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Diverse stress signals activate the C1 subgroup MAP kinases of *Arabidopsis*

Dolores Ortiz-Masia[a], Miguel A. Perez-Amador[b], Juan Carbonell[b], Maria J. Marcote[a,*]

[a] *Facultad de Farmacia, Universidad de Valencia, Avda. Vicente Andrés Estellés s/n, 46100 Burjassot, Spain*
[b] *Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas y Universidad Politécnica de Valencia, Avda. de los Naranjos s/n, 46022 Valencia, Spain*

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Abstract **Mitogen-activated protein kinase (MAPK) cascades play an important role in mediating stress responses in plants. In *Arabidopsis*, 20 MAPKs have been identified and classified into four major groups (A–D). Little is known about the role of group C MAPKs. We have studied the activation of *Arabidopsis* subgroup C1 MAPKs (AtMPK1/AtMPK2) in response to mechanical injury. An increase in their kinase activity was detected in response to wounding that was blocked by cycloheximide. Jasmonic acid (JA) activated AtMPK1/AtMPK2 in the absence of wounding. Wound and JA-induction of AtMPK1/2 kinase activity was not prevented in the JA-insensitive *coi1* mutant. Other stress signals, such as abscisic acid (ABA) and hydrogen peroxide, activated AtMPK1/2. This report shows for the first time that regulation of AtMPK1/2 kinase activity in *Arabidopsis* might be under the control of signals involved in different kinds of stress.**

Keywords: Abscisic acid; Hydrogen peroxide; Jasmonic acid; MAP kinase; Wound

1. Introduction

Mitogen-activated protein kinase (MAPK) cascades link extracellular stimuli with several cellular responses. These cascades are evolutionary conserved signalling modules present in all eukaryotes. MAPKs are serine/threonine kinases that are activated by dual phosphorylation of the threonine and the tyrosine residues at a TXY activation motif. These phosphorylations are performed by a MAPK kinase (MAPKK), which is in turn activated by an upstream MAPKK kinase (MAPKKK) [1].

In *Arabidopsis thaliana*, 20 MAPKs have been identified and classified according to their sequence homology into four major groups (A–D) [2]. Groups A–C contain the phosphorylation motif TEY whereas group D contains the motif TDY. A number of studies have demonstrated that some *Arabidopsis* MAPKs are activated by hormones, abiotic stresses, wounding, and during plant–pathogen interactions and cell division [3–5]. These studies have revealed the complexity of plant MAPK cascades and the high level of cross-talk in diverse signal pathways.

A large amount of information about MAPKs in group A and B is available but very little is known about MAPKs of subgroup C1 [6–10]. Specific changes in transcript levels during pollen or ovule development have been reported for two members of C1 subgroup, *ntf3* from tobacco [10] and *PMEK1* from *Petunia* [6]. In *Arabidopsis*, subgroup C1 is constituted by two MAPK genes: *AtMPK1* (*AT1G10210*) and *AtMPK2* (*AT1G59580*). The function of these genes remains mostly unknown, regardless of data which indicate a possible relationship between these genes and some stress responses. It has been reported that the mRNA levels of *AtMPK1* and *AtMPK2* increased slightly under salt stress [9] and are downregulated after 24 h of cold treatment [11], respectively. Gene expression data deposited in public microarray repertories indicate a very low expression of the corresponding mRNAs and few and no relevant changes in their expression under a large variety of tissues and conditions ([12] and *Arabidopsis* functional genomics consortium (AFGC) microarrays databases). No data about the regulation of the kinase activity of subgroup C1 MAPKs is available. In this article, we report the upregulation of AtMPK1 and AtMPK2 kinase activities in *Arabidopsis* leaves in response to wounding, jasmonic acid (JA), abscisic acid (ABA) and hydrogen peroxide (H_2O_2).

2. Materials and methods

2.1. Plant material and treatments

Arabidopsis plants were grown in growth chambers under a 16-h-light:8-h-dark regime and 75% relative humidity at 21 °C. *coi1-1* plants were selected in Murashige and Skoog medium containing 20 μM JA (Duchefa) as previously described in [13] and then transferred to soil. Wound lesions were generated with forceps by squeezing two to three times rosette leaves (ca. 50% of the leaf area) in 4-week-old plants. Cycloheximide treatment was performed by spraying 4-week-old plants with 100 μM Cycloheximide (solved in water), or water as a control, 1.5 h before wounding. For the JA treatments, 4-week-old plants were sprayed with 50 μM JA in 0.05% (v/v) ethanol and 0.01% (v/v) Tween 20 as a surfactant or with control solution (0.05% (v/v) ethanol and 0.01% (v/v) Tween 20). ABA and H_2O_2 treatments were performed by spraying 4-week-old plants with 100 μM ABA in 0.05% (v/v) ethanol and 0.01% (v/v) Tween 20 and 5 mM H_2O_2, respectively. Leaf samples were harvested at different times after the treatments and immediately frozen in liquid N_2 and stored at −80 °C. For root assays,

*Corresponding author. Fax: +34 963544917.
E-mail address: mariajesus.marcote@uv.es (M.J. Marcote).

Abbreviations: ABA, abscisic acid; AFGC, *Arabidopsis* functional genomics consortium; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MBP, myelin basic protein

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plates were made with 0.5× MS basal salts, 1% Suc, 1% agar and supplemented with 0, 2, 25 or 50 μM JA. Seedlings were grown in plates vertically for 14 d.

2.2. Antibody production

The peptides HPQASTLNTEL and PEAATINNNEVSEF, corresponding to the C-termini of AtMPK1 and AtMPK2, respectively, were synthesized and conjugated to keyhole limpet hemocyanin carrier. Polyclonal antisera were raised in rabbits and purified by affinity column chromatography (SIGMA-GENOSYS).

2.3. Preparation of protein extracts

Arabidopsis tissues were ground in liquid nitrogen and homogenised in extraction buffer: 25 mM Tris–HCl, pH 7.5, 75 mM NaCl, 20 mM NaF, 5 mM EGTA, 5 mM EDTA, 5 mM DTT, 20 mM β-glycerophosphate, 0.05% Triton X-100, phosphatase inhibitors (1:100 dilution, Sigma # 5726) and protease inhibitors (1:250 dilution, Sigma #P9599). Samples were centrifuged at $12000 \times g$ for 20 min at 4 °C twice and supernatants were considered as protein extracts. Protein quantitation was performed with Bradford assay (Bio-Rad Laboratories GmbH, Munich, Germany).

2.4. Immuno-complex kinase assay

For immunoprecipitation, the indicated amount of protein extracts to a total volume of 400 μl of extraction buffer, were incubated with anti-AtMPK1 or anti-AtMPK2 antibodies for 1 h on ice. After binding to 20 μl of protein A-Sepharose CL-4B (Amersham Biosciences) for 2 h at 4 °C, the immunoprecipitates were washed twice with extraction buffer and once with kinase assay buffer (30 mM Tris, pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 20 mM β-glycerophosphate). To detect kinase activity in the immunoprecipitates, Sepharose beads were suspended in 40 μl of kinase assay buffer containing 0.7 mg/ml myelin basic protein (MBP), 100 μM cold ATP, and 5 μCi of γ-^{32}P-ATP (6000 Ci/mmol) and incubated for 20 min at 30 °C. The reaction was stopped by the addition of 3× SDS gel loading buffer [14]. Reactions products were separated by electrophoresis on a 13% SDS–polyacrylamide gel, and MBP phosphorylation was analyzed by autoradiography. For quantitation, MBP bands were cut out from the gel and the radioactivity counted in a microplate scintillation counter (1450 Microbeta TRILUX). Specific activities were obtained by calculating the amount of phosphate incorporated into MBP per min and mg protein extract. The relative kinase activities are shown as the fold induction of specific kinase activities, with the level of specific kinase activity at time zero set to 1. The values of relative kinase activities shown in the graphs were equal to the relative kinase activities determined by MPB densitometry of autoradiograms in the linear range of film development. Experiments were repeated at least twice with identical results.

2.5. Mutant isolation by PCR screening

Lines (Columbia background) containing a T-DNA insertion in *AtMPK1* and *AtMPK2* (*Atmpk1* and *Atmpk2*, respectively) were identified from the SALK T-DNA collection (http://signal.salk.edu/cgi-bin/tdnaexpress), corresponding to donor stock numbers SALK_063847 and SALK_019507, respectively. To identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from 12 plants and submitted to PCR genotyping using the following primers: for *AtMPK1* 5′-GGATCCCACAAATGATGCTACTTTGGTGA-3′ and 5′GGATCCTGGTGGAGATCAGTGTCCATGAG-3′; for *AtMPK2* 5′-GGATCCGCGACTCCTGTTGATCCACCT-3′ and 5′-GGATCCTCCCTGGTGAGTAAGGGAGTG-3′. The T-DNA left border primer used was 5′-GGATCCGCGTGGACCGCTTGCTGCAACT-3′. The homozygous line *Atmpk1* was crossed with the homozygous line *Atmpk2*. Individual homozygous for T-DNA insertion in both *AtMPK1* and *AtMPK2* were identified by PCR with the primers described above.

2.6. RT-PCR

Total RNA was extracted from leaves of 2-week-old plants using a Qiagen RNeasy plant mini kit and 1 μg of the RNA solution obtained was reverse transcribed using 0.1 μg oligo$(dT)_{15}$ primer and Expand Reverse Transcriptase (Roche) to finally obtain a 40 μL cDNA solution. PCR amplifications were performed on 1-μL cDNA template using the kit PCR Master (Roche). The sequences of the primers used for PCR amplifications were the following ones: for *AtMPK1*, forward 5′-GGATCCCCGGGGAACGTCGTTGG-3′ and reverse 5′-GGATCCTGGTGGAGATCAGTGTCCATGAG-3′; for *AtMPK2*, forward 5′-GGATCCGGCGATCGTCACTGACGAAT CTG-3′ and reverse 5′-GGATCCAGAGACAGAGTATGAAG-3′; for *ACT7*, forward 5′-GGATCCAAATGGCCGATGGTGAGG-3′ and reverse 5′-GGAAAACTCACCACCACGAACCAG-3′; for *AtLOX2* (AT3G45140), forward 5′-TTGGCTGAGGAAGATAAGACCGCAGAACAT-3′ and reverse 5′-TCATTTTATCAAGAAGACAGAGATACAGAA-3′; for *AtAOS* (AT5G42650), forward 5′-GGATCCATCACAACACTCGCCACT-3′ and reverse 5′-CAGATTATACAACATTTTCTCAAAATTCACG-3′; for *AtVSP1* (AT5G24780), forward 5′-ACGTCCAGTCTTCGGCATCC-3′ and reverse 5′-GAGCTTAAAAACCCTTCCAG-3′.

3. Results

3.1. AtMPK1 and AtMPK2 kinase activities are induced by wounding

To study whether wounding induces activation of AtMPK1 and AtMPK2, we first raised antibodies against specific peptides (11-amino acid and 14-amino acid, respectively) corresponding to the unique C-terminus sequence of AtMPK1 and AtMPK2. Attempts to use anti-AtMPK1 and anti-AtMPK2 antibodies to monitor AtMPK1 and AtMPK2 protein levels in different tissues by Western blot were unsuccessful, presumably due to the low *AtMPK1/2* expression (see Section 1). However, these antibodies were successfully employed to detect changes in AtMPK1 and AtMPK2 kinase activity upon wounding. Protein extracts from wounded and unwounded wild type leaves were prepared and subjected to immuno-complex kinase assay. The results showed high levels of kinase activity in anti-AtMPK1 and anti-AtMPK2 immunoprecipitates from wounded leaves (Fig. 1a). To determine the linearity of the assay, different amounts of protein were immunoprecipitated from leaf extracts and assayed for kinase activity. Linearity is observed up to 200 μg and 800 μg of protein with anti-AtMPK1 and anti-AtMPK2 antibody, respectively (Fig. 1a). To test whether the detected kinase activity is specific of AtMPK1/2, T-DNA mutants of *AtMPK1* and *AtMPK2* (*Atmpk1* and *Atmpk2*, respectively) were identified in the Salk collection, corresponding to stock numbers SALK_063847 and SALK_019507, respectively, and the *Atmpk1 Atmpk2* double mutant was obtained (Fig. 1b and c). Plants from the lines *Atmpk1*, *Atmpk2* and *Atmpk1 Atmpk2* resembled wild type plants under standard growth conditions. Protein extracts from wounded and unwounded wild type, *Atmpk1*, *Atmpk2* and *Atmpk1 Atmpk2* double mutant leaves were immunoprecipitated with anti-AtMPK1 and anti-AtMPK2 antibodies and the kinase activity was determined. The results indicate that the kinase activities detected with the anti-AtMPK1 and anti-AtMPK2 antibodies are specific of AtMPK1 and AtMPK2, respectively (Fig. 1d). Indeed, no kinase activity in anti-AtMPK1 immunoprecipitates from wounded leaves was detected in *Atmpk1* and *Atmpk1 Atmpk2* double mutant extracts. Similarly, no kinase activity in anti-AtMPK2 immunoprecipitates from wounding leaves was detected in *Atmpk2* and *Atmpk1 Atmpk2* double mutant extracts. To gain insight of the role of AtMPK1/2 in the wounding response, the kinase activities of AtMPK1 and AtMPK2 at different times after wounding were analysed. It was found that wounding induced a transient activation of AtMPK1 and