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Low temperature perception in plants: effects of cold on protein phosphorylation in cell-free extracts

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Abstract Activities of prevalent protein phosphatases decreased by nearly 95% and those of individual protein kinases were differentially reduced at low temperature. Inhibition of phosphatase activity at temperatures below 12°C resulted in marked hyperphosphorylation of a 58-kDa protein (PP58). The temperature threshold for hyperphosphorylation of PP58 coincided with the known threshold for cold-induced calcium influx. Since calcium influx is triggered by several environmental stresses, we propose that the observed direct effects of cold on the phosphorylation of specific proteins enable cells to couple a shared calcium signal to a cold-specific transduction pathway.

Key words: Cold acclimation; Cold inactivation; Plant signal transduction; Protein phosphorylation

1. Introduction

Cold acclimation, an adaptive response leading to development of freezing tolerance in certain plants, occurs upon exposure to low, non-freezing temperatures for a period of a few days to several weeks. Cold acclimation is known to be associated with altered gene expression, production of new proteins and extensive metabolic adjustments [1,2]. In order to cold acclimate, low temperature signals have to be perceived and transduced into biochemical responses. We have previously shown that cold-triggered influx of apoplastic calcium into the cytosol [3] and cold-specific protein phosphorylation [4] are required for cold acclimation of alfalfa cells. Calcium chelators, calcium channel blockers, and inhibitors of calcium-dependent protein kinases prevent cold-induced protein phosphorylation, gene expression and cold acclimation. Thus, calcium acts as a second messenger during low temperature signal transduction. However, an increase in cytosolic calcium is also triggered by several other stresses [5,6]. Thus a question arises as to how plants distinguish between different stresses? We have suggested [3] that, in order for a plant to couple the shared calcium response to a cold-specific signal transduction pathway, cold must act directly on some components of the low temperature signal transduction cascade. In support of this suggestion is our observation that the phosphorylation level of specific nuclear proteins is rapidly and reversibly altered in organello by low temperature [7].

In the present report, we present evidence that in cell-free extracts of alfalfa, low temperature alters the phosphorylation level of some proteins through a differential inhibition of protein kinases and phosphatases.

2. Materials and methods

2.1. Plant Material and preparation of cell-free extracts

Seeds of *Medicago sativa* cv. Apica were germinated and grown in a growth chamber at 24°C and 12-h photoperiod. At 30 days after sowing, 18 g fresh weight of shoot tissue was ground with a chilled mortar and pestle in 3 ml/g fresh wt of buffer A (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 μM pepstatin A, 1 mM PMSF, 10% polyvinylpolypyrolidone, 0.1% Triton X-100 and 0.5% β-mercaptoethanol). The homogenate was successively centrifuged at $120\times g$ and $1200\times g$ for 5 min each to remove cell debris and the insoluble polyvinylpolypyrolidone, and was then centrifuged at $28\,000\times g$ for 20 min. The resulting supernatant was aliquoted and stored at −80°C. An aliquot of each fraction was used to determine protein concentration by the dye-binding method [8] using the Bio-Rad (Richmond, CA, USA) protein assay kit.

2.2. Protein kinase/phosphatase assays

Protein kinase activity was assayed after fractionation of protein on polyacrylamide gels. Alfalfa protein was extracted as previously described [4] and resolved on SDS-polyacrylamide gels containing either H1 Sigma type III-S histone or casein (0.5 mg/ ml). After electrophoresis, the gels were processed for renaturation and the kinase reaction as described [9]. The kinase buffer was 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 25 μM [γ-^{32}P]ATP (1.85 GBq/mmol). For protein phosphatase assays, seedling homogenates were prepared as described [10] and assayed for phosphorylase phosphatase activity as prescribed in the Protein Phosphatase Assay System (Life Technologies, Gaithersburg, MD, USA).

2.3. In vitro protein phosphorylation

In all cases, the reaction volume was 50 μl. An aliquot of cell-free extract (50 μg of protein in 30 μl) was allowed to thaw on ice, mixed with 10 μl solution containing 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 20 μM [γ-^{32}P]ATP (1 GBq/mmol). The phosphorylation reaction was carried out for 10 min at indicated temperatures. The reactions were stopped with the addition of 50 μl of 2×SDS protein sample buffer (0.125 Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol 0.1% bromophenol blue) and placing in boiling water for 5 min.

2.4. Electrophoresis and densitometry

SDS-PAGE for separation of radio-labeled phosphoproteins was on 11% polyacrylamide gels, whereas that for in-gel kinase assays was on 9% polyacrylamide gels. Each lane contained 30 μg of protein. The gels were stained with Coomassie blue, and dried on Whatman 3MM paper. Quantitation of autoradiograph signals was obtained by densitometry with a Model SM3 scanner interfaced with the Image-Master 1-D software (Pharmacia/pdi, Huntington Station, NY, USA). Analysis was carried out on autoradiographs which had no apparent overexposure of signals. Signals were corrected for variability due to gel loading by using as reference signals from stained protein in corresponding gel lanes.

3. Results

First, we examined the effects of low temperature on the

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activities of protein kinases. Total protein extracted from alfalfa grown at 24°C was fractionated on polyacrylamide gels containing either histone or casein as phosphorylation substrate. When assayed at 4°C, activities of both histone and casein kinases decreased markedly as compared to those at 25°C (Fig. 1A). However, different protein kinases displayed differential sensitivities to cold. The densitometric analysis presented in Fig. 1B shows that whereas one histone kinase, H2, lost more than 90% of its activity at 4°C, another, H1, retained nearly 35% of its activity. Differential inhibition at 4°C can also be seen for casein kinases (C1–C6).

We then examined the effects of low temperature on the activity of protein phosphatases. Homogenates were prepared from alfalfa grown at 24°C and assayed for phosphatase activity at either 4°C or 25°C. The phosphorylase phosphatase assay used here has been reported to reflect the activity of at least 85% of cellular phosphatases [10]. As shown in Fig. 2, activities of the most active protein phosphatases were inhibited more than 90% in reactions carried out at 4°C. Thus

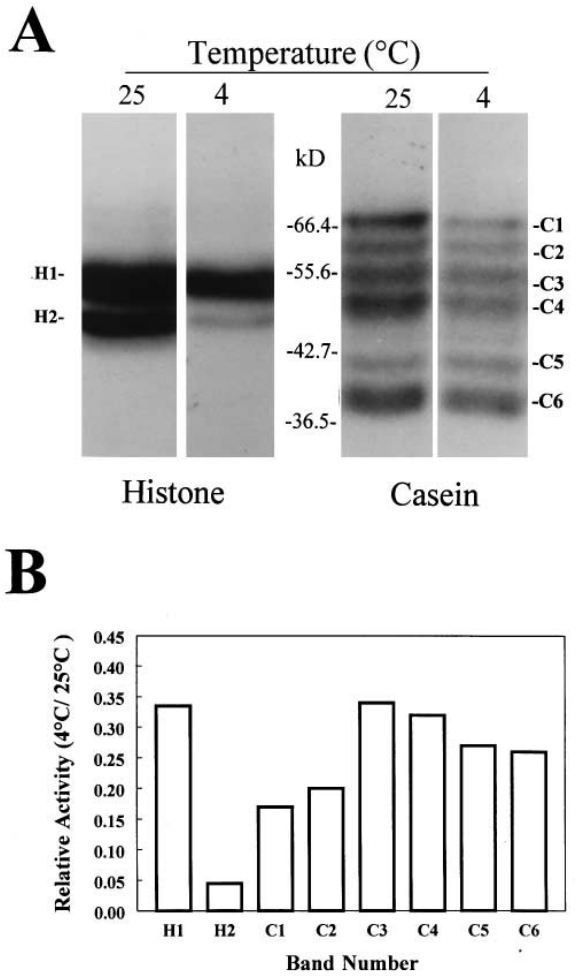

Fig. 1. Effects of low temperature on the activity of protein kinases. (A) Aliquots of total protein from alfalfa grown at 24°C were subjected to electrophoresis on gels containing SDS and either histone or casein. Protein kinase activity was detected by autoradiography of gel strips incubated in protein kinase buffer at the temperatures indicated. (B) Densitometric analysis of the activity of the protein kinases indicated by alphanumeric nomenclature. Each datum represents the fraction of protein kinase activity remaining in gel samples incubated at 4°C. Data presented are representative of experiments with similar results.

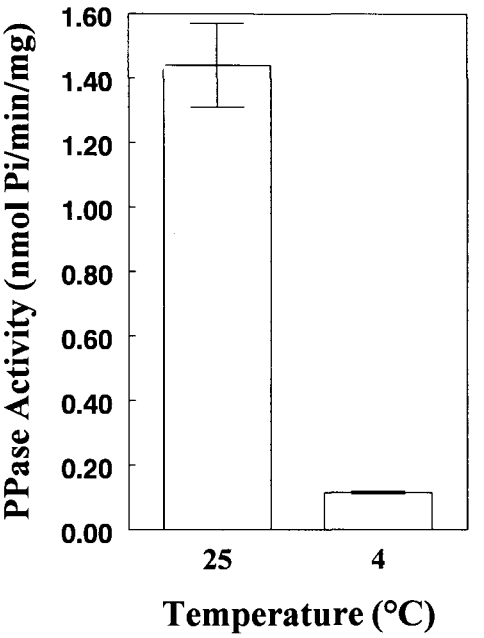

Fig. 2. Effects of low temperature on the activities of protein phosphatases. Homogenates prepared from alfalfa grown at 24°C were assayed for dephosphorylation of ^{32}P-labeled glycogen phosphorylase (SA 4.4×10^5 dpm/nmol phosphate). Specific activity was calculated after 10 min of reactivity at either of the temperatures indicated. Each datum is the average of three sample dilutions. Data presented are representative of experiments with similar results.

protein kinases and phosphatases exhibit differential sensitivity to cold.

The above results suggested that a differential sensitivity to cold of protein kinases and phosphatases could alter the equilibrium of their actions on substrate proteins and, thereby, change the phosphorylation level of the product, favoring hyperphosphorylation when phosphatase is much more inhibited than is kinase. To examine this possibility, tissue homogenates were prepared from alfalfa seedlings grown at 24°C and assayed in vitro for protein phosphorylation at a range of temperatures. As can be seen in Fig. 3A, the phosphorylation level of a 58-kDa phosphoprotein (PP58) increased sharply as the reaction temperature declined below 12°C, reaching a maximum at 0°C. The data in Fig. 3B show a comparison of the increase in phosphorylation of PP58 with that of the decrease in phosphorylation of a 34-kDa phosphoprotein (PP34). These results indicate that temperatures below 12°C favor the hyperphosphorylation of specific proteins such as PP58.

To determine whether the phosphorylation pattern of PP58 could be attributed to an inhibitory effect of low temperature on protein turnover, we pre-incubated samples for 10 min either at 25°C or 4°C, and then labeled them at 4°C or 25°C, respectively. As can be seen in Fig. 4A, pre-incubation of the protein extract did not affect the phosphorylation of PP58 at either temperature. In addition, phosphoprotein levels of PP58 did not increase in reactions carried at 25°C in presence of a cocktail of protease inhibitors (data not shown). Therefore, differential degradation of PP58 or of the enzymes responsible for its phosphorylation cannot account for the observed effect of temperature on its phosphorylation.

To ascertain whether the observed cold stimulation of PP58

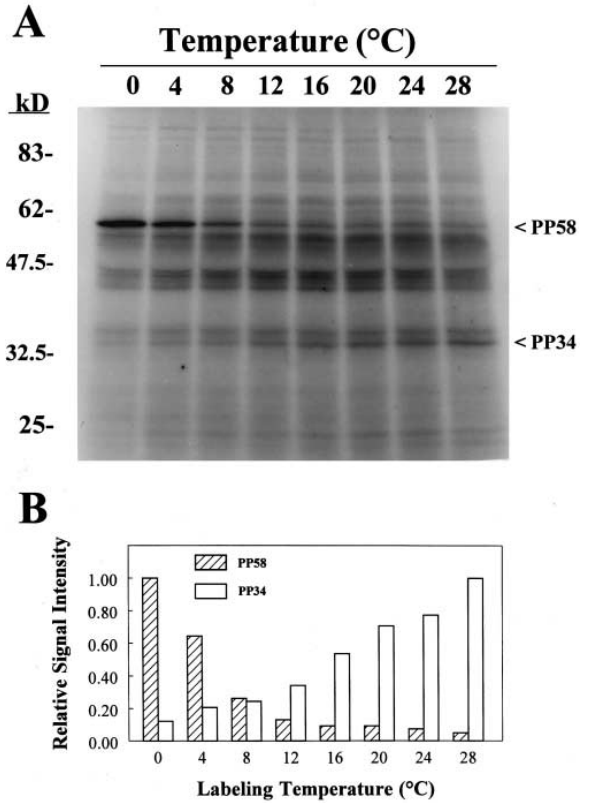

Fig. 3. Effect of a range of reaction temperatures on the in vitro phosphorylation of alfalfa proteins. (A) Autoradiogram of phosphorylated protein fractionated by SDS-PAGE. Individual reactions were carried out at the indicated temperatures in a programmable thermal bath. Similar results were obtained in independent experiments. (B) Densitometric analysis of the signals from two phosphoproteins with molecular sizes of 58 kDa (PP58) and 34 kDa (PP34), as indicated in A. The highest value in each set was considered as 1.

phosphorylation was the result of a decrease in protein phosphatase activity, [γ-^{35}S]thio-ATP was used in the labeling reactions instead of [γ-^{32}P]ATP. This thio-analog of ATP can be utilized by a variety of kinases to phosphorylate their substrates [11]. However, once its γ-thiophosphoryl group is transferred to a substrate, the modified amino acids are resistant to dephosphorylation, thus acting as a competitive inhibitor of phosphatases. As shown in Fig. 4B, the cold-induced increase in PP58 phosphorylation observed with ^{32}P, was no longer detected with ^{35}S. This suggests that hyperphosphorylation of PP58 results from cold-mediated inhibition of protein phosphatase activity. We further attempted to determine the class to which the cold-sensitive phosphatase belongs. However, neither NaF, okadaic acid, nor EGTA altered the phosphorylation level of PP58 at 25°C (data not shown). Thus, the cold sensitive protein phosphatase is not type 1, 2A, or 2B. We have not directly tested the possibility that it is a Mg^{2+}-dependent, type 2C, protein phosphatase. This is experimentally difficult due to the absence of specific inhibitors of protein phosphatase 2C activity and the Mg^{2+} requirements of protein kinases.

4. Discussion

The present study shows that the most prevalent protein phosphatases [10] are almost completely inhibited at 4°C, the temperature which is routinely used to trigger cold acclimation in the laboratory. However, different protein kinases were found to have differential sensitivity to cold and some of them can retain substantial activity at 4°C. While a differential effect of low temperature on the stability and activity of different proteins may be expected, the present observation is of particular significance because the two types of proteins responding differentially to cold, protein kinases and phosphatases, catalyze opposing reactions. Thus by shifting the equilibrium between phosphorylation and dephosphorylation, low temperature directs its signal transduction cascade through cold-specific protein phosphorylation [4] leading to cold-specific gene expression and development of freezing tolerance. As cellular temperature declines, dephosphorylation of certain proteins such as PP58 is more strongly inhibited than their phosphorylation, resulting in their hyperphosphorylation. This cold-induced inhibition of dephosphorylation may be mediated by either conformational changes [12,13] or dissociation [14] of macromolecular complexes. A test of these possible mechanisms must await the identification and purification of the protein phosphatases involved.

The data in Fig. 3 show that increasing phosphorylation of PP58 at temperatures below 12°C is not simply a Q_{10} effect. The slope of the line connecting the phosphorylation values at different temperatures suddenly changes at 12°C. Thus PP58 phosphorylation shows little change with decrease in temperature above 12°C. However, below 12°C, the level of phosphorylation nearly doubles with every 4°C decrease in temperature. For this reason, we consider 12°C as a threshold temperature at which the phosphorylation of PP58 starts increasing with further decrease in temperature. This temperature lies within the range of temperatures which trigger calcium influx into the cytosol [3], induce changes in membrane fluidity [1,2], and inflict chilling injury to chilling-sensitive plants [2,15]. This coincidence within a narrow range of temperatures triggering different processes is significant because all these processes are related to cold acclimation and may originate in a common temperature sensor.

How could the cold-promoted increase of phosphorylation of some proteins have a physiological niche? Based on evidence provided by us and others [3–6,16], we have proposed [3] that the low temperature signal transduction pathway comprises the initial perception of cold through a decrease in membrane fluidity, an activation of calcium channels and influx of cell wall calcium into the cytosol, and specific phosphorylation and dephosphorylation events leading to the induction of specific genes. However, influx of calcium has been shown to occur in response to a variety of stimuli [5,6]. Thus, it is not known how the cell decodes a calcium signal to mount a stimulus-specific response. It is now apparent that sensing of low temperature by the plasma membrane coupled with the cold-imposed modulation of the activities of protein kinases and phosphatases may allow plants to distinguish between low temperature and other stresses which also increase cytosolic calcium. Phosphorylation of proteins triggered by calcium and sustained by low temperature may prove to be a key mechanistic interaction for low-temperature signal transduction.

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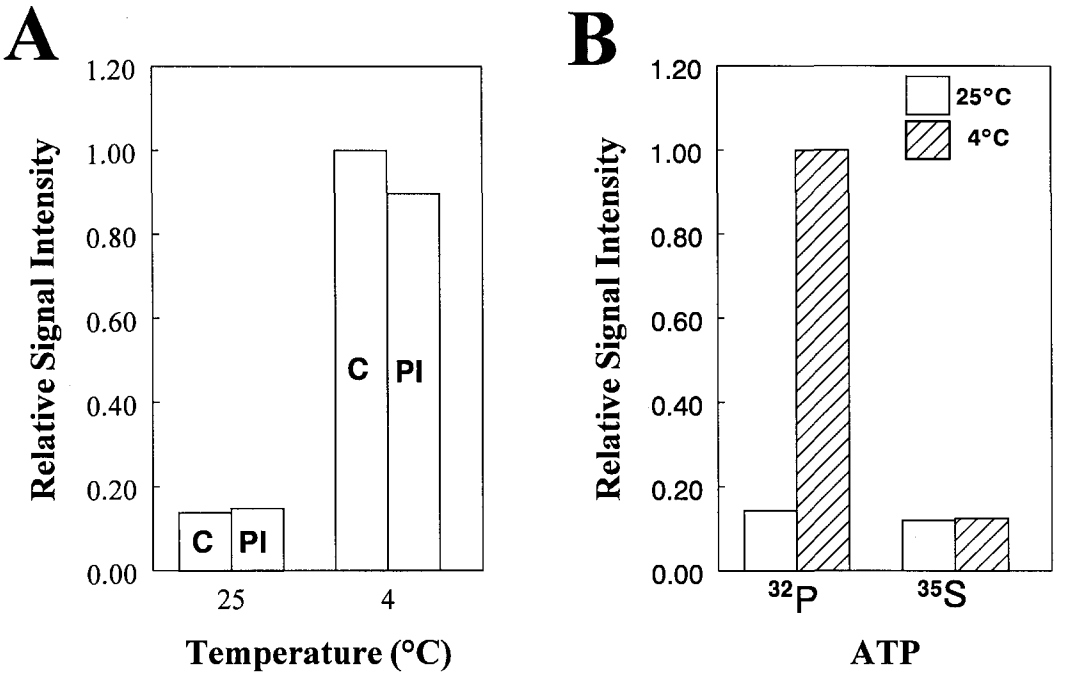

Fig. 4. Analysis of the effects of proteases and protein phosphatases on the cold-induced phosphorylation of PP58. (A) Effect of pre-incubation of samples on subsequent in vitro phosphorylation of PP58. Control reactions (C) were carried out under usual conditions at the indicated temperatures. The other samples were pre-incubated (PI) for 10 min at either 4° or 25°C in reaction buffer, and then labeled with [γ-^{32}P]ATP for 10 min at 25° or 4°C, respectively. (B) Effect of inhibition of protein phosphatase activity on the phosphorylation of PP58. In vitro phosphorylation reactions were with either [γ-^{32}P]ATP or phosphatase-insensitive [γ-^{35}S]thio-ATP at the indicated temperatures. Data were obtained by semiquantitative densitometry of autoradiographs. To assign relative values,, the highest signal intensity obtained was considered as 1. The difference in signals between the two radioisotopes used reflects the difference between their decay energies.

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