

Lucifer Yellow Uptake in Cells and Protoplasts of *Daucas carota* Visualized by Laser Scanning Microscopy

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ABSTRACT

The uptake of lucifer yellow CH by suspension-cultured carrot cells and protoplasts has been studied by laser scanning microscopy. This fluorochrome, which does not diffuse across membranes, gradually accumulates in the cell vacuole over a period of hours. In contrast, the central vacuole of protoplasts did not show lucifer yellow fluorescence. The latter was restricted, in protoplasts, to punctate sources in the peripheral cytoplasm. Confocal optics allowed the complexity of the vacuolar system to be dramatically depicted with the laser scanning microscope. Control experiments support the contention that lucifer yellow uptake, as in other eukaryotic systems, occurs via endocytosis.

Key words: Carrot cells, endocytosis, laser scanning microscopy, lucifer yellow CH, protoplasts, vacuolar apparatus.

INTRODUCTION

The last few years have seen considerable progress in light microscopic techniques. Advances in computer sciences have improved image acquisition and made digital image processing possible (Arndt-Jovin, Robert-Nicoud, Kaufman, and Jovin, 1985). One of the new powerful techniques is confocal laser scanning microscopy (LSM) which allows for optical sectioning of a specimen and electronic adjustment of its contrast (Robert-Nicoud, Arndt-Jovin, Schormann, and Jovin, 1988). The application of LSM in plants has hitherto been restricted to an analysis of the arrangement of cytoskeletal structures and the endoplasmic reticulum (Wijnaendts-van-Resandt, Ihrig, Knebel, and Quader, 1988). By virtue of its confocal optics, details that are otherwise hidden in conventional fluorescence microscopy due to fluorescent signals emanating from surrounding structures, can be visualized clearly in LSM (Wilson and Sheppard, 1984).

Lucifer yellow CH (LY) is a highly fluorescent dye (Stewart, 1981), which does not appear to diffuse across the plasma membrane (Miller, Griffiths, Lenard, and

Firestone, 1983). It has, therefore, found considerable use as a probe for fluid-phase endocytosis in animal cells (Swanson, Yirinec, and Silverstein, 1985; Swanson, Burke, and Silverstein, 1987) and in yeast (Riezman, 1985). This paper presents the results of our investigations on the capacity of carrot cells, protoplasts, and vacuoles to take up LY.

MATERIALS AND METHODS

Cells, preparation of protoplasts, isolation of vacuoles

Suspension-cultured carrot cells (*Daucas carota* L.) were grown as previously described (Andreae, Blankenstein, Zhang, and Robinson, 1988). Cells were harvested during the logarithmic growth phase by filtration and resuspended in fresh White's culture medium (Nickell and Maretzki, 1969).

Protoplasts were prepared exactly as described by Hillmer *et al.* (1986). For the isolation of vacuoles, protoplasts were ruptured osmotically by resuspending for 20 min in 100 mol m⁻³ phosphate buffer, pH 8.0, containing 1.0 mol m⁻³ EDTA (Wagner, 1986). The homogenate was centrifuged for 5 min at 500 × g and the pellet taken up in 1% (v/v) White's culture medium containing 700 mol m⁻³ sucrose. This was layered over

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Abbreviations: DMSO: Dimethyl sulphoxide; FITC: Fluorescein isothiocyanate; GIPS: Göttingen Image Processing System; LSM: Laser scanning microscopy; LY: lucifer yellow; PM: Plasma membrane.

500 mol m⁻³ mannitol (also in 1% White's culture medium) and centrifuged for 5 min at 80 × g. Vacuoles collected at the interface.

Uptake experiments

Cells were incubated in solutions of LY (Fluka, 2.5 mg cm⁻³ dissolved in White's culture medium) at 28 °C on a rotary shaker operating at 120 rev. min⁻¹. Samples were removed after various time periods up to 24 h and washed several times until the washing solution remained colourless.

In control experiments, cells were incubated with LY at 4 °C or prefixed and permeabilized prior to incubation. Fixations were carried out in fresh White's culture medium with 1% (v/v) formaldehyde and 5% (v/v) DMSO for 60 min at room temperature.

LY uptake experiments were also performed on protoplasts (conditions as described above), which were resuspended in White's culture medium containing 500 mol m⁻³ mannitol, 2.5 mg cm⁻³ LY and, in one experiment, cell wall-digesting enzymes in addition. Control experiments with protoplasts were carried out at 4 °C.

Conventional light microscopy

Cells, protoplasts, and isolated vacuoles were observed using either a Zeiss M405 inverted microscope or a Zeiss photomicroscope equipped with an FITC fluorescence filter combination, differential interference contrast and phase contrast optics.

Confocal laser scanning microscopy

Observations were made either with a laser scanning microscope (LSM) of Heidelberg Instruments (Heidelberg, FRG) (Wijnaendts-van-Resandt *et al.*, 1988) or a Zeiss LSM (Carl Zeiss, Oberkochen, FRG) interfaced to the Göttingen Image Processing System (GIPS) (Robert-Nicoud *et al.*, 1988). Both microscopes employed an argon-ion laser for excitation at 488 nm.

RESULTS

Resolution of LY-containing compartments with the laser scanning microscope

Conventional fluorescence microscopy can be used to demonstrate that LY can be taken up by suspension-cultured cells and delivered to the central vacuole. For example, Fig. 1A shows a Normarski image of a typical cell after 18 h in LY. The cytoplasm is closely appressed to the wall, and nuclei (arrowheads) and central vacuoles (asterisks) are easily identifiable. In the corresponding fluorescence image (Fig. 1B) the dye is apparently confined to the central vacuole with only the nuclear regions (arrowheads) and the cross walls being clearly non-fluorescent. However, it is not possible to obtain details of the vacuolar system in such cells by means of conventional fluorescence microscopy (Fig. 1B) or laser scanning microscopy in the *non-confocal* modus (Fig. 1C). In contrast, the small focal depth of the confocal image of the laser scanning microscope shows clearly that LY fluorescence in carrot cells (compare Fig. 1C with 1D) is not restricted to the large central vacuole, but is also present in structures spread throughout the cytoplasm having either a tubular (arrowheads) or a granular (arrows)

appearances (Fig. 1E). The confocal optics of the laser scanning microscope can be used to obtain optical serial sections through these cells (Fig. 2), from which it can be seen that both tubular and granular structures are present in the cortical cytoplasm as well as in the cytoplasm surrounding the nucleus. We are not entirely certain whether, in each case, these structures are extensions of the large central vacuolar apparatus.

Problems encountered in investigating LY uptake in carrot cells by laser scanning microscopy

Suspension-cultured carrot cells are morphologically and physiologically heterogeneous. As a result, there are often differences in fluorescence intensity between individual cells in cultures incubated for long periods (up to 24 h, see Fig. 3A). Even two interconnected cells which had clearly recently divided might often show divergent labelling. Another characteristic of some cells in long-term LY cultures is heterogeneous fluorescence intensity within a single cell. Such cells have a weakly fluorescing vacuole together with more intensely fluorescing tubular and granular structures in the surrounding cytoplasm (Fig. 3B).

Short-term uptake studies

Samples taken at different times during incubation with LY demonstrate how slowly the vacuole accumulates dye during this period. After approximately 2 h in LY a faint fluorescent signal could be detected within some cells. The gradual increase in vacuole fluorescence can best be demonstrated by comparing two extreme stages. Thus, Fig. 4A depicts a cell after 3 h LY incubation and Fig. 4B shows the cell after 18 h incubation. For this comparison the microscope was adjusted for high sensitivity and remained unchanged for both micrographs. Under these conditions the cell in Fig. 4B is not optimally imaged due to the intensity of the fluorescent signal.

Uptake studies with protoplasts

Protoplasts prepared from suspension-cultured carrot cells also had to be incubated for at least 12 h in LY in order to obtain a sufficiently clear fluorescent signal. Despite these long incubation periods LY fluorescence was not detected in the central vacuole, and fluorescence was restricted to punctate sources in the peripheral cytoplasm (Fig. 5A–C). There was no difference between protoplasts incubated in the presence or absence of cell wall-degrading enzymes (cellulase) in the LY incubation medium.

Control experiments

We have made several important observations pertaining to the origin of vacuole-based fluorescence. Firstly, cytoplasmic streaming and structural integrity of LY-treated cells observed under phase contrast optics were indistinguishable from untreated cells, indicating that the

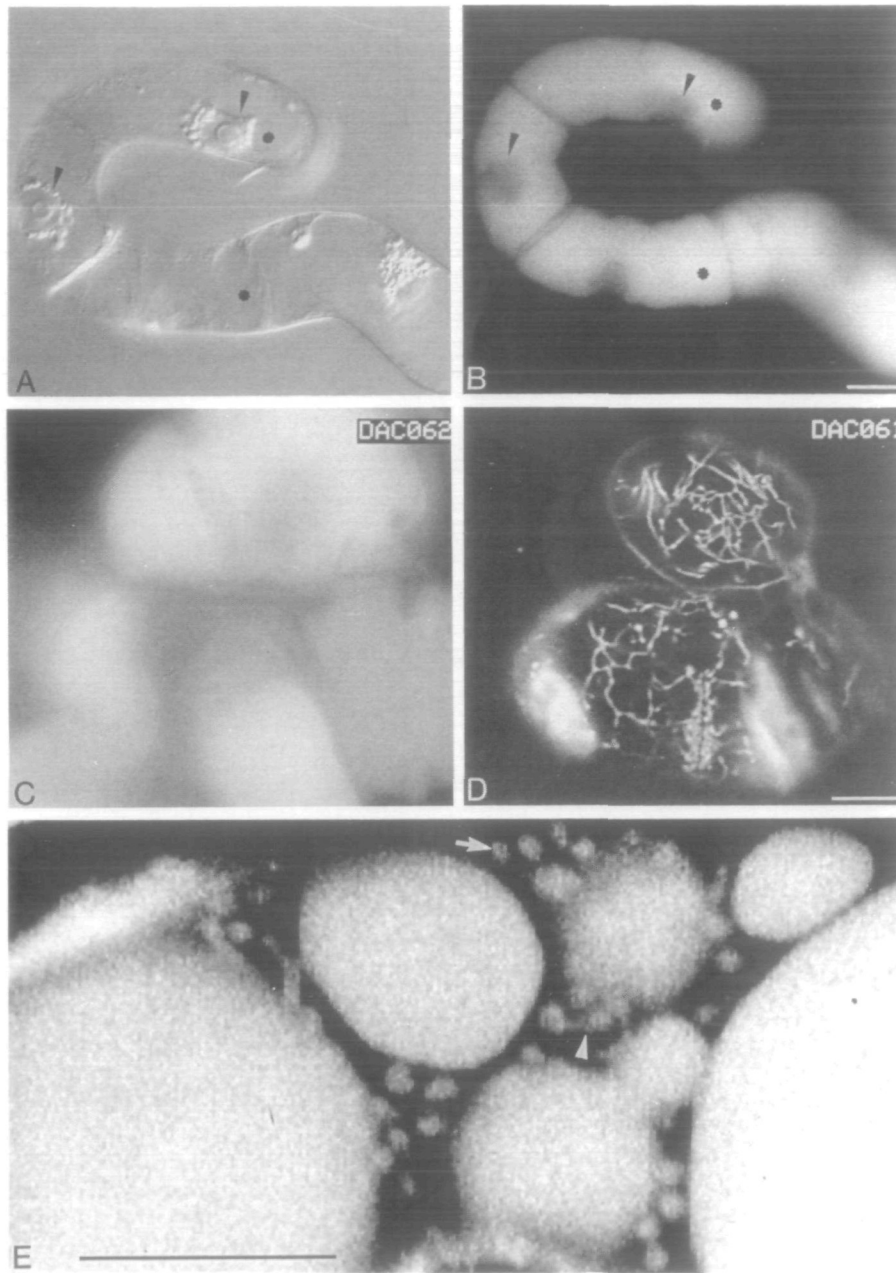


FIG. 1. Comparison of conventional light microscopy (A, B) and LSM (C–E). (A) Normarski interference contrast picture of suspension-cultured carrot cells incubated for 18 h in culture medium containing LY (Zeiss M 405 inverted microscope; bar = 10 μ m). (B) Fluorescence micrograph of the cell shown in Fig. 1A. (C) LSM [Zeiss] non-confocal image of a cell incubated for 18 h in culture medium containing LY (40 \times water immersion objective; bar = 10 μ m). (D) Confocal image of the same cell shown in Fig. 1C. (E) LSM [Heidelberg Instruments] image of a cell incubated for 18 h in culture medium containing LY (40 \times oil immersion objective; bar = 10 μ m).

cells remained healthy during a 24-h incubation period. The same was true if Normarski optics were employed, when no difference between dye-loaded and untreated cells could be observed.

Secondly, cells which were incubated with LY at 4 $^{\circ}$ C appeared normal (Fig. 6A), but fluorescence was restricted to the wall (Fig. 6B). However, prefixed and permeabilized specimens which were exposed to LY were quite different in appearance, the cytoplasm being withdrawn somewhat

from the wall and the vacuole not clearly identifiable (Fig. 6C). Fluorescence was diffuse in the region of the cytoplasm and more intense in the nucleus (arrowhead; Fig. 6D).

Finally, we have been able to show that the tonoplast of carrot cells is impermeable to LY. This was inferred from experiments with isolated vacuoles (Fig. 6F, G), which take up neutral red (Fig. 6E), but are incapable of accumulating LY.

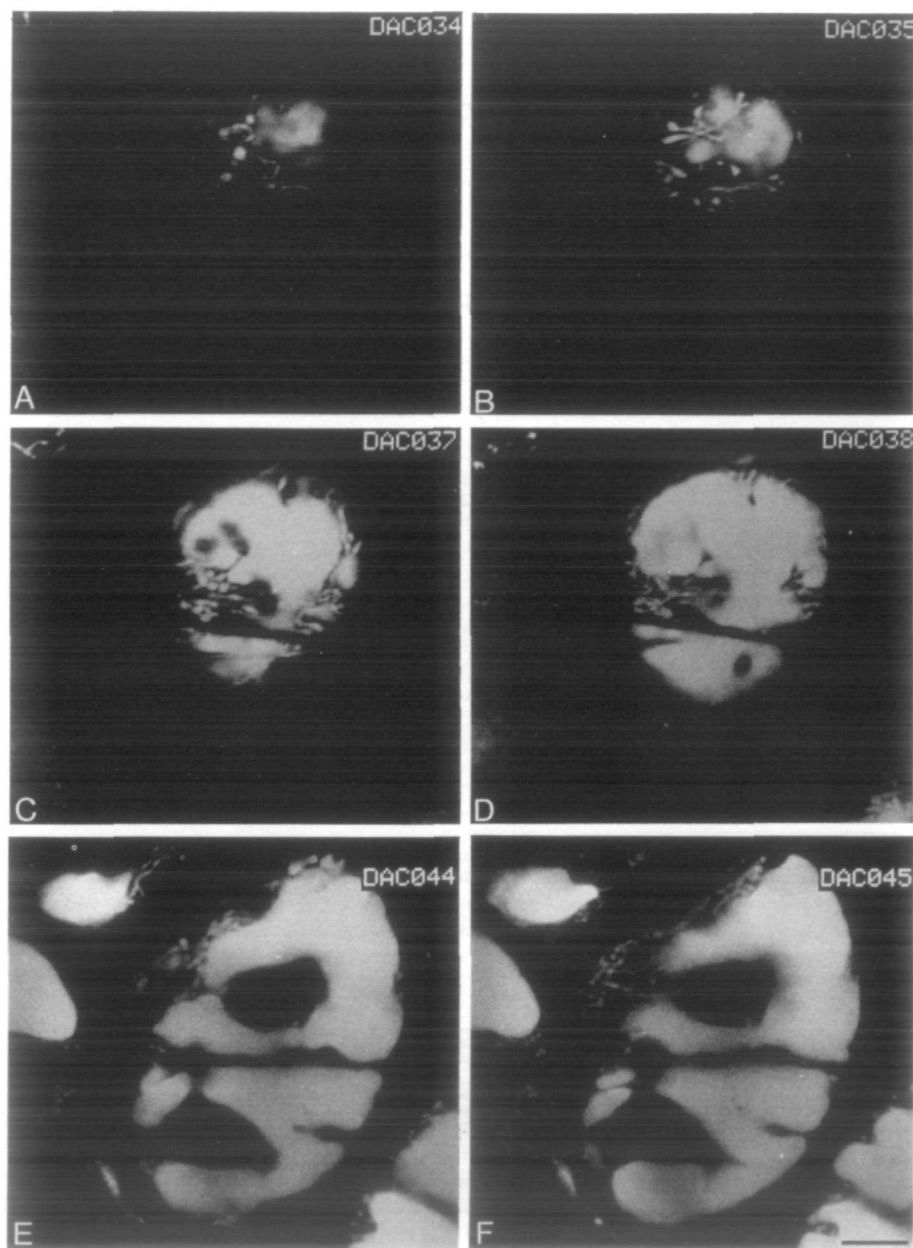


FIG. 2. Individual optical sections from a confocal LSM [Zeiss] series through dye loaded cells with changing z position (63 \times oil immersion objective; bar = 10 μ m). Distance from the top of the cell: (A) 8 μ m; (B) 10 μ m; (C) 14 μ m; (D) 16 μ m; (E) 28 μ m; (F) 30 μ m.

DISCUSSION

In this paper we have shown for the first time that plant cells can take up the fluorescent dye LY and deposit it in the vacuole. The confocal optics of the laser scanning microscope have also made it possible to observe details of the vacuolar system that would not normally be visible with conventional light microscopy. As demonstrated here, the elaborate ramifying, tubular nature of the vacuole at its periphery resembles earlier descriptions of the vacuolar apparatus as determined by neutral red staining (Guilliermond, 1929; reviewed also in Buvat, 1971). Similar images were also obtained by Palevitz, O'Kane,

Kobres, and Raikhel (1981), who exploited the fact that the vacuoles of stomatal guard cells accumulate large amounts of flavonoids (Weissenböck, Hedrich, and Sachs, 1986; Zeiger, 1981), enabling their detection by autofluorescence when excited by blue light (Zeiger and Hepler, 1979). Because this fluorescence is relatively weak Palevitz *et al.* (1981) used a video enhancement system to obtain detailed images of the vacuolar apparatus.

Since LY has been successfully used to study fluid phase endocytosis in animal and yeast cells (see above) we believe that endocytosis is also the most likely mechanism by which this dye reaches the vacuole. Because of its fixed

negative charge, LY does not leak across the plasma membrane of animal cells (Stewart 1981; Miller *et al.*, 1983). Only when the PM is permeabilized can LY freely enter the cytosol of animal cells (Steinberg, Swanson, and

Silverstein, 1988). Previous work on plant cells also suggests that LY cannot diffuse across membranes. When LY is injected into the cytoplasm it remains there and does not accumulate in the vacuole, nor does it leak out of the plasma membrane (Steinbiss and Stabel, 1983; Palevitz and Hepler, 1985). LY can also be introduced into the cytosol of plant cells by micro-injecting liposomes containing LY into the vacuole (Madore, Oross, and Lucas, 1986). These fuse with the tonoplast and release the dye into the cytosol. Such studies have established the usefulness of LY as a tracer for symplastic transport in plants (see also Erwee, Goodwin, and Van Bel, 1985). Our results are in accordance with these investigations and show that LY can only enter the cytosol when the PM is permeabilized by fixation and detergent treatments. Since the tonoplast is also impermeable to LY and vacuolar accumulation of LY is prevented at low temperatures (a property of endocytosis in animal cells; Steinmann, Mellman, Mueller, and Cohn, 1983), we propose that, in carrot cells, LY is transported to the vacuole by vesicles derived from the plasma membrane.

Despite the foregoing discussion, it might nevertheless be suggested that the vacuolar accumulation of LY in

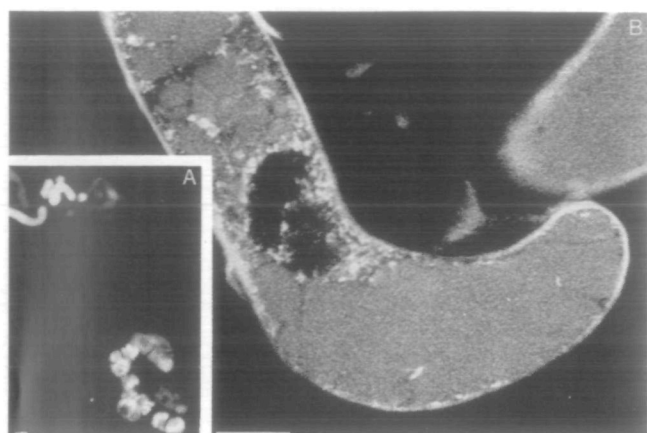


FIG. 3. (A) Fluorescence micrograph of the cells after 24 h of incubation in LY (Zeiss M 405 inverted microscope, planneofluar 16.3 \times ; bar = 10 μ m). (B) LSM (Zeiss) confocal images of cells incubated in LY at 28 $^{\circ}$ C for 18 h in LY showing differential distribution of fluorescence (40 \times water immersion; bar = 10 μ m).

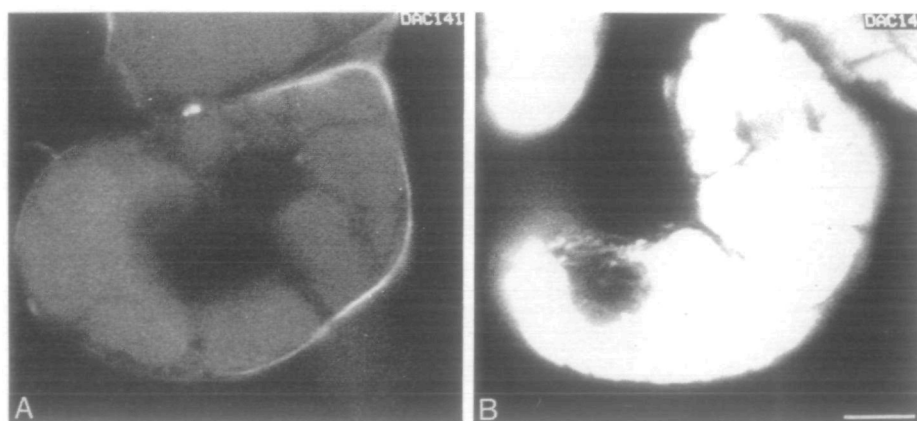


FIG. 4. (A, B) LSM (Zeiss) confocal images of cells incubated for 3 h (A) or 18 h (B) in LY at 28 $^{\circ}$ C. Settings of microscope and photographic processing are identical for both images (40 \times water immersion; bar = 10 μ m).

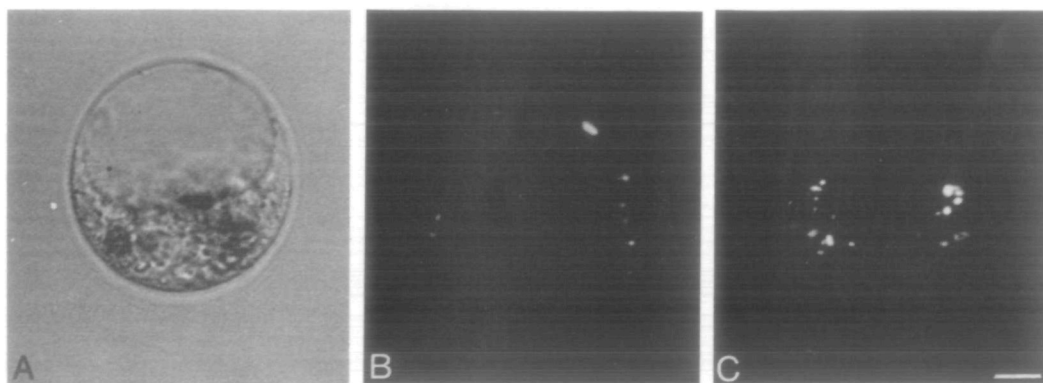


FIG. 5. LY uptake by carrot protoplasts. (A) Phase contrast image of a protoplast incubated in LY for 18 h (bar = 10 μ m). (B, C) Two different focal levels of the protoplast shown in (A) obtained with the LSM (operating with non-confocal optics; bar = 10 μ m).

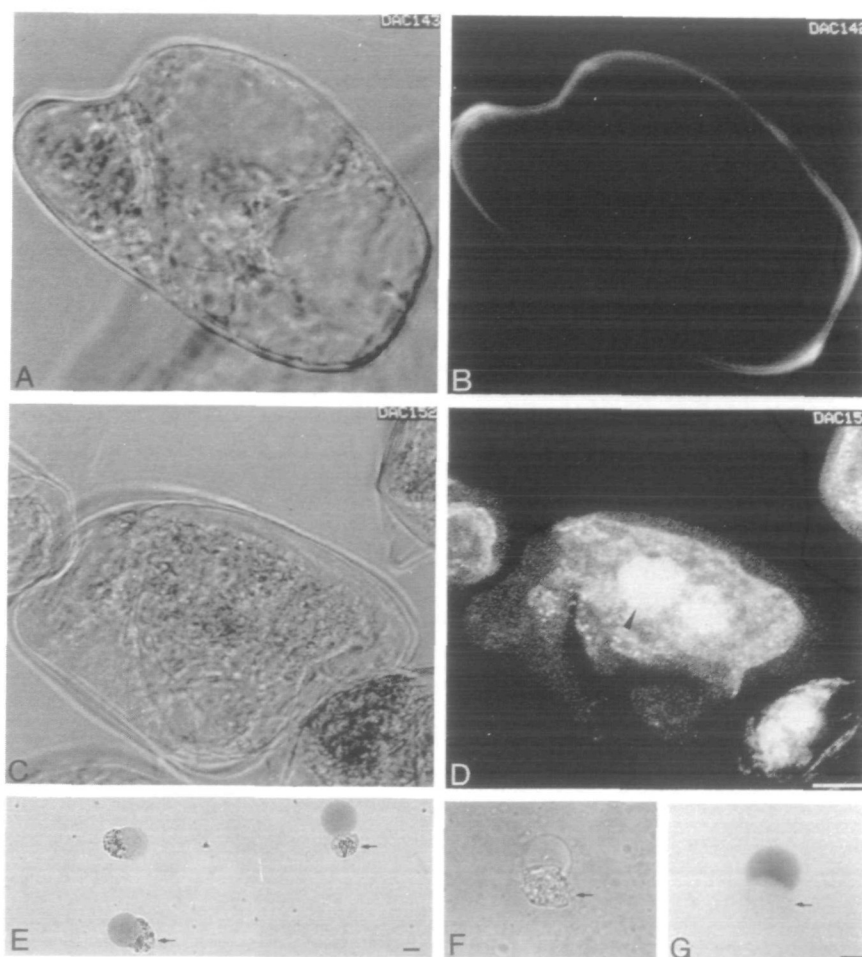


FIG. 6 Control experiments with individual cells photographed first with phase contrast optics and subsequently with confocal laser scanning microscopy [Zeiss LSM] ($40\times$ water immersion; bar = $10\ \mu\text{m}$). (A, B) Incubation with LY at $4\ ^\circ\text{C}$ for 18 h. (C, D) Cells fixed and permeabilized (formaldehyde + DMSO) prior to incubation with LY for 18 h. (E) Isolated carrot vacuoles incubated for 30 min neutral red. (F, G) Vacuoles incubated for 18 h in LY observed with phase contrast optic (F) and conventional fluorescence microscopy (G) without prior washing. As a result the unstained vacuole appears dark against a bright (fluorescing) background. Arrows indicate cytoplasmic remnants which remain attached to the vacuoles (Zeiss M 405 inverted microscope, planneofluar $16.3\times$; bar = $10\ \mu\text{m}$).

carrot cells is analogous to the general phenomenon of alkaloid storage in plant vacuoles (Matile, 1984). Such compounds are normally synthesized in the cytosol or plastids and sequestered by the vacuole of the same cell, but there are examples of both alkaloid transport between cells (Wink, 1986) and uptake by suspension cultured cells (Von Borstel and Hartmann, 1986). Whereas the accumulation of alkaloids in the vacuole was previously considered to be due to their conversion into cations, to which the tonoplast is impermeable, recent work suggests that the mechanism of vacuolar alkaloid storage is not facilitated by free diffusion, but is highly selective and species-specific (Deus and Zenk, 1982; Deus-Neumann and Zenk, 1986). In this context, it is interesting to note that the suspension-cultured carrot cells used by Deus and Zenk (1982) were essentially alkaloid-free and their vacuoles were unable to sequester alkaloids. Moreover, alkaloids which bear sulphate groups, as does LY, tend not to be taken up by vacuoles (M. Wink, personal communica-

tion). We therefore discount the possibility that LY can diffuse directly or be transported selectively across the tonoplast.

Somewhat surprising is the time-course of vacuolar LY accumulation in suspension-cultured carrot cells. Primary endocytotic events in protoplasts (coated pit formation, transfer of extracellular tracer to partially coated endoplasmic reticulum) are rapid, of the order of minutes (Joachim and Robinson, 1984; Tanchak, Griffing, Mersey, and Fowke, 1984; Hillmer, Depta, and Robinson, 1986; Tanchak, Rennie, and Fowke, 1988). This means that if LY uptake occurs via endocytosis, either the latter process occurs much less frequently in walled plant cells than in protoplasts (see Gradmann and Robinson, 1988 for a discussion on this), or there is a rate-limiting step in the endocytotic pathway prior to the vacuole. In this respect our results with protoplasts are of some significance. The failure of LY to gain access to the central vacuole of protoplasts is reflected in studies on the uptake of extra-

cellular tracers (see above). Markers such as cationic ferritin can be found in large vesicles, but the central vacuole remains free of tracer. Only in one case (Tanchak and Fowke, 1987) has it been possible to demonstrate the presence of such markers within the vacuole of protoplasts, and then only in small amounts and after prolonged incubation periods. Whether the difference in LY uptake between protoplasts and cells is due to the different osmotic parameters of these two systems has yet to be established.

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LITERATURE CITED

- ANDREAE, M., BLANKENSTEIN, P., ZHANG, Y.-H., and ROBINSON, D. G., 1988. Towards the subcellular localization of plant polyhydroxylase. *European Journal of Cell Biology*, **47**, 181–92.
- ARNDT-JOVIN, D. J., ROBERT-NICOUD, M., KAUFMAN, S. J., and JOVIN, T. M., 1985. Fluorescence digital imaging microscopy in cell biology. *Science*, **230**, 247–56.
- BUVAT, R., 1971. Origin and continuity of cell vacuoles. In *Origin and continuity of cell organelles*. Eds J. Reinert, and H. Ursprung. Springer, Berlin, Heidelberg, New York. Pp. 127–57.
- DEUS, B., and ZENK, M. H., 1982. Exploitation of plant cells for the production of natural compounds. *Biotechnology and Bioengineering*, **24**, 1965–74.
- DEUS-NEUMANN, B., and ZENK, M. H., 1986. Accumulation of alkaloids in plant vacuoles does not involve an ion-trap mechanism. *Planta*, **167**, 44–53.
- ERWEE, M. G., GOODWIN, P. B., and VAN BEL, A. J. E., 1985. Cell-cell communication in the leaves of *Commelina cyanea* and other plants. *Plant Cell and Environment*, **8**, 173–8.
- GRADMANN, D., and ROBINSON, D. G., 1988. Does turgor prevent endocytosis in plant cells? *Ibid.* (in press).
- GUILLIERMOND, A., 1929. The recent development of our idea of the vacuome of plant cells. *American Journal of Botany*, **16**, 1–22.
- HILLMER, S., DEPTA, H., and ROBINSON, D. G., 1986. Confirmation of endocytosis in higher plant protoplasts using lectin-gold conjugates. *European Journal of Cell Biology*, **41**, 142–9.
- JOACHIM, S., and ROBINSON, D. G., 1984. Endocytosis of cationic ferritin by bean leaf protoplasts. *Ibid.* **34**, 212–16.
- MADORE, M. A., OROSS, J. W., and LUCAS, W. J., 1986. Symplastic transport in *Ipomoea tricolor* source leaves. *Plant Physiology*, **82**, 432–42.
- MATILE, Ph., 1984. Das toxische Kompartiment der Pflanzenzelle. *Naturwissenschaften* **71**, 18–24.
- MILLER, D. K., GRIFFITHS, E., LENARD, J., and FIRESTONE, R. A., 1983. Cell killing by lysomotropic detergents. *Journal of Cell Biology*, **97**, 1841–51.
- NICKELL, L. G., and MARETZKI, A., 1969. Growth of suspension cultures of sugar-cane cells in chemically defined media. *Physiologia plantarum*, **22**, 117–25.
- PALEVITZ, B. A., and HEPLER, P. K., 1985. Changes in dye coupling of stomatal cells of *Allium* and *Commelina* demonstrated by micro-injection of lucifer yellow. *Planta*, **164**, 473–9.
- O'KANE, D. J., KOBRES, R. E., and RAIKHEL, N. V., 1981. The vacuole system in stomatal cells of *Allium*. Vacuole movements and changes in morphology in differentiating cells as revealed by epifluorescence, video and electron microscopy. *Protoplasma*, **109**, 23–55.
- RIEZMAN, H., 1985. Endocytosis in yeast: Several of the yeast secretory mutants are defective in endocytosis. *Cell*, **40**, 1001–9.
- ROBERT-NICOUD, M., ARNDT-JOVIN, D. J., SCHORMANN, T., and JOVIN, T. M., 1988. 3-D imaging of cells and tissues using confocal laser scanning microscopy and digital processing. *European Journal of Cell Biology*, Suppl. **25**, 49–52.
- STEINBERG, T. H., SWANSON, J. A., and SILVERSTEIN, S. C., 1988. A prelysosomal compartment sequesters membrane-impermeant fluorescent dyes from the cytoplasmic matrix of J774 macrophages. *Journal of Cell Biology*, **107**, 887–96.
- STEINBISS, H. H., and STABEL, P., 1983. Protoplasts derived from tobacco cells can survive capillary micro-injection of the fluorescent dye lucifer yellow. *Protoplasma*, **116**, 223–7.
- STEINMANN, R. M., MELLMAN, I. S., MUELLER, W. A., and COHN, Z. A., 1983. Endocytosis and the recycling of plasma membrane. *Journal of Cell Biology*, **96**, 1–27.
- STEWART, W. W., 1981. Lucifer dyes—highly fluorescent dyes for biological tracing. *Nature*, **292**, 17–21.
- SWANSON, J., BURKE, E., and SILVERSTEIN, S. C., 1987. Tubular lysosomes accompany stimulated pinocytosis in macrophages. *Journal of Cell Biology*, **104**, 1217–22.
- YIRINEC, B. D., and SILVERSTEIN, S. C., 1985. Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. *Ibid.* **100**, 851–9.
- TANCHAK, M. A., and FOWKE, L. C., 1987. The morphology of multivesicular bodies in soybean protoplasts and their role in endocytosis. *Protoplasma*, **138**, 173–82.
- GRIFFING, L. R., MERSEY, B. G., and FOWKE, L. C., 1984. Endocytosis of cationized ferritin by coated vesicles of soybean protoplasts. *Planta*, **162**, 481–6.
- RENNIE, P. J., and FOWKE, L. C., 1988. Ultrastructure of the partially-coated reticulum and dictyosomes during endocytosis by soybean protoplasts. *Ibid.* **175**, 433–41.
- VAN BORSTEL, K., and HARTMANN, T., 1986. Selective uptake of pyrrolizidine N-oxides. *Plant Cell Report*, **5**, 39–42.
- WAGNER, G. J., 1986. Methodological and other aspects of intact higher plant cell vacuoles. In *Plant vacuoles*. Ed. B. Marin. NATO ASI Series 134. Pp. 7–19.
- WEISENBÖCK, G., HEDRICH, R., and SACHS, G., 1986. Secondary phenolic products in isolated guard cell, epidermal cell and mesophyll cell protoplasts from pea (*Pisum sativum* L.) leaves: Distribution and determination. *Protoplasma*, **134**, 141–8.
- WIJNAENDTS-VAN-RESANDT, R. W., IHRIG, C., KNEBEL, W., and QUADER, H., 1988. 3-D-confocal microscopy of cytoskeleton structures. *European Journal of Cell Biology*, Suppl. **25**, 39–42.
- WILSON, T., and SHEPPARD, C., 1984. *Theory and practice of scanning optical microscopy*. Academic Press, New York.
- WINK, M., 1986. Storage of quinolizidine alkaloids in epidermal tissues. *Zeitschrift für Naturforschung*, **41c**, 375–80.
- ZEIGER, E., 1981. Novel approaches to the biology of stomatal guard cells: protoplast and fluorescent studies. In *Stomatal physiology*. Eds T. Mansfield and P. Jarvis. Cambridge University Press, Cambridge. Pp. 103–17.
- and HEPLER, P. K., 1979. Blue light-induced, intrinsic vacuole fluorescence in onion guard cells. *Journal of Cell Science*, **37**, 1–10.