Plant Physiol. (1987) 84, 1172–1176
0032-0889/87/84/1172/05/$01.00/0

Changes in Protein Patterns and Translatable Messenger RNA Populations during Cold Acclimation of Alfalfa[1]

Received for publication August 19, 1986 and in revised form April 1, 1987

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ABSTRACT

Changes in the rate and pattern of protein synthesis and in translatable mRNA population during cold acclimation of alfalfa (*Medicago falcata* cv Anik) seedlings have been examined. There appears to be a positive correlation between the increase in ability to synthesize proteins at 4°C and the increase in freezing resistance (survival at −10°C). Results obtained with three different approaches using sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern visualized by (a) staining, (b) immunoblotting and autoradiography, and (c) fluorography of *in vivo* labeled proteins, show that at least eight polypeptides are newly synthesized during cold acclimation. Results of analysis of *in vitro* translation products of mRNA from nonacclimated and acclimated seedlings show the appearance of new translatable mRNAs. It is concluded that changes in gene expression occur during cold acclimation, most probably at the transcriptional level.

Analysis of changes which occur in plants during cold acclimation are expected to be useful for understanding the mechanisms underlying freezing resistance, and for devising means to improve freezing resistance of plants. Although it has long been proposed that metabolic changes that occur during cold acclimation of plants originate from the altered expression of genes involved in the acquisition of cold resistance (11), there is still a general paucity of information on this subject. There have been several studies which demonstrate quantitative changes in protein synthesis (6, 9, 10). But relatively few qualitative changes (2–4) in the pattern of protein synthesis have been demonstrated. Evidence for altered gene expression during cold acclimation of spinach has been reported (5). In this paper we report on changes in patterns of protein synthesis and in translatable mRNA populations during cold acclimation of an alfalfa cultivar, an important forage crop plant that over-winters.

Results obtained with three different approaches, *in vivo* labeling of proteins, immunoblotting, and *in vitro* translation of mRNAs, show that cold acclimation is accompanied by changes in gene expression, most likely regulated at the transcriptional level.

MATERIALS AND METHODS

Plant Material. Seeds of alfalfa (*Medicago falcata* cv Anik) were surface-sterilized and planted in vermiculite which was presterilized and soaked with Hoagland solution. Seedlings were grown under a 12 h photoperiod (250 μE m^{-2} s^{-1}) and at 20°C. They were supplied with water every other day and with Hoagland solution once a week.

Administration of Cold Acclimation and Deacclimation. When the seedlings were 7 d old they were transferred to another growth chamber with light conditions as before but at a temperature of 4°C. Acclimation was allowed to proceed for various lengths of time as given in respective experiments. In order to deacclimate the seedlings, they were transferred from the acclimation chamber to the one at 20°C. Deacclimation was allowed for 2 d. Entire seedlings, nonacclimated (grown 7 d at 20°C), acclimated (grown 7 d at 20°C followed by up to 17 d at 4°C) or deacclimated (grown 2 d at 20°C after 17 d at 4°C), were used as experimental material. Although acclimated and deacclimated plants were older than nonacclimated controls, there was no visible developmental change during the acclimation period.

Test of Freezing Resistance. After being cold-acclimated for different times, triplicate samples of 40 to 50 seedlings each were transferred to a −10°C freezer for 3 h. They were then returned to 20°C at a rate of 2.5°C per hour. Preliminary experiments showed that more rapid thawing was detrimental. However, the rate of cooling did not seem to affect the percentage survival. After 2 d the percentage of surviving seedlings was determined. Maintenance of turgidity and resumption of growth by the seedlings were used to determine survival.

Protein Extraction. All steps of protein extraction were carried out at 4°C. Seedlings were homogenized in 2 volumes of buffer containing 25 mM Tris-HCl (pH 7.5), 10 mM KCl, 20 mM $MgCl_2$, 5 mM mercaptoethanol, and 1 mM PMSF.[2] The homogenate was centrifuged for 30 min to obtain a 40,000*g* supernatant expected to contain predominantly soluble proteins. Aliquots of this supernatant were used to determine protein content by the dye-binding method (1), radioactivity by liquid scintillation spectrometry, and protein patterns by SDS-PAGE.

Preparation of Antisera. Soluble proteins from cold-acclimated plants extracted with 25 mM Tris-HCl (pH 7.5) and 150 mM NaCl were injected into rabbits (New Zealand, White). An intramuscular injection of the extract containing 1 mg proteins and mixed with an equal volume of Freund's complete adjuvant, was administered followed by a subcutaneous injection without the use of adjuvant, next day. Similar injections were given 12 and 21 d later. A total of about 7 mg protein was injected. The antiserum obtained was enriched for IgG by ammonium sulfate fractionation.

***In Vivo* Labeling of Proteins.** Fifteen seedlings, nonacclimated, or acclimated up to 17 d, were rinsed with sterile distilled H_2O and placed in a vial containing 100 μCi of [^{35}S]methionine (1148 Ci/mmol) in 2 ml sterile, distilled H_2O, under light. Incorpora-

[1] Research supported by F.C.A.C. (Quebec) grants AS-2241 and EQ-3146 to R. S. D. and R. J. P., and by Natural Sciences and Engineering Research Council Canada grant A2724 to R. S. D.

[2] Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

tion was carried out for 3 h at 4 or 20°C as indicated in the respective experiments. At the end of incorporation seedlings were washed with sterile distilled H_2O and extraction buffer. Proteins were extracted as described above. A 2 to 5 μl aliquot of the protein extract was precipitated by 10% TCA on Whatman GF/A filters. Filters were washed with 5% TCA and then with ethanol, dried and the radioactivity on them was determined by liquid scintillation spectrometry.

Analysis of Proteins by SDS-PAGE. In case of unlabeled proteins, equal amounts of proteins from nonacclimated and acclimated seedlings were analyzed by SDS-PAGE. In the case *in vivo* or *in vitro* labeled proteins, equal amounts of protein radioactivity were loaded on gels to be separated by SDS-PAGE which was essentially according to the procedures described by Laemmli (7) except that the separating gel consisted of a 7.5 to 15% gradient of acrylamide and a stacking gel contained 4% acrylamide. After electrophoresis the gels were stained with Coomassie blue (R-250) and were photographed. For autoradiography gels were treated with EN^{3}HANCE (NEN, DuPont), dried and then exposed to preflashed Kodak XAR-5 film.

Immunoblotting. Essentially the procedures were as described previously (8). Proteins from nonacclimated and 14 d-acclimated seedlings were separated by SDS-PAGE as described above. They were electro-blotted onto nitrocellulose filter which was then incubated with an antiserum (diluted 1:50) against the proteins of 14-d-acclimated seedlings. The filter was washed extensively to remove the un-reacted antibodies and then incubated with ^{125}I-protein A. Filter was again washed to remove the unreacted ^{125}I-protein A and autoradiographed.

Preparation of Total and Poly(A)$^+$ RNA. Nonacclimated and acclimated seedlings were transferred to liquid N_2 directly from their respective growth temperatures and ground in liquid N_2 to a fine powder. The powder was thawed to 4°C and to this 2 volumes of extraction buffer (200 mM Na-acetate (pH 5.2), 10 mM EDTA, 1 mM SDS) and 2 volumes of phenol (saturated with Tris-HCl [pH 7.5]), both preheated to 60°C, were added. Following centrifugation (6000 rpm, 10 min) of the homogenate, the aqueous phase was first extracted with a mixture of equal volumes of phenol (containing 0.1% 8-hydroxyquinoline) and chloroform (containing 4% v/v isoamyl alcohol) and then with chloroform:isoamyl alcohol (24:1) at room temperature. The RNA in the aqueous phase was precipitated overnight at 4°C by adding LiCl to a final concentration of 2 M, collected by centrifugation and washed once with 2 M LiCl and twice with 70% ethanol. The RNA was stored in sterile water at −70°C until use.

Poly(A)$^+$ was isolated from the total RNA using Hybond-mAP paper (Amersham, Oakville). A piece of Hybond-mAP paper of required size was wetted with TES buffer (10 mM Tris HCl [pH 7.5], 1 mM EDTA, and 0.5 M NaCl) and then placed on a few layers of 3 MM filter paper. RNA in sterile water was heated at 70°C for 3 min and NaCl was added to a final concentration of 0.5 M. It was slowly spotted onto Hybond-mAP. The paper was then washed three times with TES, 5 min each, and once in 70% ethanol, for 2 min, to remove unbound RNA. Following removal of excess ethanol, the mAP paper was placed in sterile water and heated at 70°C for 5 min to elute the poly(A)$^+$ RNA. This RNA was translated in the rabbit-reticulocyte lysate-system (Bethesda Research Laboratories [BRL], Gaithersburg, MD) as described below.

***In Vitro* Translation of mRNA.** Poly(A)$^+$ RNA from nonacclimated and acclimated seedlings was translated *in vitro* in the rabbit reticulocyte lysate system obtained from BRL. The protocol provided by the supplier was followed. The reaction mixture with total volume of 15 μl contained, in addition to all other essential components, 1.0 μg of poly(A)$^+$ RNA and 15 μCi of [^{35}S]methionine (1148 Ci/mmol). The reaction was run at 30°C for 1 h and was terminated by placing the tubes on ice. The reaction mixture was treated with 10 μg of RNase for 15 min. A 2 μl aliquot was precipitated on Whatman GF/A filter with 10% cold TCA. The filter was washed with 5% hot TCA and then with 5% cold TCA. Finally, it was washed in 95% ethanol and dried. Its radioactivity was determined by liquid scintillation spectrometry.

Aliquots containing equal protein radioactivity were removed from various reaction mixtures corresponding to nonacclimated and acclimated seedlings, and were lyophilized. They were then used for the analysis of *in vitro* translation products by SDS-PAGE as described above.

RESULTS

The progressive increase in freezing resistance with increasing period of acclimation is shown in Table I. It is seen that 23% of the nonacclimated seedlings survive −10°C treatment. Percent survival increases with increase in the acclimation period reaching more than 50% within 8 d of acclimation and nearly 100% with 17 d of acclimation. Percent survival of seedlings which were acclimated for 17 d and then deacclimated for 2 d was 26 ± 2% (not shown in Table I), which is more or less equal to that in the nonacclimated seedlings.

The effect of increasing periods of cold acclimation on the ability to incorporate label from [^{35}S]methionine into proteins at acclimation temperature and at 20°C is also shown in Table I. The rate of incorporation at 4°C increases with the time of acclimation. For example, in the case of nonacclimated plants the rate of incorporation at 4°C is only 14% of that at 20°C. After 8 d of acclimation, this percentage increases to 58% and after 17 d of acclimation to 100% of that in the nonacclimated control. It is noteworthy that during acclimation the ability of the plants to incorporate label at 20°C first decreases as compared to that of the nonacclimated plants, then after 8 d of acclimation catches up with that of the nonacclimated plants, and by 17 d of acclimation it is more than twice (237%) of that of the nonacclimated plants.

To what extent does this incorporation of label into protein reflect changes in uptake of label rather than changes in protein synthesis? Uptake is certainly affected by temperature: total radioactivity taken up by non-acclimated plants at 4°C was 24% of that at 20°C (not shown in Table I). However, only a relatively small proportion of the label taken up was incorporated into protein: this proportion was 12% at 20°C and 8% at 4°C. This suggests that incorporation may not be limited by rate of uptake, and may therefore be an approximate measure of the rate of protein synthesis. This suggestion is strengthened by the fact that the increasing rate of incorporation of label with increasing time of acclimation (Table I) is paralleled by an accelerating increase in protein content of the seedlings (Table II). It is, therefore, concluded that with increasing period of cold acclimation there is progressive increase in the ability to survive freezing temperature of −10°C and to synthesize proteins at the temperature of acclimation. Furthermore, deacclimation for two days causes the freezing resistance to decline to the level characteristic of the nonacclimated seedlings.

The increases in protein and RNA contents during acclimation are shown in Table II. Protein content of seedlings increases by more than 70% during 17 d of acclimation. RNA content also shows a progressive increase during acclimation. While the poly(A)$^-$ RNA increases to 340% of the nonacclimated control, the poly(A)$^+$ RNA increases to 221% of the nonacclimated seedlings. It is, therefore, concluded that both total protein and total RNA increase during acclimation. The increase in total RNA is much greater than the increase in total protein, and the increase in poly(A)$^-$ RNA is considerably greater than the increase in poly(A)$^+$ RNA, especially in the early stages of acclimation.

Table I. *Changes in Freezing Resistance and in Ability to Incorporate Label from [^{35}]Methionine into Protein at 20°C and 4°C during Cold Acclimation*

Figures in parentheses represent incorporation as percent of that in the nonacclimated control seedlings at 20°C.

Acclimation Period	Survival (3 h at −10°C)	Rate of Incorporation[a]	
		20°C	4°C
d	%	*cpm × 10^{-3}/mg protein·3 h*	
0 (control)	23 ± 3	13.9 ± 1.3 (100)	2.0 ± 0.5 (14)
2	39 ± 5	7.2 ± 2.3 (52)	2.9 ± 0.7 (21)
5	45 ± 18	9.3 ± 1.6 (67)	5.6 ± 0.2 (40)
8	57 ± 7	13.7 ± 1.8 (99)	8.1 ± 1.0 (58)
11	77 ± 12	21.8 ± 2.9 (157)	9.3 ± 0.8 (67)
14	93 ± 4	25.8 ± 3.2 (186)	13.3 ± 1.3 (96)
17	97 ± 4	33.0 ± 1.7 (237)	14.0 ± 1.2 (101)

[a] Each value is a mean of three replicates ± SE.

Table II. *Increase in Protein and RNA Contents during Cold Acclimation*

Figures in parentheses represent protein or RNA content as percent of that in the nonacclimated control seedlings.

Acclimation Period	Protein Content[a]	RNA Content[a]	
		Poly(A)$^+$	Poly(A)$^-$
d		*μg/g fresh wt*	
0 (control)	2,400 ± 179 (100)	33 ± 7 (100)	200 ± 15 (100)
5	2,700 ± 133 (112.5)	42 ± 6 (127)	450 ± 22 (225)
11	3,200 ± 203 (133)	61 ± 9 (185)	575 ± 17 (288)
17	4,100 ± 247 (171)	73 ± 4 (221)	680 ± 23 (340)

[a] Each value is a mean of 3 replicates ± SE.

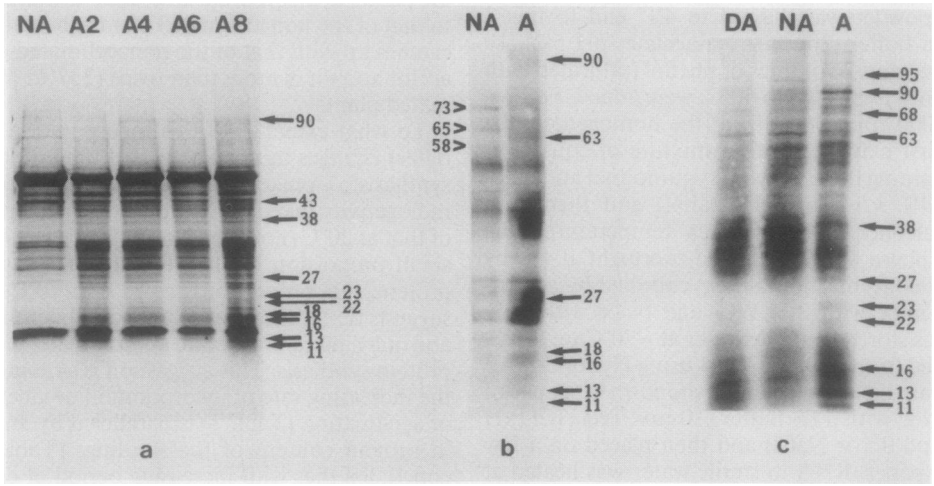

FIG. 1. Changes in pattern of protein synthesis during cold acclimation demonstrated by three different techniques: (a) SDS-PAGE gel stained with Coomassie blue. Fifty μg of proteins were loaded in each lane. Protein patterns of non-acclimated plants (NA) and of plants acclimated for 2 d (A2), 4 d (A4), 6 d (A6), and 8 d (A8) are shown. Arrows indicate polypeptides that are increasingly or newly synthesized during acclimation. Numbers with arrows indicate approximate molecular mass in kD of these polypeptides. (b) Autoradiograph of an immunoblot in which antibodies raised against plants acclimated for 14 d have been reacted with proteins from nonacclimated plants (NA) and from plants acclimated for 14 d (A). Position of arrows shows the polypeptides specific to acclimated plants and their molecular mass. (c) Fluorograph of *in vivo* labeled proteins, separated by SDS-PAGE, from seedlings acclimated for 17 d (A), nonacclimated seedlings (NA), and seedlings deacclimated for 2 d after acclimation for 17 d (DA). Incorporation was carried out at the respective growth temperatures, *i.e.* 20°C for nonacclimated and deacclimated seedlings and 4°C for acclimated seedlings. Protein sample containing 100,000 cpm was loaded in each lane. Positions of arrows indicate polypeptides specific to acclimated plants and their molecular mass. Note the decrease or disappearance of these polypeptides in deacclimated plants (DA).

Changes in pattern of *in vivo* protein synthesis during acclimation are shown in Figure 1. Results presented have been obtained by three different techniques: Coomassie blue staining of proteins separated by SDS-PAGE (a), immunoblotting (b), and fluorography of proteins labeled *in vivo* with [^{35}S]methionine and separated by SDS-PAGE (c). Figure 1a compares the pattern of nonacclimated seedlings (NA) with that of the seedlings acclimated for 2 d (A2), 4 d (A4), 6 d (A6), and 8 d (A8). Several polypeptides (13, 16, 18, 22, 23, 27, and 38 kD) appear during 2 d of acclimation. Two other polypeptides, 43 and 90 kD, appear to have increased in concentration. Another polypeptide (11 kD) appears at 8 d of acclimation. After this period no new

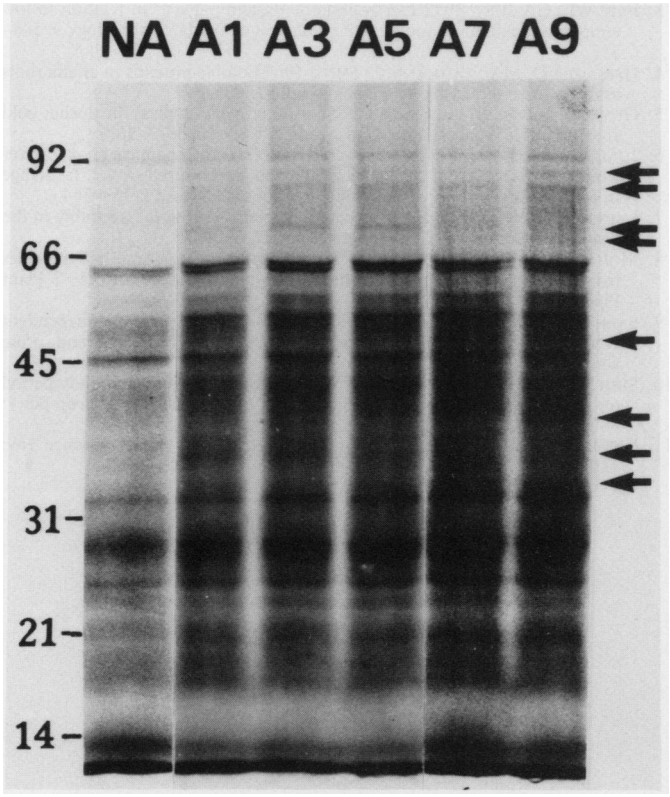

FIG. 2. Fluorograph of *in vitro* translation products, separated by SDS-PAGE, from poly(A)$^+$ RNA of nonacclimated plants (NA) and from plants acclimated for 1, 3, 5, 7, and 9 d (A1, A3, etc.). Translation products containing 50,000 cpm were loaded in each lane. Arrows indicate the position of polypeptides specific to mRNA from acclimated plants. Protein sizes are indicated in kD.

polypeptides appeared: there was only an increase in the amount of proteins which were already being synthesized (data not shown).

Results obtained with immunoblotting (Fig. 1b), where an antiserum against proteins from acclimated plants was reacted with proteins from nonacclimated (NA) or acclimated (A) plants also show that new proteins appear in acclimated plants. These proteins, shown by their M_r on the right, largely correspond with those seen in the gel stained with Coomassie blue (Fig. 1a). In addition, three polypeptides (58, 65, and 73 kD), shown on the left, decrease in amount during acclimation. Results obtained with SDS-PAGE and fluorography of the *in vivo* labeled proteins (Fig. 1c) from nonacclimated (NA), acclimated (A), and deacclimated (DA) seedlings show the appearance during acclimation of several proteins which correspond in M_r with those shown in Figure 1, a and b. In addition, other polypeptides (68 and 95 kD) are shown to appear during acclimation only by this technique. Fluorography of proteins from deacclimated (after 14 d of acclimation) seedlings (Fig. 1c; DA) shows that several proteins synthesized by acclimated seedlings are not synthesized by nonacclimated seedlings. In general, the pattern of protein synthesis in deacclimated seedlings resembles that of nonacclimated seedlings. We have determined that the freezing resistance after 2 d of deacclimation was 26 ± 2% survival at −10°C which is quite similar to that of nonacclimated seedlings.

It is, therefore, concluded that during acclimation some preexisting proteins disappear, some others increase in amount, and some new ones are synthesized. Furthermore, during 2 d of deacclimation, the pattern of protein synthesis returns to be more or less similar to that of nonacclimated seedlings.

Analysis of products of *in vitro* translation of mRNAs from nonacclimated and acclimated seedlings by SDS-PAGE and fluorography is presented in Figure 2. Eight polypeptides of 33, 36, 38, 48, 72, 86, and 90 kD indicated by arrows on the right appear during acclimation. It is concluded that the mRNA population from acclimated seedlings contains several mRNA species which do not seem to be present in the nonacclimated seedlings.

DISCUSSION

The results obtained in the present study show that during cold acclimation of alfalfa, (a) some preexisting proteins decrease while some others increase, (b) several proteins are newly synthesized, and (c) new translatable mRNAs appear. An interesting observation is the gradual increase in the ability of the seedlings during acclimation to synthesize proteins at low temperature (Table I). It is noteworthy that the increase in this ability corresponds to the increase in freezing resistance. This probably reflects a crucial role of proteins in the development of freezing resistance.

Surprisingly, during the first 2 d of acclimation when the ability to synthesize proteins at 4°C is low, the ability to synthesize proteins at 20°C is also low. The reasons for this are unclear. It is possible that some changes in the properties of the protein synthesizing apparatus have taken place such that protein synthesis at either 4°C or 20°C is temporarily impaired. Alternatively, the lower rate of incorporation of label at 20°C after 2 d at 4°C may be related to an adaptation to the lower temperature. Thus it may be significant that the temperature coefficient of incorporation, expressed as (rate at 20°C)/(rate at 4°C), falls from about 7 in the nonacclimated plants to about 2 after 2 d of acclimation, and remains at that value (within experimental error) for up to 17 d of acclimation.

Despite low rates of protein synthesis during the initial period of acclimation, most of the new polypeptides appear at only 2 d of acclimation (Fig. 1a). However, development of maximum freezing resistance requires a longer acclimation period. Perhaps the factors responsible for freezing resistance have to accumulate to a greater level in order to confer a greater freezing resistance. Interestingly, only 2 d of deacclimation are sufficient to bring down the freezing resistance to the level of nonacclimated seedlings and to cause a substantial decrease in the rate of synthesis of the acclimation-specific proteins (Fig. 1c). As shown previously for spinach by Guy *et al.* (5), we established that the incorporation of label into acclimation-specific proteins could be seen whether the incorporation itself was carried out acclimating or at nonacclimating temperatures (results not shown). Thus the effect of 2 d deacclimation (Fig. 1c) is not due to an immediate effect of temperature on the pattern of protein synthesis.

Earlier studies of changes in the electrophoretic pattern of proteins during acclimation of alfalfa (2–4) have shown much less marked changes in proteins probably because of the less sensitive techniques used. In the present study results from three different techniques, electrophoretic patterns shown by staining, immunoblotting, and fluorography of *in vivo* labeled proteins confirm that at least eight polypeptides are increasingly or newly synthesized during acclimation. Our observation that new polypeptides appear in the *in vitro* translation products of mRNA from acclimated seedlings strongly suggests that during cold acclimation changes in gene expression occurs at the transcriptional level.

Increased activity of DNA-dependent RNA polymerase and increased RNA content during cold acclimation of wheat have been reported (9). Although we have not measured RNA polymerase activity in the present study, the increased RNA content observed suggests that this enzyme activity increases also during the cold acclimation of alfalfa.

The limitations of the present study need to be pointed out. First, it does not provide definitive evidence for the transcriptional regulation of gene expression during cold acclimation. Second, there is no conclusive evidence that the proteins which are newly synthesized during acclimation are causally related to the development of freezing resistance. However, such a causal role would appear to be plausible from the parallel increases in the ability to synthesize proteins at low temperature and in the ability to survive freezing temperature, clearly demonstrated in the present study.

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