Tissue-specific immunofluorescent localization of zein and globulin in *Zea Mays* (L) seeds

Christa Dierks-Ventling and Derek Ventling

Friedrich Miescher-Institut, P.O.Box 273, 4002 Basel, Switzerland

Received 25 May 1982

Zein *Globulins* *Antibodies* *Thin sections* *Immunofluorescence* *Tissue specificity*

1. INTRODUCTION

Zein is the most abundant storage protein of maize seeds, and is made in the endosperm where it is deposited inside vesiculate protein bodies of 1–2 μm diam., as characterized by electron microscopy [1,2]. Most investigations dealing with kinetic aspects of zein synthesis utilize seeds of < 25 days after pollination. While the endosperm is almost fully developed at this time, the embryo is still very small. The expression of the zein genes has therefore been assumed to be limited to the endosperm. The possibility of zein biosynthesis occurring in embryos has in fact not been contemplated until significant amounts of zein were extracted from mature embryos [3,4]. This raised the question of whether zein was also synthesized in the embryos. Tissue-specificity is critical when studying developmental regulation of zein or hormonal control at a molecular level.

As we had raised antibodies against zein, we investigated zein localization by employing an immunofluorescent technique on thin sections of whole maize kernels:

(i) We needed to establish that anti-zein would recognize no other endosperm or embryo protein but zein;

(ii) As control we required an antibody against a protein present in both endosperm and embryo, but non-crossreacting with zein.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered physiological saline solution

We were able to fulfill these conditions: our anti-zein IgG only reacted with zein and the anti-globulin IgG we produced with either endosperm or embryonal globuline as antigens did not cross-react with zein. Here we demonstrate that zein is confined to endosperm tissue and is not present in embryos.

2. MATERIALS AND METHODS

2.1. *Plant material*

Normal and opaque-2 maize (*Zea mays* L.) seeds of the genotype W 64A were planted in a greenhouse in 1980. Plants were self-pollinated and ears were harvested at 40 days after pollination and at maturity (70 days after pollination). The kernels from 40 days after pollination were processed for the immunofluorescent studies immediately after harvesting. Mature kernels were used for the extraction of zein and globulins.

2.2. *Isolation of zein and globulin*

Zein was extracted from mature W 64A normal endosperm (yield: 10 mg/endosperm), globulins were extracted mainly from opaque-2 seeds because of their enrichment in globulins (yield: 2 mg/opaque-2 endosperm *vs* 1 mg/normal endosperm). The exact procedure for selectively extracting zeins and globulins from mature endosperms after dissecting the embryo has been described in [5]. Globulins were also extracted from mature embryos after a 4-h soaking of the kernels in ice-cold distilled water containing protease inhibitors [6]. The soaking was necessary to assure removal of embryos without contamination by endosperm. Embryos were ho-

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

mogenized in dry acetone (10 ml/10 embryos) and then selectively extracted for globulins as in [6]. The yield was of the order of 1 mg/embryo from either normal or opaque-2 source.

2.3. *Preparation of antibodies against zeins and globulins*

Antibodies against zein and globulins were produced in rabbits. Injected antigens were:

(i) zein (ICN Pharmaceuticals, Cleveland OH);
(ii) endosperm globulins from W 64A normal and opaque-2 kernels;
(iii) embryo globulins from W 64A normal and opaque-2 kernels.

Each type of storage protein was injected into 2 rabbits using the protocol in [7]. IgG fractions were purified from the antisera according to [8] and after lyophilization stored dry. Testing of the IgG fractions was done on immobilized antigens as below.

2.4. *SDS–PAGE of zein and globulins*

SDS–PAGE was done in vertical slab gels of 15% polyacrylamide –1% SDS containing 6 M urea with 100 μg globulins (or 50 μg zein)/track for 16 h at room temperature at a constant 50 V as in [9]. Gels were stained with Coomassie blue R250 for 4 h and destained electrophoretically for 15 min at 3 A in 5% methanol–7.5% acetic acid as detailed in [5].

2.5. *Immunochemical reactions of zein and globulins*

Immunochemical reactions were done on proteins immobilized on nitrocellulose sheets after SDS–PAGE. For this purpose zein or globulins (1 mg) were placed across the entire gel omitting the comb, submitted to electrophoresis as above and without fixing, electrophoretically transferred to nitrocellulose sheets as in [10]. The sheets were cut into 5 mm-wide strips which, containing identical protein bands, were incubated with anti-zein IgG or anti-globulin IgG first, then with swine anti-rabbit IgG conjugated to horseradish peroxidase exactly as in [7]. The antigen–antibody reaction was visualized by the blue color produced by the peroxidase catalyzed oxidation of 4-chloro-1-naphtol. Controls were done with IgG fractions purified from non-immunized rabbit sera.

2.6. *Preparations of thin sections from total maize kernels and immunofluorescent staining*

Freshly harvested W 64A normal or opaque-2 maize seeds (40 days after pollination), placed in distilled water at 4°C overnight, were fixed in periodate–lysine–paraformaldehyde according to [11], frozen in liquid nitrogen and stored at – 70°C until used. For sectioning the frozen seeds were mounted in Tissue Tek II (Miles Labs., Naperville IL), 10 μm sections were cut in a cryostat and the sections placed on gelatine-coated microscope glass slides. Sections were incubated with anti-zein or anti-globulin IgG and then with rhodamine-conjugated goat anti-rabbit IgG (Nordic Immunological Laboratories, Netherlands) as in [12]. All antibodies were used as 1% solutions in PBS. Sections were viewed and photographed in a Zeiss fluorescent Universal microscope with a M 35 camera attached.

3. RESULTS

3.1. *Characterization of zein*

Fig.1 shows commercial ICN zein consisting of 2 polypeptides of 19 000 and 22 500 M_r. As these M_r-values correspond to those of zeins from genetically established genotypes [2,13] this material, being readily available, was considered equally as well-suited as W 64A zein for the production of antibodies in rabbits and was used. Furthermore, ICN preparation of zein had been employed for the preparation of antibodies in rabbits with excellent results [14].

3.2. *Characterization of globulins*

Globulins can be resolved into several polypeptides on SDS–PAGE: ≥ 12 major polypeptides ranging from ~ 10 000–80 000 M_r are visible (fig.2). Globulins from endosperm and those from embryos do not have the same subunit composition although many polypeptides of similar M_r-values exist (cf. tracks 1 and 2). The patterns presented here belong to globulins from opaque-2 mutants; those obtained from normal seeds are alike and are therefore not shown.

3.3. *Immunochemical reactions of zein and globulins: Non-crossreactivity of zeins and globulins*

Zein as antigen immobilized on nitrocellulose was reacted with anti-zein IgG and produced 2 blue bands corresponding to the polypeptides obtained by SDS–PAGE (fig.3, cf. fig.1). No bands appeared when IgG from non-immunized rabbits (= normal IgG) was used or when the anti-zein IgG was reac-

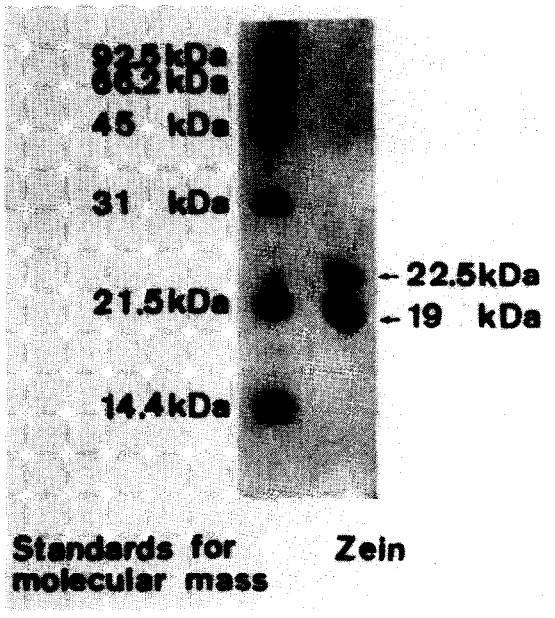

Fig.1. SDS–PAGE of zein: The zein was of commerical origin as in section 2; zein purity expressed polypeptides of 19 000 and 22 500 M_r. Zein from normal W 64A maize gave identical results.

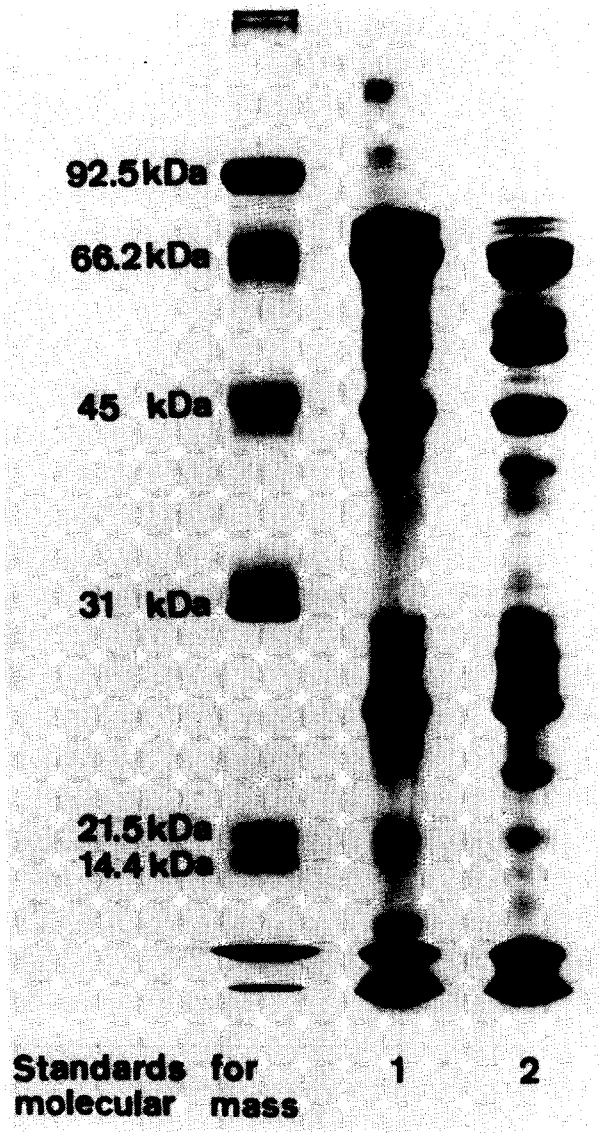

Fig.2. SDS–PAGE of mature maize W 64A opaque-2 endosperm and embryo globulins (tracks 1 and 2, respectively); for details see section 2. On each track 100 μg protein were loaded.

ted with globulins from either endosperm or embryo. Anti-globulin IgG were produced from antigens extracted from normal and opaque-2 endosperm and from normal and opaque-2 embryos. Although all 4 IgG preparations crossreacted with each other, for the sake of clarity only the results from opaque-2 kernels are shown here. However, neither anti-globulin IgG preparation reacted with zein (fig.3).

3.4. *Immunofluorescence of thin sections of whole maize kernels*

Having established non-crossreactivity of anti-zein IgG with globulins or anti-globulin IgG with zein, we proceeded to localize zein within tissue sections. When the staining of the sections was with anti-zein IgG, only the endosperm was labelled (fig.4A). The staining was exclusively between starch granules which corresponds to the distribution of protein bodies. In sections stained with anti-globulin IgG both endosperm and embryos became labelled (fig.4B).

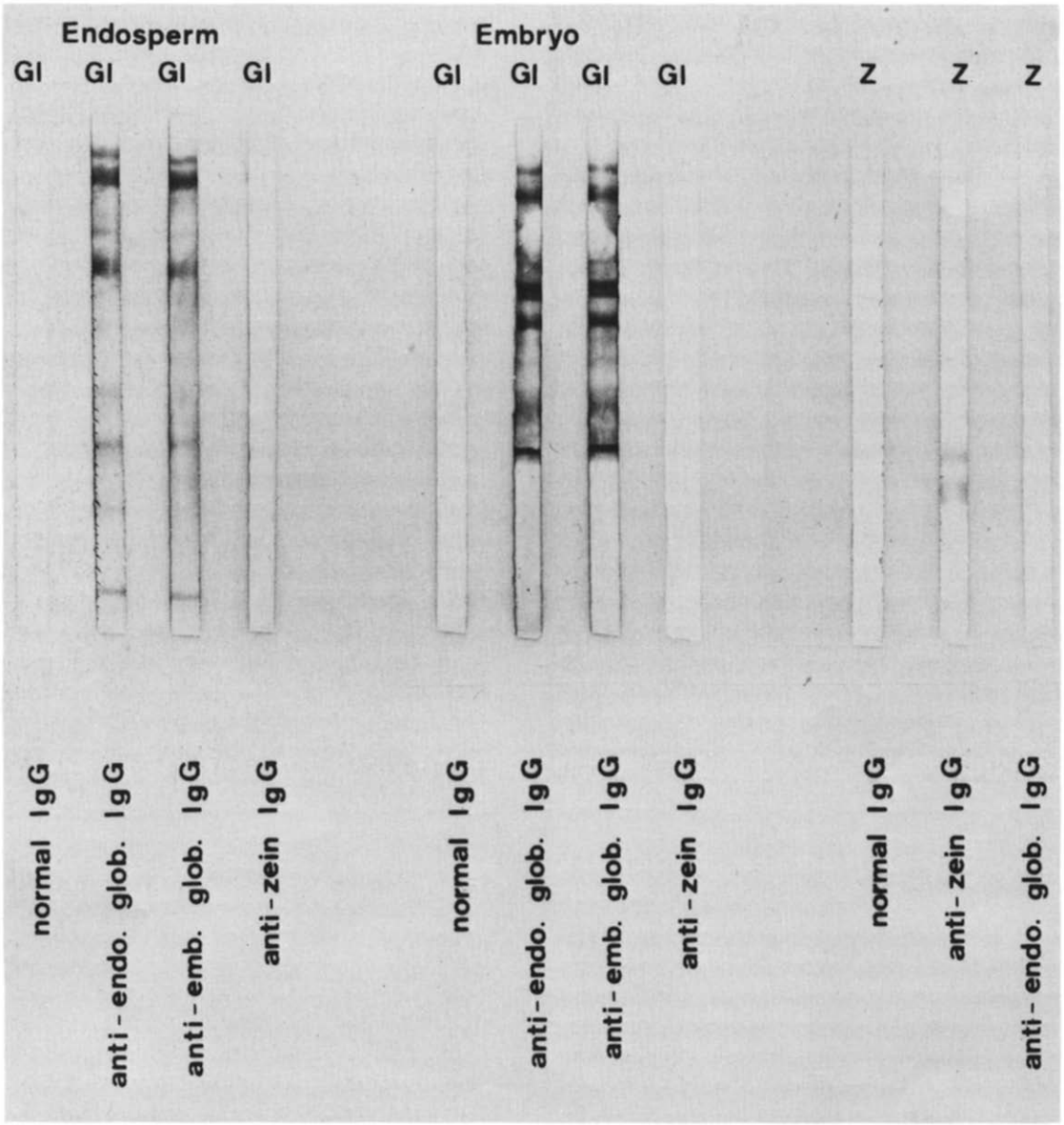

Fig.3. Immunochemical antigen–antibody reactions. Endosperm and embryo globulins (Gl) from W 64A opaque-2 seeds and zeins (Z) were separated by SDS–PAGE into individual polypeptides, then blotted on nitrocellulose sheets as in section 2. Nitrocellulose strips containing Gl or Z antigens were reacted with anti-(endosperm) globulin IgG or anti-(embryo) globulin IgG or anti-zein IgG. Controls were done with rabbit IgG from non-immunized animals.

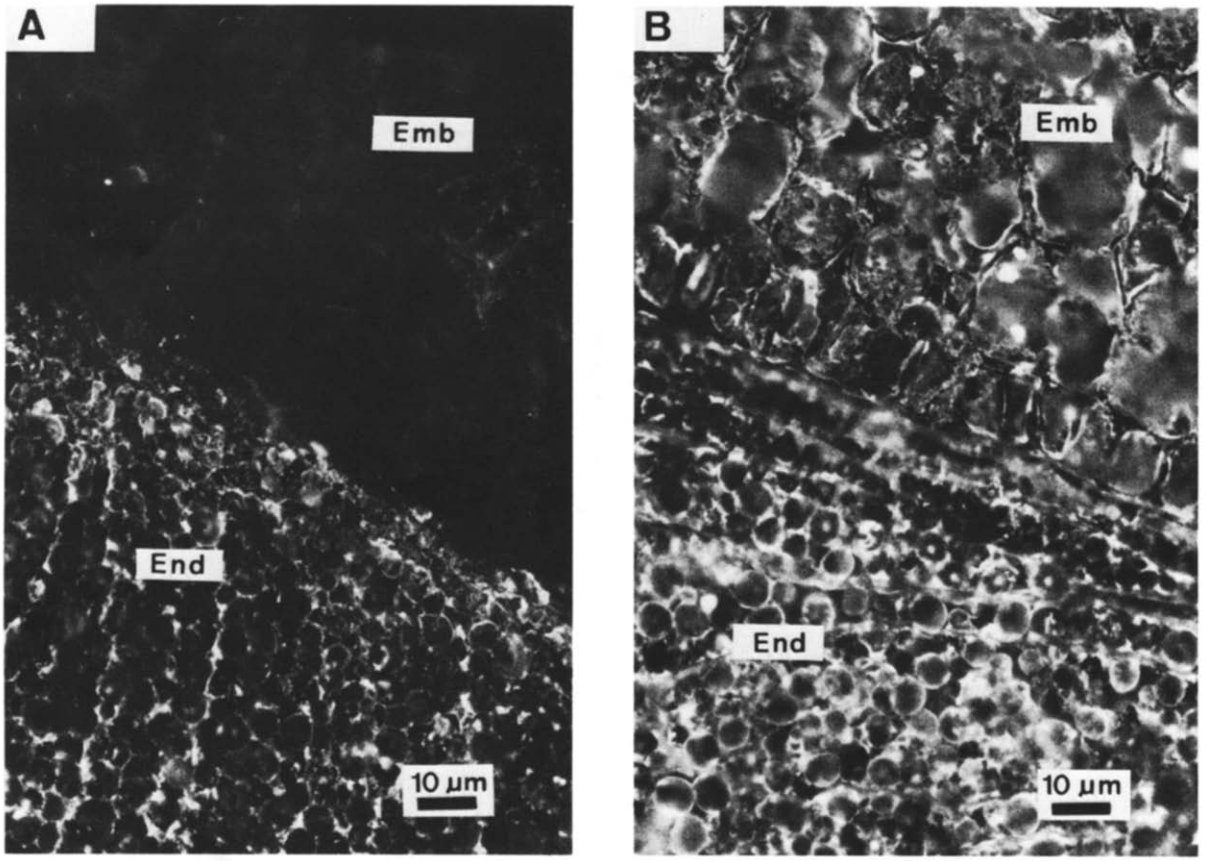

Fig.4. Tissue-specificity of zein assayed by immunofluorescence. Sections of maize kernels consisting of endosperm and embryo were treated with either anti-zein IgG (panel A) or with anti-(embryo) globulin IgG (panel B), followed by rhodamine conjugated goat anti-rabbit IgG as in section 2.

4. DISCUSSION

We have demonstrated immunochemically that:

(i) We were able to raise specific antibodies against zein and against globulins from endosperm or embryo;
(ii) Antibodies against zein only recognized zein;
(iii) Antibodies against endosperm globulins also reacted with those from embryos and vice-versa;
(iv) Antibodies against globulins did not crossreact with zein.

The immunological relatedness of endosperm and embryonal globulins was a convenient property for testing the presence of globulins in either tissue by the use of one antibody only. These results enabled us to employ the antibodies for determining the localization of specific proteins within tissue sections, by coupling the antibodies to a fluorescent dye. Specifically, we could demonstrate that zein is exclusively localized in endosperm cells. This corresponds to the distribution of protein bodies indicated as the site of zein storage [2]. Protein bodies have not been found in the aleurone layer [15] nor in embryo cells (unpublished). A remote possibility for the alleged presence of zein in embryo cells [3,4] could be that zein, an extremely hydrophobic protein, would be soluble in oil which exists in embryo cells in large amounts and therefore would not need to be deposited in the form or protein bodies. The oil could possibly prevent an antigen–antibody reaction. However, ethanol extracts of embryos,

which contain nitrogenous compounds as shown by Kjeldahl analysis, did not produce the typical zein bands when submitted to SDS–PAGE. In view of the limitations of anti-zein staining to endosperm, reports of zein in embryos may be the result of contamination of dissected embryos by endosperm.

ACKNOWLEDGEMENTS

We are most grateful to Dr A. Matus of the Friedrich Miescher-Institut for his continuing interest and help in this study. Thanks are due to Ms S. Wirth for her excellent technical assistance. We are also indebted to many of our colleagues for their criticism in preparing this manuscript.

REFERENCES

[1] Khoo, U. and Wolf, M.J. (1970) Am. J. Bot. 57, 1042–1050.
[2] Burr, B. and Burr, F.A. (1976) Proc. Natl. Acad. Sci. USA 73, 515–519.
[3] Tsai, C.Y. (1979) Biochem. Genet. 17, 1109–1119.
[4] Landry, J. and Moureaux, T. (1980) J. Agric. Food Chem. 28, 1186–1191.
[5] Dierks-Ventling, C. (1981) Eur. J. Biochem. 120, 177–182.
[6] Dierks-Ventling, C. (1982) Theoret. Appl. Genet. 61, 145–149.
[7] Dierks-Ventling, C. and Cozens, K. (1982) FEBS Lett. 142, 000–000.
[8] Goding, J.W. (1976) J. Immunol. Methods 13, 215–226.
[9] Thomas, G., Sweeney, R., Chang, C. and Noller, H.F. (1975) J. Mol. Biol. 95, 91–102.
[10] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[11] McLean, I.W. and Nakane, P.K. (1974) J. Histochem. Cytochem. 22, 1077–1083.
[12] Hökfelt, T., Kellerth, J.-O., Nilsson, G. and Pernow, B. (1975) Brain Res. 100, 235–252.
[13] Lee, K.H., Jones, R.A., Dalby, A. and Tsai, C.Y. (1976) Biochem. Genet. 14, 641–650.
[14] Wienand, U. and Feix, G. (1978) Eur. J. Biochem. 92, 605–611.
[15] Burr, F.A. and Burr. B. (1981) J. Cell Biol. 90, 427–434.