Plant Science, 53 (1987) 215–221
Elsevier Scientific Publishers Ireland Ltd.

ABSENCE OF STORAGE PROTEIN SYNTHESIS IN THE EMBRYO OF *ZEA MAYS*

DEMETRIO SÁNCHEZ-MARTÍNEZ, JORDI GÓMEZ, M. DOLORS LUDEVID, MARGARITA TORRENT, PERE PUIGDOMÈNECH and MONTSERRAT PAGÈS*

Departamento de Genética Molecular, Centro de Investigación y Desarrollo, CSIC, Jorge Girona Salgado, 18, 08034 Barcelona (Spain)

(Received June 2nd, 1987)
(Revision received July 31st, 1987)
(Accepted August 31st, 1987)

Polypeptides were identified in the total protein extract from isolated maize embryos having a similar behaviour of endosperm zeins and glutelins by means of two-dimensional gel electrophoresis. They reacted with antisera ellicited against the same proteins as seen by immunoblotting. In contrast, mRNAs coding for these proteins were not identified in the poly(A +) or poly(A −) RNA fractions prepared from embryo tissues by Northern hybridization experiments or immunoprecipitation of the in vitro translated products. 'In situ' hybridization experiments with zein cDNA clearly show that the corresponding mRNAs are confined to the endosperm and they are not present in the embryo tissues. Possible hypotheses to explain these results are discussed.

Key words: glutelins; immunoblotting; 'in situ' hybridization; Northern blot analysis; *Zea mays* embryo; zeins

Introduction

The most abundant proteins in maize seeds are zeins and glutelins, which together account for more than 75% of the total endosperm proteins in normal varieties, and they are supposed to have a protein storage function. There are different reports concerning the possibility of storage protein biosynthesis occurring in embryo tissues, as significant amounts of these proteins were extracted from embryos at different stages of development [1–3]. Values reported for the ethanol-soluble protein fraction in normal isolated embyros of *Zea mays* range from 2 to 10% [2,4]. However, when comparing the putative zein components of embyro to those from the endosperm, differences have been observed in the relative intensities of the electrophoretic bands and in the amino acid composition with respect to the endosperm counterpart [4,5]. From a qualitative point of view the identification of zeins in the total seed using an immunocytochemical approach has shown that zein proteins are confined to endosperm tissues and that they are not detected in the embryo [6]. However the possibility that zeins could not be observed due to the large amounts of oil existing in the embryo cells cannot be discarded.

In this report we have identified in the total protein complement of isolated embryos, polypeptides similar to those of endosperm zeins and glutelins by means of two-dimensional gel electrophoresis and immunoblotting with antibodies raised against zeins and glutelins. However in the Northern blot analysis of the poly(A +) RNA or total RNA extracted from embryos at different stages of development, no hybridization was observed with cDNA for zeins or glutelins. No positive result was obtained when the in vitro translation product from embryo poly(A +) RNA was immunoprecipitated with the zein or glutelin antibody. 'In situ' hybridization studies were also performed using a chemically modified probe of zein cDNA. The distribution of staining on cryostat sections of immature maize

*To whom correspondence should be sent.

0618-9452/87/$03.50

seeds clearly shows that zein mRNAs are confined to the endosperm and they are not present in the embyro tissues.

Materials and methods

Plant material and protein extraction and detection

Inbred line W64A (*Z. mays* L.) seeds were harvested at different developmental stages and stored at −80 °C. Embryos from both mature and immature seeds were carefully dissected by hand. The epithelium was removed and a control of the presence of endosperm remainings were carried out in selected embryos by observation in the light microscope. The stages used for the present study were E20 (immature seeds 20 days after pollination), E35 (35 days after pollination) and D0 (dry embryos). Five to ten embryos were ground with a mortar and a pestle in the presence of liquid N_2. The resultant powder was extracted and the proteins resolved in two-dimensional gel electrophoresis as described [7]. The proteins, separated in polyacrylamide gels, were transferred to nitrocellulose filters (BA85, Schleicher and Schuell) by means of a Trans-Blott device (BioRad), incubated with the antibodies and detected with ^{125}I-Protein A [8].

RNA preparation and analysis

Extraction of RNA [7] and purification of poly(A +) RNA was carried out by oligothymidylic acid-cellulose (Pharmacia PL Biochemicals) chromatography [9]. Translation of poly(A +) RNA was carried out by oligothy- (Amersham) [7] or wheat germ extract (Amersham) [14] was followed by immunoprecipitation, SDS polyacrylamide gel electrophoresis and fluorography. The analysis of RNA complementary to zein and glutelin cDNA clones was done by Northern analysis [10]. Filters were hybridized to nick-translated cDNA inserts and washed as described [11].

'In situ' hybridization

Frozen sections (8 μm) of embryos incubated with a 4% freshly made paraformadehyde solution for 20 min were treated as described by Haffen et al. [12] using proteinase K (0.2 mg/ml) for 10 min instead of pronase in order to better preserve the morphology of the endosperm tissues. The sections were subsequently incubated at 35 °C for 16 h in a mixture containing 50% formamide (v/v), 10% dextran sulfate (w/v), 1 × Denhart, 2 × SSC, 2 mg/ml yeast RNA and DNA (2 μg/ml) modified according to the Chemiprobe (Orgenics, Inc.) protocols. Washing was performed for 16 h with 50% formamide in 2 × SSC at 30 °C with 4 changes of buffer every 4 h. The hybridization was visualized with the use of a monoclonal antibody against the modified DNA as indicated by the manufacturer.

Results and discussion

Reaction of anti-zein antisera with embryo proteins

Antisera elicited in rabbits against the glutelin-2 fraction and the whole zein fraction from maize flour [13] were used. No reaction was observed with the antibodies with total protein extracts from maize leafs or roots. Total proteins from young embryos (20 days after pollination, E20) and dry embryos (D0) were extracted and analyzed by two-dimensional gel electrophoresis using electrophocusing (pH range 3.5 − 10) for the first dimension and SDS electrophoresis for the second one. The immunoblot from these two-dimensional gels shows that the antiserum reacts with proteins having molecular weight and isoelectric point similar to the values reported for zeins and glutelins in the endosperm [14]. In Fig. 1 the polypeptides recognized by the anti-zein serum in E20 (A) and D0 (B) embryos are shown.

Experiments of 'in vivo' and 'in vitro' labelling of polypeptides synthesized in excised maize embryos [7] failed to detect the polypeptides observed by immunoblotting with the anti-zein antiserum. In the 'in vitro' trans-

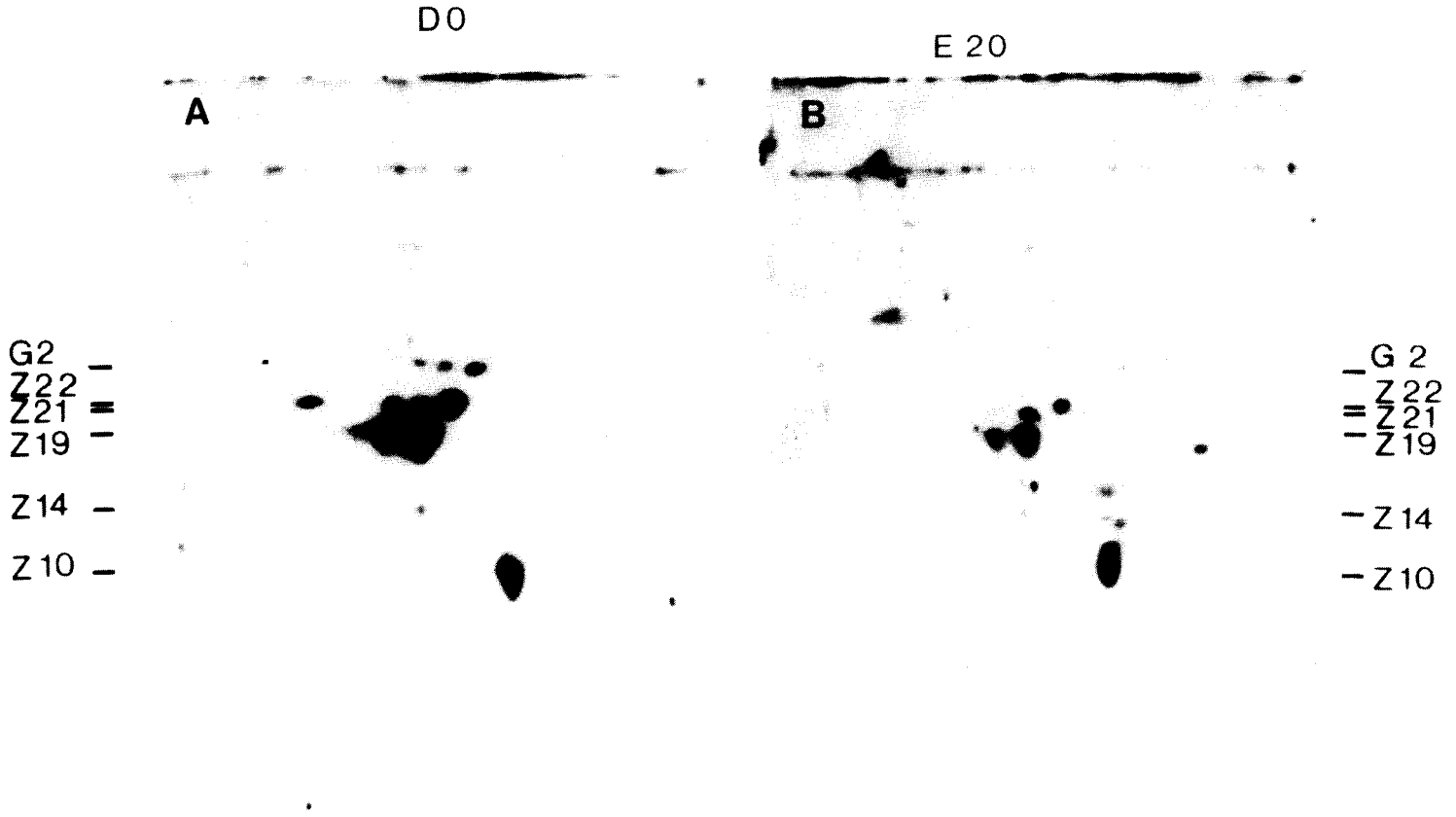

Fig. 1. Reaction of different embryo proteins with anti-zein sera assayed by immunoblot. Porteins extracted from D0 (A) and E20 (B) embryo were analyzed by two-dimensional electrophoresis, transferred to nitrocellulose filters and incubated with anti-zein sera. Filters were developed with ^{125}I-Protein A and autoradiographed. Isoelectric focusing was run in the first dimension (pH 3 to 10) and SDS electrophoresis in the second dimension (top to bottom). The position of the main endosperm proteins indicated in the margin of the gels. G2, 28 kD glutelin-2; Z, zeins, according to their molecular weight.

lation of (Poly(A +) RNA from dry embryos it was possible to detect 5 polypeptides running at a molecular weight similar to zeins, however, none of them coincides with storage proteins in isoelectric point. The proteins recognized by the antibodies and extracted from the embryo can be considered either as constituted by specific embryo proteins or by zeins and glutelins originating from contaminating endosperm tissue.

Northern hybridization studies

Using cloned cDNAs corresponding to endosperm zeins (clone A20, Ref. 15, kindly provided by Drs. F. and B. Burr, Brookhaven) and glutelins (clone pME119, Ref. 16) as probes we have investigated whether homologous mRNA sequences were present in the embryo tissues at different stages of development. The glutelin clone pME 119 was identified from a maize endosperm cDNA library by using anti-glutelin antibodies and it selected RNA that directed the synthesis of polypeptides having the same electophoretic mobility of those immunoprecipitated by the same antibodies [16]. As shown in Fig. 2 the glutelin (A) and zein (B) probes hybridized strongly to RNA samples isolated from endosperm but no hybridization was detected with poly(A +) or poly(A −) RNA (not shown) isolated from embryo tissues. Immature embryo 20 days after pollination were analysed because this is the period where the maximum abundance of mRNA coding for zeins and glutelins [11] have been detected in the endosperm while mature and dry seeds represent stages of maximum protein accumulation [8]. RNA species homologous to endosperm zeins or glutelins were detected neither in 20 days after pollination embryos nor in mature and dry stages. As zeins were derived from a large and heterogeneous multigene family, it is possible that the proteins detected by the antibody in Fig. 1 do not correspond to the translation products of mRNAs that would hybridize to the cDNAs probes used. However a similar result was obtained when poly(A +) RNA from young (E20) and dry embryos (D0) was translated in vitro. Translation products were examined by immunoprecipitation with anti-glutelin IgG and with anti-zein antiserum and resolved by electrophoresis (Fig. 2C and D). No polypeptide synthesized from embryo RNA comigrates with the products from endosperm RNA when the polypeptides are immunoprecipitated with the same antisera [14]. These results suggest that zeins and glutelins are not represented in the poly(A +) population of RNAs extracted from isolated young or dry embryos.

'In situ' hybridization studies

As we were unable to detect hybridization of the zein cDNA to mRNA isolated from immature or dry embryos using Northern analysis we performed 'in situ' hybridization experiments to frozen sections of total seeds 30 days after polination in order to assess whether hybridization was restricted to some defined cells in the embryo tissues where zein mRNA accumulates. The mRNA was hybridized with a chemically modified cDNA probe and subsequently detected using a specific antibody raised against modified DNA and visualized with the Chemiprobe Kit (Orgenics, Inc.) staining.

A representative result of the 'in situ' hybridization experiments is shown in Fig. 3. A cryostat section containing endosperm and scutellum is presented. The areas reacting with the zein cDNA probe appear in black in the photograph. In the original figure a red-brown staining was distributed throughout the endosperm cell layers with different intensity. The staining was more intense in the periphery of the endosperm than in the center. The aleurone and embryo cells were unstained. Control experiments demonstrated that the hybridization observed represents specific binding to RNA. Acridine orange staining was used to demonstrate that RNA was preserved in pre-hybridized sections in embryo and scutellum. Preincubation of replicate section with Ribonuclease A completely abolished the orange staining. RNase pre-

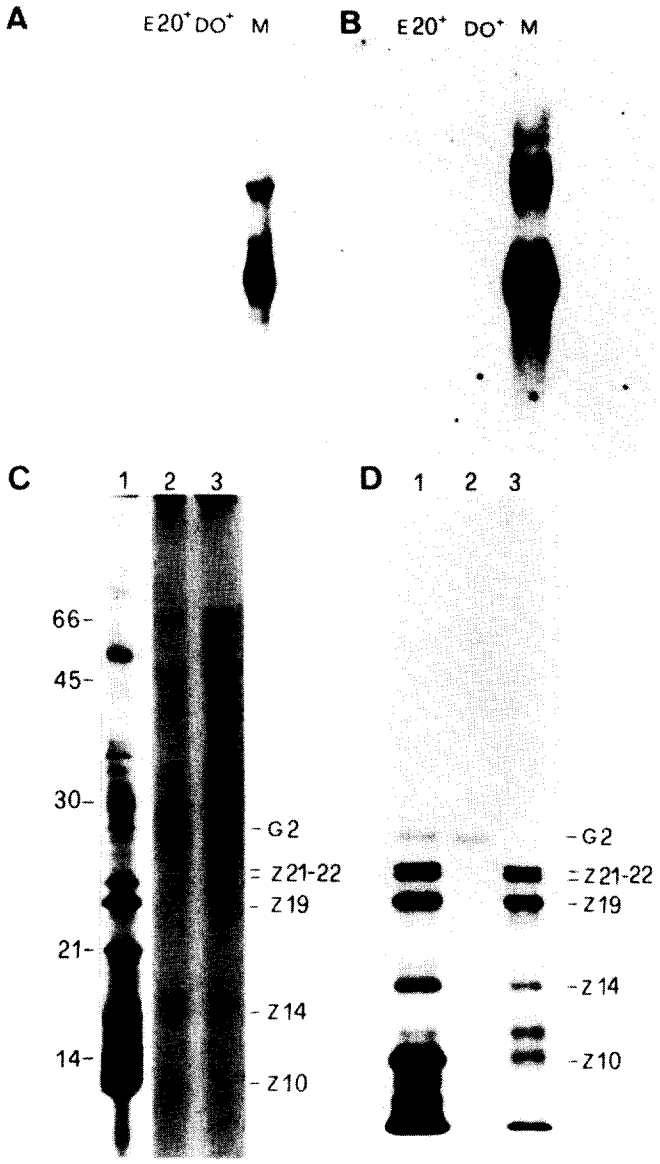

Fig. 2. Northern-blot analysis (A,B) and in vitro translation (C,D) of RNA poly(A +) from young (E20), dry embryos (D0) and endosperm (M). Filters were hybridized with cDNA inserts from glutelin-2 (pME 119) clone (A) and from zein A20 clone (B). 0.5 μg of polyA+ RNA were loaded in all filters. In vitro translation of RNA polyA+ from dry embryos (C) and immature endosperm (D). (1) Total translation products. (2) Immunoprecipitation products using anti-glutelins antibodies. (3) Immunoprecipitation products using anti-zein serum. Molecular weight markers, and position of glutelin-2 and zein polypeptides were indicated.

treatment of the tissue sections before hybridization as a control for nonspecific binding of the cDNA probe to molecules other than RNA, prevented hybridization.

Previous reports have established that zeins and glutelins are present in the global protein pattern of *Zea mays* embryo. While our results confirm this, we furthermore have

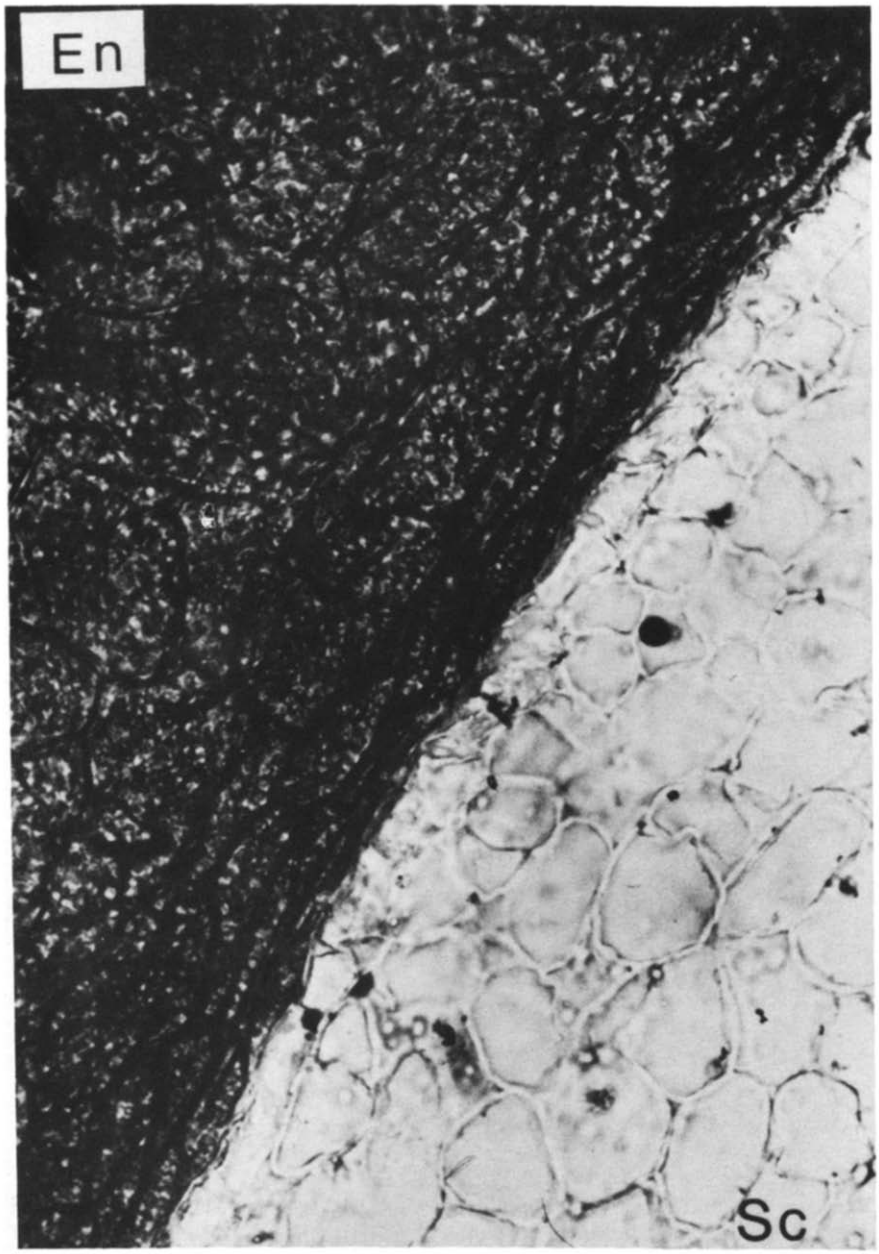

Fig. 3. Detection of zein mRNA by 'in situ' hybridization with chemically-modified cDNA and subsequent reaction with a monoclonal antibody against the modified DNA. Specific reaction was distributed throughout the endosperm (En) but the embryo axis and scutellum (Sc) were unstained.

produced evidence that at least under the conditions which were used, no detection is observed of any transcripts corresponding to zein or glutelin mRNA in the total RNA or poly(A+) RNA extracted from embryos at different developmental stages. There are several possible explanations for the presence of zeins and glutelins in the embryo. One could be the existence of a low level of gene expression for zeins and glutelins in the embryo tissues. In this case, the levels of specific mRNAs could be under the limit of detection for Northern or 'in situ' hybridization experiments. One has to consider the possibility of an extrinsic origin of these proteins in the embryo. This could be due to the existence of a transport system for some proteins and peptides through the vascular system of the seed. In this way, proteins arising from the mature endosperm cells could be mobilized and they eventually reach the embryo. However the most likely explanation is contamination, in fact the sensitivity of the immunoblotting method employed is such that a contamination with endosperm protein of only 1% of the total tissue would account for the amount of protein that we observe in the embryo.

Acknowledgements

The authors are indebted to Dr. Blanca San Segundo for the chemically-modified probe and to Consejo Superior de Investigaciones Científicas (grant 618/500) for continuous financial support.

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