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Relationship between Proline and Abscisic Acid in the Induction of Chilling Tolerance in Maize Suspension-Cultured Cells[1]

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Both proline and abscisic acid (ABA) induce chilling tolerance in chilling-sensitive plants. However, the relationship between proline and ABA in the induction of chilling tolerance is unclear. We compared the time course of the increase in chilling tolerance induced by proline and ABA, and the time course of the uptake of both into the cultured cells of maize (*Zea mays* L. cv Black Mexican Sweet) at 28°C. The plateau of proline-induced chilling tolerance preceded by 12 h the plateau of ABA-induced chilling tolerance. The uptake of exogenous ABA into the cells reached a plateau in 1 h, whereas the uptake of exogenous proline gradually increased throughout the 24-h culture period. Although the proline content in ABA-treated cells was 2-fold higher than in untreated cells at the end of the 24-h ABA treatment at 28°C, the correlation between the endogenous free proline content and the chilling tolerance in the ABA-treated cells was insignificant. Isobutyric acid treatment, which resulted in a larger accumulation of proline in the cells than ABA treatment, did not increase chilling tolerance. The induction of chilling tolerance by proline and ABA appeared to be additive. Cycloheximide inhibited ABA-induced chilling tolerance, but it did not inhibit proline-induced chilling tolerance. Newly synthesized proteins accumulate in ABA-treated cells at 28°C while the chilling tolerance is developing (Z. Xin and P.H. Li [1993] Plant Physiol 101: 277–284), but none of these proteins were observed in the proline-treated cells. Results suggest that proline and ABA induce chilling tolerance in maize cultured cells by different mechanisms.

Both Pro and ABA induce chilling tolerance in many chilling-sensitive plant species (Rikin et al., 1979; Duncan and Widholm, 1987; Xin and Li, 1992). Three lines of evidence link Pro with chilling tolerance: (a) plants accumulate Pro rapidly following exposure to low temperature in the absence of water deficiency (Chu et al., 1974), (b) treatments that increase Pro content also increase chilling tolerance (Duncan and Widholm, 1987; Flores et al., 1989), and (c) addition of Pro to culture medium increases chilling tolerance of callus (Duncan and Widholm, 1987) and cultured cells of maize (Songstad et al., 1990).

Exogenous ABA increases Pro content in leaves of barley (*Hordeum distichum* L.) (Aspinall et al., 1973; Rajagopal and Andersen, 1978; Stewart, 1980; Pesci, 1987, 1988) and rice (*Oryza sativa*) (Chou et al., 1991). ABA-induced Pro appears to depend on the activation of genes and/or protein synthesis (Stewart and Voetberg 1985; Pesci, 1987). During drought stress, an increase in ABA preceded the increase in Pro in intact barley plants (Aspinall, 1980) as well as in excised leaves (Stewart and Voetberg, 1985). Spraying cucumber (*Cucumis sativus* L.) plants with ABA or its analogs causes an increase in chilling tolerance and an increase in endogenous free Pro content (Flores et al., 1988). From these reports, it appears that ABA may influence chilling tolerance by increasing free Pro content.

Recently, Duncan and Widholm (1991) reported that Pro is not the primary determinant of ABA-induced chilling tolerance in maize callus cultures, based on findings that ABA induces chilling tolerance in Pro-free culture medium without increasing the endogenous free Pro content. The relationship between ABA and Pro in the induction of chilling tolerance is still unclear. In this report, we studied the relationship between Pro and ABA in the induction of chilling tolerance: (a) by comparing the time course of the increase in Pro- and ABA-induced chilling tolerance and the time course of the uptake of Pro and ABA in the cells, (b) by analyzing the interaction between Pro and ABA in the induction of chilling tolerance, (c) by studying the effect of CH on the inhibition of Pro-induced chilling tolerance, and (d) by examining the profiles of protein synthesis in the cells treated with Pro. Results suggest that the mechanisms by which Pro and ABA induce chilling tolerance in maize suspension-cultured cells are different.

MATERIALS AND METHODS

Culture of Maize Cells and Application of Treatments

The maize (*Zea mays* L. cv Black Mexican Sweet) cell suspension was cultured according to Green (1977). Briefly, the cells were cultured in Murashige-Skoog medium at 28°C in the dark on a rotary shaker. Cells were subcultured weekly in fresh medium. The culture medium was free of Pro except where noted.

At d 5 after subculture (late log growth phase), cells were collected on sterile Miracloth (Calbiochem, San Diego, CA). Two grams of cells (fresh weight) were transferred to fresh

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Abbreviations: CH, cycloheximide; IBA, isobutyric acid; TTC, triphenyl tetrazolium chloride.

medium containing each of various combinations of Pro and ABA. Pro (0–100 mM) and ABA (0–100 μM) were added to culture media before autoclaving. Control culture media are free from either Pro or ABA. Since mild osmotic stress has been shown to induce chilling tolerance (Duncan and Widholm 1987, 1991), experiments with 100 mM mannitol were carried out to verify if Pro-induced chilling tolerance occurs via imposition of mild osmotic stress. IBA, which has been shown to increase the endogenous Pro content (Chou et al., 1991), was used as an alternative means by which to increase the endogenous Pro in maize cultured cells. IBA was added to 1 mM in the culture medium before autoclaving. The pH of the medium was adjusted to 5.8. The treated cultures were either chilled at 4°C in the dark immediately after treatment or incubated for 3, 6, 12, or 24 h at 28°C before chilling.

Uptake of Pro and ABA

Pro uptake was estimated by the method of Bates et al. (1973). At 0, 3, 6, 12, or 24 h after Pro treatment, cells were collected by filtration through Miracloth, blotted dry, and frozen in liquid nitrogen. Frozen cells (100 mg) were pulverized with a liquid nitrogen-chilled mortar and pestle and homogenized in 10 mL of 3% sulfosalicylic acid. The extract was centrifuged for 15 min at 10,000*g*. The pellet was extracted again with 10 mL of 3% sulfosalicylic acid. The pooled supernatant was used for Pro determination.

Uptake of exogenous applied DL-*cis, trans*-[^{3}H]ABA (115 Ci mmol^{-1}, Amersham, UK) by cultured cells was estimated by determining the amount of radioactivity in the cells. Two microcuries of [^{3}H]ABA was added to each flask containing 40 mL of culture medium with 100 μM of unlabeled ABA and then autoclaved. At various times, as indicated in Figure 1B, after transferring cells into culture medium containing 100 μM unlabeled and 2 μCi of labeled ABA, 2 mL of culture was placed into a preweighed 5-mL syringe with the end plugged with a Miracloth disc. The cells were washed twice with 5 mL of fresh medium containing only unlabeled ABA. The syringe was centrifuged for 5 min at 1000*g* to remove the culture medium in the sample and weighed. The cells were transferred to a scintillation vial containing 1 mL of 0.2 M NaOH and 1% SDS and were autoclaved for 20 min at 120°C to release ABA from the cells. After neutralization with HCl, samples were counted on a Beckman scintillation counter with auto-counting-efficiency determination. Uptake of ABA was estimated by the radioactivity in the cells.

ABA Determination

Endogenous ABA was determined by an indirect, competitive immunoassay according to Walker-Simmons (1987). After Pro treatment, maize cells were collected by centrifugation through a syringe with the end plugged with a Miracloth disc. The cells were washed twice with 5 mL of fresh medium and once with deionized water and were frozen in liquid nitrogen. The frozen cells (0.3 g fresh weight) were homogenized twice for 30 s at maximum speed with a Polytron in 10 mL of cold 80% methanol containing 10 μg mL^{-1} butylated hydroxytoluene. The homogenate was shaken overnight and centrifuged for 15 min at 16,000*g* and the supernatant was collected. The supernatant was dried by Speed-Vac and redissolved in 4 mL of Tris-buffered saline (3.03 g Trizma, 5.84 g NaCl, and 0.2 g $MgCl_2 \cdot 6H_2O$ per L). The recovery rate of ABA was monitored by adding [^{3}H]ABA as an internal standard. The recovery ranged from 85 to 93%. ABA measurement was done by the procedure of Walker-Simmons (1987).

Chilling Survival Estimation

Chilling survival was estimated by relative TTC reduction (chilled/unchilled cells) and regrowth as described by Xin and Li (1992). For TTC reduction, 1 mL of unchilled or 7-d-chilled cultures was incubated with 2 mL of 0.8% TTC in 50 mM potassium phosphate buffer (pH 7.5) for 24 h at room temperature in the dark. The red formazan in the cells was extracted with 6 mL of 95% ethanol for 24 h in the dark. The A_{530} of the extract was measured. Survival was defined as the ratio of *A* of chilled to unchilled cells. For the regrowth assay, 1 mL of a 14-d-chilled cell culture containing 30 mg of cells (fresh weight) was plated on a filter paper that had been placed on 25 mL of Murashige-Skoog medium containing 1% agar and regrown at 28°C for 14 d in the dark. Survival of cells after chilling was estimated visually by the growth of the plated cells.

Interaction of Pro and ABA in the Induction of Chilling Tolerance

To determine the interaction between Pro and ABA in the induction of chilling tolerance, four levels of Pro (0, 10, 50, and 100 mM) and four levels of ABA (0, 1, 10, and 100 μM) were used in a factorial design. After cells were transferred to the culture media for treatments, cells were cultured at 28°C for 24 h before chilling exposure to 4°C.

Determination of the Effects of Pro on Met Uptake and Protein Synthesis

We have shown that 1 μM CH does not injure the cells but is sufficient to inhibit the ABA-induced chilling tolerance in maize suspension-cultured cells (Xin and Li, 1992). To compare the effects of CH on the protein synthesis and chilling tolerance in ABA- and Pro-treated cells, 2 g of cells (fresh weight) were transferred to culture medium containing 100 μM ABA, 100 mM Pro, or neither with CH at concentrations of 0, 0.5, and 1.0 μM. After incubation for 2 h at 28°C, 2 mL of cell culture were transferred to a 5-mL plastic culture tube containing 10 μCi L-[^{35}S]Met (>1000 Ci mmol^{-1}, ICN, Irvine, CA). The cell cultures were allowed to incorporate L-[^{35}S]Met for 2 h at 28°C, were rinsed three times with fresh culture medium containing 1 mM unlabeled Met, and were then frozen in liquid nitrogen and stored at −70°C. To determine the incorporation of L-[^{35}S]Met into proteins, the method described previously by Xin and Li (1992) was used. Cells were ground with a liquid nitrogen-chilled mortar and pestle with 1 mL of O'Farrell lysis buffer without ampholyte (O'Farrell, 1975). The homogenate was centrifuged for 30 min at 100,000*g*. Aliquots of 5 μL of supernatant from each sample were used to determine the amounts of total and TCA-

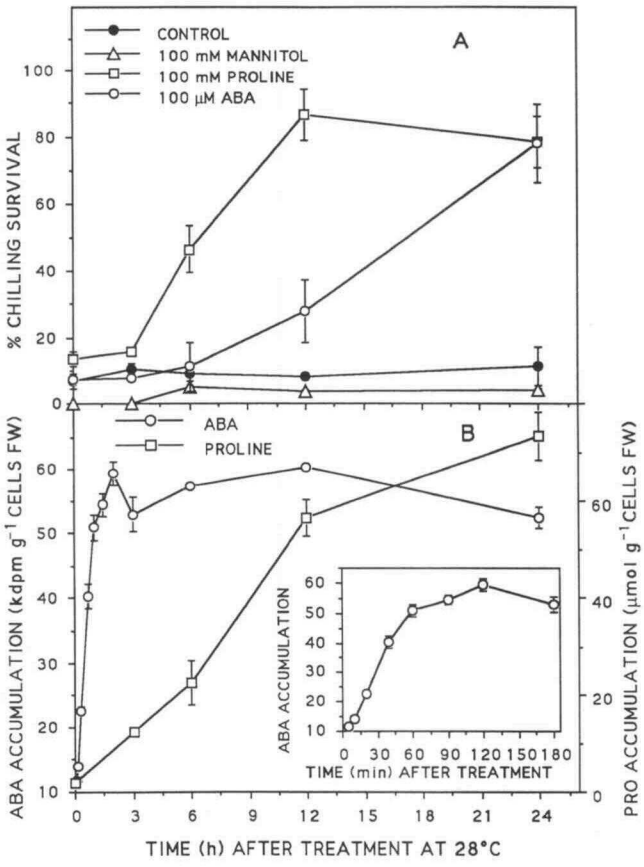

Figure 1. The time course of the development of Pro- and ABA-induced chilling tolerance (A) and the time course of the accumulation of Pro and ABA in maize cells after the cells were transferred to a medium containing either 100 mM Pro or 100 μM ABA (B). Survival was estimated by the ratio of TTC reduction of 7-d-chilled cells to unchilled cells. The vertical lines show the SE.

precipitable radioactivity. The percent incorporation of L-[^{35}S] Met into proteins was expressed by the ratio of TCA-precipitable activity to the total activity in the homogenate. The percent uptake of L-[^{35}S]Met into cells was expressed by the ratio of total radioactivity in the homogenate to the amount of radioactivity used in labeling the sample.

Protein Labeling and Electrophoresis

Protein synthesis after Pro treatment was analyzed by two-dimensional PAGE according to Xin and Li (1993). At 10 h after Pro (100 mM) treatment, 2 mL of culture was transferred to a sterile 5-mL plastic culture tube containing 100 μCi of L-[^{35}S]Met (>1000 Ci mmol $^{-1}$). After 2 h of incubation at 28°C, cells were collected for protein extraction and analysis on two-dimensional PAGE (Xin and Li, 1993).

RESULTS

Time Courses of Pro- and ABA-Induced Chilling Tolerance and Time Courses of Pro and ABA Accumulation in Cells during Pro and ABA Treatment at 28°C

The induction of chilling tolerance by Pro in maize suspension-cultured cells is presented in Figures 1 and 2. Pro increased chilling tolerance significantly and can be used at concentrations as high as 0.5 M without decreasing the viability of unchilled cells (data not shown). However, there was no further increase in chilling tolerance when cells were treated with Pro at concentrations greater than 100 mM (data not shown). Although Pro treatment increased the ability of unchilled cells to reduce TTC, relative TTC reduction (TTC reduction of chilled versus unchilled cells) is still a valid method with which to estimate chilling survival. Figure 2 shows the appearance of 14-d chilled (4°C) Pro-treated, ABA-treated, and untreated cells after 14 d of regrowth at 28°C in the dark. A regrowth assay gave results similar to relative TTC reduction.

Pro-induced chilling tolerance is time dependent. Chilling tolerance became significant at 6 h and reached a plateau at 12 h after treatment (Fig. 1A). No chilling tolerance was observed in cells treated with 100 mM mannitol for 6, 12, or 24 h at 28°C, suggesting that the Pro-induced chilling tolerance is not due to the increase in osmotic potential in the culture medium caused by adding 100 mM Pro. ABA-induced chilling tolerance was initially observed at 6 h, became significant at 12 h, and reached a plateau at 24 h after the treatment. The plateau in Pro-induced chilling tolerance preceded by 12 h the ABA-induced chilling tolerance plateau (Fig. 1A).

The time course of the induction of chilling tolerance by Pro and ABA in maize suspension-cultured cells was compared with the time course of the uptake of Pro and ABA by the cells (Fig. 1). When grown in a medium containing Pro

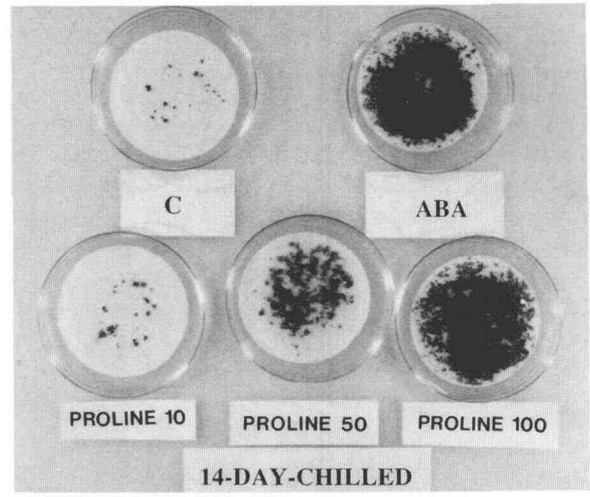

Figure 2. Appearances of 14-d-chilled (4°C) Pro- and ABA-treated cells after regrowth for 14 d at 28°C. One milliliter of chilled cells (30 mg of cells fresh weight) was plated on a filter paper that had been placed on 25 mL of Murashige-Skoog medium containing 1% agar. Before the photograph was taken, the plates were stained with 2 mL of 0.8% TTC solution in 50 mM potassium phosphate buffer (pH 7.5) and incubated for 24 h at room temperature in the dark. The darkness indicates the cell's viability. C, Untreated control cells; ABA, cells treated with 100 μM ABA and incubated for 24 h at 28°C before chilling; proline 10, proline 50, and proline 100 indicate that cells were treated with 10, 50, or 100 mM Pro, respectively, for 24 h at 28°C before chilling.

Table I. *Free Pro content (μmol g^{-1} cells fresh weight) in maize cultured cells at 28°C during a 24-h period in Pro-free media containing no ABA (control), 100 μM ABA, or 1 mM IBA*

Values are means of three replicates.

Time (h)	Control	ABA (100 μM)	IBA (1 mM)[a]
0	0.47 ± 0.14	0.37 ± 0.04	0.40 ± 0.11
3	0.59 ± 0.08	0.50 ± 0.04	0.38 ± 0.13
6	0.47 ± 0.20	0.52 ± 0.08	0.86 ± 0.21[b]
12	0.46 ± 0.06	0.56 ± 0.07	1.23 ± 0.18[c]
24	0.37 ± 0.08	0.65 ± 0.14[b]	1.67 ± 0.30[c]

[a] No increase in chilling tolerance was observed after IBA treatment. [b] Significant at 5% level by *t* test. [c] Significant at 1% level by *t* test.

at 28°C, maize cells accumulated Pro, and the accumulation increased with time of culture (Fig. 1B). After 12 h of Pro treatment, cells had accumulated free Pro content of 56.6 μmol g^{-1} cells (fresh weight) from a pretreatment level of 0.6 μmol. Pro continued to accumulate after 12 h even though Pro-induced chilling tolerance had reached a plateau at this time (Fig. 1A). During the ABA treatment at 28°C, accumulation of exogenously applied ABA in the cells reached plateau in 1 h (Fig. 1B), but the ABA-induced chilling tolerance became significant only at 12 h and reached a plateau at 24 h after ABA treatment (Fig. 1A). The degree of chilling tolerance developed in Pro-treated cells paralleled the accumulation of Pro by the cells until chilling tolerance reached a plateau. However, the development of chilling tolerance in ABA-treated cells was far behind the ABA accumulation plateau.

To determine whether ABA induces chilling tolerance in maize cells by increasing the endogenous Pro level, Pro content was measured after ABA treatment. The endogenous free Pro content in the ABA-treated cells was significantly higher than in untreated cells at the end of the 24-h ABA treatment at 28°C (Table I). The net increase was 0.28 μmol g^{-1} (fresh weight). The correlation ($r = 0.38$, $n = 15$) between Pro content and ABA-induced chilling tolerance in the ABA-treated cells was insignificant. The endogenous Pro content in 1 mM IBA-treated cells increased significantly between 6 and 24 h of treatment (Table I), but IBA treatment did not increase the cells' chilling tolerance.

To determine whether Pro treatment increases the endogenous ABA level, endogenous ABA content after Pro treatment was measured by indirect competitive immunoassay. Endogenous ABA content in maize cells was around 3 to 10 pmol g^{-1} cells (fresh weight) (Fig. 3), about one-tenth of the level in the leaves of young maize plants (Zhang et al., 1986). Pro treatment did not increase the endogenous ABA content (Fig. 3).

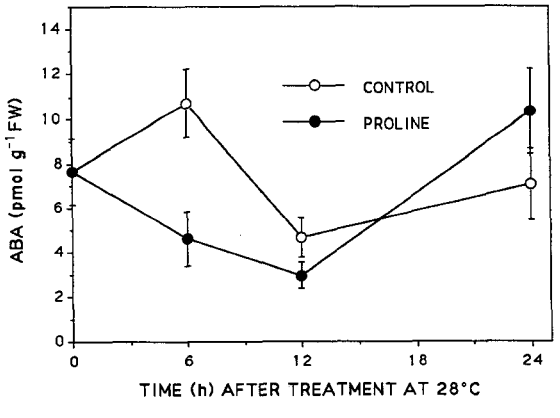

Figure 3. Endogenous ABA content in Pro-treated cells and in untreated control cells. ABA content was measured by indirect competitive immunoassay. Each sample was assayed in triplicate at three series of dilutions. The vertical lines show the SE.

Interaction between Pro and ABA in Chilling Tolerance Induction

The relationship between ABA and Pro in the induction of chilling tolerance in maize suspension-cultured cells was statistically analyzed using a factorial design. The percent survival after chilling was plotted in Figure 4 against different concentrations of Pro and ABA. Chilling tolerance increased with increasing concentrations of either ABA or Pro in the media. Variance analyses of the chilling survivals were conducted after logarithmic transformation. The *F* values for the main effects, Pro and ABA, were 55.5 and 39.4, respectively, which were very significant. The *F* value for the interaction between Pro and ABA was 3.41, which is significant but much less than the main effects. The addition of ABA to the medium containing 100 mM Pro further increased the chilling tolerance, even though the Pro-treated cells had accumulated 75 μmol g^{-1} cells (fresh weight) of Pro in 24 h of Pro treatment at 28°C. At lower concentrations of Pro (<50 mM) and ABA (<10 μM), the effects of Pro and ABA on increase in survival were additive. At higher concentrations, the combined effect of Pro and ABA was less than the sum of effects from the independent Pro or ABA treatment.

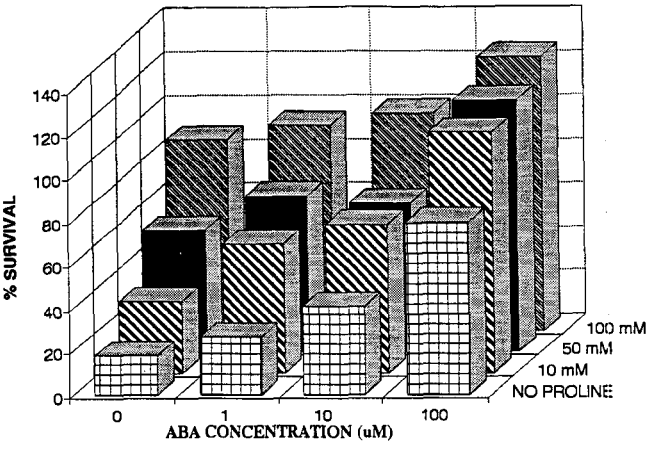

Figure 4. Chilling tolerances induced by Pro and ABA. Cells were cultured for 24 h at 28°C prior to chilling in media containing various combined concentrations of Pro and ABA as indicated on the axes. Chilling tolerance, as expressed in percent survival, is the ratio of TTC reduction of 7-d-chilled cells to unchilled cells from the same sources.

Effect of CH on Chilling Tolerance and Protein Synthesis in Pro-Treated Cells

To determine the relationship between Pro and ABA in the induction of chilling tolerance, CH was added to the culture medium containing either ABA or Pro. The CH, at 1 μM, inhibits the ABA-induced chilling tolerance by 60% in maize suspension-cultured cells with a similar degree of inhibition in protein synthesis (Fig. 5A; Xin and Li, 1992). In contrast, Pro-induced chilling tolerance was not inhibited by CH at 1 μM (Fig. 5A).

To determine whether Pro treatment altered the ability of CH to inhibit protein synthesis, the effects of CH on the uptake of L-[^{35}S]Met and incorporation of ^{35}S into proteins were measured in both Pro- and ABA-treated cells and in untreated cells. In a 2-h incubation at 28°C, 88% of the L-[^{35}S]Met was taken up by the untreated and ABA-treated cells and only 18% by the Pro-treated cells (Fig. 5B). Pro treatment also slightly decreased protein synthesis (Fig. 5C). CH inhibited the incorporation of ^{35}S into proteins similarly in the Pro-treated cells and in untreated and ABA-treated cells (Fig. 5C).

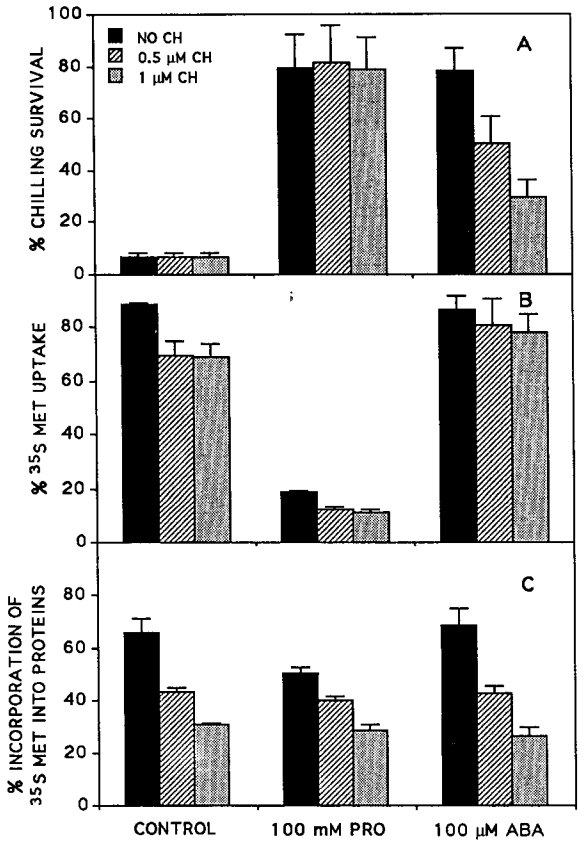

Figure 5. Effects of CH on the development of ABA- and Pro-induced chilling tolerance (A), on the uptake of [^{35}S]Met (B), and on the incorporation of ^{35}S into proteins (C). Control, No Pro or ABA; NO CH, no CH added to the medium; 0.5 μM CH and 1 μM CH, CH was added to the medium to 0.5 and 1 μM, respectively. Chilling survival was estimated by relative TTC (chilled/unchilled cells) reduction. The vertical lines show the SE.

Protein Profile in Cells Treated with 100 mM Pro

We reported that the abundance of several proteins is increased by the ABA treatment, and this increase is associated with the increase in chilling tolerance (Xin and Li, 1993). Here, we compared the protein synthesis in cells treated with 100 mM Pro and the protein synthesis in ABA-treated cells. If the induction mechanisms by Pro and ABA are related, we would expect to detect some common proteins, the abundance of which can be increased by both Pro and ABA, during the induction of chilling tolerance. Figure 6 shows that no specific alterations in protein synthesis were found in Pro-treated cells such as were observed in the ABA-treated cells during the chilling tolerance induction (Xin and Li, 1993).

DISCUSSION

Pro accumulation has been implicated in ABA-induced chilling tolerance in plants (Duncan and Widholm, 1987; Flores et al., 1988). We found that the degree of chilling tolerance development in Pro-treated cells paralleled Pro accumulation by the cells and preceded by 12 h the ABA-induced chilling tolerance (Fig. 1). Chilling tolerance development in ABA-treated cells was far behind the ABA accumulation plateau. The endogenous Pro content in the ABA-treated cells was higher than in the untreated cells at the end of the 24-h ABA treatment (Table I).

These results suggest that ABA induces chilling tolerance by increasing free Pro content. The net increase of endogenous Pro in ABA-treated cells was 0.28 μmol g^{-1} cells (fresh weight) at the end of the 24-h ABA treatment. Is this increased amount of Pro physiologically responsible for the ABA-induced chilling tolerance? Duncan and Widholm (1987) demonstrated that 530 mM mannitol, or 100 μM ABA, or a combination of the two could induce chilling tolerance in maize callus culture. This induced chilling tolerance is correlated with the accumulation of Pro by the treated callus. Later, they showed that addition of 100 μM ABA to the culture medium results in a free Pro content of 145 μmol g^{-1} maize callus (fresh weight) if the culture medium contains 12 mM Pro and about 1 μmol g^{-1} if the culture medium contains no Pro (Duncan and Widholm, 1991). Maize callus grown in medium containing Pro at various concentrations accumulated various amounts of Pro in the tissue. The callus treated with Pro also increased survival at 4°C. However, ABA-treated callus in Pro-free medium survived a longer period of exposure to 4°C than the callus treated with Pro alone. The authors concluded that endogenous free Pro content of the ABA-treated callus is not the primary factor in inducing chilling tolerance in maize callus cultures (Duncan and Widholm, 1991). We failed to detect any significant increase in chilling tolerance in maize cultured cells treated with Pro at concentrations below 10 mM in the medium (Fig. 2).

Duncan and Widholm (1991) reported that 530 mM mannitol induced a 12-fold increase in Pro content in maize callus culture and also increased the longevity of the callus at 4°C. We examined the effect of mannitol on Pro content and chilling tolerance in maize cultured cells. We did not observe any increase in endogenous free Pro content in maize cultured

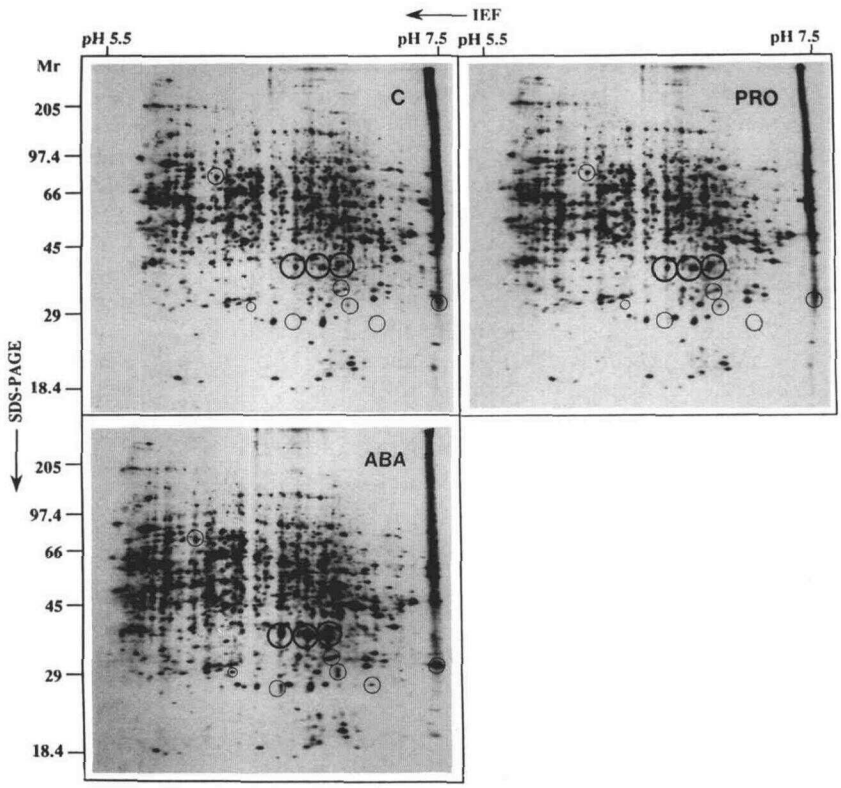

Figure 6. Fluorographs of in vivo ^{35}S-labeled proteins from cells without any treatment (C), cells treated with 100 mM Pro (PRO), and cells treated with 100 μM ABA (ABA) at 28°C for 12 h. The proteins circled correspond to proteins whose synthesis was increased by ABA treatment (Xin and Li, 1993).

cells treated with mannitol from 100 mM to 1 M (data not shown). Mannitol treatments at such concentrations decreased the cells' chilling survival (Fig. 1A). IBA has been shown to increase Pro accumulation in detached rice leaves (Chou et al., 1991). IBA at 1 mM concentration in the medium induced a larger accumulation of Pro than in ABA-treated cells (Table I), but IBA did not increase the chilling tolerance of the treated cells. Correlation analysis between the increase in Pro content and the increase in chilling tolerance in ABA-treated cells was insignificant ($r = 0.38$, $n = 15$). It appears that the increase in endogenous free Pro in ABA-treated cells is not physiologically responsible for the ABA-induced chilling tolerance.

Since the uptake of ABA in maize cells was much faster than the uptake of Pro but the occurrence of ABA-induced chilling tolerance was far behind Pro-induced chilling tolerance (Fig. 1), it is unlikely that Pro induces chilling tolerance by regulating the endogenous level of ABA. Indeed, the endogenous ABA level showed no increase after Pro treatment (Fig. 3). We have reported that ABA-induced chilling tolerance can be inhibited by CH (Xin and Li, 1992). To determine further the relationship of Pro-induced chilling tolerance to ABA, we examined the effect of CH on the Pro-induced chilling tolerance. CH, at 0.5 and 1 μM, which inhibited the development of ABA-induced chilling tolerance, failed to inhibit the development of Pro-induced chilling tolerance, even though it did inhibit the protein synthesis of Pro-treated cells (Fig. 5, A and C). We have reported that ABA increased the net synthesis of 11 proteins at 28°C (Xin and Li, 1993). None of these ABA-regulated proteins showed any detectable changes in Pro-treated cells (Fig. 6). Apparently, de novo synthesis of ABA-induced proteins is not required for the development of Pro-induced chilling tolerance. Thus, the induction of chilling tolerance by Pro in maize cultured cells is independent of ABA.

Statistical analyses of the increased chilling tolerances showed that the effects of ABA and Pro were additive in the induction of chilling tolerance, suggesting that ABA and Pro act via independent mechanisms. At high concentrations, the chilling tolerance induced by a combined treatment of Pro and ABA appears to be less than the sum induced by either ABA or Pro alone (Fig. 4). This may be due to a genetic limitation in the ability of maize suspension-cultured cells to develop the maximum tolerance.

The mechanism(s) by which Pro induces chilling tolerance is not clear. The parallel relation between Pro-induced chilling tolerance and Pro accumulation by the cells from 0 to 12 h of Pro treatment suggests that Pro may protect cellular components directly. It has been reported that Pro can protect pyruvate phosphate dikinase from maize against cold denaturation (Krall et al., 1989). Heber et al. (1973) showed that Pro at 100 mM is capable of preventing freezing-induced inactivation of membrane activities. It has been shown that plant membrane damage during chilling is related to the peroxidation of membrane lipid due to the stress-induced accumulation of free radicals (Wise and Naylor, 1987). Pro prevents the oxidation of unsaturated vegetable oils (Ahmad et al., 1983). Whether Pro prevents membrane lipid peroxidation in plant tissue by acting as an antioxidant to counteract the chilling-induced free radicals is under investigation.

Our results suggest that the mechanisms of Pro- and ABA-induced chilling tolerance in maize suspension-cultured cells

are different. ABA-induced chilling tolerance appears to be associated with the increased net synthesis of some specific proteins, whereas Pro probably alleviates chilling injury through the direct protection of cellular components from chilling stress.

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