Plant Physiol. (1992) 99, 707–711
0032-0889/92/99/0707/05/$01.00/0

Received for publication October 2, 1991
Accepted January 24, 1992

Abscisic Acid-Induced Chilling Tolerance in Maize Suspension-Cultured Cells[1]

Zhanguo Xin and Paul H. Li*

Laboratory of Plant Hardiness, Department of Horticultural Science, University of Minnesota, St. Paul, Minnesota 55108

ABSTRACT

The induction of chilling tolerance by abscisic acid (ABA) in maize (*Zea mays* L. cv Black Mexican Sweet) suspension cultured cells was examined. Cell viability during exposure to chilling was estimated by triphenyl tetrazolium chloride reduction immediately after chilling and a filter paper growth assay. Both methods yielded comparable results. Chilling tolerance was induced by transferring 5-day-old cultures (late log phase) to a fresh medium containing ABA (10 to 100 micromolar). The greatest chilling tolerance was achieved with ABA at 100 micromolar. Growth of cells was inhibited at this concentration. After a 7-day exposure to 4°C in the dark, the survival of ABA-treated cells (100 micromolar ABA, 28°C for 24 h in the dark) was sevenfold greater than untreated cells. Effective induction of chilling tolerance was first observed when cells were held at 28°C for 6 hours after adding ABA. No tolerance was induced if the culture was chilled at the inception of ABA treatment. Induction of chilling tolerance was inhibited by cycloheximide. These results indicate that ABA is capable of inducing chilling tolerance when ABA-treated cells are incubated at a warm temperature before exposure to chilling, and this induction requires *de novo* synthesis of proteins.

A chilling temperature is defined as a temperature low enough to cause plant tissue damage but not low enough to cause freezing of tissue water (13). For most chilling-sensitive plants, chilling temperatures are between 10 and 0°C (14). Maize (*Zea mays* L.) is a chilling-sensitive plant species. Exposing young maize plants to chilling at 5°C day/night for 6 d results in >60% damage of total leaf area (28). Spraying mefluidide on maize plants before chilling increases endogenous ABA and reduces chilling injury (29). Zhang *et al.* (29) proposed that increases in endogenous ABA before chilling may be an essential step in inducing chilling tolerance. Although direct application of ABA to maize plants was unsuccessful in reducing chilling injury (29), ABA has been shown to reduce chilling injury in a number of other chilling-sensitive plants (5, 10, 19–21) and in maize callus culture (4).

The mechanism of ABA-induced chilling tolerance is not understood. ABA is capable of inducing freezing tolerance in many chilling-insensitive plants (2, 3), whether or not the treatment was held at warm or cold temperature regimens. The induction of freezing tolerance can be inhibited by CH[2] (2), indicating that protein synthesis is required. It also has been shown that the induction of freezing tolerance by ABA is associated with *de novo* synthesis of proteins (9, 12, 16, 22, 27). Some of these ABA-regulated proteins are also synthesized in response to cold acclimation (9, 12, 26, 27). However, cold acclimation results in injury, including death of chilling-sensitive plants such as maize. ABA induces chilling tolerance, and the induction occurs only in a warm temperature regimen (5, 20, 21). It is not known whether the enhancement of chilling tolerance by ABA in maize cultured cells is also associated with the expression of ABA-induced genes. CH, a cytoplasmic protein synthesis inhibitor (10), was used in conjunction with ABA to address this question.

We report here a study of ABA-induced chilling tolerance in maize suspension-cultured cells. Chilling injury in intact plants is often associated with chilling-induced water stress (29). Suspension-cultured cells provide conditions in which water stress can be minimized. It also facilitates the uptake of exogenous chemicals, such as ABA and CH, into cells. That ABA-induced chilling tolerance is probably a true tolerance to chilling rather than an ability to tolerate chilling-induced secondary water stress is discussed.

MATERIALS AND METHODS

Culture of Maize Cells and Application of ABA

Maize (*Zea mays* L., cv Black Mexican Sweet) cells were cultured in Murashige-Skoog medium containing 100 mg L-asparagine, 200 mg D-glucose, and 2 mg 2,4-D per L (6). Stock solutions of 2,4-D were prepared in 2 mM KOH. Ethanol was not used in any solutions because ethanol has been shown to minimize the induction of freezing tolerance by ABA in bromegrass (18). Cell cultures were incubated at 28°C in the dark on a rotary shaker; 2.5-mL volumes of cell suspension were subcultured weekly into 40 mL of fresh medium in a 125-mL flask.

Racemic ABA (Calbiochem, San Diego, CA) stock solutions were prepared in 50 mM K_2CO_3. Equal amounts of K_2CO_3 (0.25 mM final concentration) were added to control cultures. Medium pH was adjusted to 5.8 with NaOH or HCl before autoclaving. At day 5 after subculturing (late log growth phase), cells were collected by filtration through sterile Miracloth (Calbiochem). Two grams of cells (fresh weight) each

[1] Scientific Journal Series Paper No. 19397 of the Minnesota Agricultural Experiment Station, St. Paul, MN 55108.

[2] Abbreviations: CH, cycloheximide; TTC, triphenyl tetrazolium chloride; FPGA, filter paper growth assay.

were transferred to fresh media containing various concentrations of ABA. Cells were cultured at 28°C for various durations and then chilled at 4°C.

Determination of Protein Synthesis Inhibition

The method for determining the effect of CH on protein synthesis in ABA-treated cultures was similar to that of Lam *et al.* (11). After incubation for 2 h with CH and 100 μM ABA, 2 mL cell culture was transferred to a 5-mL plastic culture tube containing 10 μCi L-[^{35}S]methionine (>1000 Ci mmol^{-1}; ICN Irvine, CA). Cell cultures were allowed to incorporate L-[^{35}S]methionine for 2 h at 28°C, rinsed with culture medium, then frozen in liquid nitrogen, and stored at −70°C. To determine the incorporation of L-[^{35}S]methionine into proteins, cells were ground in a liquid nitrogen-chilled mortar and pestle with 1 mL O'Farrell lysis buffer (17). The homogenates were centrifuged for 30 min at 100,000*g*. Aliquots of 5 μL supernatant from each sample were used to determine the total and TCA-precipitable radioactivities with a scintillation counter. The ratio of TCA-precipitable activity to the total activity in non-CH-treated ABA-containing medium was set at 100%. Relative protein synthesis rates in CH-treated samples were expressed as the percentage of non-CH-treated ABA samples. After the inhibition of protein synthesis was determined, the remaining cells were cultured at 28°C for 12 h and then chilled to determine survival.

Estimation of Survival after Chilling

Survival of cell cultures after chilling exposure was estimated by TTC reduction and FPGA. TTC reduction was conducted according to the method described in ref. 25 with slight modifications: TTC (0.8%, w/v) was dissolved in 50 mM potassium phosphate, pH 7.5; after TTC treatment for 1 d at 25°C in the dark, the red formazan was extracted from cells with 95% ethanol for 1 d at 25°C in the dark. The absorbance of the extract was measured at 530 nm. Survival was defined as the ratio of absorbance of chilled to unchilled cells. FPGA was conducted similarly to the method described in ref. 8. One milliliter of chilled cell suspension containing 30 mg (fresh weight) cells was plated on 7-cm Whatman No. 2 qualitative filter paper discs that had been placed on 25 mL Murashige-Skoog medium containing 0.8% agar in 100- × 15-mm Falcon Petri dishes. After the plated cells were incubated in the dark at 28°C for 1 d, the filter paper with the cells was transferred to another Petri dish containing 25 mL fresh agar medium; the net growth of plated cells (fresh weight) was measured. Increase in fresh weight during this period might be the result of both cell division and enlargement.

After 14 d, the fresh weight reached a plateau because of nutrient limitation. Given sufficient time, a small amount of cells survived the chilling exposure and grew to a weight comparable to unchilled cells; therefore, a standard growth assay with the unchilled ABA-treated and control cells was conducted. Growth of unchilled cells plated on agar medium reached a plateau after 14 d growth at 28°C. The 14-d regrowth period was then chosen as a standard regrowth time to estimate the survival of chilled samples. The ratio of the net growth of chilled to nonchilled cells was used to estimate the percentage survival.

RESULTS

Induction of Chilling Tolerance with ABA

Figure 1 shows the percentage survival estimated by TTC reduction and FPGA in ABA-treated and untreated cells after various days of chilling exposure to 4°C. Both assays of cell viability indicated that 100 μM ABA significantly increased the chilling tolerance of maize cells. These two methods were comparable for cell viability estimation with a correlation coefficient of 0.94** (** means significance at 0.01 level). After a 14-d exposure to chilling, the ABA-treated cells had about 40% capability to reduce TTC and to regrow. The capability for TTC reduction and regrowth was diminished in untreated cells after the same period of chilling. Figure 2 shows the appearances of the 14-d chilled controls and 14-d chilled ABA-treated cultures after a 14-d regrowth at 28°C.

Effect of ABA Concentrations

Various concentrations of ABA induced different levels of chilling tolerance (Fig. 3). At a concentration <1 μM, ABA did not induce any detectable increase in survival after chilling. At 10 μM ABA, a 20% increase in survival after chilling was observed; at 100 μM, survival was doubled as compared with the culture treated with 10 μM. After 7 d of chilling, the survival of 100 μM ABA-treated cells was 76% estimated by TTC reduction, whereas the survival of untreated cells was <10%. Concentrations >100 μM were not tested because cell growth with 100 μM ABA was inhibited at 28°C. However, when treated cells were transferred into fresh medium containing no ABA, cells resumed normal growth (data not shown).

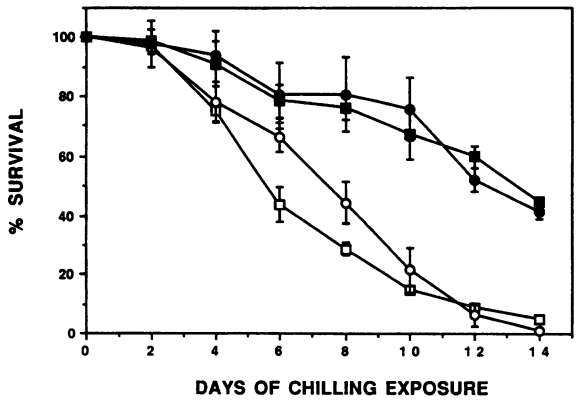

Figure 1. Survival of maize cells estimated by both relative TTC reduction and relative regrowth. Two grams of cells (fresh weight) at late log phase was transferred to fresh medium containing no ABA (○, □) or 100 μM ABA (●, ■) and were grown at 28°C for 24 h before chilling exposure. Aliquots of 1 mL cell culture each containing about 30 mg cells (fresh weight) were sampled to determine survival just before and every 2 d after initiation of chilling. Survival was estimated by TTC reduction immediately after chilling and by regrowth for 14 d at 28°C. Data are the means of four replicates. Vertical lines represent SE. Survival of control (○) and ABA-treated (●) cells was estimated by TTC reduction; survival of control (□) and ABA-treated (■) cells was estimated by regrowth.

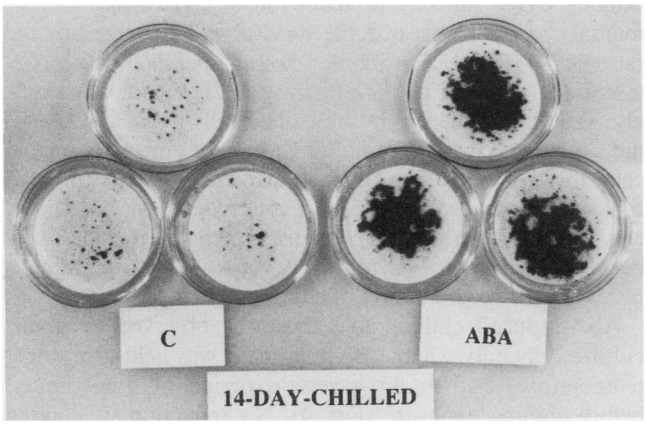

Figure 2. Regrowth of 14-d chilled ABA-treated (100 μM, 24 h at 28°C) and control cells. After a 14-d exposure to 4°C, 30 mg ABA-treated or control cells was plated as described in "Materials and Methods." After an additional 14-d incubation at 28°C, cells were incubated with 0.8% TTC in the dark at 28°C for 24 h and photographed. Darkness indicates viability of cells. C, Cells transferred to medium containing no ABA; ABA, cells transferred to medium containing 100 μM ABA.

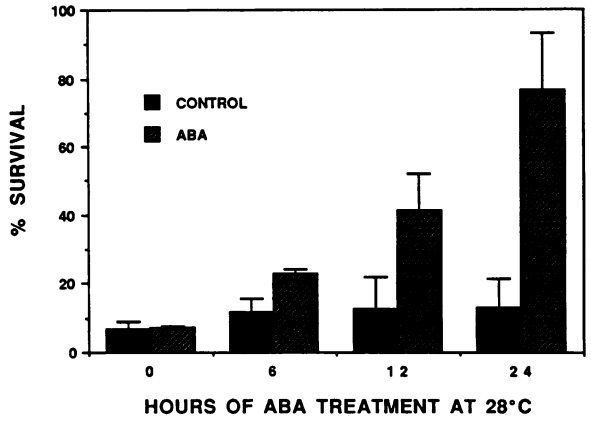

Figure 4. Increase in chilling tolerance of ABA-treated maize cells in ABA-containing medium cultured with time. Two grams of cells (fresh weight) at late log growth phase was transferred to fresh medium containing 0 (control) or 100 μM ABA. Cells were grown at 28°C for 6, 12, and 24 h and then chilled at 4°C. Following 7 d of chilling, 1-mL aliquots of cell suspension were sampled, and survival of the chilled cells was estimated by relative TTC reduction. Data are the means of four replicates. Vertical lines are SE.

Time Course of Development of ABA-Induced Chilling Tolerance

The development of chilling tolerance at 28°C in ABA-containing medium was time dependent (Fig. 4). Induction of chilling tolerance was observed when cells were held at 28°C for 6 h after ABA treatment. Chilling tolerance increased with time and reached the highest level after 24 h. No significant increase in chilling tolerance was observed with longer incubation at a warm temperature (data not shown). ABA did not increase chilling tolerance if applied at the inception of chilling exposure (Fig. 4).

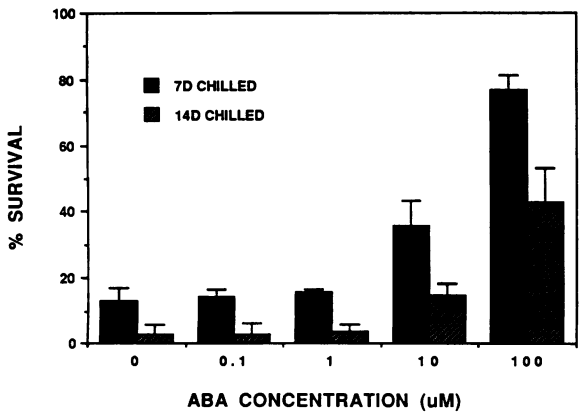

Figure 3. Effects of ABA concentrations on chilling tolerance. Two grams of cells (fresh weight) at late log phase was transferred to fresh medium containing various concentrations of ABA and grown at 28°C for 24 h before chilling at 4°C. On days 7 and 14 after chilling exposure, 1-mL aliquots of cell suspension were sampled for TTC reduction. Data are the means of four replicates. Vertical lines are SE.

Inhibition of Protein Synthesis and of ABA-Induced Chilling Tolerance by CH

To test whether ABA-induced chilling tolerance depends on the *de novo* synthesis of proteins, CH was added to ABA-containing media at concentrations from 0.5 to 10 μM before transferring cells. Up to 2 μM, CH did not decrease survival of unchilled cells (data not shown). CH was present during the entire period of ABA treatment at 28°C and throughout the chilling exposure at 4°C. Preliminary experiments showed that the presence of CH at 4°C had no significant effect on cell viability (data not shown). At 0.5 μM, CH reduced the protein synthesis rate to 47% compared to the cells treated with ABA without CH; it also reduced the survival to about half that of the non-CH-treated ABA sample (Fig. 5). Correlation analysis indicates that the percentage of protein synthesis was significantly correlated with percentage of survival after chilling with a correlation coefficient of 0.99 (significant at 0.01 level).

DISCUSSION

The results indicate that ABA is capable of increasing chilling tolerance in maize suspension-cultured cells when ABA-treated cells are held at 28°C for 6 h before chilling. Although induction of chilling tolerance with ABA in maize plants has not been demonstrated (29), ABA has been reported to increase chilling tolerance of eggplant, soybean, and cucumber seedlings (5, 15, 21), cotton cotyledonary discs (19, 20), and maize callus culture (4). The inability of ABA to induce chilling tolerance in maize plants could result from lack of uptake or biodegradation of applied exogenous ABA.

High RH during chilling exposure greatly reduced chilling injury in cucumber seedlings (21). ABA treatment reduces water loss during chilling exposure in cucumber cotyledon (21). Rikin and Richmond (21) proposed that ABA amelio-

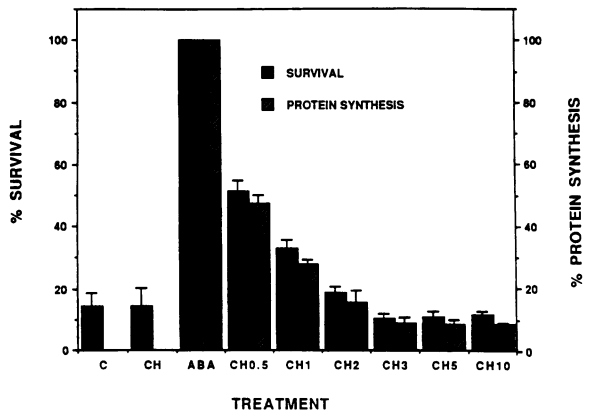

Figure 5. Effects of CH on both protein synthesis and chilling tolerance induction by ABA. CH was added to ABA-containing (100 μM) medium to concentrations of 0.5 μM (CH0.5), 1 μM (CH1), 2 μM (CH2), 3 μM (CH3), 5 μM (CH5), and 10 μM (CH10). Relative protein synthesis was defined as the ratio of percentage incorporation of L-[^{35}S]methionine into proteins in the presence of CH to the percentage incorporation in the absence of CH. After cell were sampled to determine relative protein synthesis, the remaining cells were allowed to grow at 28°C for an additional 10 h to determine survival by TTC reduction. Relative survival was expressed as the ratio of the survival in the presence of CH to the survival in the absence of CH. C, Control without ABA treatment; CH, control treated with 2 μM CH; ABA, ABA treatment without CH. The vertical lines are SE.

ration of chilling injury functioned by improving water balance. ABA improves plant water status by an increase in root hydraulic conductance (15) and/or by an early closure of stomata during chilling (5). Because the water potential of a liquid medium changes little during culture as well as during chilling exposure, the complication of water stress during chilling is greatly reduced in the maize suspension-cultured cell system. Therefore, ABA-induced chilling tolerance in maize suspension-cultured cells is likely to reflect a true tolerance to direct effects of chilling rather than amelioration of secondary stresses during chilling exposure.

Most chilling-insensitive plants synthesize new proteins and become hardy during cold (chilling) acclimation (7, 9, 12, 26). Also, ABA can induce freezing tolerance in many chilling-insensitive plants with a warm temperature regimen (3), and the induction is associated with the *de novo* synthesis of proteins (12, 16, 22, 27). ABA-mediated responses to osmotic and salinity stresses are also associated with the induction of specific gene expression (1, 23, 24).

We were interested in knowing whether ABA-induced chilling tolerance in chilling-sensitive plants is also regulated by expression of ABA-induced genes. CH was then used to inhibit the protein synthesis during the induction of chilling tolerance by ABA. This study showed that the presence of CH (0.5–10 μM) in the ABA-containing medium inhibited to various degrees the synthesis of proteins and the development of chilling tolerance (Fig. 5) and that the inhibition of chilling tolerance was highly correlated with the inhibition of protein synthesis ($r = 0.99$). It could be argued that the inhibition of protein synthesis may weaken the cell's viability and result in a decreased survival. Weakening of cell viability of unchilled cells by CH is unlikely to affect the survival in our experimental conditions because the survival was estimated by the ratio of TTC reduction (chilled *versus* unchilled cells). Any loss in viability due to the presence of CH in unchilled cells also should be reflected in the chilled cells. Also, CH (2 μM) did not reduce the survival after chilling of non-ABA-treated cells (Fig. 5). The decrease in chilling tolerance of ABA-treated cells with the presence of CH is conceivably the result of a lack of *de novo* synthesis of proteins. We, therefore, tentatively conclude that *de novo* synthesis of ABA-induced proteins is necessary for the induction of chilling tolerance.

ABA-induced chilling tolerance was observed in maize culture cells only when ABA treatment was held at a warm temperature regimen for a certain period of time (hours) before chilling exposure (Fig. 4). No reduction of chilling injury was detected when cells were exposed to chilling immediately after ABA treatment. These effects are also observed in eggplants (5) and cotton (19, 20). Eggplants that were foliar sprayed with ABA at 30°C close their stomata after 1 h of chilling; however, stomata remained open when ABA was sprayed at 6°C.

Rikin *et al.* (19) showed that ABA was ineffective in preventing chilling injury if applied to cotton cotyledonary discs at the inception of chilling, even though sufficient ABA was found in the tissue. Penetration of ABA into the tissue appeared not to be a problem at a chilling temperature. Rikin *et al.* (19) proposed that "factors" such as active metabolism may be required for an effective ABA protection. Exposure of maize seedlings to chilling (4°C) increased endogenous ABA content but had no effect on chilling tolerance (29). Mefluidide, a synthetic growth regulator, capable of inducing an increase in endogenous ABA at a warm temperature regimen (28°C), increases chilling tolerance if applied 12 h before chilling (29). It is suggested that a "protective system" may be activated by the mefluidide-induced elevation of endogenous ABA at warm temperatures (29).

Our results indicate that *de novo* synthesis of proteins after ABA treatment is associated with induction of chilling tolerance. We suggest that inability to synthesize some ABA-induced proteins may be one of the reasons that ABA cannot induce chilling tolerance when the treatment is initiated at the inception of chilling exposure. It is also possible that these proteins are synthesized but unable to function at a chilling temperature. The latter is unlikely, because the ABA-treated cells could survive for at least 14 d at 4°C. This is an interesting question to be clarified.

ACKNOWLEDGMENT

We thank D.A. Somers of the Department of Agronomy and Plant Genetics, University of Minnesota, for providing the maize cell suspension.

LITERATURE CITED

1. **Bray EA** (1988) Drought- and ABA-induced changes in polypeptide and mRNA accumulation in tomato leaves. Plant Physiol **88:** 1210–1214
2. **Chen HH, Li PH, Brenner ML** (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol **71:** 362–365
3. **Chen THH, Gusta LV** (1983) Abscisic acid induced freezing resistance in cultured plant cells. Plant Physiol **73:** 71–75

4. **Duncan DR, Widholm JM** (1987) Proline accumulation and its implication in cold tolerance of regenerable maize callus. Plant Physiol **83:** 703–708
5. **Eamus D** (1987) Stomatal behaviour and leaf water potential of chilled and water-stressed *Solanum melongena*, as influenced by growth history. Plant Cell Environ **10:** 649–654
6. **Green CE** (1977) Prospects for crop improvement in the field of cell culture. Hortscience **12:** 131–134
7. **Guy CL** (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol **41:** 187–223
8. **Horsch RB, King J, Jones GE** (1980) Measurement of cultured plant cell growth on filter paper discs. Can J Bot **58:** 2402–2406
9. **Johnson-Flanagan AM, Singh J** (1987) Alteration of gene expression during the induction of freezing tolerance in *Brassica napus* suspension cultures. Plant Physiol **85:** 699–705
10. **Krizek DT, Semeniuk P, Moline HE, Mirechi RM, Abbott JA** (1985) Chilling injury in coleus as influenced by photosynthetically active radiation, temperature and abscisic acid pretreatment. I. Morphological and physiological responses. Plant Cell Environ **8:** 135–142
11. **Lam E, Green PJ, Wong M, Chua N-H** (1989) Phytochrome activation of two nuclear genes requires cytoplasmic protein synthesis. EMBO J **8:** 2777–2783
12. **Lang V, Heino P, Palva ET** (1989) Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet **77:** 729–734
13. **Levitt J** (1980) Responses of Plants to Environmental Stresses, Ed 2, Vol 1. Academic Press, New York, pp 23–64
14. **Lyons JM, Raison JK, Steponkus PL** (1979) The plant membrane in response to low temperature: an overview. *In* JM Lyons, D Graham, JK Raison, eds, Low Temperature Stress in Crop Plants. Academic Press, New York, pp 1–24
15. **Markhart AH III** (1984) Amelioration of chilling-induced water stress by abscisic acid-induced changes in root hydraulic conductance. Plant Physiol **74:** 81–83
16. **Mohapatra SS, Poole RJ, Dhindsa RS** (1988) Abscisic acid-regulated gene expression in relation to freezing tolerance in alfalfa. Plant Physiol **87:** 468–473
17. **O'Farrell PH** (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021
18. **Reaney MJT, Ishikawa M, Robertson AJ, Gusta LV** (1989) The induction of cold acclimation: the role of abscisic acid. *In* PH Li, ed, Low Temperature Stress Physiology in Crops. CRC Press, Boca Raton, FL, pp 3–25
19. **Rikin A, Atsmon D, Gitler C** (1979) Chilling injury in cotton (*Gossypium hirsutum* L.): prevention by abscisic acid. Plant Cell Physiol **20:** 1537–1546
20. **Rikin A, Atsmon D, Gitler C** (1983) Quantitation of chill-induced release of a tubulin-like factor and its prevention by abscisic acid *Gossypium hirsutum* L. Plant Physiol **71:** 747–748
21. **Rikin A, Richmond AE** (1976) Amelioration of chilling injuries in cucumber seedlings by abscisic acid. Physiol Plant **38:** 95–97
22. **Robertson RJ, Gusta LV, Reaney MJT, Ishikawa M** (1987) Protein synthesis in bromegrass (*Bromus inermis* Leyss) culture cells during the induction of frost tolerance by abscisic acid or low temperature. Plant Physiol **84:** 1331–1336
23. **Singh NK, LaRosa PC, Handa AK, Hasegawa PM, Bressan RA** (1987) Hormonal regulation of protein synthesis associated with salt tolerance in plant cells. Proc Natl Acad Sci USA **84:** 739–743
24. **Skriver K, Mundy J** (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512
25. **Towill LE, Mazur P** (1975) Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. Can J Bot **53:** 1097–1102
26. **Tseng MJ, Li PH** (1990) Alterations of gene expression in potato (*Solanum commersonii*) during cold acclimation. Physiol Plant **78:** 535–547
27. **Tseng MJ, Li PH** (1991) Changes in protein synthesis and translatable messenger RNA populations with ABA-induced cold hardiness in potato. Physiol Plant **81:** 349–358
28. **Tseng MJ, Zhang C-L, Li PH** (1986) Quantitative measurements of mefluidide protection of chilled corn plants. J Am Soc Hortsci **111:** 409–412
29. **Zhang CL, Li PH, Brenner ML** (1986) Relationship between mefluidide treatment and abscisic acid metabolism in chilled corn leaves. Plant Physiol **81:** 699–701