

Novel MAP kinase substrates identified by solid-phase phosphorylation screening in *Arabidopsis thaliana*

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Abstract Phosphorylation of substrate proteins by mitogen-activated protein kinases (MPKs) determines the specific cellular responses elicited by a particular extracellular stimulus. However, downstream targets of plant MPKs remain poorly characterized. In this study, 29 putative substrates of AtMPK3, AtMPK4 and AtMPK6 were identified by solid-phase phosphorylation screening of a λ phage expression library constructed from combined mRNAs from salt-treated, pathogen-treated and mechanically wounded *Arabidopsis* seedlings. To test the efficiency of this screening, we performed in vitro kinase assay with 10 recombinant fusion proteins. All proteins were

phosphorylated by AtMPK3, AtMPK4 and AtMPK6, indicating the efficiency of this screening procedure. To confirm phosphorylation of isolated substrates by plant MPKs, we performed in-gel kinase assays. All test substrates were strongly phosphorylated by wounding or H₂O₂-activated AtMPK3 and AtMPK6. Three substrates, encoded by genes At2g41430, At2g41900, and At3g16770, were strongly phosphorylated, suggesting a function as AtMPK substrates. The type of screening provides a powerful way for identifying potential substrates of MAP kinases responsive to biotic and abiotic stresses.

Keywords Arabidopsis · MAP kinase · Phosphorylation · Solid-phase screening · Substrates

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Abbreviations

MPK	Mitogen-activated protein kinase
BWMK	Blast- and wound-induced MAP kinase
GST	Glutathione S-transferase
PAMP	Pathogen-associated molecular pattern
PPPDB	Plant protein phosphorylation database
MBP	Myelin basic protein

Introduction

The mitogen-activated protein kinase (MPK) cascade constitutes an essential intracellular signal transduction module that controls a multitude of cellular responses in most eukaryotes (Ahn 1993; Colcombet and Hirt 2008; Pitzschke et al. 2009). The MPK cascade is composed of three classes of molecules: MPKs, MPK kinases (MEK), and MPK kinase kinases (MEKK). MPK cascades are activated in response to a variety of internal and external stimuli. Signaling through an MPK cascade is initiated

when the Ser/Thr kinase MEKK phosphorylates and thereby activates the MEK. MEK is a dual specificity Thr/Tyr kinase. Activated MEK in turn phosphorylates and activates the Ser/Thr kinase MPK (Fiil et al. 2009). Phosphorylation by activated MPKs modulates the site of subcellular localization, protein stability, transcriptional activity, and/or interaction with other proteins in their target range of transcription factors and protein kinases. This ability of MPKs thus contributes to the generation of appropriate cellular responses.

The signaling network of MPK cascades in yeast and mammalian cells are better characterized in comparison with those of plant systems. These studies have established that MPK signaling networks are complex. The complexity of MPK signaling networks can be appreciated by considering the extracellular signal-regulated kinases 1 and 2 (ERK1/2). They represent two mammalian MPKs that transmit many extracellular signals to 160 substrates known so far including transcription factors, protein kinases, protein phosphatases, cytoskeletal elements, and a variety of signaling regulators (Yoon and Seger 2006). The signaling networks of plant MPK cascades are expected to be equally complex, considering, for example, possible combinations of 20 MPKs, 10 MEKs and 60 MEKKs in *Arabidopsis*. The number of MPK networks in plant is suggested to be much higher than the corresponding numbers in yeast and mammalian systems (Ichimura et al. 2002). The large number of MPKs and their upstream kinases enhances complexity, interconnectedness and functional redundancy in plant signaling networks.

MPKs are mainly involved in biotic and abiotic signaling, and in processes such as hormonal and developmental signaling in *Arabidopsis* (Colcombet and Hirt 2008; Sinha et al. 2011). Especially, MPK3, MPK4 and MPK6 among the 20 MPKs mainly function in a variety of distinct processes ranging from environmental stress responses to developmental processes (Colcombet and Hirt 2008). For example, MPK6 is involved in not only H₂O₂, O₃, PAMPs, osmotic shock, JA, ET and ABA signaling pathways, but also in developmental processes such as stomatal patterning and embryo development (Asai et al. 2002; Droillard et al. 2002; Ahlfors et al. 2004; Teige et al. 2004; Miles et al. 2005; Bush and Krysan 2007; Takahashi et al. 2007; Wang et al. 2007; Yoo et al. 2008). However, it is not possible to conclude that the three well-known MPKs, namely MPK3, MPK4 and MPK6 are specific, or solely responsible, for involvement in the many different signaling pathways because of a lack of exploratory tools, such as specific antibodies, to precisely analyze other MPKs.

In addition, it is difficult to identify the substrates of MPK simply by homology analysis, because their myriads of substrates are not conserved evolutionarily

(Sörensson et al. 2012). Thus, in comparison to yeast and mammalian MPKs, relatively few MPK substrates have been identified in plants. In *Arabidopsis*, substrates that have been identified and characterized to date include ACS6 (1-aminocyclopropane-1-carboxylic acid synthase), MKS1 (MAP Kinase 4 Substrate 1), EIN3 (Ethylene Insensitive 3), ERF104 (Ethylene-response factor 104), SPCH (SPEECHLESS), ZAT10, AS1 (Asymmetric Leaves 1), the MYB44, NIA2, WRKY1, WRKY33 transcription factors, VIP1 (VirE2-Interacting Protein 1), PPS3 (Protein Phosphorylated by StMPK1), and PHOS32 (Liu and Zhang 2004; Andreasson et al. 2005; Katou et al. 2005; Menke et al. 2005; Djamei et al. 2007; Lampard et al. 2008; Merkouropoulos et al. 2008; Yoo et al. 2008; Wang et al. 2010; Mao et al. 2011; Nguyen et al. 2012; Park et al. 2013). In addition, a tobacco protein, MAP65-1 (Microtubule Associated Protein 65-1) has also been identified as an MPK substrate (Sasabe et al. 2006). Although several plant MPK substrates have been identified, the steps that connect each of the *Arabidopsis* MPKs originating from an environmental stimulus to the resultant cellular responses remain unclear.

High-throughput approaches have recently been applied to analyze the complex MPK signaling networks in *Arabidopsis*. One study identified 48 and 39 putative substrates of AtMPK3 and AtMPK6, respectively, employing a protein microarray strategy (Feilner et al. 2005). A second study by screening a protein microarray reported identification of about 570 putative substrates for 10 different AtMPKs (Popescu et al. 2009). Transcription factors were the largest group of putative MPK substrates uncovered by these protein microarray analyses. However, a majority of the identified proteins have not been confirmed as real MPK substrates.

To identify novel *Arabidopsis* MPK substrates, we constructed a cDNA expression library in λ phage using RNA purified from biotic and abiotic stress-treated *Arabidopsis* seedlings. This expression library was screened using rice Blast- and wound-induced MAP kinase 1 (OsBWMK1) as the probe in a solid-phase phosphorylation screen. OsBWMK1 is an MPK that is rapidly induced in response to fungal infection and mechanical wounding (He et al. 1999; Cheong et al. 2003). OsBWMK1 showed higher kinase activity than activated AtMPK3, AtMPK4 and AtMPK6 in our system. All 29 potential MPK substrates identified by this screen were phosphorylated by AtMPK3, AtMPK4 and AtMPK6 in an in vitro kinase assay. These results provide new insights into the integration of MPK signaling pathways with the post-translational regulation of stress responses in *Arabidopsis*.

Materials and methods

Construction of λ GEX5 *Arabidopsis* cDNA library

The phage expression vector λ GEX5, a *gt11*-derived vector was modified by insertion of the *pGEX-PUC-3T* plasmid nucleotide sequence (Fukunaga and Hunter 1997). For construction of the cDNA library, total RNA was extracted from *Arabidopsis* seedlings that had been subjected to 200 mM NaCl, wounding and pathogen (*Pseudomonas syringae*) treatments. Poly(A)⁺ RNA was obtained using a mRNA separator kit (Clontech Laboratories, Palo Alto, CA, USA). Double-stranded cDNA was synthesized with oligo (dT) primer using a cDNA synthesis kit (Stratagene, La Jolla, CA, USA), and ligated to an adaptor which consisted of 5'-phosphorylated oligonucleotides, 5'-pCCAG-CACCTGCA-3' and 5'-pAGGTGCTGG-3'. cDNAs were then size-fractionated by agarose-gel electrophoresis and cDNAs larger than 400 bp were ligated to the *Sfi*I-digested GEX5 arms and packaged into bacteriophage particles. The cDNA library was amplified by passage through *E. coli* strain BB4.

Screening of the cDNA library by solid-phase phosphorylation

The *Arabidopsis* cDNA library was plated on *E. coli* BB4 at a density of 1.5×10^4 plaques per 150 mm agar plate. The screening of the cDNA library was carried out as previously described (Fukunaga and Hunter 1997). OsBWMK1, a rice blast- and wound-induced MAP kinase, was used for cDNA expression library screening (He et al. 1999). Positive clones were purified by secondary screening and the phage DNA was prepared from plate lysates. The phage DNA was digested with *Not*I and the cDNA-containing plasmid (*pGEX-PUC-3T*) was recovered by self-ligation followed by transformation of *E. coli* strain XL-1 Blue. The transformed bacteria were used for plasmid preparation and isolation of GST-fusion protein.

Analysis of phosphorylated proteins

The protein expression procedure was carried out as described previously (Park et al. 2002). An aliquot (10 μ l) of the glutathione-sepharose bound GST-fusion protein was incubated for 30 min at 30 °C with 200 ng of BWMK1 in 20 μ l of the kinase buffer [20 mM HEPES–NaOH (pH 7.4), 10 mM MgCl₂ and 1 mM DTT] in the presence of [γ -³²P]ATP (0.5 μ Ci). After phosphorylation, the reaction mixture was added to 4 \times SDS sample buffer and then separated on a 12.5% polyacrylamide gel. Phosphorylated proteins were detected by exposure of the gel to

X-ray films for 12–48 h at –70 °C with intensifying screens.

Purification of recombinant proteins

Expression and affinity purification of Glutathione S-transferase (GST) fusion proteins (OsBWMK1, AtMPK3, AtMPK4, AtMPK6) were performed as follows. *E. coli* BL21(*pLysS*) cells transformed with the GST-fusion constructs were grown for overnight at 37 °C and sub-cultured until the OD_{600nm} reached 0.5. Expression of the GST-fusion proteins was induced with 0.5 mM IPTG. Cells were incubated at 30 °C for 3 h, harvested and then lysed. Beads of Glutathione-Sepharose 4B were added to the supernatant and mixed by inversion for 30 min at 4 °C. The Sepharose beads were washed three times with 1 \times PBS. The GST-fusion proteins were eluted from the Sepharose beads with 10 mM reduced glutathione in 50 mM Tris–HCl (pH 8.0).

Plant materials and preparation of protein extracts

Arabidopsis thaliana ecotype Columbia was used. The *mpk6* knockout mutant line (SALK_062471) was obtained from the *Arabidopsis* Biological Resource Center (ABRC). Plants were grown at 22 °C in a growth chamber under 120 μ Em^{–2} s^{–1} light intensity and 16-h-light/8-h-dark photoperiod. Plants were wounded by nipping twice with a forceps. For oxidative stress, plants were sprayed with 1 mM H₂O₂. Plant samples were frozen in liquid nitrogen at the appropriate times after each treatment for 5, 10 and 15 min. For protein extraction, the frozen plants were ground in liquid nitrogen, and then thawed in extraction buffer (50 mM HEPES–KOH pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 25 mM NaF, 1 mM Na₃VO₄, 50 mM β -glycerophosphate, 20% glycerol, 2 μ g ml^{–1} leupeptin, 2 μ g ml^{–1} pepstatin A, and 2 mM PMSF). After centrifugation at 15,000 \times g for 30 min at 4 °C, the supernatants were transferred into clean tubes, frozen in liquid nitrogen, and stored at –80 °C for later use.

In-gel kinase assay

The in-gel kinase assay was performed as described previously (Zhang et al. 1993; Liu et al. 2010). 10 μ g of protein from total protein extracts of wild type and *atmpk6* mutant plants treated by wounding or application of 1 mM H₂O₂, respectively, was separated on 10% SDS–polyacrylamide gels embedded with 0.2 mg/ml myelin basic protein (MBP) as a substrate. After electrophoresis, gels were washed three times with wash buffer (25 mM Tris–HCl pH 7.5, 0.5 mM DTT, 0.1 mM Na₃VO₄, 5 mM NaF,

0.5 mg/ml bovine serum albumin, 0.1% TritonX-100). The proteins were then renatured in renaturing buffer (25 mM Tris–HCl pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4 , 5 mM NaF) for overnight at 4 °C. The gels were incubated in reaction buffer (25 mM Tris–HCl pH 7.5, 2 mM EGTA, 12 mM MgCl_2 , 1 mM DTT, 0.1 mM Na_3VO_4) at room temperature for 30 min, and then phosphorylated in 20 ml reaction buffer containing 250 nM ATP and 50 μCi [γ - ^{32}P]ATP at room temperature for 1.5 h. The reaction was terminated by transferring the gels into stop solution (5% trichloroacetic acid and 1% sodium pyrophosphate). Gels were washed five times with the stop solution for 5 h at room temperature and then dried on Whatman 3 MM paper and exposed to X-ray film (Fuji Photo Film, Tokyo, Japan) or a BAS2500 imaging plate (Fuji Photo Film, Tokyo, Japan).

In vitro kinase assays

Recombinant GST-tagged MPKs were used to phosphorylate recombinant putative substrate proteins. GST-tagged proteins were purified by using Glutathione-Sepharose 4B columns (GE Healthcare, Piscataway, NJ, USA). Purified MPKs (200 ng) and substrates (500 ng) were incubated in 20 μl of kinase reaction buffer (50 mM Tris–HCl, pH 7.5, 10 mM MgCl_2 and 10 μCi of γ - ^{32}P -ATP) at 30 °C for 30 min. The reactions were stopped by the addition of SDS-loading buffer. Phosphorylated substrates were visualized by autoradiography after electrophoresis on 12.5% polyacrylamide gels.

Results

Isolation of MPK phosphorylation substrates from an *Arabidopsis* cDNA expression library

To identify new substrates for AtMPK3, AtMPK4 and AtMPK6, we used a solid-phase phosphorylation screening method (Fukunaga and Hunter 1997). First, we established a λ phage cDNA expression library constructed from combined mRNAs from salt-treated, pathogen-treated and mechanically wounded *Arabidopsis* seedlings. After the induction and the immobilization of fusion proteins from the expression library, we performed in vitro solid-phase phosphorylation assays using MEK-activated recombinant AtMPK3, AtMPK4 and AtMPK6 as kinases. However, we could not isolate positive clones from such assays, although high amounts of MPKs (up to 10 $\mu\text{g ml}^{-1}$) were used (data not shown). Thereafter, we used a hyperactive kinase, recombinant OsBWMK1, as the probe, owing to its more than 100-fold higher kinase activity compared to AtMPK3, AtMPK4 and AtMPK6 (data not shown). Furthermore,

OsBWMK1 has been shown to exhibit strong kinase activity without activation by an upstream kinase (Cheong et al. 2003). By the solid-phase phosphorylation screening with OsBWMK1 as the kinase, 130 positive clones were isolated from 1×10^5 clones (Fig. 1). Among these 130 clones, we rescued 29 clones containing correct open reading frames in expression plasmids by sequencing. The fusion proteins were expressed in *E. coli* and purified by Glutathione S-transferase affinity chromatography. The phosphorylation of isolated fusion proteins by OsBWMK1 were verified by in vitro kinase assay (Fig. 1). As a result, 29 clones were isolated as new putative substrates of MAP kinase (Table 1). When we compared these isolates with entries in databases, 18 clones had previously identified as putative kinase substrates in the Plant Protein Phosphorylation DataBase (PPPDB; <http://www.p3db.org>), although information about which kinases phosphorylates them does not exist. The remaining 11 isolates represented novel MPK substrates (Table 1).

Confirmation of the phosphorylation of putative substrates by AtMPKs

To test the ability of putative substrates of OsBWMK1 identified by library screening to become phosphorylated by *Arabidopsis* MPKs, in vitro kinase assays with AtMPK3, AtMPK4, and AtMPK6 were carried out

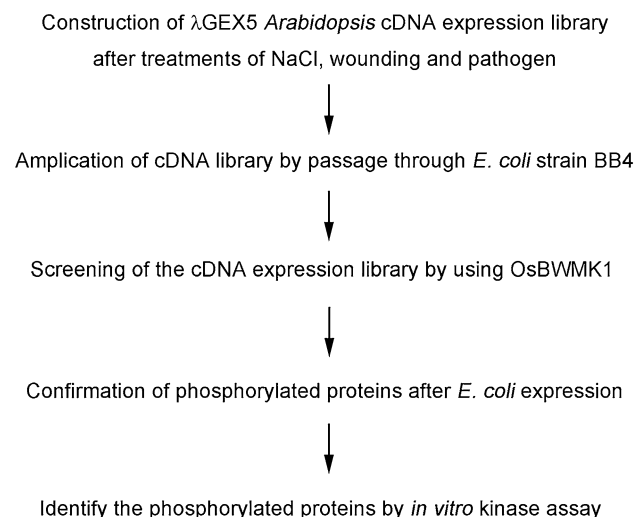


Fig. 1 Scheme for screening of MPK substrates from an *Arabidopsis* cDNA expression library by solid-phase phosphorylation. A λ phage cDNA expression library was constructed from combined mRNAs from salt-treated, pathogen-treated and mechanically wounded *Arabidopsis* seedlings. The *Arabidopsis* cDNA library was plated on *E. coli* BB4 at a density of 1.5×10^4 plaques per 150 mm agar plate. OsBWMK1, a rice blast- and wound-induced MAP kinase, was used for cDNA expression library screening. Recombinant GST-tagged MPKs were used to confirm the phosphorylation of the recombinant putative substrate proteins

Table 1 Potential AtMPK substrate proteins identified by solid-phase phosphorylation screening

No.	Blast-AGI	Protein name	Putative molecular function
1*	At1g09840	Shaggy-like protein kinase 41	Shaggy-like Ser/Thr protein kinase
2*	At1g11310	MLO2	Seven transmembrane MLO family protein
3	At1g14690	MAP65-7	Microtubule binding
4	At1g18840	IQD30	IQ domain calmodulin binding protein
5*	At1g21630	Calcium-binding EF hand family protein	Calcium ion binding protein
6	At1g27330	RAMP4	Ribosome-associated membrane protein
7*	At1g30470	SIT4 phosphatase-associated family protein	Unknown protein
8*	At1g51510	RNA-binding protein	RNA-binding protein
9	At1g74160	Unknown protein	Unknown protein
10*	At1g80180	Unknown protein	MAP kinase substrate
11*	At2g20950	Phospholipase-like protein (PEARLI 4)	Phospholipase-like protein
12*	At2g33830	Dormancy/auxin-associated family protein	Unknown protein
13	At2g41430	ERD15	Early responsive to dehydration
14*	At2g41900	CCCH-type zinc finger family protein	Nucleic acid binding/transcription regulation
15*	At2g46020	Transcription regulatory protein SNF2	SWI/SNF chromatin remodeling ATPase
16*	At3g03790	Ankyrin repeat family protein	Regulator of chromosome condensation protein
17	At3g16770	ERF72	Ethylene responsive transcription factor
18	At3g41950	Unknown protein	Unknown protein
19*	At3g43590	CCHC-type zinc knuckle family protein	Nucleic acid binding protein
20*	At3g57150	Homologue of NAP57	Pseudouridine synthase
21	At4g09320	NDPK1	Nucleoside diphosphate kinase
22*	At4g15545	Unknown protein	Unknown protein
23*	At4g32610	Unknown protein	Copper ion binding protein
24	At5g16750	TOZ (Tormozembryo defective)	WD-40 repeat family protein
25*	At5g22650	Histone deacetylase 2B	Histone deacetylase
26*	At5g27120	Pre-RNA processing ribonucleoprotein	RNA-binding protein
27	At5g27950	Nucleoside triphosphate hydrolases	ATP binding and microtubule motor activity
28*	At5g52310	Desiccation-responsive protein 29A (RD29A)	Cold and desiccation responsive protein
29	At5g63310	Nucleoside diphosphate kinase 2	Nucleoside diphosphate kinase

* These proteins are present in plant protein phosphorylation data base (<http://www.p3db.org>)

(Fig. 2a–c). Ten of 29 identified substrates of OsBWMK1 were selected including an RNA-binding protein (At1g51510), a member of the dormancy/auxin-associated family proteins (At2g33830), the EARLY RESPONSIVE TO DEHYDRATION15 (ERD15; At2g41430), AtERF72 (At3g16770), an unknown protein (At4g15545), MLO2 (At1g11310), the calcium-binding EF hand family protein (At1g21630), SIT4 phosphatase-associated family protein (At1g30470), a zinc finger (CCCH-type) family protein (At2g41900) and RD29A (At5g52310). They were expressed in bacteria and purified as GST-fused full length proteins. GST-fused MAP kinase substrate 1 (MKS1), which acts as a substrate of AtMPK4 (Andreasson et al. 2005), was used as positive control. The in vitro kinase assays showed that all tested proteins were phosphorylated by AtMPK3, AtMPK4 and AtMPK6 (Fig. 2). The

phosphorylation signal of seven of ten proteins by three AtMPKs was show higher than that observed with MKS1 (lane 2), strongly suggesting they could be authentic substrates of AtMPKs with high affinity.

Phosphorylation of putative substrates by endogenous AtMPKs

Next, we tested whether the putative substrates could be phosphorylated by plant AtMPKs by in-gel kinase assays. AtMPKs in wild type (WT) and MPK6 mutant (*mpk6*) plants were activated by the treatments of wounding or oxidative stress (H₂O₂). The activities of AtMPKs were measured by phosphorylation of MBP in a gel, a well-known general MPK substrate (Fig. 3). As a negative control, GST protein was used in assays. As previously reported, the activity of AtMPK6 is

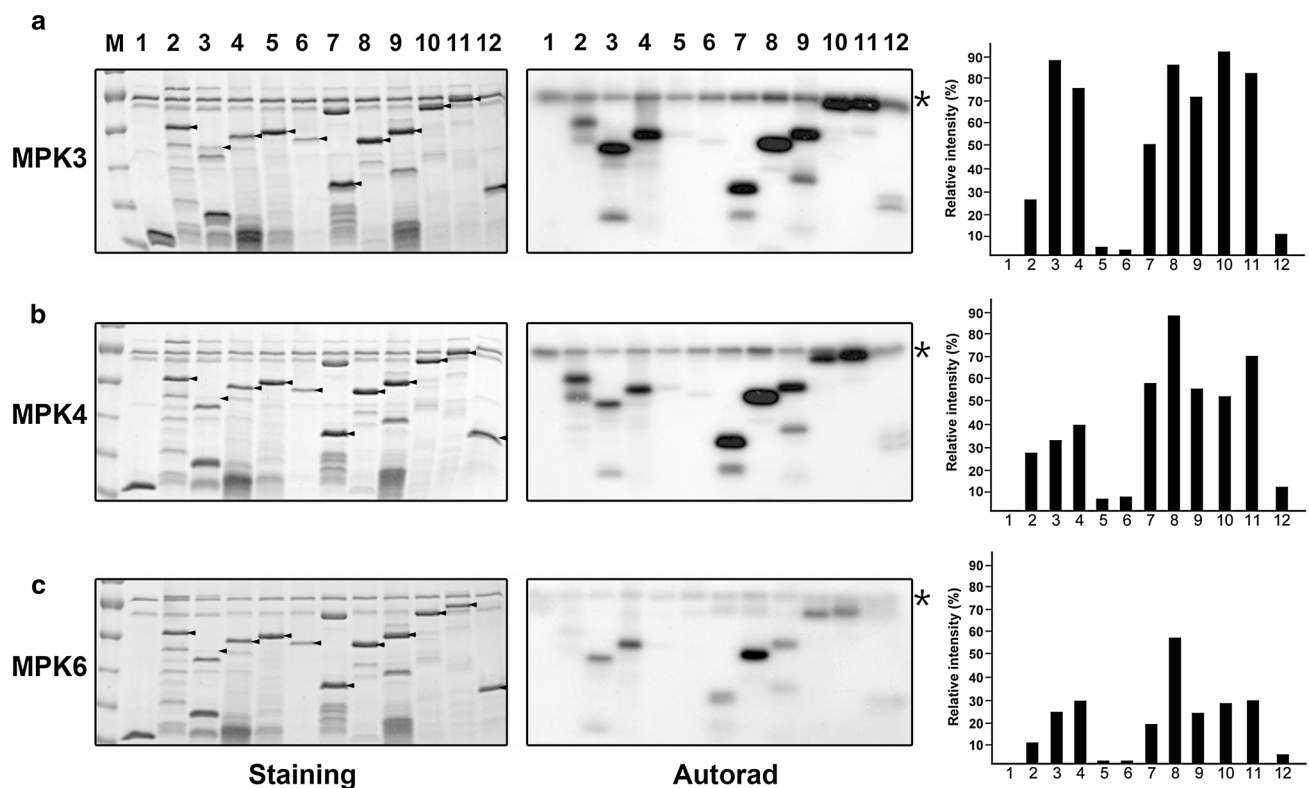


Fig. 2 Verification of potential AtMPK substrates. Phosphorylation of potential substrates by purified recombinant MPK3 (a), MPK4 (b) and MPK6 (c). Activated MPK3, MPK4 and MPK6 (500 ng) were used to phosphorylate the substrate proteins (1 μ g) in the presence of [γ - 32 P]ATP. After electrophoresis, proteins were visualized by Coomassie blue (staining), and phosphorylated proteins were visualized by autoradiography (autorad). The positions of putative substrates are indicated by an *arrowhead*. The positions of the autophosphorylated MPKs are indicated by an *asterisk*. The purified substrate

proteins used in each lane were: GST (*lane 1*, negative control), MKS1 (*lane 2*, positive control), At1g11310 (*lane 3*), At1g21630 (*lane 4*), At1g30470 (*lane 5*), At1g51510 (*lane 6*), At2g33830 (*lane 7*), At2g41430 (*lane 8*), At2g41900 (*lane 9*), At3g16770 (*lane 10*), At4g15545 (*lane 11*), and At5g52310 (*lane 12*). *Graphs in the right panel* indicate relative intensities of the [32 P] phosphoprotein bands in each lane as measured by scanning densitometry of the autoradiogram. Band intensities are expressed relative to that of phosphorylated GST. M, pre-stained protein markers

rapidly and strongly induced with the treatments of wounding or H₂O₂ (Ichimura et al. 2000; Kovtun et al. 2000). The activity of AtMPK3 was very weak in WT but significantly increased in *mpk6* plants. The reason could be attributed to compensation of AtMPK6 functions in *mpk6* plants (Menke et al. 2004). Three of the chosen substrates (At2g41430, At2g41900, and At3g16770) were strongly phosphorylated by AtMPK3 and AtMPK6. However, weak phosphorylation was observed with four substrates (At1g11310, At1g21630, At2g33830, and At4g15545), but this is possibly an effect of incomplete refolding in the reaction solution after denaturation by SDS-PAGE, although repeat experiments were carried out under optimized condition (Fig. 3).

In another study, AtMPK3 and AtMPK6 substrates show similarity in their substrates, by phosphorylating the conserved protein motif (L/P-P/X-S-P-R/K) (Sörensson et al. 2012). In addition, we compared the similarity of substrates used to substrates reported from references (Fig. 4). The results indicated that 22 of 29 substrates represented novel

substrates suggesting that the method could provide a useful tool for isolating novel substrates of MPKs in plants.

Discussion

The existence of a large family of MPKs with overlapping as well as unique substrate specificities has complicated delineation of MPK signaling pathways in plants. Although a large number of putative *Arabidopsis* MPK substrates have been identified using high-throughput protein microarray methods, the majority of them have neither been confirmed as AtMPK substrates in planta nor characterized further (Feilner et al. 2005; Popescu et al. 2009). In addition, the MPK3 and MPK6 specifically phosphorylate their substrates on specific serine residues. However, mutations in these serine residues abolish the activities of MPK, which renders them unable to phosphorylate their target substrate (Park et al. 2011).

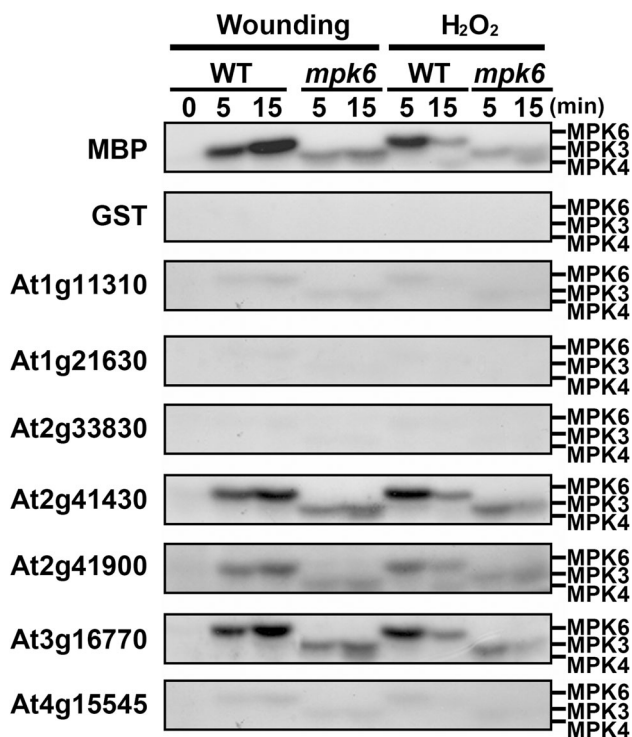


Fig. 3 Phosphorylation of putative substrates by endogenous AtMPKs. Proteins were extracted from WT and *mpk6* seedlings that had received wounding or H₂O₂ treatments (1 mM) for the indicated time periods. MPK activities were monitored with an in-gel kinase assay using substrates that were embedded at 1 mg/ml in the running gel. MBP and GST are shown as positive and negative control substrates, respectively. The positions of MPK3, MPK4, and MPK6 on the gel are indicated

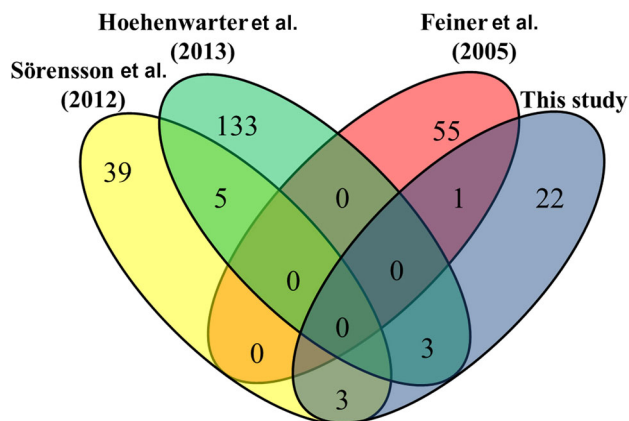


Fig. 4 Venn diagram analysis of MPK substrates identified in this study with substrates from other individual studies. The overlap segments show similar substrates in *Arabidopsis*. Different groups share similar substrates in respective studies

Our screening method differed from other approaches because it facilitated the necessary tests providing detailed characterization. We used a phage *Arabidopsis* cDNA expression library constructed in λ GEX5 vector to screen for MPK substrates. The advantages offered by the λ GEX5

vector for solid-phase phosphorylation screening by the method of Fukunaga and Hunter (1997) are: (1) isolated cDNA clones can be used directly for expression, purification and characterization of the putative substrates identified in the screen, and (2) the GST expression vector can reduce the noise of background phosphorylation. Therefore, the method facilitates identification of physiological substrates for a variety of MPK kinases in plants.

Significantly also, several studies show agreement with our findings (Feilner et al. 2005; Sörensen et al. 2012; Hoehenwarter et al. 2013). By comparing the substrates it was found that three substrates were identical to those found by Hoehenwarter et al. (2013) and Sörensen et al. (2012) and one substrate was similar to one identified by Feilner et al. (2005) (Fig. 4). In the current study, 22 of 29 substrates were identified as novel putative substrates of rice BWMK1 (Table 1).

Among the putative substrates identified in the first round of screening, 10 putative substrates were selected to study phosphorylation activities using AtMPK3, AtMPK4, and AtMPK6 as probes. Seven identified substrates were more strongly phosphorylated than MKS1 by AtMPK3, AtMPK4 and AtMPK6, indicating that they could be highly suitable substrates of AtMPKs (Fig. 2). In addition, an excellent example for the suitability of our screening method is the identification of the microtubule-associated protein 65-7 (MAP65-7) as a substrate in *Arabidopsis* (Table 1, number 3). Sasabe et al. (2006) reported that phosphorylation of NtMAP65-1, a homolog of AtMAP65-7 in tobacco plants, by an MAP kinase down-regulates its activity of microtubule bundling and stimulates the progression of cytokinesis. The results illustrate the value of the cDNA expression library for identifying putative MPK substrates.

Interestingly, nucleotide diphosphate (NDP) kinase 1 (NDPK1) and NDPK2 were obtained by the solid-phase phosphorylation screening method (Table 1, number 21 and 29). *Arabidopsis* NDPK2 was expressed by H₂O₂ treatment and overexpression of AtNDPK2 showed tolerance to multiple stresses such as cold, salt, and oxidative stress (Moon et al. 2003). In addition, AtNDPK2 interacted and activated AtMPK3 and AtMPK6 by phosphorylation (Moon et al. 2003). These data imply that NDPKs and MPKs might function equally or jointly in various plant signaling pathways.

Based on our screening protocol, we have identified several transcription factors as well as transcriptional regulators as potential AtMPKs substrates. Studies in mammalian systems have shown that phosphorylation of transcription factors by MPKs can alter their activities, localizations, and stabilities (Raman et al. 2007; Turjanski et al. 2007). Indeed, several lines of evidence suggest that transcription factors are also major targets of MPKs in *Arabidopsis*. Firstly, it has been reported that AtMPKs may

phosphorylate transcription factors or transcription-related proteins (Asai et al. 2002; Ichimura et al. 2002; Hoang et al. 2012). Secondly, transcription factors involved in development and stress responses were represented as substrates of AtMPK identified by high-throughput protein microarray approaches (Feilner et al. 2005; Popescu et al. 2009). In agreement with these findings, >30% of the putative MPK substrates identified in our screen represented transcription-related proteins, implying MPK-based phosphorylation of many *Arabidopsis* transcription factors. Finally, further biological and genetic analysis of the phosphorylation of the novel substrates identified in our study (Table 1) will be necessary to extend our understanding of the role of MPK signaling pathways on the integration of plant responses to various external and internal cues in plants.

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