

Cold resistance in rapeseed (*Brassica napus*) seedlings. Searching a biochemical marker of cold-tolerance

Resistencia al frío en plántulas de raps (*Brassica napus*)
Búsqueda de un marcador de tolerancia al frío

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The synthesis of proteins has been analyzed in young seedlings of a winter and a spring variety of rapeseed (*Brassica napus*) at two different temperatures, 18°C and 0°C. The different polypeptides were separated by two dimensional polyacrylamide gel electrophoresis. The synthesis of several polypeptides is stimulated at 0°C whereas that of others is repressed.

Similar changes in the mRNA population are also obvious when mRNAs obtained from cold-treated and control seedlings are translated *in vitro* in a cell-free system and then the translation products are compared. Several mRNAs are apparently more abundant at 0°C than at 18°C while others are less abundant or have completely disappeared. Differences were noticed in the response of the winter and spring varieties at the low temperatures. L-phenylalanine ammonia-lyase is induced specifically in the cold-tolerant cultivar, suggesting that it might be a useful biochemical marker in predicting the cold sensitivity of new variety or in improving a sensitive cultivar.

Key words: Protein synthesis, cold acclimation, rapeseed.

INTRODUCTION

Plant species are exposed to a wide variety of adverse environmental stresses in many of the geographical areas in which they are grown. Those plants adapted to warm habitats are quite susceptible to injury by low, above freezing temperatures. For such plants, a relatively brief chilling exposure can have adverse effects on germination, development and productivity. However, certain plant species have evolved the specific capacity to resist low temperatures without permanent damage. Although these cold-tolerant plants differ in their ability to withstand freezing temperatures, in several situations exposure to a moderate stress very often increases their tolerance to subsequent freezing.

Such process is known as cold acclimation or cold hardening (1). Many physiological changes and metabolic adjustments have been shown to occur during cold acclimation including a general increase of soluble proteins (2), free sugars compositions (3, 4), changes in some specific aminoacids (5), an increase in the level of unsaturation of fatty acids in membranes (6), changes in non enzymic proteins (7), isoenzyme composition of a number of enzymes or enzyme conformation (8); nevertheless cause and effect remain to be established. Based on existing data, it seems that proteins are involved in several mechanism for increasing cold-tolerance in higher plants and it has been suggested (9-11) that these changes are controlled by DNA composition and become manifest by

the subsequent sequential activity of RNA, enzymes and proteins. Indeed, many authors have reported that specific polypeptides are accumulated in hardy plant tissues (8, 10, 12) but in most situations it is not clear whether the changes in protein content and enzyme activity result from inactivation or activation of pre-existing enzymes, from changes in mRNA abundance or translation efficiency.

We have previously found changes in proteins and mRNA profiles of an overwintering freezing-tolerant rapeseed cultivar (10). Here we report a more detailed study directed to monitor the effect of low temperature on protein synthesis in two rapeseed seedlings varieties; Jet Neuf, a winter cultivar and Brutor, a spring one. Our results confirm evidences that synthesis of some specific polypeptides and the corresponding mRNAs specifically increased by the cold treatment. Among the proteins induced or increased in cold treated tissues, some were expressed at higher level in the winter cultivar, suggesting that these changes correspond to a specific adaptation more than a consequence of a general modification of the metabolism. One of these polypeptides corresponds to L-phenylalanine ammonia-lyase (EC 4.3.1.5.). The induction of this enzyme seems to be an universal alarm signal because it is very sensitive to many environmental stresses (13, 14) and it might be a useful biochemical marker in predicting the cold resistance or sensitivity of new varieties.

MATERIALS AND METHODS

Plant Material. Rapeseed (*Brassica napus*) seeds (cv. Jet Neuf and cv. Brutor) were obtained from Michele Renard (Station d'Amelioration des plantes INRA, Rennes, France).

Growth Conditions. Seeds from sensitive and resistant rapeseed varieties were germinated at 18°C in a dark incubator, on a wet filter paper. After 48 hours seed coats were removed and the seedlings samples divided in two batches. One half of germinated rapeseed seeds of 48 hours old were transferred to a cold room at 0-2°C for 48 hours. The other one was incubated at 18°C as a control. In both situation seedlings were grown in the dark.

Freezing-Tolerance. Freezing-tolerance of rapeseed seedlings was determined as reported previously (5) and expressed as the temperature required to kill 50% of the seedlings (LT₅₀).

Labeling Conditions and Extraction of Proteins. Labelings were performed with 20 seedlings in a Petri dish. Samples were incubated with 500 µl of ³⁵S methionine solution (175 µCi/ml, 1740Ci/mM) either at 0°C or 18°C during the last 6 hours of the 48 hours treatment period. After labeling, seedlings were washed with a 3% sodium hypochlorite solution for 30 seconds and then with sterile distilled water. Cotyledons were removed and samples ground in a mortar with liquid nitrogen. The powder was resuspended with 4 ml of extraction buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM unlabelled methionine, 10% sucrose, 1% 2-Mercaptoethanol and 50 µg/ml of phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 10,000 x g for 30 min. To determine ³⁵S methionine uptake, aliquots of the supernatant were spotted onto small pieces of Whatman CGF filter paper and air-dried. Filters were boiled in 10% TCA for 15 min, followed by two washes with 70% ethanol, and finally with ether and once scintillation cocktail was added, the radioactivity was recorded (15). The remaining soluble fraction was precipitated with 8 volumes of cold acetone at -20°C. Pellets were recovered by centrifugation at 15,000 x g for 15 min and dried down and resuspended in either Laemmli (16) sample buffer or in O'Farrell (17) lysis buffer (9 M urea, 2% ampholites (pH 3-10), 2% Nonidet P-40, 5% 2-Mercaptoethanol) for two-dimensional electrophoresis. Protein concentration was measured by the method of Rasmussen *et al.* (19) with BSA as standard.

RNA Extraction. Total RNA from cultivars, cold-treated and room temperature controls, was prepared as previously described from at least 100 seedlings (18). Samples were 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 precipitated was dissolved in sterile water and the mRNA solution stored at -80°C.

In vitro Protein Synthesis. Poly (A⁺) RNA was translated *in vitro* using a rabbit reticulocyte lysate system in the presence of ³⁵S methionine as previously described (10, 18). Tobacco mosaic virus was used as a control of the activity of the cell-free system. Translation products were analyzed by using one-dimensional and two-dimensional gel electrophoresis.

Gel Electrophoresis. To separate proteins in one dimension, samples containing around 50 µg of protein were applied to one dimensional 12.5% polyacrylamide gels containing 0.4% SDS. The gel and buffer solution were prepared as described by Laemmli (16). For two dimensional separations we used the O Farrel system (17). The first di-

mension was a non-equilibrium pH gradient containing Pharmacia ampholytes (pH 3.5-10). The sample was loaded at the acid end. After migration, the gel was equilibrated in second dimension buffer and placed on the top of a 12.5% SDS-polyacrylamide slab gels (16).

L-Phenylalanine Ammonia Lyase Purification (EC 4.3.1.5). The enzyme was purified to homogeneity from cut-injured potato tubers tissue, Desiree variety, using basically gel filtration, anionic exchange and affinity chromatography. This method corresponds to a modified protocol described by Havir and Hanson (20).

Antiserum against L-Phenylalanine Ammonia Lyase. Specific antiserum against purified L-Phenylalanine Ammonia Lyase was produced in a rabbit by injecting a mixture of the purified preparation (250-500 µg protein) and Freund's complete and incomplete adjuvant, for three times at 10 days intervals. The titles were followed by using Ouchterlony immunodiffusion method (21). The IgG fraction was obtained using DEAE-column chromatography essentially according to reference (22).

Immunodots and Western Blotting. Products from *in vivo* and *in vitro* synthesis from control and cold-treated cultivars were analyzed using immunodots technique (23). Proteins analyzed by electrophoresis were transferred from polyacrylamide gels to 0.2 µm nitrocellulose sheets (24). A portion of each gel was stained with Coomassie blue. The remainder of the gel was pre-equilibrated in transfer buffer (12.5 mM Tris, 96 mM glycine, pH 8.3, 20% methanol (v/v) and 0.1% SDS. The detection methodology employed primary antibody, a biotinylated secondary antibody and a preformed avidin-biotinylated horseradish peroxidase complex, essentially as described by Hsu *et al.* (25).

RESULTS

General changes induced by cold treatment

Before investigating changes at level of induction or repression of specific genes, various parameters were examined. Young seedlings of the winter and the spring variety of rapeseed were subjected to appropriate hardening conditions (5). The cold-tolerant cultivar (cv Jet Neuf) and the cold sensitive variety (cv Brutor) reached LT₅₀ values of -14°C and -1°C, respectively. Analysis of soluble proteins extracted from both cultivars were performed by SDS-PAGE. As early as 6 h of cold treatment the electrophoretic

pattern showed differences between cold-treated and control samples. The changes were more pronounced in the cold-resistant variety and remained unalterable between 24-48 h. The one-dimension gel resolution was not good enough to determine exactly how many polypeptides were affected (data not shown). Accordingly, samples were analysed using a two-dimensional gel electrophoresis (17).

*Changes in *in vivo* protein synthesis*

The uptake of ³⁵S methionine did not show any difference between both cold-treated cultivars, but its incorporation into soluble proteins showed to be less efficient at 0°C than at 18°C. Labeled soluble proteins from cold-treated and controls were analyzed using 2-D gel electrophoresis. Fig. 1 illustrates the results obtained with Jet Neuf, the winter cultivar. The intensity of a number of spots specially increased during the cold-treatment (Spots 1 to 21 on the 0°C pattern). Most of them could be detected on the 18°C pattern as faint spots; other spots on the other hand decreased and they corresponded to proteins, of which the gene seemed to be repressed at low temperature. Major examples in this category corresponded to spots 51 to 60. In order to make easier the comparison between the two patterns, a series of spots which did not change were numbered from 80 to 96. These spots were used as internal standards.

Fig. 2 shows the two patterns obtained with the spring cultivar, Brutor. Most of the spots in these patterns could be aligned with those in Jet Neuf, and we used the same numbering system. However, some spots detected on the Jet Neuf patterns could be unambiguously identified on the Brutor one, and a few new spots lighted up on the Brutor patterns (spots 22-24 and 61-62). The same observation could be made in relation to the intensity of a number of spots that increased by the cold treatment (spots 2-4, 11, 12, 14, 15, 19-24) whereas others decreased (spots 52, 54-58, 60-62). However, several of the differences noticed in the Jet Neuf patterns

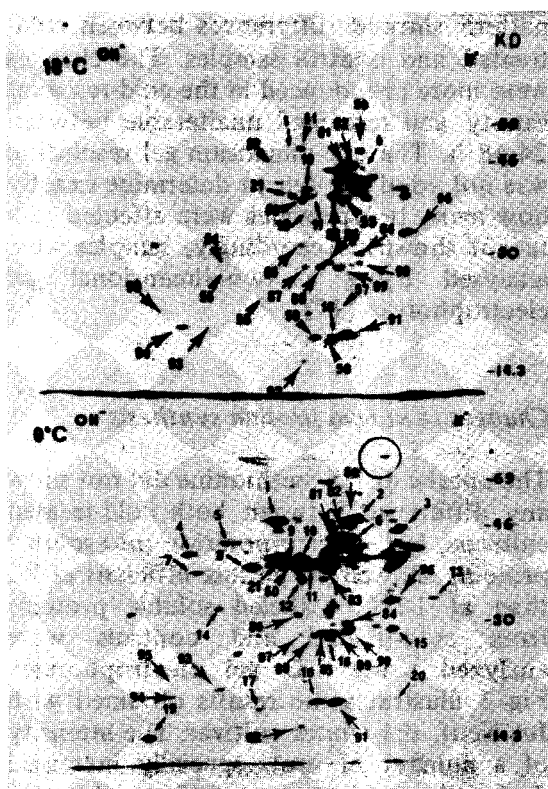


Fig. 1: Changes in pattern of *in vivo* protein synthesis in Jet Neuf seedlings grown at 18°C or 0°C. Fluorographs of *in vivo* labeled proteins separated by 2-D electrophoresis. The different types of arrow correspond to the different evolution of the spots when seedlings are shifted from 18°C to 0°C. The intensity of spots 1 to 21 increases, that of spots 51 to 60 decreases and that of spots 80 to 96 does not change when seedlings are grown at 0°C. The circled spot corresponds to PAL subunit. The ^{14}C size markers were the following: lysozyme (14.3 kD), carbonic anhydrase (30 kD), ovoalbumin (46 kD), bovine serum albumin (69 kD).

did not show up on the Brutor patterns. For instance, spots 10, 13, 16 and 17 did not change in intensity when the seedling were transferred to 0°C. Spot 6 which increased in Jet Neuf, decreased in Brutor. Two spots which did not change in Jet Neuf during this transfer were altered in Brutor: the intensity of spot 87 was decreased whereas that of spot 92 was increased by the shift from 18°C to 0°C.

We then investigated whether these changes in *in vivo* protein synthesis resulted from changes in the relative abundance of the different mRNA in the mRNA populations or from changes in their translation efficiency. Polyadenylated mRNAs were isolated from control and cold-treated

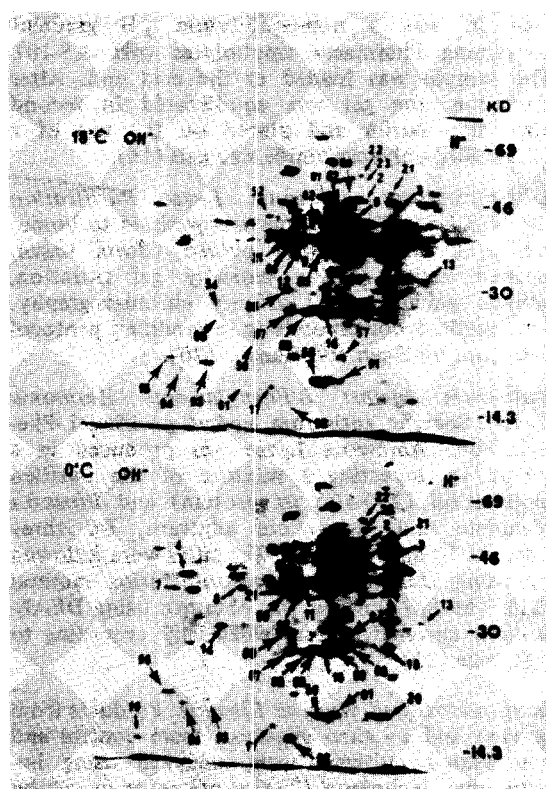


Fig. 2: Changes in pattern of *in vivo* protein synthesis in Brutor seedlings grown at 18°C or 0°C. Experimental conditions and numbering of the spots are as in Figure 1.

seedling and translated *in vitro* using the reticulocyte lysate cell free protein synthesis system.

Changes in vitro protein synthesis

We analyzed the *in vitro* protein synthesis programmed by mRNA prepared from control or cold-treated seedlings. Figure 3 corresponds to translation of Jet Neuf mRNAs. It was difficult to establish a correspondence between spots in the *in vitro* and *in vivo* protein synthesis patterns although some spots were unambiguously identical in both patterns. We therefore used a different system to label the spots. The main difficulty in comparing such patterns is that the primary translation products, detected on the *in vitro* patterns were not always identical to the mature products observed on the *in vivo* patterns.

This analysis showed that at least a dozen of spots had an increased intensity when

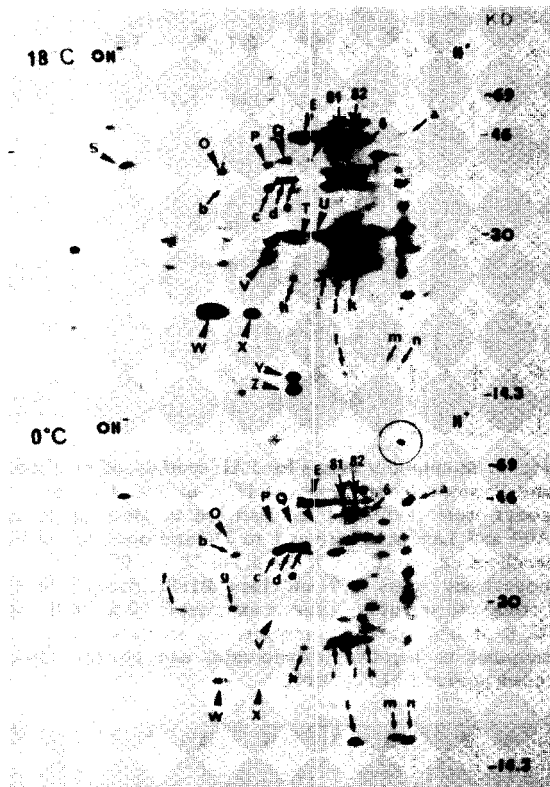


Fig. 3: Changes in pattern of *in vitro* protein synthesis programmed by polyadenylated mRNAs from Jet Neuf seedlings grown at 18°C or 0°C. Fluorographs of labeled proteins separated by 2-D electrophoresis. Lower-case letters indicate spots which have an increased intensity at 0°C and capital letters those which have decreased. The circled spot corresponds to PAL subunit precursor.

the seedling were shifted from 18°C to 0°C (spots a to n). Some of these differences were very clear cut (spots a-e, g and m) and correspond to spots which also increased on the *in vivo* pattern.

Therefore, at least for these spots the increased *in vivo* protein synthesis was accounted by an increase in the mRNA level. The relationship between *in vivo* and *in vitro* variations in the intensity of the other spots is less clear. Spots h, i, j and l increase *in vitro* but their *in vivo* counterpart is not clearly altered by the temperature shift. Among the spot on the *in vitro* pattern which decrease during the cold treatment only "o" correspond to a spot also decreasing *in vivo*. Spots P to Z have no *in vivo* correspondent. It was demonstrated previously (10) that spots W and X correspond to precursors of the small

subunits of ribulose biphosphate carboxylase.

Similar changes can be seen when the mRNAs from cultivar Brutor are analyzed (Fig. 4). However many of them are less marked than in Jet Neuf. Most of the mRNAs which increased in Jet Neuf during cold treatment seemed to remain at about the same level in Brutor at 0°C and 18°C (e.g., c, d, e or k). At 0°C many mRNAs have disappeared, as in Jet Neuf, so that mRNAs for c, d, e or k still belong to the prevalent sequences.



Fig. 4: Changes in pattern of *in vitro* protein synthesis programmed by polyadenylated mRNAs from Brutor seedlings at 18°C or 0°C. Experimental conditions and lettering system is the same as in Figure 3.

Our results suggest that some of the mRNAs were not translated with the same efficiency either *in vivo* or *in vitro*. For instance two groups of spots with similar intensity in the Jet Neuf *in vivo* protein synthesis pattern at 0°C (81, 82, 6 and 21(c), 9(d), 10(e)) strikingly differ on the *in vitro* protein synthesis pattern. The increased intensity of spots 21, 9 and 10

corresponds to an accumulation of mRNAs. Such a mechanism could not account for the increase of the *in vivo* level of spots 81, 82 and 6. This change might result from a reduced rate of turnover of these polypeptides at 0°C or for an increased *in vivo* translation efficiency of their mRNAs.

Differential expression of L-Phenylalanine ammonia-lyase

Looking for biochemical markers of cold acclimation process we attempted to identify a specific induced polypeptide of which synthesis is increased preferentially in the winter cultivar. A non-numbered prevalent spots, identified with a circle in Fig. 1 and Fig. 2, corresponds to a cold inducible polypeptide in the winter cultivar. The intensity of these spots, their position in the 80-83 kD region gel and their pI, approximately 5.5, suggested to us that they might represent the L-Phenylalanine ammonia-lyase (PAL) subunit. A non-labeled enzyme obtained from potatoe was mixed with the *in vivo* protein synthesis products from cold-treated Jet Neuf cultivar. Following two dimensional electrophoresis, the gel was stained with Coomassie blue and then fluorographed. The position of the stained polypeptide nearly co-migrate with the presumptive *in vivo* labeled PAL subunit (not shown). We checked the corresponding enzymatic activity in soluble extracts; however, the results were not conclusive and we did not investigate this point in detail. We decided also to detect it by an immunological technique. As shown in Fig. 5 an immunodot assays were performed. Soluble proteins extracted from controls and cold-treated varieties were analyzed. Comparison between both cultivars showed that the IgG fraction raised against PAL recognized the induced enzyme from Jet Neuf rapeseed seedlings. There was a faint or a negative immunological reaction when extracts from the Spring cultivar was assayed under the same conditions as above.

Results obtained in Fig. 6 further confirm the assumption that PAL synthesis was cold-induced. Products of the *in vivo*

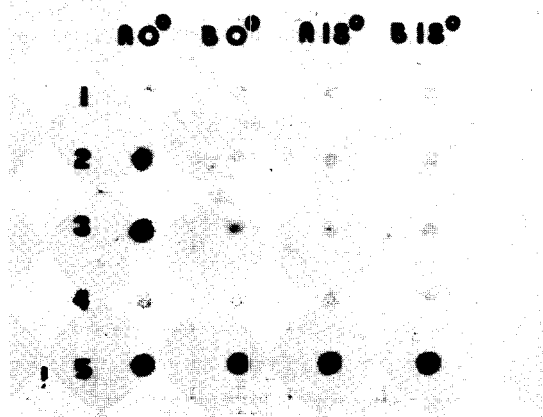


Fig. 5: Immunodot assays for PAL synthesized in Winter and Spring cultivars grown at 18°C or 0°C. Immunodot assays were performed as described in Methods. Lane A0° and Lane A18° (rows 1 to 3) correspond to winter seedlings exposed for 0 h, 24 h and 48 h at 0°C or 18°C, respectively. Lane B0° and Lane B18° (rows 1 to 3) correspond to spring cultivars exposed for 0 h, 24 h and 48 h at 0°C or 18°C, respectively. Rows 4 and 5 correspond to negative (ovoalbumin) and positive (anti-serum against PAL) controls.



Fig. 6: Identification of L-Phenylalanine ammonia-lyase subunit in winter rapeseed seedlings. Fluorograph of *in vivo* protein synthesis in seedlings exposed 48 h at 18°C (1) and 0°C (2). Lanes 3 and 4, parallel experiment as before, was electroblotted to nitrocellulose paper in which antibodies against PAL react with the PAL subunit. Fluorograph of *in vitro* protein synthesis in seedlings exposed 48 h at 18°C (6) and 0°C (7). Lanes 8 and 9, parallel experiment as in lanes 6 and 7, transferred to nitrocellulose in which the *in vivo* PAL subunit synthesis is detected immunologically. Lane 5, ¹⁴C size markers: lysozyme (14.3 kD), carbonic anhydrase (30 kD), ovoalbumin (46 kD), bovine serum albumin (69 kD).

and the *in vitro* synthesis of cold-treated Jet Neuf seedlings were subjected to one dimensional polyacrylamide-SDS gels. Following electrophoresis, they were elec-

troblotted onto nitrocellulose filters which was then incubated with IgG fraction raised against potatoe PAL. The results in both cases were essentially the same obtaining in the 80-83 kD region a positive immunological reaction.

DISCUSSION

Recently, there has been a great deal of interest to investigate the effects of the environment on plant at the molecular level. Many plants are subjected to varying climatic changes during the course of their life cycle. There is an obvious interest in improving the tolerance to severe conditions of plants of potential agronomic importance. In order to contribute to understand the molecular basis of cold-tolerance, we have analysed two rapeseed cultivars, a winter and a spring one, attempting to discriminate whether the cold-induced protein synthesis changes correspond to a specific adaptation process or merely a consequence of a general modification of the metabolism.

The results obtained in the present study show that during cold-acclimation of rapeseed, the synthesis of specific polypeptides is increased probably because the amount of the corresponding mRNA has increased. Whether this change in mRNA content results from increased synthesis or reduced degradation is not yet clear but we favour the first hypothesis. Synthesis of other polypeptides was severely reduced and we also observed a concomitant degradation of several mRNAs. In addition we observed several cases where a change in *in vivo* synthesis was not correlated with a change in mRNA amount. Changes in rates of protein turnover or in post-translation regulation mechanisms might account for these discrepancies. Interestingly, differences were observed between the two varieties and the response to cold-treatment in terms of changes in the protein synthesis pattern was much more clear cut in the winter cultivar. In the latter, some proteins

and their mRNA were clearly induced by the cold treatment but their synthesis did not seem to be stimulated in the spring variety. The appearance of these new polypeptides in the Winter cultivar, strongly suggest that cold treatment induced a differential modification at the gene expression level. Therefore, temperature stress in young rapeseed results in a complex response in specific proteins synthesis which suggest a significant degree of genotype dependency. Our data indicate that the winter variety may have specific gene or a set of genes which are expressed and regulated by the cold conditions of the short-term low temperature hardening. In order to elucidate the role of these putative freezing tolerance gene products, we have attempted to purify and characterize these specific polypeptides. This approach will facilitate the isolation of the corresponding genes, allowing in this way to know how they are regulated.

We tried several strategies to localize a good candidate whose protein synthesis was clearly cold-induced in winter variety. As a criteria, we used the intensity of the spot, the electrophoretic movility and the isoelectric point. In addition to this type of analysis we found a number of reports that pointed out to L-Phenylalanine ammonia-lyase (PAL), an enzyme belonging to the phenylpropanoid metabolism. This enzyme is altered in higher plants when they are subjected to a diversity of environmental stress conditions (e.g., UV radiation, pathogen infection, mechanical injury) (13, 14, 20). Therefore, it might be feasible that PAL expression could also be promoted by a low temperature treatment. According to our results this assumption seems to be correct, because it is possible to establish a correlation between changes in the PAL expression and the degree of cold-tolerance. Such biochemical marker of this tolerance might be useful in predicting the cold sensitivity of a new variety or in improving a sensitive cultivar. Experiments are in progress to elucidate the physiological role of PAL in the induction of freezing tolerance in *B. napus*.

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ABREVIATIONS

L-Phenylalanine ammonia-lyase: PAL, trichloroacetic acid: TCA, polyacrylamide gel electrophoresis: PAGE, sodium dodecylsulphate: SDS, bovine serum albumin: BSA.

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