A heat-activated MAP kinase in tomato: a possible regulator of the heat stress response

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Abstract **Adaptation to elevated temperatures is of major importance for the survival of plants. The role of kinases in heat stress response was studied in tomato by *in gel* and *in solution* kinase assays using myelin basic protein as substrate. The application of heat stress in a naturally occurring temperature range resulted in a fast and transient activation of a 50 kDa mitogen-activated protein (MAP) kinase both in a photoautotrophic cell suspension culture and in leaves of mature plants. The heat activation of the MAP kinase was shown to be calcium-dependent. The specific phosphorylation of tomato heat stress transcription factor HsfA3 by a partially purified preparation of the heat-activated MAP kinase supports a physiological role of the identified kinase activity in transducing the heat stress signal.**

Key words: MAP kinase; MAP kinase substrate; Heat stress; Heat shock factor; Phosphorylation

1. Introduction

Changes in environmental temperature are a highly variable parameter to which cellular functions of plants have constantly to adapt. As sessile life forms, plants have had to develop mechanisms that can sense and respond to temperature changes in a very fast and flexible way to ensure cellular functions over a surprisingly large range of temperatures. The response to heat stress is usually transient in nature and helps to maintain cellular function over a rather short time of heat exposure, such as during the daily hours of higher temperatures, but may also lead to an increased overall thermotolerance [1]. The heat stress response is characterised by a large attenuation of transcriptional and translational activity with the exception of heat shock proteins (HSPs), which accumulate in a dose-dependent manner and seem to account for a major part of thermotolerance [2,3]. The regulation of HSP activity occurs mainly on the transcriptional level, via heat shock elements (HSEs) located in the promoters of the heat shock genes. The HSEs in turn provide binding sites for the heat stress factors, transcription factors that become activated upon heat stress and drive HSP expression, with HsfA1, which is a thermo-inducible protein itself, as a central regulator [4]. However, only little is known about the molecular events leading to Hsf activation and Hsp expression to induce thermotolerance. It has been shown for higher eukaryotes that, upon heat stress, Hsfs undergo trimerisation and thereby acquire high binding affinity to HSEs and transactivation potential [5]. In addition, phosphorylation events have been implicated in the regulation of Hsfs and it has been shown that *Arabidopsis* Hsf1 is a substrate of CDC2 [6]. Mitogen-activated protein (MAP) kinases are involved in biotic and abiotic stress-mediated defence reactions [7–11] and were found to phosphorylate Hsfs in yeasts and mammals [12–14]. This study focusses on the characterisation of the very early responses of tomato plants and cell cultures to heat stress. Here we present data on the specific activation of a 50 kDa MAP kinase by heat stress and provide an indication of the possible involvement of this MAP kinase in the regulation of the heat stress response by phosphorylating a heat stress transcription factor.

2. Materials and methods

2.1. Cell culture conditions and plant growth

Photoautotrophic suspension culture cells of tomato (*Lycopersicon peruvianum*) were established by [15] and were subcultured every 2 weeks in Murashige and Skoog medium under constant illumination and in a 2% CO_2 atmosphere. Cells were used for experiments as described in [16]. Tomato plants (*Lycopersicon esculentum* cv. Moneymaker) were grown in the greenhouse under a 16 h light, 8 h dark regime. Plants were used for the experiments after 9 weeks. When experiments involved a change in the environment for the plants, they were allowed to adapt for 16 h.

2.2. Heat stress treatments

Heat stress was applied to the suspension cultures by the addition of prewarmed, preconditioned culture medium to the cells. For a single experiment, the appropriate amounts of cell cultures were mixed and redistributed into the culture flasks. Remaining cell culture was cleared and the cell-free medium was used as preconditioned medium for the experiments. Unless otherwise indicated, experiments were performed by adding 21 ml of 80°C preconditioned medium to 40 ml of cell culture, which resulted in a temperature increase of 12°C. The cells were then incubated at 26°C. To challenge plants, they were either exposed to direct sunlight, shifted from a 26°C to a 37°C growth chamber or by dipping leaves into 37°C water.

2.3. MAP kinase assays and inhibitor treatments

In gel kinase assays, *in solution* assays, immunoprecipitations and Ca^{2+} channel inhibitor treatments were performed as described in [16].

2.4. Partial purification of the heat-activated MAP kinase

To enrich the heat-activated MAP kinase for *in solution* kinase assays, crude cell extracts were applied to a ResourceQ anion ex-

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change column and processed as described in [16]. One single peak of kinase activity eluted from the column at concentrations of 150–200 mM NaCl.

2.5. HsfA1 and HsfA3 overexpression and purification

A C-terminal His-tag fusion to HsfA1 was created by insertion of a synthetic oligonucleotide linker (5′-tatgctcgtgcctcgcggcagccatcaccatcaccatcactaagcttggtac-3′) into pRTHsf8LS [17] cut with *NdeI* and *Asp718I*. The coding sequence of the fusion protein was released with *Eco*RI and *Apa*I and religated into pJC20 to create pJCA1HC, which was used for overexpression. HsfA1 was overexpressed in *Escherichia coli* BL21 by addition of 0.4 mM IPTG (isopropyl thiogalactose) followed by 3 h incubation at 30°C. The protein was purified using the HisTrap system (Pharmacia, Freiburg, Germany) by sonyfying the cells in 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES)/KOH pH 7.6, 500 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 100 μM PMSF (phenylmethylsulfonyl fluoride). Columns were washed with 20 mM Na-phosphate buffer containing 10 mM imidazole and eluted with the same buffer containing 250 mM imidazole. To overexpress HsfA3, the DNA binding domain of the factor was deleted by polymerase chain reaction (PCR) using the oligonucleotides HsfA3/MBP5′ (gaattcatctctttatatgatatgaacc) and HsfA3/MBP3′ (gcagcctgaagcttttcattaacggatcc) with pJCHN–HsfA3 [18] as a template. The resulting fragment was subcloned into pGEM-T (Promega, Madison, WI, USA) and released with *Eco*RI and *Pst*I to be ligated into pMALc (NEB, Schwalbach, Germany) to create an N-terminal in-frame fusion with the maltose binding protein. Induction of protein synthesis was carried out by addition of 0.5 mM IPTG and incubation for 16 h at 26°C. The fusion protein was purified on amylose resin (NEB) according to the manufacturer's instructions. For *in solution* kinase assays, 3 μg of each purified protein was added per reaction.

2.6. Antibody production

To generate antibodies directed against different MAP kinases a cDNA fragment spanning the 11 domains that are conserved among MAP kinases [19] was cloned from MAP kinase MMK 4 of *Medicago* [7]. Using primers OMK3 (TCCTGGATCCATGCCGATTGGTCGTGGTGC) and OMK4 (TTTTAAGCTTTAAGCATACTCAGGATTG) a 1000 bp cDNA fragment with a *Bam*HI site at the 5′ end was amplified by reverse transcriptase PCR and cloned into the *Sma*I site of pUC18. The insert of the resulting plasmid pMK3–4 was released as *Bam*HI fragment and cloned into pMalc (NEB) to generate an N-terminal in-frame fusion with the maltose binding protein. Induction of protein synthesis and purification of the fusion protein was carried out as described above. Polyclonal antibodies directed against the purified fusion protein were generated in New Zealand white rabbits as described before [20].

3. Results and discussion

3.1. A 50 kDa MAP kinase is activated in response to heat stress in tomato cell suspension cultures

In gel kinase assays using myelin basic protein (MBP) as substrates were carried out to determine the possible involvement of kinases in early heat stress signalling. To investigate fast responses to elevated temperatures in higher plants, we used photoautotrophic suspension culture cells of tomato (*L. peruvianum*) that were heat stressed by a fast increase in the temperature of the growth medium. To induce heat stress in these cell cultures, we added heated, preconditioned medium to the cells. This treatment was found to be superior to e.g. shifting the cultures to the warmer environment of a different growth chamber, since the resulting temperature changes were much more reliable in terms of extent and timing of the increase/decrease of the temperature gradient. For a first characterisation, the cells were challenged with a temperature increase from 26 to 38°C and cell extracts were tested at different time points for the activation of MBP-phosphorylating protein kinases. As shown in Fig. 1A, a protein kinase

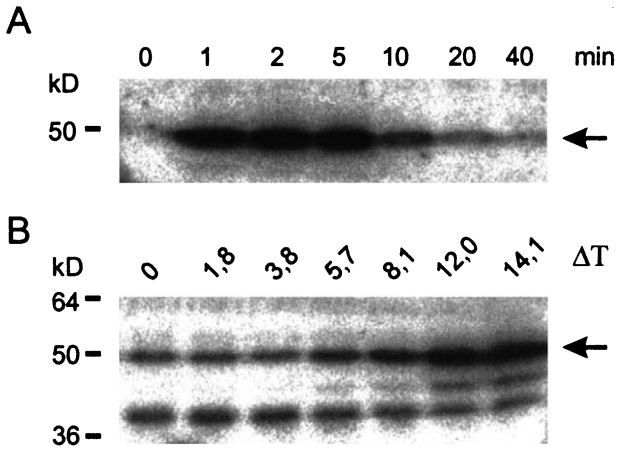

Fig. 1. Heat-induced MAP kinase activation. A: Time course of activation of kinase after adding 21 ml of 80°C preconditioned medium to a 40 ml tomato cell culture resulting in a temperature increase of 12°C. B: Dose response of increase in temperature by adding 21 ml of medium at different temperatures. Samples were taken 1 min after the temperature shift. Kinase activity was analysed by an *in gel* kinase assay with MBP as substrate.

with an apparent molecular weight of 50 kDa is transiently activated only 1 min after the treatment and is found to be deactivated approximately 10 min after the heat stress. This fast and transient activation is a typical feature of early response elements and was found to occur often in the activation patterns of stress-related MAP kinases [21–24]. To further investigate the specificity of the heat stress treatment, we conducted a dose–response experiment (Fig. 1B) which reveals that the protein kinase is not activated by temperature shifts of less than 5.7°C and that the activation is maximal at a temperature difference of 12°C. At activating temperatures, an additional but weaker phosphorylation signal becomes evident. However, since this signal is not present in the experiment shown in Fig. 1A and appears in parallel with the 50 kDa signal, it is considered as a degradation product of the activated protein kinase.

Apart from the fast and transient activation, another typical feature of MAP kinases is their phosphorylation on tyrosine residues upon activation [25]. To test whether this feature is also present in the heat-activated protein kinase, we performed immunoprecipitations using the phosphotyrosine-specific monoclonal antibody 4G10. As is shown in Fig. 2A, this antibody effectively precipitates the heat-activated protein kinase. The specificity of this reaction is demonstrated by the use of P-tyr, P-thr and P-ser as competitors, of whom only P-tyr was able to hinder binding of the antibody. To gain further support to place the heat-activated protein kinase into the group of MAP kinases, we also performed an immunoprecipitation using a polyclonal serum directed against the conserved MAP kinase domains. The antibody was raised against mmk4 MAP kinase excluding its N-terminus, and should thus be able to recognise tomato MAP kinases, since the kinase domains of mmk4 and those encoded by tomato MAPK expressed sequence tags (ESTs) are highly homologous and show several identical stretches. As depicted in Fig. 2B, this polyclonal serum effectively precipitates the heat-activated protein kinase, whereas the control reaction using the preimmune serum does not display any kinase activity in the precipitate. These various lines of experimental

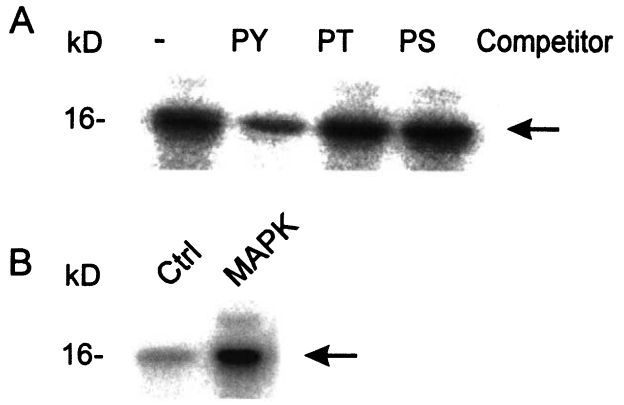

Fig. 2. Immunoprecipitation with phosphotyrosine and MAP kinase-specific antibodies. A crude extract of heat-stressed cells was subjected to immunoprecipitation and subsequent *in solution* kinase assay with MBP as substrate. A: Phosphotyrosine-specific antibody 4G10 and competition with 1 mM phosphotyrosine (PY), phosphothreonine (PT) or phosphoserine (PS). B: Preimmuneserum (Ctrl) and serum with antibodies against conserved MAP kinase domains (MAPK).

evidence support the conclusion that the heat-activated protein kinase belongs to the family of MAP kinases.

MAP kinases have also been reported to be involved in the regulation of the heat stress response of yeast and mammals [14,26]. The involvement of a MAP kinase in the heat response of plants thus demonstrates that the heat response is a conserved mechanism probably following analogous pathways in all eukaryotes.

3.2. The activation of the heat-activated MAP kinase is dependent on calcium influx

Calcium signalling is involved in several stress-related signal transduction processes. Specifically, for the induction of a heat stress response a strong dependence on extracellular calcium has been demonstrated in sugar beet cells [27]. In another report, the dependence of heat stress factor activation in rabbit heart cells on calcium was demonstrated by the use of the calcium channel inhibitor gadolinium [28]. To elucidate the underlying signal transduction pathway of heat MAP kinase activation in plant cells, we tested the involvement of calcium in heat stress signalling by the use of calcium channel inhibitors. As demonstrated by the *in gel* kinase assay shown in Fig. 3, treatment of the cells with gadolinium and lanthanium effectively inhibited MAP kinase activation by heat stress. Likewise, depletion of calcium ions from the culture medium by EGTA also hindered MAP kinase activation by heat stress, which was only retained by the concomitant addition of equal molar amounts of calcium. Therefore, the heat-activated MAP kinase acts downstream of a calcium-mediated signal transduction process.

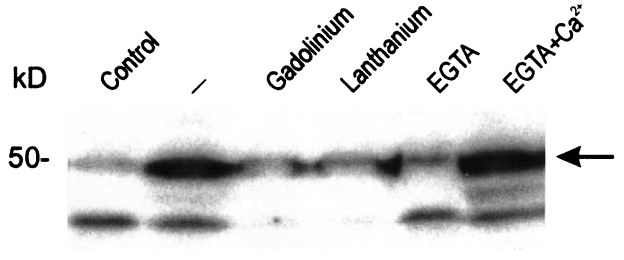

Fig. 3. Heat-induced MAP kinase activation depends on calcium signalling. Gadolinium (1 mM), lanthanium (1 mM), EGTA (8 mM) or EGTA (8 mM) and calcium (8 mM) were added 4 min before heat stress treatment (temperature increase of 14°C). Samples taken 1 min after stimulation were analysed by an *in gel* kinase assay with MBP as substrate.

3.3. Activation of the heat-activated MAP kinase in planta

To relate our observations in the cell culture system to the physiological significance *in planta*, we determined the temperature increase on leaf surfaces that were suddenly exposed to bright sunlight. Interestingly, and in accordance with the dose–response pattern observed in the cell cultures, we found this temperature increase to be approximately 11°C. *In gel* kinase assays were performed with leaves of plants that were shifted to bright sunlight. The experiments were carried out in the greenhouse to exclude the effect of UV light exposure. The results from a representative sunlight experiment are shown in Fig. 4. By analysis with *in gel* kinase assays using MBP as substrate, a 50 kDa protein kinase is found to be activated shortly after exposure to sunlight. As is the case in cell cultures, the activation of the MAP kinase is fast and transient, showing activation after 5 min and a return to steady-state activity after 30 min. Similar results for heat activation of MAP kinase activity *in planta*, although with different activation kinetics, were obtained with other heating methods, such as dipping detached leaves into warm water or moving plants to another climate chamber (data not shown). In order to exclude light as an activator of the MAP kinase [29,30] plants were shifted between two climate chambers without exposing them to daylight. This experiment also led to the activation of the heat-activated MAP kinase (data not shown). In contrast to our findings, a heat-inactivated MAP kinase has been described in heterotrophic tomato cell cultures [31]. However, our study used autotrophic cell cultures and mature leaves, which both can be considered as source organs. Interestingly, the inactivation of the MAP kinase was observed in a system rather resembling a sink organ, thus pointing towards different modes of regulation of MAP kinase(s) in response to heat stress in source and sink organs.

3.4. The heat-activated MAP kinase phosphorylates HsfA3

In the yeast *Saccharomyces cerevisiae*, the heat stress response is part of the cell integrity pathway, in which gene activation in response to heat is mediated by a MAP kinase module [14] which ultimately leads to the production of heat stress proteins (Hsps), that seem to account for a major part of induced thermotolerance [32]. One major component of this pathway is the heat stress factor 1 (Hsf1), a transcription factor that is involved in the upregulation of Hsps, which was

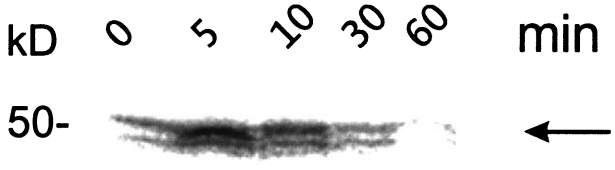

Fig. 4. Activation of heat-activated MAP kinase *in planta*. Tomato plants were moved to bright sunlight and the leaves were analysed for activation of MAP kinase at the indicated time points by an *in gel* kinase assay with MBP as substrate.

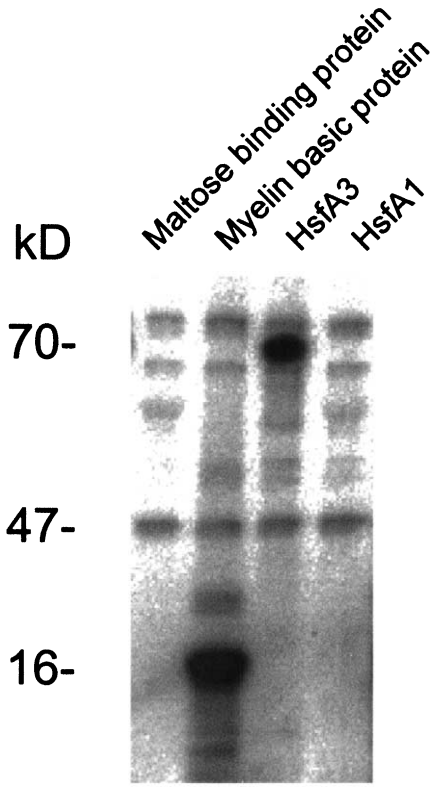

Fig. 5. Purified, recombinant heat stress factors HsfA3 and HsfA1 were used as substrates for the partially purified heat-activated MAP kinase in an *in solution* kinase assay. As controls, purified maltose binding protein alone and myelin basic protein (MBP) were also used as substrate in separate reactions.

demonstrated by temperature-sensitive mutants lacking a functional Hsf1 [33]. In plants, several homologues to Hsf1 from yeast have been described [18,34]. Interestingly, some of these Hsfs, along with another homologue from *Drosophila* can functionally complement Hsf function in yeast [35,36], which shows that the signal transduction pathway leading to the activation of a heat stress response is highly conserved among eukaryotes. All four known heat stress factors in tomato contain several copies of consensus MAP kinase phosphorylation sites ((PX)[(S/T)P]) [18,34], with HsfA1 (nine times (S/T)P, one time ((PX)[(S/T)P]) and HsfA3 (eight times (S/T)P, one time ((PX)[(S/T)P]) having the highest number of sites. The factors were thus expressed in *E. coli* as maltose binding protein (A3) or His–Tag (A1) fusions and the purified proteins were used in *in solution* kinase assays to test their potency as MAP kinase substrates. As is shown in Fig. 5, when purified HsfA3 is incubated in the presence of a partially purified heat-activated MAP kinase, a strong phosphorylation of the recombinant protein is observed at the predicted molecular mass of 70 kDa. No phosphorylated protein is observed at this position when the substrate is omitted from the reaction or is replaced by the maltose binding protein. MBP was included as a positive control. Interestingly, HsfA1 is not phosphorylated in this assay, indicating a selective recognition of HsfA3, since both proteins display MAP kinase phosphorylation sites. The specific phosphorylation of HsfA3 thus supports the idea of an involvement of a MAP kinase(s) in the regulation of the heat stress response of higher plants. This implies that not only the molecular mechanisms of transcriptional regulation but also upstream components of the heat stress-induced signalling are conserved in eukaryotes. MAP kinases were also found in mammals to be involved in heat stress signalling [12,13], although the system appears to be regulated by more complex mechanisms than in the case for yeast [37,38]. However, in yeast as well as mammals, MAP kinases have been shown to directly phosphorylate Hsf1 in vivo [39]. Although MAP kinases belonging to several subfamilies have been identified in many plant species and were shown to be involved in various signal transduction pathways [6–10], so far no physiological substrate has been identified in plants. The finding that the heat-activated MAP kinase from tomato specifically accepts HsfA3 as a substrate, but not HsfA1, gives a strong indication for the first physiological MAP kinase–substrate described for plants. Recently, it has been published that tomato HsfA1 is a central regulator of the heat stress response that seems to affect HsfA2 and HsfA3 activity [4]. The heat-activated MAP kinase could thus act as a 'safety' switch to also initiate the heat stress response. Further investigation will aim to show the direct interaction of the heat-activated MAP kinase with HsfA3 in vivo to elucidate the exact regulatory role of the heat-activated MAP kinase in the heat stress response.

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