Effect of low temperature on the protein metabolism of wheat leaves

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Abstract

The effect of low temperature on the protein metabolism of wheat primary leaves was examined. In seedlings transferred from 25 to 5 °C, total soluble protein accumulation, *in vivo* protein synthesis and breakdown, *in vitro* protein breakdown, and SDS-PAGE profiles of proteinases in gelatine-containing gels were analysed. Leaf protein content increased within a 7-d period (70 % over the initial value) in plants exposed to 5 °C. The fast protein accumulation observed on days 0 - 2 was mainly attributed to a decreased breakdown. In further days, parallelly to a slowdown in the rate of protein accumulation, the leaf proteolytic activity increased. The incubation temperature also had an influence on the proteolytic activity: Q_{10} values for the 15 - 5 °C range were 80 - 200 % higher than those observed for the 25 - 15 °C range. On the other hand, the *in vivo* protein synthesis capacity, at either 25 or 5 °C, was not significantly modified in cold-treated plants. In addition to the enhanced activities of two serine-proteinases (previously found in control plants by SDS-PAGE analysis), cold-treated plants displayed a new proteinase, which had not been detected so far.

Additional key words: chilling, proteases, protein synthesis, protein degradation, *Triticum aestivum* L.

Introduction

Growth rates are reduced in plants exposed to chilling temperatures. Soluble and membrane proteins accumulate in the leaves of chilling resistant plants, such as wheat (Brown 1978, Levitt 1980). Some newly synthesised polypeptides appear parallel to the development of freezing tolerance. Moreover, they have been considered as essential to cold-acclimation (Guy 1990). This is supported by experiments showing that inhibitors of protein synthesis impair the cold acclimation (Hatano *et al.* 1976). However, most of the proteins are common to both acclimated and non-acclimated plants. These polypeptides might be related to plant functioning at low, non-freezing temperatures, enabling them to overcome physicochemical restrictions imposed to enzyme activity (Huner 1985).

It is well known that the rate of protein synthesis is highly dependent on temperature. Rochat and Therrien (1975) found that the incorporation of radioactive amino acids into leaf proteins is reduced by 50 % when wheat plants are transferred from 22 to 3 °C. These authors also found that cold-acclimated plants are less efficient than controls in incorporating radioactive amino acids into proteins. However, Weidner and Ziemens (1975) reported that this adaptive response only takes place when labelling is performed at nearly control temperatures.

Since both protein synthesis and degradation rates are expected to decrease at low temperatures, an increase in the

Received 6 May 1999, *accepted* 4 October 1999.
Abbreviations: EDTA - ethylenediaminetetraacetic acid; f.m. - fresh matter; PMSF - phenyl-methanesulfonyl fluoride; TCA - trichloroacetic acid.
Acknowledgements: This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica (PMT-PICT0084), Comisión de Investigaciones Científicas Prov. de Buenos Aires (CIC), Universidad Nacional de Mar del Plata and International Foundation for Science. Wheat seeds were generously provided by Ing. H. Bariffi (EEA-INTA Balcarce). R.D.C. is a Researcher from CONICET and J.A.T. is a Career Researcher from CIC. We thank Ms. Marta Terrazo for assistance in preparation of the manuscript.
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protein content would indicate that degradation suffers a greater reduction than synthesis. This was proposed by Pearce *et al.* (1991), who emphasised the need of examining protein turnover at low temperatures. Several genes encoding for proteases are induced by low temperature (Schaffer and Fischer 1988, Lee and Chen 1993). However, inhibition of proteolytic activities has also been reported (Prasad 1996). In any case, as Boothe *et al.* (1995) stated, it is necessary to examine the expression not only at the transcription level but also at the protein level itself. The latter is more directly correlated with physiological functions.

This work aimed at examining the protein metabolism in leaves of wheat seedlings subjected to low temperatures.

Materials and methods

Plants: Wheat (*Triticum aestivum* L. cv. San Agustín INTA) seedlings were grown in plastic pots filled with vermiculite soaked with half-strength Hoagland solution in a chamber at a temperature of 25 ± 2 °C, an irradiance of 250 μmol(photon) m^{-2} s^{-1}, and a 14-h photoperiod. Eight days after sowing, the first leaf was completely expanded. On the 10th day, some of the pots were transferred to another chamber, where they were further cultivated for 7 d at 5 ± 2 °C. The remaining pots were kept at 25 °C as controls.

Protein content determination: Primary leaves (5 g) were homogenised in 15 cm^3 of 50 mM Tris-HCl, pH 7.5, 0.1 % (v/v) 2-mercaptoethanol and 0.6 M NaCl (buffer A), using a *VirTis 45* homogeniser *(The Virtis Co.,* Gardiner, USA). Homogenates were filtered and centrifuged (*Prepsin 75* ultracentrifuge, *MSE*, England) at 100 000 *g* and the pellet was discarded. The supernatant was mixed with TCA (up to 10 % m/v final concentration). The resulting pellet was washed twice in ethanol:ether:chloroform (2:2:1, v/v), once with acetone and, finally, with ethyl ether. Proteins were solubilised in 0.75 M NaOH and 0.04 cm^3 aliquots were processed according to the micro-biuret assay (Goa 1953).

Estimation of *in vivo* protein synthesis capacity: After removing seedlings from the growth medium, their roots were gently washed and pruned to approximately 1 cm length. The aerial part above the first leaf insertion was also removed. Later on, pruned seedlings were placed in *Eppendorf* tubes containing 0.2 cm^3 of 100 mM [^{14}C] phenylalanine (10.36 kBq cm^{-3}) at the same growing conditions as described above. In preliminary experiments, conducted as described by Lamattina *et al.* (1985), it was found that 100 mM phenylalanine allowed for 80 - 90 % saturation of the endogenous pool. This concentration was used in subsequent labelling experiments in order to overcome the differences between phenylalanine endogenous pools caused by plant treatments (Lamattina *et al.* 1985). Control or seedlings cold-treated for 2 d were left to take up this solution at either 5 or 25 °C. The incorporation of radioactivity into protein was linear during either 8 h at 25 °C or 24 h at 5 °C. Therefore, seedlings tested at 25 °C and 5 °C were harvested following either 2- or 8-h intervals, respectively. Then, they were grounded in liquid nitrogen. Soluble proteins from primary leaf blades were extracted as follows: 1 g frozen powder was mixed with 4.5 cm^3 of 100 mM sodium phosphate buffer (pH 7.5), containing 0.5 mM hydroxyquinolein, 1 mM PMSF and 1 mM EDTA. After mixing for 30 min at 4 °C, homogenates were centrifuged at 400 *g* in cones with a cribbed glass bottom. Filtered samples were centrifuged at 100 000 *g* and the pellet was discarded. The supernatant was mixed with TCA (up to 10 % m/v final concentration). The resulting pellet was washed twice in ethanol:ether: chloroform (2:21, v/v), once with acetone and, finally, with ether. The pellet was solubilised in 1 cm^3 of formic acid before mixing with scintillation liquid. This protein extraction method was adopted due to the low amount of plant material used. It yielded about 60 % of the protein mass extracted by the total soluble protein estimation method. The rates of protein synthesis were calculated according to the slope of the radioactivity incorporated into protein against time.

Measurement of *in vitro* proteolytic activity: Primary leaf blades (5 g), homogenised in 15 cm^3 of buffer A in a *VirTis 45* homogeniser were filtered and centrifuged at 100 000 *g*, as described hereabove. Supernatants were dialysed against half-strength extraction buffers and caseinolytic activity was determined as described by Pinedo *et al.* (1993). The reaction mixture (total volume 0.4 cm^3) contained 0.28 cm^3 of dialysed supernatant and 2 mg of casein dissolved in 50 mM Na phosphate (pH 7.5). A set of assays was performed in the absence of casein (autolysis), eventually incorporated just before the reaction was stopped by addition of 0.05 cm^3 of TCA 50 % (m/v). The reaction mixtures were incubated at 5, 15 or 25 °C.

Gel electrophoresis: For the detection of proteinase activity, proteins were separated by SDS-PAGE (10 % acrylamide) in slabs, containing 0.1 % gelatine as previously described (Pinedo *et al.* 1993). After SDS removal, the slabs were incubated for 4 h at 37 °C in 50 mM sodium phosphate, pH 7.4. Finally, they were stained with *Coomasie Brilliant Blue R-250.*

All experiments were repeated at least twice, bearing essentially the same results.

Results and discussion

Protein accumulation at 5 °C: Total soluble protein content of primary wheat leaves increased within a 7-d period in plants exposed to 5 °C (Fig. 1*A*) reaching on the 7th day a 70 % increase over the initial value. Protein content of plants maintained at 25 °C (control) slightly decreased. The rate of protein accumulation at 5 °C was 2.5 mg(protein) g^{-1}(f.m.) d^{-1} in the first days. Thereafter, it was reduced (Fig. 1*B*).

Considering an equal amount of tissue (fresh matter), the SDS-PAGE profile of leaf soluble proteins of plants treated for 2 days at 5 °C showed an increase in protein mass. However, the bands displayed were essentially the same as those observed in controls (Fig. 2*A*). Although some gene families, such as *cor* (cold regulated), *lea* (late embryogenesis abundant), *wcs* (wheat cold-stimulated), and *rab* (ABA-responsive) are up-regulated at low temperatures in a number of species (Thomashow 1998), the proteins they encode may not contribute significantly to the total protein mass. Therefore, they may not be detected in one-dimensional gels.

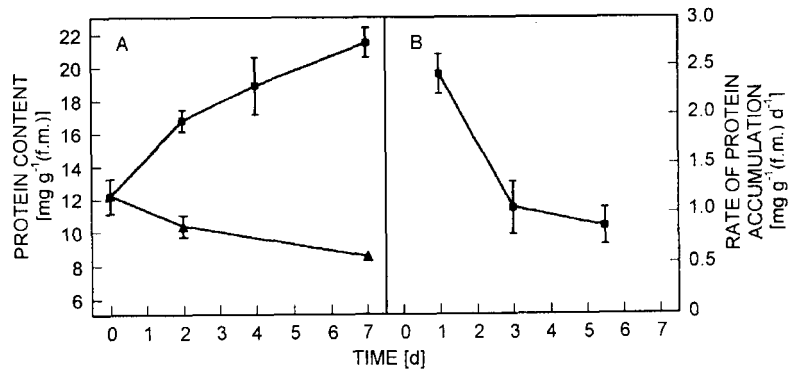

Fig. 1. Soluble protein content (*A*) in wheat leaves during cold treatment. Seedlings were cultivated at 25 °C for 10 d and subsequently grown at either 5 °C (*squares*) or 25 °C (*triangles*). *Bars* indicate standard error ($n = 6$). Rate of protein accumulation in low temperatures (*B*), calculated from data shown in *A*.

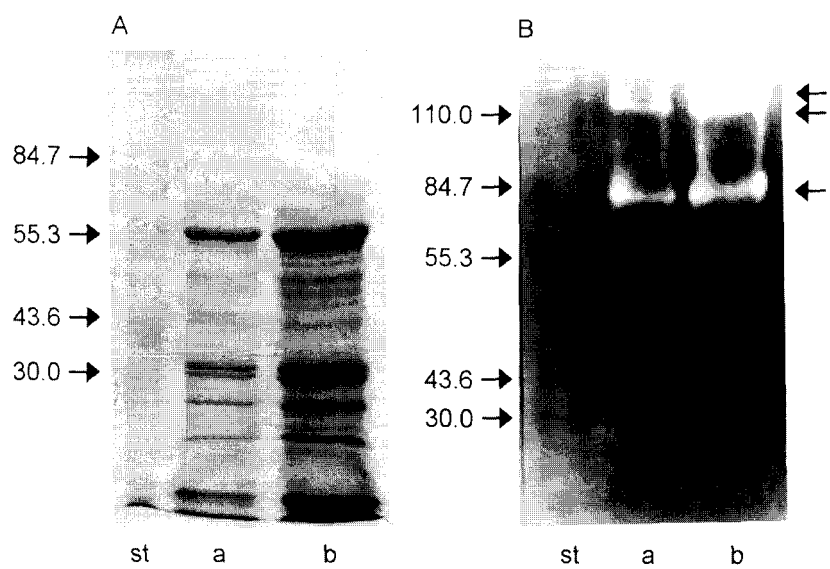

Fig. 2. SDS-PAGE of leaf proteins stained with *Coomasie Brilliant Blue* (*A*) and proteinases (*B*). Extracts corresponding to equal amounts of tissue (in fresh matter) were loaded on all lanes; *st* - molecular mass standard; *a* - control (25 °C); *b* - 2 d cold-treated. *Arrows* at the right margin indicate the presence of proteinase bands. Molecular mass in kD.

***In vivo* protein synthesis and degradation:** ^{14}C-Phe incorporation rate at 5 °C was approximately 15 % of that at 25 °C (Fig. 3). However, *in vivo* protein synthesis was not significantly affected in plants cold-treated for 2 d as

compared with controls (Fig. 3). On the other hand, experiments with ^{14}C-Phe pre-labelled wheat leaves, chased for 24 or 48 h with 100 mM unlabelled Phe at 5 °C, showed a meagre degradation of pre-existing proteins (data not shown).

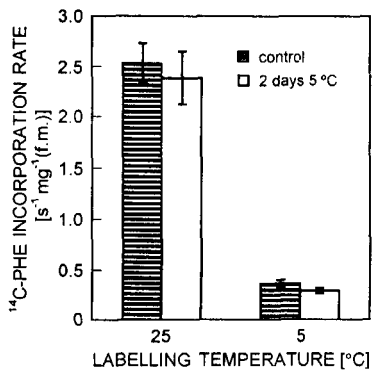

Fig. 3. Protein synthesis capacity. Rates of ^{14}C-Phe incorporation [counts s^{-1} mg^{-1}(f.m.)] during one hour of labelling into soluble protein were determined at either 5 or 25 °C in control and seedlings cold-treated for 2 d (means ± SE; $n = 6$)

***In vitro* proteolytic activity:** Proteolytic activity was detected using either the endogenous substrates (autolysis) or added casein. In both cases, it increased in cold-treated plants (Fig. 4). The incubation temperature also had a determinant effect on proteases: the proteolytic activity was more affected in the 15 - 5 °C range than in the 25 - 15 °C one. Thus, Q_{10} values in the 15 - 5 °C range were 80 - 200 % higher than those observed in the 25 - 15 °C one. Greatest Q_{10} values were observed between the 2nd and 4th day of cold treatment. As a result, it can be inferred that the leaf proteolytic activity was significantly affected soon after shifting to low temperatures (Fig. 4*D*).

The proteinase profiles of the control and 2-d-treated leaves were analysed by SDS polyacrylamide gels, containing gelatine as substrate (Fig. 2B). Three main bands of proteinase were displayed by cold-treated plants. Two of them, those with lower mobility, appeared with low intensity in control plants. These two bands correspond to those previously reported as belonging to the serine proteinase family (Pinedo *et al.* 1993). It is unlikely that

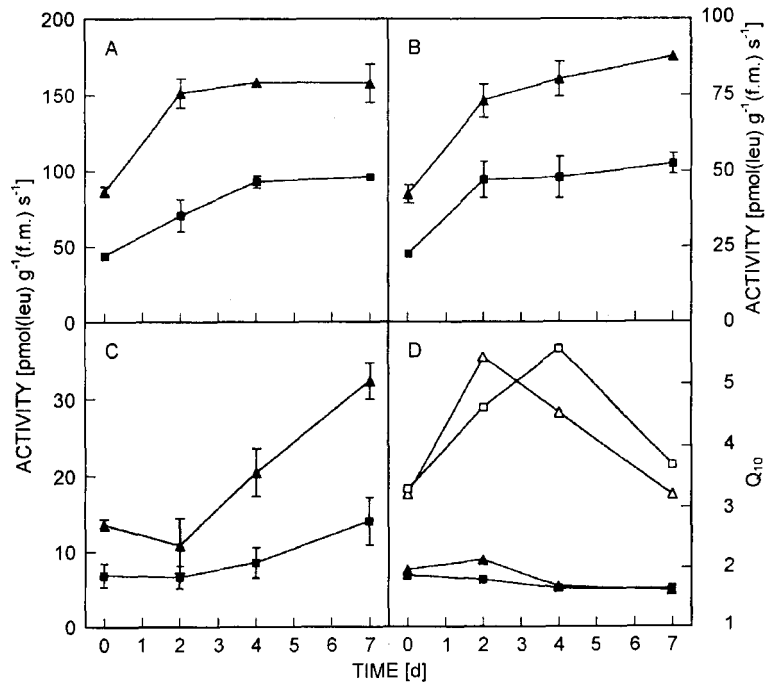

Fig. 4. *In vitro* soluble proteolytic activity during cold treatment. Leaf extracts, obtained in quadruplicate, were incubated with casein added as substrate (*triangles*) or with no substrate addition (*squares*). *A* - 25 °C; *B* - 15 °C; *C* - 5 °C; *D* - Q_{10} values for the ranges 25 - 15 °C (*closed symbols*) and 15 - 5 °C (*open symbols*). *Bars* indicate SE ($n = 5$).

enzyme activity regulators would be preserved after denaturing electrophoresis and subsequent handling for activity analysis. Therefore, the patterns displayed by cold-treated plants should reflect increases in enzyme contents. In addition, the presence of a new proteinase band in cold-treated plants appears as an interesting finding.

The information concerning protease gene expression and activity at low temperatures is relatively limited and sparse, and mainly related to chilling sensitive species. Schaffer and Fischer (1988) reported that the expression of mRNAs for thiol proteases is induced in tomato fruits. Prasad (1996) found that the protease activity in developing maize seedlings is inhibited at low temperatures and that this phenomenon is probably due to protease oxidation by reactive oxygen species. It was also reported that ubiquitin, the polypeptide of 76 amino acids which targets proteins

for cytosolic degradation, is induced in maize leaves at 4 °C (Didierjean *et al.* 1996). On the other hand, in chilling tolerant bromegrass, Lee and Chen (1993) reported a 2 - 3 fold increase in transcript levels of a gene identified as homologous to cathepsin D. Other reports on the effects of temperature on proteolysis are related to heat shock stress (Ferguson *et al.* 1990, 1994) and seasonal changes (Fischer and Feller 1994). Moreover, genes that encode proteases can also be induced by wounding and osmotic stresses (Koizumi *et al.* 1993, Schaller and Ryan 1996).

The increase in proteolytic activity at low temperatures may fulfil different physiological roles. First, it might control the rate of protein accumulation, which decreases along the cold period (Fig. 1*B*). Second, an increased proteolytic activity might help to degrade proteins that have become useless as a consequence of cold (Ingram and Bartels 1996). Third, the fast protein loss observed during plant growth recovery at increasing temperatures (Tognetti *et al.* 1989) would be facilitated by an initial high proteinase content.

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