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Characterization of chilling-acclimation-related proteins in soybean and identification of one as a member of the heat shock protein (HSP 70) family

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Abstract. Through a 5-d exposure at 14° C/8° C (day/night), soybean (*Glycine max* [L.] Merr.) was acclimated to a lower temperature of 8° C. In order to assess changes in protein synthesis related to chilling acclimation, proteins were labeled in vivo with [^{35}S]methionine, separated by two-dimensional gel electrophoresis, and the derived autoradiograms were subjected to computer analysis. Two sets of chilling-acclimation-related proteins were characterized following exposure and labeling at 8° C. One set corresponded to proteins whose synthesis was stimulated in acclimated plants in comparison with non-acclimated plants after transfer to 8° C for 2 d. The other set also displayed an enhanced synthesis in the acclimated plants versus the non-acclimated plants but after 7 d of exposure at 8° C. Most of these chilling-acclimation-related proteins were not increased during the acclimation period at 14° C. Using microsequence analysis, one of these proteins was shown to have a high sequence homology with members of the heat-shock protein (HSP 70) family.

Key words: Chilling-acclimation-related protein – *Glycine* – Heat-shock protein

Introduction

The ability to extend the cultivation of crop plants of subtropical or tropical origin into temperate zones is limited by their sensitivity to low, non-freezing temperatures. By contrast, plants of temperate origin are tolerant to these temperatures and are also able to increase their freezing tolerance when they are progressively exposed to low temperatures. This process, known as cold acclimation, occurs naturally in winter for these species (Levitt 1951; Li 1978) but can also be reproduced under controlled conditions (Levitt 1972; Li and Sakai 1982). In fact, in many temperate species, an increased cold tolerance was obtained by exposure to low, non-freezing temperatures (Guy and Haskell 1987; Gilmour et al. 1988; Weiser et al. 1990).

The freezing tolerance derived from cold acclimation is an inducible and transient character that has often been studied in order to understand tolerance mechanisms at the molecular level. For example, it has been demonstrated that exposure to acclimation temperatures resulted in alterations in protein synthesis (Guy 1990). Some genes corresponding to proteins induced during the cold acclimation have been cloned and their products display similarities to antifreeze proteins and late-embryogenesis-abundant proteins (Kurkela and Franck 1990; Gilmour et al. 1992; Orr et al. 1992).

Chilling-sensitive species of tropical or subtropical origin are not naturally submitted to a process of acclimation to low temperature. While a few studies focused on alteration in protein synthesis in response to low temperatures in these species (Ort et al. 1989; Hahn et Walbot 1989), the possibility of chilling acclimation was not taken into account.

In this study, we have examined the changes in protein synthesis in relation to chilling acclimation in soybean (*Glycine max* [L.] Merr.). The extension of soybean cultivation in Europe requires cultivars more adapted to cold conditions, particularly at young stages. This study was thus undertaken in order to identify some potential molecular markers of chilling adaptation in soybean. First, we evaluated the ability of soybean to acclimate to low temperatures. Then, we compared the protein synthesis in response to low temperature in acclimated versus non-acclimated plants. In-vivo-labeled proteins were resolved by two-dimensional gel electrophoresis and the results were computer-analysed. We report here on the characterization of chilling-acclimation-related proteins in soybean and the molecular identification of one of them.

Abbreviations: HSP 70 = heat-shock protein of 70 kDa

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Materials and methods

Plant material and growth conditions. Plants of soybean (*Glycine max* [L.] Merr.), cultivars Verdon (Tourneur Grandes Cultures, Montauban, France) and Maple Arrow (Rustica, Blagnac, France), were grown in vermiculite soaked with a nutrient solution (Bounìols et al. 1981) in growth chambers with a day/night period of 14 h/10 h and light irradiance of 300 μmol photons$\cdot m^{-2} \cdot s^{-1}$. Seedlings were first grown at 25° C/23° C (day/night) for 16 d. In order to induce chilling acclimation, plants were then transferred to 14° C/8° C (day/night) for 5 d and acclimation was revealed after a transfer to 8° C. Non-acclimated plants were directly transferred after 16 d from 25° C/23° C to 8° C.

In-vivo labeling and separation of proteins. Proteins were labeled and resolved by two-dimensional gel electrophoresis as previously described (Cabané et al. 1992). The labeling was allowed to proceed for 24 h after application of approx. 3.7 MBq [^{35}S]methionine (Amersham International, Buckinghamshire, UK) to the first trifoliate leaf. Subsequently, 33 kBq of labeled proteins were loaded on the first dimension and the proteins were detected on the gel by direct autoradiography after 24-d exposure on β-max Hyperfilm (Amersham).

Computer analysis of two-dimensional gel electrophoresis. Autoradiograms were computer-analyzed using the HERMeS II software package (Tarroux et al. 1987). For each labeling condition, four replicate gels derived from independent experiments were selected for the analysis on the basis of the quality of the separation. Twenty-four gels were then treated in parallel. After image acquisition, the background and streaks were removed and the spots were detected. The gels were matched and a gel master was constituted which contained all the spots detected in all gels (Vincens and Tarroux 1987). For each gel, the total volume of all spots was normalized based on the assumption that the total volume was constant, since the differences observed were subtle.

Principal component analysis was performed on the 24 autoradiograms to evaluate the relatedness of the 24 gels based on the relative abundance of each protein (Tarroux 1983; Tarroux et al. 1987; Lefkovits et al. 1988; Rabilloud et al. 1991). This mathematical analysis allows the representation of objects defined by a set of variables. In the present case, each protein is considered as an object described by a set of variables, each variable corresponding to its label intensity in a given condition. The principal component analysis allow the initial set of variables to be replaced by another set in which (i) each new variable is a linear combination of the initial one, and the number of obtained variables is reduced; (ii) each new variable is independent; and (iii) the new axes produced are sorted in such a way that the overall information they enclose decreases. In practise, the first axes are used since they contained almost all the information. The correlation between the variables can be studied by a projection on the new axes. A complete description of the method can be found in Benzecri (1980).

Variance analysis (Tarroux et al. 1987) was used to select spots with statistically significant variations in their intensities under different labeling conditions. The mean value of each spot volume and variance was calculated for each set of repetitive autoradiogram. A statistical test to a given marginal error (5% in our case) was performed to determine the statistical value of the variation in the mean spot volume. Only spots with significant variations in their intensities according to this test were taken into account.

Concentration and microsequencing of proteins. Selected spots were collected from 50 dried two-dimensional gels stained with Coomassie blue (Neuhoff et al. 1988). After concentration as described by Rasmussen et al. (1991), the protein was electroblotted onto Immobilon (Millipore, Saint Quentin les Yvelines, France) using 50 mM Tris, 50 mM boric acid as transfer buffer. Protein transfer was carried out for at least 10 h at 35 V. The protein transferred onto the membrane was disgested with trypsin according to Bauw et al. (1989). The tryptic peptides were separated by reverse-phase HPLC (SP 8800 chromotograph; Spectra Physics, Les Ulis, France) on a C18 column (10.6 cm long, 0.46 cm diameter) according to Rasmussen et al. (1991). The concentrated protein and the tryptic peptides were sequenced in a microsequencer (ABI 470A; Applied Biosystems, Roissy Charles de Gaulle, France).

Comparison of amino-acid sequences with protein databases. The amino-acid sequences were compared with the NBRF protein database using the programs from the software package BISANCE (Dessen et al. 1990) developed by CITI2 (Centre Interuniversitaire d'Informatique à Orientation Biomédicale, Paris, France).

Results

Cold acclimation of soybean plants. The acclimation protocol is shown in Fig. 1. Plants grown for 16 d at 25° C/23° C were transferred to 14° C/8° C for 5 d in order to induce acclimation. After this period the plants were transferred to 8° C to evaluate the chilling acclimation. In parallel, 16-d-old plants were directly transferred from 25° C/23° C to 8° C. Two cultivars, Verdon and Maple Arrow, were tested for their ability to acclimate to low temperatures. During the transfer to 14° C/8° C, plant growth was very limited as previously shown (Cabané et al. 1992). Thus, non-acclimated and acclimated plants showed similar development when transferred to 8° C. At 8° C, soybean plants were strongly affected since they stopped their growth and began to yellow rapidly. Neverthless, the yellowing was more rapid for non-acclimated plants than acclimated ones. After 22 d

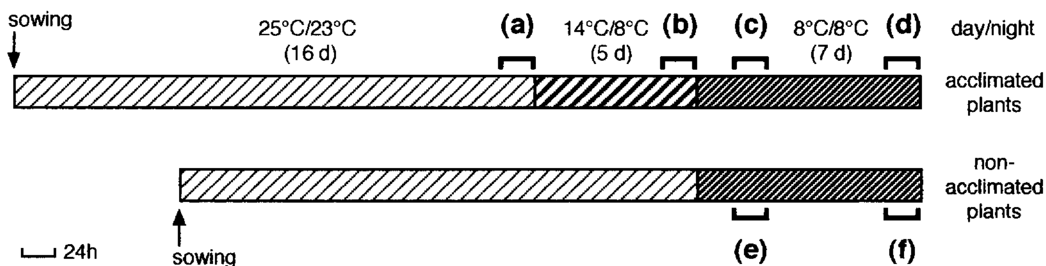

Fig. 1. Acclimation protocol. Acclimated plants: 16-d-old plants grown at 25° C/23° C were transferred for 5 d to 14° C/8° C and then mantained at 8° C. Non-acclimated plants: 16-d-old plants grown at 25° C/23° C were transferred to 8° C. Proteins were in-vivo-labeled in six different conditions: (**a**) 16-d-old plants grown at 25° C; (**b**) 5-d-acclimated plants at 14° C; (**c**) acclimated plants transferred for 2 d to 8° C; (**d**) acclimated plants transferred for 7 d to 8° C; (**e**) non-acclimated plants transferred for 2 d to 8° C; (**f**) non-acclimated plants transferred for 7 d to 8° C

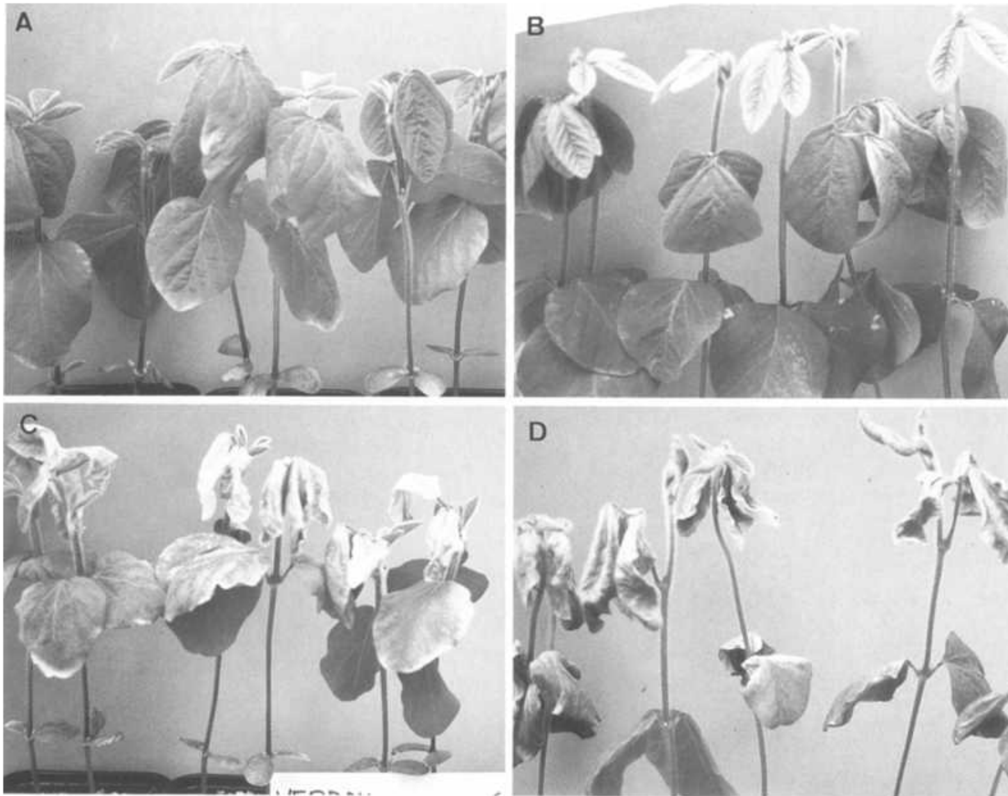

Fig. 2A–D. Soybean plants mantained for 22 d at 8° C. **A** Acclimated plants of Verdon; **B** acclimated plants of Maple Arrow; **C** non-acclimated plants of Verdon; **D** non-acclimated plants of Maple Arrow

at 8° C, non-acclimated plant showed chlorosis (Fig. 2) and acclimated plants showed a normal green colour. No differences were noticed between the two cultivars. After 27 d at 8° C, the quantity of chlorophyll was two fold higher in acclimated than in non-acclimated plants, the difference being similar in both cultivars (data not shown).

After an exposure to 14° C/8° C, soybean plants showed a better tolerance to 8° C than non-previously exposed plants. Thus soybean was able to be acclimated to low temperatures. Since the two cultivars exhibited no differences, only Verdon was used for further analysis.

Global analysis of two-dimensional gels. Leaf proteins of the Verdon cultivar were in-vivo labeled in six different conditions (Fig. 1): (i) 16-d-old plants grown at 25° C, (ii) 5-d-acclimated plants at 14° C, (iii) acclimated plants transferred to 2 d to 8° C, (iv) acclimated plants transferred for 7 d to 8° C, (v) non-acclimated plants transferred for 2 d to 8° C, (vi) non-acclimated plants transferred for 7 d to 8° C. The first trifoliate leaf which had completed its growth at theses stages, was used for labeling experiments in order to avoid any changes in protein synthesis due to differential growth velocity.

Proteins were resolved by two-dimensional gel electrophoresis and detected by direct autoradiography (Fig. 3) to allow quantification of the spots (Laskey and Mills 1975). For each labeling condition, autoradiograms from four repetitive independent experiments were computer-analyzed. The master image constituted after matching the 24 autoradiograms, contained 853 spots. The relative molecular mass (M_r) of the polypeptides ranged from 110000 to 20000 and the isoelectric points (pI) ranged from 4.5 to 8.

Principal component analysis was applied to the 24 gels on the basis of the comparison of all-spot volume (i.e. the intensity of labeling). This method has proved to be a good tool for defining the relatedness of a set of two-dimensional gels (Tarroux et al. 1987; Lefkovits et al. 1988; Rabilloud et al. 1991). The relations between the 24 autoradiograms were represented in axes 2 and 4 which contained respectively 9.4% and 4.5% of the information. This analysis (Fig. 4) revealed that the autoradiograms were clearly separated depending on the temperature conditions according to axis 2. The patterns of protein synthesis were thus specifically related to each temperature programm. Moreover, the protein pattern could be differentiated according to the exposure time at 8° C in axis 4. At 8° C, the differences between protein patterns from acclimated and non-acclimated plants were less clear. This seemed to indicate that acclimation induced subtle and limited changes in the polypeptide patterns of cold-exposed plants. The computer analysis of quantitative variations in spot intensities was then essential to characterize such changes.

Characterization of chilling-acclimation-related proteins. Computer analysis allowed us to quantify the spot intensities and to make statistical tests on the spot volumes. Only spots whose mean intensity in a set of autoradiograms was found to be different according to the variance analysis from one set to another were taken into account.

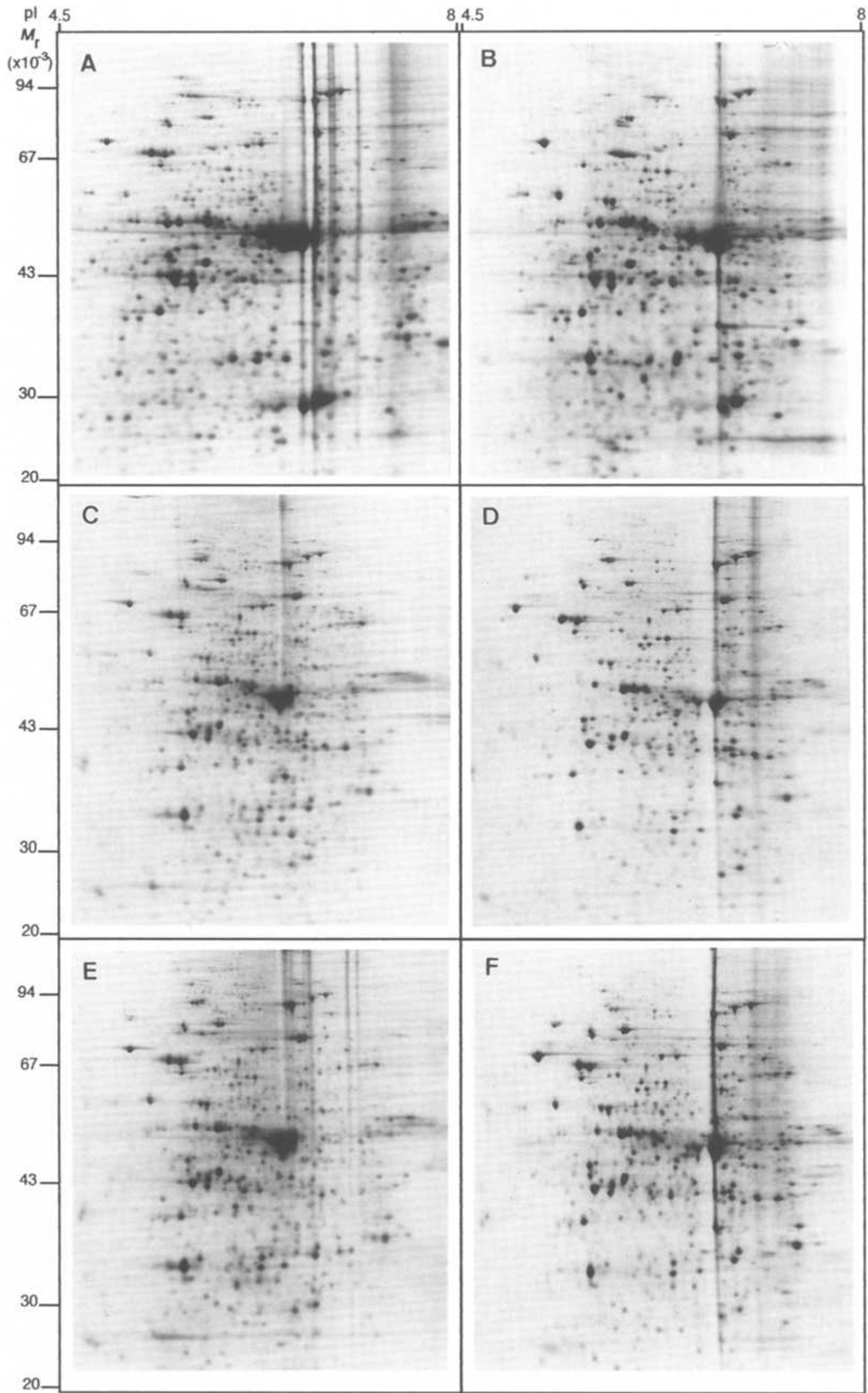

Fig. 3A–F. Autoradiograms of in-vivo-labeled soybean proteins resolved by two-dimensional gel electrophoresis; 33 kBq of proteins were loaded on the first dimension and the proteins were revealed by direct autoradiography after 24 d exposure. Labeling was allowed to proceed for 24 h in six conditions. **A** 16-d-old plants grown at 25° C; **B** 5-d-acclimated plants at 14° C; **C** acclimated plants transferred for 2 d to 8° C; **D** acclimated plants transferred for 7 d to 8° C; **E** non-acclimated plants transferred for 2 d to 8° C; **F** non-acclimated plants transferred for 7 d to 8° C

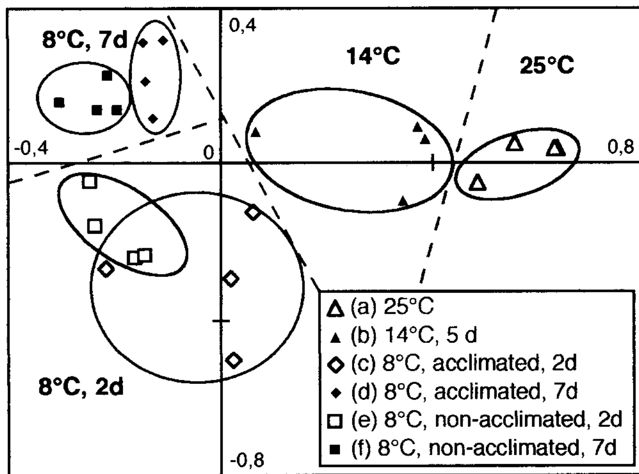

Fig. 4. Representation of the coordinates of the 24 autoradiograms in axes 2 and 4 arising from principal component analysis. Each symbol represents one of the 24 gels, and the gels corresponding to the same conditions are represented with identical symbols. The four replicate gels corresponding to one labeling condition are *circled*. The *dashed lines* shows the separation between temperature conditions and time of labeling at 8° C

In this analysis, we compared acclimated to non-acclimated plants and we looked for polypeptides whose synthesis was more abundant in acclimated than in non-acclimated plants. In plants transferred for 2 d to 8° C, 32 polypeptides (set A; Fig. 5A) were more abundantly synthesized in acclimated than in non-acclimated plants. The M_r of these polypeptides ranged from 94000 to 30000. Among these polypeptides, 15 were newly synthesized but in low abundance, and most of the others were more than two-fold increased. In plants transferred for 7 d to 8° C, 37 polypeptides (set B; Fig. 5B) with M_r ranging from 90000 to 35000 were characterized as more abundant in acclimated than in non-acclimated plants. Seventeen of these polypeptides appeared in low abundance and most of the others were more than two-fold increased. Only one polypeptide was found to be common to both sets. The polypeptides characterized from plants transferred for 2 d to 8° C were different from the ones characterized after 7 d at 8° C. The differences between the acclimated and the non-acclimated plants strongly depended on the exposure time at 8° C.

We also determined if the characterized polypeptides resulted from increased synthesis of these proteins during the acclimation period at 14° C in comparison to control (25° C). Among the set A, only six polypeptides were increased in plants cultivated at 14° C in comparison to plants grown under normal conditions. Among the set B, five polypeptides responded to this criterion. In general, the acclimation-related polypeptides were not previously increased by low temperature during the acclimation period at 14° C.

Identification of a chilling-acclimation-related protein. Polypeptide No. 54 (Fig. 5) was microsequenced because

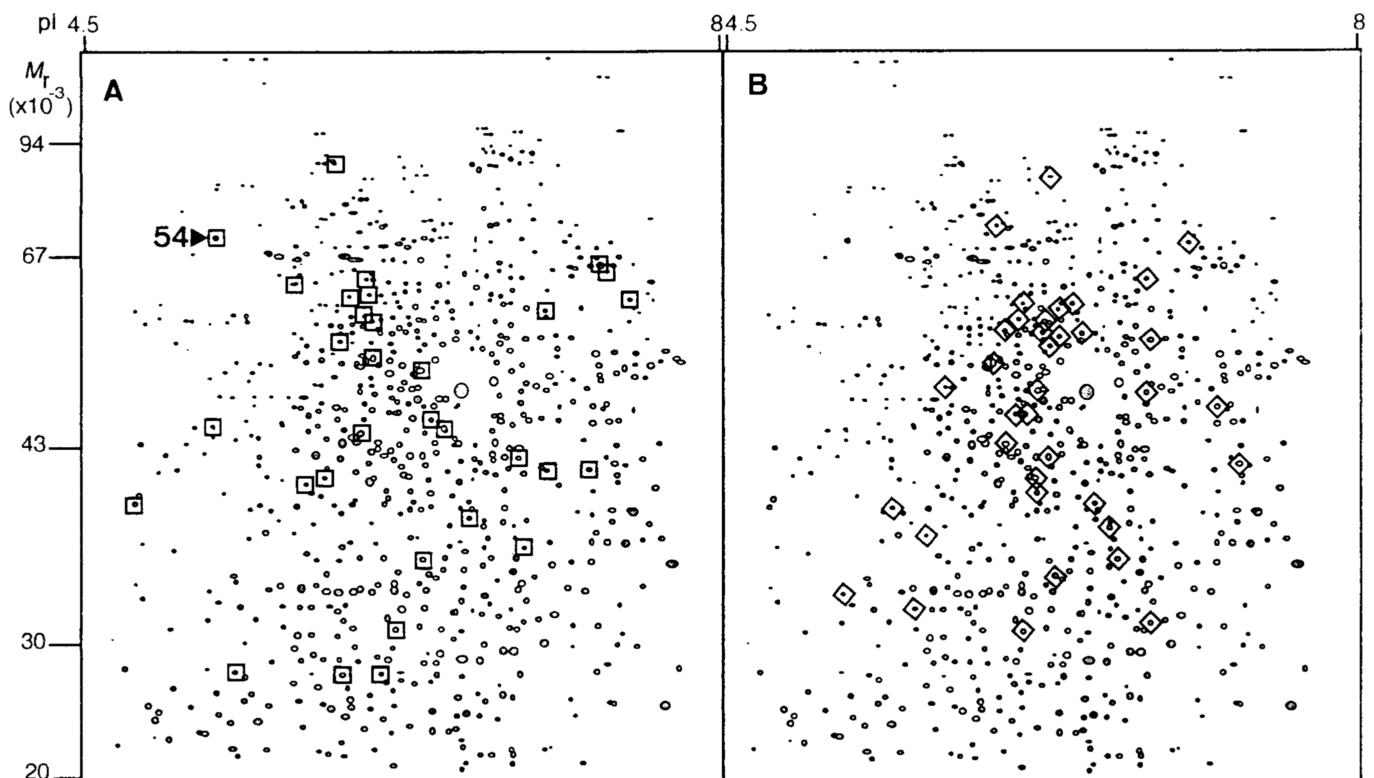

Fig. 5A–B. Localization on the master gel of two sets of acclimation-related polypeptides. **A** Polypeptides (□) more abundantly synthesized in acclimated plants than in non-acclimated plants after 2 d at 8° C. **B** Polypeptides (◇) more abundantly synthesized in acclimated plants than in non-acclimated plants after 7 d at 8° C. Polypeptide No. 54 is indicated by an *arrow*

	N-terminal	peak n°1	peak n°2
spot n°54	E K V V G I D L G	T T P S V V A Y T	Y V V L V P A Y F N D
	7 … 15	40 … 48	146 … 156
Hsc 70 tomato	G P A I G I D L G	T T P S Y V G F T	A V V T V P A Y F N D
	6 … 14	39 … 47	145 … 155
Hsp 70 soybean	G K A I G I D L G	T T P S Y V A F T	A V V T V P A Y F N D
	6 … 14	39 … 47	144 … 154
Hsp 70 maize	G P A I G I D L G	T T P S Y V G F T	A V V T V P A Y F N D
	7 … 15	40 … 48	146 … 156
Hsp 70 petunia	G P A I G I D L G	T T P S Y V G F T	A V V T V P A Y F N D
	30 … 38	63 … 71	166 … 176
SSC1 yeast	G S V I G I D L G	T T P S V V A F T	A V V T V P A Y F N D
	2 … 15	40 … 48	147 … 157
Dna K *E. coli*	G K I I G I D L G	T T P S I I A Y T	A V I T V P A Y F N D

Fig. 6. Amino-acid sequence of NH_2 terminal and two tryptic-digest peptides from polypeptide No. 54 and comparison with the sequences of HSP 70 proteins from tomato (Lin et al. 1991), soybean (Roberts and Key 1991), maize (Rochester et al. 1986), petunia (Winter et al. 1988), *Saccharomyces cerevisiae* (Craig et al. 1989) and *E. coli* (Bardwell and Craig 1984). Identical amino acids are *boxed*

of its relative abundance in the protein pattern. It has an M_r of 78000 and pI of 5. This polypeptide is a member of the set A. In plants transferred for 2 d to 8° C, it was two-fold more abundant in acclimated plants than in non-acclimated. It was synthesized in plants grown under normal conditions but its synthesis was already increased during the acclimation period at 14° C. After 7 d at 8° C, the synthesis was similar in acclimated and in non-acclimated plants, and was four-fold increased in comparison with plants grown under normal conditions.

The polypeptide No. 54 was concentrated from 50 two-dimensional-electrophoresis gels. Comparison of microsequence data for polypeptide No. 54 with protein databases revealed that this polypeptide shared homology with members of the heat-shock proteins of the 70 kDa (HSP 70) family (Fig. 6). Out of 29 residues 20–23 were identical when the peptide sequence was compared with HSP 70 proteins from plants and microorganisms. Moreover, the M_r and pI of this polypeptide were in keeping with the properties of this family of proteins.

Discussion

Cold acclimation is a well-known process in plants of temperate origin. We have shown that a subtropical species such as soybean is also able to acclimate to low temperatures. It seems, then, to be a general phenomenon since it is even found in some insects (Czajka et Lee 1990). This kind of phenomenon is very useful in increasing the understanding of the molecular mechanisms of cold tolerance, since it allows the correlation of the modifications in gene expression with cold tolerance on a single genotype.

In fact, in many plants of temperate origin, protein changes related to cold acclimation have been studied in order to understand the molecular basis of freezing tolerance. Most studies have focused on the characterization of protein changes occurring at the temperature which induces the cold acclimation in comparison with a control temperature (Guy and Haskell 1987; Gilmour et al. 1988). By contrast, we have compared the protein synthesis in acclimated and non-acclimated plants at a temperature (8° C) at which the plants display tolerance resulting from the acclimation. This allowed a comparison between plants of the same genotype under the same temperature condition (8° C) but showing different tolerance. We demonstrated that the greatest number of differences observed between acclimated and non-acclimated plants concerned proteins (57 out of 68) which were not previously increased during the acclimation period. Thus, only a few changes in protein synthesis occurring during the acclimation period (14° C) resulted in a preferential increase in acclimated plants when these were transferred to 8° C. The stimulation of these chilling-acclimation-related proteins would rather be an indirect effect of the acclimation period.

Some chilling-acclimation-related proteins (11 out of 68) were previously stimulated during the acclimation period. The HSP 70-related protein that we identified is among them. We can hypothesize that the acclimation period (14° C) induces the stimulation of these proteins and thereby increases the tolerance capacity of acclimated plants.

We characterized two sets of polypeptides (A and B) which were more abundantly synthesized in chilling-acclimated plants than in non-acclimated ones. These two sets consisted of different polypeptides depending on the time after transfer to 8° C. Polypeptide set A, synthesized after only 2 d at 8° C, was different from set B which resulted from synthesis following 7 d at 8° C. The differences in protein synthesis between acclimated and non-acclimated plants depended on the time at 8° C. Acclimation seemed to induce complex responses as far as protein synthesis is concerned. The short-term effects which could be observed after 2 d were completely different from the long-term effects which were visible after 7 d. These two kinds of effect may be involved in chilling-acclimation mechanisms. Different kinetics for the stimulation of protein synthesis were also found during low-temperature exposure (Guy 1990).

We demonstrated that one of the chilling-acclimation-related polypeptides detected after 2 d at 8° C shared a high degree of homology at the amino-acid level with members of the HSP 70 family. After 7 d at 8° C, acclimated and non-acclimated plants showed similar levels of this protein. It is likely that this protein is not rapidly enough stimulated by a direct transfer to 8° C, and the acclimation process stimulates its synthesis to a level which allows the plant to better tolerate the transfer to 8° C.

The HSP 70 proteins were first described in response to heat shock, and the induction was found in a wide range of organisms but was also caused by numerous stresses (Lindquist 1986). Nevertheless, in plants, cold stress was not reported among these (Neumann et al. 1989). In fact, many studies did not find similarities between heat-shock-induced proteins and cold-induced proteins (Guy et al. 1982; Yacoob and Filion 1986; Ougham 1987; Mohapatra et al. 1989). Here, we have described for the first time the stimulation of an HSP 70-related protein in chilling-acclimated plants. Some HSP 70-related proteins called heat-shock cognate are also found to be present under normal conditions and are not induced by heat shock. In fact, it is possible that the HSP 70 that we found to be stimulated by cold is not induced by heat stress since it is relatively abundant under normal conditions; it would thus be a heat-shock-cognate protein. In yeast, Craig and Jacobsen (1985) showed that the two heat-shock-cognate proteins of 70 kDa were stimulated under cold conditions and were necessary for growth at low temperature. Furthermore, the induction of this HSP 70 during the acclimation period at 14° C is in good agreement with the results obtained recently by Neven et al. (1992). These authors have shown an increase in the synthesis of a heat-shock-cognate protein of 70 kDa at acclimation temperature in spinach. These results are particularly interesting since, in combination with ours, they demonstrate for the first time the increased synthesis of the same family of proteins in freezing- and chilling-sensitive species. Thus the accumulation of HSP 70-cognate proteins during acclimation may have an important adaptive value.

Lin et al. (1990) reported that cold-regulated genes of *Arabidopsis thaliana* and wheat encoded polypeptides which remained stable upon boiling. It was proposed that these polypeptides could act as cryoprotectants in cold-acclimated plants. Indeed, one of them has a cryoprotective activity in in-vitro experiments (Lin and Thomashow 1992). Here, we have identified a chilling-acclimation-related protein as a heat-shock protein. Could low- and high-temperature tolerance involve similar mechanisms? Under normal conditions, HSP 70 would play a role in folding of nascent proteins and protein transport across the membranes of organelles (Vierling 1991). Under stress conditons, it was proposed that they interact with damaged proteins in order to avoid their aggregation (Pelham 1986). It is known that low temperature induces changes in protein folding that may result in aggregation (Jaenicke 1990). In cold-tolerance mechanisms, HSP 70 could play a fundamental role in protection to avoid the aggregation of proteins and prevent cells from resulting damage.

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