**Isolation of GFP-labeled endoplasmic reticulum (GFP-ER) from tobacco cultured cell, BY-2.**

To visualize the endoplasmic reticulum (ER) in tobacco cultured cell BY-2, the cells which are transformed with a chimeric gene constructed from the signal peptide of pumpkin 2S albumin, GFP and the ER-retention signal (HDEL) have been prepared and shown a strong and stable fluorescence on the ER network (Mitsuhashi et al., 2000). As reported in various plant cells, the distribution, morphology and behavior of GFP-ER in BY-2 cells also alter spatially and temporally. In interphase cells, GFP-ER is distributed throughout cytoplasmic strands emanating from the nuclear region and penetrating the vacuole (Fig. 1A). The GFP-ER in cytoplasmic strands moves and streams actively with an average velocity of about 7 μm/sec ranging from 4 to 9 μm/sec. In the cortical region, GFP-ER is arranged into mesh-like structures consisting of tubes and sacks (Fig. 1B), which are less mobile, but show shrinking, elongation and contraction-like motions. These movements and streaming are suppressed by actin-depolymerization drugs, but not by microtubule-depolymerization ones, indicating that the actin cytoskeleton is involved in these motions of ER. In mitotic cells, some portions of GFP-ER gather in the spindle (Fig. 1C), at the equatorial plane in the phragmoplast (Fig. 1D) and around daughter nuclei (Fig. 1D). Because the ER-retention signal peptide, HDEL, is incorporated into nuclear envelopes, perhaps due to the fusion of ER into them (Napier et al., 1992), bright GFP signals are also observed around nuclei (Fig. 1E and F).

In this section, the isolation procedure of GFP-ER from BY-2 cells not synchronized is introduced. The following procedures are carried out at 0 to 4 $^{o}$C. A solution A containing 8 mM EGTA, 1 mM $MgCl_2$, 1% casein, 0.3 M sucrose, 0.5 mM DTT, 1 mM PMSF, 50 μg/ml leupeptin and 40 mM PIPES-KOH (pH 7.0) is chilled on ice and centrifuged at 27,000xg for 5 min to remove undissolved casein. Three volumes [?] of the solution A are added to one volume of protoplasts prepared from BY-2 cells. The mixture is homogenized with a hand-operated Downs homogenizer. The homogenization is performed carefully, with observation and checking of the degree of breakage of the protoplasts using light microscopy. The homogenate is first centrifuged at 500xg for 3 min. The supernatant is further centrifuged at 12,000xg for 10 min. The resultant supernatant containing microsomal and cytosol fractions is applied on 0.6 M, 1.0 M and 1.5 M discontinuous sucrose density gradient made up in a solution B containing 5 mM EGTA, 1 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 50 μg/ml leupeptin and 30 mM PIPES-KOH (pH 7.0), and centrifuged at 86,000xg for 1.5 hr. Interfaces between 0.6 M and 1.0 M, and 1.0 M and 1.5 M sucrose are recovered and analyzed using fluorescent microscopy or immunoblotting with an antibody against GFP. Almost [all?] of GFP-ER are recovered in the interface between 1.0 and 1.5 M sucrose (GFP-ER fraction; Fig. 1G). To further remove cortical proteins, 3 M KCl or KI are added to ER fraction at final concentrations of 0.5 M or 0.4 M, respectively. The mixture is stood on ice for 10 to 20 min and then centrifuged at 140,000xg for 20 min. The pellet is suspended in

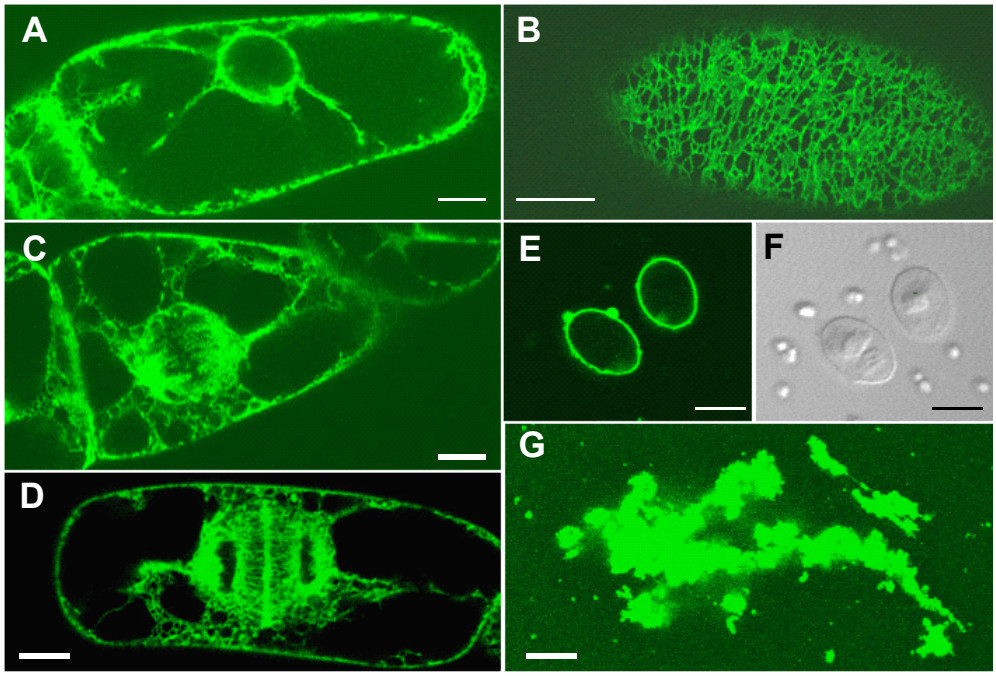
the solution B supplemented with 0.3 M sucrose with a glass-Teflon homogenizer.

References

Mitsuhasi N, Shimada T, Mano S, Nishimura M, Hara-Nishimura I (2000) Characterization of organelles in the vacuolar-sorting pathway by visualization with GFP in tobacco BY-2 cells. Plant Cell Physiol. 41: 993-1001.

Napier RM, Fowke LC, Hawes C, Lewis M, Pelham HRB (1992) Immunological evidence that plants use both HDEL and KDEL for targeting proteins to the endoplasmic reticulum. J. Cell Sci. 102: 261-271.

Fig. 1




The distribution of GFP-ER in loving BY-2 cells and isolated GFP-ER.

A, GFP-ER around nucleus and in cytoplasmic strands emanating from nuclear region in an interphase cell. B, GFP-ER in the cortical region in an interphase cell. C, GFP-ER in the spindle in a metaphase cell. D, GFP-ER in the phragmoplast in a telophase cell. The GFP-ER accumulates in the equatorial plane of phragmoplast and around daughter nuclei. E, GFP signals in nuclear envelops in isolated nuclei. F, Nomarski light microscopic image of E. G, isolated GFP-ER by the sucrose density gradient ultra-centrifugation. Bras represents 10 $\mu$ m.