Immunocytochemical Evidence for the Involvement of the Golgi Apparatus in the Transport of the Vacuolar Protein, γ-Secalin, in Rye (*Secale cereale*) Endosperm

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ABSTRACT

We resolved the alcohol-soluble prolamins of rye endosperm (secalins) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis into four groups of proteins: high molecular weight (HMW) secalins, 68,000- and 57,000-dalton γ-secalins, ω-secalins, and 33,000-dalton γ-secalins. Antibodies raised against wheat prolamins reacted specifically with γ-secalins of rye upon protein blot analysis. Thin sections of rye grains were examined immunocytochemically with these antibodies. γ-Secalins were present primarily in protein bodies. Vesicles arising from the Golgi apparatus also were specifically labeled, whereas the cytosol and other organelles, including mitochondria, lipid bodies, and amyloplasts, were not. These observations support the hypothesis that the transport of γ-secalins from the site of synthesis (rough endoplasmic reticulum) to that of deposition (protein bodies) is mediated by the Golgi apparatus in rye caryopses.

Most of the economically important cereals accumulate large amounts of alcohol-soluble proteins, termed prolamins, in the endosperm tissue (Kreis et al 1985, Shewry and Miflin 1985). Rye storage proteins are relatively poorly characterized, but in recent years they have received some experimental attention (Preston and Woodbury 1975; Field et al 1982; Shewry et al 1982, 1983, 1984). Secalins, the alcohol-soluble proteins of rye, are composed of four groups of polypeptides: high molecular weight (HMW) secalins, 75,000-dalton (Da) γ-secalins, ω-secalins, and 40,000-Da γ-secalin (Shewry et al 1983). These proteins are structurally related to the storage proteins of barley and wheat, which have been characterized extensively (Field et al 1982, Shewry et al 1984, Kreis et al 1985, Okita et al 1988).

The alcohol-soluble storage proteins of all cereals accumulate during the midstage of seed development (Shewry and Miflin 1985). They are synthesized on rough endoplasmic reticulum (ER) and transported to storage organelles, the protein bodies (Larkins and Hurkman 1978, Cameron-Mills and Von Wettstein 1980, Campbell et al 1981, Oparka and Harris 1982, Taylor et al 1985). In recent years, immunocytochemical techniques have been used to elucidate the morphogenesis and structure of protein bodies (Ludevid et al 1984; Kim et al 1988; Lending et al 1988; Krishnan et al 1986, 1988, 1989). The involvement of the Golgi apparatus in trafficking and condensing of wheat prolamins was first suggested by Buttrose (1963) and supported by Bechtel and co-workers (Bechtel et al 1982, Bechtel and Barnett 1986) and Parker and Hawes (1982). A recent immunocytochemical study also strongly supports the involvement of the Golgi apparatus in prolamin protein body formation (Kim et al 1988). Based on biochemical studies, however, Miflin and co-workers (1981) questioned the involvement of the Golgi apparatus in protein body formation. They believe that wheat and barley are similar to maize and sorghum (Larkins and Hurkman 1978, Taylor et al 1985, Lending et al 1988, Krishnan et al 1989), where the rough ER serves as the site of storage-protein synthesis and accumulation. Thus, despite numerous studies, a clear understanding of the mechanism of storage-protein deposition in the subfamily Triticeae is lacking.

Here we present results of experiments designed to explore the role of the Golgi apparatus in prolamin transport and packaging in rye endosperm tissue. We chose rye as the experimental material because of its economic importance and the fact that it has been neglected in such studies.

MATERIALS AND METHODS

Plant Material

Rye (*Secale cereale* L. cv. Blanco) and wheat (*Triticum aestivum* L. cv. Chinese spring) caryopses were obtained from P. Gustafson and E. R. Sears, University of Missouri, Columbia. Grains were germinated on moist filter paper for three days at room temperature and transplanted to small clay pots containing garden soil. The plants were grown in a glasshouse and fertilized every 15 days with Peter's (Allentown, PA) soluble fertilizer. Cereal heads were tagged on the day of flowering, and developing caryopses from the centers of panicles were harvested 15 (soft dough) and 20 (hard dough) days later.

Prolamin Extraction

Five grams of rye or wheat grains were ground to a fine powder with the help of a pestle and mortar and extracted with 50 ml of 70% aqueous EtOH at 37°C for 2 hr in an orbital shaker (150 rpm). Clear supernatant solutions were obtained from these slurries by centrifugation at 14,000 × *g* at 4°C for 15 min. Prolamins were precipitated by adding five volumes of ice-cold acetone. After incubation at −20°C for 12 hr, precipitated proteins were recovered by centrifugation as above and briefly dried in vacuo. The proteins were dissolved in a small volume of sodium dodecyl sulfate (SDS) sample buffer (120 m*M* Tris-HCl, pH 6.8, containing 4% [w/v] SDS, 10% [v/v] 2-mercaptoethanol [2-ME], and 20% [v/v] glycerol). Total proteins were extracted directly

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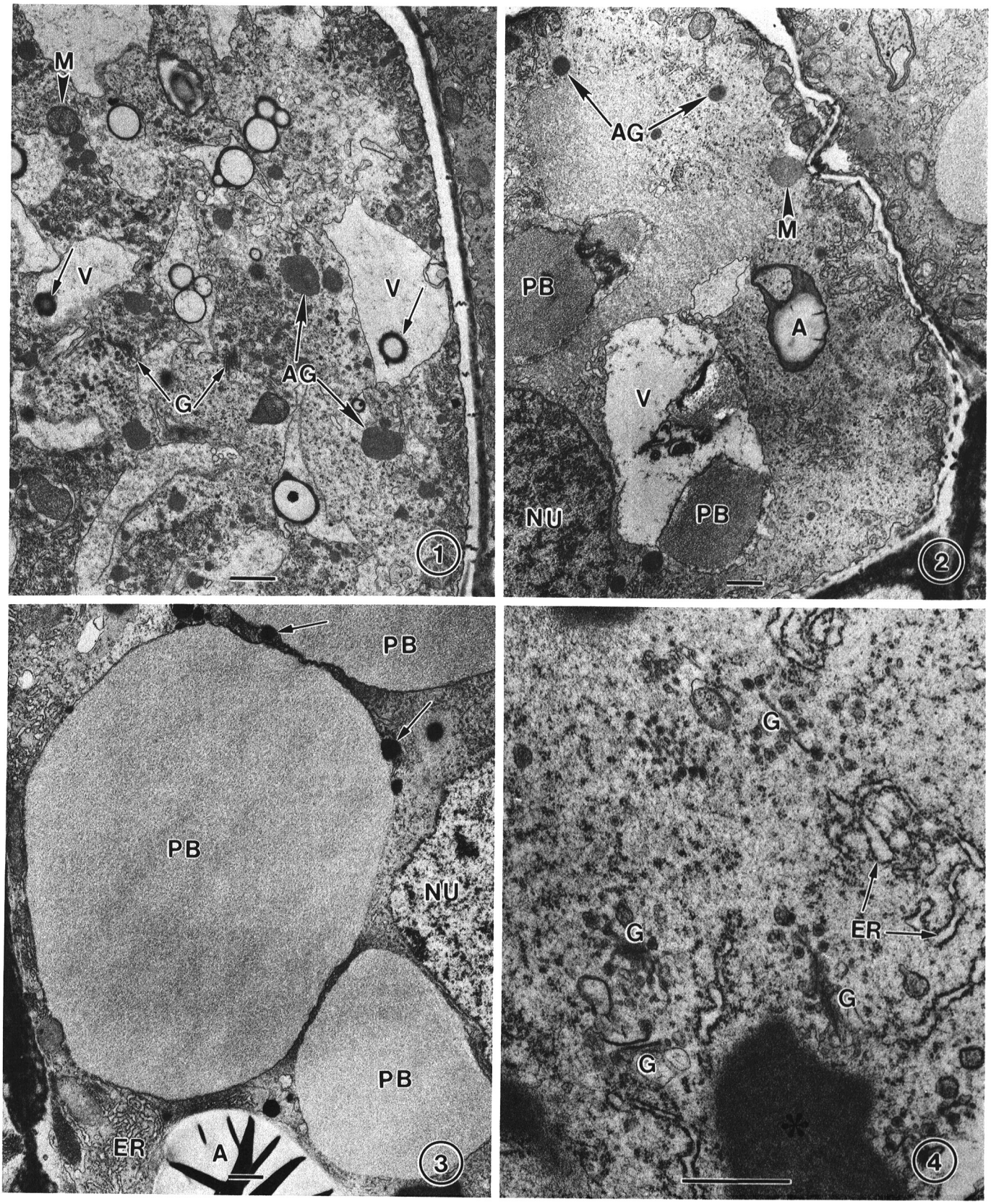


Fig. 1. Low magnification transmission electron micrograph of the rye aleurone layer, at soft dough stage. Several small vacuoles (**V**) harboring osmiophilic substances (arrows) are seen. Numerous aleurone granules (**AG**) and Golgi complexes (**G**) are present. Protein bodies and starch grains are absent in this layer. **Fig. 2.** Subaleurone layer, showing the presence of protein bodies (**PB**) inside the vacuoles. Aleurone grains in this cell type indicate its origin from the aleurone layer. Mitochondrion (**M**) and nucleus (**NU**). **Fig. 3.** Large protein bodies within endosperm cells. Note the presence of associated dark staining granules (arrows). Rough endoplasmic reticulum (**ER**) and amyloplasts (**A**) also are present in these cells. **Fig. 4.** Numerous Golgi complexes and extensive rough endoplasmic reticulum in endosperm cells. Unidentified dark staining amorphous materials are also visible (*). Scale bars = 1 μm.

from the cereal meal with SDS sample buffer and boiling for 5 min. The samples were recovered by centrifugation at 12,000 $\times g$ for 10 min at room temperature.

Gel Electrophoresis and Protein Blotting

Proteins from rye and wheat were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). Each slab gel (13.5 cm × 16 cm × 1.5 mm) was composed of a resolving gel of 12.5% (w/v) acrylamide and a stacking gel of 4% (w/v) acrylamide. Following electrophoresis, the proteins were visualized by staining with 0.25% (w/v) Coomassie Brilliant Blue R-250. Protein blot analysis was carried out essentially as described by Burnett (1981). Proteins were electrophoretically transferred to a nitrocellulose sheet and incubated with antibodies against wheat (*Triticum aestivum* L. cv. Chinese Spring) prolamin (Krishnan et al 1988), followed by incubation with 1 μCi of ^{125}I-labeled protein A from *Staphylococcus aureus*. Two different antibody dilutions (1:100 and 1:500) were used for protein blot analysis. The immunoreactive polypeptides were detected by autoradiography.

Tissue Preparation and Immunocytochemical Labeling

Small segments of rye caryopses (3–4 mm thick), collected at 15 and 20 days after flowering, and representing the soft and hard dough stage, respectively, were excised with a sharp razor blade and immediately fixed in 2.5% glutaraldehyde in 50 m*M* sodium phosphate buffer (pH 7.2) in small glass vials. These vials were left at room temperature for 1 hr and then transferred to 4°C for 14 hr. Tissue was washed thoroughly with several changes of sodium phosphate buffer (pH 7.2) and post-fixed with 2% aqueous osmium tetroxide. Samples were dehydrated in a graded acetone series and infiltrated with Spurr's resin (1969) for extended periods (Krishnan et al 1986). Thick sections (4 μm) were cut with a glass knife and stained with 1% toluidine blue. A diamond knife was used to cut thin sections, which were collected on 300-mesh nickel grids.

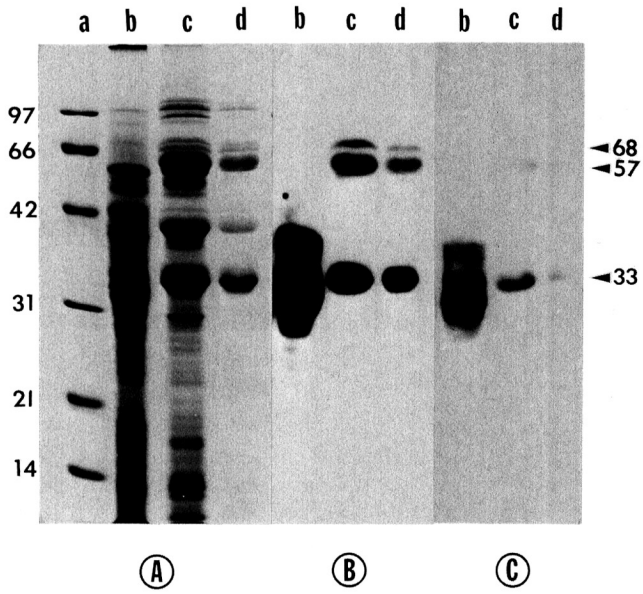


Fig. 5. Analysis of seed proteins from rye and wheat. Total proteins and proteins soluble in 70% ethanol were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie Blue (panel **A**). Panels **B** and **C**, Protein blot analyses. Wheat and rye proteins resolved by SDS-PAGE, as shown in panel A (lanes b–d), were electrophoretically transferred to a nitrocellulose filter and probed with wheat prolamin antibodies. Two concentrations of antiserum, a 1:100 dilution (panel B) and 1:500 dilution (panel C) were employed. Lanes: molecular weight markers (**a**) (phosphorylase b, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,699; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500 and lysozyme, 14,400); wheat total proteins (**b**); rye total proteins (**c**), and rye prolamins (**d**). The numbers on the sides of the figure refer to molecular weights of proteins in kilodaltons.

Immunocytochemical labeling was carried out at room temperature, essentially as described earlier (Krishnan et al 1986, 1989). Ultrathin sections mounted on uncoated nickel grids were floated over 200 μl drops of 50 m*M* Tris-HCl, pH 7.5, containing 0.15*M* NaCl, 0.1% Tween-20, and 1% (w/v) bovine serum albumin (TTBS) for 30 min. The sections then were incubated for 60 min with wheat prolamin antiserum that had been diluted 1:100 or 1:500 with TTBS. Control sections were treated with preimmune sera or TTBS instead of prolamin-specific antisera. The grids were rinsed several times with 50 m*M* Tris-HCl, pH 7.5., containing 0.15*M* NaCl and 0.1% Tween-20 to wash away nonspecifically bound antiserum. Specifically bound antiserum was visualized by incubating the sections for 30 min on a droplet containing 10-nm diameter protein A gold particles. After several rinses in TTBS and distilled water, the sections were stained sequentially with 0.5% aqueous uranyl acetate for 30 min and 0.4% aqueous lead citrate for 30 min. Sections were examined with a JEOL JEM 100B electron microscope at 100 kV.

RESULTS

Anatomy of Rye Caryopses

Ultrastructural analysis of rye caryopses at the soft dough stage reveals three distinct zones. The aleurone layer is composed of a row of rectangular cells, which in a few cases is two cells thick. Cells of the aluerone layer contain several small vacuoles that often enclose dark-staining osmiophilic substances (Fig. 1, arrows). Several aleurone grains, mitochondria, and Golgi apparatus also are apparent (Fig. 1). No protein bodies are in this layer. Frequently, the aleurone layer differentiates into subaleurone layers. Cells of such layers are easily identifiable by the coexistence of protein bodies and aleurone grains (Fig. 2). The protein bodies in these cells are commonly inside the vacuoles, near the osmiophilic granules. Unlike the aleurone layer, the cells of the subaleurone layer contain starch grains.

The starchy endosperm cells contain large protein bodies that are enclosed by limiting membranes (Fig. 3). These bodies are granular and possess filamentous inclusions. The number of protein bodies per cell is variable. Centrally located starchy endosperm cells, for example, usually have a very large central protein body. In some cases, dark staining granules are attached to the outer surface (Fig. 3, arrows). These cells contain extensive rough ER and numerous Golgi bodies (Fig. 4).

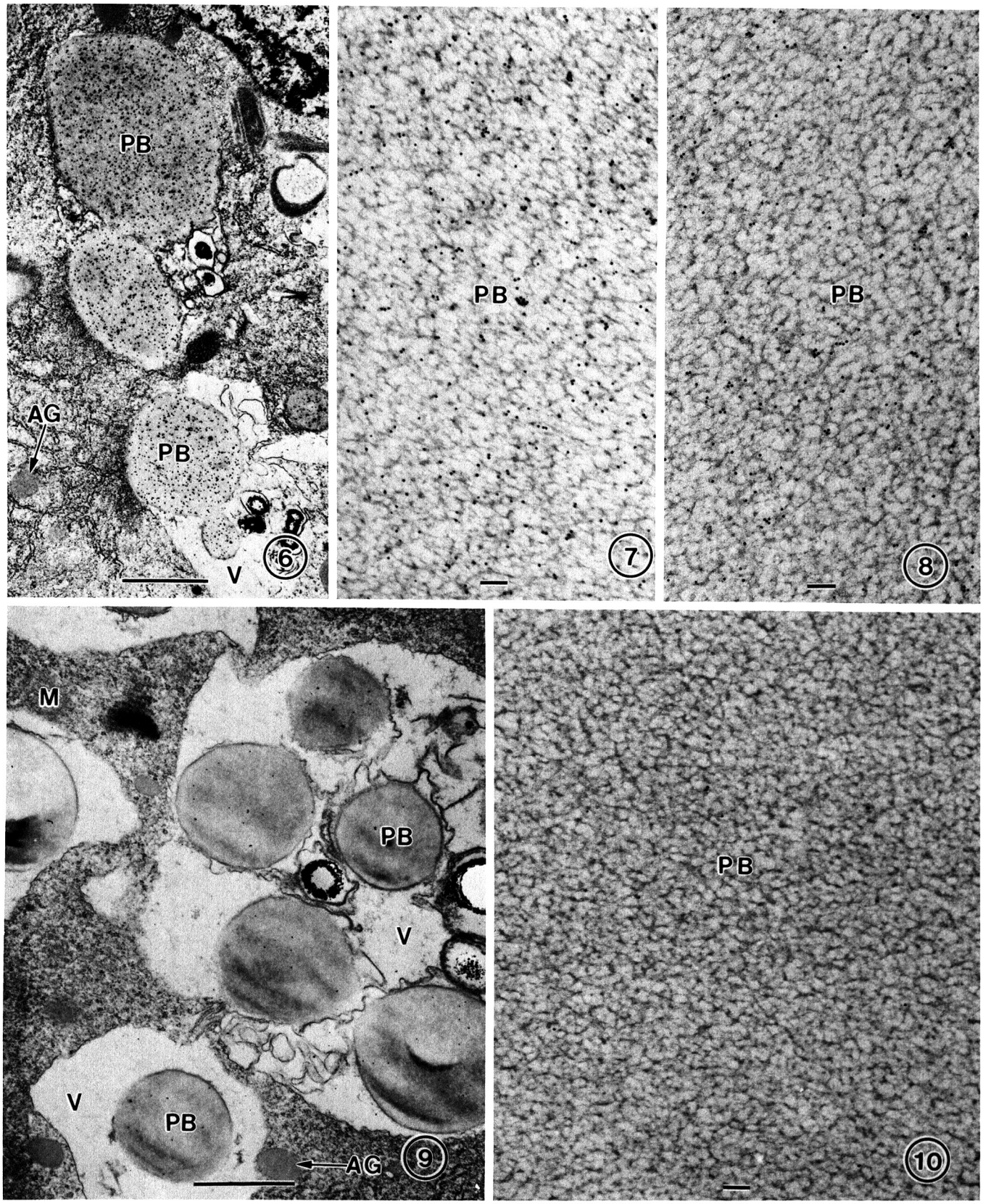
Homology Between Rye and Wheat Prolamins

Electrophoretic analysis of the total wheat and rye endosperm proteins reveals several distinct polypeptides ranging in size from 10 to 100 kDa (Fig. 5A, lane b and c). The alcohol-soluble proteins of rye are resolved into four groups of polypeptides having molecular weights of 115 and 102 kDa (HMW secalins), 68 and 57 kDa (γ-secalins), 40 kDa (ω-secalin), and 33 kDa γ-secalin) (Fig. 5A, lane c). Minor polypeptides of 22 and 13.5 kDa also are visible. In the control immunological experiments and as previously shown (Krishnan et al 1988), the wheat prolamin antibodies (diluted 1:100) react very strongly with three major wheat proteins of 30, 33, and 38 kDa (Fig. 5B, lane b). The antibodies strongly cross-react with the major rye prolamins, the 68-, 57-, and 33-kDa γ-secalins (Fig. 5B, lane c and d), but there is no cross-reactivity with the other rye proteins. At a lower concentration of antiserum (1:500 dilution) there is a marked reduction in the reactivity of the antiserum with the 57-kDa secalin (Fig. 5C, lane c and d) but significant cross-reactivity remains.

Immunocytochemical Localization of γ-Secalins

The extensive cross-reactivity of our wheat prolamin antibodies with γ-secalins prompted us to carry out an immunocytochemical localization of these proteins. Thin sections of rye endosperm were incubated with wheat prolamin antisera (diluted 1:100) and protein A gold particles. The colloidal gold was specifically and heavily deposited over protein bodies in the subaleurone layers (Fig. 6) and starchy endosperm (Fig. 7) under these conditions. Since protein blotting analysis with more diluted antiserum

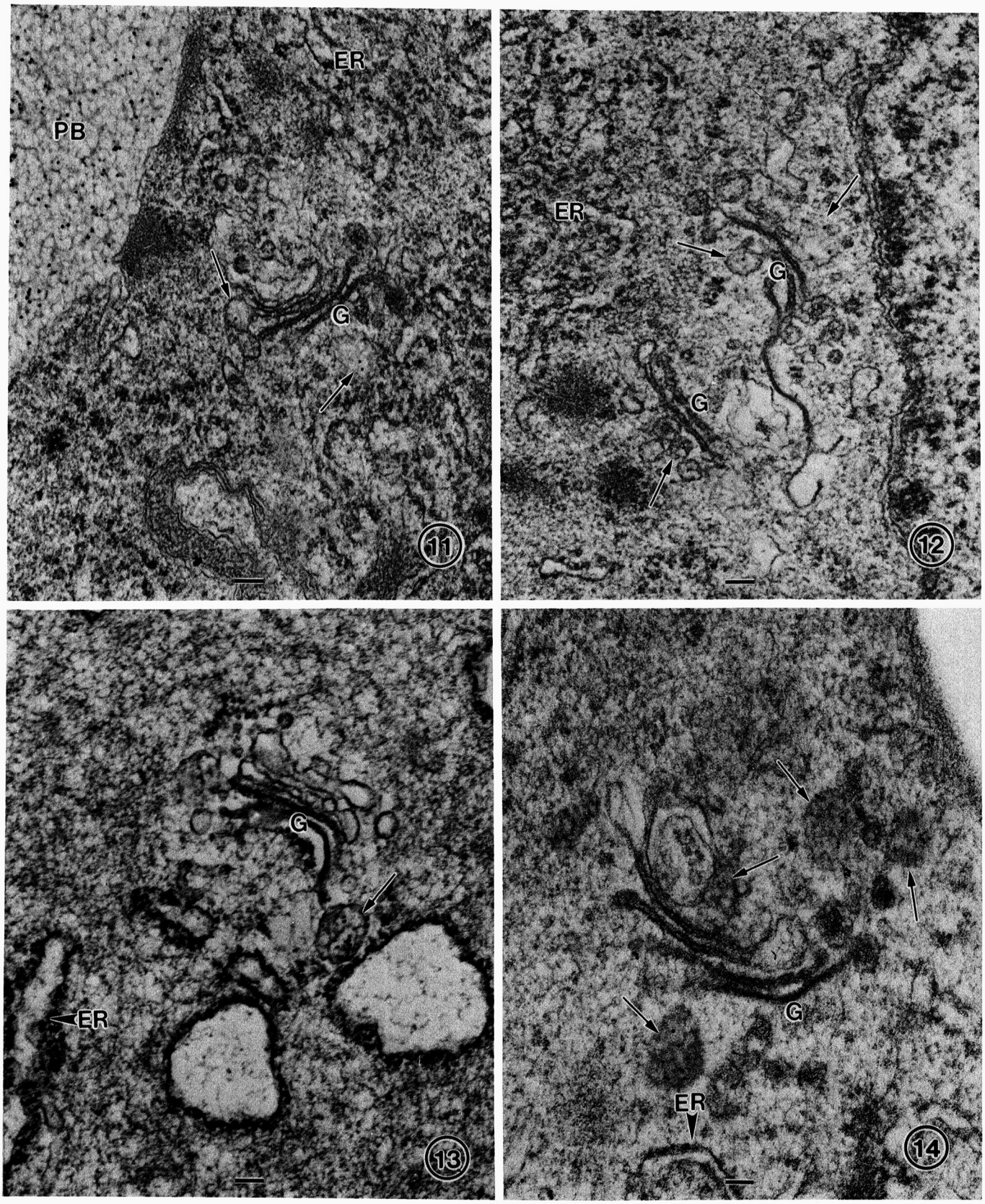
revealed some specificity toward the 33-kDa γ-secalin, we treated some sections with similarly diluted antibody. There were no obvious differences in the spatial distribution of gold particles under these conditions, though the number of gold particles over the protein bodies was reduced (compare Figs. 7 and 8). Other organelles, including the nucleus, ER, aleurone granules, and mitochondria were not labeled. Control sections incubated with preimmune sera or buffer (TTBS) replacing the prolamin antisera



Figs. 6–8. Immunocytochemical localization of prolamins in rye seeds; 6 and 7 are thin sections incubated with wheat prolamin-specific antibodies (1:100 dilution). Protein bodies (**PB**) in the subaleurone layers (Fig. 6) and endosperm cells (Fig. 7) are heavily labeled with gold particles. Scale bars = 1 and 0.1 μm, respectively. Fig. 8 is a thin section incubated with 1:500 diluted prolamin antibody. Scale bar = 0.1 μm. **Figs. 9 and 10.** Control sections treated with preimmune IgG (Fig. 9) or buffer (Fig. 10) instead of prolamin antiserum prior to gold labeling. A few particles are scattered on the protein bodies. Scale bars = 1 and 0.1 μm, respectively.

contained only a very sparse background labeling over the protein bodies (Figs. 9 and 10).

The abundance of Golgi complexes in the endosperm cells (Fig. 4) suggested that they could play a role in the transport of the rye prolamins into vacuoles. This possibility was examined by incubating thin sections of rye caryopses (soft dough stage) with prolamin antibodies and protein A gold particles. The protein A gold particles are deposited over protein bodies and Golgi-complex-associated vesicles under these conditions (Fig. 11 and 12). The labeling over the vesicles is less intense than that over



Figs. 11–14. Immunocytochemical localization of γ-secalins in the Golgi-derived vesicles. Golgi complexes are commonly seen in the endosperm tissue, both at soft dough (Figs. 11 and 12) and hard dough stages (Figs. 13 and 14). The immuno-gold particles on the vesicles are indicated by arrows. Golgi apparatus (**G**), rough endoplasmic reticulum (**ER**). Scale bar = 0.1 μm.

protein bodies, and the cisternal stacks are not labeled. Golgi complexes also are common in endosperm tissue harvested at the hard dough stage, and the vesicles at the distal phase are specifically labeled (Figs. 13 and 14). Large vesicles near the Golgi apparatus usually are more intensely labeled with protein A gold particles than are the small vesicles (compare Figs. 11 and 12 with 13 and 14).

DISCUSSION

SDS-PAGE resolved the alcohol-soluble secalins from rye seeds into four distinct classes of polypeptides (Fig. 5A), the molecular weights of which are different from those reported by Shewry and co-workers (1983). Under our electrophoretic conditions, the two major γ-secalin polypeptides had molecular weights of about 57 and 33 kDa, as opposed to 75 and 40 kDa (Shewry et al 1983). These differences could be due to differences in electrophoretic conditions that result in anomalous migration of cereal prolamins (Bunce et al 1985, Ng and Bushuk 1989). The fact that wheat prolamin antibodies cross-reacted extensively with γ-secalins is not surprising, given the fact that S-rich prolamins of wheat (γ- and α-gliadins) show sequence homology with γ-secalin of rye (Shewry et al 1984, Kasarda et al 1984, Kreis et al 1985). This homology enabled us to carry out immunocytochemical investigation of protein body formation in rye.

Mature protein bodies in rye endosperm appear to be formed by the fusion of several smaller protein granules, a process that gives rise to large, irregularly shaped protein bodies (Fig. 3). A similar situation occurs in wheat, where the different individual small protein bodies can be easily identified by their surrounding membranes. In rye, however, the individual components that comprise a large protein body are less easily resolved. Ultrastructural analysis reveals no obvious differences in the composition of materials within the protein bodies, i.e., they appear to be composed of uniformly textured filamentous proteinaceous material (Figs. 7 and 8).

Formation of protein bodies in rye appears to be analogous to that in wheat (Buttrose 1963, Bechtel et al 1982, Parker 1982, Parker and Hawes 1982, Kim et al 1988) and in some leguminous plants (Herman and Shannon 1984, Craig and Goodchild 1984, Greenwood and Chrispeels 1985, Chrispeels 1985), where the deposition of vacuolar proteins is mediated by the Golgi apparatus. This mode of prolamin protein body formation differs from that of sorghum (Taylor et al 1985, Krishnan et al 1989) maize, (Larkins and Hurkman 1978, Ludevid et al 1984, Lending et al 1988) and rice (Oparka and Harris 1982, Krishnan et al 1986). In these species, the alcohol-soluble proteins are deposited directly into the lumen of rough ER. Thus the rough ER serves as both the site of synthesis and deposition of these proteins. Even though extensive rough ER characterizes the rye endosperm, this organelle is not labeled. Although this observation suggests that rough ER does not act as the primary site of prolamin deposition in rye endosperm, we cannot exclude the possibility that this organelle has some secondary role in prolamin protein body formation.

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