Plant Physiol. (1986) 82, 733–738
0032-0889/86/82/0733/06/$01.00/0

Changes in Protein Synthesis in Rapeseed (*Brassica napus*) Seedlings during a Low Temperature Treatment[1]

Received for publication July 23, 1985 and in revised form July 7, 1986

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ABSTRACT

Changes induced by cold treatment in young rapeseed (*Brassica napus*) seedlings were investigated at the molecular level. Following germination at 18°C for 48 hours, one half of the seedlings was transferred to 0°C for another 48 hour period, the other half being kept at 18°C as a control. Newly synthesized proteins were labeled for the last 6 hours of incubation with [^{35}S]methionine. The different polypeptides were separated by two-dimensional electrophoresis in polyacrylamide gels. Newly synthesized proteins were revealed by fluorography. Protein synthesis clearly continues at 0°C and some polypeptides preferentially accumulate at this temperature. On the other hand, synthesis of several others is repressed while many are insensitive to cold treatment. Similar changes are also observed when mRNA is prepared from cold treated seedlings, translated *in vitro* in a reticulocyte cell free system and compared with the products of mRNA extracted from control samples. Among the genes which are repressed we identified the small subunit of ribulose 1,6-bisphosphate carboxylase. These changes are also detectable after shorter treatments.

During the last few years, it has been demonstrated that higher plants are able to respond to various environmental changes by inducing the synthesis of specific proteins. The best known examples are the induction of heat shock proteins (10, 11, 17) and anaerobic proteins (4, 19, 22). Other forms of stress have also been studied, such as osmotic shock, desiccation or infection by fungi or viruses. In several situations exposure to a moderate stress very often allows the plant to adapt itself and to resist to a subsequent stronger treatment. Such a mechanism seems to be used by plants to adapt to chilling or freezing temperature and it has been reported that exposure for a few hours or days at low, nonchilling temperatures can protect them against freezing (3, 8, 9, 14). Various biochemical responses of plants to low, nonfreezing temperatures have been widely documented and reviewed recently (6). They involve changes in protein content and enzyme activities (2, 3, 7, 14, 20), metabolic modifications and changes in lipid composition and membrane structure (21, 24, 25). Until recently (8), the effects of low temperatures on gene expression have not been analyzed in detail and in most situations it is not clear whether the changes in protein content and enzyme activity result from inactivation or activation of preexisting enzymes, from changes in mRNA abundance or translation efficiency, or from other mechanism. As a first step in understanding regulation of gene expression in response to a cold stress, we have investigated *in vivo* and *in vitro* protein synthesis in young rapeseed seedlings after 48 h exposure to nonchilling temperature. This species has been chosen as a model because it is very convenient to handle in a laboratory environment and because it is an important crop in many countries with temperate or cold climates. Preliminary results have indicated that this plant can adapt to freezing conditions during exposure to nonchilling temperature (9, 24) and that varieties with improved germination at low temperature can be selected (1). In addition, winter and spring cultivars are available so that it should be possible to observe various degrees of cold tolerance. This report deals with cultivar Jet Neuf, a chilling-resistant winter variety.

Our results provide clear evidence that the apparent synthesis of specific polypeptides is increased by the cold treatment whereas that of others is repressed.

MATERIALS AND METHODS

Growth Conditions. Rapeseed (*Brassica napus*) seeds (cv Jet Neuf) were germinated at 18°C, in a dark incubator, on wet filter paper without nutrient medium. After 48 h seed coats were removed and the seedling sample divided into two batches. One of them was transferred for another 48 h to a cold room (2°C) and kept on an ice tray. (This sample is referred to as the 0°C sample, the temperature being between 0 and 2°C). The other was incubated at 18°C as a control. In both situations seedlings are grown in the dark.

'In Vivo' Labeling and Protein Extraction. Labelings were carried out with 20 seedlings in a Petri dish. They were incubated with 500 μl of [^{35}S]methionine solution (175 μCi/ml, 1470 Ci/mmol) either at 0 or 18°C, during the last 6 h of the 48 h treatment period. At the end of the labeling, seedlings were washed with freshly prepared 3% calcium hypochlorite for 30 s and then rinsed with sterile distilled H_2O. They were transferred to a precooled mortar and ground under liquid N_2. The powder was resuspended in 4 ml of extraction buffer 50 mM Tris HCl (pH 8), 100 mM NaCl, 1 mM unlabeled methionine, 10% (w/v) sucrose, 1% (v/v) β-mercaptoethanol and 50 μg/ml phenyl-

[1] Supported by the CNRS (UA 565 and A.I.P. 95 31 67) in France and by grants from Direccion de investigacion (UACH S-84-29, RS-83-19 and FNC 1212-84) in Chile. Part of this work was carried out while Dr. Luis Meza-Basso was on leave from UACH and was a visiting Professor at the University Louis Pasteur in Strasbourg; M.L. Ferrero-Cadinanos was supported by a student fellowship from the exchange programme between the Spanish CSIC and the French CNRS and by the Réseau européen de Biologie moléculaire végétale.

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methanesulfonyl fluoride. The homogenate was centrifuged 10 min at 9000 rpm in a Sorvall SS-34 rotor at 2°C. Aliquots of the supernatant were taken in order to estimate [^{35}S]methionine uptake and incorporation into TCA precipitable material. Proteins were assayed by the Lowry method (15).

The remaining supernatant was precipitated with 8 volumes of acetone at −20°C. The precipitate was spun down, dried, and dissolved in the appropriate electrophoresis buffer.

mRNA and *in Vitro* Protein Synthesis. Total RNA was prepared as previously described from at least 100 seedlings (13). It was fractionated into poly(A$^+$) and poly(A$^-$) RNA by oligo-dT cellulose column chromatography. Poly(A$^+$) RNA containing fractions were pooled and precipitated with ethanol. The precipitate was dissolved in sterile water and the mRNA solution stored at −80°C. This mRNA solution was then translated with the reticulocyte lysate cell free synthesis system in the presence of [^{35}S]methionine. The reticulocyte lysate and [^{35}S]methionine were purchased from Amersham Ltd (U.K.) and used as previously described (13). Tobacco mosaic virus RNA was used as a control of the activity of the cell-free system. Translation products were analyzed by PAGE using one-dimensional and two-dimensional gels.

The precursor for the small subunit of rubisco[5] was identified by immunoprecipitation with an antibody against radish rubisco small subunit. The antibody was prepared, characterized, and given to us by P. Fourcroy (5). It was used according to published methods (16).

Gel Electrophoresis. To separate proteins in one dimension, the SDS-polyacrylamide gel system (12) was employed using 12.5% slab gels. For two-dimensional separations we used the O'Farrell system (17, 18). The first dimension was a nonequilibrium pH gradient electrophoresis run in cylindrical polyacrylamide gels containing Pharmacia ampholytes (pH 3.5–10). The sample was loaded at the acid end. After migration, the gel was equilibrated in second dimension sample buffer and placed on top of a classical 12.5% SDS-polyacrylamide slab gel (12).

The two-dimensional gels were fluorographed as already described (13). To be able to compare autoradiograms we loaded equal amounts of radioactivity on the gels and several gels with different samples were run at the same time in identical tanks. In addition, size markers were included in the second dimension gel, and several spots which coincide from one experiment to another were used as internal standards.

RESULTS

General Changes Induced by Cold Treatment. Before investigating changes at the level of induction or repression of specific genes we examined various growth parameters. This section is a summary of these preliminary observations.

The most striking effect of cold treatment is a considerable slowing of elongation of the hypocotyl. In the dark at 18°C the length of the hypocotyl very rapidly increases between 48 and 96 h of germination from about 1 cm to approximately 4 cm. In contrast, at 0°C its size increases only slightly. During this period the fresh weight almost doubles at 18°C, from 35 mg/seedling to 65 mg/seedling at 96 h, while it remains virtually unchanged during the incubation at 0°C.

Due to storage protein hydrolysis in the cotyledons, the protein amount in soluble extracts gradually decreases by about 25% in seedlings grown at 18°C during the studied period. In contrast, at 0°C the protein level remains relatively stable. Analysis of the proteins by SDS-PAGE and staining with Coomassie blue reveals clear differences at the end of the cold treatment between the cold-treated and the control sample. These preliminary results cannot be simply interpreted as a difference in the rate of degradation of storage proteins, but rather suggest that some specific polypeptides might be preferentially synthesized at 0°C. This led us to study protein synthesis using a radioactive tracer. Both cold-treated and control seedlings incorporate [^{35}S]methionine into their proteins although incorporation at 0°C seems to be less efficient than at 18°C; radioactivity incorporated into hot TCA-insoluble material representing 39% of the uptake at 18°C and only 24% at 0°C. Uptake is not significantly different at 0 and 18°C.

Since both samples actively synthesized proteins it was interesting to compare their protein synthesis patterns and to investigate mechanisms controlling expression of specific genes.

Changes in *in Vivo* Protein Synthesis. Preliminary experiments (not shown) using one-dimensional gels reveal obvious differences between protein synthesis patterns from seedlings kept at 18 or 0°C. However, the resolution of these gels was not good enough to determine how many polypeptides were affected. Thus, the samples were compared using a two-dimensional gel electrophoresis system.

The patterns obtained with proteins extracted from seedlings maintained at 18°C or transferred to 0°C for an additional 48 h incubation are shown in Figure 1 (top 18°C, bottom 0°C). As a further control, proteins were also extracted from 48 h old seedlings, just before transfer to 0°C; their pattern was almost the same as after 2 more d at 18°C (not shown).

Such patterns are rather complex and difficult to analyze. Although approximately equal amounts of radioactivity were loaded on the gels, it is often hazardous to compare directly the intensities of a given spot between two samples and it is safer to compare its intensity with that of other spots on the same autoradiogram. Fortunately, numerous spots do not change in relative intensity when the protein synthesis temperature is shifted from 18 to 0°C and can be used as references. We restricted our analysis to a few prevalent spots which show a clear cut and reproducible change in their relative intensity after a temperature shift. Spots numbered 1 to 14 (arrows) have an increased intensity after a cold treatment and the corresponding polypeptides therefore account for an increased proportion of proteins newly synthesized at the lower temperature. In contrast, the relative intensity of spots numbered 15 to 20 (open triangles) decreases and several of them are no longer detectable on the 0°C patterns. To rule out degradation artefacts, proteins were also extracted in the presence of 15% TCA. All the spots shown in Figure 1 are visible after such an extraction except those in the more basic region of the gels. To determine if these changes are detectable earlier following the temperature shift, we carried out a time course study. The relative increase in the intensity of spots 1 to 14 observed after a 48 h cold treatment is in fact detectable after only 6 h at 0°C and the patterns of protein synthesis from seedlings exposed to 0°C temperature for various times are similar to that shown in Figure 1.

We then investigated whether these changes in *in vivo* protein synthesis result from changes in the relative abundance of the different mRNAs in the mRNA populations or from changes in their translation efficiency.

Changes in *in Vitro* Protein Synthesis. Polyadenylated mRNA was isolated from control and cold treated seedlings and translated *in vitro* using the reticulocyte lysate cell-free protein synthesis system. We reproducibly observe that cold-treated seedlings contain more RNA on a per seedling basis and that the final yield in poly(A$^+$) mRNA was higher with cold treatment samples, but we did not investigate this point in detail.

The translation products corresponding to the two different mRNA samples were analyzed by two-dimensional gel electrophoresis. A zero time control at 18°C was also carried out and found identical to the 48 h control. Figure 2 corresponds to translation products of mRNA extracted from control (top) or

[5] Abbreviation: rubisco, ribulose 1,5-bisphosphate carboxylase.

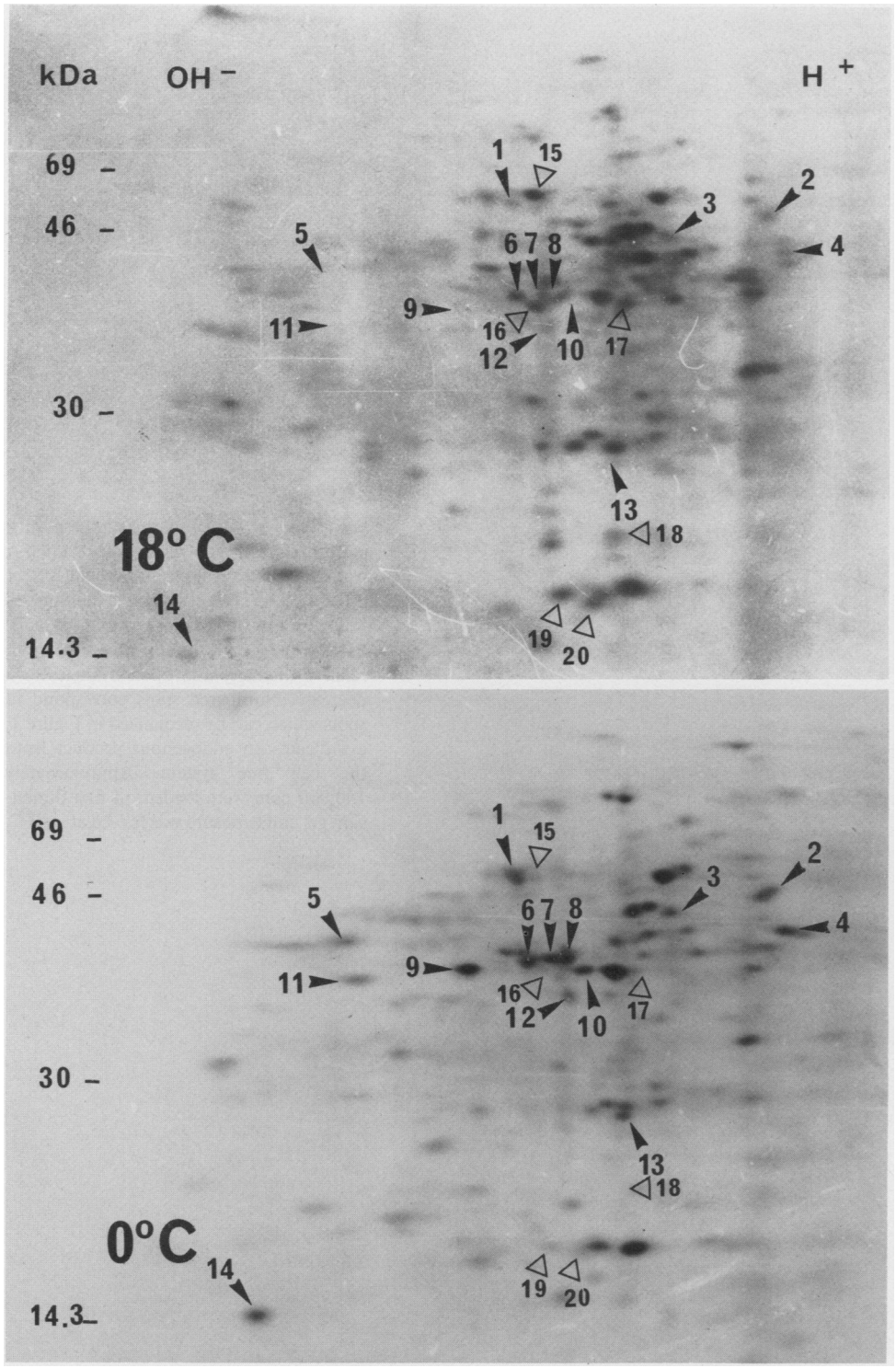

FIG. 1. *In vivo* protein synthesis in Jet Neuf seedlings grown at 18°C (top) or 0°C (bottom). *In vivo* labeled proteins were separated by two-dimensional gel electrophoresis (18). Second dimension gels were 12.5% SDS polyacrylamide gels. Approximately 180,000 cpm were loaded on each gel. The autoradiograms were exposed for 2 d at −80°C. The ^{14}C-labeled size markers were the followings: lysozyme (14.3 kD), carbonic anhydrase (30 kD), ovalbumin (46 kD), BSA (69 kD). The different types of arrows correspond to the different evolutions of the spots when seedlings are grown at 0°C. The intensity of spots 1 to 14 (black arrows) increases, that of spots 15 to 20 (open triangles) decreases.

cold treated seedlings (bottom). The *in vitro* synthesis patterns show the same type of variations as the *in vivo* patterns, the relative intensity of a given spot being either increased (arrows), unchanged or decreased (open triangles) by the temperature shift. Comparison between *in vivo* and *in vitro* patterns is not easy because primary translation products, detected on the *in vitro* pattern are not always identical to the final products which are detected *in vivo*. Nevertheless, several spots are observed at the same position on both *in vivo* and *in vitro* patterns. We have tentatively assumed that they correspond to the same polypeptides and are identified by the same number in Figures 1 and 2 (spots 2, 6–10). Several other spots cannot be identified at this stage of the experiment and they have been labeled by an arrow or a triangle without a number. These spots might correspond to mRNAs for precursor proteins which are processed *in vivo*, to mRNAs which are not translated *in vivo* at this developmental stage, or to *in vitro* translation artefacts.

Changes in the Level of Translatable mRNA for Rubisco Small Subunit. Two prevalent spots are clearly visible on the 18°C *in vitro* protein synthesis pattern in the 22 to 23 kD region. Their intensity is reduced following transfer at 0°C suggesting that the level of translatable mRNA for these two polypeptides decays

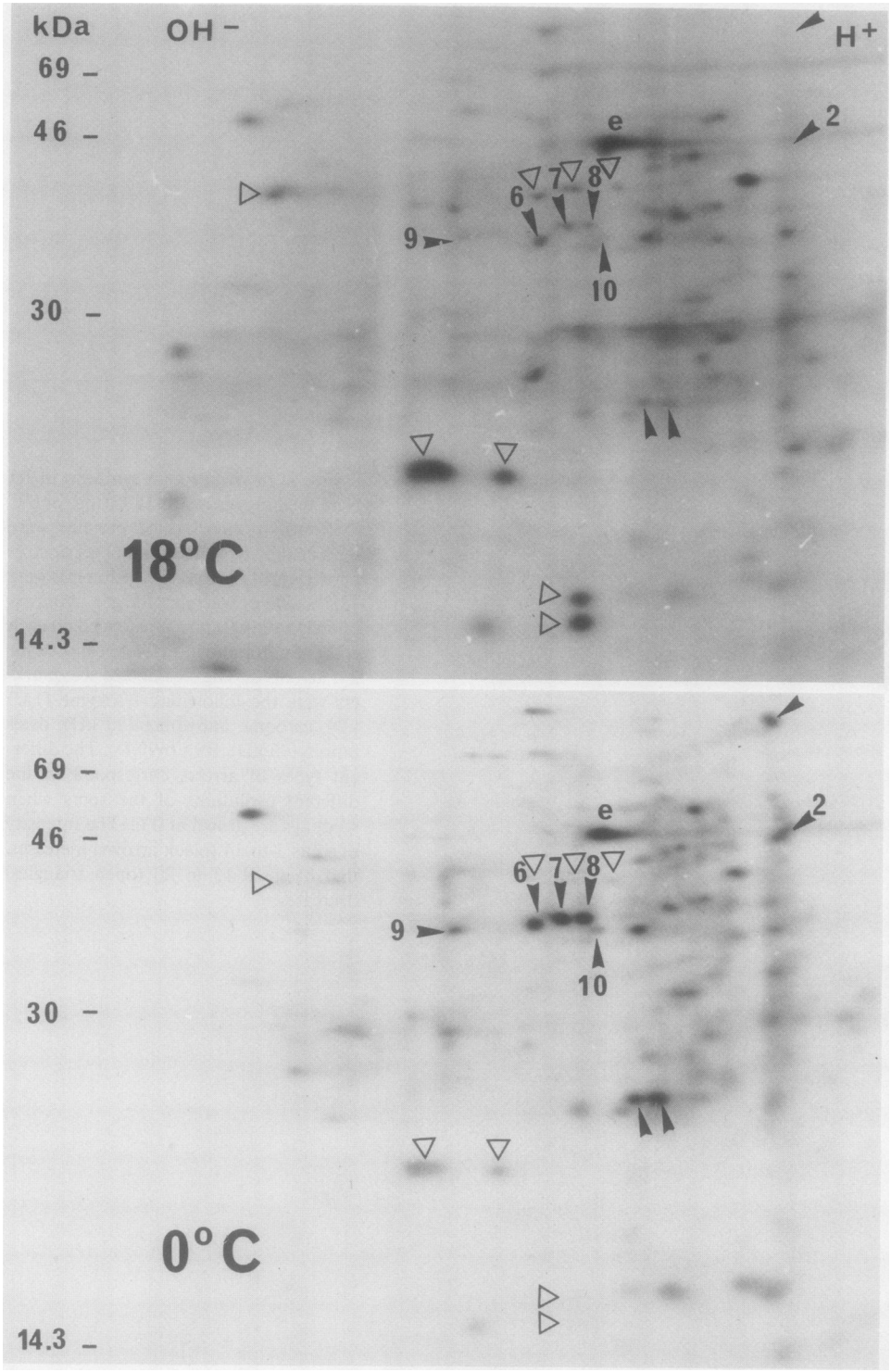

FIG. .2. *In vitro* protein synthesis programmed with polyadenylated mRNA from Jet Neuf seedlings grown at 18°C or 0°C for 48 h. The gel system is the same as in Figure 1 as well as markers and symbols. Black arrows corresponding to increase in relative intensity and open triangles to a decrease. Numbered spots correspond to spots which can be recognized in Figure 1; e indicates an endogenous product from the cell free system. Approximately 150,000 cpm were loaded on first dimension gel and exposure was for 3 d at −80°C.

after the temperature shift. The intensity of these spots, their position in the gel, and the absence of polypeptides in the same region of the *in vivo* protein synthesis pattern suggested to us that they may represent the precursors of rubisco small subunit. As shown in Figure 3 this assumption seems to be correct since the major band in this region on a one-dimensional gel comigrates with products immunoprecipitated by an anti-rubisco small subunit immune serum.

DISCUSSION

Freezing temperatures are very often a problem for many crops. In several examples, exposure to low but not chilling temperatures seems to protect plants against subsequent freezing. It is therefore essential to understand this mechanism and to look for biochemical markers of cold tolerance. They might be useful in predicting the cold hardiness of a new variety or in improving a sensitive cultivar.

This report is an attempt to find such markers at the protein and gene level. We demonstrate that important changes in gene expression do occur during the exposure of young rapeseed seedlings to a low temperature for 2 d.

Control experiments have indicated that there are no major changes in the polypeptide synthesis pattern from seedlings grown at 18°C during the studied period and therefore, the

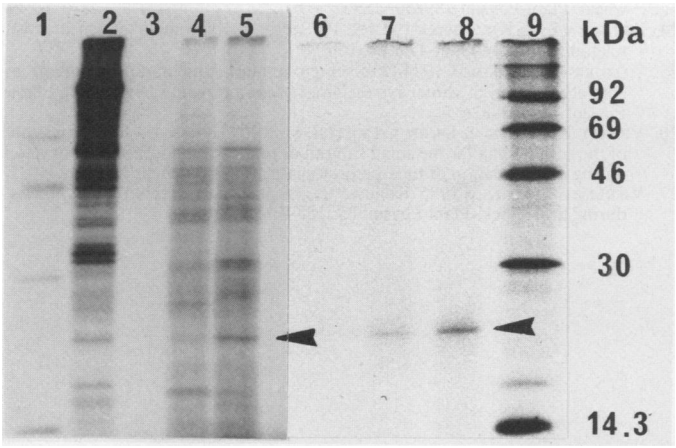

FIG. 3. Identification of rubisco small subunit among cell-free translation products. Lanes 1 and 9, mol wt markers; lane 2, translation products from TMV mRNA; lanes 3 and 6, endogenous synthesis without added mRNA; lanes 4 and 7, cell free protein synthesis programmed with mRNA from seedlings exposed for 48 h to a 0°C treatment; lanes 5 and 8, cell free synthesis programmed with mRNA from control seedlings maintained at 18°C during the same period. Lanes 6, 7, and 8 correspond to products described in lanes 3, 4, 5, immunoprecipitated with antibodies specific for rubisco small subunit. Arrows indicate the position of the 23 kD radish rubisco small subunit precursor.

modifications which have been observed following cold treatment correspond to the response to this temperature shift rather than to a developmental change.

We observed that the relative synthesis of specific polypeptides is increased at 0°C, and that the relative amount of the corresponding mRNA has increased too. Whether this change in mRNA amount results from increased synthesis or modified stability is not yet clear. Synthesis of other polypeptides is severely reduced and we also observed a concomitant degradation of several mRNAs. Therefore, our results confirm previous data (3, 6, 9, 25) that protein patterns change during cold acclimation. They extend the available information in showing that a few other proteins are apparently repressed and that these changes result from modifications in the level of translatable mRNA. Increase in total RNA amount and decrease in RNase activity have recently been associated with cold acclimation (23). Our results demonstrate that a more complex situation occurs and that the relative concentration of different specific mRNAs may either increase or decrease. Recently, a similar study on spinach leaves has provided evidence for an increase of specific mRNAs correlated with improved freezing tolerance (8).

The major drawback of the analysis of protein synthesis patterns by two-dimensional gel electrophoresis is that no function can be ascribed to a specific protein. However, this technique is presently the most sensitive to gain a general overview of protein synthesis and to analyze the expression of genes of as yet unknown function. Our results indicate that the synthesis of a limited number of proteins is altered after a shift to a cold temperature and that, in contrast to heat shock or anoxia, there is no abrupt drop in total protein synthesis (10, 11, 22). In addition, this type of analysis might help in purifying some of the polypeptides which are induced or repressed and in comparing accurately the response to cold treatment with that to other stresses.

At this stage of our investigations we are left with two main problems. The first is to identify some of the polypeptides which show an altered synthesis following the temperature shift and the second is to determine how changes in gene expression are relevant to improved cold hardiness.

The relationship between changes in gene expression and adaptation is illustrated by our observation that the amount of mRNA for rubisco small subunit is decreased after a cold treatment. Indeed this gene seems to be very sensitive to many environmental changes since synthesis of this protein is reduced immediately after osmotic or heat shock (26, 27). Therefore, this change does not seem to be specific. Another approach to elucidate this problem would be to establish a correlation between changes in the synthesis of a given protein in different cultivars and their various degrees of cold tolerance. Preliminary results obtained with a spring rapeseed suggest that this approach might be feasible.

Several strategies might be used to identify the polypeptides whose synthesis is affected. Several enzymes have been reported to undergo activity changes during cold treatment (2, 6, 7, 14, 20). They are likely candidates as polypeptides with altered synthesis. Some of them have been purified and antibodies and cloned probes will be soon available. They can be recognized in the same way we identified rubisco small subunit. A further step is the construction of a cDNA library and isolation of clones corresponding to genes which are activated or repressed. Work is now in progress to screen such a library.

Acknowledgments—The authors wish to thank Marie-Thérèse Lacoste, Yvette Chartier, and Alain Got, for their help in preparing the manuscript and during the experiments. They thank Professors J. H. Weil and P. Penon for providing laboratory facilities and for their continued interest.

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