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Heat Shock Activation of *Phospholipase C* Signaling Pathway in Tobacco Cells

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ABSTRACT

Phospholipid signaling, frequently associated with rapid responses to environmental stimuli, is well known in animal and some higher plants. Heat shock is a major component of abiotic stress and stimulates many signaling pathways. Applying heat shock stress to tobacco (*Nicotiana tabacum*) BY-2 cells activated the phospholipids signaling pathway, including protein kinase C (PKC) and diacylglycerol (DAG) kinase pathways, both of which are associated with the downstream phospholipid pathway. In addition, activation of phospholipase C (PLC) activated heat-activated MAP kinase (HAMK) and caused the accumulation of heat shock protein 70 (HSP70) as an end-point marker. In contrast, chemical inhibition of PLC, PKC or DAG kinase completely or partially inhibited HAMK activation and HSP70 accumulation during heat shock. Moreover, treatment of cells with phosphatidic acid (PA) or PKC activators led to HAMK activation and HSP70 accumulation at 25°C, as did treatment of cells with either IP_3 or cADPR, both of which are known to release Ca^{2+} from intracellular stores. We conclude that the heat shock response, as measured by HAMK activation and HSP70 accumulation, requires phospholipid signaling and mobilization of vacuolar Ca^{2+}. Thus, the PIP_2-PLC pathway appears to play a key role in thermotolerance after heat shock treatment.

Keywords: calcium influx, HAMK, heat stress, HSP70, MAP kinase, phospholipid signaling, plant cells
Abbreviations: ATP, adenosine triphosphate; **BSA**, bovine serum albumin; **BY-2**, bright yellow 2; **CDPKs**, calcium-dependent protein kinases; **DAGK**, diacylglycerol kinase; **DTT**, dithiothreitol; **EDTA**, ethylene diammine tetraaacetic; **EGTA**, ethylene glycol-*bis*(b-aminoethyl ether)*N*',*N*',*N*',*N*'-tetraacetic acid; **ERK**, extracellular signal regulated protein kinase; **HAMK**, heat activated MAP kinase; **MAPK**, mitogen activated protein kinase; **HSP**, heat shock protein; **HSF**, heat shock factor; **PA**, phosphatidic acid; **PAGE**, polyacrylamide gel electrophoresis; **PKC**, protein kinase C; **PLC**, phospholipase C; **SDS**, sodium dodecyl sulfate; **WB**, Western blot

INTRODUCTION

For signaling to occur, two components are required. First is the means to sense change in the external environment, i.e., a sensor. Second is a sequence of events that transmits the information collected by the sensor to the nucleus, where specific genes need to be activated, i.e., a signal transduction pathway. In plants, the pathways of cellular communication are governed by signaling systems which translate extracellular signals to intracellular second messengers which can serve a number of functions. These second messengers can turn on or off specific pathways and regulate intracellular calcium concentrations in the cell. Ultimately, this can lead to changes in protein expression patterns, and thus the acquisition of tolerance to a particular stress (Berridge and Michael 1993; Arsell and Lindquist 1994; Kostak *et al*. 2007).

In phospholipids signaling pathways, there are two important secondary messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), both of which control a wide variety of pathways and cellular responses (Berridge and Michael 1987). The messengers are released and metabolized by means of the phosphatidylinositol cycle which was first recognized as the "phospholipid effect" (Berridge and Irvine 1984). Initially, phospholipase C (PLC) hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate IP_3 and DAG (Liu *et al*. 2006). Each of these second messengers has been linked to downstream events in signal transduction. DAG is subsequently phosphorylated by DAG kinase (DAGK) to yield phosphatidic acid (PA) (Berridge and Michael 1987).

The phospholipid signaling pathway has been found to be involved in multiple stress signaling events in plant cells, including the responses to hyperosmotic, salt, drought, cold stresses and heat stress (Rhee and Choi 1992; Hansen *et al*. 1995; Ruelland *et al*. 2002; Mills *et al*. 2004; Liu *et al*. 2006; Misra *et al*. 2007). At the same time, transcriptome analysis of plant has revealed the involvement of factors other than classical heat stress responsive genes in thermotolerance. Additionally, an increasing number of mutants that have altered thermotolerance have extended our understanding of the complexity of the heat shock signaling pathway in plants

In the present study, the role of different components in the phospholipid signaling pathway in response to heat shock, using activation of heat shock-induced MAPK, HAMK (heat-activated MAP kinase), and accumulation of HSP70 (heat shock protein 70) as end-point markers were examined.

MATERIALS AND METHODS

Plant material

One-week-old tobacco BY-2 (*Nicotiana tabacum* cv. 'Bright Yellow 2') suspension culture cells were grown in 250 ml Erlenmeyer flasks containing 50 ml of MS basal salts media (Murashige and Skoog 1962) (Sigma Chemicals Co., Oakville, Canada) supplemented with 2.72 mM KH_2PO_4, 100 mg l^{-1} *myo*-inositol, 1 mg l^{-1} thiamine, 3% (w/v) sucrose and 0.9 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The pH of the media was adjusted to 5.0 prior to autoclaving. Cultures were incubated on a rotary shaker at 130 rpm with a 16 h photoperiod at a light intensity of 40 μmol m^{-1} sec^{-1} at 25 ± 0.5°C and 60% relative humidity. Cells were subcultured every 7 days.

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Chemical treatments

The role of PLC and DAGK in the heat stress-induced activation of HAMK and accumulation of HSP70 was studied. Cells were treated with the PLC inhibitors U-73122 (1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl]-1H-pyrrole-2,5-dione) (0-150 μM; Calbiochem, San Diego, USA) and Et-18-OCH3 (1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphorylcholine) (0-350 μM; Calbiochem) or the DAGK inhibitors diacylglycerol kinase inhibitor I (6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5H-thiazolo-[3,2-a]-pyrimidin-5-one) (0-300 μM; Sigma), and diacylglycerol kinase inhibitor II (3-[2-[4-(bis(4-fluorophenyl)methylene)-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone) (0-200 μM; Sigma). To study the role of PA in heat signaling, cells were treated with PA (150 μM). In all these experiments, cells were pretreated with the appropriate chemical for 2 h at 25°C prior to their exposure to heat shock at 37°C.

The role of the Protein Kinase C (PKC) in heat signaling was examined by using H7 (0-125 μM; Calbiochem), and bisindolylaleimide III.HCl (BIM) (0-125 μM; Calbiochem) both of which inhibit PKC activity, while PKC was activated using PKC activators, SC-10 (100 μM; Calbiochem) and 1-oleoyl-2-acetyl-Sn-glycerol (OAG) (100 μM; Calbiochem). Moreover, IP_3, cyclic adenosine diphosphate-ribose (cADPR) and ruthenium red (RR) (0-250 μM) were used to examine the role of intracellular Ca^{2+} mobilization in the activation of HAMK and the expression of HSP70. For these experiments, cells were incubated in the treatment solution for the times indicated prior to harvest. In all experiments where DMSO was used as a solvent to dissolve the chemical, DMSO was used as a control.

Cell viability

Cells from the control and variously treated samples were tested for their viability by using tetrazolium chloride (TTC) as described by Towill and Mazur (1974) and optical density was measured at 400 nm. For comparisons among several treatments, three to five replicates of each were stained and then the absorbance data was statistically analyzed. All obtained data in the experiment was subjected to analysis of variance according to Snedecor and Cochran (1967) and the comparison of means was done using LSD test at the 0.05 level of probability, as mentioned by Cochran and Cox (1957).

Heat treatment

Control and treated cells were harvested and subjected to heat shock by adding 5 ml of MS medium pre-heated at 37°C in a water bath, followed by incubation of the cells in a 37°C water bath for the indicated times. Following heat shock, cells were immediately harvested by vacuum filtration, frozen in liquid nitrogen and stored at -80°C. All experiments were performed at least twice and representative data are shown.

Protein extraction and quantification

250 mg of frozen tissue was ground in liquid nitrogen and total protein was extracted in 400 μl of extraction buffer (100 mM HEPES (pH 7.5), 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 5 mM EDTA, 10 mM DTT, 50 mM β-glycerophosphate, 5 $\mu g.ml^{-1}$ antipain, 5 $\mu g.ml^{-1}$ aprotinin, 5 $\mu g.ml^{-1}$ leupeptin, 10% (v/v) glycerol and 7.5% polyvinylpolypyrrolidone). The homogenate was centrifuged at 20,000 × *g* for 20 min at 4°C. Supernatants were quick-frozen in liquid nitrogen and stored at –80°C until analysis. Protein concentrations were determined using the Bradford dye-binding assay (BioRad Laboratories, Hercules, USA).

Measurement of PLC activity

Phosphatidyl inositol (PI)-dependent PLC activity was measured in tobacco cells heat shocked for different durations and pretreated with PLC inhibitors viz., U-73122 and ET-18-OCH_3, prior to heat shock treatment using the method described by Dwivedi and Pandey (1999) with some modifications. Briefly, the reaction was conducted by using 5 μg of protein/tube in an incubation buffer (20 mM Tris-HCl, 1 mM $CaCl_2$, and 100 mM KCl, pH 7.4) containing 10 mM lithium chloride, PIP^2 substrate (50 μM unlabeled PIP^2, 2.0 μCi/ml [3H]PIP^2, and 0.5 mg/ml cetrimide) in a total volume of 100 μl at 37°C for 10 min. The reaction was terminated by the addition of 50 μl of 1 M HCl and 500 μl of a mixture of chloroform/methanol (1:1 v/v). The tubes were vigorously mixed and centrifuged at 1,000 × *g* for 10 min. The aqueous (upper) phase was transferred to a scintillation vial containing scintillation liquid, and the radioactivity was counted in a liquid scintillation counter. Each experiment had its blank, in which the protein suspension was added after stopping the reaction with chloroform/methanol. PI-PLC activity is expressed as the amount of [3H]IP_3 formed (dpm) per minute per milligram of protein.

In-gel assay of HAMK activity

The in-gel kinase assay was performed essentially as described by Zhang and Klessig (1997). Protein extract (25 μg) from control and treated samples were fractionated in a 10% SDS-PAGE gel co-polymerized with 100 $\mu g\ ml^{-1}$ of myelin basic protein (MBP, Sigma Chemicals Co., Oakville, Canada). SDS was then removed from the gel by washing with wash buffer (25 mM Tris pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg ml^{-1} BSA, 0.1% Triton-X) followed by renaturation in 25 mM Tris pH 7.5, 1mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF at 4°C overnight. MBP kinase activity was detected by incubating the gel for 60 min in 30 ml of activity buffer (25 mM Tris pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, 200 nM ATP and 50 μCi γ-^{32}P-labelled ATP). Unincorporated γ-^{32}P was removed by washing in 5% (w/v) TCA and 1% (w/v) NaPPi. The gel was vacuum dried at 80°C and autoradiography was performed.

Immunoblot analysis for HAMKs

Proteins in cell lysates were resolved on 10% SDS-PAGE gels and electro-blotted onto Hybond™-C Extra nitrocellulose membranes (Amersham Bioscience Corp., Baie d'Urfe, QC, Canada). Membranes were blocked at room temperature with 3% (w/v) BSA in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM EDTA pH 8.0 and 0.1% (v/v) Tween-20) followed by incubation with primary antibody for 60 min at a dilution of 1:1000. The primary antibodies used were anti-phoshoERK (anti-pERK) antibody (Cell Signaling Technology) to visualize activated HAMK, and anti-ERK antibody (Cell Signaling Technology) to monitor the total level of ERK proteins. Membranes were washed in TBST and incubated for 50 min with horseradish peroxidase-linked Protein A antibody (Amersham Biosciences) diluted in TBST (1:10,000 dilution). Following more washes with TBST, bound antigen-antibodies complexes were visualized using the Supersignal® chemiluminescence kit (Pierce Biotechnology Inc, Rockford, USA).

Immunoblot analysis for HSP70

The level of HSP70 protein in the control and variously treated tobacco cells was determined by the immunoblotting method described above using anti-HSP70 antibody (Stressgen Biotechnology Corp., Victoria, Canada) diluted in TBST (1:20,000) as the primary antibody.

Semi-quantification of HSP70 by enzyme-linked immunosorbent assay (ELISA)

Protein levels of HSP70 in extracts prepared from variously treated cells was quantified using the HSP70 ELISA Kit (Stressgen Biotechnology). Samples were incubated with biotinylated antibody followed by incubation with avidin-HRP conjugate and reaction with a tetramethylbenzidine substrate. A standard curve was made using 0.78-50 ng/ml recombinant human HSP70 and optical absorbance was measured at 450 nm using a micro-plate reader.

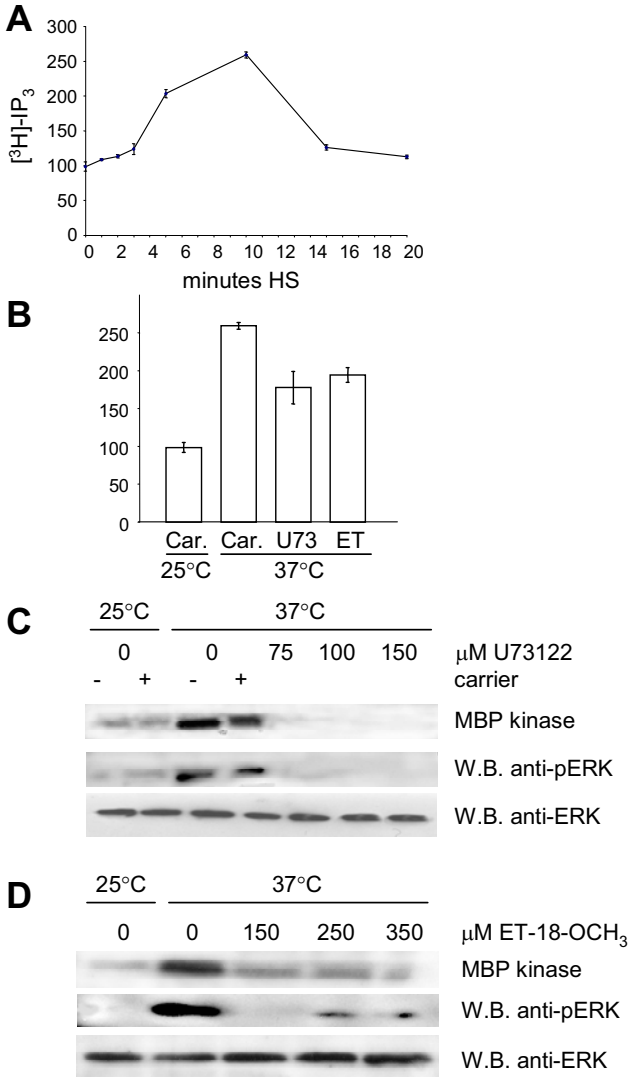

Fig. 1 Heat shock leads to PLC activation, which is upstream of HAMK activation. (**A**) Extracts from *N. tabacum* suspension culture cells exposed to 37°C for varying times were assayed for PLC activity. HS, heat shock. (**B**) Known inhibitors of PLC were tested for their ability to block PLC activation in this experimental system. Car., carrier alone; U73, U73122 (100 μM); ET, Et-18-OCH_3 (250 μM). (**C**), The ability of U73122 to prevent HAMK activation in heat shock at various concentrations was assessed by pretreating suspension culture cells with U73122 for 2 h, followed by exposure to 37°C for 10 min. Cell extracts were prepared and subjected to Western blotting to quantitate levels of MBP kinase activity and phosphorylated $p^{42/44}$ (upper and middle panels), used as measures of HAMK activation. Total $p^{42/44}$ levels did not change during treatment (lower panel). (**D**), Similar to U73122, treatment with the PLC inhibitor Et-18-OCH_3 prevents HAMK activation in heat shock.

RESULTS AND DISCUSSION

Phospholipase C (PLC) activity increases rapidly in response to heat stress

In order to investigate whether signaling via PLC is an integral element of the plant high temperature signaling pathway, PIP^2 (phosphatidylinositol-3,4-diphosphate)-dependent PLC activity was assayed in tobacco BY-2 suspension culture cells exposed to heat shock (37°C) for varying amounts of time as described in (Dwivedi and Pandey 1999). PLC activity increases rapidly with exposure to elevated temperature, reaching a peak at 10 min and then gradually returning to prior values (**Fig. 1A**). Moreover, pretreatment for 2 h with the specific and potent PLC inhibitors, U73122 (Stam *et al*. 1998) or Et-18-OCH_3, inhibited the heat-dependent activation of PLC (**Fig. 1B**).

Heat-shock-induced activation of HAMK is mediated through PLC

To investigate the role of PLC in the regulation of heat signaling, cells were treated with various concentrations of U73122 (0-150 μM) and ET-18-OCH_3 (0-350 μM) for 2 h prior to challenge by heat-shock at 37°C for 5 min. Following PLC inhibition, stimulation of heat-activated MAP kinase (HAMK) activity by elevated temperature was no longer observed. The MBP-phosphorylating activity migrated as a single band with an apparent molecular weight of ca. 46 kDa (**Figs. 1C, 1D**, upper panels). Since this size, as well as the utilization of MBP as a substrate is consistent with those of known MAPKs, and all plant MAPKs characterized to date belong to the ERK class of kinases, anti-P42/44 MAPK (ERK) and anti-phospho-P44/42 MAPK (pERK) antibodies (Cell Signaling Technology) were used to perform Western blots on proteins extracted from heat-shocked cells. The anti-pERK antibody specifically recognized an activated MAPK of 46 kDa which we named HAMK (heat-shock Activated MAPK) (Sangwan *et al*. 2002) (**Figs. 1C, 1D**, central panels). The total amount of ERK proteins remained unchanged during heat shock treatment, as determined by Western blot analysis using the anti-ERK antibody (**Figs. 1C, 1D**, lower panels). Thus, the activation of HAMK in heat-stress signaling is up-regulated through the activity of PLC.

DAG kinase and its product PA play an essential role in the heat-dependent activation of HAMK

The role of DAGK activity and subsequent PA generation in HAMK activation was initially investigated by pharmacological inhibition of DAGK. Cells were treated with various concentrations (0-200 μM) of DAGK inhibitor I (**Fig. 2A**), which potently and selectively blocks DAGK (Nunn and Watson 1987), prior to heat shock. All the concentrations tested strongly inhibited HAMK activation under heat-shock conditions, as assessed through decreases in both MBP kinase activity (**Fig. 2A**, upper panel) and pERK levels (**Fig. 2A**, central panel). The total amount of ERK proteins in each sample remained unchanged during the heat shock treatment (**Fig. 2A**, lower panel). Similar results were obtained using DAGK inhibitor II (de Chaffoy de Courcelles *et al*. 1985) (data not shown).

On the other hand, exposure of suspension culture cells to PA (150 μM) led to the rapid activation of HAMK within

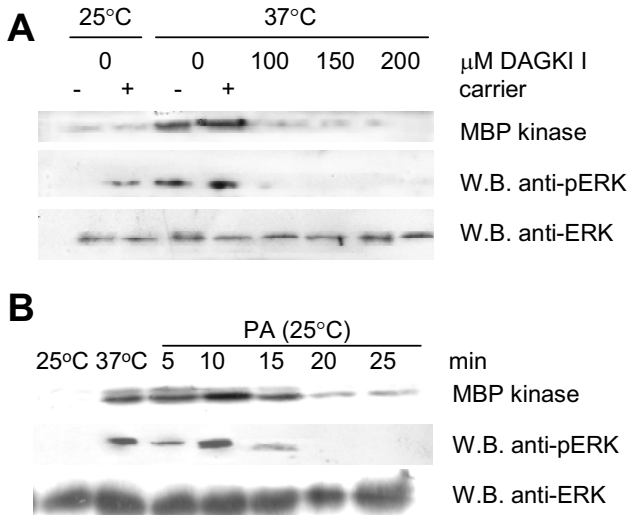

Fig. 2 The diacylglycerol kinase (DAGK) pathway is involved in HAMK activation during heat shock. (**A**) Pretreatment of suspension culture cells with an inhibitor of DAGK at 25°C inhibits subsequent activation of HAMK during exposure to 37°C. DAGKI I, DAGK inhibitor I. (**B**) Treatment with the DAGK product phosphatidic acid (PA) stimulates HAMK activation in the absence of heat shock.

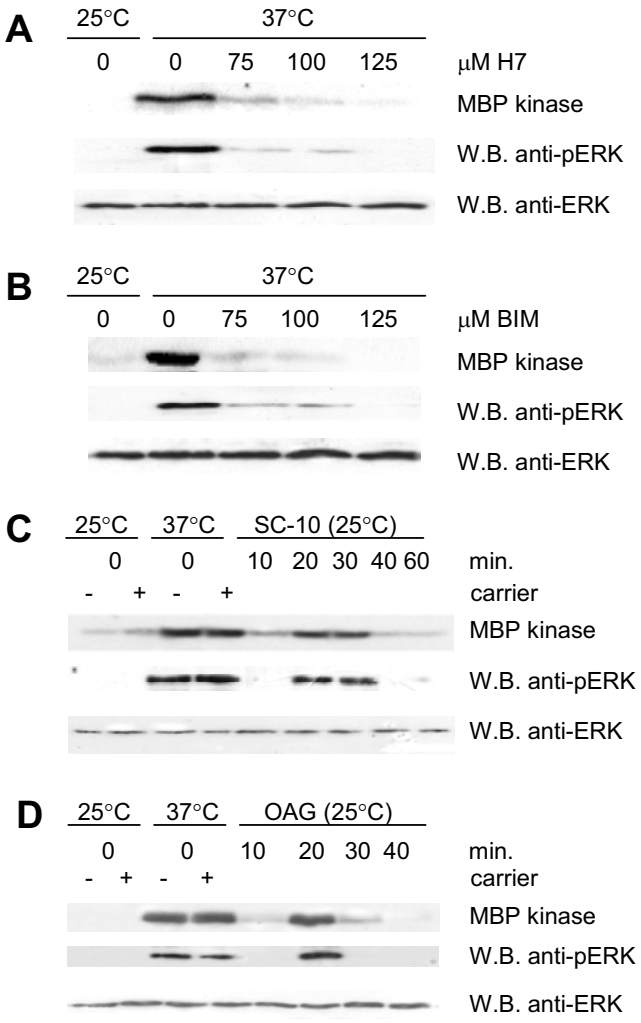

Fig. 3 Inhibitors and activators of protein kinase C (PKC) inhibit or promote, respectively, HAMK activation. (**A**) Pretreatment with the PKC inhibitor H7 inhibits the heat shock-induced increase in HAMK activity. (**B**) similarly, treatment with bisindolylaleimide III·HCl (BIM), another PKC inhibitor, also inhibites the rise in HAMK activity seen after exposure to elevated temperature. (**C**) Treatment with the PKC activator SC-10 leads to an increase in HAMK activity after 20 min at RT. (**D**) Similarly, exposure to 1-oleoyl-2-acetyl-Sn-glycerol (OAG), another PKC activator, also induces HAMK activation in the absence of elevated temperature.

5 min in the absence of heat shock, as indicated by increased MBP kinase activity (**Fig. 2B**, upper panel), as well as elevated pERK levels (**Fig. 2B**, central panel). Thus, these results clearly demonstrate the involvement of the DAGK pathway and its product, PA, in the regulation of HAMK activation.

DAG has also been implicated in the activation of the Ca^{2+} and phospholipid-dependent kinase protein kinase C (PKC). The role of PKC in high temperature signal transduction was investigated by pharmacological manipulation. Pretreatment of tobacco cell suspension culture with the PKC activators (H7) or (bisindolylaleimide III.HCl) (BIM) inhibits HAMK activation upon subsequent exposure to elevated temperatures (**Figs. 3A, 3B**), while addition of the PKC activators (SC-10 or 1-oleoyl-2-acetyl-Sn-glycerol) (OAG) led to increased HAMK activity at 25°C with similar time courses (**Figs. 3C, 3D**). Thus, it could be concluded that multiple signaling pathways downstream of PLC converge at the level of HAMK activation.

IP_3-mediated intracellular Ca^{2+} release is involved in heat-induced HAMK activation

Aside from DAG, the other product of PLC-mediated PIP_2 hydrolysis is IP_3, which has been shown to mediate the release of Ca^{2+} from intracellular stores in plant cells. The effects of elevated IP_3 levels on HAMK activation were assessed. Exposure of suspension culture cells to IP_3 (200 μM) led to an increase in HAMK activation without exposure to elevated temperature (**Fig. 4A**), as did stimulation of vacuolar Ca^{2+} release using cyclic adenosine diphosphate-ribose (cADPR) (**Fig. 4B**). On the other hand, pretreatment with ruthenium red (RR), an inhibitor of vacuolar Ca^{2+} release, inhibitd HAMK activation when cells were exposed to heat shock at 37°C (**Fig. 4C**). These results suggest that the role of IP_3 in mediating release of Ca^{2+} from intracellular stores is an integral element in the signaling pathway leading to HAMK activation under conditions of heat stress. Recently, the involvement of IP_3 in heat-shock signal transduction in *Arabidopsis* was shown (Liu *et al*. 2006).

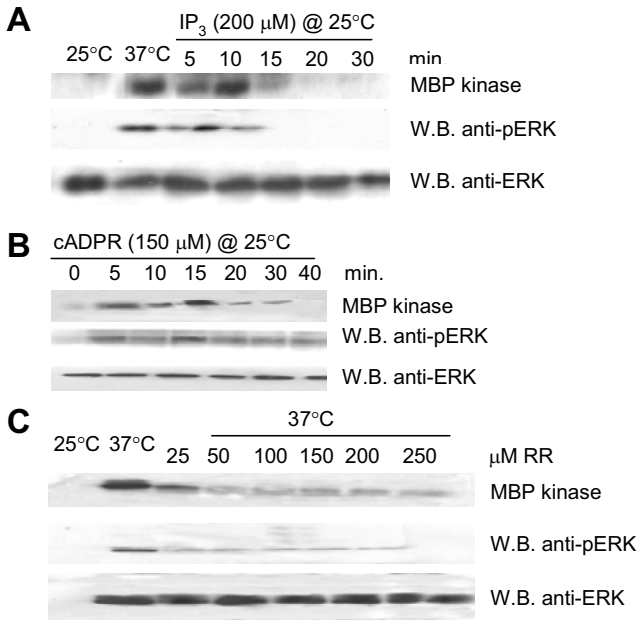

Fig. 4 Signaling via IP_3 and intracellular Ca^{2+} release is involved in HAMK activation. (**A**) Treatment of suspension culture cells with IP_3 leads to activation of HAMK in the absence of heat shock. (**B**) Induction of Ca^{2+} release from intracellular stores using cyclic adenosine diphosphate-ribose (cADPR) induces activation of HAMK at constant temperature. (**C**) Blocking the release of intracellular Ca^{2+} using ruthenium red (RR) blocks HAMK activation at 37°C.

HSP70 protein accumulation is downstream of phospholipid signaling

Although HAMK activation serves as a convenient marker to indicate the activation of the high temperature signaling pathway, the endpoint of the pathway is the increased production of proteins that aid the plant in protection against damage occurred by elevated temperatures (Kostak *et al*. 2007). One of the most abundant proteins induced by heat stress is a member of the heat shock protein family, heat shock protein 70 (HSP70). The accumulation of HSP70 was chosen to investigate whether the phospholipid signaling pathways leading to HAMK activation also induce the accumulation of HSP70. Pharmacological inhibition of PLC using U73122 or Et-18-OCH3 inhibited HSP70 accumulation after 3 h of heat shock as assessed by Western blotting with an anti-HSP70 antibody, while inhibition of DAGK with DAGK inhibitor I or DAGK inhibitor II only partially reduced HSP70 accumulation (**Fig. 5A**). These results were confirmed by quantitation of HSP70 present in treated cell extracts using the enzyme-linked immunosorbent assay (ELISA) technique (data not shown).

On the other hand, treatment with PA leads to increased HSP70 levels in the absence of heat stress (**Fig. 5B**), while pretreatment with the PKC inhibitors H7 or bisindolylalei-

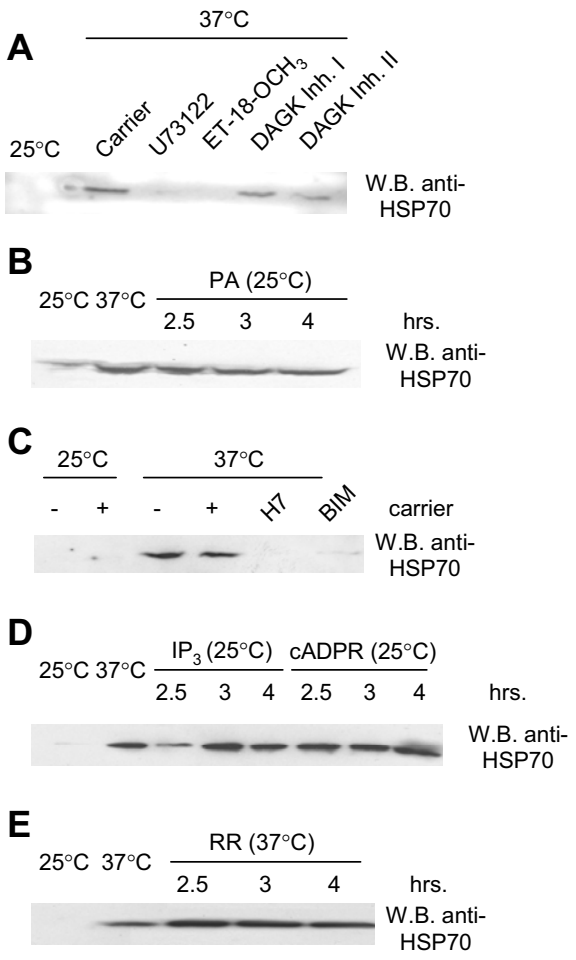

Fig. 5 Most treatments that inhibit or stimulate HAMK activation have similar effects on the levels of heat shock protein 70 (HSP70). (**A**) Pretreatment with inhibitors of PLC (U73122 and Et-18-OCH_3) and DAGK (DAGK Inh. I and DAGK Inh. II) significantly reduce heat shock-induced HSP70 accumulation, as assessed by Western blotting. (**B**) Exposure to the DAGK product PA induces HSP70 protein accumulation at RT. (**C**) Pretreatment with the PKC inhibitors H7 or BIM also inhibits the increase in HSP70 protein levels seen after exposure to elevated temperature. (**D**) Stimulation of Ca^{2+} release from intracellular stores leads to HSP70 accumulation in the absence of heat shock. E Inhibition of intracellular Ca^{2+} release does not repress HSP70 protein accumulation during heat shock.

mide III.HCl inhibits heat shock-induced elevation of HSP70 protein levels (**Fig. 5C**). Treatment with IP_3 or cADPR, leading to the release of intracellular Ca^{2+}, also can induce HSP70 accumulation under unstressed conditions (**Fig. 5D**); however, inhibition of the release of vacuolar Ca^{2+} using RR does not appear to affect heat shock-induced HSP70 protein accumulation (**Fig. 5E**). Thus, HSP70 protein levels appear to be under the control of multiple signaling pathways.

Phospholipid signaling in heat stress is independent of cytoskeleton remodeling

It has previously been demonstrated that in high temperature signal transduction, cytoskeleton remodeling is a relatively early step, lying between temperature-induced changes in membrane fluidity and the opening of plasma membrane Ca^{2+} channels (Sangwan *et al*. 2002; Johnston *et al*. 2007). Therefore, we chose to investigate whether the phospholipid signaling pathway was linked to cytoskeleton remodeling. Suspension culture cells were first treated with jasplakinolide or taxol, which act to stabilize microfilaments and microtubules, respectively, prior to exposure to either PA or IP_3 under non-stress conditions. As can be observed in (**Fig. 6**), inhibition of cytoskeletal remodeling

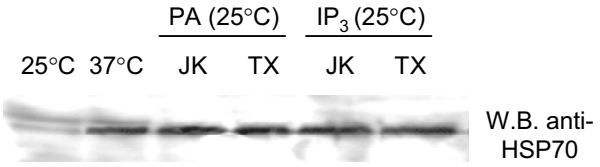

Fig. 6 Modulation of cytoskeletal rearrangement does not inhibit PA or IP3-induced HSP70 accumulation. Extracts prepared from suspension culture cells pretreated with either the microtubule stabilizer Taxol (TX) or the microfilament stabilizer jasplakinolide (JK), and subsequently exposed to PA or IP_3 at RT, exhibit increased levels of HSP70 as compared to untreated cells maintained at 25°C.

failed to inhibit the increment in HSP70 protein accumulation caused by PA or IP_3 treatment, suggesting that the phospholipid signaling pathway is initiated by signals unrelated to cytoskeletal remodeling.

Proposed model of heat signaling pathways

By investigating several upstream pathways involved in heat signaling (Mansour *et al*. 2003; Suri *et al*. 2003; Dhindsa *et al*. 2005; Mansour and Dhindsa 2005), we attempted to propose a model scheme of some pathways involved in heat signaling (Mansour *et al*. 2006). As the model shows (**Fig. 7**), by applying heat to plant cells, membrane fluidity takes place (Mansour and Dhindsa 2005) and Phospholipids C becomes activated as a primary response to heat. Both actions occurred at the same time and triggered more cascades of signals streaming. In addition, PLC enzyme hydrolyses phosphatidylinositol (PIP2) to produce two important second messengers which are inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG

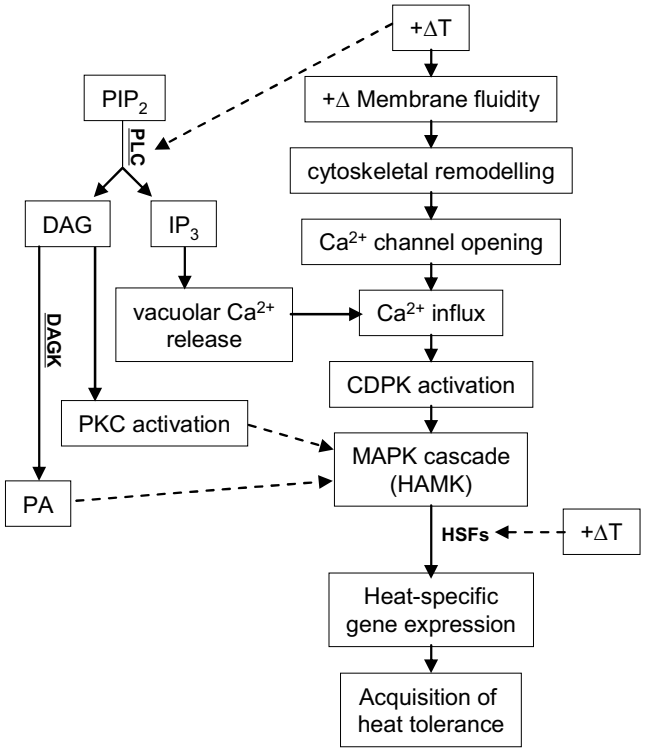

Fig. 7 General model of the signaling pathway leading to the acquisition of heat tolerance. The model illustrates the multiple pathways involved in heat signaling based on the obtained results. In this study, we have demonstrated that in tobacco suspension culture cells, exposure to elevated temperature leads to an increase in PLC activity, leading to the production of IP_3 (and subsequent release of Ca^{2+} from intracellular stores) and DAG, which directly activates PKC as well as being processed by DAGK to yield PA. Each of these pathways appears to lead in turn to the activation of the MAPK HAMK, as well as to the induction of HSP70.

are important because they control a wide variety of pathways and cellular responses, including heat shock (Mansour *et al*. 2003). Upon generation of these second messengers two signaling events take place.

First, IP_3 is released into the cytosol and acts to mobilize intracellular calcium stores from the ER, resulting in Ca^{+2} influxes. Secondly, DAG stays in the cell membrane and activates a protein kinase C (plant homologue) (Suri *et al*. 2003). This happens in a very short time frame, less than one minute. DAG kinase, which activated by heat also, phosphorylates DAG to give phosphatidic acid (PA) which in turn triggers the stimulus MAPK Cascade, specifically HAMK (Suri and Dhidsa 2008).

On the other hand, membrane fluidity is followed by many cellular events in response to heat (Sangwan *et al*. 2002; Mansour and Dhindsa 2005). Cytoskeleton remodeling, reorganization of microtubules and microfilaments, and Ca^{+2} channel openings, which causes Ca^{+2} influx, were two important cellular phenomena following the heat shock respectively. Activation of plant CDPKs followed by MAPK cascade activation, specifically HAMK, were noticed as an integral part of heat signaling transduction (Sangwan *et al*. 2002).

After several minutes, the downstream activation of heat shock factors followed by induction of heat shock genes and the accumulation of HSPs in the cells could be noticed obviously. Then, the acquisition of thermo-tolerance occurs and the cells showed more heat tolerance (Mansour *et al*. 2006) (**Fig. 7**).

CONCLUSION

The results of this investigation show that phospholipid signaling pathway plays an important role in plant heat shock response (Johnston *et al*. 2007; Misra *et al*. 2007). Independent of cytoskeletal rearrangements, PLC activity increases rapidly in response to elevated temperature, resulting in the generation of two distinct second messengers, DAG and IP_3. DAG then in turn activates PKC, leading to the activation of a MAPK cascade terminating at HAMK, while DAGK generates PA, which exerts a similar effect. IP_3, on the other hand, causes the release of Ca^{2+} from intracellular stores, resulting in increased cytoplasmic Ca^{2+} levels and most likely indirectly contributing to the activation of a MAPK cascade via the activation of calcium-dependent protein kinases (CDPKs) (Bush 1995).

Elevated DAG levels can lead to the activation of protein kinase C (PKC), while PA has been suggested to activate a mitogen-activated protein kinase (MAPK) cascade. Interestingly, PKC may also be important in providing a negative feedback signal by attenuating PLC activity. IP_3, on the other hand, has been implicated in mediating the release of Ca^{2+} from intracellular, i.e., vacuolar, stores. Ultimately, PA is recycled back to phosphatidylinositol and PIP_2 via the CDP-diacylglycerol pathway, which uses free inositol to resynthesize PI (Berridge and Irvine 1984; Berridge and Michael 1987; Berridge and Michael 1993).

On the other hand, it appears that following a prolonged exposure to elevated temperatures, HSP70 protein accumulation can occur even when DAGK is blocked. This may either reflect a direct effect of heat on the activity of HSFs, or, alternately, may suggest that the arm of the phospholipid signaling pathway which proceeds through IP_3 is capable of activating the transcription of heat shock-specific genes without involving HAMK, since while DAGK inhibition only partially reduces heat shock-induced HSP70 accumulation, it completely inhibits HAMK activation; on the other hand, inhibition of PKC completely inhibits both HAMK activation and HSP70 protein accumulation.

Based on the above mentioned results, it is evident the plants have evolved a variety of responses to elevated temperatures that minimize damage and ensure protection of cellular homeostasis. Structure and function of heat shock signaling pathway and molecular chaperones that included become available.

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