MAPKs (mitogen-activated protein kinases) are signalling components highly conserved among eukaryotes. Their diverse biological functions include cellular differentiation and responses to different extracellular stress stimuli. Although some substrates of MAPKs have been identified in plants, no information is available about whether amino acids in the primary sequence other than proline-directed phosphorylation (pS-P) contribute to kinase specificity towards substrates. In the present study, we used a random positional peptide library to search for consensus phosphorylation sequences for Arabidopsis MAPKs MPK3 and MPK6. These experiments indicated a preference towards the sequence L/P-P/X-P/K/R for both kinases. After bioinformatic processing, a number of novel candidate MAPK substrates were predicted and subsequently confirmed by in vitro kinase assays using bacterially expressed native Arabidopsis proteins as substrates. MPK3 and MPK6 phosphorylated all proteins tested more efficiently than did another MAPK, MPK4. These results indicate that the amino acid residues in the primary sequence surrounding the phosphorylation site of Arabidopsis MAPK substrates can contribute to MAPK specificity. Further characterization of one of these new substrates confirmed that At1g80180.1 was phosphorylated in planta in a MAPK-dependent manner. Phenotypic analyses of Arabidopsis expressing phosphorylation site mutant forms of At1g80180.1 showed clustered stomata and higher stomatal index in cotyledons expressing the phosphomimetic form of At1g80180.1, providing a link between this new MAPK substrate and the defined role for MPK3 and MPK6 in stomatal patterning.

Key words: Arabidopsis, At1g13390.1, At1g80180.1, At2g14850.1, At3g16770.1, mitogen-activated protein kinase substrate (MAPK substrate), random positional scanning synthetic combinatorial peptide library, stoma.

INTRODUCTION

Protein phosphorylation is one of the most common post-translational modifications in eukaryotic organisms and is involved in almost all cell biological processes. The phosphorylation of serine, threonine and tyrosine residues can affect protein structure, enzymatic activity and subcellular localization, as well as interaction with other proteins. In eukaryotes, MAPK (mitogen-activated protein kinase) pathways transduce extracellular stimuli perceived by cell-surface receptors into cellular responses [1]. The core MAPK pathway is evolutionarily conserved and consists of a three-component cascade of kinases that activate the next kinase by phosphorylation. The most upstream component in this conserved MAPK cascade is a MAP3K (MAPK kinase kinase) that is a serine/threonine protein kinase which phosphorylates MAP2Ks (MAPK kinases). The dual-specificity MAP2Ks phosphorylate MAPKs on both serine/threonine and tyrosine residues in the activation loop [2]. Once activated, MAPKs phosphorylate many evolutionarily diverged substrates on serine or threonine residues within a minimal S/T-P motif [3,4]. Specificity determinants of MAPK signalling can involve several different mechanisms. Proteins may function as scaffolds to direct the kinase signal to a more specific response as described in mammalian systems [5], a mechanism which has not yet been described in plants. Other mechanisms mediating specificity include the spatially and temporally co-ordinated expression of substrate and kinase, protein docking domains and kinase specificity involving residues surrounding the phosphorylation site.

MAPKs play an important role in the adaptations of plants to changes in their environment and are important intermediates in diverse stress and developmental signalling pathways. These responses include abiotic stresses such as low temperature, humidity, wounding and ROS (reactive oxygen species) and biotic stresses such as recognition of PAMPs (pathogen-associated molecular patterns) such as flg22 (flagellin) [6–9]. In Arabidopsis, there are 20 MAPKs, of which MPK3, MPK4 and MPK6 have been the kinases of primary interest as they are activated by many of these diverse biotic and abiotic stimuli [10]. In addition, MPK3 and MPK6 have been shown to play important roles in plant development, such as during regulation of stomatal patterning [11].

Understanding how MPK3 and MPK6 are involved in these important, but diverse, biological responses requires the identification of the downstream substrates. However, in contrast with the MAPKs themselves, the substrates of these kinases are not evolutionarily conserved throughout eukaryotes.
making it difficult to identify substrates by simple homology analysis, and only a few bona fide MAPK substrates in plants have been identified. MPK3 and MPK6 are involved in ethylene signalling via phosphorylation of EIN3 (ethylene-insensitive 3), a transcription factor mediating ethylene responses [12,13], and phosphorylation of ACS6 (aminocyclopropane-1-carboxylic acid synthase 6) [14,15], the rate-limiting enzyme of ethylene biosynthesis. In addition, MPK6 phosphorylates an ERF (ethylene-response factor), ERF104 [16] that, like ACS6, is phosphorylated on a serine residue within a P-X-S-P motif. A transcription factor involved in stomatal patterning, SPCH (SPEECHLESS), is also an MPK3 and MPK6 substrate [17].

An additional substrate phosphorylated in response to biotic stress, AtPHOS32, is a member of the USP (universal stress protein) family [18]. This protein contains an evolutionarily conserved motif surrounding the only MAPK-phosphorylated serine residue. In addition to these substrates arising from targeted studies and the evolutionarily conserved phosphorylation sites, we derived a list of potential novel substrates from Arabidopsis. We confirmed that five of the candidates are indeed substrates of MPK3 and MPK6. Therefore our results indicate that the amino acids surrounding the phosphorylated serine residue contribute to MAPK specificity. In addition, one of these new substrates, At1g80180.1, was found to influence stomatal patterning, a developmental programme regulated by MPK3 and MPK6 [11,17].

EXPERIMENTAL

PS-SCL (positional scanning synthetic combinatorial peptide library) assay

HsS-tagged MPK3 and MPK6 in pDEST17 (Invitrogen) were affinity-purified from Escherichia coli strain BL21 Rosetta-gami™ (Novagen). Before the assay, the kinases were activated by incubation in activation buffer [50 mM Tris/HCl, 150 mM NaCl, 5 mM MnCl2, 10% glycerol, 1 mM DTT] at 30 °C for 20 min. The PS-SCL used was synthesized as described in [21] and consisted of 162 peptide mixtures, each with one of 18 amino acids fixed on one of nine positions in relation to the central phosphorylation residue (serine). The remaining eight positions were degenerate. The peptides were also provided with a biotin tag, to enable binding to a biotin capture membrane, a linker and charged amino acid residues for increased solubility. The kinase assays were performed mainly as described previously [21,22]: each peptide mixture from the library was used as a substrate in a 20 μl reaction volume in one well of a 384-well polypropylene plate. The reactions were composed of 25−35 ng of kinase, 100−150 μM ATP and 0.05 μCi/μl [γ-32P]ATP in 18 μl of kinase buffer (20 mM Hepes, pH 7.5, 0.5 mM EDTA, 0.5 mM DTT, 5 mM MgCl2 and 0.1% Tween 20). The library peptide substrate was added as 2 μl of 0.5 mM aqueous peptide mixture to each reaction. The 0.5 mM aqueous peptide mixtures were prepared by diluting a 12.5 mM DMSO stock solution in 20 mM HEPES (pH 7.4). The plates were covered with adhesive tape and incubated in a water bath at 30 °C for 2 h. After incubation, the plates were chilled on ice and 2 μl of each reaction were spotted out on a SAM® Biotin Capture Membrane (Promega) with a pin tool replicator (VP Scientific). The membrane was subsequently washed twice in 0.1% SDS/TBS (Tris-buffered saline: 25 mM Tris/HCl, 150 mM NaCl and 2 mM KCl, pH 7.4), twice in 2 M NaCl, three times in 2 M NaCl/1% phosphoric acid, and twice in distilled water. After letting the membrane air-dry, it was wrapped in plastic and exposed to a phosphor storage screen for 16−17 h. The radioactive signal was detected in a Molecular Imager FX (Bio-Rad Laboratories) and signal strengths were quantified using Quantity One, version 4.6.8 (Bio-Rad Laboratories). To assess the relative importance of a certain amino acid at a certain position in relation to the phosphorylation site, the signal of each sample spot was normalized to all signals at that specific position. The calculations were made as described by Hutti et al. [23]:

$$Z_{ca} = \frac{S_m}{\sum_i S_i}$$

where $Z_{ca}$ is the normalized value of amino acid a at position c, $S_m$ is the signalling score, m is the total number of amino acids, and $\sum_i S_i$ is the sum of signalling scores from all amino acids at a certain position. Spots that gave a 2-fold or higher signal, compared with the other amino acids at that position, were considered to be preferred in the context of consensus sequence recognition.

In vitro protein kinase assay

The Gateway pENTR-D-TOPO (Invitrogen) vector was used to clone the full-length cDNAs from Arabidopsis thaliana ecotype Columbia, which were verified by sequencing. These constructs were then used for site-directed mutagenesis of serine to alanine and aspartic acid using standard methods. A previous protocol [22,24] was modified to immunoprecipitate MPK3 and MPK6 from 50 ml of uninduced and flg22-induced [18] (1 μM flg22 for 10 min) 4-day-old green Arabidopsis thaliana ecotype Landsberg erecta cell cultures grown in 1× Murashige and Skoog basal salt (pH 5.7, adjusted with KOH), 1× Gamborg’s B5 vitamin mixture, 3% sucrose, 0.5 mg/l NAA (naphthaleneacetic acid) and 0.05 mg/l BAP (1,6-benzylaminopurine). Cells were pelleted by centrifugation at 16000 g for 1 min. In the following procedures, one-fifth of the cells were used. The proteins were extracted by sonication in a water bath in extraction buffer (100 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 15 mM EGTA, 5 mM EDTA, 0.5% polyvinylpyrrolidone, 1% Triton X-100, 50 mM sodium pyrophosphate, 25 mM glycerophosphate, 25 mM sodium fluoride, 1 mM sodium molybdate, 1 mM PMSE, 10 μM leupeptin, 1 mM calyculin A and 1 mM Na3VO4) and were frozen in liquid nitrogen. This was repeated twice and the homogenate was incubated for 5 min with rotation at 4 °C. The sample was then centrifuged at 16000 g for 10 min. MPK3 and MPK6 were immunoprecipitated by specific antibodies described in [18] at 4 °C for 2 h followed by addition of 50 μl of a 50% Protein A-conjugated agarose slurry mixture, with 2 h of additional incubation. The precipitate was washed twice in 5 ml of extraction buffer including incubation for 10 min, followed by two washes in 1 ml of extraction buffer. A final wash was carried out in 1 ml of kinase buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM MgCl2, 100 μM Na3VO4 and 1 mM DTT). The immunoprecipitated kinases were left in 200 μl of kinase buffer. From the kinase slurry, 10 μl was mixed...
with 8 μl of 5× kinase buffer, 0.4 μl of 1 mM ATP, 0.2 μl of [γ-32P]ATP (GE Healthcare), 20 μl of His6-tagged protein and water to a final volume of 40 μl. Incubation with shaking was carried out at 30 °C for 15 min. The reaction was terminated by addition of 8 μl of 6× SDS buffer and heating to 65 °C for 10 min with shaking, followed by SDS/PAGE, CBB (Coomassie Brilliant Blue) staining and destaining in 20% methanol, 10% acetic acid and 7% glycerol. Then the gel was dried and the incorporation of radiolabelled ATP was detected by placing a phosphor storage screen on the dried gel for 24 h and analysis by scanning.

**Nicotiana benthamiana transient expression**

*N. benthamiana* plants were grown at 22 °C with 16 h light (90 μmol·m−2·s−1)/8 h dark in a controlled environment chamber for 4 weeks. *Agrobacterium tumefaciens* strain GV3101 containing pGGW44-At1g80180.1-HA [25] with and without pGreen0229-SIMEK100 [26] were grown in 4 ml of LB (Luria–Bertani) with appropriate antibiotics [27]. After 2 days, 25 ml of LB with appropriate antibiotics was inoculated with 50 μl of the dense culture and grown to a ΔD600 of 1. The cells were pelleted at 3500 g for 10 min and washed twice in 10 mM Mes (pH 5.6), 10 mM CaCl2, and 100 μM acetoxyerin and resuspended to a ΔD600 of 0.5. The bacteria was incubated for 4 h at 28 °C before agroinfiltration of *N. benthamiana* leaves [28].

**Plant protein extraction**

At 2 days after infiltration, six leaf discs (8 mm in diameter) were ground in 50 mM Mops/KOH (pH 7.5), 5 mM EDTA, 5% glycerol, 5 mM DTT, 5 mM ascorbic acid, 0.5% polyvinylpolypyrrolidone, 0.5% Nonidet P40, 25 mM glycerol phosphate, 1 mM Na2VO3, 25 mM sodium fluoride, 50 mM sodium pyrophosphate, 1 mM sodium molybdate, 1 mM PMSF, 10 μM leupeptin and 1 mM calyculin A with sand in a pre-chilled mortar. The homogenate was centrifuged twice at 16000 g for 10 min at 4 °C.

**Phosphatase treatment**

Proteins were extracted as described above but phosphatase inhibitors (glycerol phosphate, Na2VO3, sodium fluoride, sodium pyrophosphate, sodium molybdate, EDTA and calyulin A) were excluded from the buffer. Dephosphorylation of proteins in extracts from *N. benthamiana* was performed by treatment of lambda protein phosphatase (New England Biolabs) for 30 min at 30 °C and the reaction was stopped by addition of 6× SDS sample loading buffer and heating to 65 °C with shaking for 10 min.

**Protein gel immunoblotting**

Total protein extracts and dephosphorylated extracts were run on SDS/PAGE gels (Tris/Glycine) followed by transfer on to Hybond nitrocellulose membranes (GE Healthcare). Immunoblotting was carried out with mouse anti-HA (haemagglutinin) monoclonal antibody (Roche) and horseradish-peroxidase-conjugated goat anti-mouse secondary antibody (Pierce). Protein detection was carried out using Super Signal West Femto Maximum Sensitivity Substrate (Pierce). The protein bands were detected on a CL-XPosure Film Clear Blue X-Ray Film (Pierce).

**Arabidopsis material and transformations**

*Arabidopsis thaliana* ecotype Columbia-0 was used in all experiments. HA-containing pGGW44 vector with the 3SS promoter was used to make At1g80180.1-HA N-terminally tagged constructs. *A. tumefaciens* GV3101 containing pSOUP were used to transform pGGW44-At1g80180.1 (S105D/A) [29–31]. T1 transformants were grown at 22 °C with 16 h light (90 μmol·m−2·s−1)/8 h dark in a controlled environment chamber for 10 days. Selection was done with Basta spraying (Bayer). Epidermal peels of cotyledons of 2-week-old seedlings were analysed using ordinary light microscopy. Reporter–GUS (β-glucuronidase) cloning was performed with a 1672 bp region upstream of the At1g80180.1 coding sequence. The promoterless GUS-containing pGGW35 destination vector was used in Invitrogen Gateway cloning. X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) colorimetric stain buffer for GUS activity containing 100 mM sodium phosphate (pH 7), 1 mM EDTA, 1% Triton X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide and 2 mg/ml X-gluc was vacuum-infiltrated for 3 min into the tissue, incubated in the dark at 37 °C overnight and subsequently treated with 70% ethanol to remove chlorophyll.

**RESULTS**

**Determining MPK3 and MPK6 substrate specificities using a positional scanning peptide library**

In our previous study [18], we found that the primary sequence surrounding the phosphorylation site of AtPHOS32 appeared to be highly conserved throughout evolution. To understand whether conservation of local residues surrounding the phosphorylation site may contribute to enzymatic specificity of MPK3 and MPK6 towards potential substrates, we used a random positional scanning peptide library assay [21]. The random library consisted of 162 mixtures of sixteen amino acid long peptides where one of the 18 naturally occurring proteogenic amino acids (all except methionine and cysteine) was fixed at each of nine positions surrounding a central serine residue (Supplementary Figure S1 at http://www.BiochemJ.org/bj/446/bj4460271add.htm). In the degenerated positions, 15 of the 20 amino acids (methionine, cysteine, threonine, serine and arginine were excluded) were incorporated competitively such that all residues were equally represented. Tagged MPK3 and MPK6 were expressed and affinity-purified from *E. coli*. Activated kinase was incubated with the peptide library, and the incorporation of radiolabelled ATP into each peptide mixture was imaged by autoradiography (Figure 1A). The radioactive signals were quantified and normalized to the average value for all amino acids at the same position. A threshold value of 1.5 times the average radioactive signal indicated preferential phosphorylation of the peptide. This assay was repeated twice with each kinase with similar results (Supplementary Figure S2 at http://www.BiochemJ.org/bj/446/bj4460271add.htm). From these results, we concluded that both kinases preferentially phosphorylated the core sequence L/P-P/X-S-P-R/K (Figure 1B). These data are consistent with a mammalian minimal MAPK substrate consensus motif of P-X-S-P. The inclusion of leucine in the −2 position as well as the positively charged residues, arginine or lysine, in the +2 position, represents novel additional information regarding potential MPK3 and MPK6 preference.

**In vitro MPK3 and MPK6 substrates**

The empirical data from the positional scanning peptide library assay indicated that the sequence motif L/P-P/X-S-P-R/K may reflect a primary sequence recognized by MPK3

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were phosphorylated within the motifs P-P-R motif to an aspartic acid or an alanine and used the mutated proteins for in vitro kinase assays. In all cases except for At1g13390.1, mutating the serine to alanine abolished phosphorylation by MPK3 and MPK6 as opposed to MPK4 (Figure 2). Interestingly, MPK3 and MPK6 phosphorylated all candidate proteins more efficiently than did MPK4, even though all three kinases phosphorylated the general kinase substrate MBP (myelin basic protein) to a similar extent. As a further control, the MPK4 substrate MKS1 (MAPK substrate 1) [36] was included in these assays; and MKS1 was phosphorylated preferentially by MPK4 rather than by MPK3/MPK6. Collectively, these results indicate that substrate specificity exists for these MAPKs. Because the MPK3/MPK6 substrates examined in these experiments share similarity only within the motif of the predicted phosphorylated region, the short amino acid sequence surrounding the phosphorylation site appears to be important as a specificity-determinant for MPK3 and MPK6 substrates.

Consistent with this hypothesis, MKS1 has been shown to be a specificity-determinant for MPK3 and MPK6 substrates.

Confirmation of the phosphorylation site

To confirm that MAPK phosphorylation of the candidate proteins occurs at the predicted serine residue, we mutated the serine in the P-P-S-P-R motif to an aspartic acid or an alanine and used the mutated proteins for in vitro kinase assays. Figure 3 shows in vitro kinase assays using activated Arabidopsis MPK6 with wild-type and mutated At3g16770.1, At1g13390.1, At2g14850.1 and At1g80180.1. In all cases except for At1g13390.1, mutating the single target serine residue abolished phosphorylation by MPK6. At1g13390.1 contains an additional P-S-T-P-R motif, and when the double mutant of both P-S-S-P-R and P-S-T-P-R was used (Figure 3), phosphorylation by MPK6 was also abolished. Similar results were obtained using MPK3 (not shown). The
In plant phosphorylation of At1g80180.1 during MAPK activation

Further support for the validity of the in vitro results comes from the fact that At1g80180.1 has been found to be phosphorylated in vivo on the predicted serine residue in large-scale phosphoproteomic studies [38,39]. To examine whether the phosphorylation of At1g80180.1 occurs in planta in a MAPK-dependent manner, we used transient expression in N. benthamiana. Detection of At1g80180.1 was facilitated by expressing the protein with an HA tag. In this assay, the constitutively active MAP2K StMEK1DD was used to activate the MKP3 and MKP6 orthologues SIPK (salicylic acid-induced protein kinase) and WIPK (wound-induced protein kinase) [26,40]. The immunodetected At1g80180.1 migrated at a higher apparent molecular mass when co-infiltrated with the autoactivated StMEK1DD (Figure 4, left-hand panel). To verify that the upper band was indeed the phosphorylated form, extracts from plants expressing At1g80180.1 and MEK1DD were treated with lambda phosphatase. This treatment caused the higher-molecular-mass form to shift to a lower apparent molecular mass (Figure 4, right-hand panel). The lower panel in Figure 4 shows CBB staining as a control for migration in the gel. These findings indicate that the protein encoded by At1g80180.1 is an in planta substrate of MAPKs related to MKP3 and MKP6.

Phosphorylated At1g80180.1 influences stomatal patterning

Because At1g80180.1 was known to be phosphorylated in vivo on the predicted site and the transient expression results suggest that this in planta phosphorylation is MKP3/MKP6-dependent, we examined the potential role of At1g80180.1 in MKP3/MKP6-dependent responses. While generating transgenic plants, we noticed that seedlings of the phosphomimetic mutants died with a high frequency (for S105D, 31 out of 60 independent lines, and for S105A, three out of 58) at an early developmental stage similar to other MAPK-related stomatal clustering mutants such as plants expressing SPCH without its MAPK phosphorylation domain [17] and the double mpk3mpk6 mutants [11]. Therefore cotyledons of Arabidopsis T1 transformants expressing either unphosphorylatable (S105A) or phosphomimetic (S105D) mutants of At1g80180.1 were examined early for changes in stomatal patterning. Cotyledons of Arabidopsis plants that later died and expressing phosphomimetic At1g80180.1 consistently showed clusters of stomata, escaping the typical one-cell-spacing rule (Figure 5A). Quantification of epidermal cells in two independent transformants of Arabidopsis expressing the phosphomimetic form of At1g80180.1 gave a stomatal index (number of stomata per number of pavement cells) of 1.3 compared with 0.46 in wild-type (Figure 5B, >100 cells counted for each line). Arabidopsis expressing unphosphorylatable At1g80180.1 had a stomatal index of 0.59, not significantly different from the wild-type (Student’s t test, \( P = 0.43 \)). These results indicate that phosphorylation of At1g80180.1 positively

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**Figure 2 Radiographs of 32P incorporation into bacterially expressed proteins by MPK3, MPK4 and MKP6 purified from flag22-induced (+ flag22) or non-induced (-flag22) Arabidopsis Ler cells**

MKS1 is an MKP4 substrate. The activity of the kinases was confirmed using MBP as a substrate. The six upper proteins were preferentially phosphorylated by MKP3 and MKP6. No phosphorylation was detected by uninduced MPK3.

**Figure 3 In vitro kinase assays with wild-type and mutated proteins**

Upper panel: radiographs of 32P incorporation into bacterially expressed proteins by Arabidopsis MKP6. Phosphorylation of wild-type (wt) proteins was detected. No phosphorylation was detected in the proteins where serine was mutated to aspartic acid (S99D and S137D) or alanine (S105A). In At1g13390.1 an additional amino acid residue (threonine) was mutated to aspartic acid (T126D) to prevent phosphorylation. Lower panel: the abolishment of phosphorylation in the mutated proteins confirms that the predicted serine residue is targeted by the MAPKs. Importantly, none of the proteins were phosphorylated by MPK3 or MKP6 at any site other than the predicted serine residue, even though none of these proteins contained other minimal S/T-P MAPK motifs (V-M-T-P-P, T-G-S-P-P and V-A-S-P-S-P-S in At1g13390.1; R-V-S-P-A in At1g80180.1; R-P-S-P-L, T-G-S-S-P-S-R-S-P-P-L and A-K-S-S-P-P in At2g14850.1) and for S105A, three out of 58) at an early developmental stage similar to other MAPK-related stomatal clustering mutants such as plants expressing SPCH without its MAPK phosphorylation domain [17] and the double mpk3mpk6 mutants [11]. Therefore cotyledons of Arabidopsis T1 transformants expressing either unphosphorylatable (S105A) or phosphomimetic (S105D) mutants of At1g80180.1 were examined early for changes in stomatal patterning. Cotyledons of Arabidopsis plants that later died and expressing phosphomimetic At1g80180.1 consistently showed clusters of stomata, escaping the typical one-cell-spacing rule (Figure 5A). Quantification of epidermal cells in two independent transformants of Arabidopsis expressing the phosphomimetic form of At1g80180.1 gave a stomatal index (number of stomata per number of pavement cells) of 1.3 compared with 0.46 in wild-type (Figure 5B, >100 cells counted for each line). Arabidopsis expressing unphosphorylatable At1g80180.1 had a stomatal index of 0.59, not significantly different from the wild-type (Student’s t test, \( P = 0.43 \)). These results indicate that phosphorylation of At1g80180.1 positively
regulates asymmetric cell division in epidermis development. To verify that At1g80180.1 is expressed in cotyledons as would be expected if it is involved in regulating stomatal patterning, a 1.7-kb upstream promoter region of At1g80180.1 was used to drive expression of the GUS reporter gene. Expression driven by the At1g80180.1 promoter was indeed detected early in seedling development and to high levels in epidermis and stomata of cotyledons in all five lines inspected (Figure 5C).

DISCUSSION

MPK3 and MPK6 phosphorylation site

Most of the evidence to date indicates that MPK3 and MPK6 have redundant functions in several signalling pathways, indicating that they share common substrates [10]. However, at present, we have very little understanding of the determinants of MAPK specificity towards substrates in plants. Although a previous evolutionary comparison of the local residues surrounding the phosphorylation site of AtPHOS32 indicated that these residues may contribute to kinase substrate recognition, no empirical evidence supported this hypothesis. On the basis of the results from the random peptide library array screen in the present study, it is clear that the residues surrounding the phosphorylated residue can contribute to MPK3 and MPK6 specificity. The empirically determined motif, L/P-P/X-S/T-P-R/K, was consistent with the context of the phosphorylated residue from the MPK3/MPK6 substrate, PHOS32 [18], indicating that this motif may predict new MPK3/MPK6 substrates on the basis of the presence of this surrounding primary sequence. Indeed, we demonstrated that at least five other proteins containing this motif were good in vitro substrates for MPK3 and MPK6 and that these proteins were poor substrates for another Arabidopsis MAPK, MPK4. The kinase specificity appears to at least partially lie within the sequence surrounding the phosphorylated residue because there does not appear to be any other sequence conservation between these otherwise randomly selected substrates. The results with At1g13390.1 which contains two motifs, one with serine and one with threonine, demonstrates that threonine is an acceptable substitution as the phosphorylated residue, thereby expanding the range of possible substrates predicted by the core motif. There are, however, MPK3 and MPK6 substrates in Arabidopsis that lack the motif described in the present paper. In ethylene signalling, MPK3 and MPK6 phosphorylate and stabilize EIN3. The phosphorylated Thr174 in EIN3 is not in the context of the motif defined in the present study, but a putative docking domain was identified 70 amino acids downstream of the phosphorylation site which instead may contribute to specificity [12]. The rate-limiting enzyme in the production of the plant hormone ethylene, ACS6, is also phosphorylated by MPK6, but lacks the full core motif predicted in our present study. Therefore the motif predicted from the present study does not appear to define all possible substrates of MPK3 and MPK6. However, the fact that most of the potential MAPK substrates containing this new motif do not have obvious MAPK docking domains indicates that the L/P-P/X-S/T-P-R/K motif may be utilized in MPK3 and MPK6 substrates that lack MAPK docking motifs distant from the phosphorylation site.

Overlapping substrate phosphorylation by both MPK3 and MPK6 observed in the present study is consistent with the proposed redundant functions of MPK3 and MPK6 in some pathways. For instance, embryo development is regulated by both MAPKs as is evident from embryo lethality of mpk3mpk6 double mutants, whereas the single-knockout mutants only show minor stress-related phenotypes [11]. Therefore one possible hypothesis...
is that the core motif described in the present study is used mainly in pathways that need to have many possibilities for fine-tuning in order to maximize ecological fitness.

**MPK3 and MPK6 in vitro substrate identification**

Using the motif identified in the present study as the basis for bioinformatic prediction, candidate proteins were bacterially expressed for in vitro kinase assays to identify a number of new MPK3 and MPK6 substrates. Of the proteins identified, we know relatively little about their possible functions from the literature. At1g80180.1 is a 15 kDa highly basic protein of unknown function. At1g80180.1 and a closely related protein, At1g15400.1, are known to be in vivo phosphorylated at the predicted P-P-S-P-R motif [39]. Orthologues of At1g80180.1 in other plant species share the motif P-P-S-P-R/K around the phosphorylated serine residue, indicating that the post-translational regulation by the MAPKs is evolutionarily conserved. In addition, a quantitative phosphoproteomics study found that phosphorylation of At1g15400.1 on the P-P-S-P-R motif was reduced within minutes after sucrose resupply [41], indicating a possible link between MAPK signalling and nutrient supply. At2g43680.1 is a predicted 74 kDa protein containing a calmodulin-binding domain and five repetitive P-P-S-P-R motifs. Interestingly, this protein was found to be in vivo phosphorylated on these sites after elicitation with the biotic elicitors fgl22 and fungal xylanase [38], which would be consistent with the activation of MPK3/MPK6 during these responses. EBP (At3g16770), an ERF-type transcription factor, is the only in vitro MAPK substrate identified in the present study that was identified in one of the two large-scale screens that have been carried out with Arabidopsis MPKs [19,20]. Therefore the small level of overlap between these independent studies supports the need for pursuing multiple strategies when trying to identify kinase substrates. We have shown that integration of data from targeted screening for kinase substrates with site confirmation, evolutionary conservation and predicted substrate subcellular localization can effectively lead to identification of new kinase–substrate pairs.

**Importance of phosphorylation of At1g80180.1 in stomatal patterning**

During the investigation of possible biological roles of At1g80180.1 phosphorylation, we found that constitutively expressing the phosphomimetic form of At1g80180.1 strongly affected the normal development of Arabidopsis seedlings. These transgenic seedlings failed to develop true leaves and died early in development. Examination of cotyledons in multiple T1 transformants showed a clustering of stomata in the cotyledons. The unphosphorylatable form of At1g80180.1 did not show any effects in stomatal patterning. The result with the phosphomimetic form of At1g80180.1 is intriguing in the context of the role(s) of MPK3 and MPK6 in the regulation of stomatal patterning. In mpk3mpk6 double mutant seedlings, massive clusters of stomata without normal spacing of pavement cells are evident, indicating that the MAPKs act as negative regulators limiting stomatal division or patterning. Consistent with this hypothesis, constitutive activation of MPK3 and MPK6 prevent the formation of stomata [11]. Our results therefore appear at first to be inconsistent with MAPK signalling repressing stomatal patterning, as the constitutive phosphomimetic At1g80180.1 resulted in a higher stomatal index. However, the regulation of this developmental process is likely to be more complex than a simple linear signalling pathway. For instance, SPCH is a MPK3 and MPK6 substrate required for entry into the stomatal patterning [17,42]. The spch knockout mutant has no stomata. Plants expressing SPCH without its MAPK phosphorylation domain show moderate clustering of stomata, consistent with MPK3/MPK6 phosphorylation having a repressive role on SPCH activity. Intriguingly, phosphorylation by MPK6 of Ser193 is required for SPCH activity and production of stomata, indicating that MPK3/MPK6 phosphorylation can have both negative and positive regulatory roles in the production of stomata. Lampard et al. [17,43] and Abrash and Lampard [44] also suggest MPK3/MPK6 to be both negative and positive regulators at several steps during stomatal development. Therefore it is likely that the activity of MPK3 and MPK6 integrate several complex regulatory inputs that finely control stomatal production, and At1g80180.1 may be one of the downstream components. Although we cannot rule out an alternative explanation for this phenotype, such as a more indirect effect due to ectopic expression, we still find it likely that a link exists between this protein and the MAPK regulation of stomatal patterning as overexpression of only the phosphomimetic rather than the phospho-null mutants resulted in an obvious phenotype.

At present, even though MPK3 and MPK6 are activated in response to a wide range of stimuli, the roles of these kinases are thought to be masked by their apparent redundant functions. Conversely, the complete absence of MPK3 and MPK6 results in extreme developmental phenotypes preventing the use of this genetic approach to delve into the role(s) of these kinases. Therefore a more subtle dissection of the activity of individual MAPK substrates by using unphosphorylatable or phosphomimetic mutations may reveal activities that would otherwise be difficult to detect because of these limitations. This line of logic supports the rationale for exploiting biochemical approaches to further define additional MAPK substrate candidates, followed by targeted reverse genetic experiments to further our understanding of these complex biological processes.

**AUTHOR CONTRIBUTION**

Experimental work and experimental design was carried out by Carolin Sörensson, Marit Lennan, Simone Schopper, Jenny Veide-Vilg, Thomas Ljungdahl, Morten Grøtli and Markus Tamás. Scott C. Peck and Erik Andreasson designed the study and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Determination of primary sequence specificity of Arabidopsis MAPKs MPK3 and MPK6 leads to identification of new substrates

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Figure S1 Sequences in our PS-SCL for kinase substrate specificity studies


Figure S2 AtMPK3 and AtMPK6 peptide sequence phosphorylation preference

Preferred amino acid residues surrounding the phosphorylated serine residue (S) identified by positional scanning peptide library assay with purified Arabidopsis MPK3 (upper panel) and MPK6 (lower panel). The two sets of numbers show the results from two independent experiments.

Atg80180.1 NTGVRSPAVDPSSPRLSAFGCSAF
Atg329390.1 VTPSNRNRVSPPSPHRHPPPPPFT
Atg214850.1 KKSLGDPVPPSPKCRSFKFRDR
Atg316770.1 HPPPPNYPFPFSPRSSTDQPFPKVK
Atg13390.1 S9PRAKQPQFSSRNG3VCMAATS

Figure S3 Alignment of residues around the phosphorylation site in the confirmed in vitro substates of AtMPK3 and AtMPK6

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Table S1  *Arabidopsis* proteins containing the putative MPK3/MPK6 consensus phosphorylation sites P-P-S-P-R or P-S-S-P-R

Predicted transmembrane regions (http://aramemnon.botanik.uni-koeln.de) are indicated. Predicted nuclear or cytoplasmic localization with an AtSubP (http://bioinfo3.noble.org/AtSubP) cut-off value of 0.5. Phosphosites identified in PhosPhAt 3.0 (http://phosphat.mpimp-golm.mpg.de) database are indicated, where * denotes a phosphorylated peptide containing P-S-S-P-R, but without an exact assignment to the serine residue. Selected proteins for putative in vitro kinase test (predicted size below 50 kDa, no transmembrane regions, nuclear/cytosolic localization (× denotes yes), in vivo phosphorylation sites).

<table>
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<th>Accession number</th>
<th>Protein</th>
<th>Motif</th>
<th>Size (kDa)</th>
<th>Predicted transmembrane regions</th>
<th>Nuclear/cytosolic</th>
<th>Phosphosite identified in PhosPhAt</th>
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