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Protein synthesis during cold shock in barley tissues

Comparison of two genotypes with winter and spring growth habit

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Summary. In barley (*Hordeum vulgare* L.) seedlings, a temperature step-down from 24 °C to 6 °C (cold shock) determined a reduction in the incorporation of labeled aminoacids and modified the electrophoretic pattern of total proteins. At 6 °C some new proteins appeared and others were intensified (cold shock-induced proteins = CSPs); meantime, few proteins disappeared or were curtailed (cold-repressed proteins = CRPs). The majority of the proteins of the seedlings were labeled at about the same rate both at 6 °C and 24 °C, whereas at 0 °C only the cold shock proteins and a few others were detectable. The cold shock-induced variations of the protein profile differed in roots and in seed remnants which showed only some of the CSPs detected in roots. Total protein synthesis of barley genotypes 'Onice' and 'Georgie', which have respectively a winter and spring growth habit, were similarly inhibited by a temperature drop. The two genotypes, however, showed some differences in the CSPs and CRPs pattern. Because 'Onice' and 'Georgie' have also a different thermotolerance, the hypothesis can be made that in barley specific CSPs are involved in conferring various degrees of cold resistance.

Key words: Cold shock – Shock proteins – *Hordeum vulgare* – Cold tolerance

Introduction

In plants, the shift from 20 °C–25 °C to 40 °C–45 °C induces new synthesis of proteins. In fact, under these high temperature treatments some stress proteins are synthesized (for a review, see Mascarenhas 1984).

A role or an involvement of these heat-shock proteins in thermoprotection and acclimation has been suggested (Key et al. 1982; Lin et al. 1984). Variations in the total proteins and in the synthesis of specific proteins have also been associated with an exposure of plants to cold. The amount of soluble proteins increases in cold-hardened plants (Cloutier and Siminovitch 1982; Siminovitch and Cloutier 1982) and changes in specific protein synthesis have been detected by electrophoresis (Briggs and Siminovitch 1949; Coleman et al. 1966; Craker et al. 1969; Brown and Bixby 1975; Rochat and Therrien 1975; Faw and Jung 1976; Kacperska-Palacz et al. 1977; Cloutier 1983). Studies devoted to correlate cold-induced proteins with specific enzymes indicated modifications of the catalase and peroxidase electrophoretic profiles after hardening in alfalfa roots (Gerloff et al. 1976) and of peroxidase isozymes in winter hardened wheat (Roberts 1967; McCown et al. 1969a, 1969b). The presently available extraction and separation techniques, coupled to in vivo protein labeling, offer new opportunities for approaching the study of cold as an inducer of the synthesis of specific proteins.

Using barley seedlings we have studied the variation in the synthesis of individual proteins during a rapid temperature shift from 24 °C to temperatures as low as 0 °C. Extracts from different parts of the plants of two barley genotypes with different growth habits and thermotolerances were compared.

Materials and methods

Plant material and growth conditions

Barley seeds (*Hordeum vulgare* L. cv. 'Onice' and cv. 'Georgie', a six-rowed winter type and two-rowed spring type, respectively) were washed, surface sterilized and germinated aseptically on two layers of Whatman no. 1 filter paper wetted with 5 ml H_2O in Petri dishes for 72 h at 24 °C in the dark. Seedlings with 1 cm long coleoptiles were removed and employed for the experiments described below.

Evaluation of incorporation level of radioactivity into proteins

Samples of five seedlings with the embryonic axis protruding about 1 cm were incubated at 24 °C, 18 °C, 12 °C, 6 °C and

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0 °C for 4 h in scintillation vials containing 750 µl of a 2% (w/v) sterile sucrose solution in 10 mM K-phosphate buffer pH 6.5. L-3-5-^{3}H-tyrosine 100 µCi (spec. act. 52 Ci/mmol, 1.85 TBq/mmol) was added in the last 3 h. Incorporation of ^{35}S-methionine (spec. act. 1500 Ci/mmol, > 30 TBq/mmol) was also determined at the same temperatures. Incorporation was arrested by an excess of cold tyrosine followed by rapid freezing. The different organs (root, coleoptile and the remnant of the seed after germination) were separated, homogenized by cold mortar and pestle using 10% cold TCA, heated at 90 °C for 15 min, cooled and filtered through Whatman GF/C. The radioactivity incorporated was processed by standard procedure as described by Marmiroli and Lodi (1984).

Preparation of labeled protein extracts

Protein extracts were obtained by pre-incubating samples of 10 seedlings at the various temperatures in 3 ml of a solution consisting of 2% sucrose in 10 mM K-phosphate buffer, pH 6.5. After 1 h, 300 µCi of ^{35}S-methionine were added and the seedlings were incubated for an additional 3 h. Protein extraction from root, coleoptile and the remnant of the seed, obtained by cutting out at the edge of the germinated seed both roots and the embryonic axis, was done according to Baszczynsky et al. (1982) and was followed by dialysis against 2 changes of 5 l of a 1/5 strength extraction buffer containing 1 mM EDTA, 0.1% 2-mercaptoethanol and 25 µg/ml of the protease inhibitor phenylmethylsulfonylfluoride (PMSF). The extracts were lyophylized, freeze-dried and stored at –20 °C.

Gel electrophoresis

Proteins were solubilized in about 200µl of 2% SDS, 2% 2-mercaptoethanol and 9mM Tris (pH8.3), treated with DNaseI (10µl of a solution 2mg/ml in 0.01M Tris HCl buffer, 5mM $MgCl_2$, pH7.8) and RNase A (10µl of a solution 2mg/ml in 0.1M Tris HCl buffer, pH6.8) and activated for electrophoresis by heating at 90°C for 2min. Protein concentration was determined according to Lowry et al. (1951); ^{35}S-methionine incorporation was measured as described previously. Gel electrophoresis was performed in a 10% non-gradient discontinuous polyacrylamide SDS gel prepared according to Laemmli (1970), with the modifications described by Marmiroli and Lodi (1984). The gels were prepared for fluorography as described by Bonner and Laskey (1974).

Results

Protein synthesis activity after step-down in the temperature range 24 °C–0 °C

Activities of the protein synthesizing system of plantlets grown at 24 °C and exposed to temperature step-down in the interval 24 °C–0 °C are shown in Fig. 1. The two genotypes had a similar behaviour in this temperature interval. The data related with incorporation in seed remnants were purposefully omitted from Fig. 1 since the various temperature shift downs had a similar effect in roots and seeds in both genotypes.

Similar results were found with ^{35}S-methionine incorporation (data not shown).

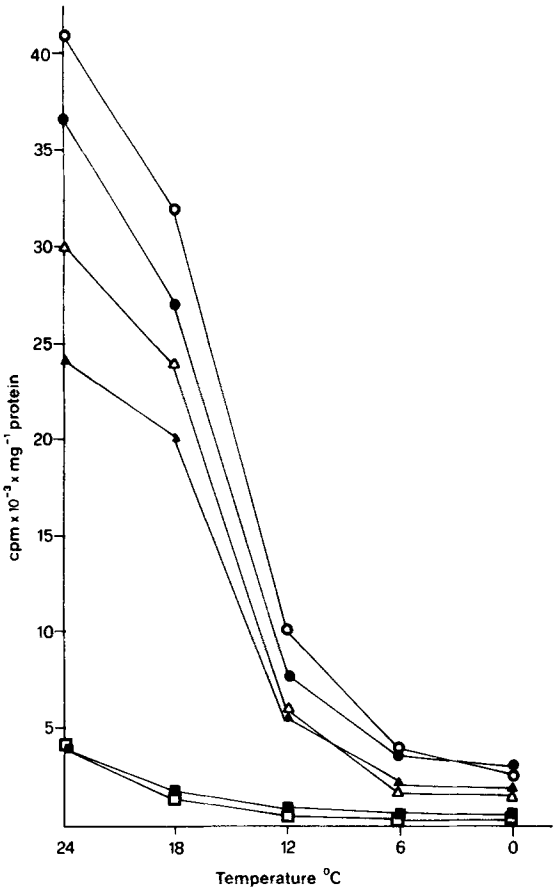

Fig. 1. Incorporation of ^{3}H-tyrosine into proteins extracted from the whole plants (–○–, –●–), roots (–△–, –▲–) and coleoptiles (–□–, –■–), determined after heating at 90 °C for 15 min and following filtration on a Whatman GF/C filter. Open and close symbols refer to cv. 'Onice' and cv. 'Georgie', respectively. Proteins were extracted by 10% cold TCA and a 100 µl sample from each temperature was precipitated, washed with ethanol, dried and resuspended in 100 µl of 10 mM K-phosphate buffer pH 6.5 and processed for determination of protein concentration according to Lowry et al. (1951)

Electrophoretic pattern of proteins synthesized in roots and in seed remnants of labeled 'Onice' and 'Georgie' plantlets, after a temperature step-down in the interval 24 °C–6 °C

'Onice'. The proteins synthesized in roots and in seeds of this genotype under normal and cold shock conditions were compared by labeling plantlets at 24 °C and after a 4 hour shift to 18 °C, 12 °C and 6 °C (Fig. 2 A, B). The electrofluorogram of Fig. 2 A depicts the pattern of protein synthesis in seeds after incubation at the various temperatures. In this organ the labeling associated with the proteins of MW 61 Kd and 67 Kd was particularly increased at 6 °C, compared to 24 °C.

Extracts from roots run on the same gel (Fig. 2 B) gave also two cold shock proteins (CSPs) of MW 67 and

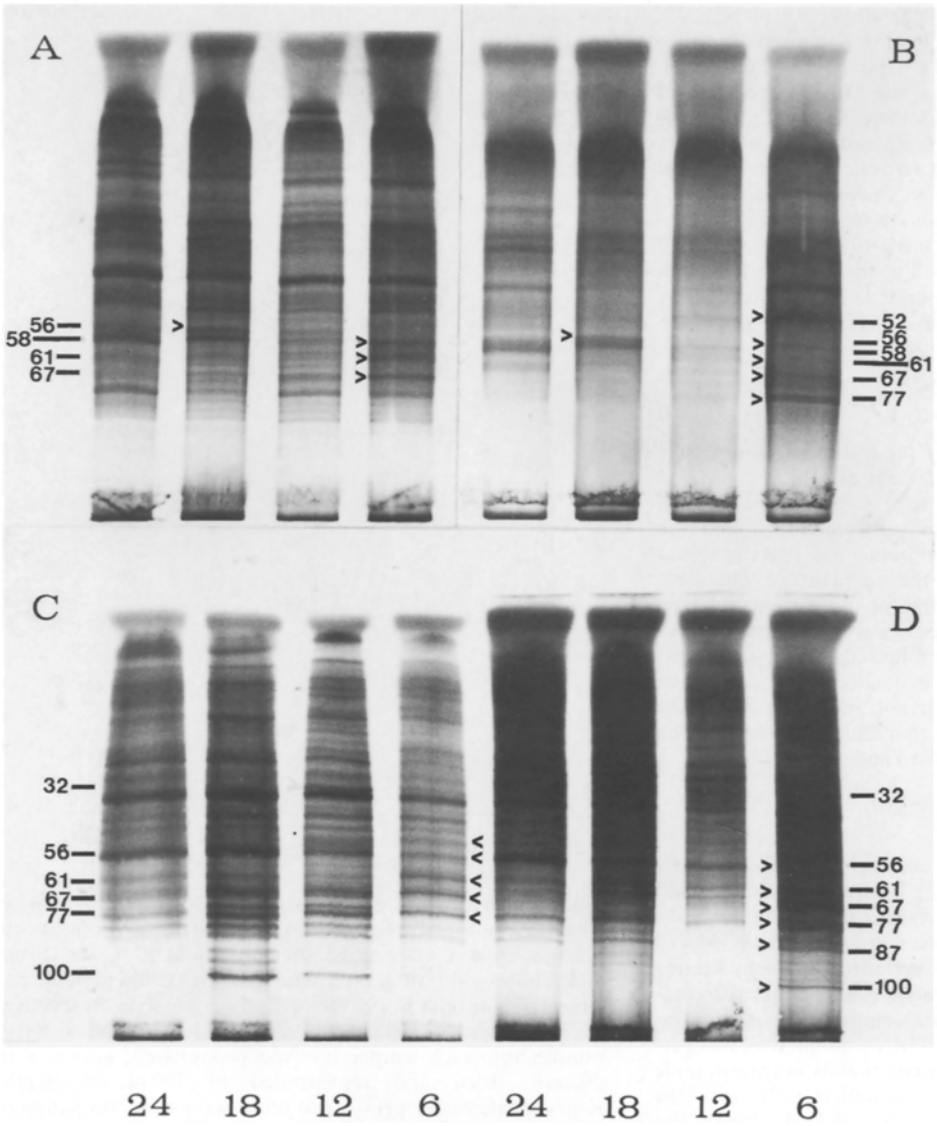

Fig. 2 A–D. Electrophoresis and fluorography of proteins extracted from seeds and roots of cv. 'Onice' and cv. 'Georgie' Plantlets were grown at 24 °C and then transferred to 24 °C, 18 °C, 12 °C, and 6 °C, five plantlets for each temperature, in phosphate buffer (pH 6.5). After 1 h of adaptation, 300 μCi/ml of ^{35}S-methionine was added and incubation carried out for 3 additional h at the scheduled temperatures. Gel electrophoresis was performed in a discontinuous non-gradient gel (stacking gel 4.5%; running gel 10%). A well, loaded with a mixture of: lysozyme (MW 14,400), soybean trypsin inhibitor (MW 21,500), carbonic anhydrase (MW 31,000), ovalbumin (MW 45,000), bovine serum albumin (MW 66,200) and phosphorylase B (MW 92,500), was alternated every 5 wells in each gel. Each 10 μl extract loaded into the gel contained about 100,000 cpm. After electrophoresis, usually 3 h at 400 V and 30 mA (for a 300 μm thick gel, at 5 °C), the gel was fixed, Coomassie-stained and treated for fluorography according to Bonner and Laskey (1970). After 7 days of exposure at −70 °C to Kodak X-Omat film, the electrofluorograms were scanned with a densitometer Mod. 1650 (BioRad) for determination of band intensity. Arrows indicate the bands which undergo major variations; molecular weights (standard plot of $\log_{10}$ MW versus rf) in Kilodaltons (Kd) have been printed on the sideboards. **A, B** Coelectrophoresis of extracts from seeds (**A**) and roots (**B**) of the cv. 'Onice'; **C, D** coelectrophoresis of extracts from seeds (**C**) and roots (**D**) of cv. 'Georgie'

61 Kd, which were induced at low temperatures, both at 12 °C and more intensely at 6 °C. Two proteins of MW 77 and 52 Kd were found as CSPs only in roots.

In roots, a reduced labeling at 6 °C of the 58 Kd protein occurred after its enhancement at 18 °C. Meantime, a lower molecular weight component (MW= 56 Kd) faded quickly at 6 °C (Fig. 2 B). The 56 Kd protein in seeds behaved as a cold repressed protein (CRP): it faded by reducing the temperature of labeling. A similar pattern of CRPs could also be found by labeling the plantlets 6 and 8 hours after the transfer at low temperature (data not shown).

The differences and the similarities in CSPs and CRPs were confirmed by running on the same lane a

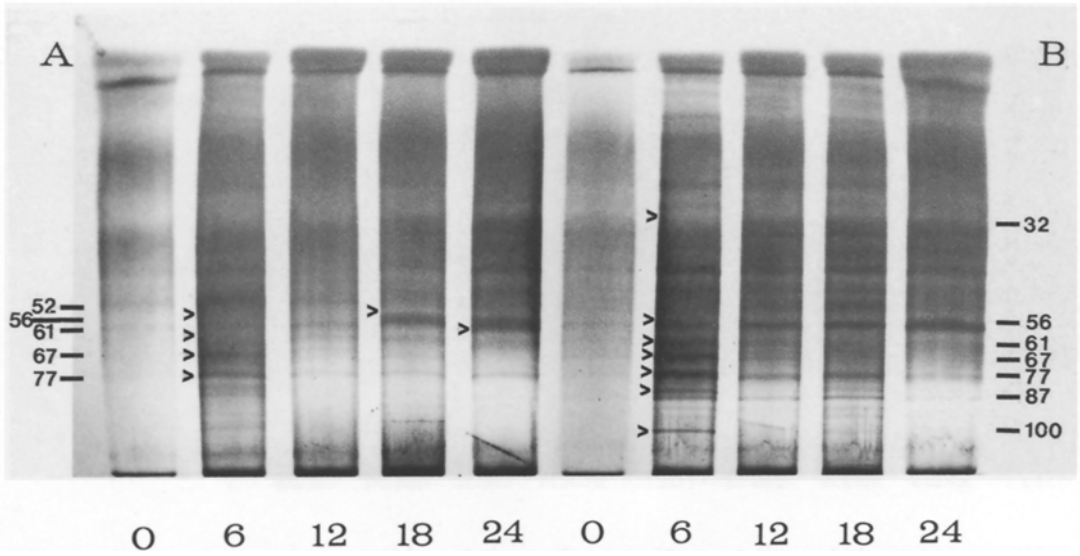

Fig. 3. Coelectrophoresis and fluorography of protein extracts from roots of (**A**) cv. 'Onice' and (**B**) cv. 'Georgie'. Labeling conditions were those reported previously; the protein pattern incorporated at 0 °C is also shown. The radioactivity content in each 10 µl sample loaded into the gel was around 300,000 cpm with the exception of the tracks corresponding to 0 °C which was about 30,000 cpm. Exposure was for 1 week at – 70 °C

mixture of extracts from the two organs and the results are summarized in Table 1.

'Georgie'. Figure 2 C shows that in plantlets labeled at 6 °C the seeds of this genotype accumulated three CSPs (MW = 55, 61 and 67 Kd). Two other CSPs (MW = 100 and 87 Kd) were induced only in roots (Fig. 2 D). Therefore, five CSPs (with MW of about 100, 87, 77, 67 and 61 Kd) could be identified by the coelectrophoresis of extracts from both roots and seeds. A particular case was presented by the 100 Kd protein classified as CSP in roots, which in seeds was enhanced at 18 °C and 12 °C (see also Fig. 3 A, B), but not at 6 °C.

Comparison of the electrophoretic patterns of proteins synthesized by 'Onice' and 'Georgie' after a temperature step-down in the interval 24 °C–0 °C

Extracts prepared from roots and seeds of the two genotypes were comigrated on the same gel to resolve differences and similarities.

Roots. Figure 3 shows the comparison between protein synthesis in roots of 'Onice' (Fig. 3 A) and 'Georgie' (Fig. 3 B), 4 h after the step-down from 24 °C to: 18 °C, 12 °C, 6 °C, and 0 °C. At 6 °C, three CSPs (MW = 77, 67, and 61 Kd) were evident both in 'Onice' and 'Georgie', one CSP (MW = 52 Kd) was induced in 'Onice' roots only, and two CSPs were typical of 'Georgie' roots (MW = 100 and 87 Kd). A protein of MW 56 Kd appeared at 18 °C and disappeared at 12 °C in 'Onice', but not in 'Georgie'. The intensity of a 32 Kd protein was reduced at low temperatures in 'Georgie'. Only a few of the normal proteins, together with the CSPs, were labeled at 0 °C.

Seeds. Co-migration of 'Onice' and 'Georgie' seeds showed two CSPs of MW 67 and 61 Kd in both the genotypes, whereas the 77 Kd protein showed an increased intensity in 'Georgie' (and no variation in 'Onice') (Fig. 4 A, B). The intensity of the 87 Kd protein, which increased in 'Georgie' roots, decreased at low temperature in seeds of both 'Georgie' and 'Onice'. The two cultivars also showed differences in the CRPs: a 56 Kd protein in 'Onice', a 56 Kd and a 32 Kd protein in 'Georgie'.

Comparison of proteins synthesized by individual seedlings of 'Onice' and 'Georgie': summary of major variations

The differences in the protein patterns found after a shift to 6 °C and 0 °C depended upon the genetic background of the two genotypes. This was shown by labeling five intact seedlings at 24 °C, 6 °C and 0 °C, and by separating roots and seeds from each individual plantlet. The electrophoretic characterization of the extracts obtained from these individual organs is reported in Table 1.

In 'Onice' roots, the shift to 6 °C determined the appearance of two polypeptides (MW 77 and 52 Kd) and the augmentation of a 67 and 61 Kd polypeptides. In 'Georgie' roots, five CSPs were noted with MW 100,

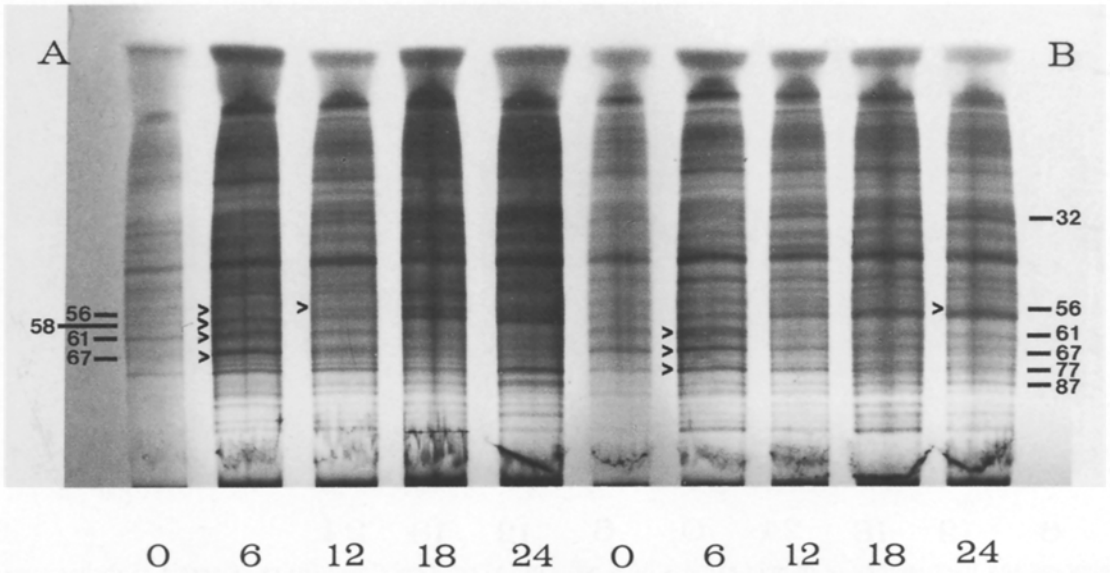

Fig. 4. Coelectrophoresis and fluorography of protein extracts from seeds of (**A**) cv. 'Onice' and (**B**) cv. 'Georgie': the protein pattern of seeds incorporated at 0 °C is also shown. The radioactivity of each 10 µl sample was around 100,000 cpm (cv. 'Onice') and 50,000 cpm (cv. 'Georgie'). The extract of the 0 °C treatment had 20,000 cpm. Exposure was for two weeks at – 70 °C

Table 1. Major variations observed in protein synthesis after a temperature shift from 24 °C to 6 °C in individual plantlets of 'Onice' and 'Georgie'

MW[a,c]	Roots[b]		Seeds[b]	
	'Onice'	'Georgie'	'Onice'	'Georgie'
100±5	○	+ + +	○	○
87±1	○	+ + +	–	–
77±3	+ + +	+ +	○	+ + +
67±2	+ +	+ +	+ + +	+ +
61±1	+	+ +	+ + +	+ +
56±2	– – –	–	– – –	– –
52±2	+ + +	○	○	○
32±1	○	–	○	– –

[a] Molecular weights were calculated on proteins denaturated by heating in the presence of SDS and 2-mercaptoethanol. A standard plot of $\log_{10}$ polypeptides molecular weight versus relative mobility was obtained by electrophoresing each time the following marker polypeptides on the same gel: Lysozyme (14,400); Soybean Trypsin Inhibitor (21,500); Carbonic Anhydrase (31,000); Ovalbumin (45,000); Bovine Serum Albumin (BSA) (66,200); Phosphorylase B (92,500)

[b] Obtained from the integration of the densitometric tracing. The area of each peak corresponding to a band in the film was determined and utilized to quantify the variations observed. The symbols indicate: (+, –) = two-fold increase/decrease in the intensity of an electrophoretic band following the shift from 24°C to 6°C; (+ +, – –) = three to five fold increase/decrease; (+ + +, – – –) = the electrophoretic band was detectable only at 6°C (+ + +) or at 24 °C (– – –); (○) = no corresponding band in one of the genotypes or no variation observed

[c] Though bands with identical MW are present in the two genotypes, they may not be encoded for by the same gene(s). On the contrary, the identification of their genetic determinants may reveal that bands with different MW in the two genotypes are encoded by homologous genes

87, 77, 67 and 61 Kd. Cold-shock induced the disappearance of a 56 Kd protein in 'Onice' roots, whereas in 'Georgie' roots proteins of MW 56 and 32 Kd were curtailed. In 'Onice' seeds, cold shock induced synthesis of proteins with MW 67 and 61 Kd, whereas at the same temperature in 'Georgie' seeds three proteins of MW 77, 67 and 61 were induced. Proteins which were curtailed or which disappeared in the seeds were of MW 56 Kd in both genotypes, and of MW 32 Kd in 'Georgie'.

The major draw-back of the in vivo labeling system of barley plantlets we have utilized was the poor incorporation into the coleoptiles. However, we had evidence suggesting the existence of two major CSPs of MW 67 and 61 Kd both in 'Georgie' and 'Onice'. The two CSPs were detectable both at 6 °C and at 0 °C.

Discussion

Previous studies on the effect of cold upon protein synthesis showed the occurrence of modifications in the protein patterns of plants during winter hardening, in relation also with their genetic constitution. In this context, differences were reported between the soluble protein fractions in genotypes of *Medicago sativa* (Coleman et al. 1966) and in three clones of *Dianthus* (McCown et al. 1969 b) and between the activities of 10 isoenzymes separated from leaf and stem of the same cultivars of *Dianthus* grown under winter and summer conditions (McCown et al. 1969 a).

Remarkably for some enzymes, the differences were found not only between genotypes but also between tissues (McCown et al. 1969 a). More recent studies in wheat and rye

(Cloutier 1983) and in alfalfa (Faw and Jung 1972; Faw et al. 1976) showed, in response to hardening, increases in soluble proteins with no major change in the electrophoretic pattern. Differences between cultivars grown in different environments were mainly quantitative even though two polypeptide band changes were related with increased freezing tolerance in wheat and rye (Cloutier 1983).

These studies considered only those proteins which were accumulated in relatively large amounts and upon a considerable period of time after transfer to the low temperature. By the use of radioactive amino acids with high specific activities, we have accumulated evidence that a temperature step-down triggers a response in barley which has been termed cold-shock since it could lead to rapid modifications of protein synthesis similar to those reported during heat shock in the same species (Marmiroli et al. 1984, 1986). Upon a shift to 6 °C, most of the normal proteins are synthesized together with cold-shock proteins (CSPs), whereas upon a shift to 0 °C the CSPs and only few normal proteins are present. The resemblance with what was observed at 40 °C, in which there is the simultaneous presence of heat shock proteins (HSPs) and most of the normal proteins, and at 42 °C, in which only HSPs and very few normal proteins are detectable (Marmiroli et al. 1986), is striking.

Focusing the attention on the proteins which are induced by cold and particularly to the differences detected between 'Onice' and 'Georgie', it is tempting to assume that peculiar CSPs have a role in conferring different cold resistance. In fact, by considering CSP synthesis in roots it appeared that a) both 'Onice' and 'Georgie' had CSPs additional to the three which they have in common, and b) the less cold-resistant genotype 'Georgie' synthesized five CSPs whereas the more cold-resistant genotype 'Onice' synthesized only four CSPs.

Preliminary experiments with barley plantlets of both genotypes indicated that the accumulation of CSPs could effectively confer protection to cold (data not shown). Our suggestion is that the cold shock proteins, as the heat shock proteins (Kimpel and Key 1985), are involved in modulating and protecting some cell functions when the temperature undergoes a sudden change and this could be consistent with the existence of a block of CSPs which are common to genotypes with different thermotolerances. On this basis, both varieties could acquire a certain degree of protection against very low temperatures if they are preventively exposed for few hours to 6 °C in order to induce synthesis of CSPs.

How the CSPs are linked with thermotolerance and with cold acclimation proteins recently detected by Cloutier (1983) in other cereals remains to be established. However, particularly interesting for their possible role are those CSPs which we found variable in the two genotypes. In order to establish the genetic basis of the cold shock response in barley, the synthesis of cold shock proteins and the disappearance of cold repressed proteins following cold shock in the progeny of 'Onice' × 'Georgie' cross is under investigation.

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