Protoplasma (2003) 222: 149–156
DOI 10.1007/s00709-003-0018-2

PROTOPLASMA
Printed in Austria

Localization of calcium in the pericarp cells of tomato fruits during the development of blossom-end rot

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Received January 13, 2003; accepted June 3, 2003; published online October 7, 2003

Summary. Blossom-end rot (BER) of tomato (*Lycopersicon esculentum*) fruits is considered to be a physiological disorder caused by calcium deficiency. We attempted to clarify the localization of calcium in the pericarp cells and the ultrastructural changes during the development of BER. Calcium precipitates were observed as electron-dense deposits by an antimonate precipitation method. Some calcium precipitates were localized in the cytosol, nucleus, plastids, and vacuoles at an early developmental stage of normal fruits. Calcium precipitates were increased markedly on the plasma membrane during the rapid-fruit-growth stage compared with their level at the early stage. Cell collapse occurred in the water-soaked region at the rapid-fruit-growth stage in BER fruits. There were no visible calcium precipitates on the traces of plasma membrane near the cell wall of the collapsed cells. The amount of calcium precipitates on plasma membranes near collapsed cells was smaller than that in the cells of normal fruits and normal parts of BER fruits, and the amount on cells near collapsed cells was small. The amount of calcium precipitates on the plasma membranes increased as the distance from collapsed cells increased. On the other hand, calcium precipitates were visible normally in the cytosol, organelles, and vacuoles and even traces of them in collapsed cells. The distribution pattern of the calcium precipitates on the plasma membrane was thus considerably different between normal and BER fruits. On the basis of these observations, we concluded that calcium deficiency in plasma membranes caused cell collapses in BER tomato fruits.

Keywords: Antimonate method; Calcium deficiency; *Lycopersicon esculentum*; Necrosis; Plasma membrane; Ultrastructure.

Introduction

Blossom-end rot (BER) is a physiological disorder that causes rotting of the tissue at the distal end of the fruit, reducing yield in tomato fruit production. BER is reported to occur under the condition of a low calcium concentration in the culture solution (van Goor 1968). BER also occurs under unsuitable growth conditions such as drought (Nishio and Morita 1991), high salinity (Ehret and Ho 1986), and high temperature (Wui and Takano 1995), even though the calcium concentration in the soils or in the culture solutions is sufficient for normal development of the fruits.

It is widely accepted that BER is caused by calcium deficiency at the distal end of tomato fruits during the initial stage of fruit development within a few weeks after anthesis (Bangerth 1979, van Goor 1968, Ward 1973). A number of other physiological disorders in plants are also caused by calcium deficiency, including bitter pit in apple fruits, internal rusting in potato tubers, internal browning of Brussels sprouts, and tip-burn in lettuce, cabbage, and tomato leaves (Ho and Adams 1989). They all display localized symptoms of necrosis, mostly found in the rapidly growing tissue of lowly transpiring organs (Ho and Adams 1989). The calcium concentration in the distal portion of tomato fruits was lower than that in the basal portion of fruits under conditions of high salinity (Adams and Ho 1992, Minamide and Ho 1993) and low moisture (Nishio and Morita 1991). A strong negative correlation was recognized between the incidence of BER and calcium contents of the water fraction in the distal half of tomato fruits (Terabayashi et al. 1988). The distal placenta and locular tissues had the lowest calcium content in the whole fruit and appeared to be the site of the earliest symptoms of BER (Adams and Ho 1992). On the other hand, no correlation was found between calcium concentration and incidence of BER in the fruit (Petersen and Willumsen 1992). Tomato fruits that had just started having blossom-end rot had a concentration of calcium ions similar to that of normal fruits (Nonami et al. 1995).

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A critical concentration of Ca^{2+} in the fruits for the occurrence of BER has not yet been documented (Saure 2001). When high-salinity stress was applied on tomato growth by a high-nutrition cultural solution in hydroponics at early stage, the rate of occurrence of BER was high (Okano and Nakano 2002). We can produce BER fruits experimentally by cultivating the plants under a high-nutrition condition where the calcium concentration is sufficient for the normal growth (Suzuki et al. 2000).

Some anatomical features of BER fruits were clarified in our previous study (Suzuki et al. 2000). The cell collapse in the epidermis and subepidermal parenchyma in fluid-soaked areas was the first symptom of the disorder observed in the blossom end of tomato fruits. In developed BER fruits, the subepidermal cells were broken down. Electron microscopic observation revealed disruption of the plasma membrane and tonoplast, a wavy-shaped cell wall, breakdown of endoplasmic reticulum, and swollen plastids. The disintegration of membrane structures and a loss of cell compartmentation occurred in calcium-deficient tissues (Hecht-Buchholz 1979). Lignin was deposited around the cells in BER regions, and peripheral cells of BER regions changed into meristematic cells like callus in the advanced stage of BER (Spurr 1959, Suzuki et al. 2000).

In this study we attempted to clarify the localization of calcium in the cells of tomato fruits by an antimonate precipitation technique. This method has been widely used in plant and animal cells (Wick and Hepler 1982). The identity of the precipitate as calcium antimonate was confirmed with energy-dispersive X-ray spectrometry in tobacco ovules (Tian and Russell 1997). Calcium-induced precipitates were formed only when calcium was sufficiently concentrated and available for ionic binding (Tian and Russell 1997). We attempted to identify the localization of calcium in BER fruits and normal fruits histochemically to determine whether there is a relationship between calcium localization and collapses of pericarp cells during the development of BER in tomatoes.

Material and methods

Plant material and hydroponics conditions

Tomato plants (*Lycopersicon esculentum* Mill. cv. House Momotaro) were grown in 10-liter pots using capillary-up hydroponics systems (Sakuma and Suzuki 1997) in greenhouse conditions (27 °C during the day and 23 °C at night) at the Okinawa Subtropical Station, Japan International Research Center for Agricultural Sciences. The culture solution was prepared by diluting Otsuka Ekihi (liquid fertilizer) Solution No. 1 and No. 2 (Otsuka Chemicals Co., Ltd., Osaka, Japan). The Otsuka Ekihi Solution No. 1 contains N, 10%; P_2O_5, 8%; K_2O, 27%; MgO, 4%; MnO, 0.10%; B_2O_3, 0.10%; Fe, 0.18%; Cu, 0.002%; Zn, 0.006%; and Mo, 0.002%; and the Otsuka Ekihi Solution No. 2 contains N, 11%; and CaO, 23%. Normal tomato fruits were produced using a mixture of 0.375 g of Solution No. 1 and 0.25 g of Solution No. 2 in 1000 ml of water (normal condition). On the other hand, BER tomato fruits were produced with a mixture of 1.125 g of Solution No. 1 and 0.25 g of Solution No. 2 in 1000 ml of water (high-nutrition condition). The BER incidence was 77% under the high-nutrition condition and only 8.7% under the normal condition (Suzuki et al. 2000).

Sample preparation for cytochemical and ultrastructural observations of calcium localization

BER fruits were sampled from hydroponically grown tomato plants under high-nutrition condition when the fluid-soaked area appeared on the blossom-end surface. Their diameters ranged from 27 to 30 mm. The control normal fruits of the same size (27–30 mm) at the rapid-growth stage and of a smaller size (8–10 mm) at the early stage were sampled from hydroponically grown tomato plants under the normal condition. They were fixed in 4% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2) containing 2% potassium antimonate. After washing in a rinse buffer (0.2 M phosphate buffer containing 2% potassium antimonate, pH 7.2), the fruits were postfixed in 1% osmium tetroxide in 0.1 M potassium phosphate buffer containing 2% potassium antimonate for 2 h at 4 °C. They were dehydrated in a graded alcohol series and embedded in epoxy resin.

Fig. 1. A light micrograph (LM) of pericarp at the early developing stage in normal fruits. Bar: 100 μm

Figs. 2–4. Transmission electron micrographs (TEMs) of pericarp cells at an early growth stage in normal fruits. Arrows, calcium precipitates; *CL* cuticular layer, *CW* cell wall, *M* mitochondrion, *N* nucleus, *P* plastid, *TE* tracheary element, *V* vacuole. Bar: 1 μm

Fig. 2. Epidermal cells

Fig. 3. Subepidermal cells

Fig. 4. Vascular bundle cells

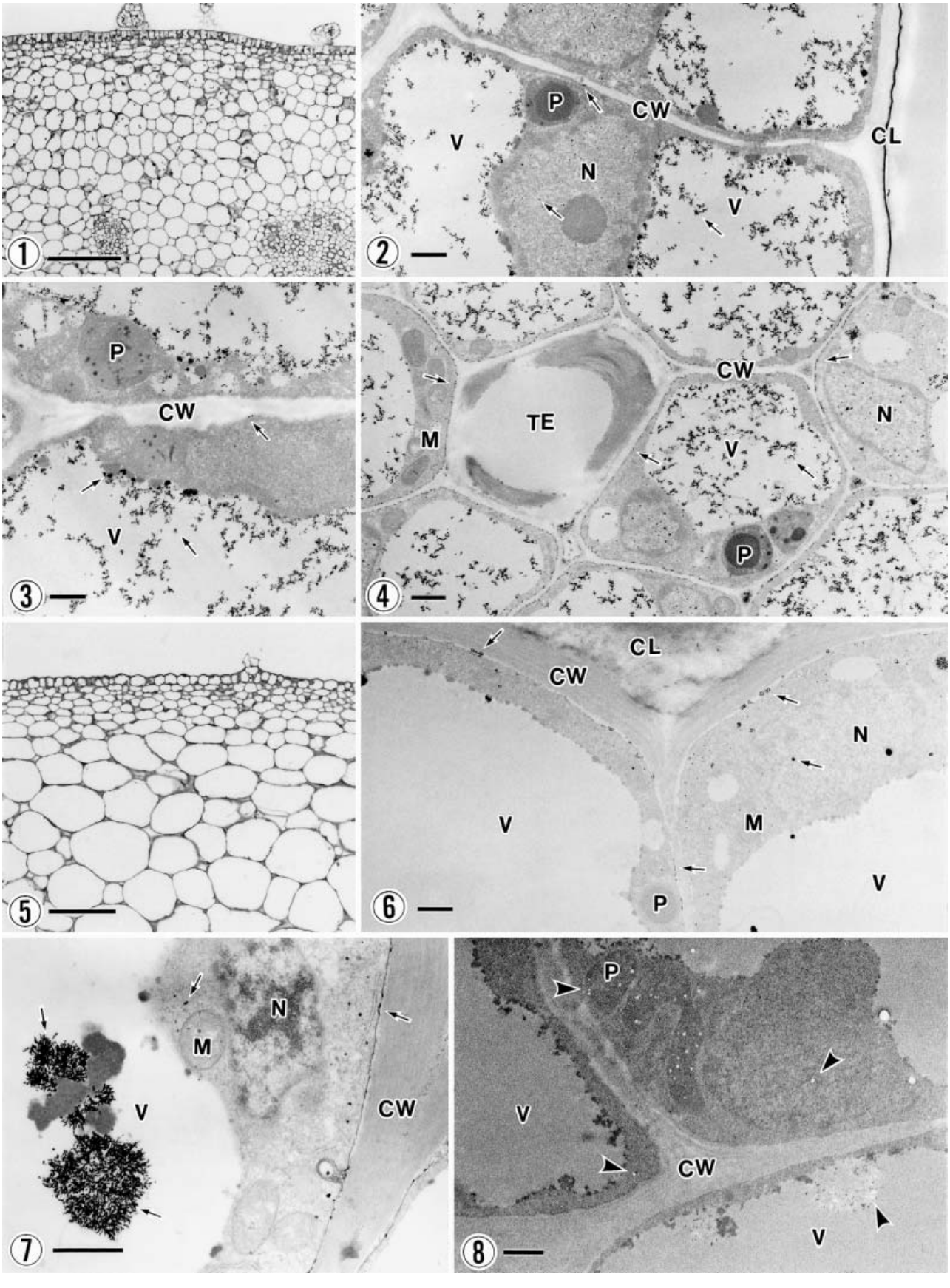
Fig. 5. LM of pericarp at the rapid-growth stage in normal fruits. Bar: 100 μm

Figs. 6–8. TEMs of pericarp cells at the rapid-growth stage in normal fruits. Arrows, calcium precipitates; *CL* cuticular layer, *CW* cell wall, *M* mitochondrion, *N* nucleus, *P* plastid, *V* vacuole. Bars: 1 μm

Fig. 6. Epidermal cells

Fig. 7. Subepidermal cells

Fig. 8. Subepidermal cells after EGTA treatment. Arrowheads, holes after chelating calcium precipitates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We prepared 2 μm thick sections and stained them with Toluidine Blue O for light microscopy observation. For the observation with the transmission electron microscope, we prepared ultrathin sections. Some grids mounted with tissue sections were immersed in a solution of 100 mM EGTA (pH 8.0), which is a chelator with high affinity for calcium ions, and incubated at 60 °C for 1 h. After treatment, the grids were rinsed in distilled water. The sections on grids were not stained. Electron micrographs were taken with an H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan) at 75 kV.

Results

Calcium localization in normal tomato fruits

Early growth stage of fruits

A pericarp of tomato fruit consists of epidermis, parenchyma, vascular bundle, and endodermis. The cells of the parenchyma were about 100 μm in length at an early stage, when the diameter of the tomato fruit was about 10 mm (Fig. 1). Calcium antimonate precipitates appeared as electron-dense particles under transmission electron microscope observation. Abundant precipitations of calcium were localized in vacuoles of the epidermal cells (Fig. 2), subepidermal cells (Fig. 3), and parenchyma cells in the vascular bundles (Fig. 4). They were also observed in vacuoles around the substances, as gray spots on transmission microphotographs (Figs. 2–4). Calcium precipitations also occasionally appeared in cytosols, nuclei and on the inside of plastids (Figs. 2–4). A small amount of precipitates appeared on plasma membranes of the epidermal and subepidermal cells. On the other hand, a large amount of calcium precipitates was detected on plasma membranes of the parenchyma cells near the tracheary elements in the vascular bundle (Fig. 4). In addition, they appeared in intercellular spaces in the vascular bundle (Fig. 4).

Rapid-growth stage of fruits

Vacuolization increased especially in parenchyma cells in the pericarp during the development of fruits. Epidermal cells and subepidermal parenchyma cells were also vacuolated, but those cells were not as large as the inner parenchyma cells. The parenchyma cells were about 200 μm in length, subepidermal cells were 100 μm long, and the epidermis cells were about 50 μm long in the normal fruits at the rapid-growth stage observed in this study (Fig. 5). Calcium precipitates increased on the plasma membranes in pericarp cells during the rapid-growth stage compared with those at an early stage (Fig. 6). They were still visible in cytosols and vacuoles. The precipitates of calcium were observed as an aggregate form around some substances in the vacuoles (Fig. 7).

The electron-dense precipitates of calcium in tomato cells were removed with EGTA, a calcium-selective chelator (Fig. 8). Small transparent holes remained in the vacuoles, cytosol, nuclei, plastids, and plasma membranes where the electron-dense grains were located before the EGTA treatment (Fig. 8).

Calcium localization in BER tomato fruits

A fluid-soaked area became visible on the surface of tomato fruits at the initial development stage of BER when the fruit diameter was about 3 cm in this variety under the high-nutrition condition. Figure 9 shows a boundary region between collapsed cells and uncollapsed cells at the fluid-soaked pericarp of BER fruits. The collapsed cells are visible on the right side in Fig. 9, and the living cells occur on the left side in this field. The collapsed cells were stained homogeneously with Toluidine Blue O, indicating that cell membranes and tonoplasts were broken in that area. Figures 10 and 11 show collapsed cells. The plasma membranes disappeared and tonoplasts were also broken in the collapsed cells (Fig. 11). In the cells damaged by BER, the cell walls had a wavy shape, the endoplasmic reticulum was broken down, and plastids were swollen. The nuclei had small electron-dense spots. Some mitochondrial and plastid membranes were also broken. Those disordered cells appeared in the fluid-soaked pericarp during the development of BER.

Fig. 9. LM of pericarp at the rapid-growth stage of BER fruits. *A* Region of collapsed cells (see Figs. 10–12). *B* Border region between areas with collapsed and with living cells (see Figs. 13 and 14). *C* and *D* Regions about 100 μm away from collapsed cells (see Figs. 15–17). Bar: 100 μm

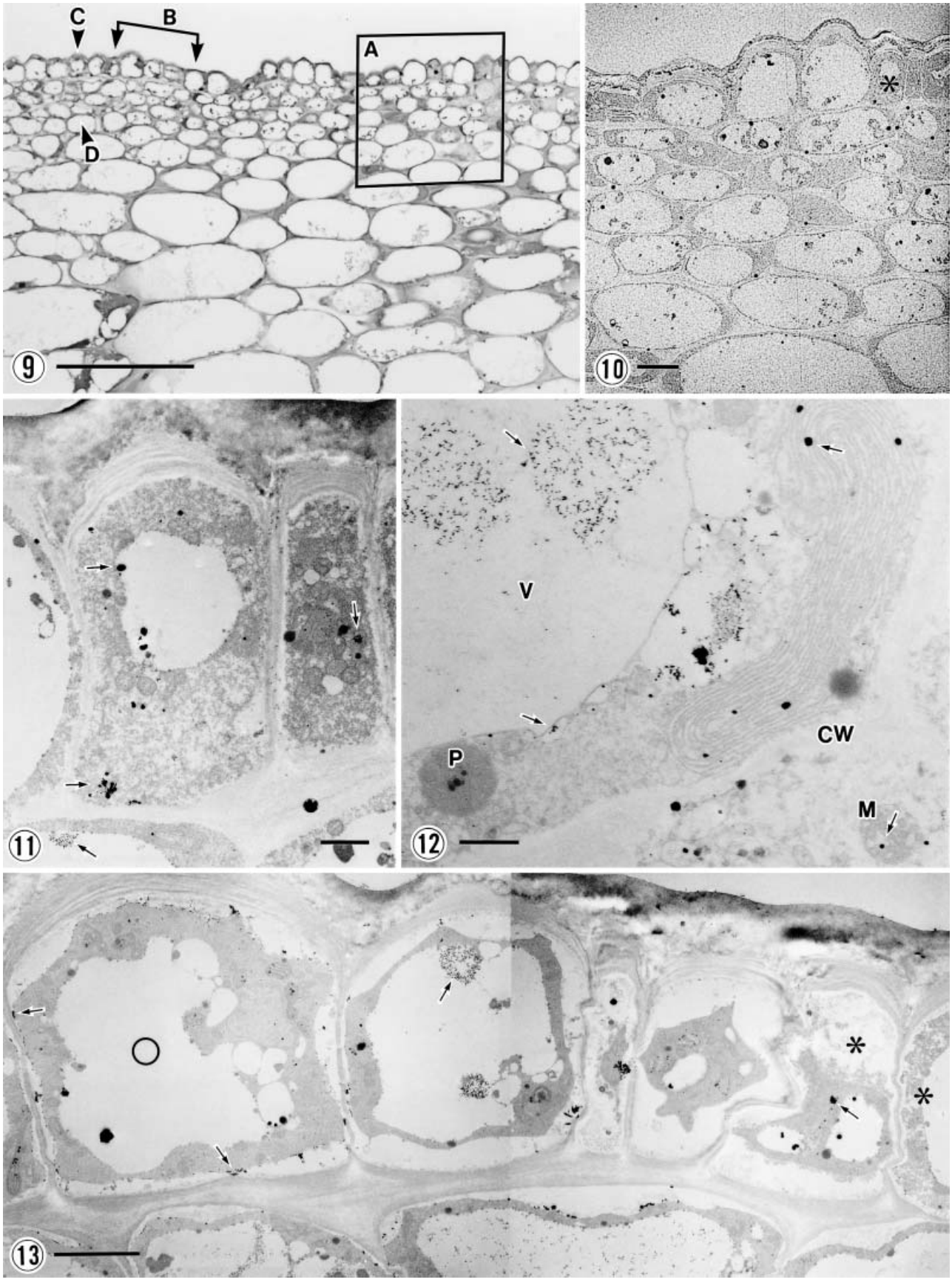
Figs. 10–17. TEMs of pericarp at the rapidly developing stage in BER fruits. Arrows, calcium precipitates; *CL* cuticular layer, *CW* cell wall, *M* mitochondrion, *N* nucleus, *P* plastid, *V* vacuole

Fig. 10. Low-magnification TEM of an area with collapsed cells (*A* in Fig. 9). Bar: 10 μm

Fig. 11. High magnification of collapsed epidermal cell indicated by an asterisk in Fig. 10. Bar: 2 μm

Fig. 12. Collapse of epidermal cell. Bar: 1 μm

Fig. 13. Border region between collapsed cells (asterisks) and living cells (circle) (*B* in Fig. 9). Bar: 5 μm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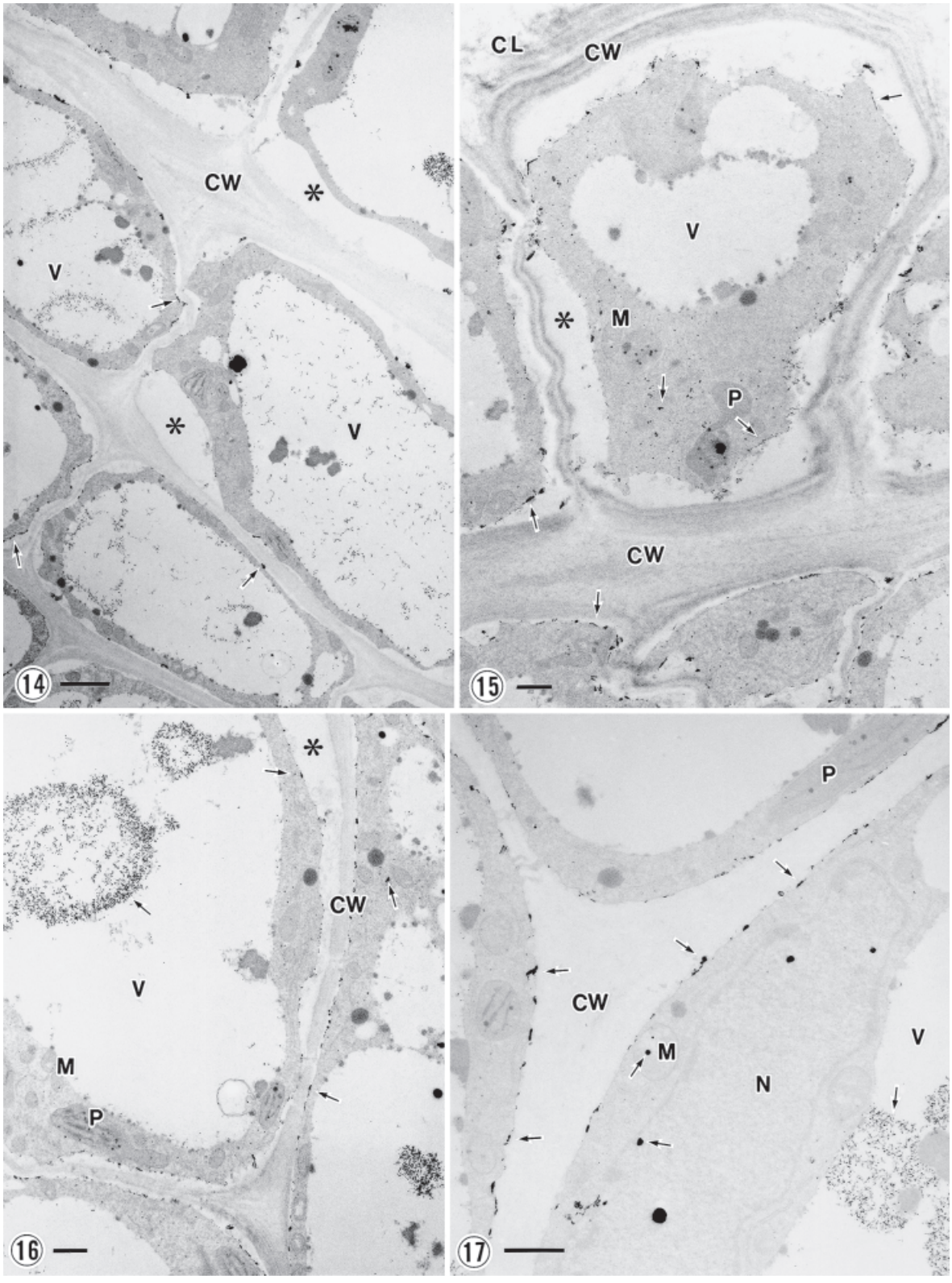

Fig. 14. Subepidermal cells around the collapsed region. Asterisks, plasmolysis. Bar: 2 μm

Figs. 15 and **16.** Epidermal cells (Fig. 15) and subepidermal cells (Fig. 16) about 100 μm away from collapsed cells of a BER region (*C* and *D* in Fig. 9). Asterisks, plasmolysis. Bar: 1 μm

Fig. 17. Subepidermal cells distant from the BER region. Bar: 1 μm

Using an antimonite precipitation method for calcium detection, we saw some calcium precipitates in the nuclei, mitochondria, plastids, and cytoplasm in collapsed cells of BER fruits (Fig. 12). As shown in Fig. 7, some aggregated precipitates existed in the vacuole in normal tomato pericarp cells at the rapid-growth stage. Those calcium precipitates were also observed in the vacuoles in a BER water-soaked area (Fig. 12). However, there were no visible calcium deposits on the traces of plasma membranes that existed near cell walls (Figs. 11 and 12).

The cells shown in Fig. 13 were located in a border region between collapsed parts and living parts. The collapsed cells are visible on the right side, and living cells occur on the left side in this field (Fig. 13). The plasma membranes were broken in the collapsed cells. The plasma membranes of the living cells were still visible, although plasmolysis occurred near collapsed cells in the epidermal cells, the subepidermal cells, and the parenchyma cells. Calcium precipitations were detected in the vacuoles, cytosol, and some organelles, and there was only a small amount of precipitates on plasma membranes of the subepidermal cells near collapsed cells (Figs. 13 and 14). The amount increased gradually as the distance from the collapsed cell region grew. The amount of calcium precipitates on plasma membranes of the cells near collapsed cells was smaller than the amount in the cells of normal fruits and normal parts of BER fruits. On the other hand, calcium precipitates were visible normally in the cytosol, organelles, and vacuoles of the cells near collapsed cells of BER fruits.

The cells shown in Figs. 15 and 16 were about 100 μm from collapsed cells. Many calcium precipitates were observed on the plasma membranes of these cells, although plasmolysis had already occurred (Figs. 15 and 16). Some calcium precipitates were observed in the cytosol and organelles (Figs. 15 and 16). Aggregations of calcium precipitates were observed in the vacuoles (Fig. 16).

Abundant calcium precipitates were deposited on the plasma membranes of the cells distant from collapsed regions in BER fruits, and no plasmolysis was observed in those cells (Fig. 17). There were aggregated calcium precipitates in the vacuoles. No obvious differences in the distribution of calcium precipitates were observed between parts distant from collapsed cells in BER fruits and normal fruits at the same stage.

Discussion

In this study we examined the localization of calcium in normal and BER tomato fruit pericarps histochemically by an antimonate precipitation method. Calcium precipitates were observed on the plasma membranes of the parenchyma cells in the vascular bundle at an early stage in normally developing tomato fruits (Fig. 4). Then, they were increased markedly on the plasma membranes of the epidermal and parenchyma cells in the rapid-growth stage (Figs. 6 and 7). On the other hand, there was a small amount of calcium precipitates on the plasma membranes in BER regions and in regions surrounding those regions. A considerable difference in the amount of calcium precipitates on plasma membranes between normal and BER fruits in the rapid-growth stage suggested that a deficiency of calcium concentration on the plasma membrane causes necrosis during the development of BER. The reduction of calcium concentration on plasma membrane occurred under the high-nutrition condition where a sufficient amount of calcium existed in the culture solution.

Calcium is an essential and major plant nutrient. Since rapidly growing tissues require a lot of calcium, it is thought that calcium deficiency occurs frequently in those tissues. Ho et al. (1993) suggested that a high incidence of BER, associated with accelerated growth, is due to an increased demand for calcium for rapid cell enlargement exceeding the supply in the susceptible fruit tissues.

Calcium is required for structural, osmotic, and signaling purposes in the plant cells (Marschner 1995, White 1998). High calcium concentrations were found in the middle lamella of the cell wall, on the exterior surface of the plasma membrane, in endoplasmic reticulum, and in the vacuole (Marschner 1995). The calcium localization on the plasma membrane has often been documented in plants (Havelange 1989, Wick and Hepler 1982, Slocum and Roux 1982, Tretyn et al. 1992). The fundamental role of calcium in the plasma membrane is thought to be the maintenance of membrane stability and cell integrity (Kirkby and Pilbeam 1984). Calcium deficiency caused as an initial effect changes in the plasma membranes in potato cells (Hecht-Buchholz 1979). We concluded that the cause of necrotic cell death in BER tomato fruits was a disorder of plasma membrane function due to calcium deficiency.

The appearance of physiological disorders caused by calcium deficiency may be closely correlated to transport systems of Ca^{2+}. There are likely to be two pathways of calcium movement, through the symplast and through the apoplast, in plant tissues (White 2001). Cellular calcium homeostasis is maintained by an ensemble of calcium transport proteins, Ca^{2+} ATPases, Ca^{2+}/H antiporters, and the Ca^{2+} channels (Bush 1995). Recently, several types of Ca^{2+}-permeable channels were reported to exist on the

plasma membrane (White 1998, 2000). Two distinct types of calcium channels, depolarization-activated and hyperpolarization-activated calcium channels, were identified in plasma membranes of plants (Miedema et al. 2001, Very and Sentenac 2002). Hyperpolarization-activated calcium channels are more specifically active in rapidly growing cells and tissues, e.g., the tip of growing root hairs (Very and Davies 2000) and the root apex (Kiegle et al. 2000). The role of calcium influx into plasma membrane in plant growth and development is twofold, including signal transduction and cell division and expansion (Kiegle et al. 2000). The regulation of the cellular Ca^{2+} concentration is an essential cell function accomplished by a complex of processes. The amount of calcium precipitates on plasma membranes decreased as the distance from collapsed cell regions in BER fruits increased when tomato plants were grown under the high-nutrition condition as indicated. The transport and regulation systems of calcium may have caused damage at the fruits' apex when BER emerges under unsuitable growth conditions.

Acknowledgment

This research was supported in part by a grant from the Bio-oriented Technology Research Advancement Institution (BRAIN), Japan.

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