Structural Changes and Fate of Crystalloplastids during Growth of Calcium Oxalate Crystal Idioblasts in Japanese Yam (*Dioscorea japonica* Thunb.) Tubers

Michio Kawasaki, Mitsutaka Taniguchi and Hiroshi Miyake

(Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan)

**Abstract**: The structural changes of crystalloplastids during calcium oxalate crystal idioblasts growth in Japanese yam tubers were examined by transmission electron microscopy. Idioblasts developed in the cortex of tubers during the tubers development were large and elliptic and contained many crystalloplastids. The crystalloplastids were shown to have novel morphological characterizations. The single or multiple obvious electron-translucent parts without membrane structures were formed in the crystalloplastids during the crystal formation. Coincidentally, the electron-dense parts containing plastid ribosomes and tubular membranes were formed at the periphery of crystalloplastids. During further progress of crystal formation, obvious electron-translucent parts enlarged and finally electron-dense parts disappeared, forming the crystalloplastids similar to small vacuoles and/or vesicles. The majority of such crystalloplastids entered and was incorporated into the central vacuoles of idioblasts during crystal formation. The plastids remaining in the cytoplasm of mature idioblasts were proplastid-like organelle. Thus, the fate of crystalloplastids with growth of idioblasts was shown in Japanese yam tubers. It was suggested that the incorporation of crystalloplastids into central vacuoles of the idioblasts was one of the processes of material transportation involved in calcium oxalate crystal formation.

**Key words**: Calcium oxalate, Crystal idioblast, Crystalloplastid, Electron microscopy, Raphide, Tuber, Yam, Yamanoimo.

Calcium oxalate crystals are observed in various tissues and organs of a large number plant species. The crystals are the most common form of biomineralization or biologically controlled mineral deposition in plants (Arnott, 1983). Although common in nature, knowledge in relation to many aspects of the mechanisms regulating the crystal formation is still unclear (Webb, 1999; Nakata, 2003).

The vast majority of plants typically deposit calcium oxalate crystals in the vacuoles of highly specialized cells called crystal idioblasts (Horner and Wagner, 1995). The development of crystal idioblasts, including the structural changes of the idioblasts and the morphological development of the crystals, has been reported for many plant species (Horner and Wagner, 1995). In crystal idioblasts, morphologically unique plastids, termed crystalloplastids (Arnott, 1966), were observed in the roots of *Vanilla planifolia* (Mollenhauer and Larson, 1966; Kausch and Horner, 1983a), *Monstera deliciosa* (Mollenhauer and Larson, 1966) and *Yucca torreyi* (Arnott, 1966; Kausch and Horner, 1984; Horner et al., 2000), and in leaves of *Typha angustifolia* (Kausch and Horner, 1983b).

The result of the L- [1-14C] ascorbic acid labeling (Horner et al., 2000) suggested that crystalloplastids in roots of *Yucca* may participate in some way in the conversion of the ascorbic acid to oxalate and in the transfer of oxalate to the cell vacuoles. Kausch and Horner (1983b) reported that the crystalloplastids may be involved in the formation of vacuolar mucilage surrounding the crystals in raphide crystal idioblasts in *Typha*. Although the plastid in the crystal idioblast is considered as an important cell organelle for the calcium oxalate crystal formation and development, the information on the structural changes during crystal formation and function of crystalloplastids are limited.

Calcium oxalate crystals in plants are considered to play a role in ion balance, in plant defense, tissue support and in detoxification (Prychid and Rudall, 1999; Nakata, 2003). However, formation of calcium oxalate crystals is disadvantageous on occasion for the quality and processing of edible yam tubers. Thus elucidation and control of the mechanisms of calcium oxalate crystal formation are important subjects in crop sciences. The purpose of our study was to understand mechanisms in relation to the crystal synthesis or formation. In this study, the structural
changes and fate of crystalloplastids during growth of the crystal idioblasts in Japanese yam tubers were focused and investigated morphologically.

Materials and Methods

1. Plant materials

Japanese yam (Dioscorea japonica Thunb.) plants were grown in the experimental field of Nagoya University at Nagoya, Japan in 2002. The tubers in elongating phase were sampled for optical and transmission electron microscopy.

2. Fixation of tubers

The small blocks from the tubers were obtained by cutting with a razor blade. The blocks were fixed in 0.05 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde and 1% paraformaldehyde at 4°C for 2 hr and then at 20°C for 2 hr. The samples were washed with 0.1 M sodium cacodylate buffer, and post-fixed in 0.15 M sodium cacodylate buffer containing 1% osmium tetroxide at 4°C for 8 hr. Fixed samples were dehydrated through a graded series of ethyl alcohol and permeated with propylene oxide. The samples were then embedded in Spurr’s resin and polymerized at 70°C for 24 hr.

3. Microtomy and microscopy

For optical microscopy, semiultrathin sections (0.7 µm in thickness) were cut with a glass knife on an ultramicrotome (Ultracut N, Reichert). Sections were stained with toluidine blue O to observe the inner structure of tubers. They were observed with an optical microscope (Optiphot-2, Nikon).

For transmission electron microscopy, ultrathin sections (80 to 90 nm in thickness) were cut with a diamond knife on an ultramicrotome (Ultracut N, Reichert) and placed on 300 mesh grids. Sections were stained with uranyl acetate followed by lead citrate to observe the inner ultrastructures of tubers. Localization of carbohydrates on sections was detected
by the following staining procedures with the periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) or basic bismuth after the uranyl acetate and lead citrate staining. For PA-TCH-SP staining, sections were incubated in 1% periodic acid at 20°C for 30 min. Then they were washed in distilled water, and incubated in 20% acetic acid containing 0.2% thiocarbohydrazide at 20°C for 90 min. After the incubation, sections were washed in 10% acetic acid, 5% acetic acid and distilled water in sequence. Then

Fig. 7. Crystalloplastid with multiple electron-translucent parts. (Bar=0.83 µm)
Fig. 8. Cytoplasm adjacent to the tip of crystals in a crystal idioblast during crystal formation. (Bar=1.25 µm)
they were incubated in 1% silver proteinate at 20°C for 30 min, and washed in distilled water. For basic bismuth staining, sections were incubated in water solution consisting of 0.1% basic bismuth, 0.5% sodium hydrate and 0.2% potassium sodium tartrate at 40°C for 30 min. Then they were washed in distilled water. Stained sections were examined at 100 kV with a transmission electron microscope (H-600, Hitachi).

Results

Fig. 1 shows the developmental process of crystal idioblasts in the cortex tissue adjacent to the tip of the tuber. The idioblasts adjacent to primary thickening meristem (left side in Fig.1) are young and small, and those near the surface of tubers (right side in Fig.1) are matured and large. The parenchyma cells surrounding the idioblasts contained starch granules although the idioblasts did not. The mature idioblasts near the surface were obviously distinguishable from surrounding parenchyma cells by their larger and more elliptic appearance. Bundles of needle-shaped calcium oxalate crystals, called raphides (Esau, 1965), were observed in central vacuoles of idioblasts, and they also enlarged as the idioblasts development (Fig. 1).

The structural changes of crystalloplastids in the idioblasts of Japanese yam tubers are shown in the transmission electron micrographs (Figs. 2-13). In young crystal idioblasts at about the beginning of crystal formation, crystalloplastids enlarged slightly or were constricted with their elongation. These crystalloplastids contained relatively electron-translucent stroma and membrane structures which invaginated from the inner plastid envelopes (Figs. 2 and 3). Subsequently, the electron-dense parts with plastid ribosomes and tubular membranes were formed in the crystalloplastids accompanied with the crystal formation in the idioblasts (Figs. 4-6). In addition, the obvious electron-translucent parts were formed in crystalloplastids during the crystal formation (Figs. 4-6). The translucent parts contained amorphous materials but did not contain membranous structures (Figs. 5-13). Central vacuoles of the idioblasts also contained amorphous materials and the materials associated with the electron-dense strands around each crystal, termed crystal chamber (Arnot and Pautard, 1970) (Figs. 5 and 12). Fig. 6 shows a part of a crystalloplastid consisting of a translucent part and dense part. There are many tubular membranes and the ribosomal compartments surrounded by tubular membranes in the dense parts of the crystalloplastid (Fig. 6). Tubular membranes comparted between the translucent parts and the dense parts in crystalloplastids.

Multiple translucent parts were also formed in a crystalloplastid during the further process of crystal formation (Figs. 7 and 8). In cytoplasm adjacent to the tip of crystals, there were plenty of crystalloplastids.
(Fig. 8). Crystalloplastids enlarged gradually as the translucent parts enlarged with the progress of crystal formation (Figs. 5 and 8). The area of the dense parts decreased as crystalloplastids enlarged and was not observed finally in the enlarged crystalloplastids (Figs. 8-10). These plastids were similar to small vacuoles...
and/or vesicles.

We also observed crystalloplastids invaginated into the central vacuoles of idioblasts during the crystal formation (Figs. 5, 9 and 10) and at about the completion of the crystal formation (Figs. 11 and 12). The crystalloplastids that had entered the central vacuoles were observed from early stage of crystal development, and increased in number as crystal formation progressed but were not observed at mature idioblasts. The envelopes of crystalloplastids which entered the central vacuoles were single membranes (Fig. 13). Therefore, crystalloplastids in the vacuoles appeared just like vesicles. The vesicles derived from crystalloplastids further enlarged and incorporated into the central vacuoles (Figs. 9-13).

Figs. 10 and 11 show crystalloplastids or the vesicles in crystal idioblasts treated with basic bismuth and PA-TCH-SP, respectively. PA-TCH-SP or basic bismuth positive material was not detected in crystalloplastids and central vacuoles during and after crystal formation. Periodic acid-Schiff-positive materials were observed neither in central vacuoles nor crystalloplastids (not shown in figure).

In the mature crystal idioblasts after the completion of crystal formation, crystalloplastids and the vesicles derived from crystalloplastids were not observed in the cytoplasm and vacuoles although few proplastid-like plastids remained occasionally in the cytoplasm (Fig. 14).

Discussion

Structural characterizations of crystalloplastids in Japanese yam were observed in this study. The electron-dense parts containing plastid ribosomes and tubular membranes were formed at the periphery of crystalloplastids from early period of crystal formation. At the same time, the obvious electron-translucent parts without membrane structures appeared in crystalloplastids. Crystalloplastids in the crystal idioblasts of *Yucca* had a circular central region containing short lamella in a somewhat electron-translucent matrix during the time of crystal formation (Horner et al., 2000). This central region was surrounded by a dense boundary that extended into electron-dense arms containing ribosome-like particles and lamellae. Crystalloplastids in *Typha* formed the enlarged parts, which were relatively electron-translucent and contained simple thylakoids, and the lobe parts, which appeared as electron-dense with many plastid ribosomes and thylakoids, after the completion of crystal production (Horner et al., 1983b). In *Vanilla*, an electron-dense layer with plastid ribosomes was formed at the periphery of crystalloplastids, and robed parts were formed after crystal formation (Kausch and Horner, 1983a). Some morphological similarities were recognized among these modified crystalloplastids although the time of their expression was not uniform.

It was novel characterization in Japanese yam that crystalloplastids were shown as the organelle similar to small vacuoles and/or vesicles, as the result of disappearance of dense parts in crystalloplastids with their enlargement. In addition, multiple translucent parts were formed in a crystalloplastids of Japanese yam during crystal formation, although the existence of such multiple translucent parts in crystalloplastids has not been reported. It is possible that cross sectional views of the lobes (Kausch and Horner, 1983a; 1983b) or arms (Horner et al., 2000) of crystalloplastids were the parts elongated to form additional translucent parts or the parts connecting between translucent parts.

The fate of crystalloplastids in idioblasts was unclear in previously investigated plant species (Kausch and Horner, 1983b). In doctoral thesis of Eilert (1974), the large-scale degeneration of enlarged crystalloplastids in the central vacuole was reported. Mollenhauer and Larson (1966) suggested that the enlarged plastids in idioblasts of *Vanilla* reverted to proplastids after crystal formation. Kausch and Horner (1983b) observed a dedifferentiation of the crystalloplastids and decrease in plastid number in idioblasts of *Typha*. In the Japanese yam tubers examined in this study, many crystalloplastids entered and incorporated into the central vacuoles of idioblasts during crystals formation and crystalloplastids finally disappeared in idioblasts. The plastids remaining in cytoplasm in mature idioblasts existed as proplastid-like organelles. The fate of crystalloplastids with growth of idioblasts was shown in Japanese yam tubers.

A feature of some raphide crystal idioblasts is the accumulation of mucilage surrounding the crystals in the central vacuoles during later stages of development, after crystals are partly formed (Horner et al., 1981; Kausch and Horner, 1983b). Analysis of the water-soluble matrix in the central vacuoles of the crystal idioblasts in leaves of *Vitis* showed the presence of an unusual polymer with novel glucuronic acid linkages (Webb et al., 1995). In addition, linkage analysis indicated the presence of 5-linked arabinitans, arabinogalactan, and various mannosyl units typical of complex carbohydrates of N-linked glycoproteins in the water-soluble matrix. Kausch and Horner (1983b) reported the existence of PA-TCH-SP positive material in translucent parts in the lobed parts of crystalloplastids and in the central vacuoles of crystal idioblasts in *Typha*. Thus, they reported that the lobed crystalloplastids may be involved in vacuolar mucilage formation in raphide crystal idioblasts. In the present study, however, PA-TCH-SP or basic bismuth positive material was not detected in plastids and the central vacuoles of the crystal idioblasts during and after crystal formation.

Intervacuolar matrices (e.g. crystal chambers) are
macromolecular structures that have been suggested to play an important role in crystal formation (Horner and Wagner, 1980; Webb and Arnott, 1983; Barnabas and Arnott, 1990; Webb et al., 1995). Crystal chambers may have unique components related to crystal nucleation or other aspects of their role in accumulating and compartmentalizing calcium and oxalate (Webb et al., 1995). Webb et al. (1995) also reported that different polypeptides associated with calcium oxalate crystal formation in Viitis. Crystals in tobacco, tomato and bougainvillea were shown to contain acidic proteins (Bouropoulos et al., 2001). The assemblage of crystal-associated macromolecules in tobacco promotes was revealed to nucleation of calcium oxalate crystals by an in vitro assay (Bouropoulos et al., 2001). Despite many electron microscopic studies showing the crystal chambers and crystal-associated materials in the vacuoles of crystal idioblasts, little was known about their origin. Recently, Li et al. (2003) reported that the matrix protein in calcium oxalate crystals was transported to the vacuoles via the Golgi apparatus. In Japanese yam, there were amorphous materials in the translucent parts of crystalloplastids. Central vacuoles also contained amorphous materials. In addition, the grater parts of plastids entered and were incorporated into the central vacuoles of idioblasts during crystal formation. It was possible that crystalloplastids play a role in transporting some materials consisting vacuolar matrix from cytoplasm to the vacuoles in the idioblasts.

Horner et al. (2000) reported that ascorbic acid is the immediate precursor of oxalate in the crystal idioblasts of Yucca torreyi primary roots from the result of L-[1,14C] ascorbic acid labeling experiment. They suggested that the plastids in the crystal idioblasts participate in some way in the conversion of the ascorbic acid to oxalate and in the transfer of oxalate to the cell vacuoles, although the system of transportation is unknown. Kausch and Horner (1984) suggested that crystalloplastids are multifunctional organelles. Therefore, it was suggested that the incorporation of crystalloplastids into central vacuoles of the idioblasts was one of the processes of material transportation involved in calcium oxalate crystal formation. We are presently attempting to identify the materials transported by crystalloplastids into central vacuoles in the idioblasts. We previously reported that the morphological characteristic and morphogenesis of amyloplasts varied among the root and tuber crops such as Japanese yam (Kawasaki et al., 1997), eddo (Kawasaki et al., 1998), potato (Kawasaki et al., 1999) and sweet potato (Kawasaki et al., 2002). Therefore, it is also necessary to study the crystalloplastids in various plant species.

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