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ENDOCYTOSIS OF LANTHANUM NITRATE IN THE ORGANIC ACID-SECRETING TRICHOMES OF CHICKPEA (CICER ARIETINUM L.)[1]

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The organic acid-secreting trichomes of chickpea (*Cicer arietinum* L.) were exposed to 2.5 mM lanthanum nitrate for 24 hr, and this concentration did not inhibit trichome secretion compared with that of controls. We subsequently used this nontoxic concentration of lanthanum to examine endocytosis. In the stalk cells of these secretory trichomes, exogenously applied lanthanum nitrate was present in cell walls and vacuoles, as well as within both invaginations in the plasma membrane and vesicles in the peripheral cytoplasm between the plasma membrane and the tonoplast. In the head cells, lanthanum nitrate was present in cell walls and in vesicles that form a layer in the cytoplasm around the edge of the head cells, but was not present in vacuoles. We propose that fluid phase endocytosis targeted to the vacuole takes place in the stalk cells and that endocytosis occurs in the head cells to remove excess plasma membrane after the fusion of secretory vesicles with the plasma membrane. This is the first demonstration of endocytosis in secretory trichomes.

Endocytosis has been examined with fluorescence microscopy in intact plant cells and protoplasts (for review see Oparka, 1991; Robinson and Hedrich, 1991) and at the ultrastructural level in walled cells (Wheeler and Hanchey, 1971; Wheeler, Baker, and Hanchey, 1972; Hübner, Depta, and Robinson, 1985; Romanenko, Kovtun, and Salyaev, 1986; Samuels and Bisalputra, 1990) and in protoplasts (Joachim and Robinson, 1984; Tanchak et al., 1984; Hillmer, Depta, and Robinson, 1986; Tanchak and Fowke, 1987; Record and Griffing, 1988). Recently, endocytosis was examined in the absorptive trichomes of the carnivorous Bromeliad, *Brochinnia reducta* (Owen, Platt-Aloia, and Thomson, 1991). However, endocytosis has not been studied in secretory trichomes.

The secretory hairs of chickpea (*Cicer arietinum* L.) are comprised of a basal cell, three long stalk cells, and a cluster of 14 head cells. Secretions collect in a subcuticular chamber at the top of the trichome and exude through pores in the cuticle (Schnepf, 1965; Lazzaro and Thomson, 1989). The trichomes secrete malic and oxalic acids, as well as chloride and calcium, and the pH of secretions is 1.0 or smaller (Lauter and Munns, 1986). Secretory trichomes are present on the leaves, stems, and pods of chickpea plants (Lazzaro and Thomson, 1989), and the secretion of strong acids by these trichomes probably deters insect herbivory (Koundal and Sinha, 1983; Rembold and Weigner, 1990). We present in this study an assay developed to determine a nontoxic concentration of lanthanum nitrate and use this concentration to examine endocytosis in both stalk and head cells of chickpea secretory hairs.

MATERIALS AND METHODS

Chickpea plants (*Cicer arietinum* L., CV UC5) were germinated from seed obtained from the Department of Agronomy and Range Science, University of California–Davis, and were grown in the glasshouse in full sunlight. Trichomes on mature leaves were infiltrated with lanthanum nitrate ($La[NO_3]_3$) according to a method modified from Campbell, Thomson, and Platt (1974). To ensure that lanthanum did not have a toxic effect on the trichomes, a toxicity assay was developed. Excised leaflets were immersed in distilled water to wash off any secretion droplets and were transferred to solutions containing either 0.0, 2.5, 5.0, 10.0, or 20.0 mM $La(NO_3)_3$ in distilled water. The pH of these solutions ranged from 6.0 to 5.5 and became more acidic with increasing concentration of $La(NO_3)_3$. Five leaflets were put in each solution, and the petiolular 2–3 mm of the leaflets was excised to allow solution uptake. The cut leaflets were floated on solutions under artificial light for 24 hr, and distilled water was periodically added to the solutions to prevent the $La(NO_3)_3$ concentrations from increasing due to evaporation. After 24 hr, the numbers of trichomes with and without secretion droplets were counted, and a percentage of secreting trichomes was calculated. An average of 1,261 ± 143 trichomes were counted for each concentration.

At 2.5 mM, $La(NO_3)_3$ had no measurable effect on secretion, with 69% ± 5% (mean ± standard error) of trichomes secreting, compared with 66% ± 7% secretion in the control (0 mM). Secretion decreased at higher $La(NO_3)_3$ concentrations (Fig. 1), which we attribute to the toxic effect of lanthanum. Leaflets from 0.0 and 2.5 mM treatments were prepared for electron microscopy. Leaflets were immersed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 50 mM phosphate buffer, pH 7.2 (Karnovsky, 1965) and cut into small pieces. As an alternative, whole pinnately compound leaves were rinsed in distilled water and floated on solutions of 0.0 or 2.5 mM $La(NO_3)_3$. The cut end of the petiole was slit longitudinally, about 1 cm, in the solution. After 24 hr, the whole leaf was immersed, and the epidermis of the petiole was peeled off in the fixative. Stalk cells from the trichomes on the epidermal peels were better fixed than were those from the leaflets. However, the ultrastructure of the head cells was the same in trichomes from either epidermal peels or leaflets. The tissue from both peels and leaflets remained in fixative for 2 hr, was rinsed in two changes of buffer for 15 min each, and was postfixed in 1% OsO_4 in

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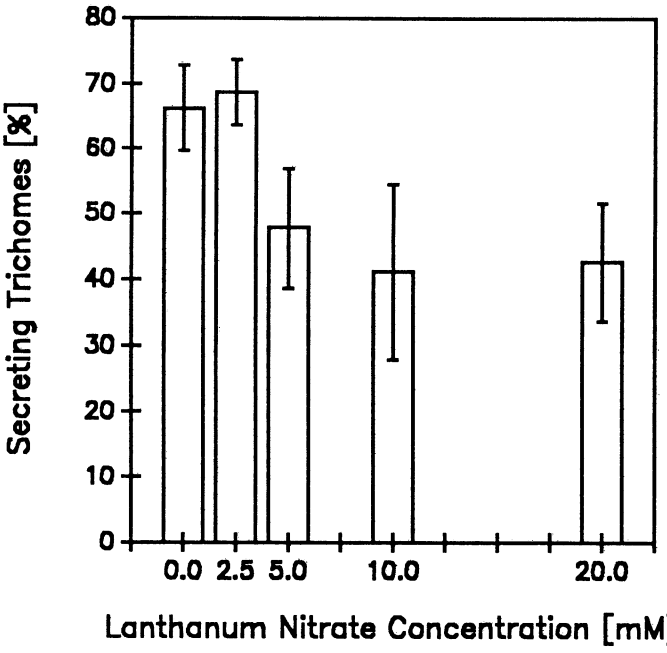

Fig. 1. Effect of lanthanum nitrate on trichome secretion. There was no significant difference in trichome secretion after 24 hr exposures to either 0.0 or 2.5 mM lanthanum nitrate. However, at higher concentrations, trichome secretion was significantly less ($P < 0.05$), according to a Least Significant Difference t-test ($t = 2.11$). Bars are means ± standard errors ($N = 5$).

buffer for 16 hr at 4 C. The tissue was dehydrated through an increasing acetone series and infiltrated with Spurr's resin (Spurr, 1969) by dropwise addition. Thin sections (90 nm) were collected on formvar-coated grids and examined unstained, except for the sections in Figs. 6 and 8, which were poststained with aqueous uranyl acetate and lead citrate.

Lanthanum crystals alone were prepared in microfuge tubes for electron microscopy by adding 200 μl of 2.5 mM $La(NO_3)_3$ in distilled water to 1,000 μl of the Karnovsky's solution described previously. After 2 hr, the tubes were centrifuged at 10,000 × g for 10 min. The pellet was rinsed in buffer for 10 min, recentrifuged, dehydrated in acetone, and infiltrated with Spurr's resin. The pellet was not postfixed in OsO_4.

Sections were examined with a Philips 400 electron microscope at 80 kV. The elemental composition of electron-dense deposits was determined with X-ray microanalysis. Thick sections (350 nm) were analyzed with an EDAX 9100 and a Philips 400 electron microscope at 60 kV, with a 0.5 μm spotsize at 24° tilt. Counts per second for lanthanum ($L_{alpha} = 4.654$ kV, $L_{beta} = 5.046$ kV) were calculated by the EDAX computer using the EDAX semi-quantitative program.

TABLE 1. *X-ray microanalysis of electron-dense deposits. Values are means ± standard deviations (N = 3). All counts for treated tissue were significantly higher than counts for controls (P < 0.05) according to a pairwise t-test (t = 2.78)*

Cellular region		Counts per second for lanthanum L_{alpha}	Counts per second for lanthanum L_{beta}
Stalk cell plasmodesmata	Treated	20.00 ± 1.43	11.47 ± 1.00
	Control	0.00 ± 0.00	0.00 ± 0.00
Stalk cell tonoplast	Treated	3.61 ± 0.32	1.99 ± 0.32
	Control	0.00 ± 0.00	0.00 ± 0.00
Head cell vesicles	Treated	1.59 ± 0.32	0.48 ± 0.38
	Control	0.00 ± 0.00	0.00 ± 0.00
Lanthanum crystals	Treated	37.97 ± 3.92	22.06 ± 2.76
	Control	0.12 ± 0.17	0.00 ± 0.00

RESULTS

X-ray microanalysis verified that the observed electron-dense deposits throughout treated trichomes were lanthanum. The same cellular locations were analyzed in control tissue. The counts per second for lanthanum were always significantly higher in treated tissue than in controls (Table 1). In addition, electron-dense deposits were observed in the test tube precipitation of lanthanum nitrate (Fig. 2), and these deposits were similar to those observed in treated cells.

Exogenously applied lanthanum accumulated in the cell walls of the stalk cells in both the long lateral walls and the short, plasmodesmata-rich transverse walls (Fig. 3). Although lanthanum seemed to occlude the plasmodesmata, cross sections through the transverse walls showed that the lanthanum deposits surrounded the plasmodesmata, while the lumen inside each plasmodesma was clear (Fig. 4). Stalk cells from control tissue lacked electron-dense deposits (Fig. 5). We observed lanthanum deposits appressed against the plasma membrane of the stalk cells (Fig. 7), within infoldings of the plasma membrane (Fig. 6), as well as in vesicles in the cytoplasm between the plasma membrane and the tonoplast (Fig. 7). Lanthanum was also present in heavy deposits inside the vacuole (Fig. 8) and was appressed in a thin layer against the vacuolar side of the tonoplast (Figs. 3, 6–8).

In the head cells of the secretory trichomes, light deposits of lanthanum were present in the cell walls (Fig. 9). In addition, lanthanum deposits were inside the cells (arrowheads; Fig. 9), within cytoplasmic vesicles (Fig. 10). There were no lanthanum deposits in the vacuoles of the

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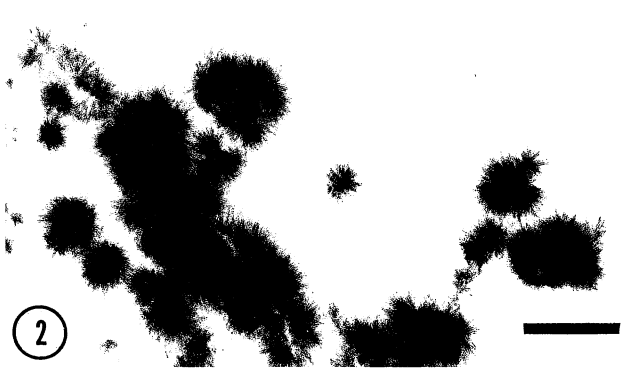
Figs. 2–8. Lanthanum in test tube and in stalk cells. **2.** Lanthanum crystals from test tube precipitate were identical to crystals observed within trichome cells. Bar = 0.5 μm. **3.** Lanthanum was observed in lateral and transverse cell walls of stalk cells (arrows), around the plasmodesmata between stalk cells (Pd), and in a thin layer along the tonoplast (t). Bar = 1.0 μm. **4.** Cross section through plasmodesmata in Fig. 3 showed that lanthanum deposits were in the wall, whereas lumens of plasmodesmata were clear (arrowhead). Bar = 0.1 μm. **5.** Control stalk cells lacked electron-dense deposits. Bar = 1.0 μm. **6.** At higher magnification, lanthanum was observed within invaginations of the plasma membrane (arrow). Bar = 0.5 μm. **7.** Lanthanum was also appressed against the plasma membrane (arrows) and was within cytoplasmic vesicles (v). Bar = 0.5 μm. **8.** Lanthanum deposits accumulated within vacuoles in large deposits (V) and in a thin layer appressed against the tonoplast (arrows). Bar = 0.5 μm.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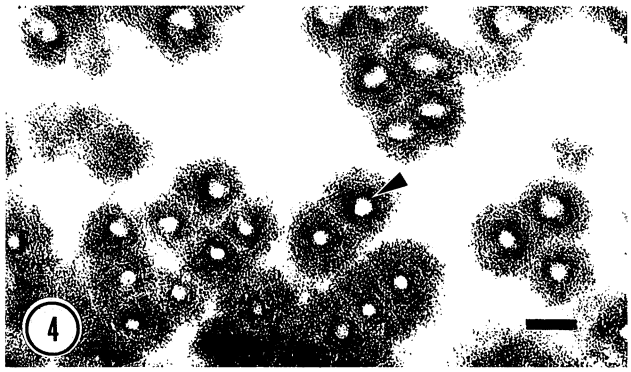
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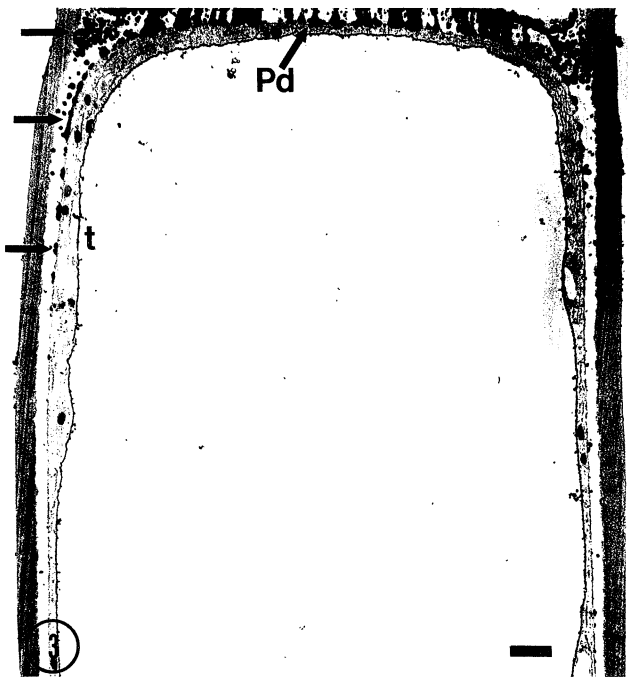
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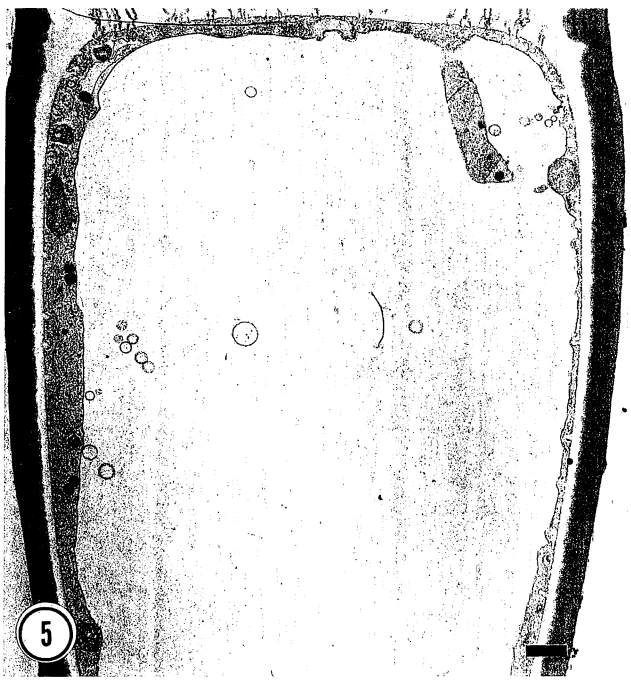
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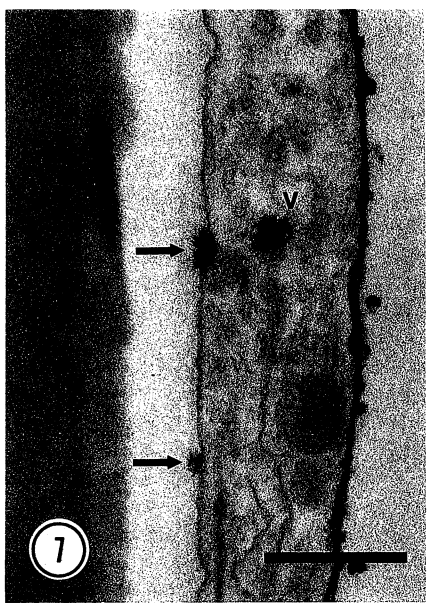
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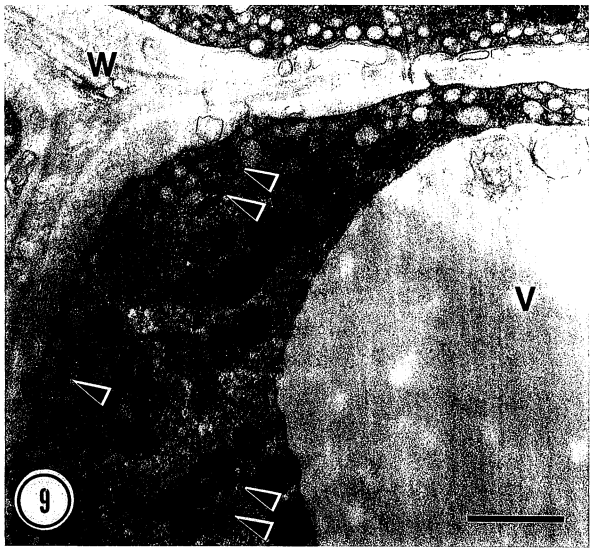

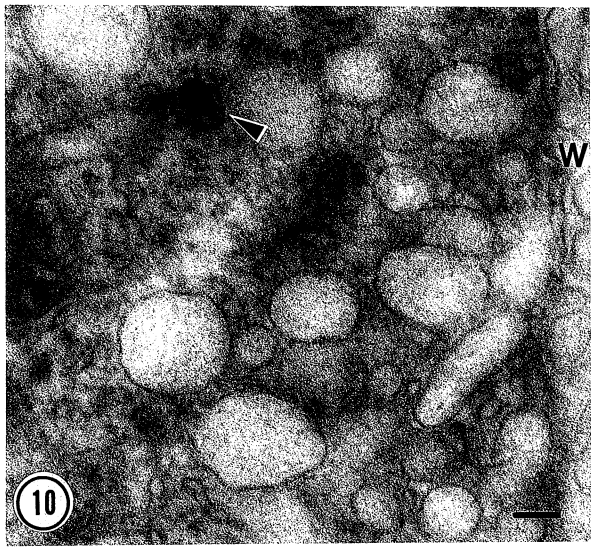

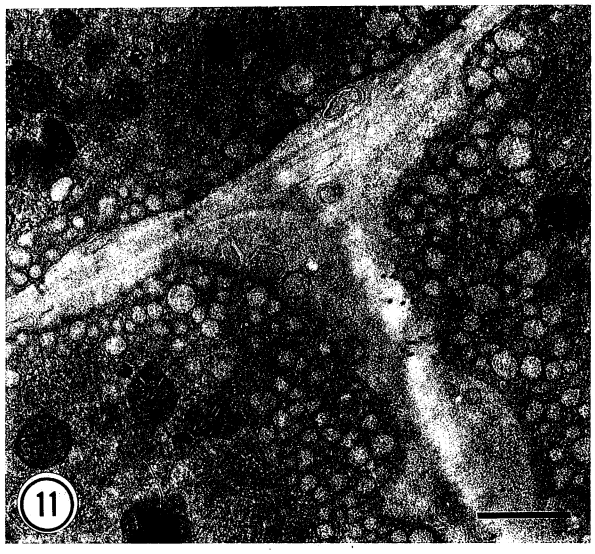

Figs. 9–11. Lanthanum in head cells. **9.** Lanthanum was observed in the cell wall (W) and within vesicles along the periphery of head cells (arrowheads), but was not present in vacuoles (V). Bar = 1.0 μm. **10.** At higher magnification, lanthanum deposits within head cells were clearly inside cytoplasmic vesicles (arrowhead), 1 μm from the cell wall (W). Bar = 0.1 μm. **11.** Control head cells lacked electron-dense deposits. Bar = 1.0 μm.

head cells (Fig. 9). Head cells from control tissue did not contain electron-dense deposits (Fig. 11).

DISCUSSION

Lanthanum nitrate is a useful apoplastic marker because it does not cross the plasma membrane and is electron dense (Revel and Karnovsky, 1967). It has been used to demonstrate endocytosis in elongating root tips of *Lobelia erinus* (Samuels and Bisalputra, 1990) and in root cap cells in maize (Hübner, Depta, and Robinson, 1985). However, lanthanum is a toxic heavy metal. In physiological studies, lanthanum had toxic effects at 0.1 to 1.0 mM on dielectric focusing and electrofusion in *Hordeum* protoplasts (Abe and Takeda, 1988) and inhibited α-amylase secretion at 0.1 mM in rice scutellar epithelium (Mitsui et al., 1984). In contrast, lanthanum at 0.025 mM increased the influx of potassium ions into corn root segments after 1 hr, although it initially decreased K^+ influx (Leonard, Nagahashi, and Thomson, 1975). Similarly, lanthanum at 0.02 to 50 mM stimulated elongation in oat coleoptiles after an initial inhibition (Harmet, 1979). In the present study, we developed an assay to test whether the exogenously applied lanthanum nitrate was toxic to the secretory trichomes. Secretion in trichomes infiltrated with 2.5 mM lanthanum for 24 hr was not inhibited compared with that in controls, whereas secretion was inhibited at higher concentrations. We subsequently used this low concentration (2.5 mM) for the ultrastructural study and conclude that our results are based on a nontoxic concentration of lanthanum nitrate.

Ultrastructural evidence for endocytosis in plant cells has been demonstrated in protoplasts (Joachim and Robinson, 1984; Tanchak et al., 1984; Hillmer, Depta, and Robinson, 1986; Tanchak and Fowke, 1987; Record and Griffing, 1988) and in walled plant cells (Hübner, Depta, and Robinson, 1985; Samuels and Bisalputra, 1990; Owen, Platt-Aloia, and Thomson, 1991). In all these studies, electron-dense tracers were endocytosed in coated vesicles that migrated to the dictyosome, although Samuels and Bisalputra (1990) found that more smooth vesicles than coated vesicles contained lanthanum. In contrast, the vesicles containing lanthanum in the stalk and the head cells of chickpea trichomes were not clathrin coated, nor were they associated with dictyosomes.

In the stalk cells of chickpea trichomes, lanthanum nitrate was observed in cell walls and vacuoles, but not in cytoplasm. In addition, lanthanum was present within both invaginations of the plasma membrane and vesicles in the peripheral cytoplasm between the plasma membrane and the vacuole. Fluid phase endocytosis (or pinocytosis) has been demonstrated with the fluorescent probe Lucifer yellow CH, although recent work has suggested that Lucifer yellow may be transported across the plasma membrane and the tonoplast by a probenecid-sensitive organic anion transporter, and thus may not be a reliable marker for endocytosis directed into the vacuole (see reviews by Oparka, 1991; Robinson and Hedrich, 1991).

Pinocytosis has also been demonstrated with uranium, an electron-dense marker (Wheeler and Hanchey, 1971; Wheeler, Baker, and Hanchey, 1972; Romanenko, Kovtun, and Salyaev, 1986). However, the pinocytotic events described are attributed to exposure to toxic levels of uranyl salts (Wheeler and Hanchey, 1971; Wheeler, Baker, and Hanchey, 1972; Romanenko, Kovtun, and Salyaev, 1986). We propose in the present study, based on the localization of a nontoxic concentration of lanthanum nitrate, that fluid phase endocytosis targeted to the vacuole takes place in the stalk cells of chickpea trichomes.

Chickpea trichomes secrete organic acids, primarily malate and oxalate (Lauter and Munns, 1986), but neither stalk nor head cells of the trichomes contain chloroplasts (Lazzaro and Thomson, 1989). Although the basal cell does contain small chloroplasts, they are ten times smaller in diameter than the chloroplasts in adjacent mesophyll cells and have less-developed grana (Lazzaro and Thomson, 1989). Therefore, the carbon substrate needed for organic acid secretion may be provided by the mesophyll cells. Since there are no plasmodesmata connecting the basal cell with the adjacent epidermal and mesophyll cells (Lazzaro and Thomson, 1989), fluid phase endocytosis in the stalk cells may play a role in the uptake of carbon substrate into the trichomes.

In the head cells of chickpea trichomes, exogenously applied lanthanum was present in the cell wall and within the vesicles along the edges of the head cells, but not in the vacuoles. In addition to organic acids, the trichomes of chickpea secrete chloride, calcium, and hydrogen ions, and the pH of secretions is 1.0 or less (Lauter and Munns, 1986). We have shown that calcium is present in the vesicles around the edges of the head cells, as well as in the cell wall space and subcuticular secretion chamber (Lazzaro and Thomson, in press). In addition, calcium-containing vesicles were observed fusing with the plasma membrane of the head cells, and it has been proposed that these vesicles are involved in the secretion of calcium via exocytosis (Lazzaro and Thomson, in press). In a review of plasma membrane turnover in plant cells, Steer (1988) proposed that membrane turnover occurs as a consequence of secretion and that a large part of this turnover should take place via endocytotic vesiculation. In addition, membrane recycling has been demonstrated in pollen tube tips of *Tradescantia* (Picton and Steer, 1983), in epidermal cells of auxin stimulated, elongating coleoptiles of *Avena sativa* (Phillips, Preshaw, and Steer, 1988), and recently by the uptake of lanthanum into smooth and coated vesicles in elongating root tip cells of *Lobelia erinus* (Samuels and Bisalputra, 1990). We propose that the presence of lanthanum in select vesicles in the head cells suggests that after exocytosis of secretions, endocytotic vesicles form to retrieve excess plasma membrane.

In conclusion, this study provides further evidence for endocytosis in walled plant cells and, to our knowledge, is the first demonstration of endocytosis in secretory trichomes.

LITERATURE CITED

ABE, S., AND J. TAKEDA. 1988. Effects of La^{3+} on surface charges, dielectrophoresis, and electrofusion of barley protoplasts. *Plant Physiology* 87: 389–394.

CAMPBELL, N., W. W. THOMSON, AND K. A. PLATT. 1974. The apoplastic pathway of transport to salt glands. *Journal of Experimental Botany* 25: 61–69.

HARMET, K. H. 1979. Rapid growth responses of *Avena* coleoptile segments to lanthanum and other cations. *Plant Physiology* 64: 1094–1098.

HILLMER, S., H. DEPTA, AND D. G. ROBINSON. 1986. Confirmation of endocytosis in higher plant protoplasts using lectin-gold conjugates. *European Journal of Cell Biology* 41: 142–149.

HÜBNER, R., H. DEPTA, AND D. G. ROBINSON. 1985. Endocytosis in maize root cap cells. Evidence obtained using heavy metal salt solutions. *Protoplasma* 129: 214–222.

JOACHIM, S., AND D. G. ROBINSON. 1984. Endocytosis of cationic ferritin by bean leaf protoplasts. *European Journal of Cell Biology* 34: 212–216.

KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *Journal of Cell Biology* 27: 137A–138A.

KOUNDAL, K. R., AND S. K. SINHA. 1983. Evaluation of the significance of malic acid secretion in chickpea. *Physiologia Plantarum* 58: 189–192.

LAUTER, D. J., AND D. N. MUNNS. 1986. Water loss via the glandular trichomes of chickpea (*Cicer arietinum* L.). *Journal of Experimental Botany* 37: 640–649.

LAZZARO, M. D., AND W. W. THOMSON. 1989. Ultrastructure of organic acid secreting trichomes of chickpea (*Cicer arietinum*). *Canadian Journal of Botany* 67: 2669–2677.

———, and ———. In press. Ultrastructural localization of calcium in the organic acid secreting trichomes of chickpea (*Cicer arietinum*). *Canadian Journal of Botany.*

LEONARD, R. T., G. NAGAHASHI, AND W. W. THOMSON. 1975. Effect of lanthanum on ion absorption in corn roots. *Plant Physiology* 55: 542–546.

MITSUI, T., J. T. CHRISTELLER, I. HARA-NISHIMURA, AND T. AKAZAWA. 1984. Possible roles of calcium and calmodulin in the biosynthesis and secretion of α-amylase in rice seed scutellar epithelium. *Plant Physiology* 75: 21–25.

OPARKA, K. J. 1991. Uptake and compartmentation of fluorescent probes by plant cells. *Journal of Experimental Botany* 42: 565–579.

OWEN, T. P., K. A. PLATT-ALOIA, AND W. W. THOMSON. 1991. Ultrastructural localization of Lucifer yellow and endocytosis in plant cells. *Protoplasma* 160: 115–120.

PHILLIPS, G. D., C. PRESHAW, AND M. W. STEER. 1988. Dictyosome vesicle production and plasma membrane turnover in auxin-stimulated outer epidermal cells of coleoptile segments from *Avena sativa* (L.). *Protoplasma* 145: 59–65.

PICTON, J. M., AND M. W. STEER. 1983. Membrane recycling and the control of secretory activity in pollen tubes. *Journal of Cell Science* 63: 303–310.

RECORD, R. D., AND L. R. GRIFFING. 1988. Convergence of the endocytotic and lysosomal pathways in soybean protoplasts. *Planta* 176: 425–432.

REMBOLD, H., AND C. WEIGNER. 1990. Chemical composition of chickpea, *Cicer arietinum,* exudate. *Zeitschrift für Naturforschung* 45C: 922–923.

REVEL, J. P., AND M. J. KARNOVSKY. 1967. Hexagonal arrays of subunits in intercellular junctions of the mouse heart and liver. *Journal of Cell Biology* 33: C7–C12.

ROBINSON, D. G., AND R. HEDRICH. 1991. Vacuolar Lucifer yellow uptake in plants: endocytosis or anion transport; a critical opinion. *Botanica Acta* 104: 257–264.

ROMANENKO, A. S., G. Y. KOVTUN, AND R. K. SALYAEV. 1986. Effect of metabolic inhibitors on pinocytosis of uranyl ions by radish root cells: probable mechanisms for pinocytosis. *Annals of Botany* 57: 1–10.

SAMUELS, A. L., AND T. BISALPUTRA. 1990. Endocytosis in elongating root cells of *Lobelia erinus*. *Journal of Cell Science* 97: 157–165.

SCHNEPF, E. 1965. Licht- und elektronenmikroskopische Beobachtungen an den Trichom-Hydathoden von *Cicer arietinum*. *Zeitschrift für Pflanzenphysiologie* 53: 245–254.

SPURR, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructural Research* 26: 31–43.

STEER, M. W. 1988. Plasma membrane turnover in plant cells. *Journal of Experimental Botany* 39: 987–996.

TANCHAK, M. A., AND L. C. FOWKE. 1987. The morphology of multivesicular bodies in soybean protoplasts and their role in endocytosis. *Protoplasma* 138: 173–182.

———, L. R. GRIFFING, B. G. MERSEY, AND L. C. FOWKE. 1984. Endocytosis of cationized ferritin by coated vesicles of soybean protoplasts. *Planta* 162: 481–486.

WHEELER, H., B. L. BAKER, AND P. HANCHEY. 1972. Pinocytosis in root cap cells exposed to uranyl salts. *American Journal of Botany* 59: 858–868.

———, AND P. HANCHEY. 1971. Pinocytosis and membrane dilation in uranyl-treated plant roots. *Science* 171: 68–71.