*Plant Cell, Tissue and Organ Culture* **40:** 17–24, 1995.


# Ultrastructural and protein changes in cell suspension cultures of peach associated with low temperature-induced cold acclimation and abscisic acid treatment

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Received 7 January 1994; accepted in revised form 29 June 1994

*Key words:* freezing tolerance, $LT_{50}$, nonacclimated, *Prunus persica*

## Abstract

Cell suspension cultures were initiated from callus derived from xylem tissues of peach [*Prunus persica* (L.) Batsch]. Cold acclimation was induced ($LT_{50}$ of-13°C) in cell suspensions at 3°C in the dark for 10 days. Freezing tolerance returned to the level of nonacclimated cells ($LT_{50}$ of -4.5°C) when cold-acclimated cells were transferred to 24°C (in dark) for 3 days. Addition of 75 μM abscisic acid (ABA) to the growth medium failed to induce cold acclimation after cells were cultured for 5 days at 24°C. Microvacuolation, cytoplasmic augmentation and disappearance of starch grains were observed in cells that were cold-acclimated by exposure to low temperature. Similar ultrastructural alterations were not observed in ABA-treated cells. Several qualitative and quantitative changes in proteins were noted during both cold acclimation and ABA treatment. Both the ultrastructural and protein changes observed during cold acclimation were reversed during deacclimation. The relationship of these changes to cold acclimation in peach cell-cultures is discussed.

*Abbreviations:* ABA – abscisic acid, 2,4-D – 2,4-dichlorophenoxyacetic acid, IBA – indole-3-butyric acid, MS – Murashige & Skoog, PMSF –phenylmethylsulfonyl fluoride, $LT_{50}$ or Freezing Tolerance – temperature that resulted in 50% decrease in TTC reduction, TTC – 2,3,5-triphenyltetrazolium chloride

## Introduction

Cell suspension cultures derived from plants capable of cold acclimation can also be hardened to withstand freezing, when they are exposed to low, nonfreezing temperatures (Chen & Gusta 1982; Borochov et al. 1989; Lee et al. 1992). In addition, exogenous application of abscisic acid (ABA) at nonhardening temperatures can also induce cold acclimation in cell suspensions derived from various herbaceous plant species (Chen & Gusta 1983; Orr et al. 1986; Reaney & Gusta 1887). Few data are available, however, on low-temperature-induced cold acclimation in cell suspensions derived from woody plants (Hellergren 1983; Tremblay et al. 1992), particularly deciduous fruit trees (Wallner et al. 1986). Furthermore, to our knowledge, no study has been conducted on ABA-induced cold acclimation in cell suspensions derived from deciduous fruit trees. Data are available, however, on the accumulation of ABA in the deciduous woody perennials in response to naturally occurring cold temperatures (Perry & Hellmers 1973).

The purpose of this study was to determine the effect of low temperature (3°C) and ABA applied at warm temperatures (23°C) on the induction of cold tolerance in a suspension culture of peach cells. In addition, changes in protein composition and ultrastructure in response to the applied treatments were also monitored.

## Material and methods

### *Cell culture*

Callus obtained from dormant xylem tissue of peach [*Prunus persica* (L.) Batsch cv. Sunhigh] (Schiavone & Wisniewski 1990) was used to initiate cell suspension cultures. Attempts to grow callus from the bark tissue of peach were unsuccessful (Schiavone & Wisniewski 1990). Three to four friable callus colonies (about 3 cm in diameter) were transferred to 50 ml of liquid medium that contained basal Murashige & Skoog (MS) salts and vitamins (Sigma Chemical Company, St Louis, MO) supplemented with 9 μM (2 mg $l^{-1}$) 2,4-D, 0.5 μM (0.1 mg $l^{-1}$) IBA and 3% (w/v) sucrose. Cultures were aerated by shaking at 115 revolutions per min on a controlled environment incubation shaker (New Brunswick Scientific, Edison, NJ, USA), maintained at 24°C in the dark, and subcultured every 14–20 days by transferring 10 ml of suspension into 50 ml of liquid medium in 250-ml Erlenmeyer flasks. After 2 months, 2,4-D concentration was reduced to 1 mg $l^{-1}$, IBA was excluded from the liquid medium, and 10 ml of suspension was subcultured every 10 days.

### *Determination of growth characteristics*

A growth curve was established by the daily measurement of packed cell volume obtained by centrifuging duplicate samples of 1 ml of cell suspension in a graduated tube at 250 *g* for 5 min. These measurements were repeated three times.

### *Hardening protocol and freezing tests*

For cold hardening treatment, cell suspensions, 7 days after sub-culture, were placed on a gyratory shaker at 3°C in the dark for 10 days. Non-acclimated cells continued to grow at 24°C in dark. Deacclimation was carried out by transferring flasks after 10 days of cold-hardening treatment to a nonacclimating regime for 3 days. In a companion study, cell suspensions, 5 days after sub-culture, were treated with a final concentration of 75 μM filter-sterilized ABA (Chen & Gusta 1983; Lee et al. 1992) and allowed to grow at 24°C for another 5 days at which time they were evaluated for freezing tolerance. This ABA treatment has been found to be optimum for the induction of freezing tolerance in cell suspensions derived from wheat, rye and bromegrass (Chen & Gusta 1983) and woody plants, e.g. birch and alder (Tremblay et al. 1992). The ABA (Sigma Chemical Company, St Louis, MO) stock solution was prepared as described by Robertson et al. (1987).

Freezing stress was imposed as described by Tanino et al. (1990) with few modifications. Cells were harvested on Miracloth under slight vacuum using a Buchner funnel and were washed with deionized water (about one liter) to remove the potential cryoprotective effects of the medium during freezing tests. Since excess extracellular water within the cell clumps has been suggested to significantly affect freezing tolerance of cells (Tanino et al. 1990), extracellular water was removed by blotting with Whatman No. 1 filter paper. One hundred mg (fresh weight) of cells was placed in 16 × 125 mm culture tubes, equilibrated in a glycol bath (Forma Scientific Inc., Marietta, OH, USA) at -1°C and nucleated with ice crystals. Following equilibration (about 1 h), samples were cooled at the rate of 0.5°C $(30\ min)^{-1}$ to -4°C, 1.0°C,$(30\ min)^{-1}$ to -6°C and at a rate of 2.0°C $(30\ min)^{-1}$ thereafter. Samples were removed from the bath after the desired temperature was reached and thawed overnight on ice. Unfrozen control samples were kept on ice in dark.

Thawed samples were held at 4°C for 1 h before viability was estimated using the TTC reduction method (Towill & Mazur 1975). Cells were incubated in 2 ml of TTC solution (0.16% TTC, 50 mM potasium phosphate buffer, pH 7.4) for 22–24 h in the dark. The reduced TTC was extracted in 3 ml of 95% ethanol for 48 h. TTC-reduction was determined by measuring absorbance at 485 nm. Three samples were analyzed for each temperature and treatment. Freezing injury was expressed as the percent decrease in TTC reduction as compared to unfrozen control. The temperature that resulted in a 50% decrease in TTC reduction is defined as freezing tolerance ($LT_{50}$). Each experiment was conducted twice.

### *Electron microscopy*

Cells were fixed in 1.25% (v/v) glutaraldehyde in 25 mM sodium phosphate buffer (pH 6.8) and postfixed in 2% (w/v) osmium tetroxide prepared in the same buffer. Cells were then rinsed at least three times with buffer. After removal of the buffer 1% (w/v) Bacto-agar (DIFCO Laboratories) (warmed to 60°C) was added to the cells. The cells were then pipetted as a layer (3 mm) onto glass slides. After cooling, the agar was cut into blocks (1 $mm^2$). The blocks of agar were placed in a test tube, dehydrated in a graded ethanol series and embedded in Spurrs Epoxy resin

(Polysciences, Warrington, PA, USA). Sections were cut with a diamond knife on an LKB Ultratome IV (Reichert-Jung, Vienna, Austria), mounted on copper grids, stained with a saturated solution of uranyl acetate in 50% (v/v) methanol and 0.2% (w/v) lead citrate, and viewed with a Hitachi H-600 transmission electron microscope (Nissei Sangyo American, Mountain View, CA, USA) at 75 kV.

*Protein extraction and SDS-PAGE*

Proteins were extracted using a modification of the method of Robertson et al. (1988). Washed and filter paper-blotted cells (10–12 g fresh weight) were transferred to Braun homogenization flasks containing 25 g of 1.00 mm glass beads and 7 ml of homogenization buffer [450 mM NaCl, 10 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM sodium metabisulphite, 1mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4]. Cells were disrupted by shaking them for four 60-sec periods in a $CO_2$-cooled, Braun homogenizer. The homogenate was centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant was filtered through 0.4- and then 0.2-μm filters, respectively, and centrifuged at 48,000 *g* for 2.5 h. The supernatant was retained as a soluble protein fraction. The protein content was assayed using a modified micro-Lowry protein assay kit (Sigma, St. Louis, MO, USA).

Samples for SDS-PAGE were prepared essentially as described by Arora et al. (1992). Discontinuous SDS-PAGE was performed with a PROTEAN II electrophoreis unit (Bio-Rad Laboratories, Richmond, CA, USA) using 4% stacking gel and a 12.5% running gel. Gels were stained with 0.1% Coomassie Brilliant Blue R in methanol : water : acetic acid (4.2:4.2:1.6, v/v/v) and destained with water : methanol : acetic acid (8:1:1, v/v/v).

## Results

*Growth rate of peach cell suspensions*

The growth characteristics of the cell suspension are presented in Fig. 1. They indicate a short lag phase (approximately 1 day) followed by a linear increase in growth for about 9 days (growth phase). On the 10th day, the cells entered a stationary phase.

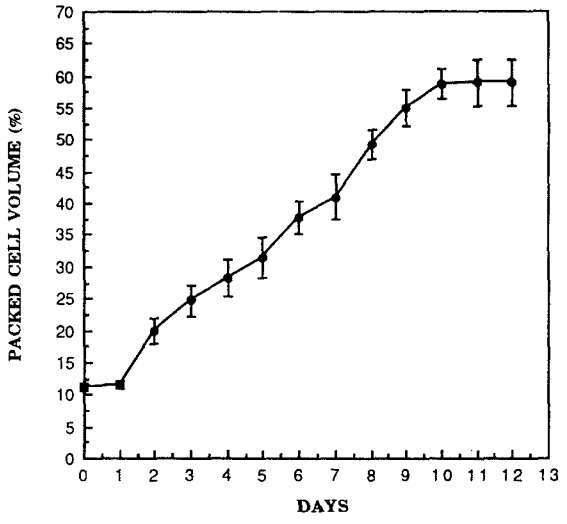


*Fig. 1.* Growth curve of peach cell suspension cultures. Values are mean ± S.E. of three experiments, each with duplicate samples.

*Cold acclimation in peach cell suspensions*

The ability of peach cell suspensions to cold acclimate was assessed by determining freezing tolerance of nonacclimated cells (i.e. cells grown at 24°C continuously for 11–13 days) and those that were exposed to either cold hardening treatment or 75 μM ABA for 5 days (Fig. 2). The $LT_{50}$ of nonacclimated suspension cultures was -4.5°C. When 7-day-old cells were transferred to 3°C for 10 days (cold hardening treatment), the freezing tolerance increased to -13°C. Preliminary experiments indicated that maximum freezing tolerance was attained when cell cultures in the mid-to late-stage of rapid growth (Fig. 1) were transferred to cold hardening conditions. There was also no further increase in hardiness after 10 days of exposure to acclimating conditions (data not shown). There was no increase in the hardiness noted when 5-day-old cell cultures were treated with 75 μM ABA for 5 days (Fig. 2). Five days exposure to 50 μM ABA or 4 days exposure to 75 μM ABA also did not increase cold tolerance of cells, whereas exposure longer than 5 days resulted in severe browning of cells (data not shown). When cold-acclimated cells were transferred to a nonacclimating temperature (24°C), the $LT_{50}$ returned to approximately the same level as that in nonacclimated cells within 3 days (Fig. 2).

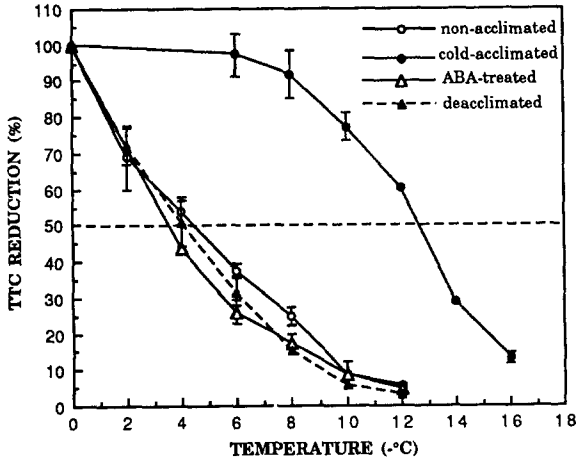


*Fig. 2.* Freezing tolerance of nonacclimated, cold-acclimated, deacclimated and ABA-treated peach cell cultures estimated by the TTC reduction method. Values are means ± S.E. of two experiments, each with triplicate samples.

*Cytological changes during cold acclimation*

Nonacclimated cells were characterized by a large central vacuole and peripheral cytoplasm (Fig. 3A, E). Numerous amyloplasts containing starch grains were visible in these cells. In cold-acclimated cells, the large central vacuole was replaced by numerous smaller ones (Fig. 3B, F). The cytoplasm appeared relatively more dense and mitochondria appeared to be more abundant. Starch grains were essentially absent in the cold-acclimated cells. A greater abundance of rough endoplasmic reticulum was also evident in cold-acclimated cells (Fig. 3F) as compared with nonacclimated cells (Fig. 3E). In deacclimated cells, a large vacuole and starch grains were again observed (Fig. 3C, G). In cells treated with 75 μM ABA, essentially no change was observed after 5 days of treatment (Fig. 3D, H). These cells, in general, looked similar to nonacclimated cells. The cytological changes observed in this study were observed in about 75% of the total number of cells of each treatment.

*Protein changes during cold acclimation and ABA trentment*

SDS-PAGE analysis of soluble proteins extracted from cell cultures after various treatments was repeated three times with similar results. Data from a single, representative analysis are presented in Fig. 4. There was an increased accumulation of 19 kD and 25 kD polypeptides during low temperature-induced cold acclimation. In addition, two other polypeptides with estimated molecular masses of 26 and 30 kD were apparent in the cells that were cold hardened by a low temperature treatment. These latter polypeptides were not detected in non-acclimated cells. Data further indicated that the protein profiles from deacclimated cells looked similar to those of nonacclimated cells, both quantitatively and qualitatively. The most apparent change in the protein profile of cells treated with 75 μM ABA was an accumulation of 26 kD polypeptide and a reduction of 19 kD polypeptide. Finally, a 60 kD polypeptide, which appeared to constitute the major portion of total protein, and a 16 kD polypeptide were the two major polypeptides commonly apparent in protein profiles from all the treatments.

## Discussion

*Cold acclimation in peach cell cultures*

In the present study, an increase in the freezing tolerance of peach cells was achieved when cultures were exposed to 3°C in the dark for 10 days (Fig. 2). Similar data have also been obtained by Wallner et al. (1986) on cold acclimation of pear cells. Our results are in contrast with those of Tremblay et al. (1992) who reported that low temperature (4°C) exposure did not induce cold acclimation in cell suspensions of birch (*Betula papyrifera* Marsh). Birch cells were grown at 4°C throughout the experiment. In the present study, however, peach cells were initially grown for 7 days at 24°C, and then subjected to low temperature treatment. Our results are also in contrast with those of Hellergren (1983) who reported that cold acclimation in cell suspensions derived from *Pinus sylvestris* needles was possible only when cells were exposed to both low temperature (2°C) and short photoperiods (8-h) at the same time. No increase in the freezing tolerance of pine cell suspensions was observed when only one type of stimulus was present. It has been shown, however, that intact plants of *Pinus sylvestris* do respond to individual stimulus during cold acclimation (Christersson 1978).

Maximum hardiness attained by cell cultures during cold acclimation in the present study was about -13°C (Fig. 2), as compared to -50°C and-36°C in the bark and xylem tissues, respectively, of field-grown peach trees (Arora et al. 1992). A similar difference in the hardening capacity between cell cultures and intact plants of *Pinus sylvestris* has been reported (Hellergren

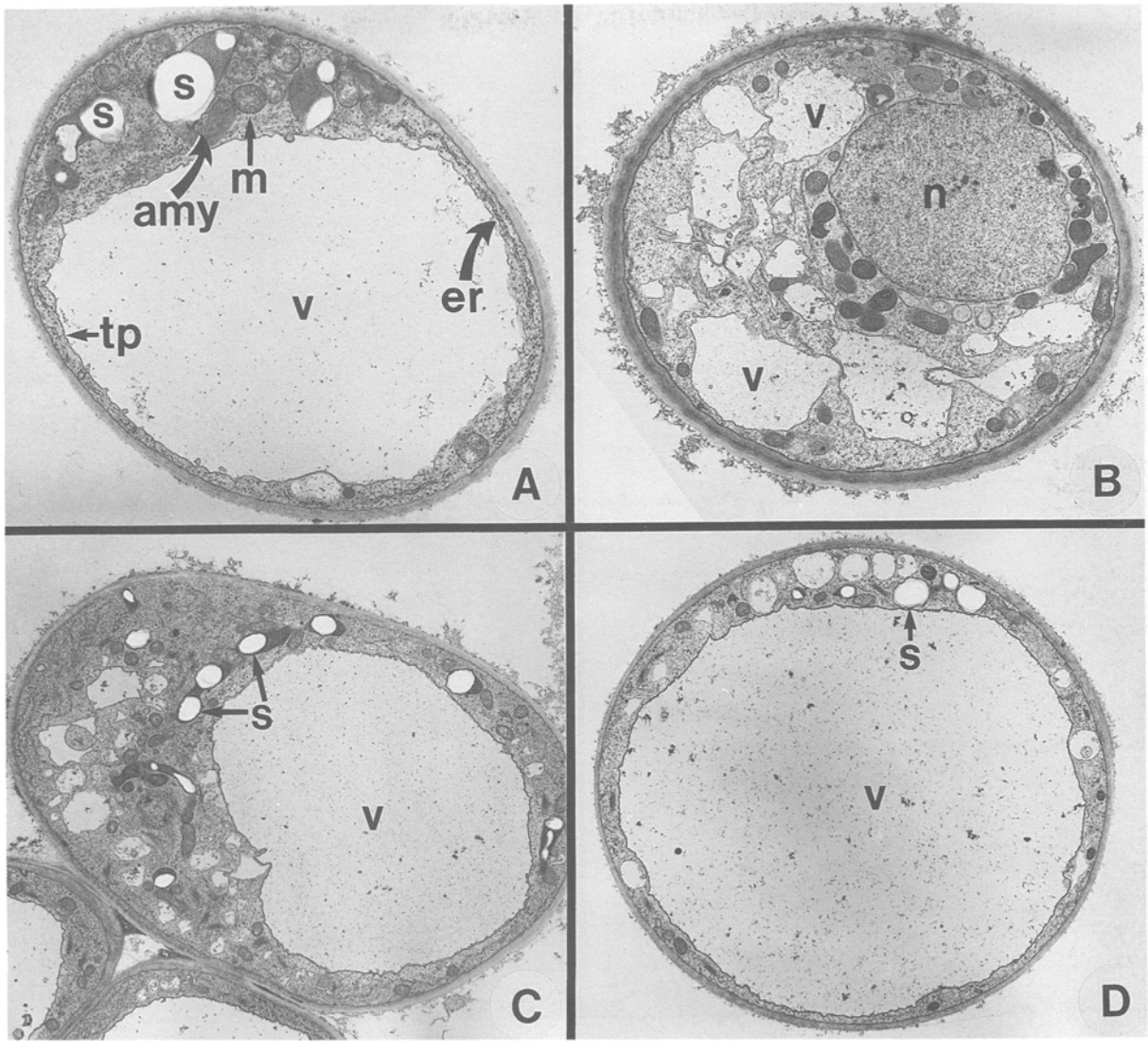


*Fig. 3.* Electron micrographs of nonacclimated (*A, E*), cold-acclimated (*B, F*) deacclimated (*C, G*) and ABA-treated (*D, H*) peach cell suspensions. amy: amyloplast; er: endoplasmic reticulum; m: mitochondria; n: nucleus; s: starch; tp: tonoplast; v: vacuole. Bar in A through D is 2 μm and E through H is 1 μm.

1983). These differences between cell cultures and intact woody plants in their responsiveness to various environmental stimuli and in cold acclimation potential suggest the importance of the organization of cells into tissues for full expression of cold hardiness in woody plants.

Several studies have reported an induction of cold acclimation in cell suspensions of various herbaceous and woody plant species at nonhardening temperatures when they were exposed to exogenous ABA (Chen & Gusta 1983; Orr et al.1986; Reaney & Gusta; 1987, Tremblay et al. 1992). In our study, however, no increase in the freezing tolerance was observed when peach cell suspensions were treated with ABA. Although the effect of various concentrations of ABA, and duration of ABA treatment was not evaluated in detail, the initial data indicate that cell suspensions derived from peach do not respond to ABA by developing increased cold tolerance. The reason for the apparent lack of responsiveness of peach cell cultures to ABA is not clear in that the concentration, and the duration of exposure, of ABA used in our study has

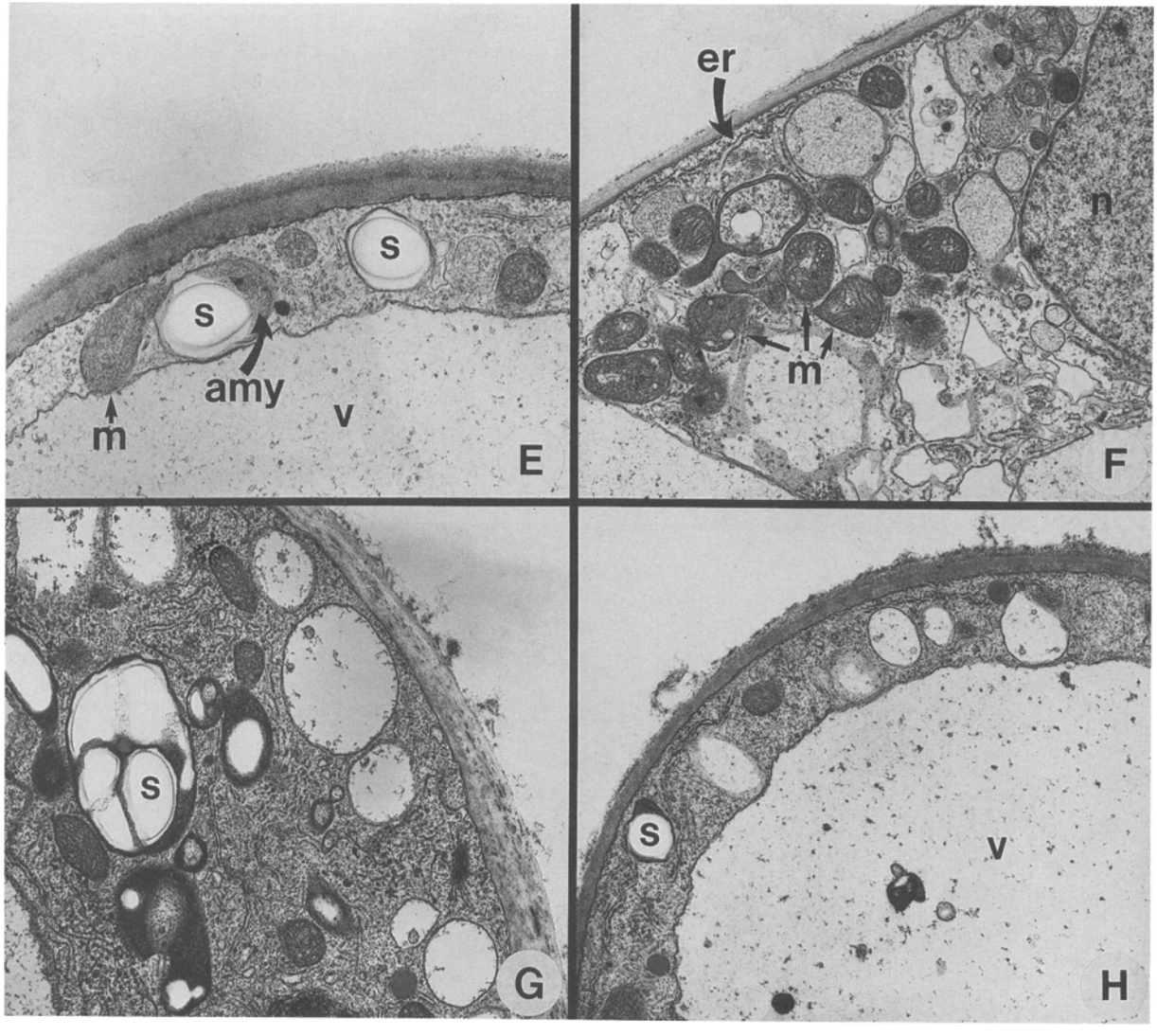


*Fig. 3.* Continued.

been shown to induce cold acclimation in many other cell suspension systems. On the other hand, this result is not very surprising because it seems that the response of woody-plant cell suspensions to ABA treatment is variable. Recently, it was reported that identical ABA-treatment ($10^{-5}$ M for 7 days at 24°C) increased the freezing tolerance of birch cells from -9.1°C (untreated control) to -16.9°C, whereas it resulted in a substantially smaller increase of 1.9°C (from -7.3°C to -9.2°C) in the cell suspension cultures of alder (Tremblay et al. 1992).

*Cytological changes during cold acclimation*

Pomeroy & Siminovitch (1971), in a study of bark tissues of black locust (*Robinia pseudoacacia* L.), noted a seasonal augmentation of total cytoplasm paralleled by increased cold hardiness in autumn and winter. The most striking feature of the cytoplasmic augmentation was a distinct presence of microvacuolation and increase in volume of cytoplasm. It was also accompanied by an accumulation of cellular material (mitochondria, ER, etc.) and denser protoplasm than in summer. Niki & Sakai (1981) and Kuroda & Sagisaka (1993) later extended these observations to cortical cells of mulberry (*Morus bombyciz* Koidz) and apple

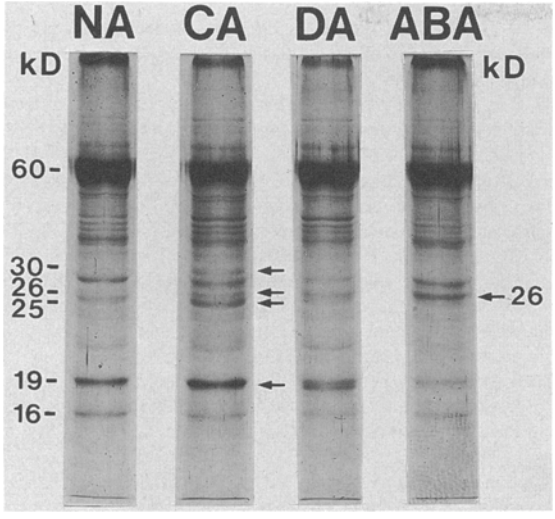


*Fig. 4.* SDS-PAGE profiles of soluble proteins from nonacclimated, cold-acclimated, deacclimated and ABA-treated cell cultures. An equal amount of protein (50 μg) was applied in each lane. NA: nonacclimated; CA: cold-acclimated; DA: deacclimated; ABA: ABA-treated. Molecular weights are indicated on the left and right of the lanes.

(*Malus pumila* Mill.) twigs. Ultrastructural changes observed in the present study in cold-acclimated ($LT_{50}$ -13°C) peach cell suspension cultures (Fig. 3B, F) were similar to those in previous reports. These changes were reversed during deacclimation ($LT_{50}$ returned to the level of nonacclimated cells) (Fig 3C). Our results also closely conform to the seasonal changes, in vivo, in cortical cells of peach twigs during cold acclimation and deacclimation (Wisniewski & Ashworth 1986). Recently, Tanino et al. (1991) also observed cytoplasmic augmentation and microvacuolation during ABA-induced cold acclimation in bromegrass cell suspensions.

Although the role of cytoplasmic augmentation is obscure, the shift toward microvacuolation and denser cytoplasm during cold acclimation would result in the reduction of large areas of free (freezable) water present in the large vacuoles of nonacclimated cells. Tanino et al. (1991) have suggested that an increased number of smaller vacuoles would also serve to increase the surface/volume ratio of these vacuoles. This could help prevent or reduce injury to tonoplast membranes, during extracellular freeze-dehydration, by increasing their interaction with remaining free water within the cell. A shift toward microvacuolation has also been observed in cell cultures of *Panicum maximum* when cells were dehydrated in preparation for cryopreservation (Gnanapragasam & Vasil 1992).

During cold acclimation, organelles involved in protein synthesis and ATP-generation, such as endoplasmic reticulum (ER) and mitochondria, were more abundant in peach cells, as compared to nonacclimated cells. This change in protein-synthetic system during cold acclimation suggests that the synthesis of proteins may be associated with the development of cold hardiness. Indeed, there are numerous reports on the synthesis of proteins during cold acclimation in various plant species (Guy 1990 and references therein). The ATP-generating system, however, may be needed not only for the energy requirement for protein sysnthesis but also for the maintenance of membrane integrity at low temperatures.

Starch content is known to decline during cold acclimation with concomitant quantitative increase in free saccharides in various temperate woody perennials (Siminovitch et al. 1953; Sakai & Yoshida 1968; Sauter & van Cleve 1991). The presence of free sugars, primarily sucrose, in cold-acclimated cells results in an increase in the osmotic potential of cell sap (Sakai & Yoshida 1968), an adaptive response that helps protect cells from dehydration caused by freezing. Although a disappearance of starch during cold acclimation (compare Fig. 3A, E with Fig. 3B, F) was observed in the present study, a significant increase in the osmolality of cell sap was not (data not shown). This result is in accordance with the findings of Borochov et al. (1989). These authors noted a small but significant decrease in the osmotic concentration of cold-acclimated alfalfa (*Medicago sativa* L.) cell suspensions compared to that of nonacclimated cells.

*Protein changes during cold acclimation*

The most apparent changes in proteins observed during cold acclimation was the accumulation of 19 kD and 25 kD polypeptides followed by a subsequent decrease during deacclimation (Fig. 4). In addition, two polypeptides with estimated molecular masses of 26 and 30 kD were apparent in cold-acclimated cells but not in nonacclimated and deacclimated cells. Both qualitative and quantitative changes in protein content have been reported during cold-induced or ABA-induced cold acclimation in callus or in cell cultures derived from various plant species (Johnson-Flanagan & Singh 1987; Robertson et al. 1987; Borochov et al. 1989; Lee et al. 1992). The present report, to our knowledge, is the first one on a deciduous fruit tree species. Although increases in existing proteins and the appearance of new proteins during cold acclimation

have been widely documented (Guy 1990), whether or not these proteins are specifically associated with cold acclimation or with metabolic adjustment to low temperature is not well understood. It remains to be determined whether the 19, 25, 26 and 30 kD polypeptides found to be associated with cold acclimation in the present study are related to proteins with similar molecular weights reported by others.

## Acknowledgements

Technical assistance by Wilbur Hershberger for maintaining cell cultures and Glen Davis for electron microscopy is greatly appreciated. We thank Drs. K. Tanino, T. H. H. Chen and A. J. Robertson for their helpful suggestions throughout this work.

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