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Communication

Alterations in Membrane Protein-Profile during Cold Treatment of Alfalfa[1]

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ABSTRACT

Changes in pattern of membrane proteins during cold acclimation of alfalfa have been examined. Cold acclimation for 2 to 3 days increases membrane protein content. Labeling of membrane proteins *in vivo* with [^{35}S]methionine indicates increases in the rate of incorporation as acclimation progresses. Cold acclimation induces the synthesis of about 10 new polypeptides as shown by SDS-PAGE and fluorography of membrane proteins labeled *in vivo*.

The cellular membranes (2) including the plasma membrane (9) are intimately involved in cold acclimation and freezing injury. Thus, it is suggested that the membranes have to undergo certain changes during cold acclimation in order to withstand freezing stress. There are several reports on membrane changes during cold acclimation (2, 3, 9, 11, 16). However, most of these studies focused on changes in chemical and biophysical properties such as composition and fluidity of the lipid component of membranes (3, 11, 16). Altered pattern of synthesis of membrane proteins has not received much attention (15, 16).

We recently reported changes in protein patterns and translatable mRNA populations during cold acclimation of alfalfa seedlings (7, 8). The proteins examined in these studies were from a 40,000*g* supernatant and were, probably, mostly soluble proteins with some contamination by membrane proteins. Here we report changes in the pattern of synthesis of membrane proteins isolated from alfalfa crown and root tissue during cold acclimation. The results obtained show that cold-treatment induces changes not only in the soluble proteins (156,000*g* supernatant) but also in the membrane protein profile.

MATERIALS AND METHODS

Plant Material and Cold Acclimation. Seedlings of alfalfa (*Medicago falcata* cv Anik) were grown and cold-acclimated as described previously (7). Briefly, seedlings grown for 7 d at 20°C were transferred to another growth chamber maintained at 4°C and right conditions as before. Acclimation time of only up to 3 d was used since most of the new acclimation specific soluble proteins become detectable during this time (7).

Labeling *In Vivo*. Thirty seedlings (weighing about 0.5–1 g), nonacclimated or cold-acclimated were rinsed with sterile distilled H_2O and labeled *in vivo* with [^{35}S]methionine as described previously (7) except that the labeling continued for 12 h for cold-acclimated seedlings. Long labeling period was necessary to obtain sufficient radioactivity incorporated into membrane proteins to make the analysis possible.

Preparation of Proteins. Proteins were extracted from crown plus root tissue excised after labeling, essentially as described (14). Crown and root tissues were used as the source of protein extract since they constitute the overwintering part of the seedling. The seedlings were homogenized in liquid N_2 to powder using mortar and pestle, thawed to 4°C, and the powder was resuspended in 0.5 ml of extraction buffer (50 mM Tris HCl [pH 8.0], 5 mM EGTA, 50 mM NaCl, 5 mM β-mercaptoethanol, 5 mM DTT, 1 mM spermidine, 0.5 M sucrose, and 1 mM Phenylmethyl sulfonyl fluoride). The homogenate was centrifuged at 14,000*g* for 15 min and the supernatant obtained was recentrifuged at 156,000*g* to obtain the membrane pellet which was used as the source of membrane proteins. The supernatant was used as the source of soluble proteins.

Gel Electrophoresis. For SDS-PAGE, the membrane proteins were solubilized in the sample buffer (62.5 mM Tris HCl [pH 6.8], 2% [v/v] SDS, 10% [v/v]). Glycerol, 5% (v/v) β-mercaptoethanol and heated at 100°C for 5 min. The solubilized protein extracts were then obtained by centrifugation at 100,000*g* for 15 min. Aliquots of this supernatant were used to determine the protein content by the dye-binding method (1). TCA precipitatable radioactivity was determined by liquid scintillation spectrometry. Equal amounts of protein radioactivity were separated by SDS-PAGE essentially by procedures of Laemmli (6) modified as described elsewhere (7) and the gels were then fluorographed (7).

RESULTS

Membrane protein content increases during cold acclimation (Table I). This increase is about 25% after 2 d of acclimation and more than 50% after 3 d of acclimation. Changes in the rate of membrane protein synthesis (rate of [^{35}S]methionine incorporation) at 4°C are also shown in Table I. Nonacclimated seedlings show a rate of incorporation of 1900 cpm/mg protein/h. The rate increases to 4200 cpm after 2 d of acclimation and 6500 cpm after 3 d of acclimation. It may be concluded that during cold acclimation the ability to synthesize membrane proteins at 4°C increases as acclimation continues. This leads to an increase in the membrane protein content of the tissue. Acclimation of 2 to 3 d at 4°C increases the freezing tolerance of seedlings. It is seen (Table I) that 22% of the nonacclimated seedlings survive −10°C

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Table I. *Increase in Membrane Protein Content and Rate of Synthesis during Cold Acclimation of Alfalfa*
Figures in parenthesis represent protein content as percent of that in the nonacclimated control.

Acclimation Period	Survival (3 h at −10°C)	Protein Content[a]	Rate of Incorporation (temperature)
d	%	*mg/g fresh wt*	*cpm × 10⁻³/mg protein · h*
0 (control)	22 ± 2	3.16 ± .23 (100)	16.3 ± 1.8 (20°C)
			1.9 ± 0.7 (4°C)
2 d—acclimated	36 ± 5	3.93 ± .67 (124)	4.2 ± 0.9 (4°C)
3 d—acclimated	40 ± 3	4.95 ± .76 (156)	6.5 ± 0.9 (4°C)

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

treatment. Percent survival increases to 40% after 3 d of acclimation.

In soluble fraction (Fig. 1a) used here as a control, several polypeptides (marked by thick, short arrows) are synthesized specifically during acclimation period by crown and root tissue. Many changes in the pattern of membrane proteins are detectable after 2 or 3 d of cold acclimation. Six polypeptides which decrease during cold acclimation are indicated by thin, long arrows. There are several polypeptides which are either newly synthesized or the synthesis of which is considerably increased (thick short arrows). Four polypeptides (marked by stars) appear to show gradual increases in their rates of synthesis as the seedlings are acclimated for 2 or 3 d. It is concluded that during cold acclimation some proteins are decreasingly synthesized while others are increasingly or newly synthesized.

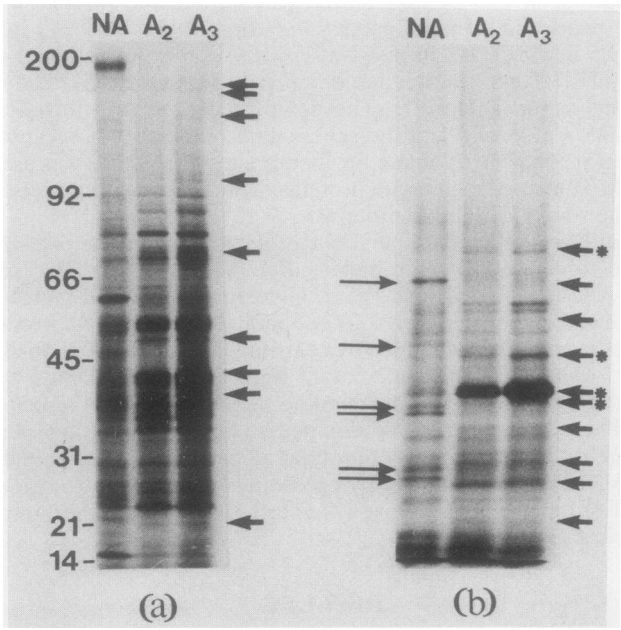

FIG. 1. Fluorographs of soluble (a) and membrane (b) proteins labeled *in vivo* and separated by SDS-PAGE from nonacclimated (NA) seedlings and seedlings acclimated for 2 d (A2) and 3 d (A3). Incorporation was carried out at the respective growth temperatures, *i.e.* 20°C for nonacclimated seedlings and 4°C for acclimated seedlings. Following labeling, crowns and roots were excised from the seedlings, proteins extracted, and a sample containing 150,000 cpm in case of soluble (a) and 100,000 cpm in case of membrane (b) fraction was loaded in each lane. Numbers in the left margin indicate M_r of the marker proteins in kilodaltons. Positions of arrows with small tails indicate polypeptides specific to acclimated seedlings. Positions of arrows with long tails indicate the polypeptides that are specific to the nonacclimated seedlings. Stars indicate the polypeptides which show increases in their rate of synthesis with cold acclimation.

DISCUSSION

The present study demonstrates that membrane protein content increases during cold treatment of alfalfa seedlings. This increase is due to increased synthesis of several specific proteins.

Membrane augmentation including protein increases during cold acclimation has been reported by others (4, 10). But the reasons underlying the increase in protein content are not understood. Since the rate of protein synthesis at 4°C is only about 12 to 40% of that at 20°C during 2 to 3 d of acclimation, a decreased breakdown due to low temperature may play a role in bringing about the observed increase in membrane protein content.

A few studies of qualitative changes in membrane protein patterns during cold-acclimation have been reported (15, 16). A major problem in these analyses of membrane protein profiles was the use of conventional staining procedures which do not provide good resolution (13). Moreover, analysis of membrane proteins from plants acclimated for 30 d may have introduced developmental changes in the plants (12). These problems have been circumvented in the present study by labeling of membrane proteins *in vivo* after 2 to 3 d of cold-acclimation. The changes in protein patterns are detected by separation of the labeled products using SDS-PAGE and fluorography. Clearly this procedure provides better resolution for visualization of membrane protein changes.

Analysis of soluble proteins from crown and root tissues shown here as control, reveals some additional polypeptide changes in the protein patterns compared to the earlier report (7). This may have significance in that some proteins may be expressed in a tissue-specific manner. It is known that several specific mRNAs and soluble proteins, the levels of which can be correlated with the development of freezing tolerance, appear as early as 1 to 2 d of cold acclimation (5, 7). Although development of maximum freezing tolerance requires a longer acclimation period, some increase in percent freezing survival has been noted after 2 to 3 d of acclimation (Table I). Perhaps the proteins have to accumulate to a greater level in order to confer maximum freezing tolerance. The possibility that some of these proteins may represent "cold shock" proteins cannot be ruled out. Although because of increased rates of synthesis of some specific proteins (Fig. 1b), it seems unlikely.

Because changes in both soluble and membrane proteins occur simultaneously during acclimation, involvement of membrane proteins in development of freezing tolerance is difficult to demonstrate by a time course analysis (7). To assign a role to these polypeptides in development of freezing tolerance changes in membrane protein patterns have to be compared in freezing tolerant and freezing sensitive cultivars. Alternatively, changes in membrane protein patterns may be compared after acclimation induced by low temperature, ABA, and desiccation treatment. The latter two are also known to induce freezing tolerance in plants.

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