Subcellular localization of type I thionins in the endosperms of wheat and barley

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Summary. Thionins are cysteine-rich polypeptides of about 5,000 Da. Localization at the subcellular level of type I endosperm thionins has been carried out by immunogold labeling, using an antibody that recognizes type I thionin variants. In developing wheat and barley caryopses, sectioned at different times between 13 and 24 days after flowering, this type of thionins was only detected around protein bodies from cells of the starchy endosperm, using light microscopy. Electron microscopy revealed that these proteins were located in electron-dense spheroids in the periphery of protein bodies, at the earlier stages, whereas later the label appeared also as a thin layer around these organelles.

Keywords: Endosperm; Protein bodies; Thionins.

Abbreviations: DAF days after flowering; RER rough endoplasmic reticulum.

Introduction

In cereal caryopses, endosperm is a specialized tissue that is mobilized during germination to nourish the growing seedling and is the target for enzymes secreted by the aleurone layer and the scutellum. It contains starch granules and protein bodies, distinct organelles where storage proteins are located. This tissue also contains other types of abundant proteins, one of which is represented by the thionins, a protein family that has received considerable attention because of its toxicity towards plant pathogens and other heterologous systems (see García-Olmedo et al. 1989).

Thionins are cystein-rich polypeptides of about 5 kDa that are also present in cereal leaves (Bohlman and Apel 1987, Gausing 1987) and in a wide range of plant species (García-Olmedo et al. 1989, 1992). Known thionin amino-acid sequences have been classified into five structural types, the first of which (type I) corresponds to the original thionins identified in the endosperms of wheat (Balls et al. 1942a, b) and barley (Redman and Fisher 1969). One genetic variant per haploid genome ($\alpha 1$, $\alpha 2$, β) in hexaploid wheat (*Triticum aestivum*) and two variants (α, β) in barley (*Hordeum vulgare*) have been found so far for type I thionins (Fernández de Caleya et al. 1976, Redman and Fischer 1969). Preliminary subcellular fractionation studies, carried out with developing and mature barley endosperm, suggested that type I thionins were associated with the endoplasmic reticulum and the protein bodies (Carbonero et al. 1989, Ponz et al. 1983), whereas type II thionins have been reported to be associated with cell walls and vacuoles in barley leaves (Bohlman et al. 1988, Reimann-Philipp et al. 1989). The only other thionin type in wheat and barley, type V, has only been characterized at the DNA level (Castagnaro et al. 1992) and no subcellular location studies have been carried out yet. We now report an immunolocalization study of type I thionins in wheat and barley endosperms which shows that these proteins are present in electron-dense spheroids in the periphery of protein bodies.

Materials and methods

Plant material

Wheat (*Triticum aestivum* L. cv. Chinese Spring) was obtained from E. R. Sears (Columbia, Mo.). The cultivated barley *Hordeum vulgare* L. cv. Bomi was the gift of H. Doll (Risø Laboratory, Denmark). Plants were grown under standard green house conditions. Developing seeds were harvested from plants at 13, 15, 17, 20, and 24

days after flowering (DAF). Thionin variants purified by high-performance-liquid-chromatography (HPLC) were the gift of A. Molina (Madrid, Spain).

Tissue preparation

Transverse sections of grains (1 mm thick blocks) were fixed for 2 h at 4 °C with 2.5% glutaraldehyde or 4% paraformaldehyde in 25 mM K-phosphate buffer, pH 7.2. The tissue was washed with three changes of excess phosphate buffer at 4 °C. The best results were obtained with seeds fixed in 4% paraformaldehyde. Samples were dehydrated in a graded ethanol series on ice: 5 min each in 10%, 30%, 50%, 70%, and 100% ethanol. After infiltration in 50% LR white resin in ethanol (Polysciences, Northampton, U.K.) for 1 h, the samples were placed in 100% LR White resin for 3 days with changes. Polymerization was performed with fresh resin at 55 °C for 24 h.

Silver-colored thin sections were obtained with a glass knife and mounted on nickel grids coated with collodion and carbon (200 mesh; Vesco, Eerbeek, Holland) for electron microscopy. For light microscopy, 1 µm-thick sections were extended on poly-L-lysine-coated slides.

Preparation of monospecific antibody and Western-blot procedure

Rabbit antiserum against purified β-thionin from barley had been previously raised (Ponz et al. 1983). Purification of antibody was accomplished by passage through a thionin-Sepharose affinity column according to Shapiro et al. (1974). Seeds were ground to a fine powder and extracted with 0.05 N H_2SO_4 and protein precipitated with 9 volumes of acetone. Extracts from barley and wheat, as well as five purified variants of thionins, were subjected to sodium-dodecyl-sulphate electrophoresis (SDS-PAGE) on 4–20% polyacrylamide-gradient gels, and to immunoblot analysis according to the manufacturers' instructions (BioRad, Richmond, Calif., U.S.A.).

Immunocytochemical staining

Ultrathin sections mounted on coated nickel grids were blocked with 1% ovalbumin in Tris-buffered saline (TBS; 20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl, pH 7.5 and 500 mM NaCl) for 10 min at room temperature. The grids were then incubated overnight at 4 °C with affinity-purified β-thionin-specific antibody in TBS with 1% ovalbumin. Controls were done in parallel using preimmune serum or TBS with 1% ovalbumin. Non-specifically bound antibodies were removed by washing the grids five times on a drop of TBS for 5 min. The grids were then floated for 1 h on a drop of 10 nm diameter protein A-gold (Janssen Biotech, Olen, Belgium) diluted 1:20 in TBS-1% ovalbumin. The incubation was followed by three washes with TBS and another three washes in distilled water. Grids were stained with a saturated uranyl acetate solution for 15 min. The same protocol was followed for the 1 µm thick sections for light microscopy, except the last step. After washing the second antibody, silver enhancement of colloidal-gold immunolabelling was performed, following the manufacturers' protocol (Janssen Biotech). A Jeol 1100 transmission electron microscope and a Nikon M-35 S microscope were used.

Results

Antibody specificity

The specificity of an antibody raised against barley β-thionin and purified by affinity chromatography on a
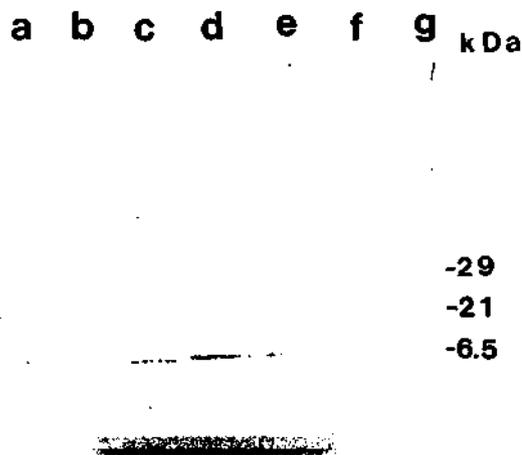

Fig. 1. Western blot analysis of the specificity of the β-thionin antiserum. Protein extracts from the endosperms of barley (*a*) and wheat (*g*). *b* and *c* α- and β-thionins from barley; *d–f* α 1, α 2, and β thionins from wheat, respectively. After transfer to an immobilon membrane, the immunoreactive bands were visualized by incubation with protein A-gold and silver enhancement

thionin-Sepharose column (Ponz et al. 1983) was tested by Western-blot analysis of purified genetic variants and total protein extracts from wheat and barley (Fig. 1). All pure wheat and barley endosperm variants of type I thionin reacted with the antibody, and in total endosperm extracts, only the thionin band was recognized by it (Fig. 1). Thionins of types II and V do not react with these antibodies (not shown). The cross-reactivity among type I thionins is in agreement with their close sequence similarity.

Immunolocalization of type I thionins under the light microscope

Developing endosperms from wheat and barley were sectioned at different moments between 13 and 24 days after flowering (DAF) to localize thionins and to analyze their accumulation and deposition during development. This period was chosen because thionins had been previously shown to be synthesized during the first half of endosperm development, with a maximum concentration of messenger at 13–16 DAF (Ponz et al. 1983, Rodriguez-Palenzuela et al. 1988). Beyond 24 DAF, the tissue became very difficult to section because of the accumulation of starch granules, specially in the case of barley, where the cells were largely filled-up with starch granules as early as 17 DAF (Fig. 2 d–f). In wheat, protein bodies of different sizes appeared either alone or forming clusters, which became larger and more numerous with time, while at least some
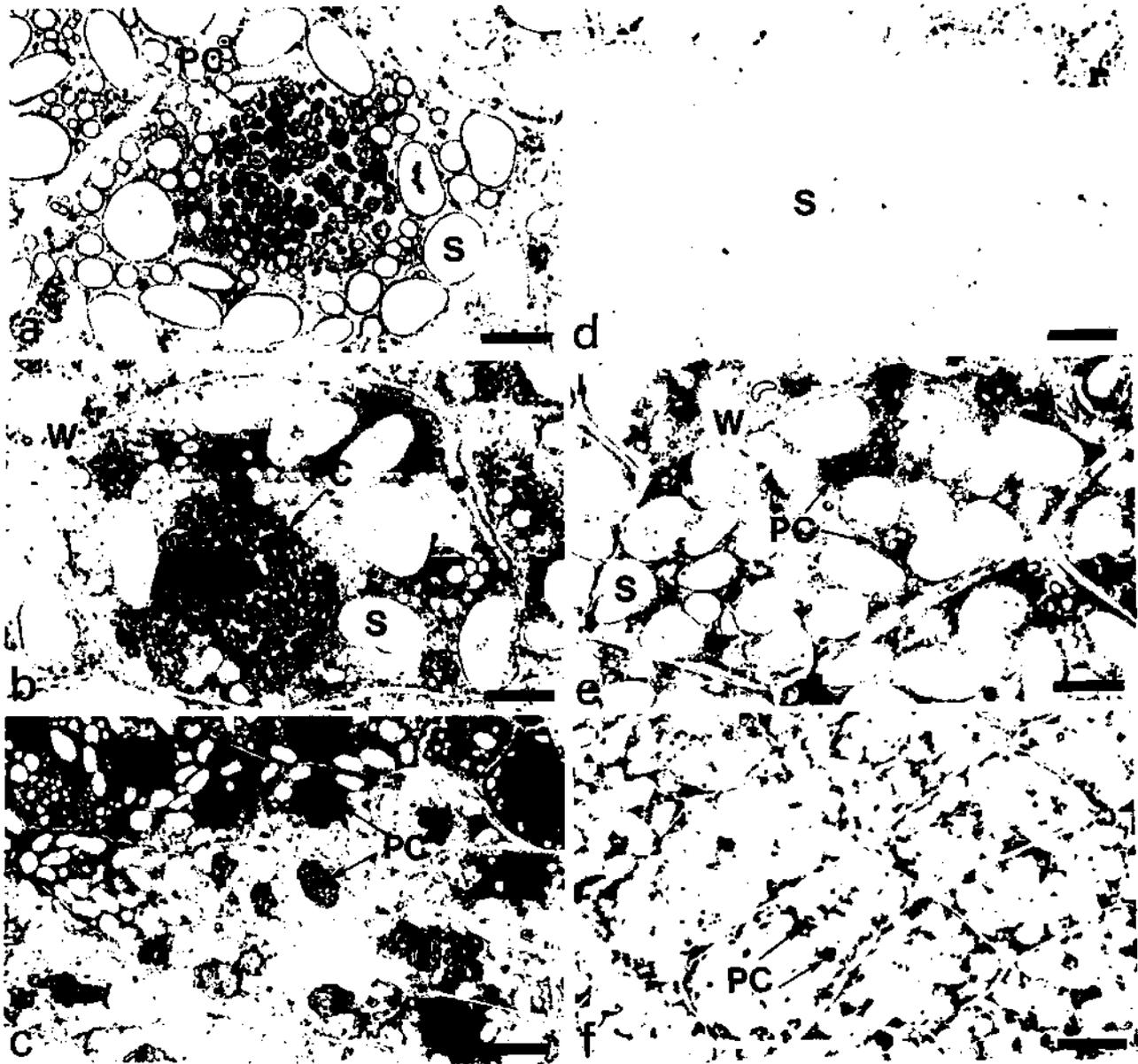
Fig. 2. Immunolocalization of wheat and barley thionins under the light microscope. Photographs correspond to a 24 DAF endosperm (wheat) and to a 17 DAF endosperm (barley). *S* Starch grain; *W* cell wall; *PC* protein body cluster. Bars: a, b, d, and e, 40 µm; c and f, 100 µm. **a** Control section of wheat endosperm incubated with preimmune serum; **b** wheat endosperm section treated with β-thionin antibody. Specific label was observed around protein-body clusters (PC); **c** low-magnification view of wheat section treated with β-thionin antibody. **d** Control section of barley endosperm incubated with preimmune serum; **e** barley endosperm section incubated with β-thionin antibody. Intense label appeared around isolated protein bodies and small clusters; **f** low-magnification view of barley section treated with β-thionin antibody

protein bodies seemed to fuse among each other (Fig. 2 a–c). In barley, the process was similar, but the clusters were smaller and more scattered in the cell (Fig. 2 e, f). Immunolabeling with the β-thionin-antibody/protein A-gold reagent showed staining around isolated protein bodies and protein-body clusters of starchy endosperm cells, both in wheat (Fig. 2 b, c) and in barley (Fig. 2 e, f), with no labeling appearing in other cell structures or in cells from the aleurone and coat layers. No labeling was observed when preimmune serum was used (Fig. 2 a, d).

Electron-microscopic localization of type I thionins

Electron microscopy of immunogold-labeled thin sections from wheat endosperms at the earlier stages of development (13 DAF) allowed the observation of gold particles specifically located in electron-dense spheroidal masses, which were normally associated with the
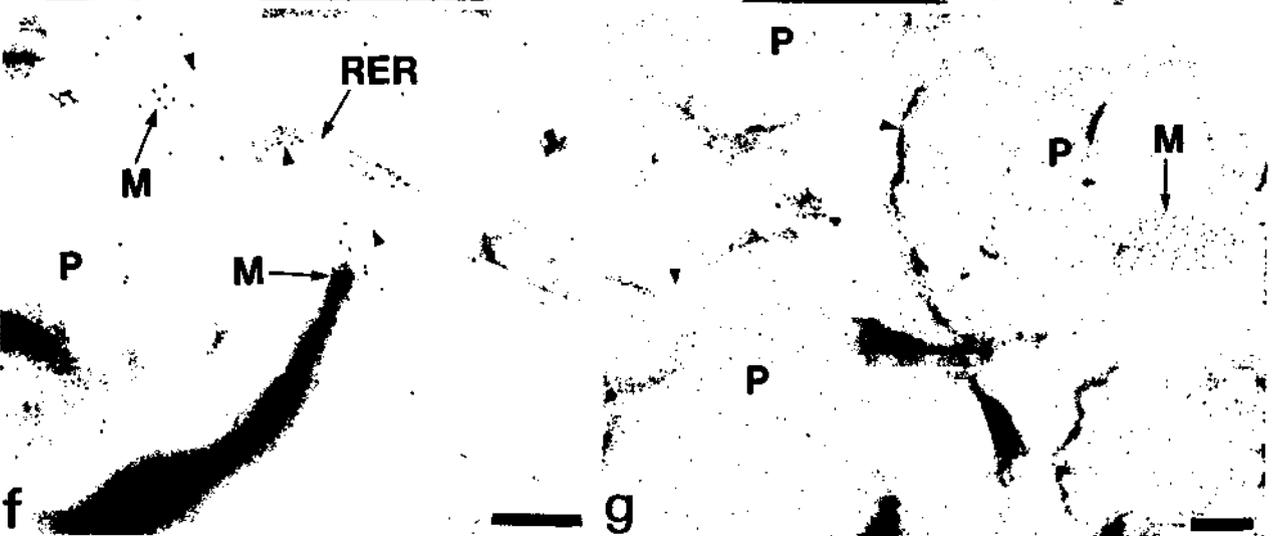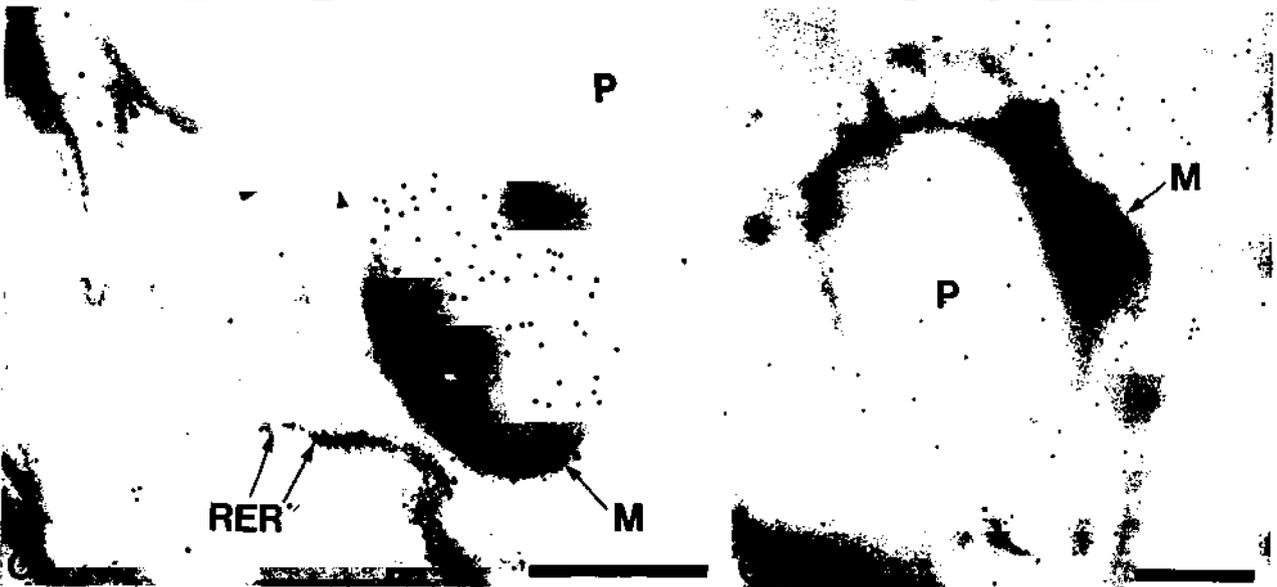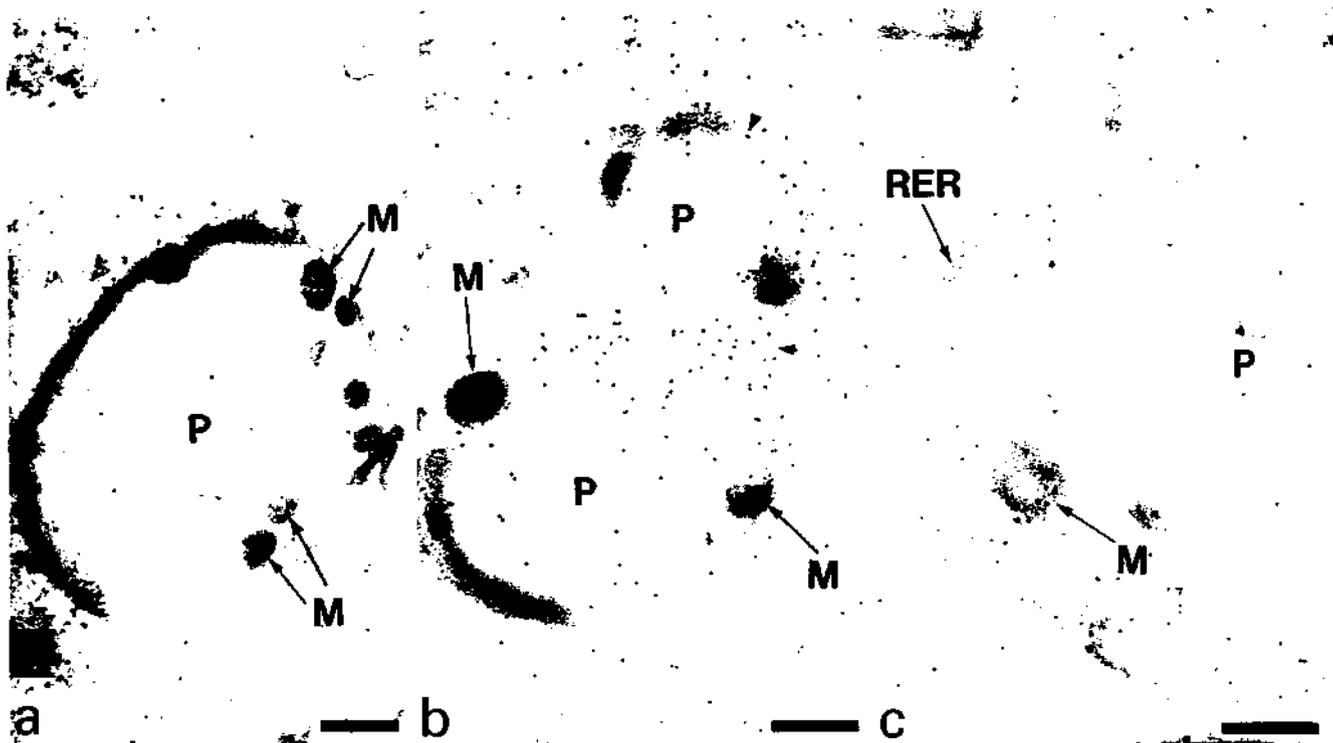
Fig. 4. Electron micrographs showing immunolocaliaztion of thionins in barley endosperm at 15 DAF. *M* Spheroidal masses; *P* protein body; *RER* rough endoplasmic reticulum. Bars: 0.5 µm. **a** Control section of endosperm incubated with preimmune serum; **b** section immunostained with β-thionin antibody. Label appeared in spheroidal masses and on the periphery of protein bodies (arrowheads); **c** labeled spheroidal mass localized in the vicinity of a protein body and surrounded by RER

periphery of both single and clustered protein bodies and only rarely appeared by themselves in their proximity (Fig. 3 a–d). These masses appeared occasionally as flattened against the protein body surface (Fig. 3 e). The number and sizes of the spheroids varied among the different protein bodies. At later stages, they were less frequent and the gold label appeared not only in them, but throughout the membrane-like structure surrounding protein bodies (Fig. 3 g). Similar observations were made in barley endosperm, where the number of spheroidal masses per protein body seemed to be greater (Fig. 4 a–c).

Rough endoplasmic reticulum (RER) was always detected in the vicinity of the spheroidal masses, whether they were associated to the protein bodies or close to them (Figs. 3 d, f and 4 c). No label was detected in other cellular membranes.

Discussion

The immunocytological data presented here show that endosperm thionins (type I) are located in the periphery of protein bodies. These observations suggest that thionins become integrated into the electron-dense spheroids in the surface of the protein bodies and that the spheroids eventually spread out as a layer around these organelles. Electron-dense spheroids associated with protein bodies have been previously described

Fig. 3. Electron micrographs showing immunolocalization of thionins in wheat endosperm. *RER* Rough endosplasmic reticulum; *P* protein body; *M* spheroidal masses. Bars: 0.5 µm. **a** Control section incubated with preimmune serum (13 DAF). Arrowheads point to background protein A-gold particles; **b** section immunostained with β-thionin antibody. Label appeared in spheroidal masses associated to the periphery of protein bodies, (13 DAF); **c** a field of protein bodies which differ in the number and size of electron-dense spheroids (13 DAF); **d** high-magnification view of electron-dense spheroid, protein body, and RER (13 DAF). Note the connections between them (arrowheads); **e** spheroidal mass spreading along the periphery of protein body (13 DAF); **f** micrograph showing RER, protein body membrane, and spheroidal masses (arrowheads) at 20 DAF; **g** electron micrograph showing immunolocalization of thionin in wheat endosperm at 24 DAF. Intense label appeared on the surface of individual protein bodies of a cluster (arrowheads)

both in barley (Munck and von Wettstein 1976, Cameron-Mills and von Wettstein 1980, Rasmussen et al. 1990) and in wheat (Buttrose 1963, Parker 1980, Campbell et al. 1981, Kim et al. 1988, Bechtel et al. 1989, Stenram et al. 1991). The wheat spheroids were not labeled by gliadin or glutelin antibodies (Kim et al. 1988, Stenram et al. 1991). However, Bechtel et al. (1989) have reported that these spheroids also contain triticin, a legumin-like protein. No such spheroids are present in leaf tissues, where it has been claimed that type II thionins are located in the cell wall (Bohlman et al. 1988) and in the vacuoles (Reimann-Philipp et al. 1989). As the synthesis of type I thionins appears to start and to level off somewhat earlier than the main reserve proteins (Ponz et al. 1983), the electron-dense spheroids could serve as transport and/or temporary storage structures from which the final deposition around the protein body would take place.

As it has been suggested that thionins could function as secondary thiol messengers in the redox regulation of enzyme activity and that they may cooperate in the rearrangement of disulphide bridges (Johnson et al. 1987), their location in the periphery of protein bodies allows to speculate that they may play a role either in packaging storage proteins into these organelles or in their mobilization during germination. In this context, it has been shown that the predominant proteases released by barley aleurone during germination, to mobilize the storage proteins, are cysteine proteases, whose proper conformation seems to be stabilized by disulphide bonds (Fincher 1989, Koehler and Ho 1990). Rearrangement of disulphide bonds has been specifically claimed in connection with protein-body growth in maize, where cysteine-rich proteins have been also identified in an electron-dense layer around protein bodies (Ludevild et al. 1984, Lending et al. 1988, Lending and Larkins 1989).

Acknowledgements

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