperms 20 days after pollination were isolated as described [6]. Enriched suspensions of chloroplasts were obtained from 30-day-old green leaves following the method of Nobel [7]. Controls of the quality of the preparations were carried out by optical microscopy.

2.2 Extraction of proteins

Tissues were ground in liquid nitrogen and protein solubilized in the extraction buffer, containing 0.25 M Tris-HCl, pH 8, 0.4 % sodium dodecyl sulfate (SDS), 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 % β-mercaptoethanol, by mixing and boiling for 5 min. Debris was sedimented by centrifugation (12 000 *g*, 10 min) and the proteins in the supernatant precipitated with 15 % w/v trichloroacetic acid (TCA) at 4 °C for 30 min. The resulting pellet (400 *g*, 30 min) was washed several times, with methanol containing 0.4 M ammonium acetate, and vacuum dried. Chloroplast suspensions were precipitated with 15 % w/v TCA and processed as above. Protein content was assessed by a modification of the Lowry method [8] using bovine serum albumin as standard. Average protein yield in the extraction procedure for one gram of initial fresh weight of tissue was 10 mg for green leaves, 135 µg for chloroplasts, 7 mg for coleoptiles and 2.5 mg for roots.

2.3 Electrophoretic procedure

Protein samples were solubilized in 9.5 M urea, 2 % v/v Nonidet P-40, 100 mM dithiothreitol (DTT), 0.8 % carrier ampholytes (pH 3.5–10 and/or pH 6–9, Pharmacia) and 1 mM PMSF by incubation 1 h in an ultrasonic bath at 4 °C, and insoluble material removed by centrifugation (10 000 *g*, 5 min). Samples were loaded at the acidic end of the electrofocusing tube gels [9] which contained 3.7 % w/v acrylamide, 0.21 % w/v N,N'-methylenebisacrylamide, 9.15 M urea, 2 % v/v Nonidet P-40 and 2 % v/v carrier ampholytes (pH 3.5–10

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and/or pH 6–9) and covered with the overlay solution (8 M urea, 0.4 % v/v carrier ampholytes, 5 % v/v Nonidet P-40 and 5 % β-mercaptoethanol). Two different sizes of tubes were used: 2.5 × 100 mm and 1.5 × 75 mm, which were electrofocused at 100 V for 30 min followed by 200 V for 45 min, for large size gels by 300 V for 160 min and 500 V for 120 min, and for the smaller gels by 300 V for 75 min and 500 V for 60 min. The gel rods were equilibrated in 60 mM Tris-HCl, pH 6.8, 10 % v/v glycerol, 5 % v/v β-mercaptoethanol and 2 % w/v SDS for 30 min, and loaded onto SDS-polyacrylamide slab gels [10], 1.5 mm thick, of 16 × 16 cm (Protean, Bio-Rad) or 6 × 8 cm (Mini Protean Bio-Rad). Electrophoresis was carried out at a constant voltage (20 v/cm) until the front dye reached the end of the gel and subsequently the gels were silver stained [11], processed for fluorography or immunoblotted.

2.4 Immunoblotting

Following electrophoresis, the separated proteins were electrophoretically transferred for 2 h at 60 V at 10 °C to nitrocellulose membranes as described [12] by using a Bio-Rad Trans-Blot device. The filters were incubated with antisera raised against maize endosperm glutelin-2 and zein-1 fractions [13]. Antibody specificity was assessed with goat anti-rabbit peroxidase conjugate using *o*-chloro-1-naphthol as substrate.

2.5 Polyribosomes and poly(A$^+$)RNA isolation

Membrane-bound polyribosomes from endosperms 20 days after pollination were isolated as described [14]. RNA from coleoptiles was prepared according to Maniatis [15] and poly(A$^+$)RNA purification was carried out by oligo dT-cellulose (Pharmacia) chromatography [16].

2.6 *In vitro* translation

Polyribosomes and poly(A$^+$)RNA were *in vitro* translated in a wheat-germ cell-free system (Amersham). Incubation mimixtures of 30 µL contained 15 µL of wheat-germ extract, 150 mM potassium acetate, 0.2 mM unlabeled amino acids, 500–1000 µCi/ml L-[^{35}S]methionine or L-[^{35}S]cysteine (Amersham) and 10–15 A_{260} units/mL polysomes or 0.8 µg poly A$^+$ RNA. Maximal incorporation at 25 °C was attained after an incubation period of 60 min. Incorporation of radioactive amino acids into proteins was determined [17] and the remaining reaction mixture was analyzed by SDS polyacrylamide gel electrophoresis or 2-D electrophoresis.

3 Results and discussion

One of the first steps in detecting genes with an organ-specific expression may be to observe proteins specifically present in the tissue of interest. We first analyzed total protein samples from maize organs and compared the patterns of coleoptile and root (Fig. 1). Total proteins were extracted with low-salt buffer in the presence of SDS and a reducing agent and were visualized by silver staining. The patterns have a high proportion of proteins characteristic of dividing tissues, which are common to both extracts. In addition, at this developmental stage (7-day-old seedlings) tissue-specific spots are also apparent. The proteins of adult leaf (Fig. 2) consist of a protein population distinct from that of young coleoptiles (Fig. 1A). Some of the differences in the protein pattern of different tissues may be due to posttranslational modification of the polypeptides or to proteins present in organelles such as chloroplasts. The proteins from chloroplasts can easily be identified in the extracts of leaf tissue (Fig. 2B). The pattern of enriched chloroplast suspensions shows a limited number of

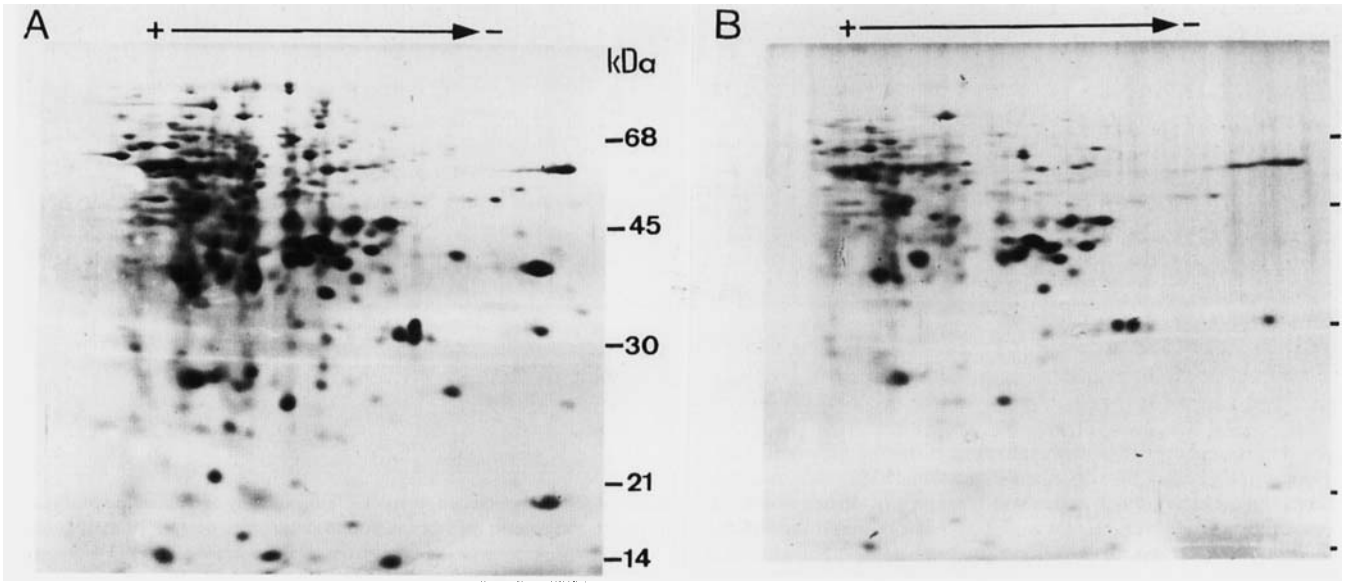

Figure 1. 2-D pattern of total protein extracts of maize (A) coleoptiles and (B) roots. Separation was carried out in a minigel apparatus (Bio-Rad) in pH 3.5–10 range carrier ampholytes in the first dimension and 15 % acrylamide for the SDS-polyacrylamide gel of the second dimension. Proteins were visualized by silver staining. Total protein load was 6 µg (A) and 5 µg (B).

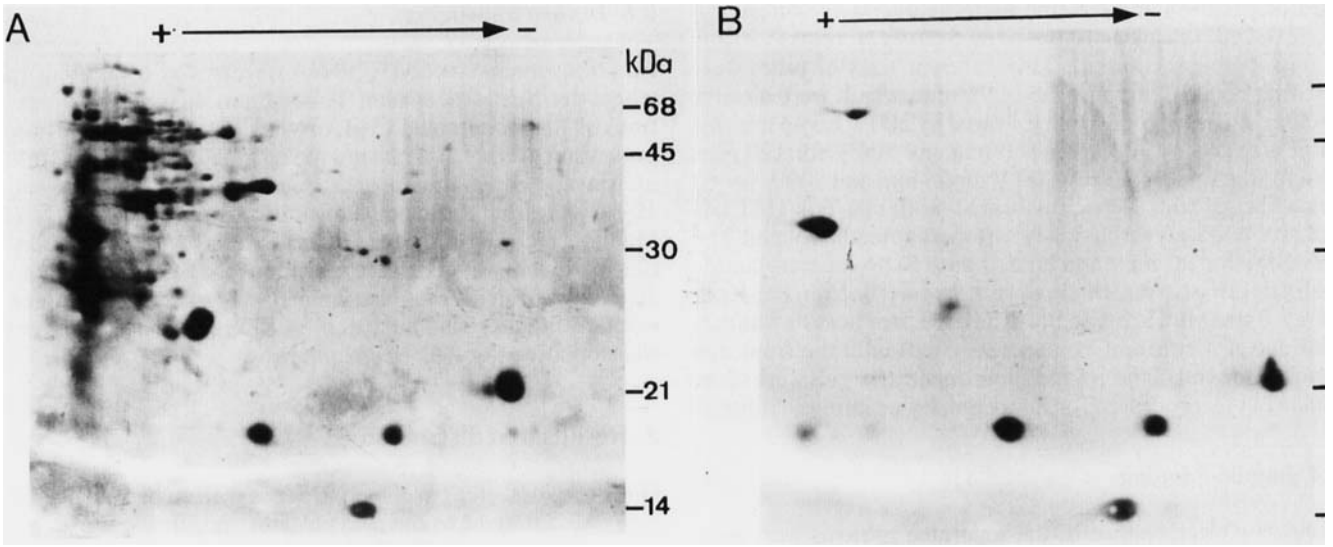

Figure 2. 2-D pattern of protein extracts from (A) green leaves and (B) enriched chloroplasts suspension. Electrophoretical conditions the same as in Fig. 1. Total amount of protein loaded was 8 μg (A) and 4 μg (B).

spots, corresponding to the most abundant polypeptides of the organelle. However, a differential loss of stromal proteins in the medium during isolation steps cannot be excluded. An example of proteins where the 2-D electrophoretical pattern has been useful to identify and clone a cDNA corresponding to a gene with interesting regulatory features is for abscisic acid-induced proteins in maize embryo [3]. In this case a group of polypeptides was detected in the *in vivo* and *in vitro* pattern of proteins synthesized in defined stages of maize embryo development. These proteins were shown to be phosphorylated [18]. The approach to clone a cDNA related to the polypeptides was to screen a stage-specific cDNA library with labelled cDNA synthesized from RNA having the

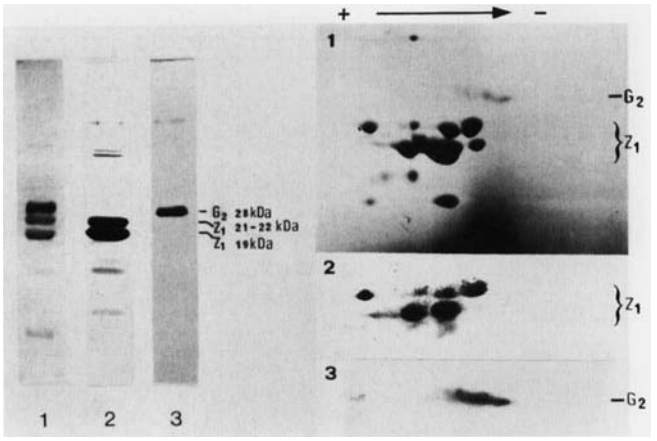

Figure 3. Electrophoresis and immunoblotting of storage proteins from endosperm protein bodies. On the left one-dimensional SDS-polyacrylamide gel electrophoresis of protein bodies. (1) Total protein stained with Coomassie Brilliant Blue. (2) Proteins immunodetected with antisera anti-zein-1 (dilution 1:1000). (3) Protein immunodetected with antisera anti-glutelin-2 (dilution 1:400). On the right 2-D electrophoresis analysis of the same samples. Carrier ampholytes pH 3.5–10 and pH 6–9 were used in identical proportions in the first dimension and 12.5 % acrylamide in SDS-polyacrylamide gel electrophoresis.

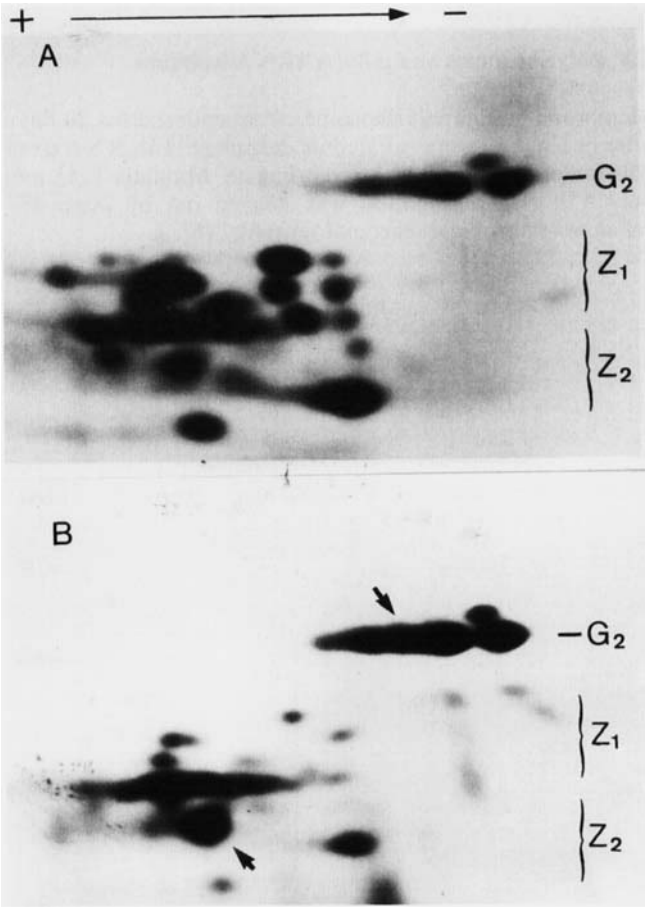

Figure 4. 2-D gel electrophoresis and fluorography of translation products of maize endosperm membrane-bound polysomes using [^{35}S]methionine (A) and [^{35}S]cysteine (B) as precursors. Carrier ampholytes pH 6–9 were used in the first dimension and 12.5 % acrylamide in the second dimension. The positions of endosperm maize storage proteins are indicated on the right. The arrows show the 28 kDa G2 and 16 kDa Z2 polypeptides enhanced by using [^{35}S]cysteine as radioactive precursor.

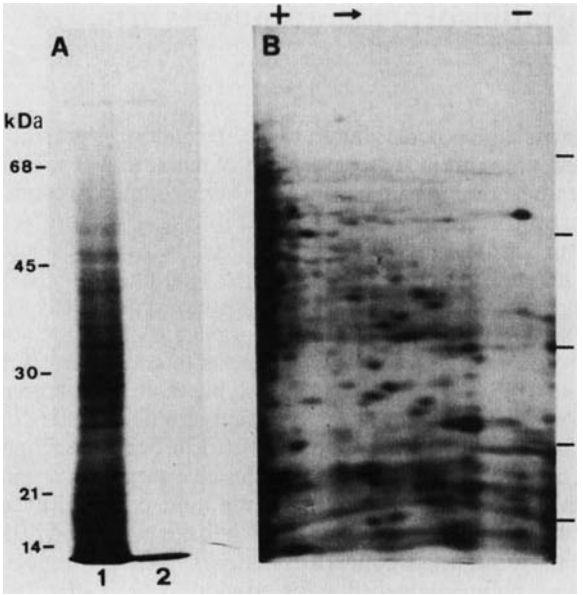

Figure 5. In vitro translation of poly(A⁺)RNA from maize coleoptiles. (A) SDS-polyacrylamide gel electrophoresis (12.5 % acrylamide) and fluorography of total translation products precipitated with 20 % w/v TCA (70 000 cpm). (B) 2-D electrophoresis of the same sample (200 000 cpm). Carrier ampholytes pH 3.5–10 were used in the first dimension and 12.5 % acrylamide in the second dimension. Positions of molecular weight markers are indicated. [^{35}S]Methionine was used as labeled precursor.

size that may be deduced from the molecular weight of the proteins. This approach was possible in part due to the fact that the proteins had a molecular weight range (between 25 000 and 28 000) that was relatively free of other proteins in maize embryo [3].

One of the groups having a clearer pattern of organ-specificity in cereals are the proteins present in endosperm. In maize two main groups of proteins are present in the seed: zeins, the maize prolamins, and glutelins. In one-dimensional gels the protein pattern is simple, consisting of three main bands with molecular weights between 19 000 and 28 000 and with less abundant bands at 10 000 and 14 000–16 000. The use of 2-D gel electrophoresis, either using IEF in the first dimension or urea-Triton [19] results in a pattern of about 20 reproducible spots. When antibodies against zeins or glutelins are raised in rabbits, these two groups of proteins can be distinguished [13]. One-dimensional and 2-D gels of endosperm proteins have been blotted and incubated with anti-zein and anti-glutelin antibodies against the two groups of polypeptides (Fig. 3). The glutelins appear as elongated spots, probably due to the poor resolution of these proteins in the IEF gels. Another interesting feature of these proteins is their different relative content in sulfur-containing amino acids. *In vitro* translation of endosperm membrane-bound polysomes using either [^{35}S]methionine or [^{35}S]cysteine as precursors produces a different pattern due to the relatively high content of cysteine in glutelins and in low molecular weight zeins (Fig. 4). This may also be a means to distinguish proteins having similar electrophoretic properties.

In general, *in vitro* translational products of poly(A⁺)RNA have a clearer pattern of organ-specific proteins. This may be due either to the high proportion of constitutive proteins having a low turnover in the different organs or to the higher resolution of 2-D gels in the absence of non-protein contaminants (Fig. 5). This is also an important control of the quality of the RNA prior to the construction of cDNA libraries. In conclusion, 2-D gel electrophoresis is an essential tool for the analysis of organ-specific protein patterns in plants. The NEPHGE method used shows a good resolution of the proteins, especially in the cathodic regions. In addition, it has the advantage of simple handling and fast separation. An added interest in this methodology is that the 2-D pattern shows an adequate resolution to attempt protein purification. The method is well suited for an analysis of *in vivo* or *in vitro* translation polypeptides and can also be used in combination with immunological detection of proteins with specific antisera. Thus, the 2-D gel analysis of organ-specific maize proteins may prove an invaluable approach prior to cloning.

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