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Alteration of Gene Expression Associated with Abscisic Acid-Induced Chilling Tolerance in Maize Suspension-Cultured Cells[1]

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ABA induces chilling tolerance in maize (*Zea mays* L., cv Black Mexican Sweet) suspension-cultured cells at 28°C when ABA was added to the culture medium at least 6 h prior to chilling (4°C), and this induction can be inhibited by blocking protein synthesis with cycloheximide treatment (Z. Xin, P.H. Li [1992] Plant Physiol 99: 707–711). De novo synthesis of proteins and changes in poly(A$^+$) RNAs were investigated during the ABA induction of chilling tolerance at 28°C as well as during chilling exposure. At 28°C, ABA increased the net synthesis of 11 proteins. Five of these proteins, whose net synthesis was also increased by chilling (4°C), were called group I ABA-induced proteins; the remaining six proteins, whose net synthesis was not altered by chilling, were called group II ABA-induced proteins. Chilling suppressed the net synthesis of three proteins. ABA treatment prior to chilling did not alleviate this suppression. ABA applied at the inception of chilling induced neither chilling tolerance nor accumulation of any of the group II proteins; however, once the group II proteins appeared, they were continually synthesized even in a chilling regimen. ABA induced seven in vitro translation products at 28°C. Three of these products could also be induced by chilling; the remaining four were induced by ABA only at 28°C. These results suggest that ABA-induced alteration of protein synthesis at 28°C is associated with an increased chilling tolerance in maize suspension-cultured cells.

Many plant species native to tropical or subtropical habitats are prone to injury at chilling temperatures (15 to 0°C) and display abrupt reductions in the rates of physiological processes when chilled (Lyons et al., 1979). Below some threshold temperatures, such chilling-sensitive species are restricted in germination, growth, and/or reproduction.

Application of ABA prior to chilling exposure reduces chilling injury in a number of chilling-sensitive plant species (Rikin and Richmond, 1976; Rikin et al., 1979, 1983; Duncan and Widholm, 1987; Eamus, 1987; Xin and Li, 1992). The mechanism by which ABA reduces chilling injury is not well understood, however. The proposed roles of ABA in amelioration of chilling injury are (a) increasing membrane permeability to facilitate water flux through the root system (Markhart, 1984), (b) rapid closing of stomata upon chilling exposure to reduce water loss (Eamus, 1987), (c) stabilizing membranes to suppress electrolyte leakage (Rikin and Richmond, 1976), and (d) stabilizing microtubule cytoskeleton networks to strengthen cell organization (Rikin et al., 1979, 1983).

Xin and Li (1992) characterized the induction of chilling tolerance by ABA in maize suspension-cultured cells. Chilling tolerance of maize cells was measured by their survival after chilling using the percentage of triphenyl tetrazolium chloride reduction of 7-d chilled to unchilled cells. Chilling tolerance was induced by transferring 5-d-old cells (late log phase) to a fresh culture medium containing ABA (10–100 μM) at 28°C. The greatest chilling tolerance was achieved with ABA at 100 μM. The induction of chilling tolerance was time dependent. Chilling tolerance was first observed after 6 h of ABA treatment, increased with time, and reached the highest level of tolerance after 24 h of treatment at 28°C. No chilling tolerance was induced if ABA treatment was initiated at the inception of chilling. The induction of chilling tolerance was inhibited by cycloheximide. Xin and Li (1992) suggested that the synthesis of ABA-regulated proteins at 28°C prior to chilling is required for the induction of chilling tolerance.

In this report we examined the alterations in protein synthesis and poly(A$^+$) RNA populations that occur during the acquisition of ABA-induced chilling tolerance at 28°C, as well as the changes that occur during chilling exposure in maize suspension-cultured cells. The results suggest that ABA causes specific alterations of gene expression at 28°C. The relationship between changes in protein synthesis and poly(A$^+$) RNA population and the ABA-induced chilling tolerance is discussed.

MATERIALS AND METHODS

Culture of Maize Cells and Application of ABA

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, and 2.5 mL of cell suspension was subcultured weekly with 40 mL of fresh medium. After 5 d of subculture, late log growth phase, the cells were collected by filtration through sterile Miracloth (Calbiochem) and 2 g of cells each (fresh weight) were transferred to fresh culture

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Abbreviations: BHT, butylated hydroxytoluene; Chaps, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; ID, identifying number arbitrarily assigned to a protein or translation product; pI, isoelectric point; UKC, urea-potassium carbonate-Chaps buffer.

media containing either no (control) or 100 μM ABA (treatment). The cell cultures were either chilled at 4°C immediately after transfer or incubated for 6, 12, and 24 h at 28°C prior to chilling.

In Vivo Labeling and Extraction of Total Proteins

Two hours prior to the end of the 6-, 12-, or 24-h ABA incubation periods, 100 mg (fresh weight) of cells in 2 mL of culture medium were transferred to a sterile 5-mL plastic culture tube containing 100 μCi of L-[^{35}S]Met (>1000 Ci mmol^{-1}; ICN). After 2 h of incubation at 28°C, 20 μL of unlabeled Met (1 M) was added to each tube and the incubation was continued for 5 min. In experiments performed at 4°C, a 12-h labeling period ensured that a sufficient amount of radioactive Met had been incorporated into proteins. After labeling, cells were collected by filtration through Miracloth, washed with 10 mL of 1 mM cold Met, frozen in liquid nitrogen, and stored at −80°C.

Protein was extracted with TCA-acetone according to Granier (1988) with some modification. Dry crushed powder was mixed with 10 mL of cold acetone containing 10% TCA, 0.1% BHT, and 0.1% β-mercaptoethanol and kept at −20°C for 1 h. After 15 min of centrifugation at 35,000*g*, the supernatant was removed and the pellet was washed at −20°C with 10 mL of cold acetone containing 0.1% BHT and 0.1% β-mercaptoethanol. After removing acetone, the pellet was vacuum dried and resuspended in a UKC buffer containing 9.5 M urea, 5 mM K_2CO_3, 4% Chaps (Sigma Chemical Co.), 2% ampholines (4 parts pH 5–7 and 1 part pH 3–10, Bio-Rad) at 50 μL UKC/mg dried pellet. Insoluble substance was removed by centrifugation at 100,000*g* for 1 h. Protein was quantified according to Polacheck and Cabib (1981). An aliquot of 30 μL or less of the resuspended protein sample containing 250,000 TCA-precipitable disintegrations per min was loaded on an IEF tube gel. The total proteins loaded on each gel were less than 100 μg.

Gel Electrophoresis

Two-dimensional PAGE was carried out according to O'Farrell (1975) with modifications according to Hochstrasser et al. (1988). IEF gel solution containing 9.5 M urea, 1.6% Chaps, 0.4% Triton-100 (Bio-Rad), 2% ampholines (4 parts pH 5–7 and 1 part pH 3–10), and 4.5% acrylamide: methylene-bisacrylamide (30:0.8, w/w) was poured into glass capillary tubes (i.d., 1.7 mm, Fisher). The gels were allowed to polymerize at 25°C for 2 h. During this period, catholyte (20 mM NaOH) and anolyte (6 mM H_3PO_4) were prepared and thoroughly degassed. After loading protein samples on the basic side of the tube gels, all samples were overlaid with catholyte to the top of the tubes. IEF was performed at 25°C with a constant voltage of 200 V for 2 h, 500 V for 5 h, and then 800 V for 16 h. After extrusion from the capillary tubes, IEF gels were equilibrated at 25°C for 5 min in 0.5 mL of modified O'Farrell sample buffer (O'Farrell, 1975) in which the 10% (v/v) β-mercaptoethanol was substituted with 10% (w/v) DTT. After equilibration, gels were either subjected to electrophoresis in a second dimension or stored at −80°C. Six identical linear gradient (8–12%) slab gels (0.75 mm) were cast with a Protein II casting chamber and a Model 395 gradient former (Bio-Rad). Stacking gels were cast according to O'Farrell (1975). Second dimension slab gels were run in a Protein II 2D Multi-Cell gel apparatus (Bio-Rad) with a constant current of 18 mA/gel at 25°C. Gels were stained with Coomassie blue (Bio-Rad) to stain the mol wt markers (Sigma).

After overnight fixation in 50% methanol containing 0.1% formalin, gels were infiltrated with 2,5-diphenyloxazole according to Laskey and Mills (1975). The gels were dried and exposed to Kodak X-Omat AR x-ray film at −80°C. All experiments were repeated a minimum of three times and only the reproducible changes in labeling were reported.

In Vitro Translation

Total and poly(A$^+$) RNAs were isolated following the method of Ausubel et al. (1989). Poly(A$^+$) RNA was translated in vitro with rabbit reticulocyte lysate (Promega, Madison, WI) essentially according to the manufacturer's instructions except that the translation products were precipitated with 1 mL of cold acetone containing 10% TCA, 0.1% BHT, and 0.1% β-mercaptoethanol for 1 h at −20°C. The translation products were microfuged for 5 min, washed twice with cold acetone containing 0.1% BHT and 0.1% β-mercaptoethanol, and vacuum dried. The dried pellet was resuspended in 100 μL of UKC buffer and the insoluble substance was removed by centrifugation for 1 h at 100,000*g*.

RESULTS

Two-Dimensional PAGE

We modified the two-dimensional PAGE method of Granier (1988) by substituting 0.5% SDS in UKC with 4% Chaps. This modified UKC buffer was very effective in solubilizing in vivo labeled proteins as well as in vitro translation products. With the modified buffer, we can resolve over 880 proteins, and smearing and streaking in both IEF and SDS-PAGE were greatly reduced. This improvement was also effective in analyzing proteins from potato and tomato leaves (data not shown). This modification also performed well with protein samples extracted from a few hundred guard cell protoplasts (data not shown).

Alteration of Protein Synthesis during ABA-Induced Chilling Tolerance at 28°C

ABA induces chilling tolerance in maize suspension-cultured cells when cells are cultured in the medium containing 100 μM ABA at 28°C for 6 h or longer prior to chilling (Xin and Li, 1992). The synthesis of proteins during the induction of chilling tolerance by ABA at 28°C was investigated. After a 6-h incubation, three proteins (IDs 12, 13, and 14) with pIs at 6.6 and M_rs at 25.5, 25, and 24.5 became labeled (Fig. 1B); labeling of these proteins was not detected in untreated cells (Fig. 1, A, C, and E). These proteins were transiently accumulated and the net synthesis was undetectable after 24 h of ABA incubation (Fig. 1F). One protein (ID 10, M_r 27, pI 6.3) was induced 6 h after ABA application and continually synthesized throughout the 24-h incubation (Fig. 1). Net

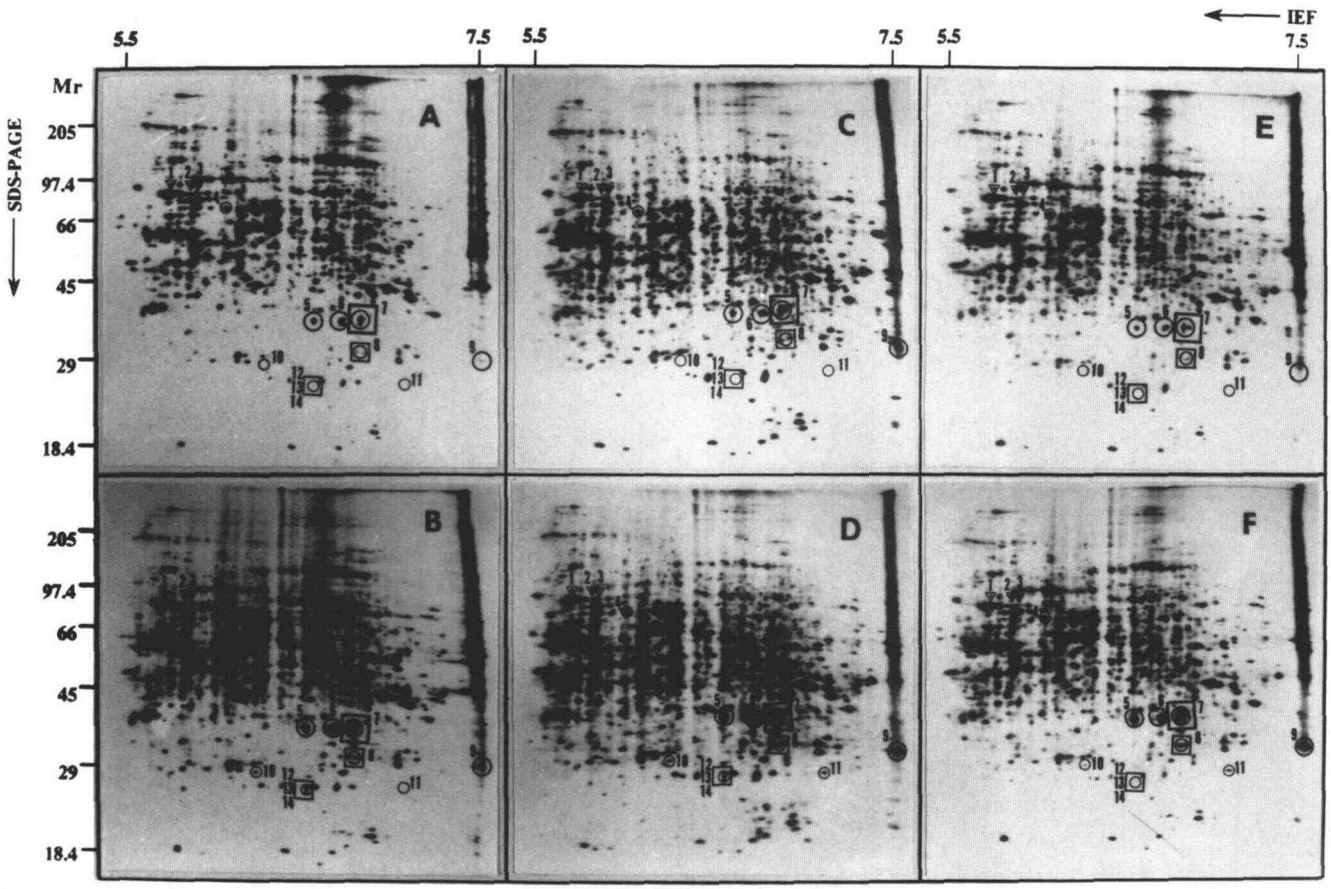

Figure 1. Fluorographs of in vivo ^{35}S-labeled proteins from cells not treated with ABA (A, C, E) and cells treated with 100 μM ABA (B, D, F) at 28°C for 6 (A ,B), 12 (C, D), and 24 h (E, F). ○, ABA-induced (increased in abundance) proteins; □, chilling-induced (increased in abundance) proteins; ▽, chilling-suppressed (decreased in abundance) proteins.

synthesis of three proteins (IDs 4, 9, and 11) with M_r/pIs of 68/6.1, 29/27.5, and 25/7.1, which were induced at 12 h, was still detectable at 24 h. Protein 4 migrates to a position adjacent to another protein that does not change with ABA treatment. One protein (ID 8, M_r 29, pI 6.9) and a group of three proteins (IDs 5, 6, and 7) with M_rs at 35 and pIs at 6.6, 6.7, and 6.9, which were synthesized in untreated cells, exhibited increased net synthesis following ABA treatment.

Alteration of Protein Synthesis at 4°C

Chilling greatly reduced overall net protein synthesis rates; however, after chilling, all but three proteins were still detectable on fluorograms of two-dimensional PAGE gels (Fig. 2). Net synthesis of these three proteins (IDs 1, 2, and 3) with M_rs at 78 and pIs at 5.8, 5.85, and 5.9 was rapidly decreased to an undetectable level (Figs. 2 and 3). ABA treatment for 24 h at 28°C prior to chilling had no effect on the suppression of synthesis of these three proteins (Fig. 3). Among the 11 proteins that exhibit increased labeling in response to ABA treatment at 28°C, net synthesis of five of them (IDs 7, 8, 12, 13, and 14) was increased by chilling (Fig. 2), and these five proteins were referred to as group I ABA-induced proteins. During chilling exposure, the pattern of the synthesis of group I proteins in ABA-pretreated (24 h at 28°C) cells was similar

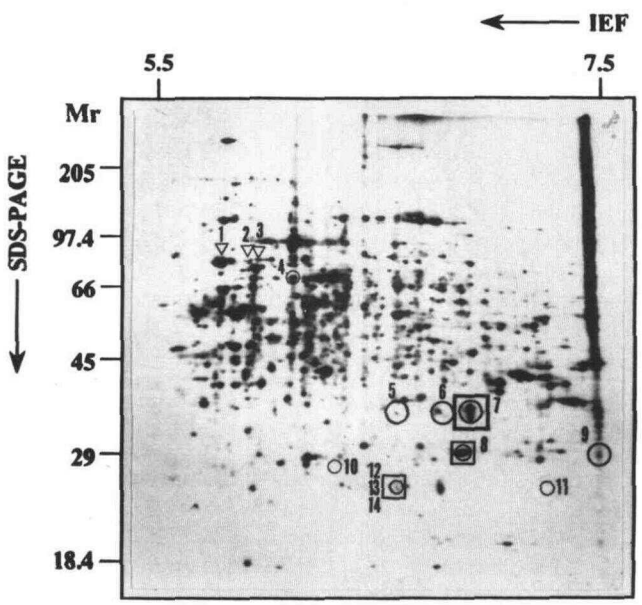

Figure 2. Fluorograph of in vivo ^{35}S-labeled proteins from control cells chilled for 24 h. □, Chilling-increased protein; ▽, chilling-suppressed protein; ○, corresponding to ABA-increased protein.

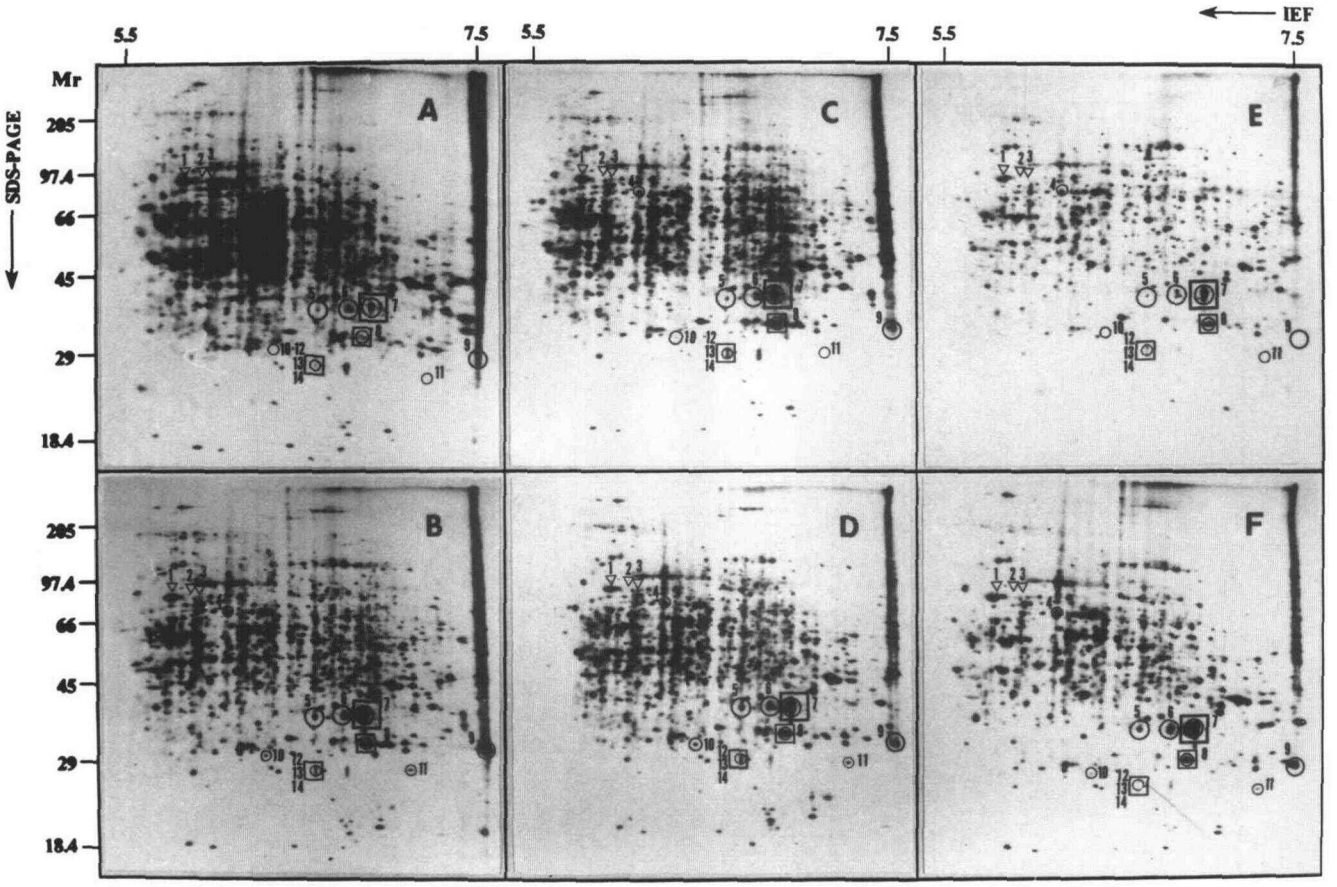

Figure 3. Fluorographs of in vivo ^{35}S-labeled proteins from cells treated with ABA at the inception of chilling exposure (A, C, E) and cells treated with ABA for 24 h at 28°C prior to chilling exposure (B, D, F). Cells were then chilled at 4°C for 12 h (A ,B), 2 d (C, D), and 4 d (E, F). ○, ABA-increased protein; □, chilling-increased protein; ▽, chilling-suppressed protein.

to that of control cells (Figs. 2 and 3). The net synthesis of the remaining six proteins (IDs 4, 5, 6, 9, 10, and 11) was not altered by chilling (Fig. 3) nor by ABA applied at the inception of chilling exposure (Fig. 3, A, C, and E). These six proteins were referred as group II ABA-induced proteins. However, once induced at 28°C by ABA, these group II proteins were continually synthesized even after a 4-d chilling exposure (Fig. 3). Table I summarizes the alteration of net protein synthesis during ABA-induced chilling tolerance at 28°C and during chilling exposure.

Alteration in Translatable Poly(A$^+$) RNA Population by ABA and Chilling

Figure 4 shows that seven new in vitro translation products (IDs 1, 2, 3, 4, 5, 6, and 7; M_r/pIs 94/6.6, 37/7.0, 25.5/7.0, 25/6.5, 25/6.9, 25/7.0, and 20/6.4) appeared during ABA-induced chilling tolerance at 28°C. Three of the seven products (IDs 2, 3, and 7) were also induced by a 24-h period of chilling (Fig. 5A). These translation products were referred to as group I translation products. The remaining four products could not be induced by chilling nor ABA applied at the inception of chilling (Figs. 4 and 5) and were called group II translation products. One of the group II products (ID 4) was induced after 6 h of incubation, whereas the other three were only detected after 12 h of incubation. When ABA was added to the culture medium at the inception of chilling, none of the group II translation products was detected (Fig. 5B). Table II summarizes the in vitro translation products induced by ABA and chilling.

DISCUSSION

It has been well documented that during low (chilling) temperature exposure, most chilling-insensitive plant species synthesize new sets of proteins that are correlated with the increase in cold hardiness (Guy, 1990). Chilling, in maize suspension-cultured cells, although it lowered the overall protein synthesis rate, suppressed the net synthesis of three proteins (IDs 1, 2, and 3) and enhanced the net synthesis (or accumulation) of five (group I) proteins (IDs 7, 8, 12, 13, and 14) that were also enhanced by ABA treatment at 28°C (Figs. 2 and 3). These alterations of protein synthesis in response to chilling temperature were not affected by the ABA treatment at 28°C.

We were unable to establish a relationship between the

Table I. *Summary of de novo synthesis of proteins regulated by ABA and chilling exposure*

Proteins			Regulation by Different Treatments	Effect of Long-Term Chilling on the Net Synthesis of These Specific Proteins	
ID[a]	M_r	pI		I[b]	II[c]
1	78	5.8	C[−d]	n[e]	n
2	78	5.85	C[−]	n	n
3	78	5.9	C[−]	n	n
7	35	6.9	C[+],[f] A6[g]	y[h]	y
8	29	6.9	C[+], A6	y	y
12	25.5	6.6	C[+], A6	y	y
13	25	6.6	C[+], A6	y	y
14	24.5	6.6	C[+], A6	y	y
4	68	6.1	A12[g]	n	y
5	35	6.6	A6	y	y
6	35	6.7	A6	y	y
9	29	>7.5	A12	n	y
10	27	6.3	A6	n	y
11	25	7.1	A12	n	y

[a] ID numbers correspond to the numbers shown in Figures 1 to 3. [b] I, ABA was added to medium at the inception of chilling, but no chilling tolerance was induced. [c] II, ABA was added to medium at 28°C 24 h prior to chilling, and the highest chilling tolerance was achieved. [d] C[−], The net synthesis of these proteins was greatly reduced by chilling. [e] n, Proteins were not detected during chilling exposure. [f] C[+], The net synthesis of these proteins was increased by chilling. [g] A6, A12, The increase in net synthesis of these proteins was first observed at 6 or 12 h of ABA treatment at 28°C, respectively. [h] y, Proteins were continually being synthesized after 12 h, 2 d, and 4 d of chilling exposure.

changes in chilling-regulated proteins and the status of chilling tolerance because no increase in chilling tolerance was observed during chilling exposure. Using one-dimensional PAGE, Yacoob and Filion (1986, 1987) found that cold-shock of maize seedlings for 2 h or conditioning for 18 h at 4°C induced several proteins with M_rs ranging from 14 to 94 (Yacoob and Filion, 1986). Comparing the synthesis of cold-shock proteins in maize cultivars that differ in chilling tolerance shows no relationship between the synthesis of cold-shock proteins and chilling tolerance. In maize suspension-cultured cells, we only observed the net synthesis of proteins with M_rs at 35, 29, 25.5, 25, and 24.4 in response to chilling. Cooper and Ort (1988) and Ort et al. (1989) found that synthesis of a 27 M_r Chl binding protein was inhibited with a concomitant synthesis of a 35 M_r protein in cucumber and tomato. Spinach, a chilling-insensitive plant, does not synthesize the 35 M_r protein when exposed to a similar temperature condition (Ort et al., 1989). Ort et al. (1989) suggested that the synthesis of the 35 M_r protein is not correlated with chilling tolerance, but rather, correlated with the chilling sensitivity of photosynthetic system in chilling-sensitive plants. Martino-Catt and Ort (1992) found that chilling interrupts circadian regulation of the transcriptional activity in tomato, and they proposed that chilling-induced mistiming of gene expression is a central factor underlying the chilling sensitivity of photosynthesis in chilling-sensitive plants. The induction of a thiol protease in tomato fruits has been shown to require less chilling in a chilling-sensitive variety than in a less chilling-sensitive one (Schaffer and Fischer, 1988, 1990). Exposure of rice plants to 15/10°C or 11/6°C induced the de novo synthesis of proteins even though several abundant proteins, e.g. subunits of Rubisco, were suppressed (Hahn and Walbot, 1989). Chilling-sensitive rice cultivars exhibit greater quantitative changes in protein synthesis as compared to less chilling-sensitive cultivars, but qualitative changes were similar. Comparison of cold night-induced accumulation of mRNAs between chilling-sensitive and less chilling-sensitive tomatoes showed that the increased mRNA species in chilling-sensitive tomatoes are likely to be involved in stress response rather than degree of tolerance (Vallejos, 1991). These results indicate that cold-induced alterations of gene expression are a general plant response to a chilling temperature rather than related to chilling tolerance.

Exposure of cultured maize cells to 4°C in the dark for 1 week decreased the survival below 10%. Incubation of maize suspension-cultured cells in culture medium containing 100 μM ABA for 24 h at 28°C prior to chilling exposure to 4°C in the dark for 1 week increased the chilling survival to 76% (Xin and Li, 1992). The chilling-reduced survival and the survival improvement by ABA treatment are unlikely to be involved in circadian mechanism, as suggested by Martino-Catt and Ort (1992), because maize cells were cultured and chilled in the dark. The induction of chilling tolerance can be inhibited by cycloheximide, and the degree of inhibition correlated with the degree of inhibition of protein synthesis (Xin and Li, 1992).

These results led us to investigate the changes in protein synthesis during ABA-induced chilling tolerance. At 28°C, ABA induced de novo synthesis of some specific proteins that were not detectable in untreated cells, and increased the net synthesis of some other specific proteins (Fig. 1). De novo synthesis of the six group II proteins (IDs 4, 5, 6, 9, 10, and 11; see Fig. 1 and Table I) coincided with the induction of chilling tolerance. Three (IDs 5, 6, and 10) of the group II proteins were detectable 6 h after ABA treatment, when increase in chilling tolerance was first observed. The remaining three proteins (IDs 4, 9, and 11) were detectable after 12 h of ABA treatment. When ABA treatment was initiated at chilling temperature, no chilling tolerance was observed (Xin and Li, 1992) and no increase in net synthesis of group II proteins was detected (Fig. 3, A, C, and E; Table I). However, once induced at 28°C, these proteins continued to be synthesized during chilling exposure (Fig. 3). Flores et al. (1988) reported that a terpenoid analog of ABA, LAB 173711, increased chilling tolerance of cucumber. In maize cell culture, this analog also induced chilling tolerance. In addition, the synthesis of group II proteins was observed in LAB 173711-treated maize cells (data not shown). The results suggest that group II proteins are involved in chilling tolerance induction.

In vitro translation of poly(A$^+$) RNAs from ABA-treated and untreated cells at 28°C showed that seven novel translatable RNA species appeared during ABA treatment. Three (group I) of the seven translation products (IDs 2, 3, and 7)

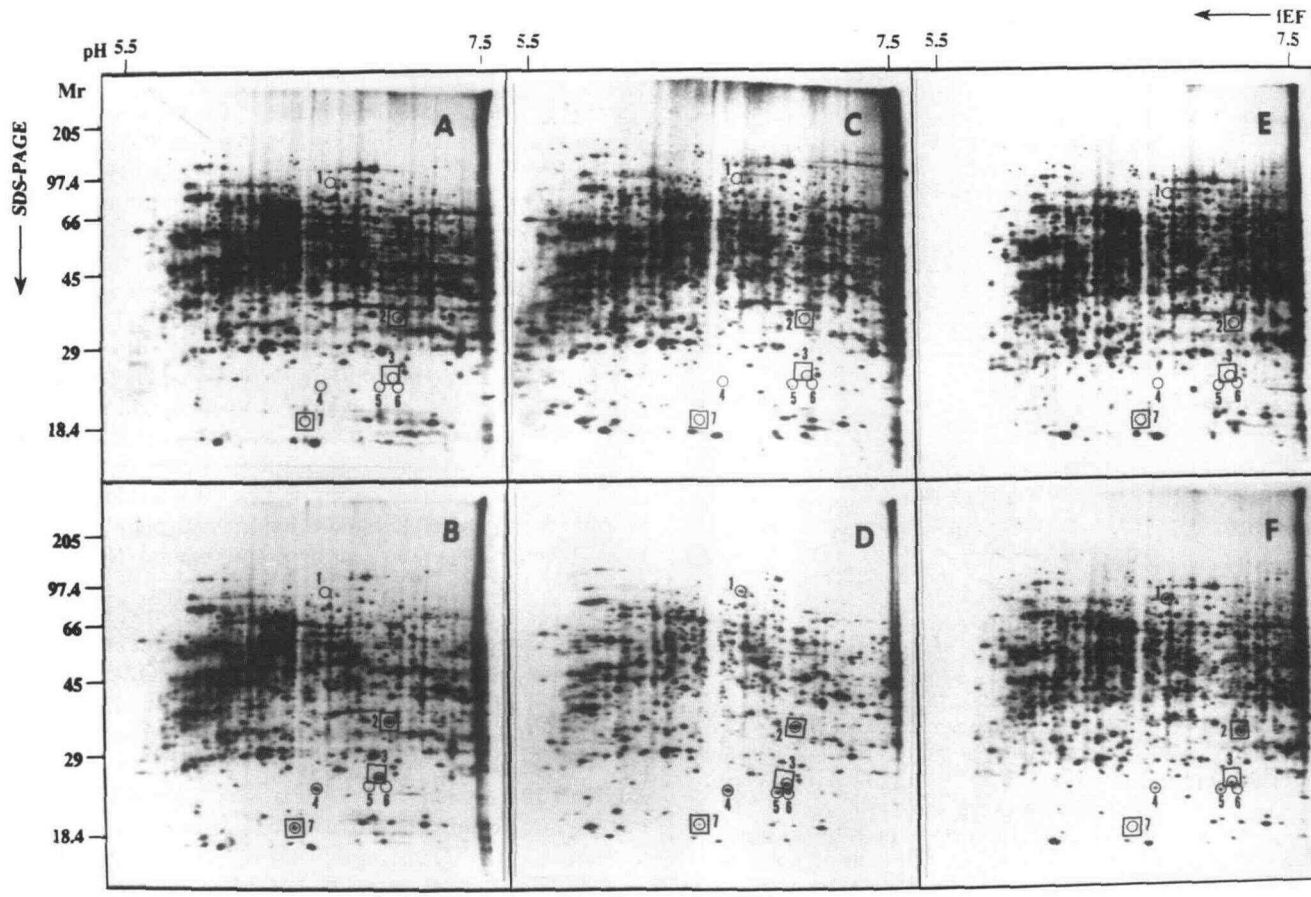

Figure 4. Fluorographs of in vitro translation products of poly(A⁺) RNAs from control (A, C, E) and ABA (B, D, F) treatment at 28°C for 6 (A, B), 12 (C, D), and 24 h (E, F). ○, ABA induced; □, chilling induced.

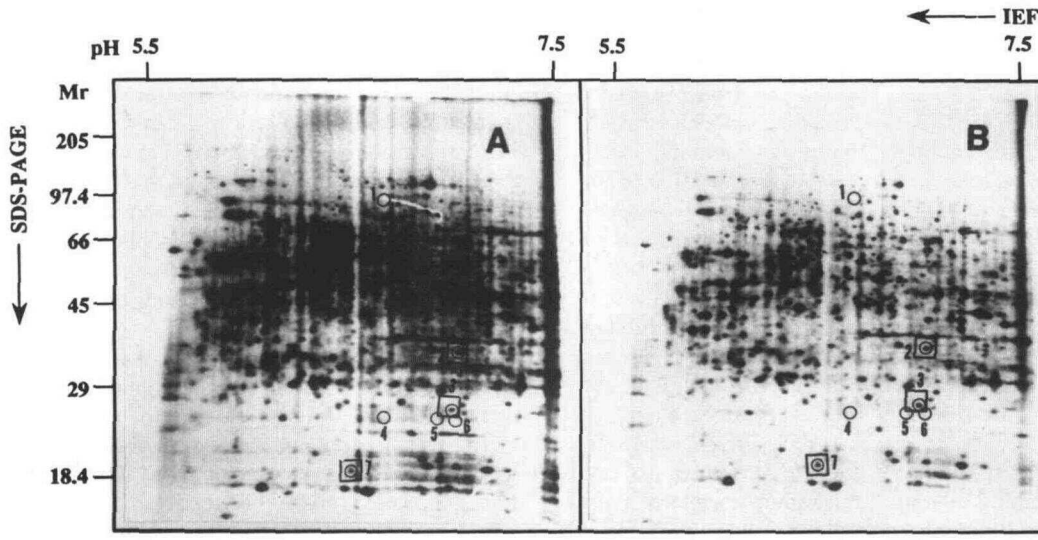

Figure 5. Fluorographs of in vitro translation products of poly(A⁺) RNAs from 24-h chilled control cells (A), and cells treated with ABA at the inception of chilling temperature (4°C) (B). ○, ABA induced; □, chilling induced.

Table II. *Summary of in vitro translation products regulated by ABA treatment and chilling*

Translation Products			Changes in mRNAs after Varied Period of ABA Treatment at 28°C			Changes in mRNAs after 24 h of ABA Treatment at 4°C	
ID[a]	M_r	pI	6 h	12 h	24 h	Control	ABA
2	37	7.0	+[b]	+	+	+	+
3	25.5	7.0	+	+	+	+	+
7	20	6.4	+	+	+	+	+
1	94	6.6	n[c]	+	+	n	n
4	25	6.5	+	+	+	n	n
5	25	6.9	n	+	+	n	n
6	25	7.0	n	+	+	n	n

[a] ID numbers correspond to the numbers shown in Figures 4 and 5. [b] +, Induced or increased in abundance. [c] n, Not detected.

could also be observed in untreated cells when exposed to 4°C for 1 d, whereas the remaining four (group II) (IDs 1, 4, 5, and 6) were only detected in treated cells at 28°C (Figs. 4 and 5). It appears that the induction of group II translation products is associated with induction of chilling tolerance. In vitro translation product 6 (M_r 25, pI 7.0) appears to be similar, with protein 11 (M_r 25, pI 7.1) found in in vivo labeling (Figs. 1 and 4). Both of them were induced at 12 h after ABA treatment at 28°C, and neither was detected if ABA treatment was initiated at the inception of chilling. However, the M_rs of other translation products do not correspond to the M_rs of de novo synthesized proteins. These discrepancies may be due to posttranslational modifications or they may represent different gene products.

ABA-induced chilling tolerance was observed in maize-cultured cells only when ABA-treated cells were held at 28°C for a certain period of time (minimum 6 h) (Xin and Li, 1992). No reduced chilling injury was detected when cells were exposed to chilling immediately after ABA treatment. These observations have been reported in cucumber (Rikin and Richmond, 1976), cotton (Rikin et al., 1979), and eggplants (Eamus, 1987). The reason for the inability of ABA to induce chilling tolerance at chilling temperature is not known. Rikin et al. (1979) ruled out decreased ABA penetration into the tissue at chilling temperature. We propose that this inability may be due to the lack of induction of the gene(s) responsible for chilling tolerance at low temperature because ABA induces neither chilling tolerance nor group II proteins or group II translatable RNAs when ABA treatment is initiated at the inception of chilling exposure.

We further propose that there may be two groups of genes. The first group (I) of genes can be triggered by either chilling or ABA at warm temperature, and the second group (II) of genes can be triggered by ABA only at warm temperature. Group I genes are chilling responsive but play no role in tolerance induction or are simply not sufficient to confer chilling tolerance. Group II genes alone or together with group I genes are responsible for the increased tolerance. However, group II genes cannot be expressed at chilling temperature. Why the group II genes cannot be initially expressed when ABA applied at the inception of chilling exposure is not known. Additional work is needed to unravel the nature of their impaired expression at low temperature.

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