Biochemistry (Moscow), Vol. 69, No. 5, 2004, pp. 575-579. Translated from Biokhimiya, Vol. 69, No. 5, 2004, pp. 706-711.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM03-255, April 18, 2004.

Initial Stages of Low-Temperature Induction of Cabbage Cold Shock Protein Gene *csp5*

F. R. Gimalov*, A. Kh. Baymiev, R. T. Matniyazov, A. V. Chemeris, and V. A. Vakhitov

Institute of Biochemistry and Genetics, Ufa Scientific Center, Russian Academy of Sciences, pr. Oktyabrya 69, Ufa 450054, Russia; fax: (3472) 35-6088; E-mail: molgen@anrb.ru; gimalov@anrb.ru

Received November 4, 2003
Revision received December 29, 2003

Abstract—Some stages of low-temperature signal transduction causing appropriate cold stress response in plants are considered. The effects of Ca^{2+} chelators, Ca^{2+} channel blockers, and protein kinase inhibitors on protoplasts and plants of cabbage suggest that the initial stages of cold signal transduction are the change in membrane fluidity followed by the activation of calcium channels and elevation of Ca^{2+} influx into the cytoplasm. Increased concentration of Ca^{2+} in cytoplasm activates calcium-dependent protein kinase most likely participating in induction of transcription factors necessary for the expression of cold-regulated genes, in particular *csp5*. The protein kinase inhibitors staurosporine and wortmannin insignificantly repress the expression of *csp5*.

Key words: cold shock, calcium channel, protein kinase, protein kinase inhibitor, signal transduction, *Brassica oleracea*

Evaluation of molecular mechanisms responsible for the perception of changes in environmental temperature followed by metabolic changes in plants can be of practical significance for agriculture, particularly for the development of cultivars resistant to unfavorable temperatures. Diverse biochemical processes triggered by low temperature in the cells of cold-resistant plants prepare them for negotiation of cold stress [1]. The cold acclimation is accompanied by induction of some genes, protein synthesis *de novo*, and corresponding physiological changes [2, 3]. Many cold-induced genes have been identified in various plant species. Previously, we have cloned the gene encoding the cabbage cold shock protein Csp5, which is induced in the plants exposed to low temperature [4]. However, the processes preceding expression of this gene and similar genes of other plants are not yet well understood. Change in membrane fluidity, which drops with decrease and elevates with increase in temperature [5], is known as a direct and possibly the primary consequence of the influence of temperature variations on the cell [1]. These observations suggest that the plasma membrane acts as a primary thermal sensor, due to dynamic variation in its physical parameters in response to the changes in temperature [6, 7].

In the study presented, we investigated the initial stages of the mechanism of low-temperature signal transduction from the environment to intracellular structures providing an adequate stress response, using induction of the gene *csp5* encoding the cabbage cold shock protein as the final link of signal-transmitting chain. Taking in account the significance of the cell membrane in perception of various outer signals, we also studied how expression of this gene depends on biochemical features of the cell membrane.

MATERIALS AND METHODS

Seedlings of cabbage *Brassica oleracea*, cultivar Amager, grown on the nutrient solution of Murashige and Skoog (Serva, Germany) [8] and protoplasts obtained from four-week-old cabbage plants were the objects of the study. Cold stress was induced in seedlings and protoplasts by placing them into a thermostat at 5°C for intervals of 15 min to several hours.

Protoplasts were obtained from the cabbage leaves by enzymatic treatment with cellulase and macerozyme [9].

The calcium chelators EGTA (at final concentration of 10 mM) and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (2 mM), calcium ionophore A23187 (10 μM), calcium channel blockers nifedipine (3 μM) and cinnarizine (5 μg/ml), and the protein kinase inhibitors staurosporine (1 μM) and wortmannin (1 μM) were added 1 h before exposure to low temperature.

* To whom correspondence should be addressed.

0006-2979/04/6905-0575 ©2004 MAIK "Nauka/Interperiodica"

Total RNA was isolated using guanidine thiocyanate buffer, pH 8.0 [10]. RNA concentration was determined on an SP-46 spectrophotometer (LOMO, Russia).

5′-Terminal labeling of DNA fragments was carried out using [γ-^{32}P]ATP and the polynucleotide kinase of phage T_4 [11].

Dot blotting of RNA was performed according to a standard protocol [12]. The [^{32}P]dATP-labeled DNA encoding the cabbage *csp5* gene was used as a probe. The RNA was immobilized on filters followed by hybridization with the radioactively labeled probe, drying, and exposure of the filters on RM-V X-ray film (Tasma, Kazan, Russia). RNA in hybridization spots was quantified by scanning of the X-ray film in transmitted light on a Chromoscan 3 densitometer (Joyce Loeble, UK).

For protein isolation, the seedlings were ground with a mortar and pestle in liquid nitrogen and the powder obtained was extracted with buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% polyvinylpyrrolidone PolyClarAT (Serva), 0.1% Triton X-100, and 0.5% 2-mercaptoethanol. Insoluble material was removed by centrifugation at 14,000*g* for 5 min. Protein was determined by the Bradford method [13].

Electrophoresis of proteins in 10% polyacrylamide gel with SDS was performed by the method of Laemmli [14].

Protein phosphorylation *in vivo* was determined as follows. The cabbage seedlings growing in liquid nutritive medium at room temperature were supplied with ^{32}P-labeled orthophosphate (30 MBq per sample) with subsequent incubation for 24 h. Then the seedlings were transferred into a cool chamber (5°C) and incubated for different time intervals. Proteins were isolated from the seedlings and determined by PAGE. Gels were dried and subjected to autoradiography.

Protein kinase activity *in vitro* was determined as described by Monroy et al. [15]. After the incubation at low temperature, the cabbage plants were ground with a mortar and pestle in liquid nitrogen and homogenized in buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 μM pepstatin A, 1 mM PMSF, 10% polyvinylpyrrolidone PolyClarAT, 0.1% Triton X-100, and 0.5% 2-mercaptoethanol. Cell debris and insoluble polyvinylpyrrolidone were removed by centrifugation for 5 min at 1200*g*. Then another centrifugation was carried out for 20 min at 28,000*g*, and the supernatant was divided into aliquots and stored at −80°C. The protein extract (30 μl) was mixed with 10 μl of buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol (DTT), 5 mM glycerol, 1 mM PMSF, 1 mg/ml histone III-SS (Sigma, USA), and 5-50 μCi [γ-^{32}P]ATP. Phosphorylation was carried out for 15 min and stopped by addition of 50 μl of buffer containing 0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.1% bromophenol blue and by boiling for 3 min.

RESULTS AND DISCUSSION

The adaptation of plants to growth conditions requires the existence of an effective mechanism for perception and transformation of environmental stimuli. The mechanisms mediating the cold signal transduction from the environment with following expression of cold-induced genes are not well studied, despite considerable attention attracted to this problem. The effect of the cold on suspended cell culture of alfalfa [7] and on rape [16] and arabidopsis plants [17] is evidenced in a dramatic increase in Ca^{2+} concentration in the cytoplasm preceding enhanced expression of cold-induced genes. As this takes place, the rigidity of cell membrane increases, as it is evident from measurements of polarization index of isolated protoplasts [7]. The same condition of membranes, which imitates the effect of the cold, can be achieved by their treatment with dimethylsulfoxide (DMSO) at room temperature [7, 16]. The opposite effect is achieved when membranes are treated with benzyl alcohol (BA) [7].

The protoplasts we used in our experiments were prepared from leaves of four-week-old cabbage plants. To increase the fluidity of plasma membranes we incubated the cabbage protoplasts (10^6 cells/ml) in medium containing 5 or 10 mM BA (and in the control medium without BA). Then RNA for dot blotting was isolated from these protoplasts. Dot blotting showed that expression of cold shock protein is significantly lower in protoplasts incubated at 5°C in presence of BA compared with control (Fig. 1a). A decrease in fluidity of plasma membrane was achieved by the treatment of protoplasts with DMSO solution (1, 2, or 3% DMSO). Dot blotting of mRNA isolated from the protoplasts (10^6 cells/ml) treated or not treated with DMSO and incubated at different temperatures was performed using a ^{32}P-labeled fragment of cabbage *csp5* gene as a probe and revealed enhanced expression of this gene in both the protoplasts treated with DMSO at 25°C and protoplasts incubated at 5°C (Fig. 1b).

Change in plasma membrane fluidity apparently results in corresponding change in membrane tension force, which contributes to opening of cold-activated calcium channels (a kind of mechanosensitive ion channels) [17]. That is, a decrease in plasma membrane fluidity increases the Ca^{2+} influx into plant cells. Earlier, it was shown that modulation of the intensity of this flow in rape seedlings caused by various agents was reflected at the expression level of genes regulated by cold stress [16]. When cabbage protoplasts were treated with the Ca^{2+} chelator EGTA at 5°C, the expression level of *csp5* significantly decreased compared with the expression level of this gene in the cells not treated with EGTA under cooling (Fig. 2). However, complete inhibition was not observed. This fact can be attributed to the binding of extracellular calcium ions by EGTA and to the elevation

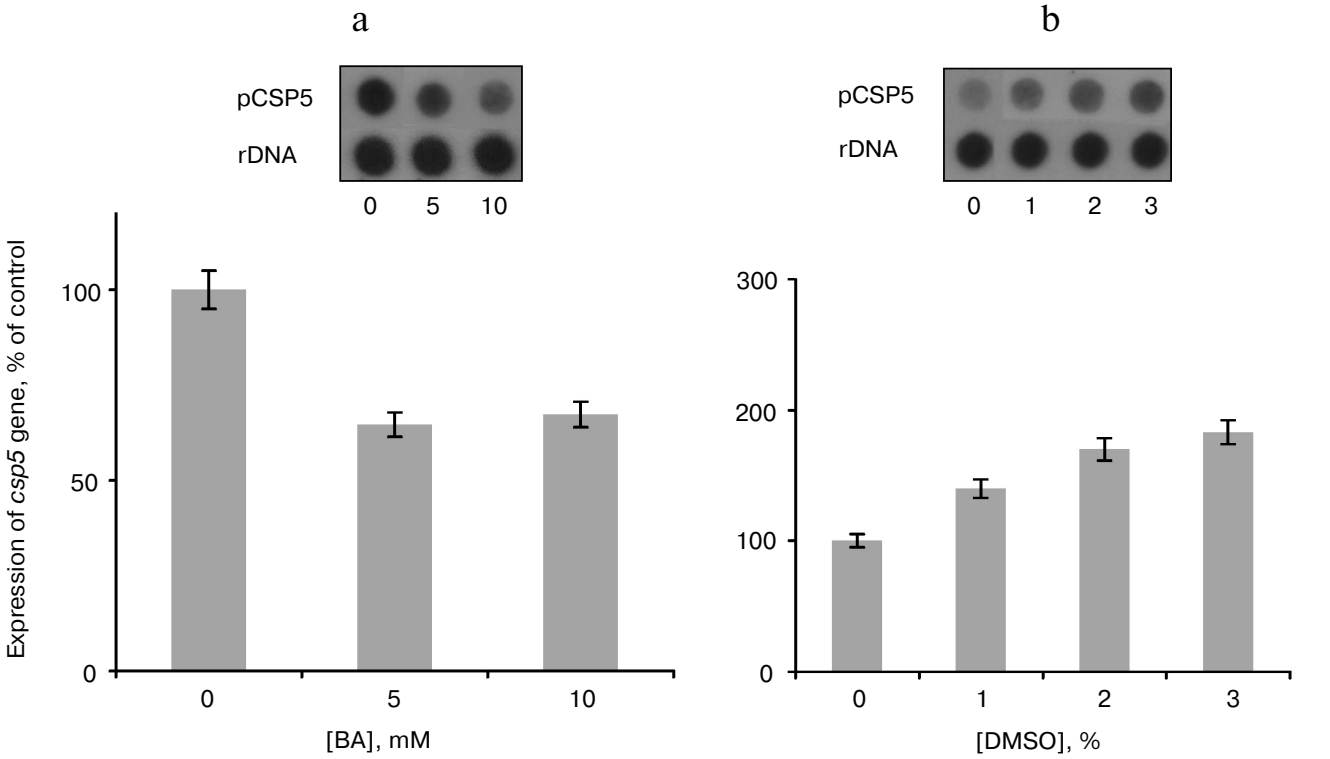

Fig. 1. Expression of *Br. oleracea* cold shock protein gene *csp5* in protoplasts treated with benzyl alcohol (BA) (a) or DMSO (b) at 5 (a) or 25°C (b). The data from dot blotting are shown at the top (plasmid coding *csp5* gene (pCSP5) and ribosomal RNA gene fragment (rDNA) are used as probe).

of total Ca^{2+} concentration in cytoplasm under the stress due to the release of Ca^{2+} from various depots. The Ca^{2+}-ionophore A23187, which stimulates the influx of calcium ions bound on the cell wall into the cytoplasm, induced a significant elevation in expression level of *csp5* in the cells at 25°C (Fig. 2a). We also studied the effect of the Ca^{2+}-channel blockers nifedipine and cinnarizine on the expression of *csp5* (Fig. 2b). Nifedipine, a blocker of

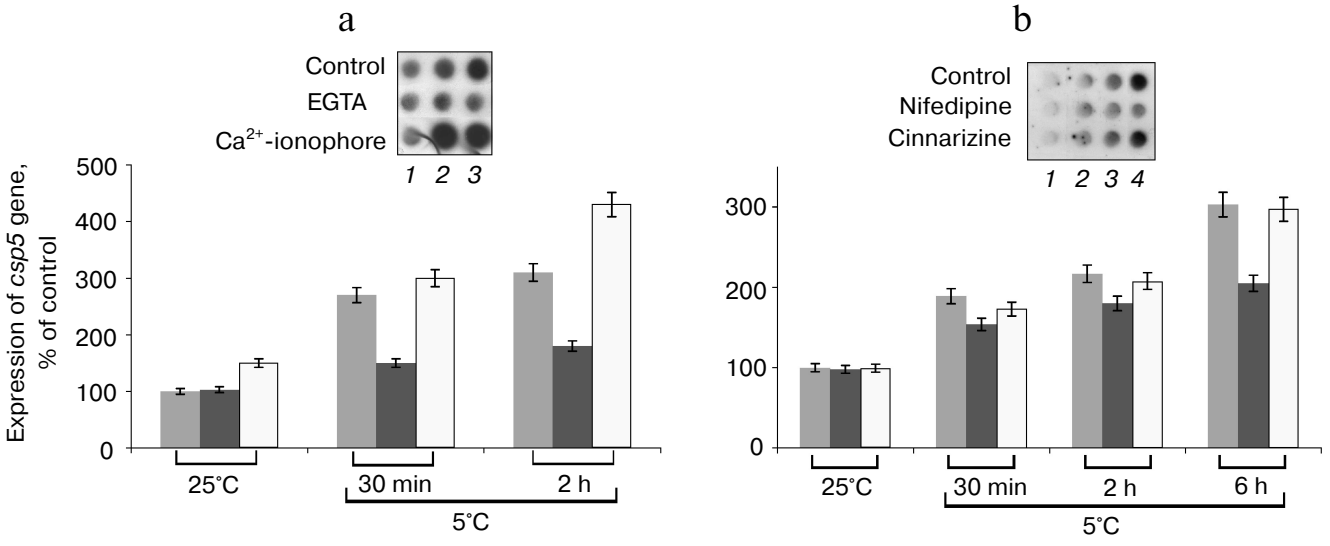

Fig. 2. Expression of *csp5* gene in untreated cells (gray rectangles) and cells treated with EGTA (black rectangles) and Ca^{2+}-ionophore (white rectangles) (a) or with nifedipine (black rectangles) and cinnarizine (white rectangles) (b) upon change in Ca^{2+} influx into the cytoplasm. The data of dot blotting are shown on the top (plasmid coding *csp5* gene is used as probe).

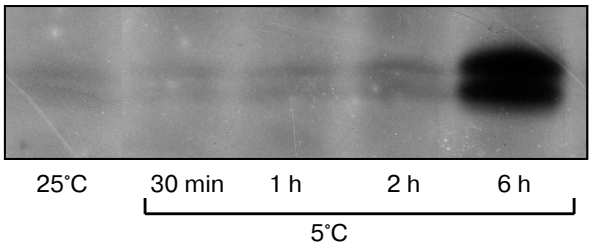

Fig. 3. Phosphorylation of histone III-SS in cell extracts of plants subjected to low-temperature stress.

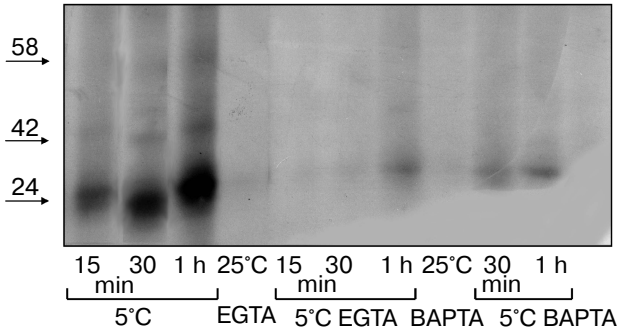

Fig. 4. Phosphorylation of proteins (on the left, molecular masses in kD) in plants exposed to low temperature.

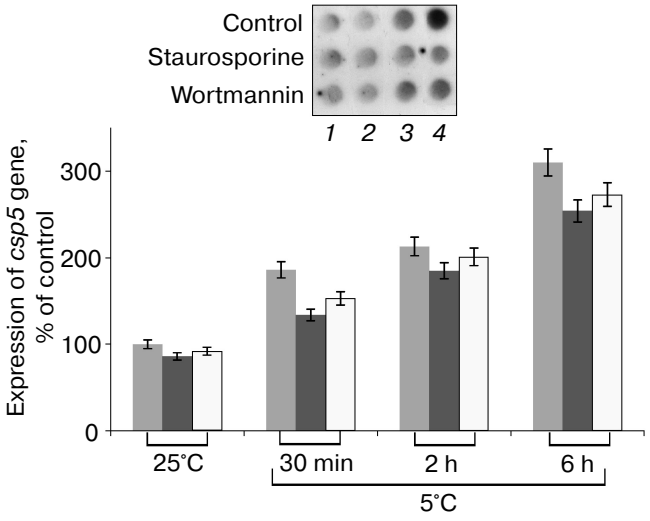

Fig. 5. Effect of protein kinase inhibitors on the expression of *csp5* gene: staurosporine (black rectangles) and wortmannin (white rectangles); control (gray rectangles). The data from dot blotting are shown at the top (plasmid coding *csp5* gene is used as probe).

calcium channels of plasma membrane, repressed the expression of *csp5*, whereas cinnarizine, a blocker of the channels of intracellular calcium depots, had no significant effect on the expression of *csp5*.

Hence, a change in cytoplasmic Ca^{2+} concentration directly reflects the expression level of cabbage *csp5* gene.

Cold stress is known to be accompanied by changes in phosphorylation of certain proteins [18]. The signal for the phosphorylation is apparently the increment in cytoplasmic Ca^{2+} concentration following the stress [19]. In protein extracts from the cabbage plants that underwent cold stress compared with non-stressed ones, the phosphorylation degree of a histone taken as a protein kinase substrate was significantly changed (Fig. 3). The increment in phosphorylation of histone was observed already 30 min after the cold stress. A rapid and dramatic increase in protein kinase activity following exposure of cabbage seedlings to cold was found in experiments on protein phosphorylation *in vivo*. Proteins with molecular weights of 24, 42, and 58 kD were preferably phosphorylated (Fig. 4). When the seedlings were preincubated in medium containing the calcium ion chelator EGTA or BAPTA, the phosphorylation degree of the 24-kD protein significantly decreased, and the 42- and 58-kD proteins became invisible on autoradiography, thus supporting calcium-dependent character of the phosphorylation of these proteins under cold stress. BAPTA (chelating Ca^{2+} from intracellular depots) compared with EGTA has less expressed inhibitory effect on protein phosphorylation, thus indicating a greater contribution of calcium ions incoming from the environment to the activation of protein kinases under cold stress. Our experiments with protein kinase inhibitors are also confirmative of the necessity of phosphorylation of certain proteins for the expression of the *csp5* gene. Figure 5 shows that plants treated with the generic protein kinase inhibitors staurosporine or wortmannin are characterized by some decrease in expression of *csp5* under cold stress. However, the difference in expression levels was not statistically significant, so we only see a tendency of the expression of *csp5* gene to decrease under the influence of the given protein kinase inhibitors.

Thus, low-temperature signal transduction from the environment resulting in corresponding stress response of the plant is a multistage process. In both protoplasts and whole plants of cabbage, the early stages of this process are shown to be the activation of calcium channels, which results from the change in condition of cell membranes, and the growth of calcium ion influx into cytoplasm, which is evident from the effect of calcium ion chelators. The increment of cytoplasmic Ca^{2+} concentration leads to the activation of calcium-dependent protein kinases involved in induction of transcription factors required for the expression of cold-regulated genes of stress response. Future studies will elucidate the next stages of the low-temperature signal transduction.

This study was supported by the Russian Federal Program on Support of Leading Scientific Schools (grant No. SS-2217.2003.4).

REFERENCES

1. Levitt, J. (1983) in *Cold Resistance of Plants* [in Russian], Moscow, pp. 10-22.
2. Hughes, M. A., and Dunn, M. A. (1996) *J. Exp. Bot.*, **47**, 291-305.
3. Thomashow, M. F. (1998) *Plant Physiol.*, **118**, 1-7.
4. Baimiyev, A. Kh., Gimalov, F. R., Chemeris, A. V., and Vakhitov, V. A. (1999) *Russ. J. Plant Physiol.*, **46**, 525-529.
5. Alonso, A., Queiroz, C. S., and Magalhaes, A. C. (1997) *Biochim. Biophys. Acta*, **1323**, 75-84.
6. Murata, N., and Los, D. A. (1997) *Plant Physiol.*, **115**, 875-879.
7. Orvar, B. L., Sangwan, V., Omann, F., and Dhindsa, R. S. (2000) *Plant J.*, **23**, 785-794.
8. Murashige, T., and Skoog, F. (1962) *Physiol. Plant.*, **15**, 473-497.
9. Draper, G., Scott, R., Armitage, F., Dury, G., Jacob, L., Walden, R., Kutar, A., Jefferson, R., and Hetil, G. (1991) *Plant Gene Engineering* [Russian translation], Mir, Moscow, p. 143.
10. Boothe, J. G., Sonnichsen, F. D., de Beus, M. D., and Johson-Flanagan, A. M. (1997) *Plant Physiol.*, **113**, 367-376.
11. Maxam, A., and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560-564.
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, p. 1626.
13. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
14. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
15. Monroy, A. F., Labbe, E., and Dhindsa, R. S. (1997) *FEBS Lett.*, **410**, 206-209.
16. Sangwan, V., Fould, I., Singh, J., and Dhindsa, R. S. (2001) *Plant J.*, **27**, 1-12.
17. Ding, J. P., and Pickard, B. G. (1993) *Plant J.*, **3**, 713-720.
18. Monroy, A. F., Sarhan, F., and Dhindsa, R. S. (1993) *Plant Physiol.*, **102**, 1227-1235.
19. Tahtiharju, S., Sangwan, V., Monroy, A. F., Dhindsa, R., and Borg, M. (1997) *Planta*, **203**, 442-447.