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Ultrastructural localization of Lucifer Yellow and endocytosis in plant cells

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Summary. The fluorescent dye Lucifer Yellow CH (LYCH) was localized at the ultrastructural level with a precipitation method using barium chloride. Applying this technique, endocytosis of LYCH was examined in the nutrient absorptive trichomes of a carnivorous bromeliad. After a two hour incubation, the electron dense reaction product was localized in the membrane compartments of the endocytotic system. These structures included coated regions of the plasma membrane, coated and smooth vesicles, dictyosomes, partially coated reticulum, and smooth endoplasmic reticulum. This procedure demonstrates for the first time at the ultrastructural level endocytosis in whole plant cells, using a non-toxic compound.

Keywords: *Brocchinia reducta* (Bromeliaceae); Coated vesicle; Dictyosome; Endocytosis; Lucifer Yellow.

Abbreviations: ER endoplasmic reticulum; $BaCl_2$ barium chloride; LYCH Lucifer Yellow CH; PCR partially coated reticulum.

Introduction

The endocytotic pathway has been successfully shown at the ultrastructural level in plant cell protoplasts using cationic ferritin (Joachim and Robinson 1984, Tanchak et al. 1984, Tanchak and Fowke 1987, Record and Griffing 1988) and lectin-gold conjugates (Hillmer et al. 1986). The electron dense tracers were seen to enter the endomembrane system from the plasma membrane surface in coated vesicles and migrate to the dictyosome. Also, some evidence for endocytosis in whole plant cells has been shown to occur in roots using toxic heavy metal solutions of lanthanum, lead and uranium salts (Wheeler and Hanchey 1971, Hübner et al. 1985, Romanenko et al. 1986) and in abscising petal cells using latex microspheres (Wiatr 1982).Endocytosis has been speculated to occur in plant cells for some time. However, because the cell wall presents a formidable barrier not encountered by animal biologists, definitive evidence for endocytosis in most plant cells having cell walls is lacking.

Recently, good evidence at the light microscopic level for endocytosis in plants has been obtained using the highly fluorescent dye Lucifer Yellow (Oparka et al. 1988, 1990; Hillmer et al. 1989; Wright and Oparka 1989). This dye has two advantages: it is nontoxic at useful concentrations (Stewart 1981), and does not cross the plasma membrane due to the negative charge on its two sulfonic acid groups (Stewart 1978). However, Lucifer Yellow has been limited in its usefulness in ultrastructural studies due to its lack of electron density. While Lucifer Yellow can be photobleached (Buhl and Lübke 1989) and reacted with diaminobenzidine to form an electron opaque reaction product (Maranto 1982), the deposits are diffuse and precise localization is not possible. We present here a precipitation reaction of Lucifer Yellow with barium chloride that forms an electron dense product for in situ localization studies. The endocytotic pathway chosen to examine is that from the nutrient absorptive hairs of the carnivorous bromeliad *Brocchinia reducta* Baker. These trichomes are known to contain the membranous elements accociated with the pathway (Owen et al. 1988) and to absorb compounds such as amino acids when applied to the leaf surface (Benzing et al. 1985, Owen and Thomson 1988).

Materials and methods

Basal leaf segments of *Brocchinia reducta* were incubated with 1% (w/v) LYCH dipotassium salt (Sigma) on the adaxial surface for 2 h in a humid chamber. Leaf material was prepared for electron microscopy by cutting the incubated tissue into small pieces in 1.25%

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glutaraldehyde containing 40 mM $BaCl_2$ in 50 mM sodium cacodylate buffer at pH 7.0, and fixed for 4.5 h at 20 °C. Some treatments had 80 mM $BaCl_2$ added directly to the LYCH and were fixed in glutaraldehyde without the $BaCl_2$. Next, the tissue was washed in buffer for 20 min, postfixed in cacodylate-buffered 1% OsO_4 overnight at 4 °C, and infiltrated with Spurr's resin (Spurr 1969) after acetone dehydration. Thin sections were cut with an RMC MT6000 ultramicrotome and stained with 2% (w/v) uranyl acetate in either distilled water or 50% ethanol, and followed by lead citrate (Reynolds 1963). Sections were observed using a Philips EM400 operated at 80 kV. As a control, tissue was incubated with distilled water and fixed as above or, alternatively, LYCH incubated material was prepared in fixative without the $BaCl_2$. Test tube reactions were performed by adding equal volumes of 1% LYCH and 80 mM $BaCl_2$ in an Eppendorf tube. After 15 min, the precipitates were collected by centrifugation and prepared as above for electron microscopy. It is important to note that phosphate buffers can not be used as barium precipitates the phosphate.

Selected area electron diffraction patterns were obtained from unstained sections of trichomes that had LYCH-Ba deposits, using an approximate camera length of 450 mm and an accelerating voltage of 100 kV. Diffraction patterns from sections of unstained test tube precipitates were used as a comparison. X-ray microanalysis was performed on identical deposits in similarly prepared cotton roots (unpubl.). Semi-thin sections were mounted on copper grids and analyzed using a Philips 400-EDAX 9100 system, with an accelerating voltage of 60 kV and a take off angle of 24 °.

Results

The localization of LYCH with $BaCl_2$ was predicted based on the chemical formula of LYCH and the chemistry of barium. LYCH has two sulfonic acid groups, which can react with barium ions to form a precipitate. This was shown in the test tube reaction where an orange precipitate immediately formed. Compounds of barium sulfonate are highly insoluble and thus will not dissolve during fixation. Furthermore, barium is known to be opaque when examined in the electron microscope (Hopsu-Havu et al. 1967), and thus is a good marker for cellular localization. Further, when the precipitate formed by the reaction of $BaCl_2$ with LYCH was examined in the electron microscope it was electron dense (not shown).

The ultrastructure of the trichomes has been described previously (Owen et al. 1988). For this study it is important to note that the only place where exogenously applied solutions can enter the trichome is along the outermost cell layer, as the subtending cells are bounded by a thick cuticle (Owen et al. 1988). These outer cap cells are easily identified as they possess an unusual labyrinthine-like cell wall (Fig. 1). The outermost cells of the trichomes contained many dictyosomes, rough and smooth ER, PCR, coated and smooth vesicles, and coated regions of the plasma membrane (Fig. 1). Trichomes incubated with LYCH followed by the addition of $BaCl_2$ before fixation had electron dense deposits within the irregular interstices of the outer cell wall matrix, and adjacent to the plasma membrane (Fig. 2). However, when the material was treated by this procedure the cells were often plasmolyzed. Structural preservation was significantly better when the tissue was fixed with the $BaCl_2$-glutaraldehyde solution; however, LYCH deposits were rarely observed in the cell walls compared to the treat-

Fig. 1. Portion of an outer trichome cap cell which was treated with LYCH and illustrates the surface where LYCH entry occurs (★). Coated invaginations of the plasma membrane (arrows), a dictyosome (*D*) and smooth ER (*ER*) are seen within the cell. *CW* Cell wall. × 20,000

Fig. 2. Outer trichome wall (*CW*) incubated with LYCH and treated with $BaCl_2$ before fixation. The electron dense deposit (arrow) is within the irregular cell wall matrix. × 70,000

Fig. 3. Coated invagination of the plasma membrane in the outer cell of the control tissue (arrow). × 85,000

Fig. 4. LYCH-Ba deposit (arrow) within a coated invagination of the plasma membrane. × 100,000

Fig. 5. Slightly larger LYCH-Ba precipitate (arrow) within a coated plasma membrane invagination. × 100,000

Fig. 6. Coated vesicles and a segment of smooth ER (◀) in the control tissue showing the lack of an electron dense deposit within the lumina. × 95,000

Fig. 7. Coated vesicle from LYCH-treated tissue containing the electron dense LYCH-Ba marker (arrow). × 105,000

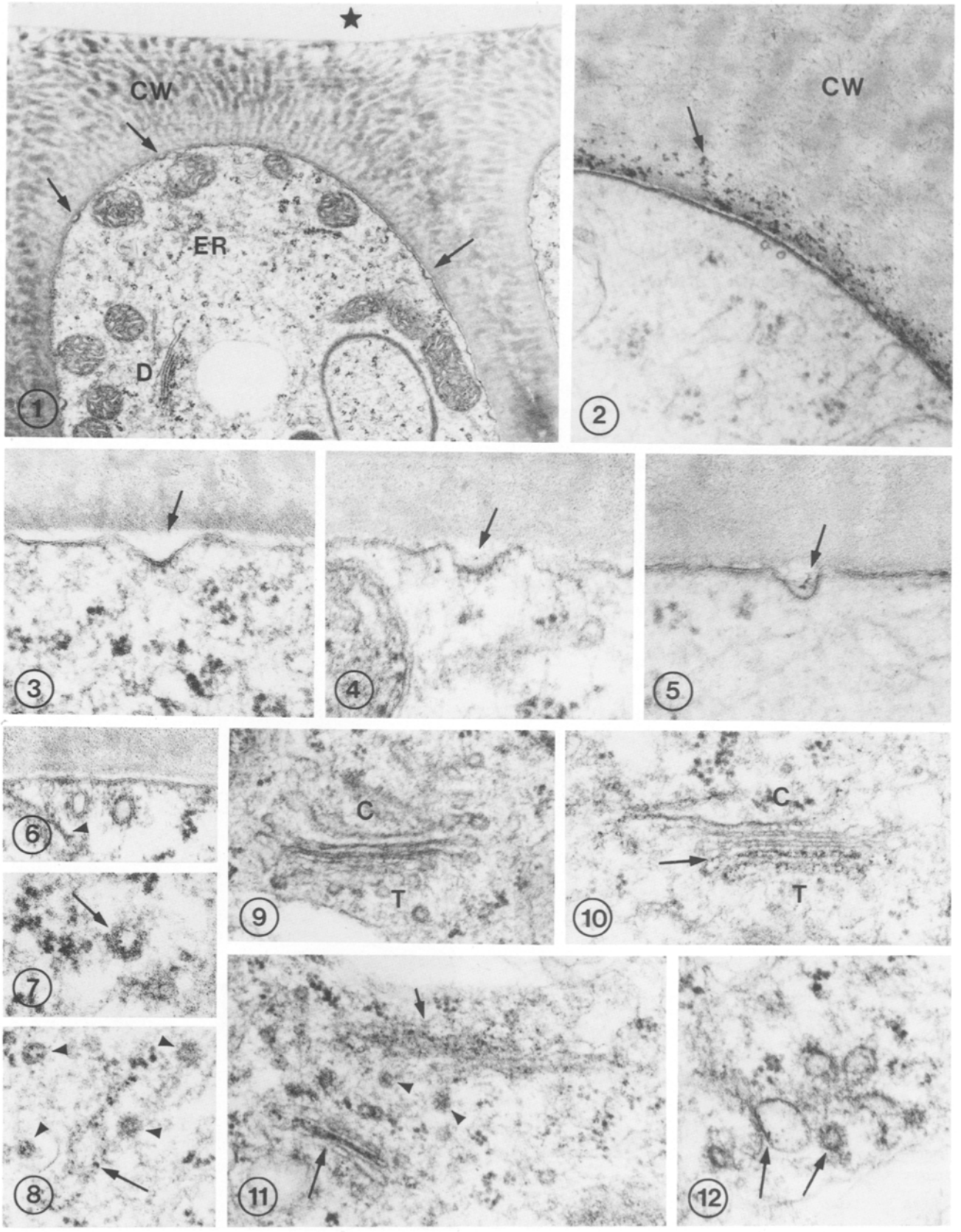
Fig. 8. Four coated vesicles (◀) and a segment of tubular smooth ER (arrow) containing the LYCH-Ba precipitate. The two vesicles on the left of the ER appear only partially coated. × 75,000

Fig. 9. Dictyosome and associated vesicles from the control tissue. *C* Cis-face, *T* Trans-face. × 60,000

Fig. 10. LYCH-Ba localization within the dictyosome cisternae. The marker is deposited throughout the lumina in both the cis- (*C*) and trans- (*T*) faces. × 65,000

Fig. 11. Two LYCH-Ba-labelled dictyosomes (arrows) with associated smooth and coated vesicles (◀). × 60,000

Fig. 12. PCR from a trichome incubated with LYCH. The blebs of the coated regions and reticulum lumen contain the endocytotic marker (arrows). × 75,000


CW
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ment where the precipitation reaction occurred before fixation.

Those trichomes prepared with the fixative containing $BaCl_2$ had many granular, electron dense deposits in compartments of the proposed endocytotic pathway. Electron dense precipitates were seen in coated invaginations of the plasma membrane (Figs. 4 and 5). While similar invaginations in conventionally fixed material often had some electron opacity, the LYCH-$BaCl_2$-treated material had definite electron-dense particles similar to those seen in the cell wall spaces (Fig. 2). Identical deposits were also observed in coated (Figs. 7, 8, and 12) and partially-coated vesicles (Fig. 8) which were either free or associated with dictyosomes (Figs. 11 and 13) and the PCR (Fig. 12) as defined by Pesacreta and Lucas (1985). The dictyosomes had deposits of tracer within the cisternae of both faces (Figs. 10, 11, and 13). Occasionally, similar deposits were seen within tubular and swollen elements of the smooth ER (Figs. 8, 14, and 15). These deposits were not observed in either the trichomes that were incubated with LYCH alone, or in distilled H_2O treated tissue with the glutaraldehyde that contained $BaCl_2$ (Figs. 3, 6, and 9).

Analyses of electron diffraction and X-ray microanalysis were performed on the electron-dense precipitates to confirm their identification. The selected area diffraction pattern from the LYCH-Ba deposits in the tissue had two indistinct rings. A comparison with the identically prepared test tube precipitates showed the diffraction rings exactly matched (Fig. 16) indicating that the two precipitates were identical. The energy-dispersive X-ray microanalysis was performed on cotton roots as larger precipitates were available for analysis compared to the trichome tissue. The analysis showed both barium ($L\alpha_1 = 4.47$, $L\beta_1 = 4.83$, $L\beta_2 = 5.16$) and sulfur ($K\alpha = 2.31$) were present (Fig. 17).

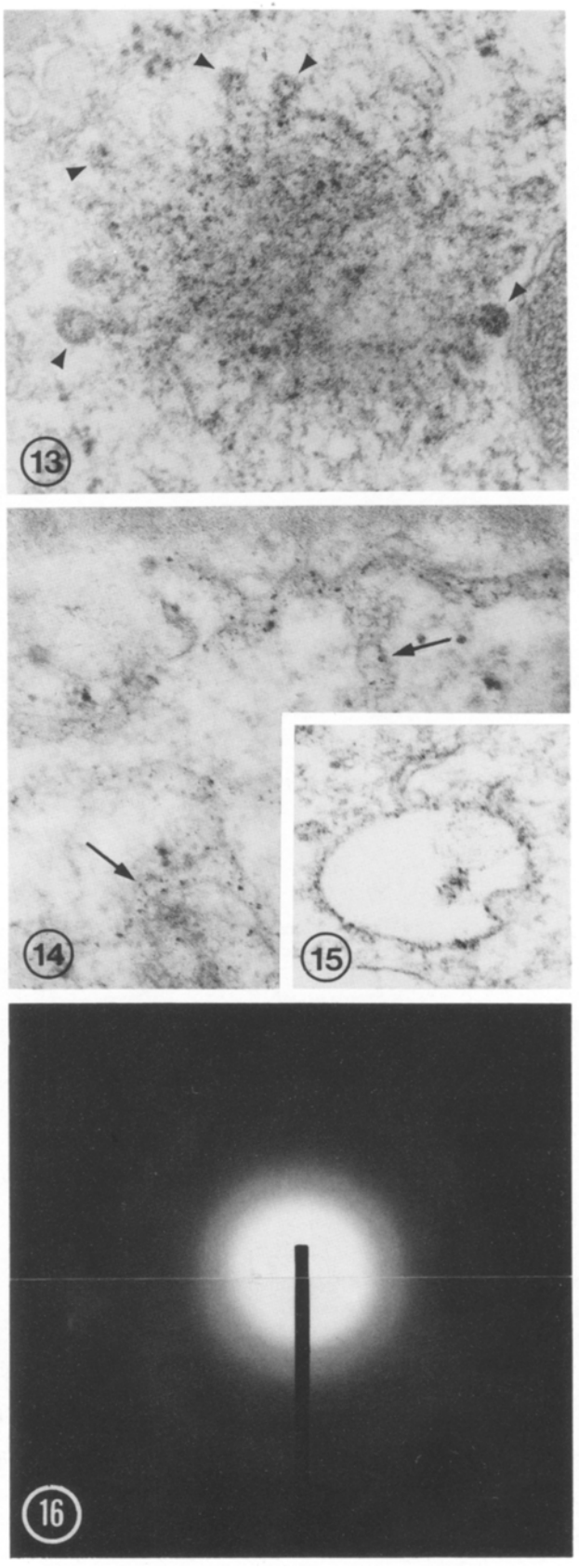

Fig. 13. Glancing section through a dictyosome that is completely labelled with the LYCH-Ba deposits. Also note the associated labelled vesicles (◀). × 85,000

Fig. 14. Smooth ER with LYCH-Ba deposits within the lumen (arrows). × 80,000

Fig. 15. Large, swollen area of smooth ER that had the LYCH-Ba deposit adjacent to the membrane, and in a small cluster within the lumen. × 90,000

Fig. 16. Selected area electron diffraction patterns. The top half is from the electron-dense precipitate within a dictyosome and the lower half is from a precipitate obtained in the test tube control precipitate. The diffraction rings match exactly

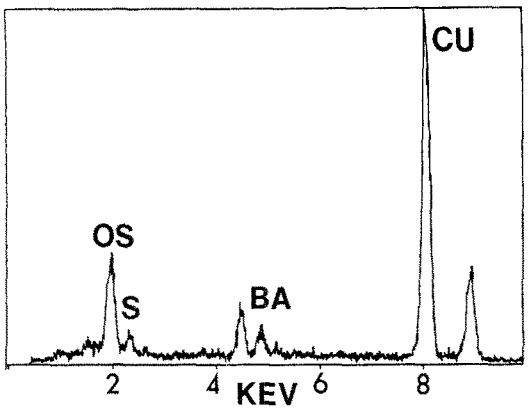

Fig. 17. X-ray microanalysis spectrum from the electron dense precipitate in LYCH and $BaCl_2$-treated cotton roots. Both barium (4.47, 4.83, 5.16 keV) and sulfur (2.31 keV) are present

Discussion

In this study, we demonstrate the fluorescent tracer LYCH can be localized at the ultrastructural level using $BaCl_2$ to show endocytosis in plant cells. This procedure has several advantages: it can be performed on whole plant cells rather than protoplasts as required in other techniques to examine endocytosis (Joachim and Robinson 1984, Tanchak et al. 1984, Tanchak and Fowke 1987), and LYCH is non-toxic (Stewart 1978) thus avoiding possible artefacts when heavy metals are used (Wheeler and Hanchey 1971, Hübner et al. 1985, Romanenko et al. 1986). Further, LYCH binds with glutaraldehyde and the dye does not redistribute during fixation (Stewart 1978). Presumably, LYCH performs in the same manner when reacted with the $BaCl_2$-containing glutaraldehyde. Moreover, the LYCH-Ba marker is easily identified which permits normal staining methods unlike the use of cationic ferritin (Tanchak et al. 1984). The best results were obtained when the $BaCl_2$ was in the fixative. While much of the LYCH appears to have been washed out of the wall spaces when using this procedure compared to the precipitation of LYCH before fixation (Fig. 2), cellular preservation was greatly improved. This may be from either an adverse osmotic stress or a toxic effect on the unfixed cells when $BaCl_2$ was applied directly.

The electron-dense deposits were identified as LYCH-Ba precipitates by selected area diffraction (Fig. 16). The diffraction rings produced from precipitates in the cells matched those from test tube prepared precipitates of LYCH and $BaCl_2$ that were fixed and prepared identically to the tissue. The diffraction pattern had only a few indistinct rings, yet closely resembled published patterns (Arstila et al. 1966). Weak patterns often occur in biological specimens, which is caused by a high background interference and the low amount of material being analyzed (Misell and Brown 1987). While these weak patterns prevent absolute structural determination, they are sufficient to compare against a known control.

Our results confirm studies using plant cell protoplasts (Tanchak et al. 1984) and root cap cells (Hübner et al. 1985) where markers for endocytosis are seen in coated vesicles (Figs. 7 and 8), PCR (Fig. 12), and in peripheral vesicles of the Golgi apparatus (Figs. 11 and 13). However, the localization of LYCH was not restricted to dictyosome associated vesicles but was distributed throughout the length of the cisternae (Figs. 10, 11, and 13). This difference in localization may, in part, be caused by the binding of cationic ferritin to the vesicle membrane (Danon et al. 1972, Ottosen et al. 1980) and not being released into the cisternal lumen. Also, the LYCH-Ba complex was found in both the cis- and trans-faces of the dicytosomes (Fig. 10). This contrasts with Hübner et al. (1985) who found internalized lead deposits only in the trans-face terminal cisternae. Thus, our localization of LYCH within the dictyosome lumina suggests a flow of LYCH through the Golgi apparatus.

There is evidence indicating that endocytosis in plant cells can involve a trafficking via multivesicular bodies to the vacuole (Record and Griffing 1988, Tanchak and Fowke 1987). However, multivesicular bodies were infrequently seen in the cap cells of the trichomes and vacuolar accumulation of the reaction product was not observed. LYCH-Ba deposits, however, were observed in tubules of smooth ER (Figs. 8, 14, and 15) and in smooth vesicles (Fig. 8). It was not possible to discern if the LYCH precipitate localized in the smooth vesicles were in a separate vesicle system, or if the labelled coated vesicles had lost their coating (Altstiel and Branton 1983).

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