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Subcellular Localization of Calcium in the Crystal-Forming Sclereids of *Nymphaea tetragona* Georgi

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ABSTRACT: By using a modified osmium tetroxide-potassium pyroantimonate (OTPA) precipitation technique, calcium ions were subcellularly localized in the crystal-forming sclereids of the leaf of *Nymphaea tetragona* Georgi. In young leaf laminae, the mesophyll parenchyma cells contained calcium antimonate precipitates in the vacuoles, mitochondria, nuclei, endoplasmic reticulum, dictyosome and the secretary vesicles. Besides, calcium precipitates were also associated with the plasmalemma and plasmodesmata. From the rudimentary mesophyll cells, the sclereid idioblasts originated sporadically. In these sclereid initial cells, many prismatic oxalate crystals bounded by the crystal sheaths were observed in the crystal chambers between the plasmalemma and primary cell wall. The calcium antimonate precipitates were densely presented on the crystal sheaths, however, only few calcium precipitates were found in the central vacuoles of the sclereid initial cells. After the formation of crystals, the secondary wall was deposited and then the crystals were embedded between the primary and secondary cell walls. In the mature sclereid, calcium antimonate precipitates were obviously associated with the crystal sheaths, primary cell wall, plasmalemma, and also accumulated in the central vacuole.

KEY WORDS: Subcellular calcium localization, Antimonate precipitates, Foliar sclereids, Calcium oxalate crystals, *Nymphaea tetragona*.

INTRODUCTION

Various functions have been attributed to the biological formation of crystal idioblasts (Arnott and Pautard, 1970; Borowitzka, 1984). For plants, the reversible calcification with oxalic acid, may play an important role in the regulation of calcium ionic equilibrium in the cells or tissues (Franscheschi and Horner, 1980; Borchert, 1986). In *Nymphaea* (Gandet, 1960; Kuo-Huang, 1990) calcium crystals are extracellularly deposited in the cell walls of sclereids. The development of foliar sclereids in *Nymphaea tetragona* Georgi has been anatomically and ultrastructurally studied (Chiang and Huang, 1984; Kuo-Huang, 1992). However, the localization of calcium ions in these crystal-forming sclereids is not jet investigated.

Calcium effects on numerous cellular reactions, processes, and structural components (Borowitzka, 1984; Schroeder and Thuleau, 1991; Kuo-Huang and Zindler-Frank, 1998). Direct measurement of the dynamics of free calcium ions in the cells is technologically difficult. However, there are many methods applied for the study of indirect cellular calcium localization (Wick and Hepler, 1982). Some calcium-sensitive probes, such as

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metallochromic dyes, luminescent proteins, and fluorescent chelates, have been used for the identification of calcium dynamics in living cells. But these macromoleculars have a rather limited application to the single cells or small groups of cells. For *in situ* localization of calcium, X-ray microanalytical techniques in connection with electron microscopy have been developed (Yang, 1986; Kuo-Huang, 1990). Besides, several modifications of the antimonate precipitation techniques are applied to the studies of cellular distributions of calcium in the tissues and organs. In the present work, by using the modified osmium tetroxide-potassium pyroantimonate (OTPA) precipitation procedures, the localization of calcium in the developing and mature foliar sclereids was ultrastructurally investigated. This method has been used successfully to localize cellular calcium in plant tissues (Wick and Hepler, 1980, 1982).

MATERIALS AND METHODS

Plants of *Nymphaea tetragona* Georgi were grown in the aquaria in the greenhouse of the Department of Botany, National Taiwan University. The leaves in various developmental stages were collected. Portions of leaf laminae containing sclereids (Chiang and Huang, 1984) were dissected into small pieces and fixed in 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.6) with 2% (w/v) potassium antimonate and 0.1% tannic acid for 2-4 hours (Slocum and Roux, 1982), and postfixed with 2% potassium antimonate in 1% osmium tetroxide in the same buffer for 2 hours, and then dehydrated in an acetone series, infiltrated and embedded in Spurr's resin under vacuum (Spurr, 1969). Thin sections for electron microscopy were cut on a Ultracut E by using a diamond knife. The sections were double stained with uranyl acetate and lead citrate, and then examined with a Hitachi H-600 TEM at 75 kV.

RESULTS

By using the cytochemical procedures in this study, it was shown that in the young and still submerged leaf laminae of *Nymphaea tetragona*, the precipitates of calcium antimonate were observed in vacuoles, mitochondria, nuclei (Figs. 1a, b), endoplasmic reticulum, dictyosome and secretory vesicles of the rudimentary mesophyllous cells (Figs. 1c, d). Besides, calcium deposits also occurred densly between the plasmalemma and cell walls. In the just emerged leaf of *Nymphaea tetragona*, the sclereid idioblasts originated sporadically from the mesophyll parenchyma cells. The sclereid initial cells with large nucleus were found first in the spongy mesophyll closed to the midribs and then they also occurred in the palisade tissues. When sclereid initial cells underwent hypertrophic growth, the neighboring ordinary parenchyma cells were divided and differentiated into photosynthetic mesophyll cells.

The differentiated palisade cell (Fig. 2a) had a large central vacuole, so that the dense cytoplasm containing the chloroplasts was confined to a thin layer around the cell periphery. In the palisade cells, the precipitates of calcium antimonate were predominantly localized within the central vacuoles. Some calcium deposits also located in the plasmodesmata (Fig. 2a).

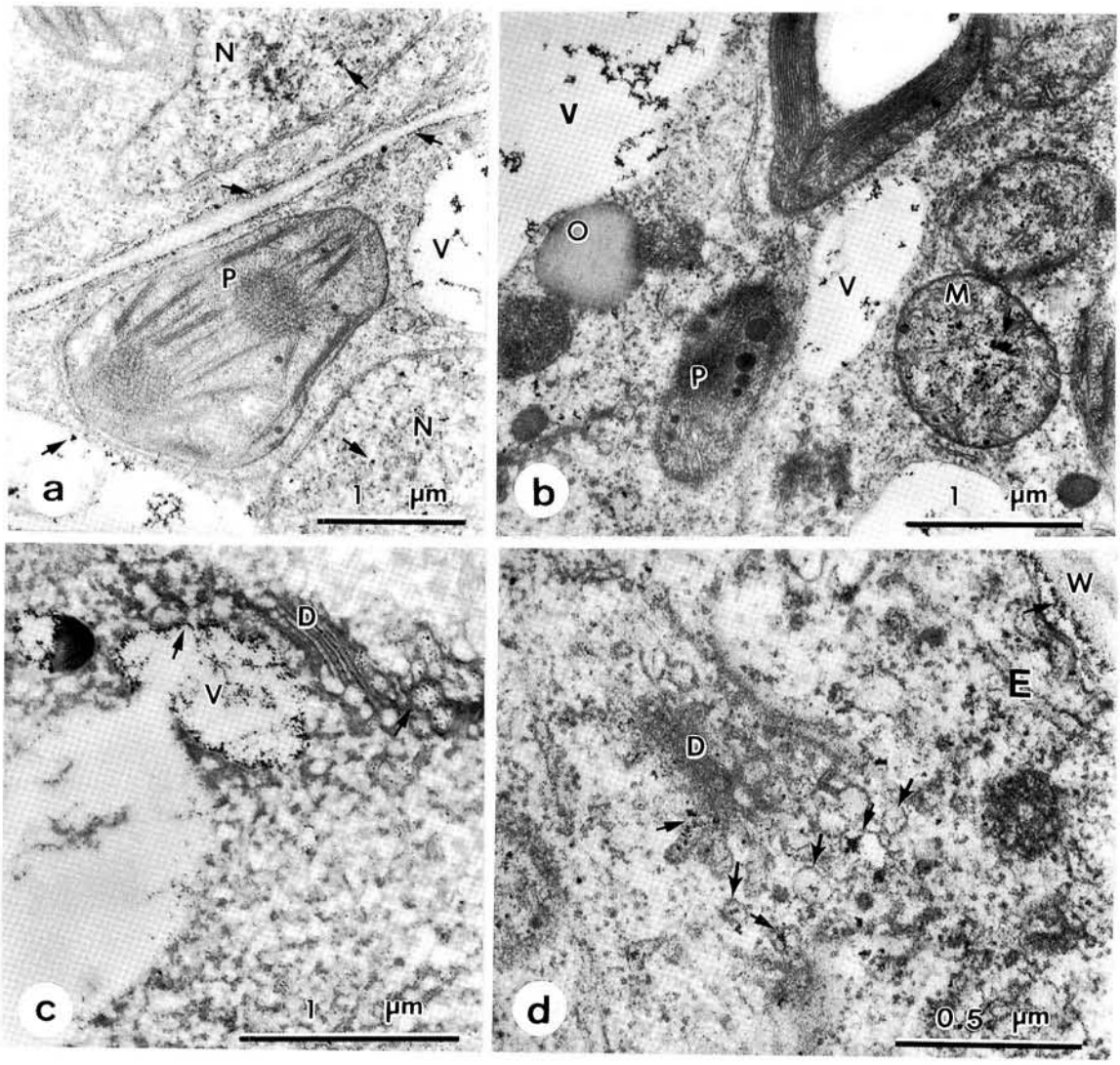

Fig. 1. Subcellular localization of calcium in the rudimentary mesophyll cells of *Nymphaea tetragona*. a and b: Calcium antimonate precipitates (arrows) were mostly observed in the nuclei, vacuoles, mitochondria, and between the plasma membrane and cell walls. c and d: The calcium deposits (arrows) were associated with the tonoplast, endoplasmic reticulum, dictyosome and the secretory vesicles. D: dictyosome; E: endoplasmic reticulum; M: mitochondrium; N: nucleus; O: oil drop; P: plastid; V: vacuole; W: cell wall.

After the cessation of hypertrophic and branching growth, the sclereid initial cells formed their calcium crystals. Between the plasmalemma and primary wall, many crystal chambers were found sporadically and almost simultaneously (Figs. 2b and c). Each crystal bounded by crystal sheath was located in a crystal chamber. The crystal sheath was in connection with plasmalemma (Fig. 2d). At this stage, the sclereid initial cells contained large undulated nucleus and central vacuole. The large central vacuole was found associated with some small vacuoles. Cytoplasm containing rich organelles was confined to the cell periphery. All the plastids in sclereid initial cells were small and only with few thylakoids and plastoglobuli, however, the plastids of palisade parenchyma cells were filled with stacks of thylakoids, plastoglobuli and large starch grains (Fig. 2c).

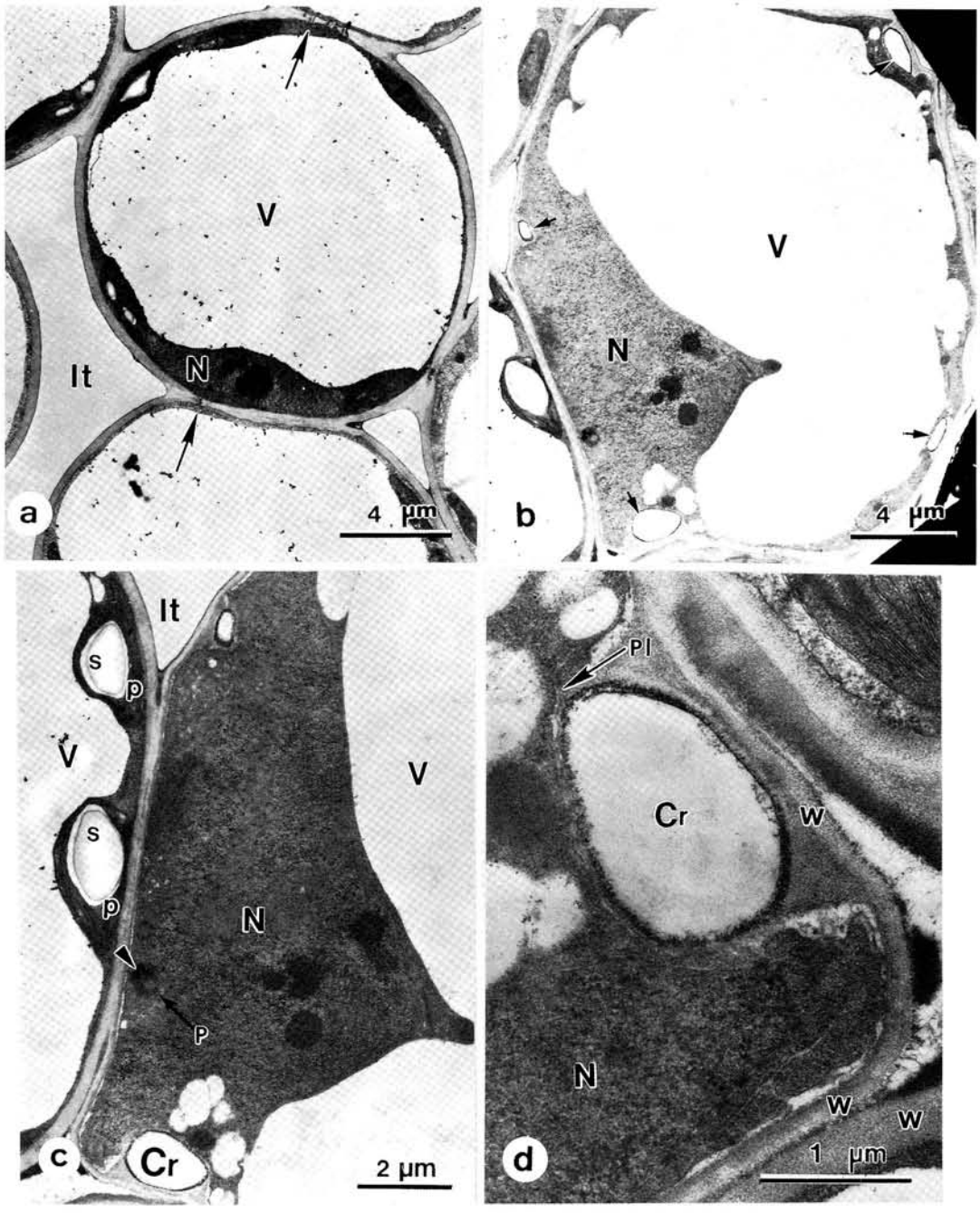

Fig. 2. Paradermal section of palisade tissue in young leaf laminae showing the distribution of calcium antimonate precipitates. a: In the central vacuole of the palisade parenchyma cells, many antimonate precipitates were found. Precipitates were also located in the plasmodesmata (arrows). b: In the sclereid initial cell, large nucleus with prominate nucleoli were observed. Besides, the large central vacuole was associated with some small vacuoles. In the central vacuole, there were only few calcium antimonate precipitates. Some crystal chambers (arrows) were found between the plasmalemma and cell wall. c and d: Enlargements of a portion of Fig. 2b. The dense cytoplasm was confined to a thin layer around the cell periphery. The plastids (arrow) of sclereid initial cell (left cell) were small and with only few thylakoids and plastoglobuli (arrowhead), however, the plastids of palisade parenchyma cell (right cell) were filled with stacks of thylakoid, plastoglobuli and large starch grains. Dense fine antimonate precipitates were found especially around the crystal sheath that was in connection with the plasmalemma (arrow). Cr: crystal; It: intercellular space; N: nucleus; P: plastid; Pl: plasmalemma; S: starch grain; V: vacuole; W: cell wall.

In the sclereid initial cells, during the crystal forming stage, fine calcium antimonate precipitates were accumulated densely around the crystal sheath and plasmalemma (Figs. 2c and d). There were also some calcium precipitates in the small vacuoles near the crystal chambers. However, in the large central vacuole of the sclereid initial cell only few antimonate precipitates were found (Fig. 2b). Nevertheless, amounts of precipitates resided in the central vacuoles of the neighboring mesophyll cells.

Almost after the formation of calcium crystals, the sclereids started to form secondary cell walls. During the process of wall thickening, the cytoplasm of sclereid was still dense with mitochondria, rough endoplasmic reticulum, dictyosome, and secretory vesicles (Figs. 3a and b). Calcium antimonate precipitates were found to be mostly associated with the plasmalemma, crystal sheaths and primary cell walls of the sclereids. Besides, many antimonate precipitate were located in the central vacuoles of the sclereids as well as the neighboring parenchyma cells (Fig. 3b). In the mature sclereids, calcium crystals were embedded between thin primary and thick layered secondary cell walls (Figs. 3c and d).

DISCUSSION

The modified osmium tetroxide-potassium pyroantimonate precipitation method in conjunction with electron microscopy is used consistently specific for the calcium ions in plant cells (Slocum and Roux, 1982; Moore, 1986; Hilaire *et al.*, 1995). Meanwhile, it gives a more precise subcellular localization. Besides, the addition of tannic acid to the aldehyde-antimonate fixative results in an improved preservation of ultrastructural morphology. Therefore the antimonate precipitate is the indicator for the localization of calcium ions. In the most cell types, the concentrations over which calcium is precipitated by 20mM antimonate are about 10^{-8} to 10^{-3} M calcium concentration range (Wick and Hepler, 1982). The ability of antimonate to precipitate calcium ions *in situ* appears to be dependent on the relative calcium content of a given organelle or cell type, as seen in this study, the ordinary mesophyll cells *versus* calcium crystal-forming sclereids.

In the ordinary mesophyll cells of *Nymphaea tetragona*, calcium antimonate precipitates were mostly found in central vacuoles or were associated with plasmalemma and plasmodesmata. Besides, some deposits also occurred in mitochondria, nuclei, endoplasmic reticulum, dictyosome, and secretory vesicles. This kind of calcium distribution pattern might indicate that in the ordinary mesophyll cells of *Nymphaea tetragona*, the central vacuoles are the main pool of calcium in the plant cells. Besides, during the differentiation of mesophyll, transport of calcium between the cells may be through the endoplasmic reticulum within the plasmodesmata. Such kind of intercellular calcium movement occurs also between the columella cells of the roots of *Melilotus alba* (Hilaire *et al.*, 1995) and *Zea mays* (Moore, 1986). Otherwise, the calcium ions might be translocated intracellularly between the organelles, e.g. nucleus, endoplasmic reticulum, dictyosome or plasmalemma, and secretory vesicles, and then stored in the central vacuole. In the leaves of many plant species, e.g. *Phaseolus* (Kuo-Huang and Zindler-Frank, 1998), *Morus* (Wu and Kuo-Huang, 1997), and *Lemna* (Franceschi, 1987), the calcium oxalate crystals are presented within the central vacuoles of crystal idioblasts. The formation of these insoluble calcium crystals is interpreted as a solution for maintaining low calcium concentration in the cytoplasm of plant cells (Franceschi and Horner, 1980).

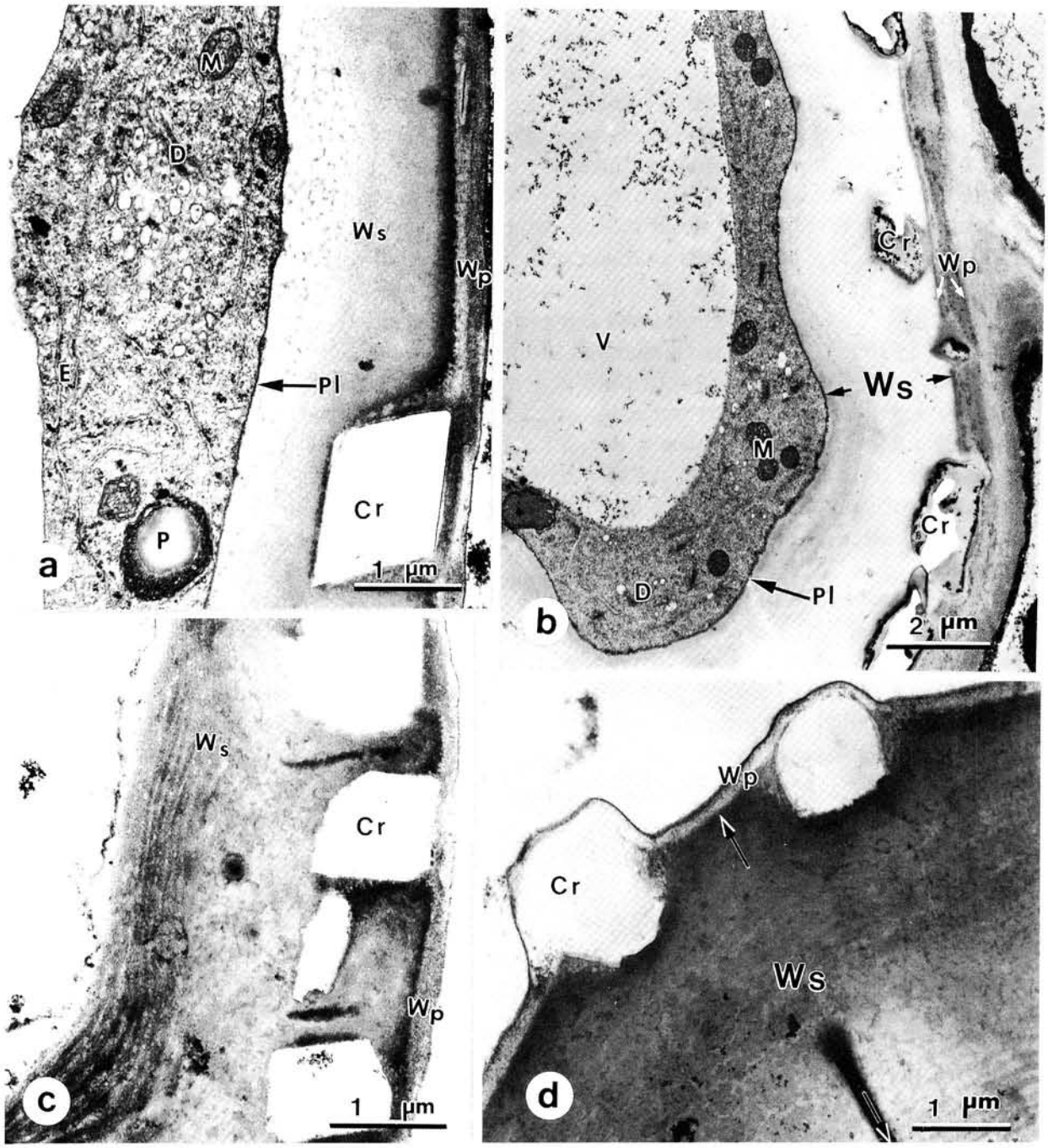

Fig. 3. Transections of foliar sclereids with calcium oxalate crystals between primary and secondary cell walls. a and b: In the sclereids, the dense cytoplasm was with many mitochondria, rough endoplasmic reticulum, dictyosomes, and secretory vesicles. Calcium antimonate precipitates were associated with the plasmalemma, crystal sheaths and primary cell walls. Besides, many calcium deposits were located in the central vacuoles. c and d:The mature sclereid showing the crystals embedded between the thin primary and the thick layered secondary cell wall. Cr: crystal; D: dictyosome; E: endoplasmic reticulum; M: mitochondrium; P: plastid; Pl: plasmalemma; V: vacuole; Wp: primary cell wall; Ws: secondary cell wall.

In the leaf laminae of *Nymphaea tetragona*, prismatic calcium oxalate crystals occur extracellularly in the cell walls of sclereids (Kuo-Huang, 1992). These sclereids formed their calcium crystals within the crystal sheaths located in the crystal chambers. The crystal chambers were found randomly and almost simultaneously between the plasmalemma and primary wall. When the sclereid idioblasts formed their calcium crystals, fine antimonate precipitates were accumulated densely on the crystal sheaths, plasmalemma, and in the small vacuoles near the crystal chambers. But in the large central vacuole of the sclereids only few antimonate precipitates were found, nevertheless, there were many precipitates in the central vacuoles of the neighboring mesophyllous cells. These results suggest that during the crystal forming stage, the sink of calcium ions in the crystal-forming sclereids of *Nymphaea tetragona* is changed from the central vacuole to the crystal chambers in the wall. In plants, calcium is an essential apoplastic nutrient and serves as a structural component of the cell wall (Bush, 1993). So that calcium stored in the central vacuoles may be translocated to the cell wall and affect its mechanical and structural properties. The redistribution of excess calcium ions, through the cytoplasmic organelles to the plasmalemma as well as to the connected crystal sheath and finally accumulated in the extracellular crystal chambers, may induce the formation of calcium crystals in the cell walls of sclereids of *Nymphaea*. After the formation of calcium crystals, the thick layered secondary cell wall deposited and then the excess calcium ions, as the neighboring parenchyma cells, were accumulated in the central vacuoles.

For many cellular processes, the presence of definite free calcium in the cytoplasm is considered as an important regulator (Schroeder and Thuleau, 1991; Bush, 1993). Although our results are too preliminary to speculate on the causal regulating mechanisms of the changed amounts of calcium precipitates associated with the central vacuoles, but it is worthy mentioning that plasmalemma and tonoplast are the most likely ones to control the intra- or extracellular excessive calcium displacement.

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睡蓮含晶體之厚壁細胞內鈣的次細胞定位

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摘　　要

本研究是透過銻酸/焦銻酸鉀（OTPA）與細胞內鈣離子作用產生微細沉澱，再經由電子顯微鏡技術的觀察，來探究睡蓮(*Nymphaea tetragona* Georgi) 葉部厚壁細胞發育過程中，鈣離子的次細胞定位。在葉片柵狀組織之葉肉薄壁細胞內，焦銻酸鈣的沉澱物分佈於細胞膜與細胞壁之間、細胞核、粒線體、液胞、內質網、高爾基氏體及分泌囊胞內。厚壁細胞分散地始源自未分化的葉肉薄壁細胞，許多結晶腔位於其初生細胞壁與細胞膜之間。晶體鞘膜包圍草酸鈣晶體，並與細胞膜相連接。厚壁始源細胞內，微細的鈣沉澱物主要密集地堆積於晶體鞘膜上，但在中央液胞內，則只可觀察到少許鈣的沉澱物。厚壁細胞之細胞壁內的晶體形成後，即進行次生細胞壁的堆積，並將晶體包埋於初生與次生細胞壁之間。成熟厚壁細胞的液胞內、細胞膜、初生細胞壁及晶體鞘膜上均可明顯觀察到焦銻酸鈣的沉澱物。

關鍵詞：鈣的次細胞定位、銻酸沉澱、葉部厚壁細胞、草酸鈣晶體、睡蓮。

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