In vivo and in vitro activation of temperature-responsive plant map kinases

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Abstract **Alfalfa cells possess two temperature-responsive Mitogen-Activated Protein Kinases (MAPKs), SAMK (Stress-Activated MAP Kinase) activated at 4°C and HAMK (Heat shock-Activated MAP Kinase) activated at 37°C. Both are inactive at 25°C. We show here that SAMK is activated when cells are transferred from 37°C to 25°C, and HAMK is activated when cells are transferred from 4°C to 25°C. Moreover, we show that heat activation of HAMK also occurs in cell-free extracts. We conclude that (i) SAMK or HAMK activation does not require a particular temperature but a relative temperature shift, and (ii) that either HAMK itself or one or more of its upstream activators can sense temperature change directly.**

Key words: Cold; Heat; Temperature; MAPK; Plant; Alfalfa

1. Introduction

The ability to sense temperature change has been shown to be essential for a plant to launch the processes underlying acclimation [1,2]. Among the many events that occur during the perception and transduction of the temperature signal is the activation of specific MAP kinases [3]. Thus in alfalfa cells, SAMK (Stress-Activated MAP Kinase) is activated at 4°C [4,3], and HAMK (Heat shock-Activated MAP Kinase) is activated at 37°C [3]. Such activation of SAMK or HAMK is transient [4,3] and is undetectable after 24 h. Both SAMK and HAMK belong to the ERK (Extracellular signal-Regulated Kinase) family of protein kinases. They have similar molecular mass and cannot be distinguished by gel electrophoresis. However, they are immunologically distinct and the anti-SAMK antibody does not recognise HAMK [3]. Although HAMK has yet to be purified and characterised at the molecular level, it is recognised by the anti-phospho-ERK antibody, establishing its identity as a MAPK (Mitogen-Activated Protein Kinase) belonging to the ERK family [3].

Temperature is a continuously variable factor and cold and hot are, therefore, relative terms. Thus cells acclimated to any temperature are expected to exhibit a cold shock or heat shock response with a sudden downward or upward shift in temperature, respectively, provided that the shift in temperature is of sufficient magnitude. Furthermore, temperature is a pervasive thermodynamic factor and should, therefore, be perceived at many places in the cell. We have previously shown that isolated nuclei perceive temperature change by reversible alteration of their phosphoprotein profile [5]. Temperature-dependent changes in protein conformation and activity [6,7] have been demonstrated. Therefore, we wanted to determine if the temperature-responsive MAPKs show their responsiveness in cell-free extracts.

The results of this study show that although neither SAMK nor HAMK is active at 25°C, SAMK is activated when cells maintained at 37°C are transferred to 25°C, and HAMK is activated when cells maintained at 4°C are transferred to 25°C. Furthermore, we show that temperature activation of HAMK also occurs in cell-free extracts.

2. Materials and methods

2.1. Plant material and administration of temperature treatment

Alfalfa (*Medicago sativa* spp. falcata cv. Anik) cells from five-day-old suspension cultures, previously described in [8], were used in all experiments. Temperature shock was administered by either cooling the cells from 25°C to 4°C (cooling rate 1.2°C/min), or by heating them to 37°C (heating rate 0.9°C/min) under low light (20 μmol m^{-2} s^{-1}) illumination. In order to acclimate the cells to cold or heat, cells were transferred to and kept at either 4°C or 37°C for 24 h, by which time SAMK or HAMK had become inactive following a transient activation. Identification of SAMK (4) and HAMK (3) as MAPKs has been established previously.

2.2. Protein extraction and in gel MAP kinase assay

In gel MBP kinase assay was performed essentially as previously described [9,3]. Briefly, 0.2 g of cells were filtered through Miracloth (Calbiochem) and proteins extracted by addition of 2 volumes (v/w) of buffer containing 100 mM HEPES (pH 7.5), 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM phenlymethylsulfonyl fluoride, 5 μg/ml antipain, 5 μg/ml aprotinin, 5μg/ml leupeptin, 10% glycerol, 7.5% polyvinyl-polypyrrolidone. Cells were sonicated twice, 15 s each time, and centrifuged for 20 min at 13 000 rpm. Supernatants were transferred to another tube, frozen in liquid nitrogen and stored at −80°C. Protein amount was assayed using the Bradford assay (BioRad), and 10 μg of total protein run on two 12% SDS–PAGE gels, one with and the other without 0.25 mg/ml myelin basic protein (MBP, Sigma). Gels that did not contain MBP were Coomassie stained and used as loading controls. Gels containing MBP were washed three times for 30 min each in buffer containing 25 mM Tris (pH 7.5), 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg/ml BSA, 0.1% Triton X-100 (v/v), followed by two 15 min washes in renaturation buffer [25mM Tris (pH 7.5), 1 mM DTT, 0.1mM Na_3VO_4, 5 mM NaF] at 4°C. Renaturation was carried out overnight at 4°C. The in gel kinase assay was performed at room temperature by placing the gel in 30 ml buffer containing 25 mM Tris (pH 7.5), 2 mM EGTA, 12 mM

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Abbreviations: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein

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$MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, 200 nM ATP and 185×10^4 Bq [γ-^{32}P]ATP (11.1×10^7 MBq/mmol) for 60 min. The reaction was stopped by washing in stop buffer [5% Trichloroacetic acid (TCA; w/v), 1% NaPPi (w/v)], and unincorporated [γ-^{32}P]ATP was removed by washing at least six times in stop buffer. Gels were then dried and autoradiographed. Prestained molecular weight markers (NEB) were used to calculate the molecular mass of the protein kinases detected.

For testing in vitro response of HAMK to heat shock, cells maintained at 25°C were divided into three lots, one was maintained at 25°C as control (25/25), second was moved to 4°C for 60 min (25/4), and the third was moved to 37°C for 30 min (25/37). Protein extract was prepared from each and SAMK was removed from the extract by immunoprecipitation with anti-SAMK antibody [4]. An aliquot each of SAMK-free 25/25, 25/4 and 25/37 extracts was retained for in gel kinase assay. Then the remaining 25/4 extract was moved to 37°C for 30 min (4/37) and the remaining 25/37 extract was moved to 4°C for 60 min (37/4) with a 15-min stopover at 25°C in each case. This stopover at 25°C was introduced to allow any residual MAPK activity due to previous temperature treatment to disappear. In gel kinase assays were carried out on 25/25, 4/37, 37/4, 25/4 and 25/37 extracts.

3. Results

3.1. Activation of MBP kinases during recovery from cold or heat stress

Alfalfa cells from five-day-old suspension cultures grown at 25°C were acclimated at either 4°C or 37°C for 24 h, by which time the transiently expressed activity of SAMK at 4°C and of HAMK at 37°C declines to negligible levels. When the cells are moved from either 37°C (Fig. 1a) or 4°C (Fig. 1b) to 25°C, a 44-kDa MBP kinase is activated within 10 min. This activation of MBP kinase is short-lived in cells that were moved from 37°C to 25°C and declines to basal level by 60 min (Fig. 1a). In the case of cells moved from 4°C to 25°C, the activity of the 44-kDa MBP kinase persists undiminished at 60 min (Fig. 1b).

It has been previously shown that different MAPKs are activated when alfalfa cells are subjected to cold or heat shock

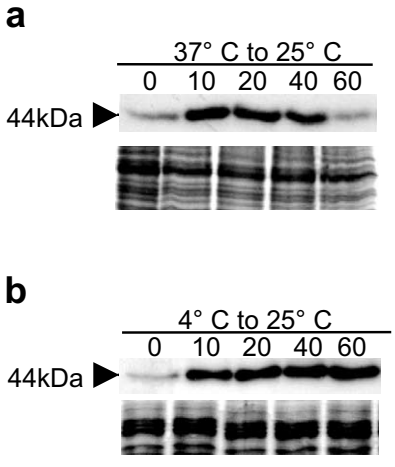

Fig. 1. MBP kinases are activated during recovery from cold or heat stress. Cells were acclimated for 24 h at either 4°C or 37°C, and then moved to 25°C. Cells were harvested at indicated times and protein extracts prepared. In gel kinase assays were performed on total protein extracts. Lower panel in each figure shows the amount of total protein loaded for the in gel assay, as determined by Coomassie blue staining. a: Cells acclimated to 37°C and then transferred to 25°C show a time-dependent increase in the activity of a 44-kDa MBP kinase. b: Cells acclimated to 4°C and transferred to 25°C show a time-dependent activation of a 44-kDa MBP kinase.

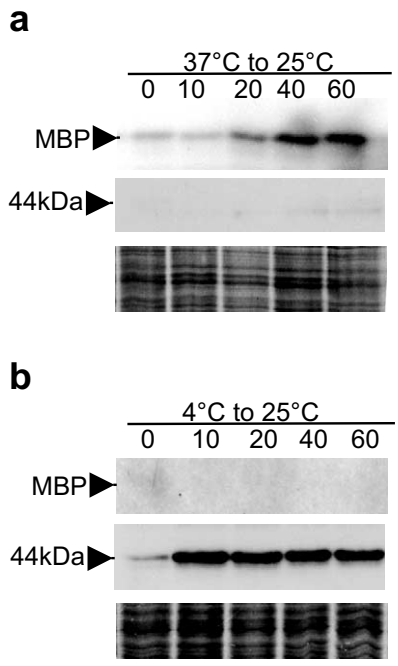

Fig. 2. SAMK is activated during recovery from heat stress and HAMK is activated during recovery from cold stress. Cells were placed at either 4°C or 37°C for 24 h and then moved to 25°C for times indicated. Protein extracts were prepared and fractionated into SAMK-containing fraction and SAMK-depleted fraction by immunoprecipitation with anti-SAMK antibody. The in vitro kinase assay was used to determine SAMK activity in the SAMK-containing fraction. The in gel kinase assay was used to determine the HAMK activity in the SAMK-depleted fraction. a: Upper panel represents SAMK activity determined by in vitro MBP phosphorylation. Middle panel shows the lack of any MAPK activity in the SAMK-depleted extract as determined by in gel assay. Lower panel shows a total protein profile visualised by Coomassie staining to indicate the amount of protein used for immunoprecipitation. b: Upper panel shows the lack of SAMK-catalysed MBP phosphorylation as determined by in vitro kinase assay on the SAMK-containing immunoprecipitate. Middle panel shows the activity of a 44-kDa HAMK as determined by in gel kinase assay on SAMK-depleted extract. Lower panel shows the total protein profile revealed by Coomassie staining to indicate the amount of total protein used in immunoprecipitation.

[3]. The results described above show that a 44-kDa protein kinase, capable of phosphorylating MBP, is activated during recovery from cold or heat stress.

3.2. Activation of SAMK and HAMK during recovery from heat stress and cold stress respectively

To investigate the identity of MBP kinases activated when cells are moved from 4°C or 37°C to 25°C, proteins extracted from cells treated as above were subjected to immunoprecipitation with anti-SAMK antibody [4]. SAMK is activated by cold shock but not by heat shock [3,4]. In vitro kinase assays were performed on the immunoprecipitate to assay for SAMK activity. To assay for HAMK activity, in gel kinase assays were performed on SAMK-depleted extracts as previously described [3]. It can be seen from Fig. 2a that MBP is phosphorylated in vitro by the SAMK-containing immunoprecipitate (Fig. 2a, upper panel). However, the in gel assays on the SAMK-depleted supernatant (remaining after SAMK immunoprecipitation) reveals no 44-kDa MBP kinase (Fig. 2a, middle panel). The protein profile of the total cell homogenate is

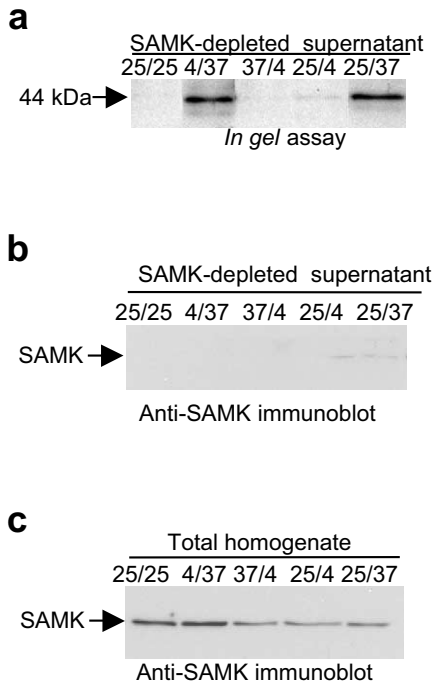

Fig. 3. HAMK is activated at 37°C in cell-free extracts. Cells grown at 25°C were either maintained at 25°C (25/25) as control, or placed at 4°C for 60 min (25/4) or at 37°C for 30 min (25/37). Protein extracts were prepared from 25/25, 25/4, and 25/37 cells and SAMK removed from each extract with anti-SAMK antibody. Aliquot of each of these SAMK-free extracts was retained for subsequent in gel kinase assay. Remaining 25/4 extract was transferred to 37°C for 30 min (4/37) and the remaining 25/37 extract was transferred to 4°C for 60 min (37/4). Each of these two transfers included a 15-min stopover at 25°C to allow any residual MAPK activity to disappear. All extracts were subjected to in gel kinase assay. a: In gel activity of 44-kDa MAPK in SAMK-depleted extracts after indicated temperature treatments. b: Immunoblot analysis showing that SAMK is, indeed, absent from SAMK-depleted extracts. c: Immunoblot analysis showing that SAMK is present in the total homogenate, before SAMK depletion.

shown to indicate that similar amounts of protein were used for immunoprecipitation for each lane (Fig. 2a, lower panel). Thus it may be concluded that SAMK is activated when cells are moved from 37°C to 25°C, suggesting that cells perceive the temperature shift from 37°C to 25°C as cold shock.

Similar analysis of the MBP kinase activated in cells on transfer from 4°C to 25°C was then carried out (Fig. 2b). Homogenates from these cells were subjected to immunoprecipitation with anti-SAMK antibody to obtain SAMK-containing immunoprecipitate and SAMK-free supernatant. The SAMK-containing immunoprecipitate shows no MBP phosphorylating activity in an in vitro kinase assay, suggesting that SAMK is not active in cells transferred from 4°C to 25°C (Fig. 2b, upper panel). On the other hand, an in gel kinase assay on the SAMK-free supernatant shows that a 44-kDa kinase is activated within 10 min at 25°C and its activity persists undiminished at 60 min (Fig. 2b, middle panel). The protein profile of the total cell homogenate is shown (Fig. 2b, lower panel) to indicate that similar amounts of protein were used for immunoprecipitation at each time point. It may, therefore, be concluded that when cells are transferred from 4°C to 25°C, a 44-kDa MBP kinase other than SAMK is activated. The activity profile of this kinase is similar to the one for heat-activated MBP kinase (Fig. 1b) and to the one reported for HAMK [3]. It suggests that cells perceive the temperature shift from 4°C to 25°C as heat shock.

3.3. Activation of HAMK in cell-free extracts

In order to study the ability of either SAMK or HAMK to sense temperature changes in vitro, proteins were extracted from cells transferred from 25°C to either 4°C for 60 min (25/4) or to 37°C for 30 min (25/37) as well as from cells continuously maintained at 25C (25/25). Each extract was then subjected to immunoprecipitation with the anti-SAMK antibody to remove SAMK. An aliquot of each SAMK-free extract (25/25, 25/4 and 25/37) was retained for further analysis. The remaining 25/4 extract was then moved to 37°C for 30 min (4/37) and 25/37 extract was moved to 4°C (37/4) for 60 min, each with a 15 min stopover at 25°C. All extracts were then subjected to either an in gel kinase assay for HAMK activation or an in vitro kinase assay for SAMK activation. Because SAMK activation was not observed in cell-free extracts (data not shown), only results for HAMK activation are shown. As seen in Fig. 3a, 44-kDa HAMK activity was observed in SAMK-depleted cell extracts that were moved from 4°C to 37°C (4/37) and in extracts from cells moved from 25°C to 37°C (25/37). No activity was observed in SAMK-depleted extracts continuously maintained at 25°C (25/25). Also, no activity was observed in cell extracts moved from 25°C to 4°C (25/4) or 37°C to 4°C (37/4), because these assays were conducted on SAMK-free supernatant. These results show that transfer of cells from 25°C to 37°C (25/37) or the transfer of cell extracts from 4°C to 37°C (4/37) is perceived as heat shock and HAMK is activated. That all these extracts were depleted of SAMK protein is shown by the data from immunoblot analysis presented in Fig. 3b. That SAMK protein was present in the total cell extract prior to immunodepletion of SAMK is shown by data in Fig. 3c. Therefore, it may be concluded that HAMK is activated in cell-free extracts.

4. Discussion

MAPKs are believed to be ubiquitously involved in eukaryotic responses to extracellular signals [10]. Thus two temperature-responsive MAPKs have been demonstrated in alfalfa cells, cold shock-activated SAMK [3,4] and heat shock-activated HAMK [3]. As temperature is a continuously variable factor, cold shock and heat shock are relative terms. Thus we reasoned that alfalfa cells maintained at 4°C would experience heat shock on transfer to 25°C. Likewise, the cells maintained at 37°C would experience cold shock on transfer to 25°C. The results of present study show that this is, indeed, the case.

That the cells experience cold shock when transferred from 37°C to 25°C is convincingly demonstrated by the activation of SAMK, a well-characterised MAPK known to be activated by cold but not by heat [3,4]. Because HAMK has not yet been characterised at the molecular level, specific probes for establishing homology of unknown MAPKs with HAMK are not available. However, the expression profile of the 44-kDa MBP kinase activated in cells transferred from 4°C to 25°C, is similar to that reported for HAMK [3]. A single activity band at 44 kDa is observed in heat-shocked alfalfa cells [3]. Moreover, just as cells transferred from 37°C to 25°C experience cold shock, it is expected that cells transferred from 4°C to

25°C would experience heat shock. Interestingly, a MAPK, ZmMPK5, is activated during recovery from cold stress in maize [11]. It is quite possible that ZmMPK5 is a HAMK and would be activated by heat shock. It should be noted, however, that our data could not rule out the possibility that the single activity band may contain more than one MBP kinase.

The activity profile of SAMK in Fig. 2a is somewhat different in time course from that shown in Fig. 1a. While the reasons for this difference are presently unclear, it should be noted that the profile in Fig. 1a was obtained on the whole cell homogenate by in gel kinase assay and the profile in Fig. 2a was obtained from SAMK-containing immunoprecipitate by in vitro kinase assay. Thus the latter profile is more specific to SAMK. It is possible that the profile in Fig. 1a may contain MAPKs other than SAMK.

In our experiments reported in Fig. 3, cell extracts were transferred between 4°C and 37°C with a 15-min stopover at 25°C. This stopover was introduced to allow the residual MAPK activity due to the preceding exposure to 4°C or 37°C to disappear. It is quite likely that the transfer of extracts from either 4°C or 37°C to 25°C would also activate HAMK or SAMK, respectively, as it does in the case of intact cells. However, we did not determine MAPK activity during the 15-min stopover because of the confounding effect of the persisting residual activity. Furthermore, the principal objective of conducting these experiments was to determine if SAMK or HAMK could be activated in cell-free extracts. Our data show that HAMK can, indeed, be activated in cell-free extracts.

Our observation that cell-free extracts are capable of perceiving heat shock and activate HAMK in vitro raises several intriguing questions. We have argued elsewhere [5] that because temperature is a pervasive thermodynamic factor it would be perceived at many places in the cell. Thus we have demonstrated temperature perception by isolated nuclei [5]. There are now several reports of direct effects of temperature on protein conformation [6] and activity [7]. Thus a heat shock transcription factor of *Drosophila* [7] and yeast [6] perceives heat shock and activates the transcription of heat shock genes in vitro. We have previously shown the temporal sequence of events involved in the transduction of the temperature signal [1,3]. Thus the events leading to heat shock activation of HAMK consist of an increase in membrane fluidity, followed by reorganisation of cytoskeleton, calcium influx and action of calcium-dependent protein kinases, in this order [3]. Furthermore, activation of a MAPK involves the action of MAPKK and MAPKKK. The question arises as to how HAMK is activated in cell-free extracts. Since the integrity of cellular membranes and partitioning of Ca^{2+} are likely to be disrupted during the preparation of cell-free extracts, some component of the signal transduction chain leading to HAMK activation must be able to sense the temperature increase directly. As mentioned earlier, the direct effects of temperature on the conformation and activity of proteins are now well-documented. Therefore, the possibility cannot be ruled out that either HAMK itself or one or more of its upstream activators can sense the temperature change directly. It is relevant to mention here that low temperature causes alterations in protein phosphorylation in cell-free extract [12].

Why is SAMK not activated by cold shock in cell-free extracts? The reasons for this are presently unclear. It is possible that once it forms a complex with its antibody, it is unable to sense the temperature directly or it becomes incapable of being activated by its upstream activators. Further investigation is needed to explore these possibilities.

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References

[1] Örvar, B.L., Sangwan, V., Omann, F. and Dhindsa, R.S. (2000) Plant J. 23, 785–794.
[2] Sangwan, V., Foulds, I., Singh, J. and Dhindsa, R.S. (2001) Plant J. 27, 1–12.
[3] Sangwan, V., Örvar, B.L., Beyerly, J., Hirt, H. and Dhindsa, R.S. (2002) Plant J. 31, 554–563.
[4] Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N.S. and Hirt, H. (1996) Proc. Natl. Acad. Sci. USA 93, 11274–11279.
[5] Kawczynzski, W. and Dhindsa, R.S. (1996) Plant Cell Physiol. 37, 1204–1210.
[6] Lee, S., Carlson, T., Christian, N., Kristi, L., Kedzie, J., Reilly, J.P. and Bonner, J.J. (2000) Mol. Biol. Cell 11, 1753–1764.
[7] Zhong, M., Orosz, A. and Wu, C. (1998) Mol. Cell 2, 101–108.
[8] Monroy, A.F. and Dhindsa, R.S. (1995) Plant Cell 7, 321–331.
[9] Zhang, S. and Klessig, D.F. (1997) Plant Cell 9, 809–824.
[10] Hirt, H. (2000) Results Probl. Cell Differ. 27, 1–9.
[11] Berberich, T., Sano, H. and Kusano, T. (1999) Mol. Gen. Genet. 262, 534–542.
[12] Monroy, A.F., Labbé, E. and Dhindsa, R.S. (1997) FEBS Lett. 410, 206–209.