Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways

Veena Sangwan1, Björn Lárus Órvar1,², John Beyerly2, Heribert Hirt2 and Rajinder S. Dhindsa1,*
1Department of Biology, McGill University, 1205 Avenue Docteur Penfield, Montreal, Quebec, H3A 1B1, Canada
2Institute of Microbiology and Genetics, Vienna Biocenter, Dr Bohr-Gasse 9, A-1030, Vienna, Austria

Summary
Mitogen-activated protein kinases (MAPKs) appear to be ubiquitously involved in signal transduction during eukaryotic responses to extracellular stimuli. In plants, no heat shock-activated MAPK has so far been reported. Also, whereas cold activates specific plant MAPKs such as alfalfa SAMK, mechanisms of such activation are unknown. Here, we report a heat shock-activated MAPK (HAMK) immunologically related to ERK (Extracellular signal-Regulated Kinase) superfamily of protein kinases. Molecular mechanisms of heat-activation of HAMK and cold-activation of SAMK were investigated. We show that cold-activation of SAMK requires membrane rigidification, whereas heat-activation of HAMK occurs through membrane fluidization. The temperature stress- and membrane structure-dependent activation of both SAMK and HAMK is mimicked at 25°C by destabilizers of microfilaments and microtubules, latrunculin B and oryzalin, respectively; but is blocked by jasplakinolide, a stabilizer of actin microfilaments. Activation of SAMK or HAMK by temperature, chemically modulated membrane fluidity, or by cytoskeleton destabilizers is inhibited by blocking the influx of extracellular calcium. Activation of SAMK or HAMK is also prevented by an antagonist of calcium-dependent protein kinases (CDPKs). In summary, our data indicate that cold and heat are sensed by structural changes in the plasma membrane that translates the signal via cytoskeleton, Ca²⁺ fluxes and CDPKs into the activation of distinct MAPK cascades.

Keywords: Cold shock, heat shock, signalling, membrane fluidity, Ca²⁺, cytoskeleton, MAPKs

Introduction
Plant tolerance to low or high temperature is a highly desirable agronomic trait. Thus, many plants are capable of developing freezing tolerance through a prolonged exposure to low, but non-freezing, temperatures during the onset of winter — a phenomenon known as cold acclimation (Levitt, 1980). Similarly, acclimation to high temperatures can occur through exposure to non-lethal heat treatment (Levitt, 1980). The processes involved in temperature acclimation are initiated by the perception of temperature signals and transduction of these signals into biochemical processes that finally lead to the development of freezing or heat tolerance. Signal transduction frequently involves the generation of second messengers, e.g. Ca²⁺, and changes in protein phosphorylation. Ca²⁺ influx and phosphorylation of specific pre-existing proteins have been implicated in both cold (Komatsu and Kato, 1997; Monroy et al., 1993) and heat (Krishnan and Pueppke, 1987) signalling in plants. Phosphorylation level of a given protein is determined by the equilibrium between the activities of the related protein kinase and protein phosphatase. Thus eukaryotic cells contain a multitude of protein kinases and protein phosphatases constituting a complex but highly organized network that has been aptly called a ‘central processing unit (CPU)’
(Hardie, 1999). This bioinformatic CPU accepts the input information from external stimuli through a variety of sensors and receptors, and then delivers the output information to downstream targets. An important part of the signal transduction network is constituted by mitogen-activated protein kinase (MAPK) cascades. Activation of a MAPK cascade involves sequential phosphorylation of a functionally interlinked 3-member cassette of protein kinases. A MAPK is activated through phosphorylation by a MAPK kinase (MAPKK) which, in turn, is activated through phosphorylation by a MAPKK kinase (MAPKKK). The MAPKKKs are activated through phosphorylation by other protein kinases. Such MAPK cascades are now believed to be ubiquitously involved in eukaryotic responses to external stimuli. Using the yeast two-hybrid system and complementation of yeast mutants, Ichimura et al. (1998) and Mizoguchi et al. (1998) have identified a possible plant MAPK cascade comprising of ATMEKK1 (MAPKKK), MEK1 (MAPKK)/ATMKK2 (MAPKKs) and ATMPK4 (MAPK). Plant MAPKs are activated in response to hormones, pathogen attack and such abiotic stresses as cold, drought, salt, etc. (Hirt, 2000; Ichimura et al., 2000). To date, no heat shock-activated MAPK has been reported from plants. In Arabidopsis, three MAP kinases, 43-kDa ATMPK4, 47-kDa ATMPK6 and a 44-kDa MAPK are activated by cold, water deficit, wounding and touch, but not by heat (Ichimura et al., 2000). A stress-activated MAP kinase (SAMK) is activated by the same stimuli in alfalfa cells (Jonak et al., 1996). How these stresses activate these MAPK cascades is unclear.

In the present investigation, we have identified the first plant heat shock-activated MAPK (HAMK) from alfalfa cells. To understand the mechanism by which cold or heat activates SAMK or HAMK, we assumed that the physical state of the membrane, which is directly and reversibly affected by temperature, might be the initial trigger. Also, we have recently shown that the cold-induced expression of alfalfa gene cas30 (Örvar et al., 2000) and Brassica napus gene BN115 (Sangwan et al., 2001), requires membrane rigidification, cytoskeleton remodelling and Ca²⁺ influx. Therefore, we have examined the role of membrane fluidity, cytoskeleton stability and Ca²⁺ influx in the activation of SAMK and HAMK.

The results of the present investigation show that a MAPK, named HAMK is activated by heat shock in plant cells. The heat activation of HAMK and cold activation of SAMK are triggered by opposite changes in membrane fluidity, but both involve remodelling of cytoskeleton, Ca²⁺ influx and action of Ca²⁺-dependent protein kinases (CDPKs). Thus the potential role of SAMK or HAMK in temperature signalling is downstream of the roles of membrane fluidity, cytoskeleton, Ca²⁺ influx and CDPKs.

Results

Cold and heat activate distinct MAPKs in alfalfa cells

To address the question of whether specific MAPKs are involved in sensing cold and heat, Medicago sativa cells were transferred from 25°C to either 4°C or 37°C. MAPKs are known to be activated on phosphorylation at both tyrosine and threonine residues, and can phosphorylate MBP. Therefore, cell extracts were subjected to immunoprecipitation to fractionate active MAPKs with anti-phosphotyrosine antibody. The specificity of the antibody binding was confirmed by using carrier phosphotyrosine, phosphothreonine and phosphoserine as competitors during immunoprecipitation (data not shown). The immunoprecipitate obtained was used as a source of active MAPKs for in-gel assays using MBP as substrate. The results revealed that both cold shock (Figure 1a) and heat shock (Figure 1b) activated a 44-kDa MAPK(s). Under cold shock, MAPK activation was detectable at 10 min, reached a maximum at 60 min, but returned to basal levels after 6 h (Figure 1b). Under heat shock, MAPK activation was detected at 20 min, peaked at 30 min, and persisted even after 6 h (Figure 1b). Thus the lag time for MAPK activation is different for cold- and heat-shock. The results presented in the lower panels in Figure 1a,b show that the level of SAMK protein in cells does not change with temperature, and that similar amounts of protein were used for immunoprecipitation of all samples.

Are the MAPKs induced by cold and heat same or different? These two activities were indistinguishable by 1- or 2-dimensional SDS-PAGE (data not shown). Therefore, an anti-SAMK antibody (Jonak et al., 1996) was used to deplete SAMK from cell extracts, and all subsequent assays involving heat-stressed cells were carried out on the SAMK-depleted extracts. The removal of SAMK from the extract was confirmed by immunoblotting using the anti-SAMK antibody. The results show that SAMK protein is absent in the SAMK-depleted extract of control (C), cold-shocked (CS) and heat-shocked (HS) cells, but is present in similar amounts in the undepleted extracts of these cells (Figure 1c). Immunokinase assays performed in vitro on the SAMK-containing immunoprecipitate revealed that MBP was phosphorylated, indicating the presence of active SAMK only in cold-shocked cells (Figure 1d). In contrast, the SAMK-depleted extracts of only heat-shocked cells contained an active 44-kDa MAPK, which we have named HAMK (Heat-Shock Activated MAPK; Figure 1e). Therefore, it may be concluded that both cold and heat activate 44-kDa MAPKs that are immunologically distinct.

Identity of SAMK as a MAPK has been previously established (Jonak et al., 1996). However, since not all MBP-phosphorylating tyrosine phosphorylated proteins are necessarily MAPKs, we considered it important to confirm that HAMK is indeed a MAPK. For this, protein
extracts from either cold- or heat-shocked samples were immunodepleted using the anti-SAMK antibody and immunoblotted using the antiphospho-ERK (ERK, Extracellular signal-Regulated Kinase) antibody (Figure 1f). Immunoblot analysis using the anti-pERK antibody confirms that both cold shock and heat shock activate ERKs, but upon depletion of SAMK, only heat shocked samples possess pERK activity (Figure 1f). This anti-pERK antibody specifically recognizes the phosphorylated TEY motif found on MAPKs, and has been used by Samuel et al. (2000) and Kiegerl et al. (2000) previously to identify ERK-related MAPKs in plants. We conclude that since HAMK can phosphorylate MBP, is tyrosine phosphorylated, and is recognized by the anti-pERK antibody, it is indeed a MAPK.

Opposite changes in membrane fluidity lead to activation of SAMK or HAMK

Membrane fluidity is directly, rapidly and reversibly affected by temperature. Therefore, we examined the possibility that the temperature activation of SAMK or HAMK is mediated by changes in membrane fluidity. The effects of cold were countered by treating the cells with the membrane fluidizer benzyl alcohol (BA), and were mimicked at 25°C by treating the cells with the membrane rigidifier dimethylsulfoxide (DMSO). The effects of heat shock were countered by treating the cells with DMSO, and were mimicked at 25°C by treatment with BA. BA is known to prevent the cold-induced membrane rigidification whereas DMSO is known to rigidify the membranes at 25°C in alfalfa cells (Orvar et al., 2000). The results of immunokinase assay of SAMK activity show that DMSO induces the SAMK activation at 25°C (Figure 2a, upper panel) but BA inhibits its activation at 4°C (Figure 2b, upper panel). In-gel kinase assays on the SAMK-depleted cell extract revealed that at the highest concentration, 3%, DMSO activated an unidentified MAPK activity band (Figure 2a, lower panel). The activation of HAMK at 37°C is inhibited by DMSO (Figure 2c, upper panel), and can be mimicked by treatment with BA at 25°C (Figure 2d, upper panel). Since pre-treatment with DMSO was started before administering the heat shock, SAMK is also activated (Figure 2c, lower panel).

Membrane fluidity can be chemically modulated by fluidizers and rigidifiers

The effects of BA and DMSO on membrane fluidity in relation to heat shock were confirmed by fluorescence

![Figure 1. Cold and heat induce two distinct 44 kDa MAPKs, SAMK and HAMK, in alfalfa cells.](image1)

(a-b) In-gel kinase assays were performed on equal amounts of proteins immunoprecipitated with an antiphosphotyrosine antibody from extracts of cells treated as indicated. The lower panel represents the level of SAMK protein present in cell extract used for the immunoprecipitation with antiphosphotyrosine antibody. (a) Incubation of cells at 4°C leads to the time-dependent activation of the 44 kDa SAMK. (b) Exposure of cells to 37°C results in the activation of a 44-kDa MAPK, HAMK. (c-e) Cells were treated at 4°C or 37°C for 60 and 30 min, respectively, and cell extracts were prepared. Immunoprecipitation with anti-SAMK antibody was carried out, and immunokinase assays on the immunoprecipitate and in-gel assays on the supernatant were performed. (c) Immunoblot with anti-SAMK antibody showing depletion of SAMK in the supernatant but its presence in cell extract before immunoprecipitation. (d) Immunokinase assays on SAMK-containing immunoprecipitate from control (C), cold-shocked (CS), or heat shocked (HS) cells. MBP is phosphorylated by the immunoprecipitate only from cold-shocked cells. (e) In-gel kinase assays on the SAMK-depleted supernatant and undepleted cell extracts. A 44-kDa MAPK activity is seen in the SAMK-depleted supernatant of only heat-shocked cells. This activity was attributed to HAMK. A 44-kDa MAPK activity was present in the undepleted cell extract from cold-shocked or heat-shocked cells. The activity in cold-shocked cells was attributed to SAMK and activity in heat-shocked cells was attributed to HAMK. (f) HAMK is a MAPK. Heat treatment of plants at 37°C results in the activation of ERKs in both the homogenate and SAMK-depleted supernatant.
polarization measurements (Table 1). Polarization index \( p \) is inversely related to membrane fluidity. It can be seen that both heat stress and BA increase membrane fluidity (or decreases \( p \)). We have previously demonstrated that DMSO rigidifies the membranes of alfalfa cells at 25°C (Örvar et al., 2000). Here we show that DMSO-induced membrane rigidification prior to heat shock inhibits membrane fluidization at 37°C. The above results suggest that cold activation of SAMK is mediated by membrane rigidification whereas heat activation of HAMK is mediated by membrane fluidization.

**Rearrangements of cytoskeleton are involved in the activation of SAMK and HAMK**

The actin cytoskeleton is known to be connected to the plasma membrane and to be associated with a variety of signalling complexes (Volkmann and Baluska, 1999). In animal cells, Ras/ERK signalling pathway is activated by cytoskeleton remodelling (Irigoyen et al., 1997). Since cold is known to reorganize the cytoskeleton in animal (Hall et al., 1993) and plant (Mazars et al., 1997; Örvar et al., 2000; Sangwan et al., 2001), we examined the possibility that temperature-induced changes in membrane fluidity activate SAMK and HAMK through reorganization of the actin cytoskeleton. Pre-treatment of *M. sativa* cells with the microfilament stabilizer jasplakinolide (JK) blocked SAMK activation by cold (Figure 3a, left panel) or DMSO (Figure 3a, right panel), as well as HAMK activation by heat (Figure 3b, left panel) or BA (Figure 3b, right panel). These results suggest that rearrangements of the actin cytoskeleton occur downstream of the changes in membrane fluidity. Since destabilization of both microfilaments and microtubules is known to increase Ca\(^{2+}\) influx in cold shocked protoplasts (Mazars et al., 1997; Örvar et al., 2000), we examined the effects of microtubule stabilizer taxol on SAMK and HAMK activation. Taxol pre-treatment prevented the activation of SAMK at 4°C (Figure 3d). The effects of JK on SAMK and HAMK activation and of taxol on SAMK activation suggest that reorganization of the cytoskeleton is necessary for the activation of these MAPKs. In such a case, destabilization of the microfilaments or microtubules should activate both SAMK and HAMK at 25°C. The data in Figure 3c show that the microfilament destabilizer latrunculin B (LatB) or the microtubule destabilizer oryzalin (Ory) activates both

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**Table 1.** Fluorescence polarization index (P) as an inverse measure of membrane fluidity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temp. (°C)</th>
<th>P</th>
<th>Treatment</th>
<th>Temp. (°C)</th>
<th>P</th>
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<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>0.158 ± 0.012</td>
<td>Control</td>
<td>25</td>
<td>0.166 ± 0.070</td>
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<tr>
<td>Heat-shock</td>
<td>37</td>
<td>0.126 ± 0.002</td>
<td>BA (5 mM)</td>
<td>25</td>
<td>0.148 ± 0.002</td>
</tr>
<tr>
<td>DMSO (1%)</td>
<td>37</td>
<td>0.147 ± 0.011</td>
<td>BA (10 mM)</td>
<td>25</td>
<td>0.143 ± 0.013</td>
</tr>
<tr>
<td>DMSO (2%)</td>
<td>37</td>
<td>0.177 ± 0.033</td>
<td>BA (20 mM)</td>
<td>25</td>
<td>0.085 ± 0.023</td>
</tr>
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</table>

Each value is a mean of at least 3 replicates ± standard deviation. Values are significantly different between a and b, and between c and d, at a confidence interval of 95% or greater as determined by Student’s t-test. Experiments 1 and 2 are independent of each other, and the values between them are not comparable. Fluorescence polarization index, P, is inversely related to membrane fluidity. All heat-shock and BA treatments were for 30 min, and all DMSO pre-treatments were for 60 min.
SAMK (upper panel) and HAMK (lower panel) at 25°C. Another known microfilament destabilizer, cytochalasin D (CD), had similar effects as those of Lat B (data not shown). Calcium influx is required for SAMK and HAMK activation and is downstream of changes in membrane fluidity and cytoskeleton

Influx of extracellular Ca\(^{2+}\) into cytosol is required for cold acclimation and cold-induced gene expression in *M. sativa* (Monroy and Dhindsa, 1995; Monroy *et al.*, 1993), and *Arabidopsis* (Tähtiharju *et al.*, 1997), and for ERK activation in animal cells (Malaguti *et al.*, 1999). CD or DMSO treatment causes an increased Ca\(^{2+}\) influx into the cytosol in hepatocytes (Yamamoto, 1989), parathyroid cells (Nygren *et al.*, 1987) and alfalfa cells (Örvar *et al.*, 2000), whereas CD and oryzalin cause Ca\(^{2+}\) influx in cold-shocked *Nicotiana plumbaginifolia* protoplasts (Mazars *et al.*, 1997).

Figure 3. Cytoskeletal rearrangements regulate cold- and heat-induced MAPK activity. Cells were pre-treated with the microfilament stabilizer jasplakinolide (JK), the microfilament destabilizers latrunculin B (Lat B), the microtubule stabilizer taxol (Tax), or the microtubule destabilizer oryzalin (Ory), at 25°C for 60 min. C represents the control sample.
(a) Cells treated with 0.2 μM JK were placed at 4°C or treated with 280 mM DMSO at 25°C for 60 min.
(b) Cells treated with JK were either heat shocked at 37°C or treated with BA at 25°C for 30 min.
(c) Cells were treated with 10 μM latrunculin B (Lat B), or 100 μM oryzalin (Ory) at 25°C for 60 min.
(d) Cells treated with 50 μM taxol were placed at 4°C for 60 min. MBP phosphorylation represents SAMK activation, whereas the 44-kDa activity represents HAMK activation.

Figure 4. Calcium influx is required for MAPK induction by cold, heat, BA, DMSO, Lat B or oryzalin. Cells were pre-treated with 50 mM EGTA (EG), 2 mM BAPTA (BP), 1 mM lanthanum (La), or 1 mM gadolinium (Gd) at 25°C for 60 min. C represents control.
(a) Cells pre-treated as above were placed at 4°C or treated with 280 mM DMSO at 25°C for 60 min.
(b) Cells pre-treated as above were placed at 37°C or treated with 20 mM BA for 30 min.
(c) Cells were pre-treated as above, followed by addition of 10 μM Lat B or 100 μM oryzalin for 60 min to all samples. MBP phosphorylation represents SAMK activation, whereas the 44-kDa activity represents HAMK activation.

To test if temperature-dependent activation of SAMK and HAMK requires Ca\(^{2+}\) influx, cells were pre-treated with the Ca\(^{2+}\) chelator EGTA (ethyleneglycol-bis-(beta-amino-ethyl ether)-N,N’-tetraacetic acid) or BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N,N’-tetraacetic acid), or with the Ca\(^{2+}\) channel blockers lanthanum (La\(^{3+}\)) or gadolinium (Gd\(^{3+}\)). These chemicals have previously been shown to prevent Ca\(^{2+}\) influx into the cytosol (Ding and Pickard, 1993; Knight *et al.*, 1996; Monroy and Dhindsa, 1995). Cold- or DMSO-induced activation of SAMK (Figure 4a) and heat- or BA-induced activation of HAMK (Figure 4b) were inhibited by Ca\(^{2+}\) chelators and channel blockers. Ruthenium red, a known blocker of Ca\(^{2+}\) release from intracellular stores (Allen *et al.*, 1995), had no effect (data not shown). These data suggest that cold shock- or heat shock-triggered Ca\(^{2+}\) influx is downstream of the membrane component of temperature signalling. To determine whether the Ca\(^{2+}\) influx lies upstream or downstream of rearrangements in the cytoskeleton, cells at 25°C were treated with either Lat B (Figure 4c, left panel) or oryzalin (Figure 4c, right panel) with or without pre-treatment with EGTA, BAPTA, La\(^{3+}\) or Gd\(^{3+}\). It can be seen that Lat B- or oryzalin-induced activation of SAMK (upper panel) and of HAMK (lower panel), is inhibited by Ca\(^{2+}\) chelators and channel blockers.
used, indicating that Ca\textsuperscript{2+} influx is downstream of the remodelling of cytoskeleton.

**CDPKs are involved in the activation of SAMK by cold and of HAMK by heat**

The results described above show that Ca\textsuperscript{2+} is required for SAMK and HAMK activation. One possible role of Ca\textsuperscript{2+} may be through Ca\textsuperscript{2+}-dependent protein kinases (CDPKs), some of which are up-regulated by cold (Monroy and Dhindsa, 1995; Tähtiharju et al., 1997). Interestingly, some CDPKs mimic some features of PKC activity (Farmer and Choi, 1999). CDPK antagonist W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride) and PKC inhibitor H7 (1-(5-isquinoline-sulfonyl)-2-methylpiperazine dihydrochloride) are known to inhibit cold-induced gene expression and cold acclimation (Monroy et al., 1993; Tähtiharju et al., 1997). Therefore, we examined the effects of W7 and H7 on SAMK and HAMK activation.

The data in Figure 5 demonstrate that both W7 and H7 inhibit the cold-activation of SAMK and heat-activation of HAMK, W7 being more effective than H7. However W5 (N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride), an ineffective analogue of W7, and the protein kinase A inhibitor H8 (N-[2-(methylamino)ethyl]-5-isquinoline sulfonamide dihydrochloride) do not have any effect (Figure 5). These data suggest that CDPKs and PKCs may be involved in the activation of SAMK and HAMK pathways.
alcohol (BA) (Carratu et al., 1996; Kitagawa and Hirata, 1992; Örvar et al., 2000; Sangwan et al., 2001), or with membrane rigidifiers such as dimethylsulfoxide (DMSO) (Lyman and Preisler, 1976; Örvar et al., 2000; Sangwan et al., 2001). Our data show that experimental rigidification of the membranes at room temperatures mimics the effects of cold shock whereas fluidization mimics the effects of heat shock. Treatment of alfalfa cells (Örvar et al., 1992; Örvar et al., 2000) in DMSO results in the expression of cold-inducible genes and enhances freezing tolerance at 25°C. In the present study, it should be noted that 3% DMSO activates, in addition to the 44-kDa SAMK, another MAPK of a slightly lower molecular mass (Figure 2a, lower panel) that is not activated by cold. The nature of this MAPK is presently unclear. Interestingly, a 43-kDa MAPK, ATMPK4, is activated by cold in Arabidopsis (Ichimura et al., 2000).

Cytoskeleton has been implicated in signal transduction in response to diverse stimuli. Re-organization of cytoskeleton during growth and development and during responses to environmental signals has been demonstrated (Volkmann and Baluska, 1999). Some MAP kinases, CDPKs and protein phosphatases are reportedly associated with cytoskeleton (Trewavas, 2000). Rearrangements in cytoskeleton are known to regulate gene expression in plants (Örvar et al., 2000; Sangwan et al., 2001). We have recently shown that destabilizers/depolymerizers of microfilaments and microtubules cause Ca\(^{2+}\) influx (Örvar et al., 2000) and the expression of cold-inducible genes at 25°C (Örvar et al., 2000; Sangwan et al., 2001). Cytoskeleton is also considered the site of transduction of biophysical forces into biochemical response (Schmidt and Hall, 1998).

Changes in membrane fluidity are expected to alter the tensile forces operating in the plane of the membrane and cause alterations in cytoskeleton organization. Thus it is noteworthy that SAMK activity has been shown to be mechanosensitive (Bögre et al., 1996). The results of the present investigation show that both the cold activation of SAMK and heat activation of HAMK requires changes in membrane fluidity and cytoskeleton remodelling. It is tempting to suggest that HAMK is also a mechanosensitive enzyme. This suggestion is supported by the observation that Gd\(^{3+}\), a blocker of mechanosensitive Ca\(^{2+}\) channels, prevents the activation of SAMK and HAMK. Our data show that the activation of SAMK or HAMK is downstream from the temperature-triggered changes in membrane fluidity, cytoskeleton organization and Ca\(^{2+}\) influx.

Published reports on the interaction between Ca\(^{2+}\) and plant MAPKs or MBPKs (myelin basic protein kinases) are somewhat contradictory. While Hoyos and Zhang (2000) have found that activation of the osmotic stress activated MBPK, HOSAK is independent of Ca\(^{2+}\) influx, Lebrun-Garcia et al. (1998), Cazale et al. (1999) and Samuel et al. (2000) have shown MAPK activation dependent on Ca\(^{2+}\).

There are other reports of MAPK activation requiring specific ion fluxes through channels. For example, MAPK activation in parsley cells by elicitor glycoprotein fragment, Pep-13, requires specific ion fluxes through channels (Ligerink et al., 1997). The present study shows that Ca\(^{2+}\) influx is required for SAMK and HAMK activation. It should be noted that the inhibition of BA-induced MAPKs by inhibitors of Ca\(^{2+}\) influx is not as efficient as that of heat shock-induced MAPKs. The reasons for this are presently unclear. However, it is possible that the effect of BA on membrane fluidity leads to the activation of not only HAMK, but also of other MAPKs not subject to regulation by Ca\(^{2+}\) influx.

Intriguingly, plant cells activate two distinct MAPKs in response to cold and heat. Initially, we found it tempting to assume that the same MAPK might be sensing both low and high temperatures and that changes in response to heat would comprise a reversal of the changes occurring during the cold response. The reality is particularly intriguing because the events involved in SAMK and HAMK activation downstream from the required opposite changes in membrane fluidity, are similar in that both require remodelling of cytoskeleton and Ca\(^{2+}\) influx. It should be noted, however, that our data do not rule out the possibility that the precise nature of the cytoskeleton rearrangements and the Ca\(^{2+}\) signatures involved may be different in the two cases.

How are SAMK or HAMK cascades activated? The effects of W7, a CDPK antagonist, on the activation of SAMK and HAMK suggest that the MAPKKKs corresponding to SAMK or HAMK may be activated through the action of CDPKs either directly or through the inhibition of protein phosphatases. We have previously shown that some CDPKs are up-regulated by cold (Monroy and Dhindsa, 1995). Moreover, protein phosphatase 2 A (PP2A) is rapidly inactivated by cold and this inactivation requires Ca\(^{2+}\) influx (Monroy et al., 1998). Activation of SAMK and HAMK is also prevented by the protein kinase C (PKC) inhibitor H7. Although PKC-like protein kinase genes have been reported (Subramaniam et al., 1997), this enzyme is not well characterized in plants. Furthermore, some CDPKs have been shown to mimic PKC-like activities (Farmer and Choi, 1999). Thus the possibility cannot be ruled out that the CDPKs involved in temperature sensing may be of the type that mimic some features of the PKCs. W7 is a general antagonist of CDPKs and its inhibition of SAMK and HAMK does not imply that the same CDPK may activate both these MAPK cascades. CDPKs are known to be involved in Ca\(^{2+}\) signalling in response to various stimuli and the question has been raised whether there is a CDPK for every Ca\(^{2+}\) signal (Harmon et al., 2000). However, since the downstream targets of CDPKs and PKCs are not well characterized, the above suggestions need to be tempered with caution.
M. sativa

Five-day-old alfalfa (M. sativa cv. Anik) cell suspensions were used as source of active MAPKs and used for in-gel assays of MAPK activity. Protein extract was prepared from 0.2 g of cells as described (Jonak et al., 1998). SAMK, which is also activated by wounding, is inactivated by the protein phosphatase type 2A (PP2A) and is not known whether PP2A is involved in the SAMK or HAMK cascade. The majority of MAPK activity in alfalfa cells, measured by immunokinase assay of SAMK activity was as described (Jonak et al., 1998). It should be noted that in the in-gel assay, MBP is included in the gel and the detected 44-kDa band refers to the Myelin Basic Protein (MBP, Sigma, Oakville, Ontario, Canada) as a substrate, as described (Zhang and Klessig, 1997).

In-gel assay of MAPK activity

To determine SAMK activity by immunokinase assay, the supernatant was depleted supernatant that remained after removing SAMK with fractionated from the extract by immunoprecipitation with anti-SAMK antibody. Absence of SAMK in the supernatant was confirmed with immunoblotting. The down-regulation of a MAPK cascade is generally confirmed with immunoblotting.

Concentration of various chemicals used in different experiments were provided in the corresponding figure legends. All experiments were performed at least three times and yielded similar results each time.

Chemical treatments

To investigate the effects of various chemicals on MAPK activity, MAPK was activated in alfalfa cells through Miracloth®. (Calbiochem-Novabiochem, San Diego, CA, USA) and freezing them immediately in liquid nitrogen. In all experiments where a solvent other than water was used to dissolve the chemical, control incubation medium (C) contained the same solvent concentration as in the treatment medium.

Determination of SAMK activity by immunokinase assay

To determine SAMK activity by immunokinase assay, the supernatant was depleted supernatant that remained after removing SAMK with fractionated from the extract by immunoprecipitation with anti-SAMK antibody. Absence of SAMK in the supernatant was confirmed with immunoblotting. The down-regulation of a MAPK cascade is generally confirmed with immunoblotting.

To verify this possibility we carried out experiments on the effects of PD98059, an inhibitor of MAPKK, and of Apigenin, a MAPK inhibitor, on cold-induced SAMK in-gel activity. Unfortunately, the results of these experiments were inconclusive. Thus the definitive proof of causal involvement of SAMK or HAMK in cold or heat acclimation must await the results of studies using the techniques of antisense, gene suppression or over-expression. It should also be pointed out that these studies are applicable to whole plants awaits further investigation.

Whether PP2A is involved in the SAMK or HAMK cascade is not known. The down-regulation of a MAPK cascade is generally confirmed with immunoblotting.

In conclusion, we have identified the first heat shock-induced SAMK in-gel activity. Unfortunately, the results of these experiments were inconclusive. Thus the definitive proof of causal involvement of SAMK or HAMK in cold or heat acclimation must await the results of studies using the techniques of antisense, gene suppression or over-expression. It should also be pointed out that these studies are applicable to whole plants awaits further investigation.

Whether PP2A is involved in the SAMK or HAMK cascade is not known. The down-regulation of a MAPK cascade is generally confirmed with immunoblotting.
MAPK. In the immunokinase assay on the other hand, MBP is phosphorylated by SAMK-containing immunoprecipitate in an in vitro reaction. Phosphorylated MBP is visualized through SDS-PAGE coupled with autoradiography. All immunokinase assays reported here refer to SAMK activity.

**Immunoblot analysis**

Level of SAMK protein in the total cell extract was determined by immunoblotting with anti-SAMK antibody as described (Jonak et al., 1996). Proteins were separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes (0.45 μm) immunoblotting with anti-SAMK antibody as described (Jonak et al., 2000). Antibody concentrations used were as follows: anti-SAMK antibody (1 : 10 000), antiphospho-ERK (New England Biolabs, Beverly, MA, USA, 1 : 2500) and a goat antirabbit HRP-conjugated secondary antibody (Transduction Laboratories, Lexington, KY, USA) was used at a 1 : 5000 dilution. Blots were visualized using the Supersignal™ chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

**Determination of membrane polarization index p as an inverse measure of membrane fluidity**

Fluorescence polarization of protoplast membranes was determined as previously described for alfalfa cells (Örvar et al., 2000). Polarization index (p), an inverse measure of membrane fluidity, was calculated as described (Raymond and Plaa, 1996).

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