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Accumulation of Small Heat-Shock Protein Homologs in the Endoplasmic Reticulum of Cortical Parenchyma Cells in Mulberry in Association with Seasonal Cold Acclimation[1]

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Cortical parenchyma cells of mulberry (*Morus bombycis* Koidz.) trees acquire extremely high freezing tolerance in winter as a result of seasonal cold acclimation. The amount of total proteins in endoplasmic reticulum (ER)-enriched fractions isolated from these cells increased in parallel with the process of cold acclimation. Protein compositions in the ER-enriched fraction also changed seasonally, with a prominent accumulation of 20-kD (WAP20) and 27-kD (WAP27) proteins in winter. The N-terminal amino acid sequence of WAP20 exhibited homology to ER-localized small heat-shock proteins (smHSPs), whereas that of WAP27 did not exhibit homology to any known proteins. Like other smHSPs, WAP20 formed a complex of high molecular mass in native-polyacrylamide gel electrophoresis. Furthermore, not only WAP20 but also 21-kD proteins reacted with antibodies against WAP20. Fractionation of the crude microsomes by isopycnic sucrose-gradient centrifugation revealed that both WAP27 and WAP20 were distributed on a density corresponding to the fractions with higher activity of ER marker enzyme, suggesting localization of these proteins in the ER. When ER-enriched fractions were treated with trypsin in the absence of detergent, WAP20 and WAP27 were undigested, suggesting localization of these proteins inside the ER vesicle. The accumulation of a large quantity of smHSPs in the ER in winter as a result of seasonal cold acclimation indicates that these proteins may play a significant role in the acquisition of freezing tolerance in cortical parenchyma cells of mulberry trees.

Cold acclimation is a complex adaptive process by which plants increase their tolerance to equilibrium freezing (Levitt, 1980). During cold acclimation, diverse intracellular and extracellular changes, including ultrastructural changes in cytoplasmic organelles (Niki and Sakai, 1981; Fujikawa and Takabe, 1996), compositional changes in plasma membranes (Steponkus, 1984; Yoshida, 1984; Zhou et al., 1994), accumulation of intracellular compatible osmolytes (Hare et al., 1998), increased rigidity of cell walls (Rajashekar and Lafta, 1996), and even compositional changes in apoplastic solutions (Griffith and Antikainen, 1996), occur in plant cells. Although all of these diverse changes due to cold acclimation are associated with the acquisition of freezing tolerance in many plant cells, the significance of these changes in the acquisition of freezing tolerance is still unclear. Efforts to clarify the molecular basis of cold acclimation in plants may lead to an understanding of the mechanisms of freezing tolerance as a result of cold acclimation. Studies along this line have led to the identification of numerous cold-induced genes and gene products.

Various genes encoding signal transduction and regulatory proteins have been shown to be up-regulated in response to low temperature (Guy, 1990; Hughes and Dunn, 1996). A number of enzymes that contribute to freezing tolerance, such as fatty acid desaturase and Suc phosphate synthase, are also induced in response to low temperature (Guy, 1990; Hughes and Dunn, 1996). A growing number of genes that encode hydrophilic and boiling-stable polypeptides (Lin et al., 1990; Gilmour et al., 1992; Kazuoka and Oeda, 1992; Neven et al., 1992; Thomashow, 1994, 1998; Kaye and Guy, 1995) have been reported to be cold induced, and many of these belong to one of a few multigene families, particularly the late-embryogenesis abundant/dehydrin family (Kaye et al., 1998). It has been suggested that these hydrophilic and boiling-stable polypeptides might contribute to freezing tolerance by mitigating the effects of dehydration associated with freezing (Thomashow, 1998). Cold acclimation also induces accumulation of antifreeze proteins, which inhibit or reduce extracellular ice-crystal growth in the apoplastic spaces of plants, suggesting their possible contribution to the acquisition of freezing tolerance (Griffith and Antikainen, 1996).

Recently, a class of proteins that accumulate in response to low temperature was identified as HSPs (Neven et al., 1992). The genes and gene products of HSP70 are induced in spinach (Neven et al., 1992; Anderson et al., 1994; Guy et al., 1998) and soybean (Cabané et al., 1993), and those of HSP90 are induced in *Brassica napus* (Krishna et al., 1995) and rice (Pareek et al., 1995), in response to low temperature. Low-temperature stress also stimulates smHSP gene expression in potato (van Berkel et al., 1994) and heat-stressed tomato fruits (Sabehat et al., 1998). Different HSPs may have different functional properties, but common to all of them is their capacity to interact with other proteins and to act as molecular chaperones (Jakob et al., 1993; Schöffl et al., 1998). It has been speculated that HSPs might contribute to chilling resistance (Guy et al., 1998) as well as

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Abbreviations: CBB, Coomassie Brilliant Blue R-250; HSP, heat-shock protein; smHSP, small HSP; WAP20 and WAP27, winter-accumulating 20- and 27-kD proteins, respectively.

to freezing tolerance (Thomashow, 1998) by stabilizing proteins against these stresses. To understand the general role of HSPs in relation to cold acclimation of plants, however, more studies are necessary.

Seasonal periodic temperature changes produce large seasonal differences in the freezing tolerance of cortical parenchyma cells of mulberry (*Morus bombycis* Koidz.) trees. The freezing tolerance of cortical parenchyma cells of mulberry trees growing in Sapporo, Japan, is above −5°C in summer (June–August), increases gradually in autumn (September–November), reaches a maximum of below −50°C in winter (December–March), and then decreases gradually in spring (April–May) (Niki and Sakai, 1981; Sakai and Larcher, 1987; Fujikawa, 1994). In the present study, we examined seasonal changes in proteins of ER-enriched fractions of cortical parenchyma cells of mulberry trees. Our results show that in association with the process of seasonal cold acclimation there is a large accumulation of smHSP homolog (WAP20) in ER-enriched fractions, suggesting that smHSP is related to the acquisition of freezing tolerance by cortical parenchyma cells of mulberry trees.

MATERIALS AND METHODS

Plant Material

One-year-old twigs were collected from mulberry (*Morus bombycis* Koidz.) trees growing on the campus of Hokkaido University (Sapporo, Japan) from August 25, 1995, to June 20, 1996.

Isolation of ER-Enriched Fractions by Isopycnic Linear Suc-Density Gradient Centrifugation

Fresh cortical tissues (25 g fresh weight) were removed from twigs, cut into small pieces, and homogenized in 180 mL of the homogenizing medium using a polytron (model T-20, Kinematika, Lucerne, Switzerland). The homogenizing medium consisted of 300 mM Suc, 75 mM Mops-KOH, pH 7.6, 5 mM EGTA, 2 mM EDTA, 3% (w/v) PVP (molecular weight 24,500), 10 μg mL^{-1} 2,6-di-*t*-butyl-*p*-cresol, 5 mM potassium metabisulfite, 1.5% (w/v) polyvinylpolypyrrolidone, and 2 mM PMSF. The homogenates were filtered through three layers of gauze. The filtrates were centrifuged at 10,000*g* for 15 min, and the supernatants were obtained. The supernatants were centrifuged at 200,000*g* for 20 min, and the precipitates were suspended in a resuspension medium containing 10% (w/w) Suc, 15 mM Hepes-bis tris propane, pH 7.3, 1 mM EGTA, 0.1 mM EDTA, and 2 mM DTT and centrifuged again at 200,000*g* for 20 min. The final precipitates were mixed with the resuspension medium to make 4.5 mL in total.

Four milliliters of the crude microsome was overlaid on 28 mL of a continuous Suc gradient (15%–50%, w/w) containing resuspension medium and centrifuged at 84,000*g* in a rotor (model RPS-27, Hitachi, Tokyo) for 17 h. After centrifugation, the gradient was fractionated from top to bottom of the gradient into 1.2-mL aliquots using a density gradient fractionator (model-640, ISCO, Lincoln, NE).

Activity of marker enzymes was measured in each fraction for vanadate-sensitive H^+-ATPase (plasma membranes), nitrate-sensitive H^+-ATPase or pyrophosphatase (tonoplasts), antimycin A-insensitive NADH Cyt *c* reductase (ER), UDPase (Golgi membranes), and Cyt *c* oxidase (mitochondria inner membranes), as described by Koike et al. (1997). The fractions that exhibited the highest activity of antimycin A-insensitive NADH Cyt *c* reductase were used as ER-enriched fractions.

Analysis of Protein Amount

The amount of proteins in each fraction was determined by a protein-assay kit (Bio-Rad) with γ-lactoglobulin as a standard.

Gel Electrophoresis

SDS-PAGE on 14% (w/v) polyacrylamide gels was conducted according to the procedure described by Laemmli (1970). Samples were solubilized in 4× SDS-lysis buffer containing 60 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 8% (v/v) 2-mercaptoethanol, 50% (v/v) glycerol, and 0.01% (w/v) bromphenol blue and heated at 70°C for 10 min. The apparent molecular masses of proteins were estimated by comparison with the mobility of M_r standard proteins (Daiichi-Kagaku, Tokyo, Japan) on the gel after SDS-PAGE.

IEF for the first dimension of two-dimensional electrophoresis was performed basically according to the method of O'Farrell (1975). Protein samples for IEF were dissolved in 8 M urea, 5 mM 2-mercaptoethanol, 2% (v/v) Triton X-100 (Nacalai Tesque, Kyoto, Japan), and 2% (v/v) Pharmalyte (1.5% [v/v], pH 3.0–10.0, 0.5% [v/v], pH 4.0–6.5) (Pharmacia). Proteins on the gel after SDS-PAGE for the second dimension were visualized by silver staining, as described by Koike et al. (1997).

Native-PAGE was performed on 5.0% to 20.0% polyacrylamide linear-gradient gels prepared by the buffer system of Laemmli (1970) without SDS. Protein samples were treated with sonication or 0.1% Triton X-100 and ultracentrifuged at 200,000*g* for 20 min. The supernatant was mixed with the same volume of buffer containing 20 mM Tris-HCl, pH 6.8, 50% glycerol, and 0.01% bromphenol blue and analyzed by native-PAGE. Electrophoresis was carried out at 5.0 V cm^{-1} at 4°C for 48 h to equilibrium.

Immunoblot Analysis

Electrotransfer of proteins onto PVDF membranes was performed using a semidry transfer apparatus (Bio-Rad) as described previously (Koike et al., 1997). Immunological detection of proteins on the PVDF membranes was carried out using a primary antibody and alkaline phosphatase-conjugated anti-rabbit IgG antibodies from goat (Bio-Rad) as a secondary antibody. The proteins, which reacted with primary antibodies, were visualized with a reaction medium containing 20 mM Tris-HCl, pH 9.2, 100 mM NaCl, 5 mM $MgCl_2$, 0.165% (w/v) nitroblue tetrazolium, and 0.083% (w/v) 5-bromo-4-chloro-3-indolyl phosphate.

Isolation of Proteins

After ER-enriched fractions were subjected to SDS-PAGE, proteins in the gels were stained with 0.25% (w/v) CBB containing 25% methanol and 10% acetic acid. After destaining, the bands corresponding to the 20- and 27-kD proteins were excised from the gels, and the gel slices were soaked in electroelution buffer containing 25 mM Tris, 192 mM Gly, and 0.1% SDS. Each protein was electroeluted from the gel slices using an elecroeluter (model 422, Bio-Rad) with the same buffer system. When both protein fractions were analyzed by SDS-PAGE, each fraction contained a single protein band corresponding to the 20- and 27-kD proteins, respectively. The isolated proteins were used for antibody production.

Antibody Production and Purification

Each of the isolated proteins was mixed with the same volume of the complete adjuvant and injected into New Zealand White female rabbits to generate antibodies, and antiserum was taken after the second immunization using the incomplete adjuvant. Preimmune serum was taken from rabbits before immunization and used for the control experiments. Antibodies were affinity purified from antiserum using the isolated antigens, which were electroblotted on PVDF membranes after two-dimensional electrophoresis. Immunoblot analysis after two-dimensional electrophoresis revealed that purified antibodies reacted with each isolated antigen.

Analysis of Amino Acid Sequences

After two-dimensional electrophoresis of the ER-enriched fraction collected in December (approximately 0.8 mg), the proteins in the gel were electrotransferred onto a PVDF membrane, stained for 1 min with 0.1% (w/v) CBB in 50% (v/v) methanol, and destained with 10% (v/v) acetic acid containing 50% (v/v) methanol until the background was lowered. After sufficient washing with distilled water, the membrane was dried and the protein spots were excised for analysis of the N-terminal amino acid sequence. Edman degradation was performed with a gas-phase sequencer (model PPSQ-10, Shimazu, Kyoto, Japan). The homology searches of the amino acid sequences were performed with the Basic Local Alignment Search Tool using Netscape network services (GenomeNet).

Trypsin Digestion of ER-Enriched Fractions

Trypsin digestion of 1 μg of ER-enriched fractions was performed with 0.1 μg μL^{-1} trypsin (Sigma) in the presence or absence of 0.1% Triton X-100 and 2 M urea at 4°C for 90 min. After digestion, samples were mixed with 4× SDS-lysis buffer, and the proteins were detected by immunoblot analysis using these antibodies.

RESULTS

Changes in Protein Amount in ER-Enriched Fractions

The amount of total proteins in microsome fractions of cortical parenchyma cells of mulberry trees increased from 25.7 mg/25 g fresh weight in August to 69.7 mg/25 g fresh weight in December. It has also been reported that the total protein amount in microsome fractions obtained from cortical tissues of black locust increased as a result of seasonal cold acclimation (Siminovitch et al., 1968).

Analysis by isopycnic linear Suc-density gradient centrifugation also showed an increase in the amount of total protein in ER-enriched fractions from August to December (Fig. 1). The seasonal increases of total proteins in crude microsome fractions, as well as proteins in ER-enriched fractions, are approximately parallel with the gradual in-

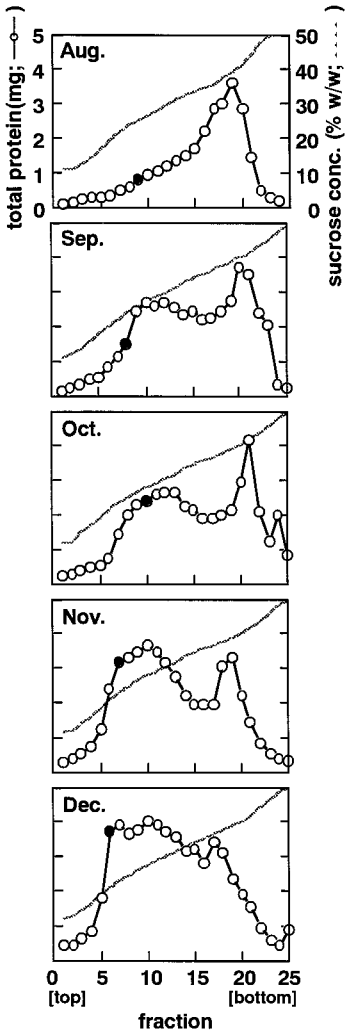

Figure 1. Seasonal changes in the amount of total proteins (○) and changes in the peak fraction of ER marker enzyme activity (●) in microsome fractions isolated from cortical parenchyma cells of mulberry tree. Microsome fractions were prepared from 25 g fresh weight of cortical tissues in each sample and were subjected to isopycnic Suc-density gradient centrifugation. Marker enzyme activities were measured as described in the text.

crease in freezing tolerance of cortical parenchyma cells of mulberry trees as a result of seasonal cold acclimation (Fujikawa, 1994).

Seasonal Changes in Protein Profiles of ER-Enriched Fractions

Examination by SDS-PAGE showed distinct seasonal changes in protein profiles in the ER-enriched fractions (Fig. 2A). The major seasonal differences in the protein profiles were a decrease in protein bands with high molecular mass and an increase in protein bands with relatively low molecular mass in association with the process of seasonal cold acclimation. Two protein bands corresponding to molecular masses of about 27 and 20 kD, respectively (indicated by arrowheads in A), specifically increased in winter. In this study, we focused on the analysis of these two proteins, named WAP27 and WAP20.

Immunoblot analysis using antiserum raised against these two proteins confirmed the increases in WAP27 and WAP20 in winter corresponding to the process of cold acclimation (Fig. 2B; see also Fig. 6). The band of WAP27 was faint in summer (June–August), increased gradually in autumn (September–October), reached a maximum in winter (November–March), and decreased again in spring (April–May). On the other hand, the band of WAP20 was barely detectable in summer (especially in August), gradually became apparent in autumn (September–October), reached a maximum level in winter (November–April), and gradually decreased from spring (May) to summer (June). In addition, anti-WAP20 antibodies also reacted with the 21-kD protein in a manner similar to the seasonal appearance of WAP20 (Fig. 2B).

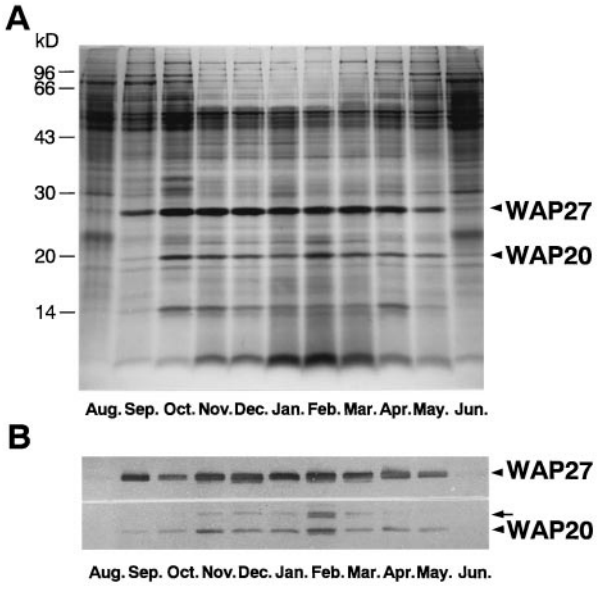

Figure 2. Seasonal changes in the protein profiles in ER-enriched fractions in cortical parenchyma cells of mulberry trees. A, SDS-PAGE was performed using 40 μg of proteins in each lane and staining with CBB. Arrowheads show WAP27 and WAP20. B, Immunoblot analysis was performed with anti-WAP27 and anti-WAP20 antibodies; 5 μg of proteins was used in each lane. The arrow shows 21-kD proteins cross-reacted with anti-WAP20 antibodies.

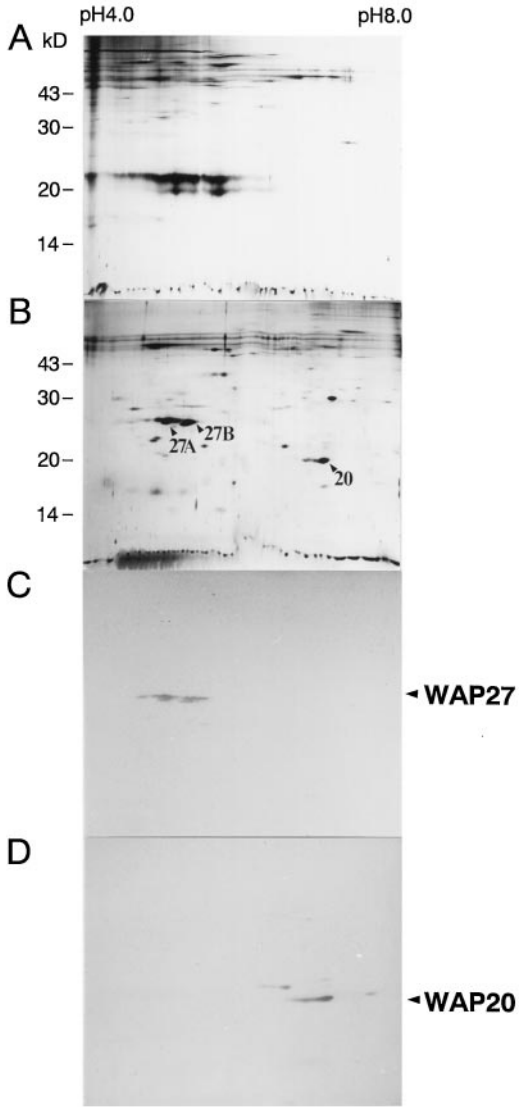

Figure 3. Two-dimensional gel electrophoresis profiles of ER-enriched fractions prepared from August (A) and December (B–D) samples. A and B, Protein profiles visualized by silver staining. Arrowheads in B show WAP27A, WAP27B, and WAP20. C and D, Profiles of immunoblot analysis. SDS-PAGE was performed with 30 μg (C) and 100 μg (D) of proteins and with anti-WAP27 (C) and anti-WAP20 (D) antibodies.

To characterize the nature of WAP27 and WAP20, two-dimensional gel electrophoresis and immunoblot analysis were performed (Fig. 3). Anti-WAP27 antibodies reacted with at least two protein spots with pI values of 4.8 and 5.0 (Fig. 3C). This indicates that the 27-kD protein band in one-dimensional SDS-PAGE includes at least two proteins, WAP27A (pI 4.8) and WAP27B (pI 5.0). On the other hand, WAP20 exhibited a main spot at pI 7.0 and a few spots were detected around the main spot (Fig. 3D). One of them, probably the spot of pI 6.8, seemed to be the 21-kD protein detected in one-dimensional SDS-PAGE.

Primary Structures of WAP27 and WAP20

N-terminal amino acid sequences of WAP27A, WAP27B, and WAP20 were determined (Fig. 4). WAP20 had homology to ER-localized smHSPs (Helm et al., 1993). WAP27A and WAP27B did not have homology to any known proteins. The partial sequences of these proteins, however, were very similar to each other, suggesting that they are isoforms.

To determine the native structures of WAP27 and WAP20, native-PAGE was performed (Fig. 5). After proteins trapped in ER-enriched fractions were released either by treatment with 0.1% Triton X-100 or by sonication, they were analyzed by native-PAGE. Proteins were detected by CBB staining and by immunoblot analysis using anti-WAP27 and anti-WAP20 antibodies. Immunoblot analysis with anti-WAP20 antibodies after native-PAGE revealed a single main band with a high molecular mass of approximately 260 to 300 kD, suggesting that WAP20 constituted a high-molecular-mass complex in the native form. In the case of the anti-WAP27 antibodies, two strongly immunoreactive bands of approximately 100 kD were detected. The profiles of proteins released by sonication (data not shown) were very similar to those of proteins released by treatment with Triton X-100. These main protein bands corresponding to WAP27 and WAP20 were also detected in CBB-stained gels.

Subcellular Localization of WAP27 and WAP20

To confirm the ER localization of WAP27 and WAP20, immunoblot analysis with anti-WAP27 and anti-WAP20 antibodies was performed on proteins in crude microsome fractions, and the results were compared with the distribution of marker-enzyme activity in the ER, tonoplast, and Golgi apparatus (Fig. 6). The results showed that fractions with higher activity of the ER marker enzymes corresponded to the higher intensity of WAP27 and WAP20 immunoreactive bands in all of the months examined. In contrast, the higher intensity of the WAP27 and WAP20 bands did not correspond to higher activity of the tonoplast and Golgi marker enzymes. Peak activity of marker enzymes for plasma membranes and mitochondria was detected in the fractions of a much higher Suc concentration

A

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WAP20          SLLPFIDRT-------------DPKLVLEQLPLGLEKD
PsHSP 22.7     SLLPFIDSPNTLLSDLWSDRFPDPFRVLEQIPYGVEKH
Potato CI19    SLLPLIPPI-TLLADLWSDRFPDPFKVLEQIPYGLENR
GmHSP 22.0     SLLPFMDPPITLLAGLWSDRFFDPFRVLEHIPFGVDKD
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B

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WAP27A         SGVGHVPSTTXXGRDF
WAP27B         SGVGH---TTEEGRDFEEVKERAQQTQNKA
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Figure 4. N-terminal amino acid sequences of WAP20 (A), WAP27A (B), and WAP27B (B). Comparisons were made to heat-induced smHSP in pea (Helm et al., 1993), cold-induced smHSP in potato tuber (van Berkel et al., 1994), and heat-induced smHSP in soybean (Helm et al., 1993). Homologous amino acids are enclosed in boxes. Gaps (–) are indicated to produce optimal alignment.

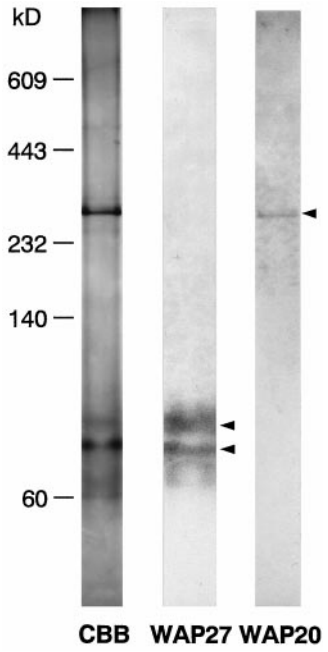

Figure 5. Immunoblot analysis of ER-enriched fractions after native-PAGE analysis. In the CBB-stained lane, 50 μg of proteins was loaded. In immunoblot lanes probed with anti-WAP27 and anti-WAP20 antibodies, 10 and 20 μg of proteins were loaded, respectively. The arrowheads in immunoblot lanes of WAP27 and WAP20 indicate the main protein bands of WAP27 and WAP20, respectively. Indicated molecular masses (in kD) represent, in ascending order, the migration of BSA (60 kD), lactate dehydrogenase (140 kD), catalase (232 kD), ferritin (443 kD), and thyroglobulin (609 kD).

(data not shown). These results suggest that WAP27 and WAP20 are predominantly located in the ER.

Detection of WAP27 and WAP20 in the ER

To examine the localization of WAP27 and WAP20 in the ER, immunoblot analysis of ER-enriched fractions after trypsin treatment was performed (Fig. 7). Both WAP27 and WAP20 in the ER-enriched fractions were protected from trypsin digestion in the absence of the detergent (Fig. 7A). However, both WAP27 and WAP20 became susceptible to trypsin in the presence of the detergent, although a slight, lower-molecular-mass band of WAP20 remained undigested. The remaining WAP20 was susceptible to trypsin digestion in the presence of both detergent and urea (Fig. 7B). These results suggest that both proteins are located inside of the ER vesicles.

DISCUSSION

In cortical parenchyma cells of hardwood species, including mulberry (Niki and Sakai, 1981; Fujikawa and Takabe, 1996), black locust (Pomeroy and Siminovitch, 1971), willow (Roberds and Kidwai, 1969), and horse chestnut (Rao and Catesson, 1987), which exhibit extremely high freezing tolerance in winter, unique seasonal ultrastructural changes in the ER have been reported. Similar sea-

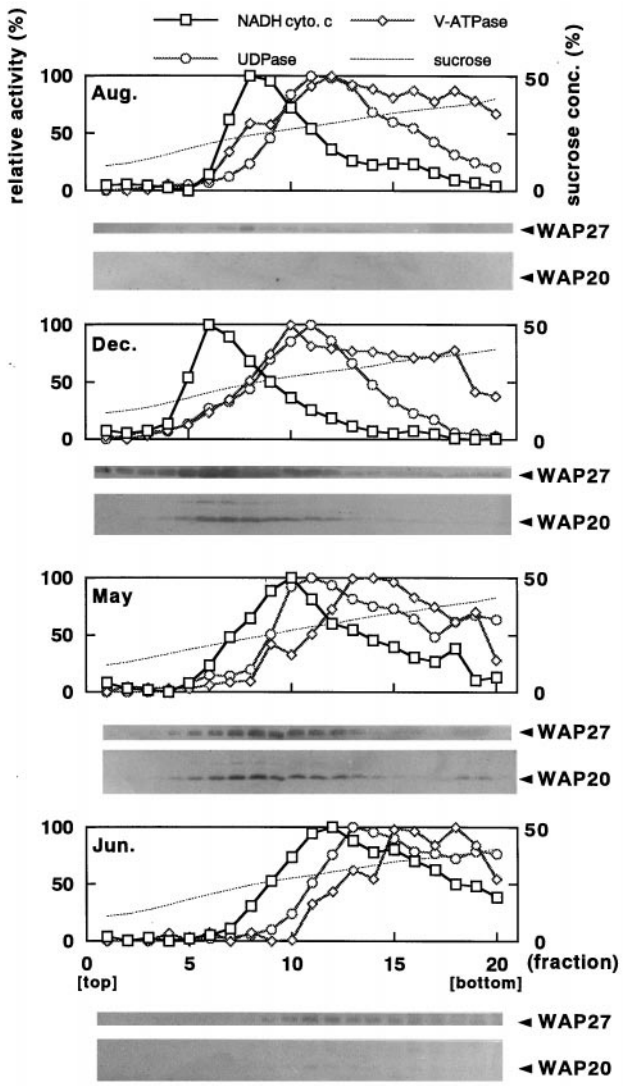

Figure 6. Seasonal changes in the localization of WAP27 and WAP20 in the crude microsome fractions and the relation with marker-enzyme activities in three organelles (ER, tonoplast, and Golgi). SDS-PAGE of fractionated proteins by isopycnic Suc-density gradient centrifugation of microsome fractions prepared from 25 g fresh weight of cortical tissues was performed using 6-μL samples in each fraction. Immunoblot analysis of proteins was performed with anti-WAP27 and anti-WAP20 antibodies. Marker-enzyme activities were measured as described in the text.

sonal changes in the ER are also found in xylem ray parenchyma cells in cold-hardy hardwood species, including *Populus* × *canadensis* (Sauter et al., 1996) and *Stylax obassia* (Fujikawa et al., 1997). The morphology of the ER in these parenchyma cells changes from flattened cisternae in summer to small vesicles in winter in parallel with the acquisition of freezing tolerance as a result of seasonal cold acclimation. It has been suggested that vesiculation of the ER in winter may be directly related to the acquisition of freezing tolerance (Fujikawa and Takabe, 1996; Sauter et al., 1996).

Seasonal ultrastructural changes in the ER, as well as seasonal changes in the amount of total proteins in crude microsomes, have been reported for cortical parenchyma cells of black locust (Siminovitch et al., 1968). The present study using mulberry trees showed that the amount of proteins in ER-enriched fractions, as well as total proteins in the crude microsome fractions, increased in parallel with the process of cold acclimation (Fig. 1). However, ER density was reduced during cold acclimation. This might be a result of increased lipid content in the ER. The increase of phospholipid in the plasma membrane has been reported for mulberry trees during cold acclimation (Yoshida, 1984).

Our results showed that the profiles of proteins in ER-enriched fractions changed in parallel with the process of cold acclimation (Fig. 2). There was a decrease of high-molecular-mass protein bands and an increase of low-molecular-mass bands, with prominent increases of the two proteins, WAP27 and WAP20, in ER-enriched fractions in winter. Our examination showed that the N-terminal amino acid sequence of WAP20 exhibited homology to ER-localized smHSPs, whereas that of WAP27 did not exhibit homology to any known proteins. We are currently continuing our examinations to analyze the entire amino acid sequences of WAP20 and WAP27.

Induction of genes or gene products of HSPs by cold acclimation has been shown in only a few herbaceous plant species (Neven et al., 1992; Cabané et al., 1993; Anderson et al., 1994; Krishna et al., 1995; Guy et al., 1998). To our knowledge, however, the only reported accumulation of HSPs in woody plants by cold acclimation has been that of HSP70 by Wisniewski et al. (1996). In that paper, seasonal changes in the accumulation of HSP70 were reported for crude extracts from bark tissues of four hardwood species, indicating apparent winter accumulation of HSP70 only in peach. Wisniewski and coworkers also examined seasonal changes in BiP, an ER-luminal heat-shock cognate 70, and they found the presence of heat-shock cognate 70 in all seasons without distinct seasonal differences in the eight hardwood species they tested.

The smHSPs are synthesized in all eukaryotes and even in some prokaryotes in response to heat-shock stress; they have molecular masses ranging from 15 to 30 kD, with a conserved C-terminal region (Vierling, 1991). The smHSPs have been classified as cytosol-localized (class I and II), chloroplast-localized, ER-localized, and mitochondria-localized smHSPs (Vierling, 1991; Lenne and Douce, 1994).

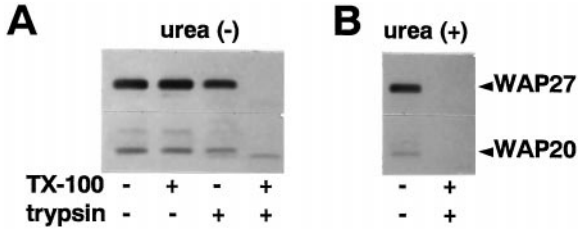

Figure 7. Localization of WAP27 and WAP20 in the ER prepared from February samples. ER-enriched fractions were treated with 0.1 μg μL^{-1} trypsin in the presence or absence of 0.1% Triton X-100 and 2 M urea, as described in the text. SDS-PAGE analysis of treated samples was performed using 15 μg (WAP27) and 20 μg (WAP20) of proteins. Immunoblot analysis of proteins was performed with anti-WAP27 and anti-WAP20 antibodies.

At present, only higher plants are known to have ER-localized smHSPs (Boston et al., 1996). A common feature of eukaryotic smHSPs is their tendency to assemble into complexes of high molecular masses ranging from 200 to 800 kD (Lee et al., 1995). In mulberry tree, WAP20 also formed complexes of high molecular mass of approximately 260 to 300 kD (Fig. 5). Furthermore, it has been reported that in soybean, ER-localized smHSPs are encoded by at least two related genes, *GmHSP22* and *GmHSP22.5* (LaFayette et al., 1996). In heat-stressed pea, two heat-stressed products with similar molecular masses, 20- and 21-kD proteins, are accumulated (Helm et al., 1993). In mulberry trees, antibodies raised against WAP20 reacted with two proteins with molecular masses of 20 and 21 kD (Fig. 2). Although we have not yet analyzed the entire amino acid sequence of WAP20, it is thought that WAP20 belongs to the smHSP family.

Our results showed that WAP20 is located predominantly in the ER. The higher activity of the ER marker enzyme corresponded to fractions of higher intensity of WAP20 (Fig. 6). Endomembrane localization of the smHSPs PsHSP22.7 and GmHSP22.0 has been found in heat-stressed pea and heat-stressed soybean, respectively (Helm et al., 1993). In potato tubers, a cold-induced gene, *C119*, has been shown to have homology to genes of heat-shock-induced smHSPs localized in the ER of pea (Helm et al., 1993), suggesting that the *C119* gene product may exist in the ER (van Berkel et al., 1994). In Arabidopsis, endomembrane localization of smHSPs has been suggested (Helm et al., 1995). Cell-fractionation experiments have also suggested that specific smHSPs may be located in cellular compartments, including the endomembrane system (Cooper and Ho, 1987; LaFayette and Travis, 1990; Sticher et al., 1990). Our results further suggested the localization of WAP20 inside of the ER vesicles by indicating that WAP20 in ER-enriched fractions was not digested by trypsin (Fig. 7).

At present, the significance of the abundant accumulation of smHSPs in the ER of cortical parenchyma cells of mulberry trees in winter as a result of cold acclimation is unclear. However, it has been found that smHSPs have protective effects against chilling injury. High-temperature stress to plants has been found to have protective effects against chilling injury in a number of fruits and vegetables, such as avocado (Woolf et al., 1995), cucumber (Lafuente et al., 1991; Junnings and Salveit, 1994; McCollum et al., 1995), pepper (Mencarelli et al., 1993), tomato (Lurie and Klein, 1991; Sabehat et al., 1996), and mung bean hypocotyls (Collins et al., 1995). In cucumber and mung bean, the loss of protective effects against chilling is correlated with the disappearance of HSPs from the tissues (Lafuente et al., 1991; Collins et al., 1995). These results suggest that smHSPs induced by high-temperature stress may protect plants against chilling injury by preventing denaturation of proteins from chilling (Sabehat et al., 1998).

The analysis by immunoprecipitation of spinach HSP70 indicates that low temperature causes an increased association of some proteins with the cytosolic or ER-luminal HSP70 (Guy et al., 1998). It has also been suggested that cold-responsive HSP genes, *hsp70* in spinach (Anderson et al., 1994) and *hsp90* in *Brassica napus* (Krishna et al., 1995), might contribute to freezing tolerance by functioning as a molecular chaperone to stabilize proteins against freezing-induced denaturation (Thomashow, 1998). Like other HSPs, smHSPs may play a role in protecting proteins and cells against freezing-induced damage.

Induction of mRNA of smHSPs is also evident, independently of temperature stresses, during embryogenesis from somatic cells, microspore genesis, and pollen development in alfalfa and tobacco (Györgyey et al., 1991; Zarsky et al., 1995). Furthermore, in zygotic embryos, expression of mRNA of smHSPs occurs during the maturation stage of the seed, when cell division has ceased and seeds have adapted to desiccation (Coca et al., 1994). In sunflower, expression of mRNA of cytosol-localized class I and II smHSPs seems to parallel seed desiccation (Coca et al., 1994). Thus, it has been suggested that smHSPs are also important for desiccation tolerance (Schöffl et al., 1998). Cold acclimation of plants also results in reduction of cellular water content (Sakai and Larcher, 1987). In cortical parenchyma cells of mulberry trees, the osmotic concentration increases from 0.25 M in summer to 0.85 M in winter, in close association with the process of cold acclimation (Sakai and Yoshida, 1968).

Accumulation of ER-localized smHSPs (WAP20), which are thought to act as a molecular chaperone to stabilize proteins, might have possible roles in the adaptation to low temperature in winter, in water reduction as a result of cold acclimation, and in freezing-induced dehydration in cortical parenchyma cells of the mulberry tree. Further studies are needed to demonstrate the precise roles of the specific ER proteins that accumulate during winter in relation to the acquisition of high freezing tolerance in the cortical parenchyma cells of the mulberry tree.

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LITERATURE CITED

Anderson JV, Li Q-B, Haskell DW, Guy CL (1994) Structural organization of the spinach endoplasmic reticulum-luminal 70-kilodalton heat-shock cognate gene and expression of 70-kilodalton heat-shock genes during cold acclimation. Plant Physiol **104:** 1359–1370

Boston RS, Viitanen PV, Vierling E (1996) Molecular chaperones and protein folding in plants. Plant Mol Biol **32:** 191–222

Cabané M, Calvet P, Vincens P, Boudet AM (1993) Characterization of chilling-acclimation-related proteins in soybean and identification of one as a member of the heat shock protein (HSP 70) family. Planta **190:** 346–353

Coca MA, Almoguera C, Jordano J (1994) Expression of sunflower low-molecular-weight heat-shock proteins during embryogenesis and persistence after germination: localization and possible functional implications. Plant Mol Biol **25:** 479–492

Collins GG, Nie XL, Saltveit ME (1995) Heat shock proteins and chilling sensitivity of mung bean hypocotyls. J Exp Bot **46:** 795–802

Cooper P, Ho T-HD (1987) Intracellular localization of heat shock proteins in maize. Plant Physiol **84:** 1197–1203

Fujikawa S (1994) Seasonal ultrastructural alterations in the plasma membrane produced by slow freezing in cortical tissues of mulberry (*Morus bombycis* Koidz. cv. Goroji). Trees **8:** 288–296

Fujikawa S, Kuroda K, Ohtani J (1997) Seasonal changes in dehydration tolerance of xylem ray parenchyma cells of *Stylax obassia* twigs that survive freezing temperatures by deep supercooling. Protoplasma **197:** 34–44

Fujikawa S, Takabe K (1996) Formation of multiplex lamellae by equilibrium slow freezing of cortical parenchyma cells of mulberry and its possible relationship to freezing tolerance. Protoplasma **190:** 189–203

Gilmour SJ, Artus NN, Thomashow MF (1992) cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. Plant Mol Biol **18:** 13–21

Griffith M, Antikainen M (1996) Extracellular ice formation in freezing-tolerant plants. Adv Low-Temp Biol **3:** 107–139

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol **41:** 187–223

Guy CL, Haskell D, Li Q-B (1998) Association of proteins with the stress 70 molecular chaperones at low temperature: evidence for the existence of cold labile proteins in spinach. Cryobiology **36:** 301–314

Györgyey J, Gartner A, Németh K, Magyer Z, Hirt H, Heberle-Bors E, Dudits D (1991) Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol Biol **16:** 999–1007

Hare PD, Cress WA, van Staden J (1998) Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ **21:** 535–553

Helm KW, LaFayette PR, Nagao RT, Key JL, Vierling E (1993) Localization of small heat shock proteins to the higher plant endomembrane system. Mol Cell Biol **13:** 238–247

Helm KW, Schmeits J, Vierling E (1995) An endomembrane-localized small heat-shock protein from *Arabidopsis thaliana*. Plant Physiol **107:** 287–288

Hughes MA, Dunn MA (1996) The molecular biology of plant acclimation to low temperature. J Exp Bot **47:** 291–305

Jakob U, Gaestel M, Engel K, Buchner J (1993) Small heat shock proteins are molecular chaperones. J Biol Chem **268:** 1517–1520

Jennings P, Saltveit ME (1994) Temperature and chemical shocks induce chilling tolerance in germinating *Cucumis sativus* (cv. Poinsett 76) seeds. Physiol Plant **91:** 703–707

Kaye C, Guy CL (1995) Perspectives of plant cold tolerance: physiology and molecular responses. Sci Prog **78:** 271–299

Kaye C, Neven L, Hofig A, Li Q-B, Haskell D, Guy CL (1998) Characterization of a gene for spinach CAP160 and expression of two spinach cold-acclimation proteins in tobacco. Plant Physiol **116:** 1367–1377

Kazuoka T, Oeda K (1992) Heat-stable COR (cold-regulated) proteins associated with freezing tolerance in spinach. Plant Cell Physiol **33:** 1107–1114

Koike M, Takezawa D, Arakawa K, Yoshida S (1997) Accumulation of 19-kDa plasma membrane polypeptide during induction of freezing tolerance in wheat suspension-cultured cells by abscisic acid. Plant Cell Physiol **38:** 707–716

Krishna P, Sacco M, Cherutti JF, Hill S (1995) Cold-induced accumulation of hsp90 transcripts in *Brassica napus*. Plant Physiol **107:** 915–923

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

LaFayette PR, Nagao RT, O'Grady K, Vierling E, Key JL (1996) Molecular characterization of cDNAs encoding low-molecular-weight heat shock proteins of soybean. Plant Mol Biol **30:** 159–169

LaFayette PR, Travis RL (1990) Soluble and membrane-associated heat shock proteins in soybean root. Protoplasma **156:** 174–182

Lafuente MT, Belver A, Guye MG, Saltveit ME Jr (1991) Effect of temperature conditioning on chilling injury of cucumber cotyledons. Possible role of abscisic acid and heat shock proteins. Plant Physiol **95:** 443–449

Lee GJ, Pokala N, Vierling E (1995) Structure and *in vitro* molecular chaperone activity of cytosolic small heat shock proteins from pea. J Biol Chem **270:** 10432–10438

Lenne C, Douce R (1994) A low molecular mass heat-shock protein is localized to higher plant mitochondria. Plant Physiol **105:** 1255–1261

Levitt J (1980) Responses of Plants to Environmental Stresses, Vol 1: Chilling, Freezing and High Temperature Stresses, Ed 2. Academic Press, New York

Lin C, Guo WW, Everson E, Thomashow MF (1990) Cold acclimation in *Arabidopsis* and wheat. A response associated with expression of related genes encoding 'boiling-stable' polypeptides. Plant Physiol **94:** 1078–1083

Lurie S, Klein JD (1991) Acquisition of low-temperature tolerance in tomatoes by exposure to high temperature stress. J Am Soc Hortic Sci **116:** 1007–1012

McCollum TG, Doostdar H, Mayer RT, McDonald RE (1995) Immersion of cucumber fruit in heated water alters chilling-induced physiological changes. Postharv Biol Technol **6:** 55–64

Mencarelli F, Ceccantoni B, Bolini A, Anelli G (1993) Influence of heat treatment on the physiological response of sweet pepper kept at chilling temperature. Acta Hortic **343:** 238–243

Neven LG, Haskell DW, Guy CL, Denslow N, Klein PA, Green LG, Silverman A (1992) Association of 70-kilodalton heat-shock cognate proteins with acclimation to cold. Plant Physiol **99:** 1362–1369

Niki T, Sakai A (1981) Ultrastructural changes related to frost hardiness in the cortical parenchyma cells from mulberry twigs. Plant Cell Physiol **22:** 171–183

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021

Pareek A, Singla SL, Grover A (1995) Immunological evidence for accumulation of two high-molecular-weight (104 and 90 kDa) HSPs in response to different stresses in rice and in response to high temperature stress in diverse plant genera. Plant Mol Biol **29:** 293–301

Pomeroy MK, Siminovitch D (1971) Seasonal cytological changes in secondary phloem parenchyma cells in *Robinia pseudoacacia* in relation to cold hardiness. Can J Bot **49:** 787–795

Rajashekar CB, Lafta A (1996) Cell-wall changes and cell tension in response to cold acclimation and exogenous abscisic acid in leaves and cell cultures. Plant Physiol **111:** 605–612

Rao KS, Catesson A-M (1987) Changes in the membrane components of nondividing cambial cells. Can J Bot **65:** 246–254

Robards AW, Kidwai P (1969) A comparative study of the ultrastructure of resting and active cambium of *Salix fragilis* L. Planta **84:** 239–249

Sabehat A, Lurie S, Weiss D (1998) Expression of small heat-shock proteins at low temperatures. Plant Physiol **117:** 651–658

Sabehat A, Weiss D, Lurie S (1996) The correlation between heat-shock protein accumulation and persistence and chilling tolerance in tomato fruit. Plant Physiol **110:** 531–537

Sakai A, Larcher W (1987) Frost Survival of Plants: Responses and Adaptations to Freezing Stress. Springer-Verlag, Berlin

Sakai A, Yoshida S (1968) The role of sugar and related compounds in variations of freezing resistance. Cryobiology **5:** 160–174

Sauter J, Wisniewski M, Witt W (1996) Interrelationships between ultrastructure, sugar levels, and frost hardiness of ray parenchyma cells during frost acclimation and deacclimation in poplar (*Populus* × *canadensis* Moench robusta) wood. J Plant Physiol **149:** 451–461

Schöffl F, Prändl R, Reindl A (1998) Regulation of the heat-shock response. Plant Physiol **117:** 1135–1141

Siminovitch D, Rheaume B, Pomeroy K, Lepage M (1968) Phospholipid, protein, and nucleic acid increases in protoplasm and membrane structures associated with development of extreme freezing resistance in black locust tree cells. Cryobiology **5:** 202–225

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol **35:** 543–584

Sticher L, Biswas AK, Bush DS, Jones RL (1990) Heat shock inhibits α-amylase synthesis in barley aleurone without inhibiting the activity of endoplasmic reticulum marker enzymes. Plant Physiol **92:** 506–513

Thomashow MF (1994) *Arabidopsis thaliana* as a model for studying mechanisms of plant cold tolerance. *In* E Meyerowitz, C Sommerville, eds, Arabidopsis. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 807–834

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol **118:** 1–7

van Berkel J, Salamini F, Gebhardt C (1994) Transcripts accumulating during cold storage of potato (*Solanum tuberosum* L.) tubers are sequence related to stress-responsive genes. Plant Physiol **104:** 445–452

Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol **42:** 579–620

Wisniewski M, Close TJ, Artlip T, Arora R (1996) Seasonal patterns of dehydrins and 70-kDa heat-shock proteins in bark tissues of eight species of woody plants. Physiol Plant **96:** 496–505

Woolf AB, Watkins CB, Bowen JH, Lay-Yee M, Maindonald JH, Ferguson IB (1995) Reducing external chilling injury in stored 'Hass' avocados with dry heat treatments. J Am Soc Hortic Sci **120:** 1050–1056

Yoshida S (1984) Chemical and biophysical changes in the plasma membrane during cold acclimation of mulberry bark cells (*Morus bombycis* Koidz. cv Goroji). Plant Physiol **76:** 257–265

Zarsky V, Garrido D, Eller N, Tupy J, Vicente O, Schöffl F, Heberle-Bors E (1995) The expression of small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Environ **18:** 139–147

Zhou BL, Arakawa K, Fujikawa S, Yoshida S (1994) Cold-induced alterations in plasma membrane proteins that are specifically related to the development of freezing tolerance in cold-hardy winter wheat. Plant Cell Physiol **35:** 175–182