Native and Artificial Reticuloplasmins Co-Accumulate in Distinct Domains of the Endoplasmic Reticulum and in Post-Endoplasmic Reticulum Compartments


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We compared the subcellular distribution of native and artificial reticuloplasmins in endosperm, callus, and leaf tissues of transgenic rice (Oryza sativa) to determine the distribution of these proteins among endoplasmic reticulum (ER) and post-ER compartments. The native reticuloplasmin was calreticulin. The artificial reticuloplasmin was a recombinant single-chain antibody (scFv), expressed with an N-terminal signal peptide and the C-terminal KDEL sequence for retrieval to the ER (scFvT84.66-KDEL). We found that both molecules were distributed in the same manner. In endosperm, each accumulated in ER-derived prolamine protein bodies, but also in glutelin protein storage vacuoles, even though glutelins are known to pass through the Golgi apparatus en route to these organelles. This finding may suggest that similar mechanisms are involved in the sorting of reticuloplasmins and rice seed storage proteins. However, the presence of reticuloplasmins in protein storage vacuoles could also be due to simple dispersal into these compartments during protein storage vacuole biogenesis, before glutelin deposition. In callus and leaf mesophyll cells, both reticuloplasmins accumulated in ribosome-coated vesicles probably derived directly from the rough ER.

Plant proteins targeted to the secretory pathway can be directed to many different intracellular sites, including the endoplasmic reticulum (ER), Golgi apparatus (GApp), vacuoles, tonoplast, and plasma membrane. They may also be directed to the extracellular space (apoplasma) or cell wall matrix, as well as to the external environment. Therefore, the secretory pathway is of fundamental importance, not only for those proteins destined ultimately to be secreted, but also for those with functions in the compartments en route. The ER is the point of entry into the secretory pathway, and proteins targeted to the ER are distinguished from cytosolic proteins by the presence of an N-terminal signal peptide that associates with a ribonucleoprotein structure called the signal recognition particle (for review, see Vitale and Denecke, 1999). The signal recognition particle enables cytosolic ribosomes to bind to the ER membrane, and proteins destined for the secretory pathway are cotranslationally translocated into lumen of the ribosome-coated (rough) ER, a process conserved among all eukaryotes (for review, see Galili et al., 1998). Once inside the ER lumen, the signal peptide is removed from the nascent polypeptide and the cleaved polypeptide associates with ER-resident enzymes and molecular chaperones that help to fold it into its native conformation. Other posttranslational modifications, such as multisubunit assembly and glycosylation, also begin in the ER lumen, with the assistance of molecular chaperones.

Many proteins entering the ER have a particular function at specific points in the secretory pathway. Such proteins are targeted to their intended destinations by signals within the polypeptide chain, in the form of specific amino acid sequences or particular structural determinants (for review, see Neuhaus and Rogers, 1998). For example, most soluble, ER-resident proteins (known as reticuloplasmins) contain a C-terminal retrieval signal comprising the consensus tetrapeptide H/KDEL, allowing the proteins that escape to the GApp to be taken back into the ER (for review, see Vitale and Denecke, 1999).

In plants, the ER system appears more versatile than in animals and yeast. The plant ER is, for example, important for the biogenesis of protein storage organelles and oil bodies. Furthermore, the ER plays an important role in cell-cell communication because...
it can form a continuous network among cell populations through plasmodesmata (for review, see Staehelin, 1997). Investigation of the ER system has revealed a complex collection of distinct subdomains that correspond to particular cellular functions. Therefore, the secretory pathway of plants has been the subject of intensive investigation, particularly with respect to seed storage proteins because of their nutritional importance for both plants and animals (for review, see Müntz, 1998). Furthermore, because the stable expression of heterologous proteins at high levels can depend on their accumulation in appropriate organelles, such studies may also assist the optimization of recombinant protein synthesis in transgenic plants. In this respect, much remains to be learned about the functional organization of the ER, e.g., pertaining to the distribution of reticuloplasmins and the mechanisms of protein trafficking from the ER along the rest of the secretory pathway. Ultrastructural studies have shown that reticuloplasmins, such as molecular chaperones, can also be found in post-ER compartments of the seed, such as protein bodies (PBs) or protein storage vacuoles (Levanony et al., 1992; Robinson et al., 1995). The presence of chaperones such as binding protein (BiP) in PBs or protein storage vacuoles (PSVs; where storage proteins are assembled) raises questions about the functional significance of chaperone distribution in post-ER compartments.

To determine how reticuloplasmins are distributed among the post-ER compartments and whether this distribution is governed entirely by their function, or whether passive diffusion also plays a role, we investigated the distribution of two reticuloplasmins: a recombinant mammalian protein targeted to the ER (an artificial reticuloplasmin, with no function in the plant cell) and a native plant reticuloplasmin with an important biological role. The artificial reticuloplasmin was a single-chain antibody fragment (scFvT84.66) carrying the KDEL signal (scFvT84.66-KDEL) for ER retrieval (Torres et al., 1999; Stöger et al., 2000). ScFvT84.66-KDEL is derived from mAbT84.66, a monoclonal antibody specific for the NA3 epitope of the carcinoembryonic antigen (Tsang et al., 1995). We compared its distribution with that of the ER-resident molecular chaperone calreticulin. Rice (Oryza sativa) was chosen as a model system due to the existence of differential sorting pathways for the two major seed storage proteins, prolamines and glutelins, reflecting the existence of functionally distinct subdomains of the ER (Okita et al., 1998). Prolamines accumulate in PBs inside the rough ER, whereas glutelins accumulate in protein storage vacuoles, and are conveyed to these organelles by transport vesicles budding from the GApp. We also followed the distribution of reticuloplasmins in two other tissues, leaf and callus, allowing us to investigate any possible links between the distribution of reticuloplasmins and the metabolic function of the plant tissue. We showed that the artificial and native reticuloplasmins have identical distribution profiles within the ER network of each tissue. In endosperm, both reticuloplasmins are found alongside prolamine PBs. However, they are also found alongside glutelins in PSVs. Glutelins pass through the GApp before accumulating in PSVs; therefore, the presence of reticuloplasmins in such vacuoles was unexpected. We discuss the implications of our results in terms of functional subdivision of the ER network in plants, and possible mechanisms by which the accumulation of reticuloplasmins in post-ER organelles could occur.

RESULTS

Subcellular Localization of scFvT84.66-KDEL and Calreticulin in the Endosperm of Rice Seeds

Ultrastructural analysis of rice endosperm from 14 d after anthesis (DAA) immature embryos revealed an extensive cisternal ER network distributed homogeneously through the cytoplasm. Endosperm cells also contained a large number of membrane-delimited structures filled with electron dense deposits resembling glutelin and prolamine PBs (Fig. 1). The fixation method using 4% (w/v) formaldehyde was chosen to compromise between a good structural preservation and antigen preservation. Fixation using 2% (w/v) glutaraldehyde with 4% (w/v) formaldehyde permitted a slightly better preservation of the tissues (Fig. 2) but was not compatible with immunogold labeling. To provide the best possible structural preservation, the processing of the samples was carried out at low temperature. With such a fixation protocol, the classical structures of the different tissues analyzed were clearly recognizable.

Immunogold labeling showed that the antibody fragment did not accumulate in the cisternal ER, but did accumulate in the two types of PBs observed. The labeling was visible inside spherical bodies delimited by ribosome-associated membranes (Fig. 3) previously described by others as prolamine PBs (Bechtel and Juliano, 1980; Krishnan et al., 1986; Li et al., 1993a). The recombinant antibody fragment was also localized in dense, irregularly shaped structures (Fig. 3), resembling glutelin PBs (Krishnan et al., 1986).

The distribution of scFvT84.66-KDEL in the endosperm secretory system was further characterized by double immunogold labeling with antisera specific for the ER-resident chaperone calreticulin. The secondary antibody used to visualize calreticulin was coupled to 15-nm gold particles to distinguish these from the 10-nm particles used to label scFvT84.66-KDEL. This experiment showed that calreticulin was also found predominantly in prolamine and glutelin PBs (Fig. 4A). Labeling of these PBs with the anticalreticulin antiserum was observed not only in the endosperm of transgenic seeds but also in the endosperm of wild-type seeds (Fig. 4B). A light labeling
for calreticulin was also observed inside the cisternal ER (Fig. 4A).

Double labeling for scFvT84.66-KDEL and the storage proteins prolamine and glutelin was used to characterize the distribution of the recombinant antibody in protein storage organelles. Again, 15-nm gold particles were used to label the storage proteins.

Double immunogold labeling for scFvT84.66-KDEL and prolamine showed that the 10- and 15-nm gold particles colocalized in the spherical structures...
thought to be prolamine PBs (Fig. 5A). This result confirmed that the recombinant antibody had accumulated in prolamine PBs, which are known to be formed inside the ER. In addition, double labeling for scFvT84.66-KDEL and glutelin showed colocalization of 10- and 15-nm gold particles in the amorphous structures thought to represent glutelin PBs (Fig. 5B). Finally, Golgi-associated vesicles (SV-GApps) that were clearly labeled by the antiglutelin antiserum (Fig. 5C) were not labeled with the antiprolamine (Fig. 5A), the anti-scFvT84.66 (Fig. 5, A and C), or the anticalreticulin antisera. These vesicles appeared closely associated with lamellar structures (Fig. 5C) clearly identified as belonging to the GApp using a rabbit anti-Lewis antiserum (Fitchette et al., 1999; data not shown). No background was observed for the scFvT84.66-KDEL antibody and no labeling of scFvT84.66-KDEL was observed in wild-type samples.

Localization of scFvT84.66-KDEL and Calreticulin in Callus and Mesophyll Cells

Callus and leaf mesophyll cells were also examined to determine the distribution of scFvT84.66-KDEL and the endogenous reticuloplasmin in non-storage tissues. Callus cells had a dense cytoplasm rich in ribosomes, but only a sparse cisternal ER and Golgi network (Fig. 6A). They also contained numerous vesicles filled with dense deposits (Fig. 6B). These vesicles appeared to form a network within the cytoplasm and we observed interconnection between vesicles, suggesting that large vesicles might be formed by the fusion of smaller ones (Figs. 6B and

Figure 2. Section of an endosperm cell from tissue fixed with a mixture of 4% (w/v) formaldehyde and 2% (w/v) glutaraldehyde. The addition of glutaraldehyde in the fixation solution allowed a slightly better fixation of the tissue in comparison with a fixation protocol using only formaldehyde as for the tissue shown in Figure 1. However, the use of glutaraldehyde was not compatible with immunogold labeling. A, Amyloplast; CER, cisternal ER; Glu-PB, glutelin PB; n, nucleus; Pro-PB, prolamine PB. Bar = 0.5 μm.
7A). Ribosomes appeared associated with the membrane delimiting these vesicles in certain areas (Figs. 6B and 7A). The labeling of scFvT84.66-KDEL on sections from transgenic material was restricted to the vesicles (Fig. 6B), whereas no labeling was observed in the cisternal ER or in other cell compartments.

In the mesophyll cells, most of the cytosolic volume was occupied by a single large vacuole, whereas other organelles were restricted to the cell periphery. The mesophyll cells, unlike those of callus tissue, contained fully differentiated chloroplasts (Fig. 6C). Vesicles filled with electron-dense deposits, similar to those seen in the callus cells, were present in the mesophyll. However, the mesophyll vesicles did not appear to form extensive interconnections, so that the vesicle size was more homogeneous. Furthermore, the vesicles generally appeared in close proximity to plasmodesmata (Fig. 6C). Immunolabeling with scFvT84.66-specific antiserum revealed that the scFvT84.66-KDEL had accumulated in the same type of vesicles as seen in callus cells (Fig. 6, B and C). Gold particles were also observed occasionally between the inner and outer membranes of the nucleus (Fig. 6D).

Double labeling using anti-scFvT84.66 and anticalreticulin antisera clearly showed colocalization of the two proteins in the vesicles present in callus (Fig. 7A) and mesophyll cells (Fig. 7B).

DISCUSSION

Distribution of Reticuloplasmins in the Cisternal ER of Endosperm Cells

Endosperm cells contained an abundant cisternal ER network, very different from the limited network seen in callus and leaf cells as discussed below. This probably reflected the differential metabolic activity of the tissues: Developing seeds are engaged in intensely active protein biosynthesis, requiring a more extensive ER network, whereas the other tissues we examined would be expected to show less active protein biosynthesis. Despite the abundance of the cisternal ER, there was a consistent lack of scFvT84.66-KDEL labeling, although some light labeling for calreticulin was observed. It is unlikely that this reflects genuinely different sorting of the endogenous reticuloplasmin and recombinant antibody. Rather, it is likely that some scFvT84.66-KDEL does remain in the cisternal ER, but at a level that is below the detection threshold of the immunological technique used. The detection of only small amounts of calreticulin probably indicates that the resolution...
of our detection methodology only allows the detection of soluble proteins when they concentrate into aggregates such as in PBs or PSVs.

**Accumulation of Reticuloplasmins in Prolamine PBs**

Immunogold labeling of endosperm tissue clearly showed accumulation of the recombinant antibody inside prolamine PBs. We propose that the antibody is incorporated into the PBs due to its presence in the subdomain of the ER from which the PBs arise. The PBs are formed inside the ER following aggregation of prolamine in the lumen, with no specific transport mechanism involved (Li et al., 1993a). The native chaperone calreticulin was also detected inside the prolamine PBs. Considering the strong biological and physical associations between calreticulin and another soluble chaperone BiP (Crofts et al., 1998), the presence of calreticulin inside prolamine PBs is in accordance with the presence of BiP in these structures as previously shown (Li et al., 1993b; Muench et al., 1997). The presence of BiP at the periphery of prolamine PBs has been linked clearly with its involvement in the biogenesis of these structures through a role in polypeptide translocation across the ER membrane and polypeptide folding. The presence of another chaperone, calreticulin, also involved in protein folding, is also likely to reflect its biological role. However, the accumulation of a recombinant mammalian protein such as scFvT84.66-KDEL inside prolamine PBs almost certainly reflects a passive trapping phenomenon during PB biogenesis because this protein has no function in the plant cell.
Accumulation of Reticuloplasmins in Glutelin Protein Storage Vacuoles

The detection of scFvT84.66-KDEL in glutelin storage vacuoles was unexpected because glutelins are known to pass through the GApp before deposition into vacuoles (for review, see Muench and Okita, 1997). The colocalization of scFvT84.66-KDEL with glutelins could reflect imperfect retention of the recombinant antibody, leading to a partial release from...
the ER despite the presence of a KDEL signal. H/KDEL retrieval signals are sufficient to confer ER localization when fused to non-ER proteins but their effectiveness can vary (Zagouras and Rose, 1989; Herman et al., 1990; Denecke et al., 1993; Pueyo et al., 1995; Gomord et al., 1997). It is possible that adjacent sequences or the overall three-dimensional structure of the protein may influence retention efficiency by influencing the accessibility of the H/KDEL signal to the receptors involved in the retrieval mechanism.

We consider this “leaky retention” model insufficient to explain the presence of scFvT84.66-KDEL in PSVs because the endogenous reticuloplasmin, calreticulin, was also localized to glutelin storage vacuoles. The presence of calreticulin in glutelin PSVs is not associated with the expression of scFvT84.66-KDEL because the same localization pattern was observed in wild-type material. There have been no reports of leaky retention of calreticulin except when the protein was heavily overexpressed, in which case

Figure 6. Immunolocalization of scFvT84.66-KDEL in rice callus and leaf mesophyll cells. A, Ultrastructure of callus cells at low magnification; B, higher magnification picture showing a network of small vesicles surrounded by ribosome-coated membranes (see arrows) where scFvT84.66-KDEL accumulates. C, In mesophyll cells, scFvT84.66-KDEL labeling is detected in similar vesicles that are found mainly in close proximity to plasmodesmata. D, Section of mesophyll cell revealing gold particles between the inner and outer membranes of the nucleus (see arrows). C, Chloroplast; CT, cytosol; CW, cell wall; LV, lytic vacuole; N, nucleus; NE, nuclear envelope; PLA, plasmodesmata; Vs, vesicles. Bar = 0.5 μm.
it is likely that the retrieval machinery in the cell became saturated (Crofts et al., 1999). In our case, protein overexpression cannot explain the presence of both native and artificial reticuloplasmins inside post-ER compartments. In the transgenic lines, scFvT84.66-KDEL was expressed at only 30 μg g⁻¹ fresh weight in endosperm, corresponding to 0.1% of the total soluble proteins, which is too low to be described as “overexpression” (Stöger et al., 2000). In addition, there is no indication that the distribution of calreticulin is linked to its overexpression in transgenic tissue expressing scFvT84.66-KDEL because the distribution pattern of the chaperone was found to be identical in corresponding wild-type tissues.

Our data could suggest the existence of a Golgi-independent route to PSV formation in rice endosperm. Such a pathway has been proposed for prolamine accumulation in developing wheat (Triticum aestivum) endosperm (Levanony et al., 1992) and progblobulin accumulation in maturing pea (Pisum sativum) and pumpkin (Cucurbita pepo) seeds (Robinson et al., 1995; Hara-Nishimura et al., 1998). Wheat prolamines and pea and pumpkin globulins are known to pass through the GApp, but the presence of BiP in the resulting PSVs has led to suggestions that an alternative pathway may bypass the Golgi. Like wheat prolamines and most pea and pumpkin globulins, rice glutelins are not glycosylated (Shewry et al., 1995; Müntz, 1998) and passage through the Golgi may not be obligatory for correct posttranslational modification. Despite these reports, we found no direct evidence for an alternative pathway for glutelin transport. For example, we did not observe the presence of storage protein aggregates inside the ER lumen, as described for pea and pumpkin, or specialized transport vesicles closely associated with the ER, as described for pumpkin. Another possible explanation is the incorporation of both native and artificial reticuloplasmins in PSVs independently from glutelin transport. Considering the interconnection between the different ER subdomains, it is conceivable that some reticuloplasmins could escape their site of synthesis—the rough ER—by a simple dispersal phenomenon. This would allow them to reach adjacent regions of the smooth ER from where PSVs are thought to originate (Staehelin, 1997). The absence of labeling for scFv and calreticulin inside Golgi-associated vesicles, which appear to transport glutelin from the Golgi to the PSVs, could represent indirect evidence for the independent transport of glutelins and reticuloplasmins to PSVs. We cannot, however, eliminate the possibility that calreticulin and scFvT84.66-KDEL could be present...
in these vesicles at a level below the detection limit of the immunological technique used.

**Distribution of Reticuloplasmins in Callus and Leaf Mesophyll Cells**

In callus and leaf mesophyll, there was a much-reduced cisternal ER network reflecting the lower metabolic activity of the callus and leaf mesophyll compared with endosperm. In both tissues, comparison of the distribution of scFvT84.66-KDEL and calreticulin indicated colocalization of the proteins in ribosome-coated vesicles, and their absence from the limited cisternal ER. In a few mesophyll samples, the antibody was also detected inside the nuclear envelope, confirming the continuity of this nuclear domain with the ER in many cell types (Staehelin, 1997). This phenomenon of nuclear retention, which is attributable to partial redirection due to the retrieval effect of KDEL, has been described for other proteins fused to KDEL, such as a recombinant phytohaemagglutinin (Herman et al., 1990) and overexpressed calreticulin (Crofts et al., 1999).

In callus and leaf mesophyll cells, vesicles were the main sites of scFvT84.66-KDEL and calreticulin accumulation. These structures were filled with electron-dense deposits, and in callus cells they appeared to fuse together to generate larger structures. Both these observations indicated a role in protein deposition. In mesophyll cells, the presence of a large central vacuole may have prevented the fusion of these smaller vesicles. Although probably involved in protein deposition, these structures were not typical PSVs because PSVs are only found in vegetative tissues specially adapted for protein accumulation (for review, see Marty 1999). Moreover, the presence of calreticulin inside the vesicles, and of ribosomes coating their membrane in some locations, suggests the vesicles are derived directly from the rough ER and are involved in protein synthesis as well as deposition.

Protein accumulation in the rough ER has been reported in vegetative tissues following the overexpression of recombinant proteins, leading to the formation of ER-derived PBs (Wandelt et al., 1992; Bagga et al., 1995, 1997). However, the vesicles we observed are not generated in response to scFvT84.66-KDEL expression because identical structures were present in wild-type mesophyll cells. We are unaware of previous reports describing the emergence of organelles from the ER of vegetative tissues, with the exception of specific examples concerning rubber and oil bodies (Gallili et al., 1998). The identification of ER-derived organelles combining synthetic and storage activities in callus and mesophyll cells is an interesting finding that could add to the already extensive list of functional ER subdomains (Staehelin, 1997).

In summary, we have investigated the distribution of an artificial reticuloplasmin, a recombinant single-chain antibody carrying a KDEL signal for ER retrieval, in the secretory pathway of rice endosperm and two other tissues, callus and leaf. We showed that the distribution of the antibody was very similar to that of the endogenous reticuloplasmin calreticulin, whose function is of great importance to the plant cell. We found that the antibody and calreticulin were deposited into both ER-derived prolamine PBs and glutelin PSVs, but we did not find any clear evidence for an alternative glutelin transport pathway bypassing the Golgi that could explain the presence of reticuloplasmins in post-ER compartments. Therefore, it is possible that the simple dispersal of reticuloplasmins within the ER network, and more precisely within the smooth ER, could lead to their trapping inside emerging PSVs. The codistribution of the artificial reticuloplasmin and calreticulin strongly indicates that the distribution of reticuloplasmins within, and even outside, the ER network is not necessarily driven by their biological function, but could also result to some extent from a simple dispersal phenomenon.

**MATERIALS AND METHODS**

**Plant Material**

Transgenic rice (*Oryza sativa*) callus cultures and plants expressing the single chain recombinant antibody fragment scFvT84.66-KDEL were generated by particle bombardment as described previously (Torres et al., 1999; Stöger et al., 2000). The transformation construct was designed such that the recombinant antibody was expressed under the control of the constitutive maize ubiquitin-1 promoter (plus first intron) and contained an N-terminal signal peptide derived from the murine immunoglobulin heavy-chain cDNA, and a C-terminal ER retrieval signal, KDEL. These two peptides ensured that recombinant antibodies were targeted to the secretory system and retained in the ER lumen.

**Specimen Processing for Electron Microscopy**

Immunolocalization experiments were performed on ultrathin tissue sections from plant material isolated from our best expressing scFvT84.66-KDEL transgenic lines (Torres et al., 1999; Stöger et al., 2000). Endosperm was dissected from developing immature seeds, 14 DAA. Callus tissue was sampled after 3 weeks in subculture, and leaves were harvested when fully expanded. Controls were taken from wild-type plant tissue at the equivalent developmental stages.

Tissue samples were cut into small pieces with a razor blade in phosphate-buffered saline (PBS) buffer (140 mM NaCl, 3 mM KCl, 4 mM Na2HPO4, and 2 mM KH2PO4 pH 7.4). They were then fixed overnight in 4% (w/v) formaldehyde in PEM buffer (50 mM PIPES [1,4-piperazinediethanesulfonic acid]/KOH, pH 6.9; 5 mM EGTA, and 5 mM MgSO4, pH 7.4) at 4°C. After washing in PBS, the tissue samples were transferred to 30% (v/v) ethanol for 1 h at 4°C and...
then to a cold box (Thor Industrial Cryogenics, Oxford) for further processing at −20°C. The specimens were dehydrated through an ethanol series (50%, 70%, and 100% [v/v], with a 1-h incubation in each solution) and infiltrated with LR white resin containing 0.5% (w/v) benzoin methyl ether as the catalyst (London Resin Company Ltd., Berkshire, UK). The resin was diluted 1:1, 1:2, and 1:3 (ethanol:resin, v/v) and a 1-h incubation was allowed for each dilution. The specimens were then incubated overnight in undiluted resin and transferred to precooled Beem capsules (Agar Scientific, Stansted, UK) filled with freshly prepared resin. The resin was allowed to polymerize for 24 h under indirect UV light at −20°C followed by 16 h under indirect UV light at room temperature. From these specimens, 100-nm sections were prepared using an Ultratcut E ultramicrotome (Leica Microsystems, Wetzlar, Germany). The sections were collected on 4% (w/v) pyroxylon and carbon-coated 200-mesh grids. Ultrastructural observations were carried out using a JEOL 1200 transmission electron microscope running at 80 kV.

Single Immunogold Labeling

The grids were initially floated on drops of PBS and then transferred to 3% (w/v) bovine serum albumin in PBS for 5 min at room temperature to block nonspecific binding sites. The grids were then incubated for 1 h at room temperature with anti-scFvT84.66, anticalreticulin, antiprolamine, or antiglutelin antisera applied at dilutions of 1:400, 1:2,500, 1:30,000, and 1:40,000, respectively, in PBS. Anti-scFvT84.66 antiserum was obtained from immunized chickens as described (Vaquero et al., 1999). Antiprolamine and antiglutelin antisera were kindly provided by Dr. Thomas Okita (University of Washington, Pullman) and were obtained from rabbits injected with rice prolamines or rice glutelins, respectively (Krishnan and Okita, 1986). Anticalreticulin antiserum from rabbits was a gift from Dr. Jürgen Denecke (University of Leeds, UK) and was obtained as described (Crofts et al., 1999). To remove any cross-reacting antibodies prior to the labeling procedure, the antisera were preabsorbed using an acetone extract of proteins from wild-type tissue according to Sambrook et al. (1989). We then carried out controls by western blots on protein extracts from all three types of tissue obtained from wild-type rice plants to confirm the absence of any cross-reaction of the antisera with rice proteins other than the ones against which they were directed. After incubation in the appropriate primary antiserum, the grids were rinsed briefly in PBS and transferred to the appropriate rabbit anti-chicken or goat anti-rabbit secondary monoclonal antibodies conjugated to 10-nm-diameter gold particles (diluted 1:25 in PBS) to label the antiglutelin, antiprolamine, or anticalreticulin primary antibodies. The grids were rinsed in PBS and incubated for a further 45 min at room temperature with the secondary anti-chicken antibody conjugated to 10-nm gold particles (diluted 1:25 in PBS) to label the anti-scFvT84.66 primary antibodies. The grids were then washed and stained as described above.

Double Immunogold Labeling

Double immunogold labeling experiments were carried out using a combination of anti-scFvT84.66 antisera and either the antiprolamine, antiglutelin, or anticalreticulin antisera. The same concentrations and incubation conditions were used as described for the single labeling experiments above. After washing off the primary antisera with PBS, the grids were incubated for 45 min at room temperature with the secondary goat anti-rabbit antibody conjugated to 15-nm gold particles (diluted 1:25 in PBS) to label the antiprolamine, antiglutelin, or anticalreticulin primary antibodies. The grids were rinsed in PBS and incubated for a further 45 min at room temperature with the secondary rabbit anti-chicken antibody conjugated to 10-nm gold particles (diluted 1:25 in PBS) to label the anti-scFvT84.66 primary antibodies. The grids were then washed and stained as described above.

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