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ULTRASTRUCTURAL STUDY ON MECHANISM OF INCREASED FREEZING TOLERANCE DUE TO EXTRACELLUAR GLUCOSE IN CABBAGE LEAF CELLS

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Summary

Glucose caused increased tolerance to equilibrium slow freezing of cabbage leaf tissues, with a decrease in the temperature for the loss of 50% of electrolytes from -5℃, in untreated controls, to -17℃ in samples that had been pre-incubated with a 1 M solution of glucose for 10 min. The brief incubation with glucose induced plasmolysis. The increased freezing tolerance of plasmolyzed cells was related to a reduction in the frequency of occurrence of ultrastructural changes in plasma membranes that were produced by the close apposition of membranes, a well characterized cause of freezing injury. It is suggested that reduction in the extent of the close apposition of membranes might be due principally to the mitigation of severe deformation of cell walls during freezing by the presence of glucose in periprotoplasmic spaces.

Key words : Cabbage leaves, freezing tolerance, cryopreservation, plasma membrane ultrastructure, glucose

Introduction

Many plant cells become tolerant to freezing as a consequence of acclimation to low temperatures. Cold acclimation produces many physiological and metabolical changes in plant cells (13). In particular, the accumulation of endogenous soluble carbohydrates during cold acclimation is clearly related to the acquisition of freezing tolerance in woody (14) and herbaceous (10) plants.

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The extracellular addition of sugars can increase the freezing tolerance of plant cells and it is widely used in procedures for cryopreservation of plant materials (7, 9). For successful cryopreservation, a long-term pre-incubation, generally for 1 to 5 days, with a solutions of sugar as a cryoprotectant is usually necessary (9). In the case of apices of asparagus shoots, increased freezing tolerance is related to the incorporation of sugars by cells during pre-incubation (7). In cultured cells of *Eucalyptus*, a cryoprotective effect was also detected only after the incorporation of sugars into cells (11).

While some studies, as mentioned above (7, 11), have shown that the protective effects of sugars at the cellular level depend on the accumulation of such sugars inside cells, other studies have suggested the protective effects of sugars against freezing even when the sugars are in the extracellular environment. Sakai and Yoshida (12) showed that brief incubation of cabbage leaves with glucose increased their freezing tolerance, suggesting a protective effect of glucose in the extracellular milieu. The protective effects of sugars and other cryoprotectants, as osmolytes, have also been reported for wheat cells in suspension cultures (16) and in cultured potato cells (17), but the mechanism of such protective effects remain to be determined.

In the present study, we reexamined the effects of extracellular glucose on the increased tolerance to freezing of cabbage leaves, and we studied the possible mechanism of the increased freezing tolerance at the ultrastructural level.

Materials and Methods

Material: Cabbages (*Brassica oleracea* L. cv. Gold Sour) were purchased from a local market. Prior to purchase, cabbages had been stored at 4℃ for 3 or 4 months. Small pieces (2 × 5 × 1 mm^3) were removed from mesophyll tissues in leaves and used as experimental material.

Incubation with solutions of sugars: The pieces of leaf tissue were incubated with solutions of various sugars in distilled water for specified times at room temperature (22℃). After incubation with a sugar solution, both surfaces of each piece of tissue were blotted with filter paper to remove excess moisture (non-washed samples). Alternatively, pieces of tissue were rinsed three times with fresh distilled water and then residual moisture was removed with filter paper as described above (washed samples).

Analysis of soluble carbohydrates: Carbohydrates were analyzed by the method of Kaneko et al. (8). The cited values are means of results from three preparations.

Equilibrium freezing: For assays of survival, five pieces of leaf were placed in a 10-ml test tube. For observations by electron microscopy, two to four pieces were placed in a metal holder for electron microscopy. Samples were first equilibrated, seeded with ice and kept for 1 hr at -3℃. Samples were then transferred to a cold chamber at -5℃ for 1 hr. Further cooling was performed by stepwise transfer of the samples to cold chambers that were cooled down in steps of -5℃ to the desired temperature. The samples were held for 1 hr at each temperature.

Assessment of freezing injury: After equilibrium freezing to the desired temperature and incubation at that temperature for 1 hr, samples were thawed at 4℃ for 1 to 5 hr. The release of electrolytes into distilled water was measured and expressed as a percentage, as described previously (3). We took values of electrolyte leakage of 100% and 0% as evidence of 100% and 0% injury, respectively. EL_{50} was defined as the temperature at which leakage of 50% of

electrolytes occurred.

Electron microscopy: After equilibrium freezing to the desired temperature and incubation at that temperature for 1 hr, the samples for electron microscopy were cryofixed in Freon 22 at -160℃. For controls, which had not been exposed to equilibrium freezing, samples were cryofixed directly from room temperature. The procedures for cryo-scanning electron microscopy and preparation of freeze-replicas were the same as those described previously (2). The frequency of cells with ultrastructural changes in the plasma membrane was calculated as the percentage of cells with such changes in fracture faces relative to the total number of fracture faces examined. The values cited in the Results were obtained from observations of more than 30 fracture faces at each temperature and for each treatment.

Results

Effects of sugars on cell survival

Cabbage leaves without sugar treatment had an EL_{50} of -5℃ when they were subjected to equilibrium freezing (Fig. 1). Pre-incubation of leaves for 10 min with 1 M solution of sugars, such as maltose, sucrose, sorbitol, galactose and glucose, increased the freezing tolerance (EL_{50}) to about -17℃. There were no distinct differences among the various sugars in term of the improvement in freezing tolerance.

The effect of the concentration of glucose during pre-incubation was examined with a fixed incubation time of 10 min (Fig. 2). The ability of glucose to increase freezing tolerance was apparently concentration-dependent. A clear protective effect was observed after pre-incubation with solutions of glucose at concentrations above 0.5 M. At 0.5 M glucose, the EL_{50} was -13℃.

The effect of the duration of pre-incubation in a solution with a fixed concentration of glucose, namely 1 M, was examined (Fig. 3). The protective effect of glucose gradually increased with increases in the duration of the pre-incubation, at least to 180 min, but the protective effect was already apparent after pre-incubation for only 10 min.

The protective effect of pre-incubation with 1 M glucose for 10 min was almost completely eliminated by washing the samples (Fig. 4). After washing, the EL_{50} fell to -5℃, which was similar to the value for control samples that had not been pre-incubated with glucose. By contrast, the protective effect of pre-incubation with 1 M glucose for 180 min was unchanged after washing; the EL_{50} remained at -17℃, resembling that of non-washed, glucose-treated samples.

The sugar content of the cabbage leaves was analysed (Fig. 5). The samples without pre-incubation contained endogenous soluble carbohydrates, such as glucose, fructose and a small amount of sucrose. In samples that had been pre-incubated with 1 M glucose, the glucose content after washing increased with increases in the duration of the pre-incubation. Even after a 10 min incubation, the glucose content increased to 170% of the control value (the total amount of soluble sugars increased to 135% of the control). After incubation for 180 min, the glucose content was 330% of the glucose content in controls (the total amount of soluble sugars increased to 166% of the control), with an increase in the amount of sucrose and a decrease in that of fructose.

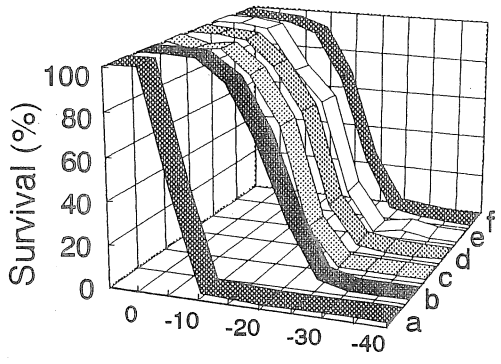

Fig. 1.

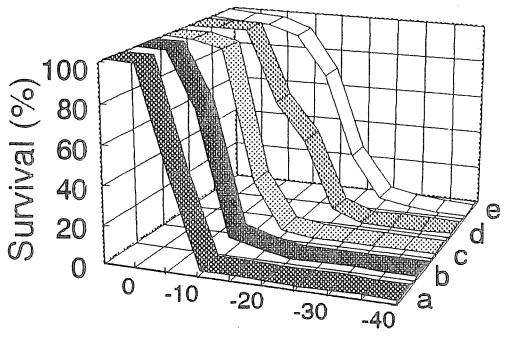

Fig. 2.

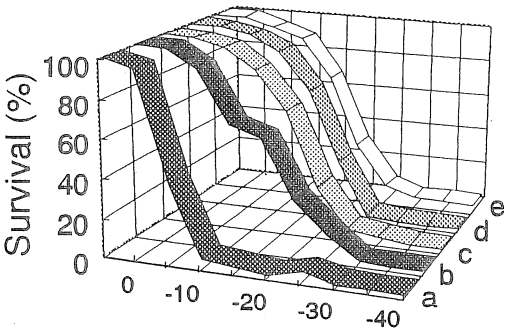

Fig. 3.

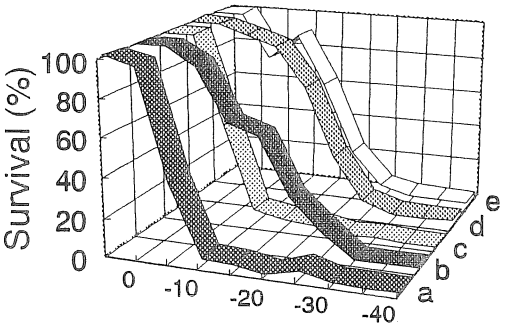

Fig. 4.

Fig. 1. Effects of pre-incubation with various sugars on freezing tolerance. All pre-incubations were performed with sugars at 1 M for 10 min. (**a**) Control without pre-incubation. (**b**) Sorbitol. (**c**) Maltose. (**d**) Sucrose. (**e**) Galactose. (**f**) Glucose. Survival (%) above 0℃ indicates that of unfrozen control, throughout Figs. 1 to 4 and 10. One hundred % of Survival corresponds to 0 % injury.

Fig. 2. Effects of pre-incubation for 10 min with glucose at different concentrations on freezing tolerance. (**a**) Control without pre-incubation. (**b**) 0.05 M. (**c**) 0.1 M. (**d**) 0.5 M. (**e**) 1 M.

Fig. 3. Effects of pre-incubation with 1 M glucose for different times on freezing tolerance. (**a**) Control without pre-incubation. (**b**) 10 min. (**c**) 30 min. (**d**) 60 min. (**e**) 180 min.

Fig. 4. Effects of washing of samples that had been pre-incubated with 1 M glucose for different time periods on freezing tolerance. (**a**) Control without pre-incubation. (**b**) Pre-incubation for 10 min, without washing. (**c**) Same as **b**, but with washing after pre-incubation. (**d**) Pre-incubation for 180 min, without washing. (**e**) Same as **d**, but with washing after pre-incubation.

Effects of glucose on cell ultrastructure

The cells of samples of cabbage leaf that had been cryofixed directly from room temperature (controls, without pre-incubation with glucose and without equilibrium freezing)

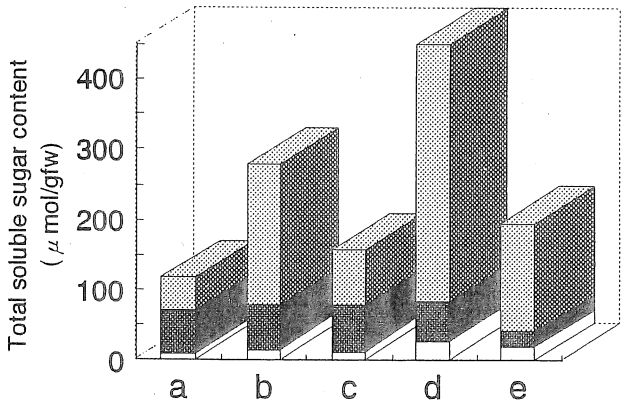

Fig .5. Total levels of soluble sugars after various pre-incubations. (a) Control without pre-incubation. (b) Pre-incubation with 1 M glucose for 10 min. (c) Same as b, but with washing after pre-incubation. (d) Pre-incubation with 1 M glucose for 180 min. (e) Same as d, but with washing after pre-incubation. □, Sucrose; ▩, fructose; ▨, glucose.

each had a large central vacuole and a narrow cytoplasmic region (Fig. 6a). No periprotoplasmic spaces were visible between cell walls and protoplasts (Fig. 6b). The distribution of intramembrane particles (IMPs) on plasma membranes was uniform over entire fracture faces (Fig. 6c).

The cells of samples of cabbage leaf that had been cryofixed from room temperature after pre-incubation with 1 M glucose for 10 min were plasmolyzed (Fig. 7a). Protoplasts were shrunken and had separated, at least locally, from cell walls (Fig. 7b). Periprotoplasmic spaces were filled with glucose solution. The freezing pattern of the glucose in the periprotoplasmic spaces, as a result of cryofixation, was similar to that of the glucose in the apoplastic spaces such as extracellular spaces (not shown). This result indicated that glucose had almost completely permeated the cell walls during the 10 min pre-incubation. Pre-incubation with 1 M glucose for 10 min did not cause any ultrastructural changes in the plasma membrane (not shown). The ultrastructure was same as in the above-described controls (see, Fig. 6c).

Equilibrium freezing of samples of cabbage leaf induced dehydration and the consequent deformation of cells (Fig. 8). In samples without pre-incubation, equilibrium freezing resulted in the distinct deformation of cells, with both cell walls and protoplasts deformed together. Locally, cell walls were severely deformed and pushed into close proximity with one another (Figs. 8a and b). The micrographs suggested the close apposition not only of plasma membranes but also of plasma membranes and membranes of cytoplasmic organelles in these areas. In samples that had been pre-incubated with 1 M glucose for 10 min, by contrast, the cell walls and protoplasts were, more or less, independently deformed as a result of plasmolysis. The existence of a freezing-concentrated solution of glucose in the periprotoplasmic spaces reduced, at least locally, the deformation of cell walls, as compared with that in non-plasmolyzed cells, at any given temperature (Figs. 8c and d).

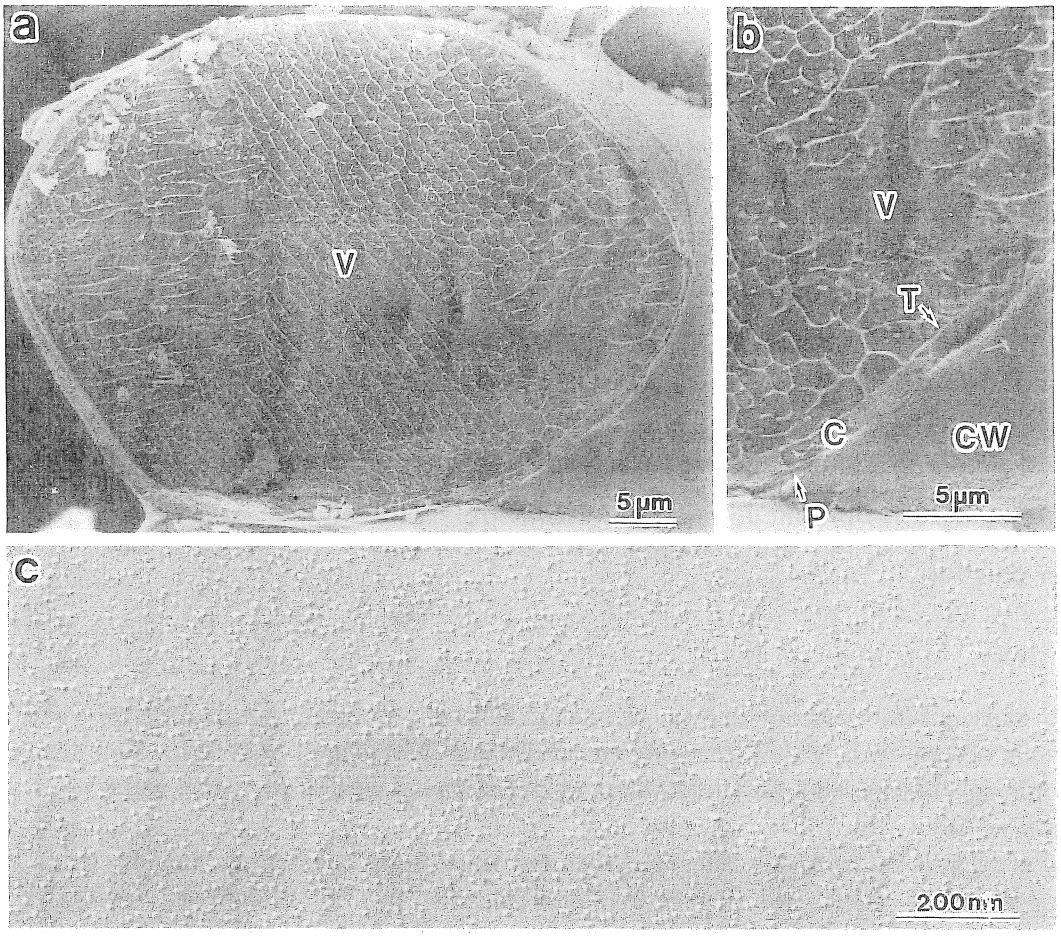

Fig. 6. Ultrastructure of leaf cells in a control sample (cryofixed from room temperature without pre-incubation with glucose and without equilibrium freezing). (a) Cryo-SEM image of a cross-fractured cell. Cryofixation produced large ice crystals, especially in the vacuole (V). (b) Part of a, showing the close association between the cell wall (CW) and the protoplast. P, Plasma membrane; T, tonoplast; C, cytoplasmic region. (c) Freeze-replica of a protoplasmic fracture face (PF) in a plasma membrane. Note the uniform distribution of intramembrane particles (IMPs).

Equilibrium freezing resulted in ultrastructural changes in plasma membranes in both glucose-treated and untreated cells (Fig. 9). The ultrastructural changes in the plasma membrane included the formation of aparticulate domains accompanied by fracture-jumps. The latter are sites at which the fracture path through the plasma membrane deviates locally to include the closely apposed membranes. The closely apposed cytoplasmic membranes were also aparticulate.

The occurrence of these ultrastructural changes in the plasma membranes depended upon the final temperature of equilibrium freezing and it was closely associated with freezing injury (Fig. 10). The temperature of equilibrium freezing that produced ultrastructural changes in the

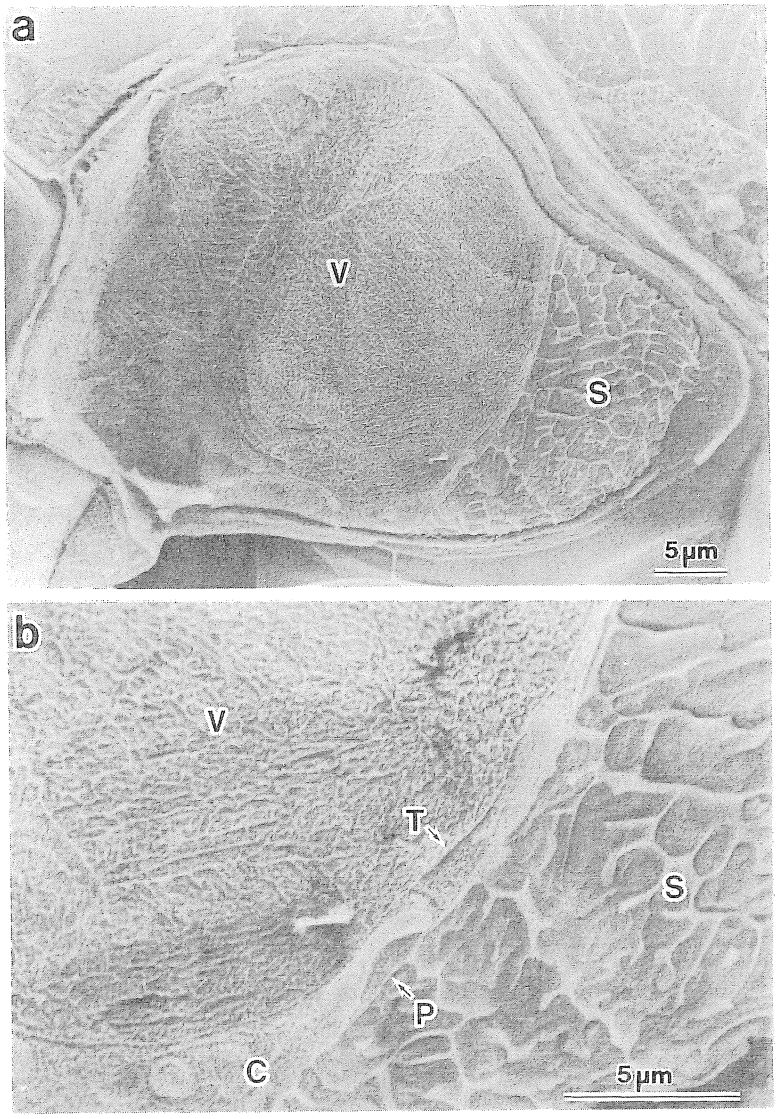

Fig. 7. Ultrastructure of leaf cells that had been pre-incubated with 1 M glucose for 10 min and then cryofixed from room temperature without equilibrium freezing. (a) Cryo-SEM image of a cross-fractured cell, showing plasmolysis. Cryofixation produced ice crystals both in the protoplast, in which the size of ice crystals was reduced because of dehydration by pre-incubation with glucose, and in the glucose solution in the periprotoplasmic space (S). (b) Part of a. For abbreviations, see legend to Fig. 6.

plasma membranes, as well as freezing injury, was different for glucose-treated and untreated samples. In untreated samples, the temperature at which 50% of the cells exhibited ultrastructural changes was -5℃, whereas in glucose-treated samples it was -20℃. After washing of glucose-treated samples (1 M for 10 min) with water, the temperature at which ultrastructural changes occurred was -7℃, approaching that for untreated samples.

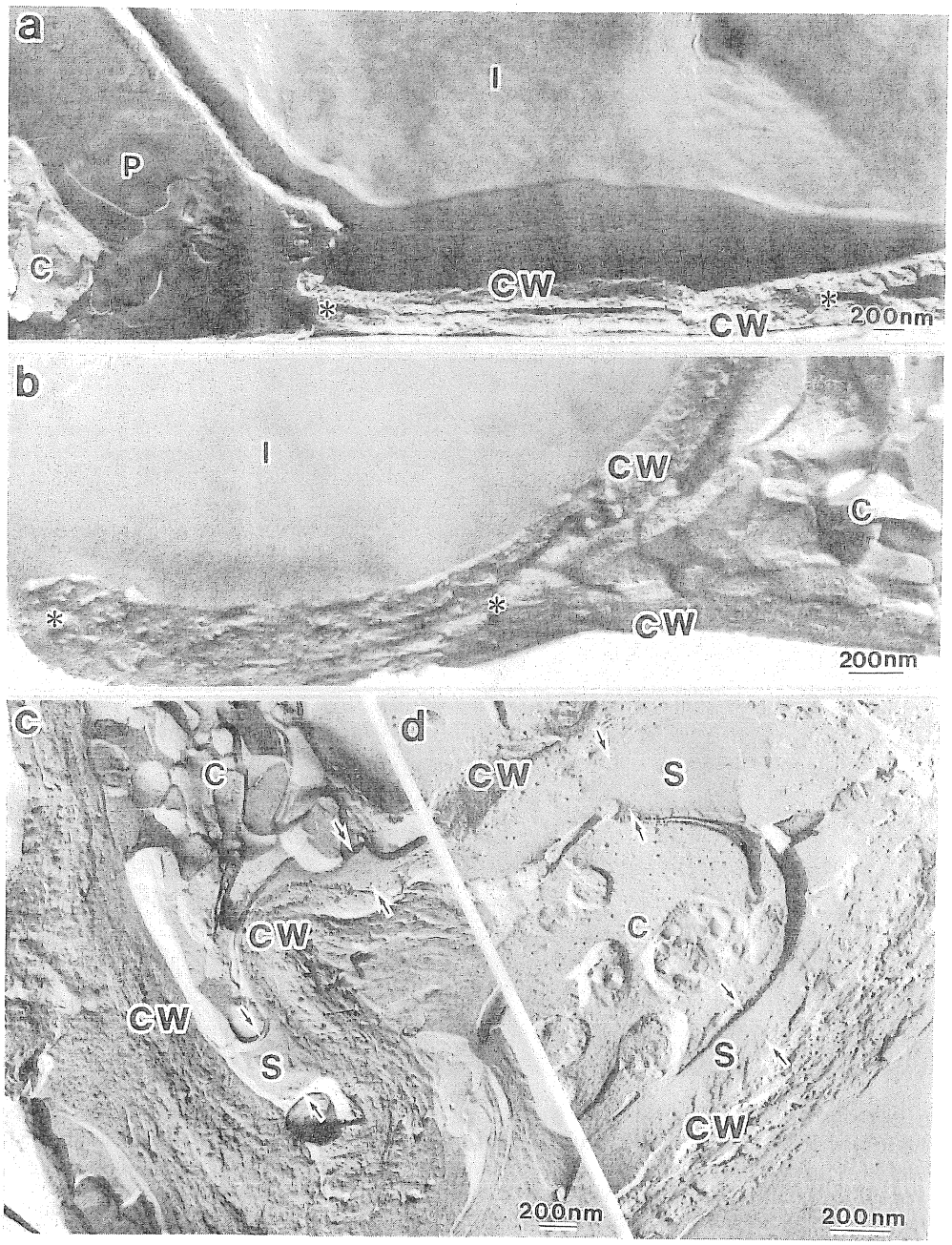

Fig. 8. Deformation of leaf cells by equilibrium freezing. All photographs show freeze replicas. (a) and (b) Controls without pre-incubation with glucose, frozen to -10℃. The regions between asterisks (*) are region in which cell walls are in close proximity. I, Extracellular ice crystals. (c) and (d) Samples that had been pre-incubated with 1 M glucose for 10 min and then frozen to -20℃. Periprotoplasmic spaces (between arrows) are filled with concentrated glucose. For abbreviations, see legend to Fig. 6.

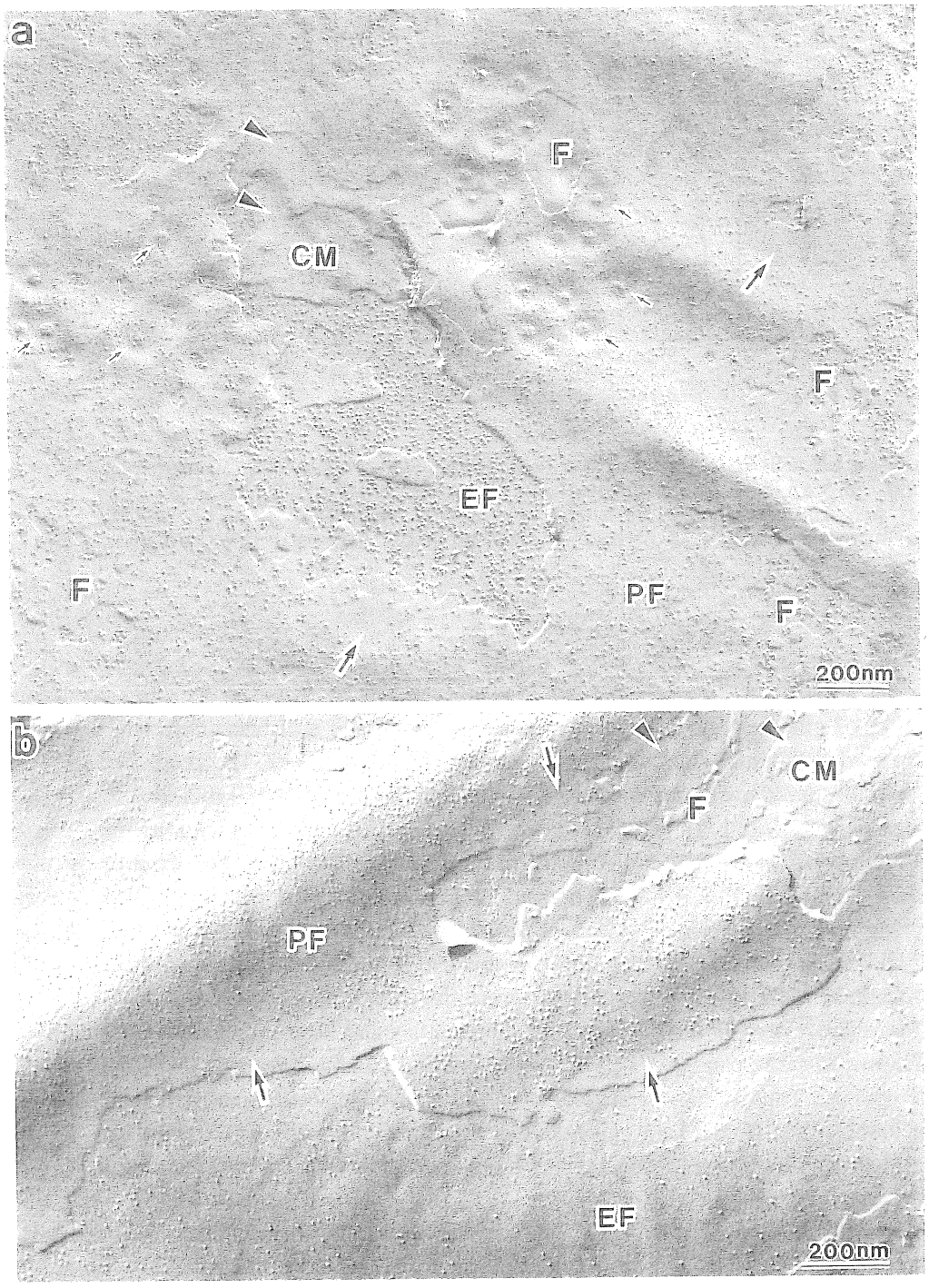

Fig. 9. Ultrastructural changes in the plasma membrane produced by equilibrium freezing. All photographs show freeze replicas. (a) Control without pre-incubation with glucose, frozen to -10℃. (b) Pre-incubation with 1 M glucose for 10 min and freezing to -30℃. F, Fracture-jump; PF, protoplasmic fracture face of plasma membrane; EF, exoplasmic fracture face of plasma membrane; CM, cytoplasmic membrane. Arrows indicate aparticulate domains on the plasma membrane. Arrowheads indicate aparticulate domains on cytoplasmic membranes. Small arrows indicate plasmodesmata in the plasma membrane.

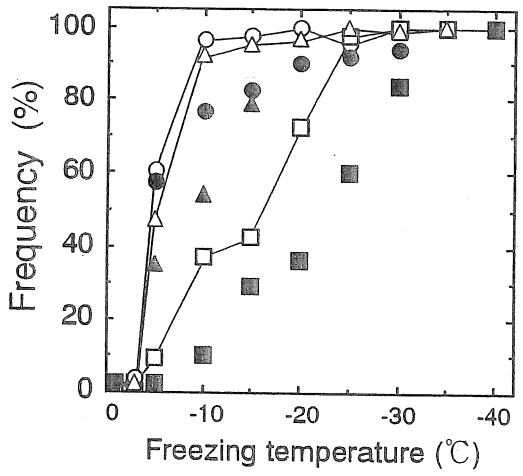

Fig. 10. Frequency of cells in which ultrastructural changes in plasma membranes were observed (●, ■, ▲), and its relationship to freezing injury (○, □, △). ●, ○ : Control without pre-incubation. ■, □ : Pre-incubation with 1 M glucose for 10 min. ▲, △ : Pre-incubated with 1 M glucose for 10 min and subsequent washing.

Discussion

Extracellular addition of glucose, and also of other carbohydrates, had a distinct protective effect against damage due to equilibrium freezing in cabbage leaves (Fig. 1). The protective effect of glucose was observed even after a brief pre-incubation with glucose (10 min) when the concentration of glucose was 1 M (Figs. 2 and 3). Washing of the leaves eliminated the protective effect of the brief pre-incubation (10 min), but did not alter the protective effect of longer pre-incubation (180 min ; Fig. 4). Incorporation of sugars acts at the cellular level to improve the freezing tolerance of shoot apices of asparagus (7) and of cultured cells of *Eucalyptus* (11). The present results also suggested that glucose permeated the cells in cabbage leaves during long-time pre-incubation to exert its protective effect, but not during brief pre-incubation. The glucose content after washing of samples that had been pre-incubated for 180 min was sufficient to cause changes in levels of pre-existing soluble carbohydrates (Fig. 5). Although the glucose content also increased in washed cabbage leaves that had been pre-incubated for a short time (Fig. 5), the glucose did not have a protective effect (Fig. 4). Hence, it is suggested that the protective effect of brief pre-incubation with 1 M glucose might be mainly due to extracellular glucose.

After brief pre-incubation (10 min) with 1 M glucose, cabbage leaf cells exhibited distinct evidence of plasmolysis (Fig. 7). It has been suggested that plasmolyzing solutions protect cells from freezing injury in some plants (16, 17). Tao et al. (1983) showed that plasmolysis, induced by treatment with the hypertonic non-permeable cryoprotectant polyethylene glycol (PEG 1000), reduced freezing injury in cultured potato cells (17). They suggested that plasmolysis might reduce the generation of mechanical stress on the plasma membranes, which might be produced by deformation of the cell wall during extracellular freezing. Hence, freezing injury is reduced in plasmolyzed cells, as compared to intact cells in which the cell wall and plasma membrane are in close proximity. It has also been shown that protoplasts

prepared from cultured potato cells exhibit greater freezing tolerance than cells with intact cell walls (17). Such an effect has also been demonstrated with poorly cryoprotectable cultured rice cells (19). These effects might possibly be due to the elimination of mechanical stress that results from deformation of the cell wall during freezing.

The present study showed that freezing injury in cabbage leaf cells was associated with the formation of aparticulate domains, accompanied by fracture-jumps, on the plasma membranes (Figs. 9 and 10). Such aparticulate domains, accompanied by fracture-jumps in the plasma membranes, are produced by the close apposition of membranes that is due to freezing-induced dehydration and deformation, and they are closely related to freezing injury in a wide variety of plants (5, 18) and bacterial cells (3). It has been suggested that a fracture-jump is site of membrane fusion and that non-physiological fusion of membranes leads to freezing injury (4). It seems likely that the increased freezing tolerance in plasmolysed cells of cabbage leaf might be a reflection of the reduction in the freezing-induced close apposition of plasma membranes.

In non-plasmolyzed cells of cabbage leaf, freezing produced distinct deformation of cell walls (Figs. 8a and b), with the apparent close apposition of membranes. In plasmolyzed cells, by contrast, freezing reduced the deformation of the cell walls (Figs. 8c and d). The reduction in the extent of local deformation of cell walls in plasmolyzed cells was apparently due to the presence of freezing-concentrated glucose in the periprotoplasmic spaces (Figs. 8c and d). Thus, we suggest that one of the main roles of extracellular glucose in protecting cabbage leaf cells against freezing injury might be to act as a mechanical support in the periprotoplasm where it might reduce the extent of the close apposition of membranes by preventing deformation of cell walls.

The role of extracellular glucose to reduce freezing injury is not exerted in plant species that exhibit no protection effect by short time incubation with soluble sugars (7, 11) as well as, possibly, in plant species in which the protoplasts do not provide increased freezing tolerance as compared with freezing of intact cells (1, 6, 15). The causes of these differences remain unknown at the present time.

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