



Study on the laminar hydathodes of *Ficus formosana* (Moraceae) IV. Coated vesicles endocytosis is one of the retrieval mechanisms of epithem during guttation

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ABSTRACT: We used lanthanum nitrate as an apoplastic tracer to examine possible mechanisms of retrieval nutrient in the epithem of laminar hydathodes of *Ficus formosana* Maxim. Cytochemical data showed that vesicles, of constant size about 100 nm, containing the precipitation of lanthanum were present in the cytoplasm and pericytoplasm. These lanthanum-containing vesicles were associated with the elongated profiles of endoplasmic reticulum (ER). Lanthanum deposits were also observed in other organelles such as mitochondria and peroxisomes. Lanthanum nitrate, an apoplastic tracer, can't penetrate the plasmalemma hence its distribution in the vesicles was via endocytosis. In particular, we found that lanthanum precipitation was not present in the plasmalemmasomes, which are the structures of the fluid-phase endocytosis of plasmalemma. From SEM/EDX results, we proposed that the lanthanum ion (La^{3+}) or calcium ion (Ca^{2+}) was absorbed and transported into cell by the coated vesicles endocytosis, not through the fluid-phase endocytosis. The coated vesicle endocytosis, a selective pathway, is a possible mechanism for the uptake of metal ions, which then are further transported into other organelles.

KEY WORDS: Calcium ion, Coated vesicle endocytosis, Epithem, Hydathodes, Fluid-phase endocytosis, Lanthanum nitrate.

INTRODUCTION

The phenomenon of guttation is caused by root pressures in xylem, which depends on the accumulation of salt ions in root xylem, and exudes solution through hydathodes. In general, the salt concentration of guttated solution is very low. Klepper and Kaufmann (1966) suggested that leaves of guttation plants play an important role in removing salt from the xylem. The process of guttated solution passively passing through hydathodes seems to provide a valuable system, with intact plants and using non-destructive sampling techniques, for study of ions transport out of plant roots (Dieffenbach *et al.*, 1980).

The role of hydathodes in plant is an interesting topic for botanists. Both water and nutrients are important for plant growth. Why do plants absorb nutrients by root but also exude them through the hydathodes? What's the function of hydathodes during guttation? There are some reports discussing these issues. Sperry (1983) observed guttation on *Blechnum lehmannii* and suggested that one mechanism of secretion from fern hydathodes is the passive transmission of xylem sap from vein endings to the plant surface through the apoplast of the hydathode under a pressure gradient induced by root pressure. Canny (1990) studied guttation in *Pilea microphylla* by feeding sulphorhodamine into the transpiration stream and noted that crystals of this fluorochrome dye accumulated preferentially in the epithem. He called epithem cells "scavenging cells" because these cells appeared to remove

solutes from the transpiration stream. Moreover, Wilson *et al.* (1991), using the apoplastic tracer Sulphorhodamine G (SR), observed that the tracer accumulated quickly in the hydathodes of the teeth of *Populus balsamifera* L., and SR was taken up by phloem parenchyma and epithem cells of the hydathode. These reports suggested that the epithem of hydathodes act collectively as a filter apparatus during guttation and retrieve solutes from the transpiration stream. However, mechanisms for nutrient retrieving of epithem are little known.

Previously, in a serial of studies, we examined the ultrastructure and the morphogenesis of hydathodes of *Ficus formosana* and presented salt damage occurring in the epithem cells (Chen and Chen, 2005; 2006; 2007). The epithem of hydathodes consists of sinuous thin-walled cells, with variable shapes, among which a ramifying system of large intercellular spaces development. A special plasmalemmasomes structure, coated vesicles and fluid-phase endocytosis were observed in these cells. In this study, we used lanthanum nitrate, an apoplastic dye, to investigate whether the formation of coated vesicles provides a special pathway for absorbing lanthanum or calcium ions into epithem cells. The results showed that lanthanum deposits were presented in the coated vesicles in epithem cells after leaves being incubated with lanthanum nitrate solution. A possible function of coated vesicles endocytosis in epithem as a special mechanism for retrieving nutrients or metal ions, especially for La^{3+} or Ca^{2+} , during guttation is discussed.



MATERIALS AND METHODS

Incubation with lanthanum nitrate solution by mid-vein loading

In vivo study, the leaves of *Ficus formosana* Maxim plant were incubated with lanthanum nitrate solution (Peterson *et al.*, 1986). Material preparation was as follows: pricking and wounding the basal region of leaf midrib with a needle, applying moisten cotton (immersed in the modified Hoagland solution, replacing ammonium phosphate with equal concentrations of ammonium sulfate, containing the 23 mM $\text{La}(\text{NO}_3)_3$ (pH 7.0) on the wound sites, and incubating the treated leaves for overnight. In control experiment, leaves were incubated in the plain Hoagland solution (pH 7.0). Subsequently, the treated leaves were cut into 1 mm square pieces, which contain the hydathodes.

Transmission Electron Microscopy (TEM)

Samples were fixed in 0.1 M phosphate buffer (pH 7.0) containing 2.5% glutaraldehyde for 3-h at room temperature, washed with 0.1 M phosphate buffer 3 times of each 15-min. Then, they were post-fixed with 1% OsO_4 in 0.1 M phosphate buffer for 3-h at room temperature, washed with 0.1 M phosphate buffer 3 times of each 15-min, dehydrated in an acetone series and embedded in Spurr's resin (Spurr, 1969). Resins containing samples were polymerization at 70 °C for 8-h and plastic blocks were cut by using a diamond knife on a Reichert UltraCut E. Thin sections were collected on grids, air-dried and doubly stained with uranyl acetate and lead citrate, and finally viewed and photographed under a Hitachi H-600 transmission electron microscope at 75 KV.

Scanning Electron Microscopy coupled with Energy Dispersive X-ray (SEM/EDX) Spectroscopy

Ultrathin-sections in 70 nm thick were cut and picked upon 50 meshes Cu grids. The sections were viewed in the JSM-6500F field emission scanning electron microscope and analyzed the elements by energy dispersive X-ray (EDX) detector.

RESULTS

The coated vesicles of epithem cell

The ultrastructure of epithem of leaves treated with plain Hoagland solution was shown in Figure 1A. The wavy plasma membranes performed in the epithem cells and several coated vesicles were observed. There are two types of coated vesicles in the cytoplasm, one contained electron dense material (arrowheads) and the other one was empty (large-arrow).

Lanthanum phosphate precipitation in the coated vesicles of epithem cells

In the section of leaf samples treated with Hoagland

solution containing lanthanum nitrate, most of the lanthanum granules were bound to the cell wall, and accumulated in the special regions nearby the ectoplasmic surface of plasmalemma. A small amount of lanthanum granules were accumulated and conspicuously observed in the intercellular vesicles, which sizes were ranging from 80 to 100 nm (Fig. 1B–1F). In addition, the lanthanum deposits were also observed in the cisterna of the mitochondria (Fig. 1D, arrow). Moreover, several coated vesicles containing lanthanum deposits also occurred in the cytoplasm of guard cells of water pores beside the epithem cells (Fig. 1E). From section with the tangential view nearby plasmalemma, the pericytoplasma region near plasmodesmata (PD) has the partially coated reticulum structure presented associated with Golgi apparatus (large-arrow) and, at same time, several coated vesicles contain lanthanum deposits performing near plasma membrane (arrowheads), as shown in Figure 1F.

Particularly, the lanthanum deposits-containing coated vesicles presented near the fluid-phase endocytosis were observed in the epithem cells (Fig. 2A). The fluid-phase endocytosis, another type of endocytosis, frequently performed in the epithem cells. It is very interesting, the lanthanum deposits have never been found accumulated in multiple-vesicular structures of fluid-phase endocytosis (Fig. 2A, 2B). The lanthanum deposits were strongly bound to the fiber matrix of the cell wall and which didn't go into vesicles formed by the fluid-phase endocytosis (large-arrowhead). However, the lanthanum deposits could entry into coated vesicles (small-arrowhead). In addition, the coated vesicles in the cytoplasm could further fuse with endoplasmic reticulum (ER) (Fig. 2C). The coated vesicles containing electron dense materials and many secretory vesicles distributed nearby plasma membrane were also observed (Fig. 2D). Furthermore, many grey electron dense material particles were present in the cisterna of mitochondria (large-arrow in Fig. 2E) and in the matrix of peroxisomes (large-arrow in Fig. 2F).

Characterization of the coated vesicles containing lanthanum granules in epithem cells using SEM/EDX analysis

For the analysis of elements, ultrathin-sections, thickness of 70 nm, of epithem of leaves treated with Hoagland solution containing lanthanum nitrate were observed in the JSM-6500F type Field Emission Scanning Electron Microscope and analyzed by Energy Dispersive X-ray (EDX) detector. As shown in Figure 3A, most of the lanthanum deposits were present in intercellular space of epithem. Also, some lanthanum deposits were observed in the coated vesicles (arrowhead). The presence of lanthanum deposited was confirmed by using Energy Dispersive X-ray (EDX). Figure 3B showed the spectra with characteristic peaks of the elements, conspicuous Ca, P, Cu and La, distributed in the detected regions.

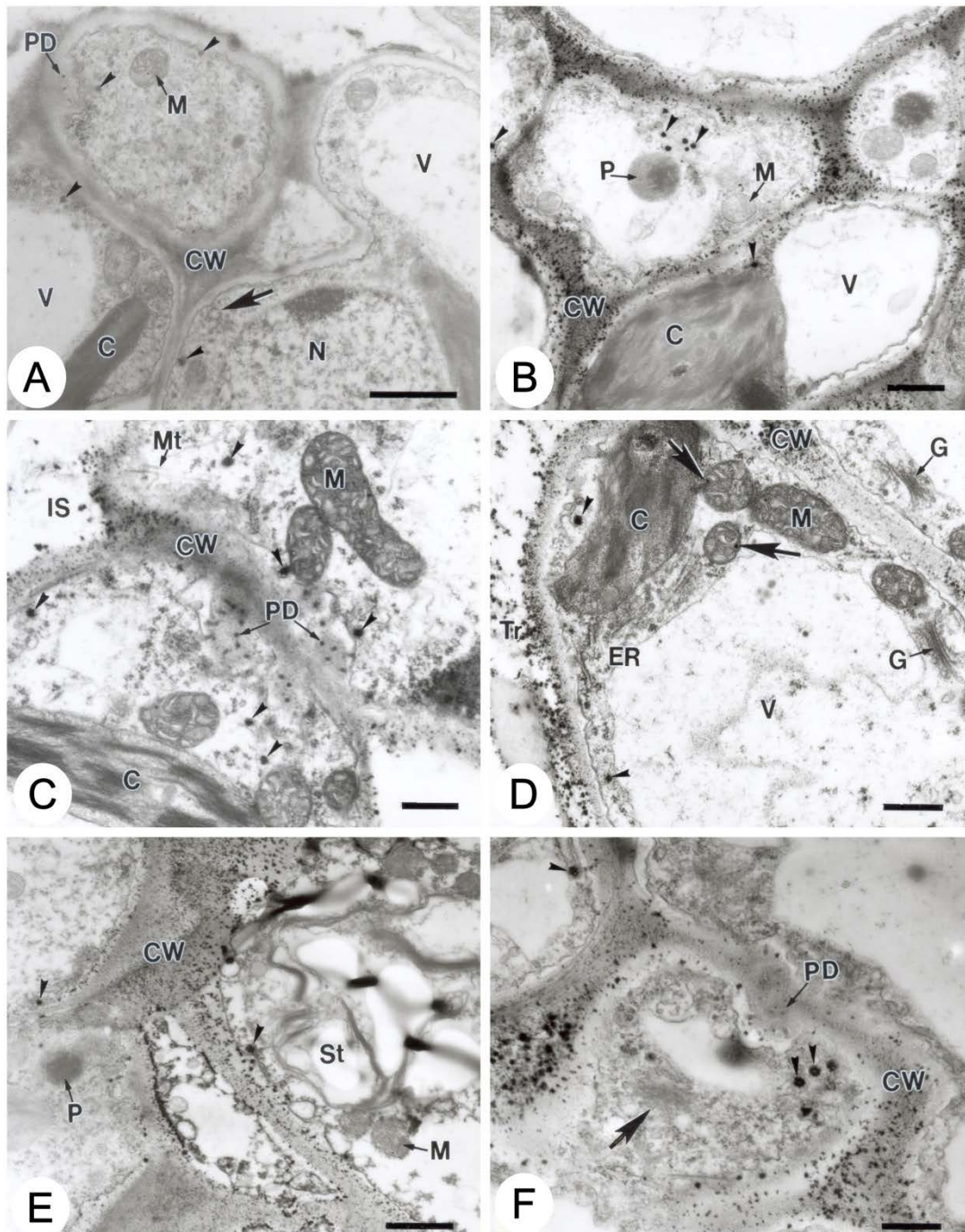


Fig. 1. Ultrastructures and distribution of lanthanum precipitates in epitherm. **A:** Transmission electron microscopic (TEM) micrograph of epitherm cell show the ultrastructure of cells of control leaves treated with plain Hoagland solution. Arrowheads indicate the coated vesicles in epitherm cells of leaves inside contain electron dense materials. Large-arrow indicate the other type of vesicle without electron dense materials. **B–F:** TEM micrographs showing the ultrastructure of epitherm cells treated with lanthanum nitrate and the precipitation of electron dense lanthanum phosphate. **B.** The coated vesicles preformed from plasmalemma and transported into cytoplasm. Arrowheads point the coated vesicles with the lanthanum deposits. **C.** The coated vesicles performed nearby the plasmodesmata between two epitherm cells. Arrowheads indicate the coated vesicles with the lanthanum deposits. **D.** The coated vesicles occurring in the epitherm cells near the tracheid cell. Arrowheads indicate the coated vesicles with the lanthanum deposits. Large-arrows indicate the lanthanum deposits present in the mitochondria. **E.** The coated vesicles occurred in the epitherm cells and water pores. Starch grain-containing chloroplasts (St) present in guard cells of water pore. Arrowheads indicated the coated vesicles with the lanthanum granules. **F.** Paradermal section nearby the pericytoplasm of epitherm showing coated vesicles and Golgi apparatus (Large-arrow indicates a dictyosome cisternum in face view of section). Arrowheads indicate the coated vesicles with the lanthanum deposits. Scale bars: **A–B, E** = 1 μm ; **C–D, F** = 0.5 μm . (**Abbreviations:** C, chloroplast; CW, cell wall; ER, endoplasmic reticulum; G, Golgi body; IS, intercellular space; M, mitochondrion; Mt, microtubule; N, nucleus; P, peroxisome; PD, plasmodesmata; St, starch grain-containing chloroplast; Tr, tracheids; V, vacuole).

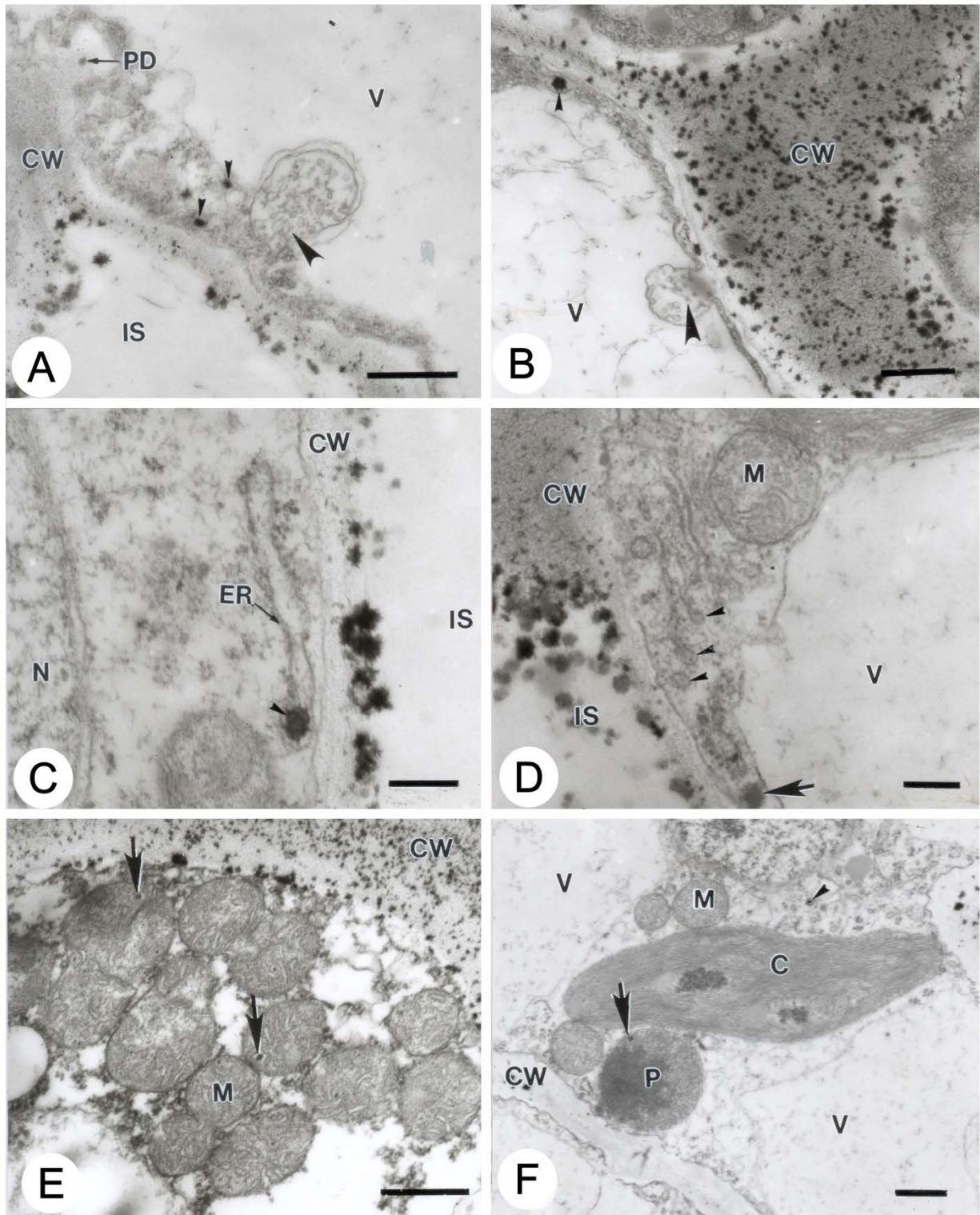


Fig. 2. Ultrastructures and distribution of lanthanum precipitates in epithem. **A–D.** TEM micrographs showing endocytosis, both coated endocytosis and fluid-phase endocytosis, occurred in the epithem cells. Large arrowheads indicate the fluid-phase endocytosis without the lanthanum granules and small arrowheads indicated the coated vesicles with the lanthanum granules. Scale bar = 0.5 μ m. **A:** Multiple-vesicular vesicles occurring near the intercellular space. **B:** Multiple-vesicular vesicles formed by fluid-phase endocytosis occurred near the intercellular cell wall. **C:** Coated vesicle (arrowhead) associated with ER in epithem cells. **D:** Secretory vesicles (arrowheads) fused with plasmalemma and the coated vesicles formation (large-arrow) in epithem cell. **E:** Mitochondria contain some grey electron dense materials in the matrix (Large-arrows). **F:** Partial cytoplasm of epithem showed the chloroplast, mitochondria and peroxisomes. There is electron dense particle in peroxisomes (large-arrow). Arrowhead indicates the coated vesicles with the lanthanum granules.

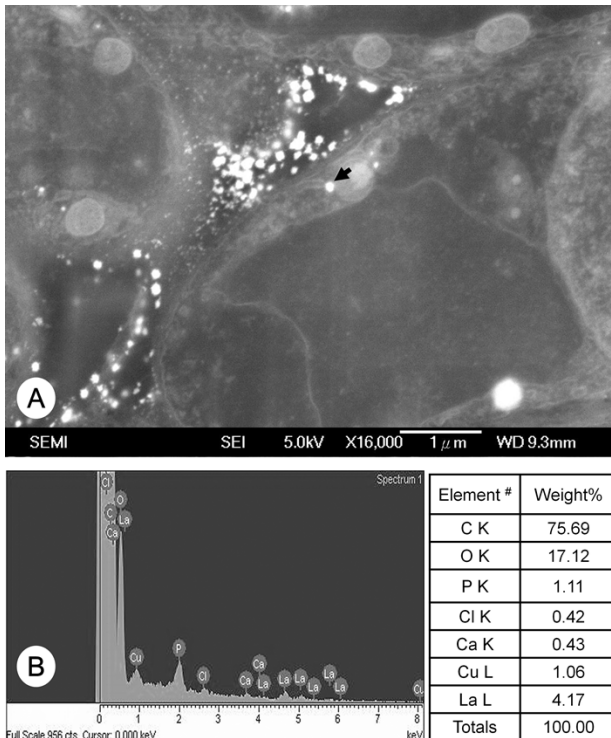


Fig. 3. Analysis of the distribution of lanthanum granules in epithem cells using SEM/EDX. **A:** SEM micrograph showing the ultrastructure of epithem cells of leaves treated with lanthanum nitrate solution. Arrowhead indicates the coated vesicles with the lanthanum granules. Scale bar: A = 1 μ m. **B:** An Energy-dispersive X-ray microanalysis (EDX) spectrum with characteristic peaks and the estimate contents of the elements distributed in the region pointed by an arrowhead in A. (# K and L, those corresponding to x-rays generated by emission from different energy-level shells (K and L) in different elements.)

DISCUSSION

Lanthanum deposits were localized in coated vesicles by endocytosis, but not in the fluid-phase endocytosis of epithem cells

From the results of TEM, we found that a large of electron-dense lanthanum deposits were precipitated in the cell wall and few were present in the invagination vesicles of plasmalemma and even associated with ER or ER-derived vesicles (Figs. 1B-G, 2C). Lanthanum nitrate as an aploplastic tracer was used to study endocytosis in plant cells. Using lanthanum to study aploplastic solute flux were reported in *Hordeum vulgare* L., *Salicornia virginica* L., *Spartina alterniflora* Loisel., *Zea mays* L. and rice roots (Peterson *et al.*, 1986; Nishizawa and Mori, 1978). The localization of lanthanum in ER and ER-derived vesicles and vacuoles, but not in cytosol offers evidence supporting the occurrence of endocytosis in plant cells. Lanthanum nitrate was also used in studying the organic acid-secreting trichomes of chickpea (Lazzaro and Thomson, 1992).

In our examination data, lanthanum deposits were

present in coated vesicles but not in the fluid-phase endocytosis (Fig. 2A, 2B). There are two possible reasons for this result. Lanthanum being trivalent cations could have high affinity for cell wall, which consists of many anion groups. In the microenvironment between cell wall and plasmalemma, the charge effect is important in affecting interaction between lanthanum ions and cell wall (Lettvin *et al.*, 1964; Sattelmacher, 2001). Another reason might be that lanthanum ions bind to plasmalemma through some special receptors, which used to absorb some metal or cation ions. The lanthanum might compete with ligands on receptors at coated pit region of the plasma membrane, be invaginated to form coated vesicles, then be transferred into the cytoplasm (Leonard *et al.*, 1974; Zeng *et al.*, 2000; Huang *et al.*, 2003). It is interesting, the electron-dense granules were also present in the cisterna of mitochondria (Fig. 1D). At the same time, the grey electron dense materials were also present in cytoplasm and organelles (mitochondria and peroxisomes) (Fig. 1E and F). Since Calcium and Calmodulin (CaM) both involving in coated vesicle endocytosis are well documented (Yamashita, 2012; Sun *et al.*, 2010; Yao and Sakaba, 2012). So the lanthanum ions might compete with calcium ions for binding proteins or receptors on plasma membrane. Some calmodulin-like proteins are targeted to mitochondria and peroxisomes (Chigri *et al.*, 2012). We suggest that lanthanum ion might compete with calcium ion presented at these grey electron-dense materials of those organelles.

The coated vesicles endocytosis is a possible retrieval mechanism for calcium ions in the epithem cells

As mentioned above, where the location of La^{3+} ion implied the existence of Ca^{2+} ion. Lanthanum ion (La^{3+}) was generally regarded as a calcium antagonist or surrogate and used as calcium channel blocker (Friedman *et al.*, 1998; Lewis and Spalding, 1998; Liu *et al.*, 2012; Curtis *et al.*, 1986; Hu *et al.*, 2006). The effects caused by La^{3+} ion on ion-absorption are similar to those by Ca^{2+} , and La^{3+} affects the plant physiological mechanism by regulating Ca^{2+} levels in the plant cells (Zeng *et al.* 2000). Because La^{3+} shows greater affinity for the Ca^{2+} binding sites, La^{3+} can function as a competitor for Ca^{2+} (Ono, 2000).

Calmodulin (CaM), Calcium-binding proteins, play a critical role in decoding calcium signatures and transducing signals by activating specific targets and corresponding metabolic pathway, and is also an essential Ca^{2+} transducer in plants (Ranty *et al.*, 2006; Hashimoto and Kudla, 2011). CaM can activate plant microsomal Ca^{2+} uptake (Dieter and Marme, 1980), and can interact with clathrin-coated vesicles, triskelions, and light-chains (Pley *et al.*, 1995). Moreover, subcellular localization of CaM has been found in the nucleus, the outer surface of the plasma membrane, in peroxisome and chloroplast (Dauwalder *et al.*, 1986;



Yang and Poovaiah 2002; Jarrett *et al.* 1980; Bussemer *et al.*, 2009). In addition, lanthanum has substituted calcium ion on the positions of calcium binding protein (CaM), four binding sites of La^{3+} on CaM were identified as the same as the binding sites of Ca^{2+} on CaM (Hu *et al.*, 2004). In another word, the distribution of lanthanum phosphate precipitation was also referred to as Ca^{2+} location in cells, according La^{3+} can compete and substitute the Ca^{2+} binding sites.

SEM/EDX analysis is a powerful method to study heavy metal uptake and elemental composition in plant tissues (Qi *et al.*, 2003; He and Kirilak, 2014). The energy dispersive spectrum of the electron dense spot in the coated vesicles (shown in Fig. 3A) revealed conspicuous Ca, P, Cu and La peaks (Fig.3 B). Interestingly, a conspicuous Ca peak could be identified from energy dispersive spectrum of these deposits. At the electron microscope level, the location of calcium in cell organelles can be achieved through cytochemical techniques including calcium precipitation by oxalate, phosphate, fluoride, or pyroantimonate (Nicaise *et al.*, 1989). In this experiment, we used phosphate ion to precipitate the lanthanum, at the same time calcium ions were also precipitated. Consequently, Ca, La and P signals were detected in the coated vesicles deposits. Moreover, depending on duration of lanthanum nitrate treatment, different ratios of La and Ca signals were found in coated vesicle deposits detected by SEM/EDX (data not shown). Accordingly under electron microscopy image, heavy electron dense deposit could mean high La/Ca ratio, then the less electron dense deposit might be with low La/Ca ratio.

Gao *et al.* (2003) found that La^{3+} could enter into plant cells through the Ca^{2+} channels and bind to the membranes of chloroplast, mitochondrion, cytoplasm, and karyon. The appearance of Ca^{2+} in situ in several cell and tissue types has been reported (Wick and Hepler, 1982; Nicaise *et al.*, 1989). All cells possess organelles that can function as sarcoplasmic reticulum equivalents by alternately sequestering and releasing Ca^{2+} to regulate free Ca^{2+} levels in the cell. Our experiments showing the presence of lanthanum in different organelles, such as in chloroplast, mitochondria, peroxisomes, and ER (Figs. 1D, 2C, 2F, 2G) that provided an evidence about an information on sequestration of calcium ion in plant cells.

The result that lanthanum ions were deposited in coated vesicles implied the possible pathway for absorbing the metal or cation ions in the epithem. In normal epithem cells, we observed that several coated vesicles contained electron dense materials and similar electron dense granules were also present in the mitochondria and peroxisomes. These granules may be the essential metal ions used as cofactors for forming metal-protein in these organelles. It is possible that the lanthanum ions substituted these ions, were then be

invaginated into the coated vesicles, and subsequently were transported into these organelles. Accordingly, we suggested that the coated vesicles play a possible mechanism for the retrieval of nutrients from the guttation solution.

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