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Changes in leaf ultrastructure and carbohydrates in *Arabidopsis thaliana* L. (Heyn) cv. Columbia during rapid cold acclimation

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Summary. We studied cell ultrastructure and carbohydrate levels in the leaf tissue of *Arabidopsis thaliana* L. (Heyn) cv. Columbia during rapid cold acclimation. Freezing tolerance of the leaves from 26 day old plants was determined after 48 h and 10 days at 4 °C. Acclimation treatment of 48 h decreased the lethal freezing temperature from − 5.7 °C to − 9.4 °C. Freezing tolerance was not altered further by acclimation at 4 °C for 10 days. Ultrastructural changes in the parenchyma cells were evident after 6 to 24 h of cold acclimation. The plasma membrane showed signs of extensive turnover. Evidence of membrane invaginations and sequestering of membrane material was observed. In addition, numerous microvesicles, paramural bodies, and fragments of endoplasmic reticulum were noticed in the vicinity of plasma membrane. Modifications in the structure of cell membranes were evident after 5 days of exposure to low temperature. Small, darkly stained globules were seen on the plasma membrane, tonoplast, chloroplast envelope membrane, mitochondrion outer membrane, dictyosome cisternae membrane, and microvesicle membrane. As far as we are aware, this type of membrane modification has not been described previously in plant cells exposed to low temperature. We propose to call these structures membraglobuli. Acclimation treatment also increased the concentrations of soluble sugars and starch. These observations suggest that cold acclimation in *A. thaliana* induces changes in both plasma membrane properties and carbohydrate composition.

Keywords: *Arabidopsis thaliana*; Cell ultrastructure; Cold acclimation; Starch; Sugars.

Introduction

Plants become more resistant to freezing temperatures when first exposed to low nonfreezing temperatures, a process known as cold acclimation (Levitt 1980). Cold acclimation is a complex process involving many changes in physiology and metabolism. For instance, cold acclimation is accompanied by increases in sugar (Koster and Lynch 1992) and soluble protein content (Guy 1990), increased osmotic potential, alterations in hormonal balance (Levitt 1980, Sakai and Larcher 1987), altered membrane properties (Steponkus 1984), and changes in gene expression (Guy 1990).

It is generally accepted that cell membranes, in particular plasma membrane, play a key role in cold acclimation (Levitt 1980, Steponkus et al. 1983, Steponkus 1984). Thus, it is likely that plasma membrane undergoes biochemical changes during exposure to acclimation temperatures. Indeed low temperature-induced alterations in plasma membrane lipid and protein composition have been observed in *Morus bombycis* Koidz. (Yoshida 1984), *Dactylis glomerata* L. (Yoshida and Uemura 1984) and *Secale cereale* L. (Uemura and Yoshida 1981).

Cold acclimation is associated with fundamental changes in plant cell ultrastructure. During seasonal cold acclimation, plasma membrane undergoes modifications in structure and conformation (Niki and Sakai 1981, 1982; Wisniewski and Ashworth 1986), cells become enriched with dictyosomes, small vacuoles (Wisniewski and Ashworth 1986) and endoplasmic reticulum (ER) cisternae (Sauter and Kloth 1987). It has been suggested that alterations in cell ultrastructure during cold acclimation represent processes related to the changes in plasma membrane composition (Niki and Sakai 1981, Wisniewski and Ashworth 1986).

Arabidopsis thaliana L. (Heyn) can cold acclimate rapidly. Nineteen-day old plants of *A. thaliana* cv. Columbia, grown at 22 °C to 24 °C, increased in freezing tolerance from − 3 °C to − 5.5 °C after only 12 h at 4 °C,

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and full acclimation was achieved after 24 to 48 h (Gilmour et al. 1988). The increase in freezing tolerance of *A. thaliana* has been shown to be associated with changes in protein synthesis, and gene expression (Gilmour et al. 1988, Kurkela et al. 1988, Lin et al. 1990). However, little is known about the ultrastructural changes of leaf parenchyma cells arising from exposure of *A. thaliana* to acclimation temperatures. It is reasonable to expect that rapid modifications in cell ultrastructure, primarily plasma membrane, must occur during cold acclimation. The objective of this study was to examine the ultrastructure of leaf parenchyma cells of *A. thaliana* cv. Columbia during cold acclimation using transmission electron microscopy (TEM). We also examined total sugar and starch levels in the leaf tissue of *A. thaliana* during exposure to acclimation temperature.

Materials and methods

Plant material and experimental setup

Seeds of *Arabidopsis thaliana* L. (Heyn) cv. Columbia were sown in plastic pots in a mixture of peat moss : perlite : top soil (2 : 2 : 1, v/v/v), and maintained on a mist bench for 7 days. Plants were then transferred to a controlled environment chamber (constant illumination from incandescent and cool white fluorescent lights; 120 µmol/m^2/s PPFD; 22 °C to 23 °C constant temperature; 70% RH), and were subirrigated daily with distilled water. Twenty-six day old plants were divided into control and experimental groups. Plants from the control group remained within the controlled environment chamber, while plants from the experimental group were cold acclimated in a cold room set at 4 °C (constant illumination from incandescent and cool white fluorescent lights; 120 µmol/m^2/s PPFD).

Assessment of cold hardiness

Cold hardiness of control and experimental plants was assessed after 48 h (3 experiments), and 10 days (1 experiment) of acclimation at 4 °C using the method of Sukumaran and Weiser (1972). From each group, 44 fully expanded leaves were taken from 16 plants and placed in stoppered glass tubes (1 leaf per tube) containing 0.4 ml deionized water. The tubes were transferred to the controlled freezing bath (RTE-210; Neslab Instruments, Inc., Newington, NH) set at 0 °C. After 30 min equilibration period, the samples were cooled at 2 °C/h to − 12 °C. Extracellular freezing of the leaf tissues was initiated at 0 °C by the addition of ice chips to each tube. Four tubes from each group were withdrawn from the bath at 0, − 2, − 3, − 4, − 5, − 6, − 7, − 8, − 9, − 10, and − 12 °C, placed on ice and thawed overnight. After thawing, 10 ml deionized water was added to each tube, and tubes were shaken gently overnight at 4 °C. The electroconductivity of the bathing solution was measured at room temperature using a conductivity meter (YSI Model 35; Yellow Springs Instrument Co., Ohio). The samples were then boiled for 1 h, shaken overnight at 4 °C, and electroconductivity of the bathing solution subsequently determined. The electroconductivity before boiling was calculated as a percentage of that after boiling to give relative electroconductivity, and the mean % relative electroconductivity of 4 leaves was determined. A plot of freezing temperature versus relative electroconductivity was used to determine the LT_{50} (50% electrolyte leakage).

Transmission electron microscopy

Samples for TEM were prepared using chemical fixation and freeze substitution techniques. Leaf samples from 5 plants were chemically fixed and processed as described by Ristic and Cass (1992). Samples (about 1 mm^2) were taken from fully expanded leaves after 0, 6, 12, 18, 24, 36, and 48 h, and after 5, 10, and 29 days of exposure at 4 °C. Specimens were fixed with 4% glutaraldehyde and 4% paraformaldehyde, in 25 mM phosphate buffer (pH 6.9), for 6 h at 4 °C, and postfixed with 2% osmium tetroxide in the above buffer for 2 h at room temperature. Samples were dehydrated using a graded series of acetone (30%, 50%, 70%, 80%, 90%, 100%) and left, first in a mixture of Spurr embedding medium (Spurr 1969) and 100% acetone (1 : 1, v/v) for 2 h at room temperature and then in pure epoxy resin overnight at room temperature. The embedding was completed in 24 h at 60 °C.

Leaf samples for freeze substitution were collected 10 days after exposure to acclimation temperature. Leaf pieces (about 1.5 mm × 1 mm) were quench-frozen in melted Freon 12, then quickly transferred to liquid nitrogen. Specimens were transferred from liquid nitrogen to vials containing freeze substitution fluid (2% osmium tetroxide in 100% methanol) which had been previously cooled to − 80 °C. The vials were held at − 80 °C for 7 days, and then warmed to room temperature over 24 h. Specimens were dehydrated and embedded as described for chemical fixation.

Thin sections (70–90 nm) were cut on a Reichert Ultracut E ultramicrotome using a diamond knife. Sections were double stained, first with 4% uranyl acetate in 70% ethanol for 20 min and then with 0.2% aqueous lead citrate for 2 min (Venable and Coggeshall 1965). Specimens were viewed with a Philips 400 TEM at 80 kV.

Quantitative analysis of cell ultrastructure

Quantitative measurements of cell ultrastructures were made using TEM photographic negatives (104 mm × 78 mm). Five to eight cells from each of five plants were examined. Cell ultrastructures were examined after 0 [total number of examined cells (n) = 33], 6 (n = 30), 12 (n = 30), 18 (n = 27), 24 (n = 39), and 48 h (n = 26) of exposure to low temperature. The following parameters were examined: (1) frequency of plasma membrane invaginations, membrane vesicles, fragments of ER, and paramural bodies (Marchant and Roberts 1968), (2) size of globules associated with cell membranes (for explanation on globules see Results), and (3) size of starch grains. Frequency of plasma membrane invaginations was determined by counting structures present on TEM photographic negatives, and expressing the counts per unit length (0.1 mm) of the cell wall. Data obtained from each of five plants were averaged and used for statistical analysis. Frequency of membrane vesicles, and fragments of ER was assessed using a visual rating scale where "0" indicated no detection of membrane vesicles/ER fragments and "4" indicated abundance of membrane vesicles/ER fragments. Frequency of paramural bodies was estimated by counting cells in which paramural bodies were observed. Size of the globules associated with cell membranes was determined by analyzing digitized images taken from TEM photographic negatives using a computer image analysis system (Quantimet 570; Leica Inc., Deerfield, IL) and Image Acquisition and Processing Program (IAPP).

Starch grain size and cell wall length were determined as described by Ristic and Cass (1991). The images of the starch grains and cell

walls from TEM photographic negatives were copied by drawing on transparency film PP2500 3M (215 mm × 279 mm). The copies of the images were digitized and processed using IAPP. Processed images were used for measurements of starch grain area and cell wall length. Thirty starch grains from each plant were analyzed. The mean starch grain area for each plant was calculated and used for statistical analysis.

Analysis of variance and χ^2 test (SAS PROC ANOVA) were employed to test the statistical significance of differences in ultrastructural characteristics of leaf parenchyma cells between cold acclimated and control plants.

Carbohydrate analysis

The effect of cold acclimation on the levels of total sugars and starch was determined. Tissue was collected from independent replicate experiments after 0, 6, 12, 18, 24, 36, 48 h, 5 days and 10 days exposure to 4 °C. At each time, all the leaves from 8 plants were excised, frozen in liquid nitrogen, and freeze dried. Freeze dried tissue was ground into a powder and stored at − 80 °C until analyzed.

Total sugars were analyzed using a modification of the procedure described by Smith (1981). Briefly, duplicate samples of approximately 35 to 40 mg freeze dried leaf tissue were extracted with 1.5 ml 80% (v/v) ethanol. Samples were centrifuged at 10,000 **g** for 10 min, and the supernatant collected. The pellet was extracted three additional times, and the supernatants combined and brought to a total volume of 10 ml with 80% ethanol. The concentration of glucose equivalents in triplicate aliquots of the ethanol extract was determined using anthrone (Van Handel 1968).

The ethanol-extracted pellet was analyzed for starch content as described by Rose et al. (1991). The pellets were dried at 80 °C overnight, resuspended in 50 mM sodium acetate buffer (pH 5.2) and digested with a combination of α-amylase and amyloglucosidase (400 units/ml α-amylase and 2 units/ml amyloglucosidase; Sigma Chemical Co.) in 50 mM sodium acetate buffer (pH 5.2). Following digestion, samples were centrifuged at 10,000 **g** for 10 min and the supernatant collected. The glucose produced by enzymatic digestion was quantified in triplicate using the Sigma (St. Louis, MO) Diagnostics Glucose Trinder reagent (Trinder 1969).

Results

Cold hardiness

Exposure to 4 °C for 48 h increased cold hardiness in *A. thaliana* cv. Columbia (Fig. 1 A). The nonacclimated plants had an LT_{50} value of approximately − 5.7 °C, whereas acclimated plants had LT_{50} of about − 9.4 °C. Prolonged exposure to acclimation temperature (for 10 days) did not significantly alter cold hardiness; nonacclimated and acclimated plants had LT_{50} values of about − 3.5 °C and − 8.2 °C, respectively (Fig. 1 B). Ion leakage at temperatures below − 4 °C increased over a narrower temperature range in nonacclimated plants than in acclimated ones.

Cell ultrastructure

Leaf parenchyma cells from the control plants grown continuously at 22–23 °C had a large centrally located

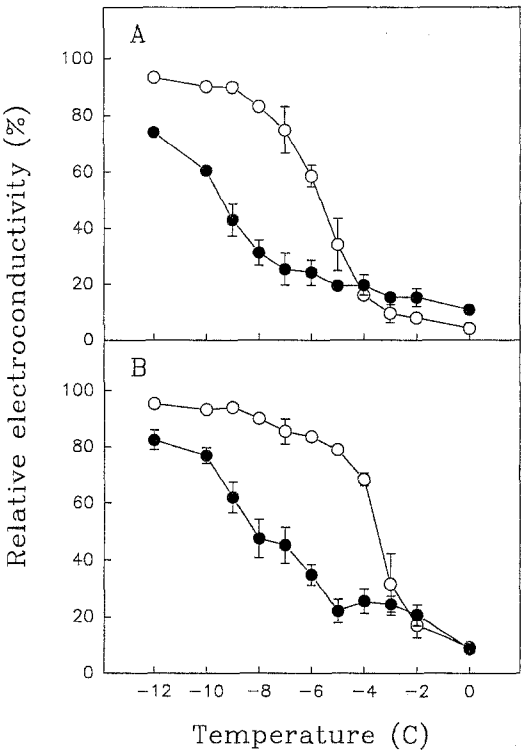


Fig. 1. Effect of cold acclimation on freezing tolerance of *Arabidopsis thaliana* cv. Columbia. Twenty-six-day old plants were cold acclimated (●) at 4 °C for 48 h (**A**) and 10 days (**B**). ○ Non acclimated plants. Freezing injury was estimated by determining relative electronconductivity of the bathing solution of the leaf tissue. Bars represent standard errors; A, n = 12; B, n = 4

vacuole (Fig. 2 a). Chloroplasts were numerous and well developed, and many had several starch grains (Fig. 2 b and d). Plasma membrane and tonoplast were distinct and well preserved (Fig. 2 c). Mitochondria (Fig. 2 c), dictyosomes and fragments of ER (Fig. 2 e) were occasionally observed in the vicinity of the plasma membrane.

Rapid ultrastructural changes occurred in the leaf parenchyma cells during cold acclimation, and these changes were apparent after only 6 to 24 h of exposure to low temperature (Figs. 3–5, Tables 1 and 2). Evidence of plasma membrane invaginations (endocytotic vesicle formation) and sequestering of membrane material was observed throughout the acclimation period (Figs. 3 c, f, and 4 e). Plasma membrane vesiculation increased during cold acclimation, reaching a maximum after 18 h (Fig. 5). After 48 h the vesiculation decreased; on the 5th, 10th, and 29th day of low temperature treatment, evidence of plasma membrane vesicle formation was rarely observed (data not presented). Furthermore, protrusion of plasma membrane invaginations along with their contents into nearby

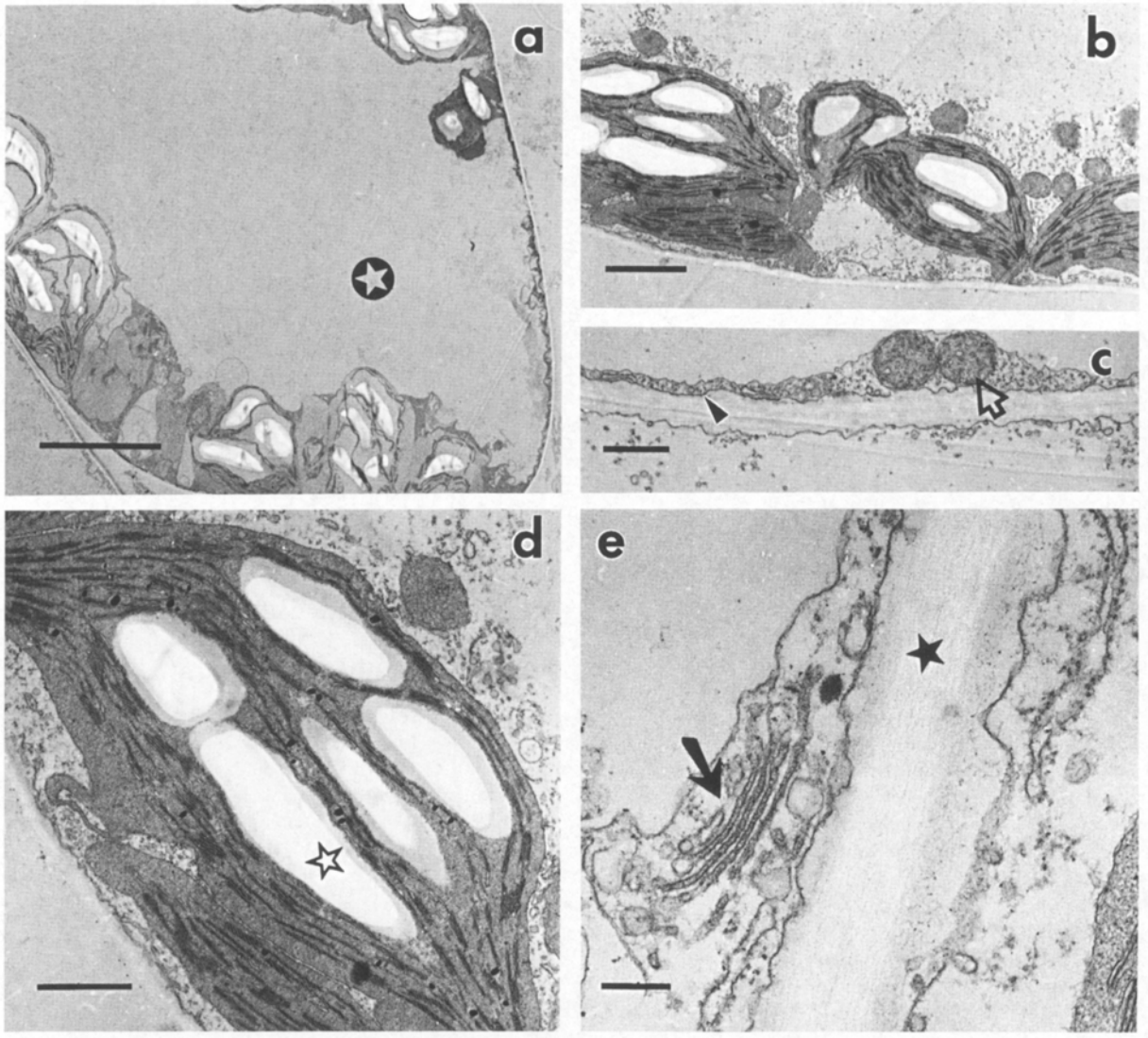


Fig. 2. Electron micrographs of leaf parenchyma cells of *Arabidopsis thaliana* cv. Columbia grown at 22 °C to 23 °C. Bars: a, 10 µm; b, 2 µm; c and d, 1 µm; e, 0.2 µm. **a** Star in the black circle, vacuole. **b** Chloroplasts. **c** Arrowhead, plasma membrane, arrow, mitochondrion. **d** Chloroplast; ☆ starch grain. **e** ★ Cell wall; arrow, dictyosome; note fragments of endoplasmic reticulum along the plasma membrane

vacuoles (Figs. 3 c and 4 f) and tonoplast invaginations (Fig. 4 f) were also noticed.

After 6 h of exposure to acclimation temperature, cells became enriched with membrane vesicles (microvesicles) and fragments of ER (Table 1), and after 24 h with paramural bodies (Table 2). Microvesicles, ER fragments, and paramural bodies were frequently observed in the vicinity of plasma membrane. Moreover, numerous microvesicles were noted nearby the dictyosomes, and some of these vesicles were associated with dictyosome cisternae (Fig. 3 f, g). Increase in size of starch grains was evident. The average starch grain area more than doubled following only 6 h of exposure to low temperature (Fig. 5). Thereafter, the size of starch grains decreased, then increased again reaching the maximum after 48 h. Throughout the 29 days of exposure to 4 °C, no changes in the number of vacuoles were noticed. Cells were characterized by a single large centrally located vacuole (Figs. 2 a, 4 c, and 6 a), although vacuoles appeared smaller in some of the cells. On the 29th day of low-temperature treatment, plasma membrane and membranes of microvesicles were discontinuous and not distinctive indicating membrane injury (Fig. 7 c).

Acclimation treatment did not affect chloroplast structure. Chloroplast envelope, grana, and fret membranes

Table 1. Effect of cold acclimation on frequency of membrane vesicles and fragments of endoplasmic reticulum (ER) in leaf parenchyma cells of *Arabidopsis thaliana* cv. Columbia

Rating scale	Hours at 4 °C											
	Membrane vesicles[a]						ER fragments[b]					
	0	6	12	18	24	48	0	6	12	18	24	48
0	8	0	0	1	0	0	19	9	3	7	9	5
1	21	13	15	7	11	7	14	11	19	10	16	14
2	4	10	6	10	14	11	0	6	8	7	12	5
3	0	4	8	8	9	8	0	4	0	3	2	2
4	0	3	1	1	5	0	–	–	–	–	–	–

Frequency was assessed using a visual rating scale where "0" indicated no detection of membrane vesicles/ER fragments and "4" indicated abundance of membrane vesicles/ER fragments. For details see Materials and methods. Values indicate number of cells within each rating category

[a] $\chi^2 = 67.358$; DF = 20; significant at probability level = 0.05

[b] $\chi^2 = 36.149$; DF = 15; significant at probability level = 0.05

Table 2. Effect of cold acclimation on frequency of paramural bodies (Marchant and Robards 1968) in leaf parenchyma cells of *Arabidopsis thaliana* cv. Columbia

	Hours at 4 °C					
	0	6	12	18	24[a]	48[b]
Total number of observed cells	33	30	29	27	39	26
Number of cells with paramural bodies	4	11	12	7	15	14

Frequency was assessed by counting cells in which paramural bodies were detected. For details see Materials and methods

[a] $\chi^2 = 3.826$; DF = 1; significant at probability level = 0.05

[b] $\chi^2 = 6.279$; DF = 1; significant at probability level = 0.01

were well defined (Figs. 3 a, b, and 4 a, b, d). In addition, the frequency of plastoglobuli appeared to be similar between chloroplasts from the acclimated and non-acclimated plants.

Modifications in the morphology of the cell membranes were noticed after prolonged (> 2 days) exposure to acclimation temperature (Figs. 6 and 7). Numerous small, darkly stained globules located on the cell membranes were observed. It appeared that these globules were an integral part of the cell membranes. They were ellipse shaped, and randomly distributed along the membranes. The majority of globules had major and minor axes (James 1976) ranging from 0.03 to 0.07 µm and 0.02 to 0.05 µm, respectively (Fig. 8). The globules were first evident 5 days after exposure to low temperature on the plasma membrane (Fig. 6 b and c), and the membrane of microvesicles nearby the plasma membrane (Fig. 6 d). After the 10th day of exposure to 4 °C, globules were observed on the tonoplast (Fig. 6 e), and chloroplast envelope membrane (Fig. 6 f); and after 29 days these structures could be detected on the mitochondrion outer membrane and dictyosome cisternae membrane (Fig. 7 b). In addition, on the 29th day of cold acclimation globules appeared smaller, but more frequent (Fig. 7 a–c).

Sugars and starch

Cold acclimation of *A. thaliana* was accompanied by an increase in the level of total soluble sugars (Fig. 9). The amount of sugars doubled after only 6 h of exposure to 4 °C, increasing from 30.6 to 63.9 mg/g dry weight. The level of sugars continued to increase at 4 °C, and reached the maximum of 148.6 mg/g dry weight after 5 days. Thereafter, the level of sugars decreased; on the 10th day of cold acclimation the sugar level was 79.5 mg/g dry weight.

Concomitant with the increase in soluble sugars there was an increase in the level of starch during cold acclimation of *A. thaliana* (Fig. 9). The amount of starch more than doubled after 6 h of exposure to acclimation temperature, then continued to increase reaching the value of 177.4 mg/g dry weight after 12 h. Thereafter, the starch level decreased slightly, then increased again after 36 h of exposure to 4 °C. After 48 h, 5 and 10 days

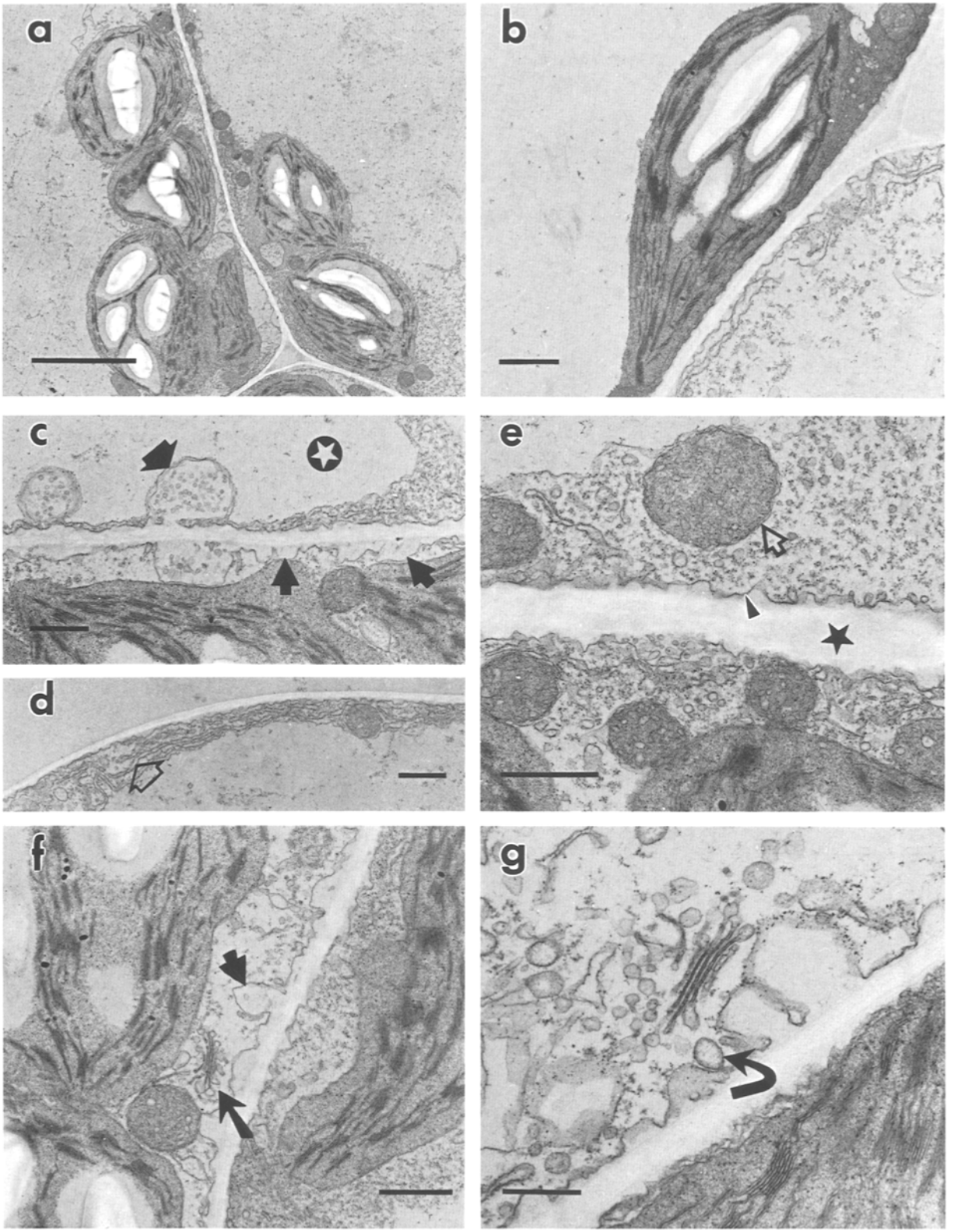


Fig. 3. Electron micrographs of leaf parenchyma cells of *Arabidopsis thaliana* cv. Columbia after 6 h (**a–f**) and 18 h (**g**) of exposure to acclimation temperature (4 °C). Bars: a, 5 μm; b–f, 1 μm; g, 0.5 μm. Formation of endocytotic vesicles and paramural bodies, and protrusion of vesicles into the vacuoles were noted. Mitochondria, dictyosome, and fragments of endoplasmic reticulum were observed in the vicinity of plasma membrane. **a** Chloroplasts with starch grains. **b** Chloroplast. **c** Arrow, invagination of the plasma membrane; fat arrow, protrusion of the paramural body into the vacuole; star in the black circle, vacuole. **d** Arrow, fragments of endoplasmic reticulum. **e** ★ Cell wall; arrowhead, plasma membrane; arrow, mitochondrion. **f** Short arrow, invagination of the plasma membrane; long arrow, dictyosome. **g** Curved arrow, microvesicle

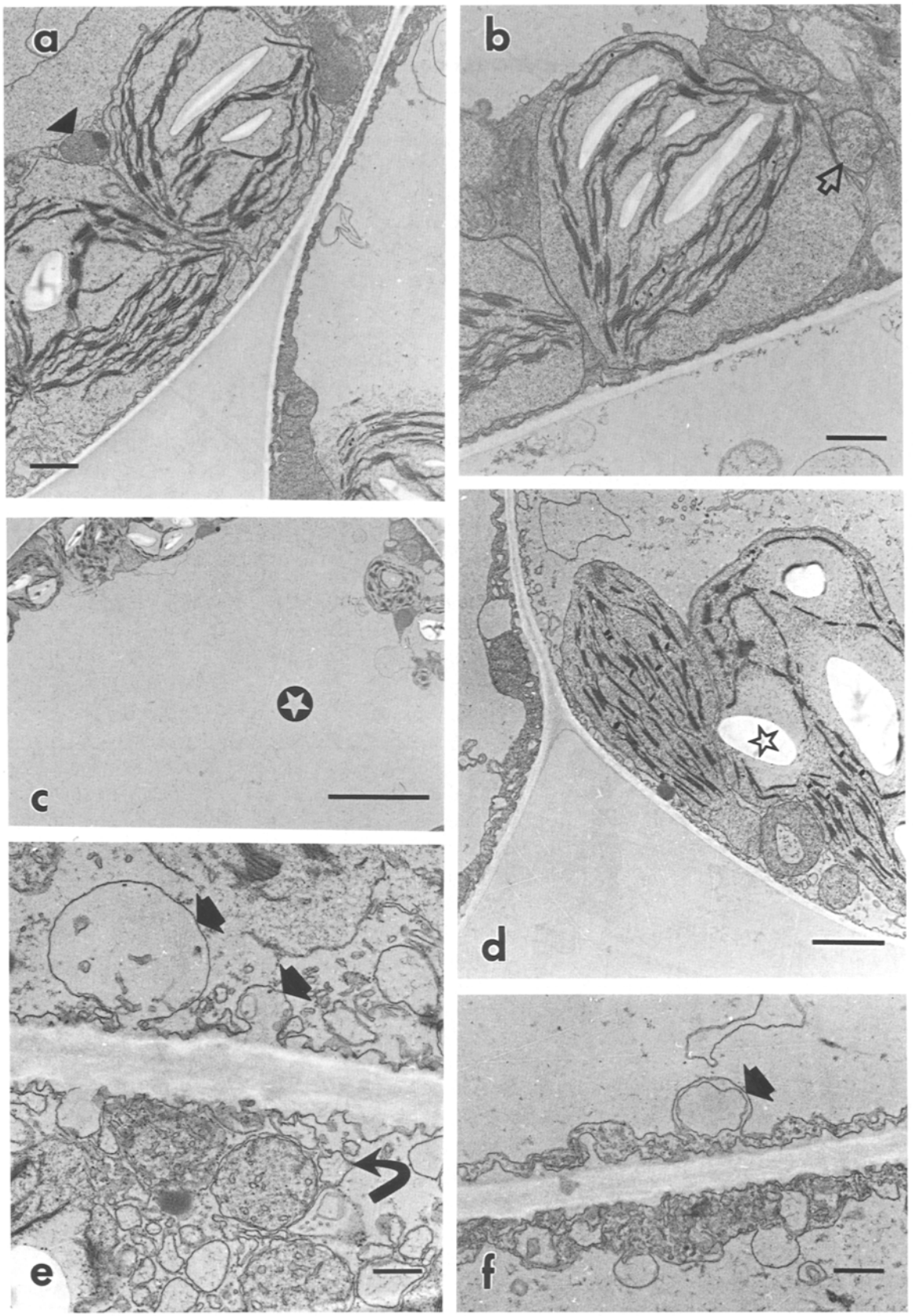


Fig. 4. Electron micrographs of leaf parenchyma cells of *Arabidopsis thaliana* cv. Columbia after 36 h (**a** and **b**) and 48 h (**c–f**) of exposure to acclimation temperature (4 °C). Changes in cell ultrastructure after 36 and 48 h were similar to those after 6 h (for explanation see Fig. 3). Bars: a and b, 1 µm; c, 10 µm; d, 2 µm; e and f, 0.5 µm. **a** Arrowhead, microbody (Tolbert 1971). **b** Arrow, mitochondrion. **c** Star in the black circle, vacuole. **d** ☆ Starch grain. **e** fat arrows, paramural body; curved arrow, microvesicle. **f** Arrow, protrusion of microvesicle into the vacuole

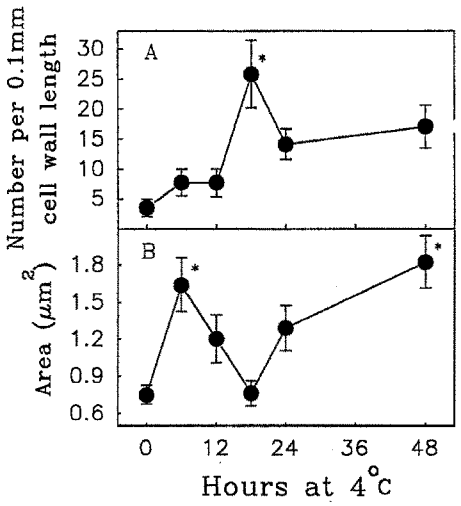


Fig. 5. Effect of cold acclimation on frequency of plasma membrane invaginations (**A**) and size of starch grains (**B**) of leaf parenchyma cells of *Arabidopsis thaliana* cv. Columbia. Twenty-six-day old plants were cold acclimated at 4 °C for 48 h. Leaf samples from 5 plants were chemically fixed and processed as described in Materials and methods. Cell ultrastructure was examined after 0, 6, 12, 18, 24, and 48 h of exposure to 4 °C. Frequency of plasma membrane invaginations was obtained from TEM negatives by visual counting, and expressed per 0.1 mm cell wall length. Starch grain area and cell wall length were determined as described by Ristic and Cass (1991). Total number of examined cells: n = 33 at 0 h, n = 30 at 6 and 12 h, n = 27 at 18 h, n = 39 at 24 h, and n = 26 at 48 h exposure to 4 °C. Bars indicate standard errors; * significant at probability level = 0.05

of cold acclimation the levels of starch were 170.8, 190.9, and 218.2 mg/g dry weight, respectively.

Discussion

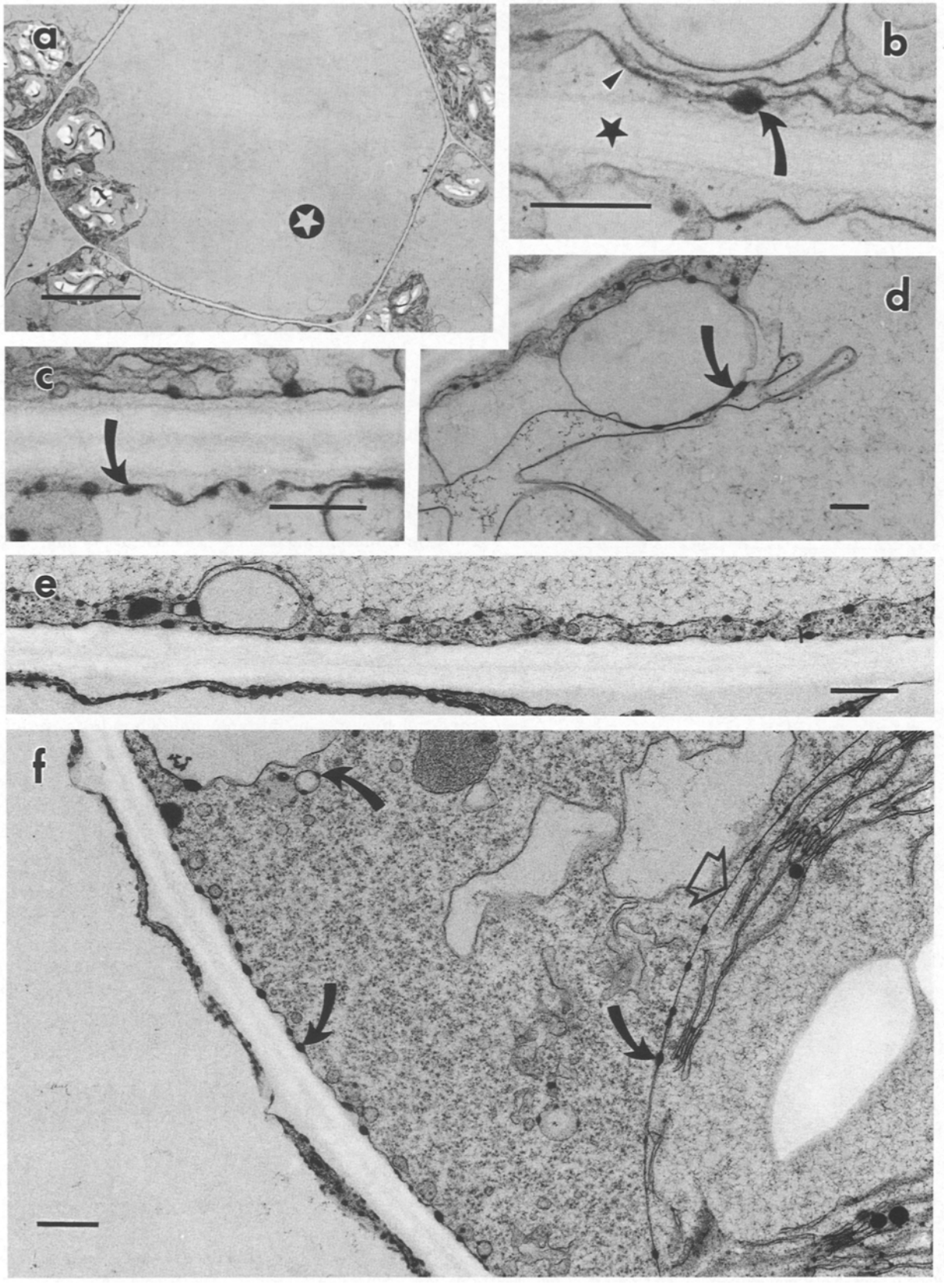
Our results confirmed previous findings (Gilmour et al. 1988, Kurkela et al. 1988) that *Arabidopsis thaliana* cv. Columbia cold acclimates rapidly. Exposure of 26 day old plants to 4 °C for 48 h decreased lethal freezing temperature, as indicated by LT_{50} values. Prolonged exposure up to 10 days at 4 °C, however, did not further increase the cold hardiness of *A. thaliana*.

Cold acclimation of *A. thaliana* was accompanied by rapid modifications in leaf cell ultrastructure. Plasma membrane underwent structural and conformational modifications. Cells became enriched with paramural bodies, and an abundant population of ER cisternae. Cells also contained more microvesicles, that were either associated with dictyosome cisternae or located in the vicinity of dictyosome cisternae. Similar alterations in cell ultrastructure have been observed in bark tissues of *Robinia pseudoacacia* L. (Pomeroy and Siminovitch 1971) and *Morus bombycis* (Niki and Sakai 1981, 1982), and cortical and xylem ray parenchyma cells of *Prunus persica* (L.) Batsch. during seasonal cold acclimation (Wisniewski and Ashworth 1986).

The ultrastructural changes of leaf parenchyma cells of *A. thaliana* likely represent processes related to plasma membrane turnover during cold acclimation. Plant cells incorporate new plasma membrane material with a turnover time of less than 4 h (Robinson and Quader 1981). In our study, modifications in cell ultrastructure were apparent after 6 h of exposure to low temperature. Niki and Sakai (1981) and Steer (1988) have identified two processes involved in plasma membrane turnover: (1) retrieval of membrane material, and (2) insertion of new membrane material. Flow of the membrane material into the cell interior occurs via formation of endocytotic vesicles. Two pathways have been detected for the retrieval of material, one to the vacuole via protrusions of single vesicles and paramural bodies (Niki and Sakai 1981, Steer 1988), the other to the dictyosome cisternae (Steer 1988). New membrane material is delivered to the plasma membrane by dictyosome (Steer 1988) and ER secretory vesicle formation (Niki and Sakai 1981). Thus, the ultrastructural changes observed in leaf parenchyma cells of *A. thaliana* were similar to the pathway of plasma membrane turnover suggested by Niki and Sakai (1981) and Steer (1988).

Exposure to acclimation temperature induces a number of biochemical and biophysical changes. These include alterations in plasma membrane lipid and protein composition (Yoshida 1984, Yoshida and Uemura 1984, Uemura and Yoshida 1984), and changes in membrane behaviour and fluidity (Steponkus 1984, Yoshida 1984). It is possible that the observed alterations in the

Fig. 6. Electron micrographs of leaf parenchyma cells of *Arabidopsis thaliana* cv. Columbia after 5 days (**a–d**) and 10 days (**e** and **f**) to exposure to acclimation temperature (4 °C). Bars: a, 10 μm; b–d, 0.1 μm; e and f, 0.4 μm. Modifications in the structure of the cell membranes were noticed. Small darkly stained globules, membraglobuli, located on the cell membranes were observed (**b–f**). **a** Star in the black circle, vacuole. **b** ★ Cell wall; arrowhead, plasma membrane; curved arrow, globul on the plasma membrane – membraglobul. **c** Curved arrow, membraglobul. **d** Curved arrow, membraglobul on the microvesicle membrane. **e** Membraglobuli on the plasma membrane and tonoplast. **f** Fat arrow, chloroplast envelope membrane; curved arrows, membraglobuli on the plasma membrane, chloroplast envelope, and microvesicle membrane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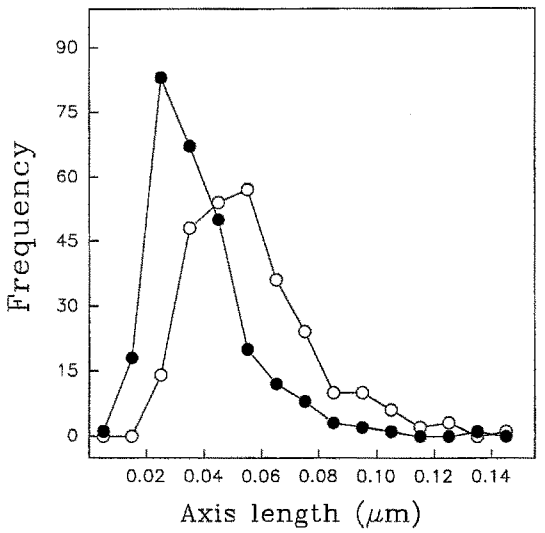


Fig. 8. Frequency of ellipse shaped membraglobuli (for explanation on membraglobuli see text and Fig. 6). ○ Major axis, ● minor axis. Images of 266 membraglobuli from 8 negatives were digitized and analyzed using Computer Image Analysis System

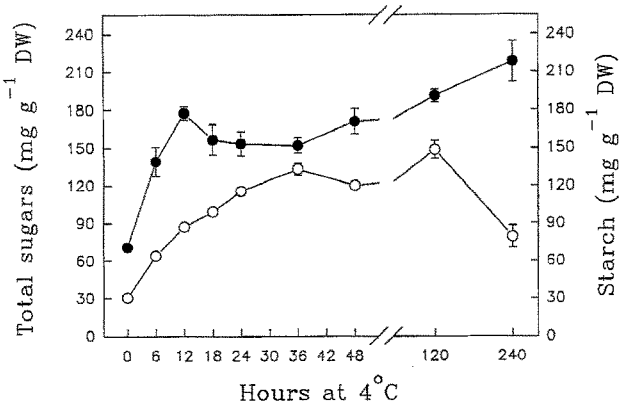


Fig. 9. Sugars (○) and starch (●) levels in the leaf tissue of *Arabidopsis thaliana* cv. Columbia during cold acclimation. Twenty-six-day old plants were exposed to 4 °C for 10 days (240 h). Total soluble sugars, and starch levels were determined using the methods of Smith (1981) and Rose et al. (1991), respectively. Bars represent standard errors; n = 4

ultrastructure of leaf parenchyma cells of *A. thaliana* represent processes related to changes in plasma membrane properties.

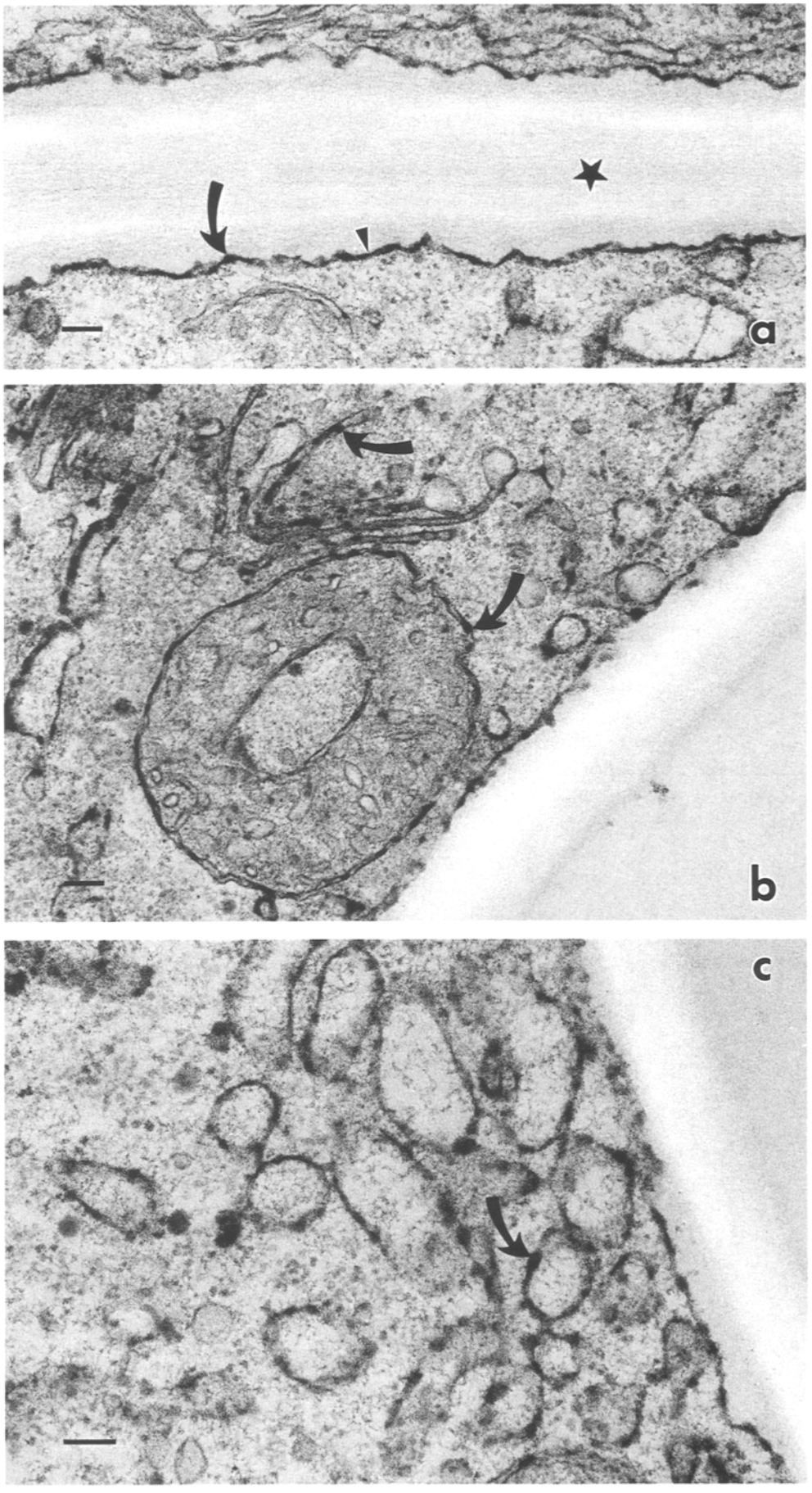
Wisniewski and Ashworth (1986) conducted seasonal studies of ultrastructural changes of bark and xylem parenchyma cells of *Prunus persica* (L.) Batsch, and noted that cold acclimation was associated with a reduction in the number and size of vacuoles; a large, centrally located vacuole in nonacclimated cells during summer, was replaced with several small vacuoles during winter. Similar changes in the number and size of vacuoles in xylem ray parenchyma cells of *Populus* × *canadensis* Moench have been reported (Sauter and Cleve 1991). It has been suggested that the reduced size of vacuoles results from the decline in tissue water content associated with cold acclimation (Wisniewski and Ashworth 1986).

Our results on the number of vacuoles in *A. thaliana* during cold acclimation contrast with those of Wisniewski and Ashworth (1986) and Sauter and Cleve (1991). Both nonacclimated and acclimated cells of the leaf tissue had one large centrally located vacuole, and this was apparent throughout the entire period of exposure to low temperature. In some acclimated cells, however, the vacuoles appeared smaller compared to those in nonacclimated cells. At least in *A. thaliana*, reduction in the number and size of vacuoles is not necessarily associated with increased cold hardiness.

Exposure to acclimation temperature for a period longer than 2 days resulted in modifications in the structure of cell membranes. Small, darkly stained globules located on the plasma membrane, tonoplast, chloroplast envelope membrane, mitochondrion outer membrane, dictyosome cisternae membrane and microvesicle membrane (Figs. 6 b–f and 7) were apparent after 5 days of exposure to 4 °C. The globules appeared to be an integral part of the membranes. We named this (these) globule(s) membraglobul(i). To our knowledge, this type of membrane modification has not been described previously in plant cells exposed to low temperature.

It could be argued that the membraglobuli may represent an artifact created by chemical fixation. We believe that this is unlikely since the membraglobuli were never observed in control tissues. To clarify this further, we employed a freeze-substitution technique to prepare leaf tissue for TEM. The membraglobuli were visible in tissues prepared for TEM using freeze-substitution technique (data not presented). Thus, we believe that

Fig. 7. Electron micrographs of leaf parenchyma cells of *Arabidopsis thaliana* cv. Columbia after 29 days of exposure to acclimation temperature (4 °C). Bars: 0.1 μm. Note modifications in the structure of cell membranes. **a** ★ Cell wall; arrowhead, plasma membrane; curved arrow, globule on the plasma membrane – membraglobul. **b** Curved arrows, membraglobul on the mitochondrion outer membrane, and dictyosome cisternae membrane. **c** Curved arrow, membraglobul on the microvesicle membrane. For more details see Fig. 6


a
b
c

the observed membraglobuli are not an artifact of chemical fixation, but instead represent a unique membrane structure which develops during low temperature exposure.

The appearance of darkly stained globules associated with cell membranes has been reported previously (Shi et al. 1991 a, b). Shi et al. (1991 b) described the development of osmiophilic droplets in root vessel contact cells and adjacent parenchyma cells in *Gossypium barbadense* L. plants infected by the fungus *Fusarium oxysporum* f. sp. *vasinfectum*. In the early stages of infection, the osmiophilic droplets were small, and associated with dilations of ER cisternae, mitochondrion envelope membranes, and occasionally chloroplast envelope membranes and nucleus membrane. As the infection proceeded, the droplets became larger and less electron-dense, and many of them were secreted through the plasma membrane into the infected vessels. The authors concluded that the appearance of osmiophilic droplets could represent defense mechanism against fungus infection.

The membraglobuli in *A. thaliana* seem to differ from the osmiophilic droplets described by Shi et al. (1991 a, b). First, the membraglobuli appear to be much smaller than the osmiophilic droplets. Second, the membraglobuli did not become larger and did not exhibit any changes in electron density in response to longer exposure to low temperature. In addition, no evidence of membraglobul secretion through the plasma membrane into the apoplast was noted.

The accumulation of membraglobuli does not appear related to increased freezing resistance since they became apparent after maximum freezing resistance was established. Several questions concerning the nature and significance of membraglobuli remain unanswered. What is the chemical composition of membraglobuli? Do the membraglobuli represent signs of low-temperature injury or signs of cell response to low temperature? Do they disappear after returning to normal growth temperature (22 °C to 23 °C)? Current studies are underway to address these questions.

The accumulation of soluble sugars during cold acclimation has been documented in a number of species including: *Triticum aestivum* L. (Perras and Sarham 1984); *Opuntia ficus-indica* (L.) Miller (Goldstein and Nobel 1991); *Secale cereale* (Koster and Lynch 1992) and *Populus* × *canadensis* (Sauter and Cleve 1991). Our results on the level of soluble sugars in the leaf tissue of *A. thaliana* during cold acclimation are consistent with those from previous studies. Although the relationship between cold acclimation and sugar accumulation has not been established, it is generally believed that sugar accumulation plays a role in cold hardiness.

Sauter and Kloth (1987) and Sauter and Cleve (1991) studied starch-sugar conversion in xylem ray parenchyma cells of *Populus* × *canadensis* during seasonal cold acclimation. They found that increase in the level of soluble sugars was accompanied by a concomitant decrease in the level of starch. Similarly, Pollock and Lloyd (1987) reported that in *Lolium temulentum* L., *Dactylis glomerata, Pisum sativum* L., and a few other species incubated at 5 °C for 6 h the accumulation of starch was greatly reduced. In contrast, we observed an increase in both sugars and starch during cold acclimation. Starch content increased during the first 12 h of exposure to 4 °C. After prolonged exposure to low temperature (5 and 10 days), the starch level continued to increase and this increase was accompanied by a decrease in sugar level.

In some species, carbon dioxide fixation continues at temperatures below the threshold for vegetative growth. Pollock et al. (1983) studied growth, and photosynthetic capacity in *Lolium temulentum* exposed to chilling temperatures. They found that dry weight gain and leaf elongation were greatly reduced at 5 °C. In contrast, the photosynthetic capacity, as indicated by the rate of oxygen evolution, was significantly increased at 5 °C. Our visual observation of the plants of *A. thaliana* exposed to low temperature indicated that the vegetative growth was markedly reduced. Even though we do not know the rate of carbon dioxide fixation in our plants, it is possible that plants exposed to 4 °C continued to photosynthesize, and part of the assimilate was converted into starch. This could account for the increase in starch content during exposure to acclimation temperature.

In summary, the ultrastructural study of the leaf tissue of *A. thaliana* showed that rapid changes in cell ultrastructure, primarily plasma membrane, occurred during cold acclimation. Cell membranes underwent modifications in their structure after prolonged exposure to low temperature. Small darkly stained globules, membraglobuli, were observed 5 days after exposure 4 °C. Furthermore, sugars and starch levels increased during cold acclimation. Observations suggest that cold acclimation in *A. thaliana* induces both changes in plasma membrane properties and in carbohydrate composition.

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