

## Microbial Elicitors Induce Activation and Dual Phosphorylation of the *Arabidopsis thaliana* MAPK 6\*

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Protein kinases related to the family of mitogen-activated kinases (MAPKs) have been established as signal transduction components in a variety of processes in plants. For *Arabidopsis thaliana*, however, although one of the genetically best studied plant species, biochemical data on activation of mitogen-activated protein kinases are lacking. *A. thaliana* MAPK 6 (AtMPK6) is the *Arabidopsis* orthologue of a tobacco MAPK termed salicylate-induced protein kinase, which is activated by general and race-specific elicitors as well as by physical stress. Using a C terminus-specific antibody, we show that AtMPK6 is activated in elicitor-treated cell cultures of *A. thaliana*. Four different elicitors from bacteria, fungi, and plants lead to a rapid and transient activation of AtMPK6, indicating a conserved signaling pathway. The induction was equally rapid as medium alkalization, one of the earliest elicitor response observed in cell cultures. A similarly rapid activation of AtMPK6 was observed in elicitor-treated leaf strips, demonstrating that recognition of the elicitors and activation of the MAPK pathway occurs also in intact plants. We demonstrate by *in vivo* labeling that AtMPK6 is phosphorylated on threonine and tyrosine residues in elicited cells.

Mitogen-activated protein kinase (MAPK)<sup>1</sup> cascades are among the most conserved signal transduction systems in eukaryotes (1). Homologues of MAPKs as well as upstream kinases have been found in fungi, invertebrates, vertebrates, and plants. Activation of MAPKs requires phosphorylation on threonine and tyrosine residues (2) by upstream dual specificity kinases, termed MAPK kinases or MAPKKs (3). MAPKKs, in turn, are activated through phosphorylation by a highly diverse group of MAPKK kinases. A remarkable feature of this three-tiered cascade is that a relatively small number of MAPKs and MAPKKs appear to be coupled to a vast range of extracellular stimuli to give tissue-specific responses. Specificity is thought to be achieved by scaffolding of the cascade components, differences in signaling kinetics, and co-activation of other pathways (4, 5).

The biological processes in which MAPKs are involved are

best understood in yeast and mammals. Five MAPK modules have been described in yeast that regulate mating, filamentous growth, high osmolarity response, cell wall remodeling, and sporulation (6). In mammals, the extracellular signal-regulated kinases ERK1 and ERK2 regulate growth and differentiation, while stress responses are signaled through p38 and c-Jun N-terminal kinase (1, 7).

Knowledge about homologues of MAPKs and their upstream kinases in plants is increasing rapidly (8–10). Based on the induction of gene expression or a kinase activity with MAPK-like characteristics, MAPKs have been proposed to play a role in cell cycle/cell division (11–13), hormone signaling (14), and transduction of stimuli related to physical (15–19) and biological (20–23) stress. More than 20 cDNAs encoding putative MAPKs have been cloned from different plant species, which can be grouped into four or five classes according to sequence similarity (9, 10). Within each class, the high degree of sequence similarity appears to reflect a functional conservation between plant species (10).

Despite extensive evidence for the induction of MAPK-like activity in plants, the identification of a particular class of MAPK-like plant kinases induced by a particular stimulus has been difficult and has successfully been performed only in two cases. During cell cycle and cytokinesis, specific changes in transcript levels, kinase activities, and subcellular localization were reported for two members of one class, Ntf6 from tobacco and MMK3 from alfalfa (12, 13).

The second and most thoroughly studied class of MAPKs is that induced in stress and elicitor responses, in particular salicylate-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) in tobacco. SIPK was originally identified as a 48-kDa kinase induced in tobacco suspension culture by salicylic acid (24). Increased SIPK activity has been demonstrated after treatment with general and race-specific elicitors (22, 26), tobacco mosaic virus infection (23), and wounding (25). The transcript encoding WIPK accumulates in tobacco leaves after wounding (27). Similarly, transcript levels of the highly related genes *MMK4* and *AtMPK3* increase in wounded alfalfa plants (19) and cold/drought-treated *Arabidopsis* plants (16), respectively. Recently, the induction of WIPK kinase activity has been reported in wounded tobacco (28) and Avr9-treated suspension cells of tobacco expressing the *Cf9* gene (26).

For the study of the responses of plants to pathogens, *Arabidopsis thaliana* is a well established model organism (29, 30). Although a large collection of mutants with altered pathogen responses is available, the question remains open which “classical” signal transduction pathways, if any, play a role in pathogen sensing. Nine MAP kinases, five MAPKKs, and eight MAPKK kinases have been cloned from *Arabidopsis* (9, 31), but it is not known which of these kinases is involved in elicitor signal transduction. The high degree of sequence similarity between the AtMPK6 and SIPK indicates that the *Arabidopsis*

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<sup>1</sup> The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAP, mitogen-activated protein; SIPK, salicylate-induced protein kinase; WIPK, wound-induced protein kinase; AtMPK1, -2, -3, and -6, *A. thaliana* MAP kinase 1, 2, 3, and 6, respectively; flg22, flagellin peptide; MBP, myelin basic protein; MES, 4-morpholineethanesulfonic acid.

protein might perform a function similar to that of SIPK in tobacco.

We have used previously characterized elicitors (32) and the peptide flg22 (33, 34) to study the induction of kinase activities in *A. thaliana*. flg22, a peptide derived from the most conserved domain of the eubacterial flagellin protein, is a potent elicitor of defense responses in cell cultures of several plant species (33) and *Arabidopsis* seedlings (34). Here, we demonstrate that at least four elicitors are able to induce AtMPK6 activity in *Arabidopsis* suspension cultures. Activation of AtMPK6 is rapid (*i.e.* concomitant with the other earliest observable responses) and correlated with its phosphorylation on threonine and tyrosine residues.

#### EXPERIMENTAL PROCEDURES

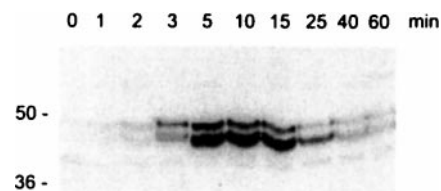
**Cell Cultures and Elicitors**—Suspension-cultured cells of *A. thaliana*, ecotype "Landsberg erecta" were maintained as described previously (35) and used for experiments 7 days after subculture. Flagellin peptide (flg22) was synthesized by F. Fischer (Friedrich-Miescher-Institut, Basel, Switzerland). Hexa-*N*-acetylchitohexaose was obtained from Seikagaku Corp. (Tokyo, Japan), and xylanase from *Trichoderma viride* was purchased from Fluka (Buchs Switzerland). Pectic fragments were prepared by incubation of citrus pectin (Roth) at 60 mg/ml (adjusted to pH 5 with NaOH) with pectolyase Y23 (Seishin) at 30  $\mu$ g/ml for 20 min at room temperature. After digestion, the solution was boiled for 5 min and centrifuged at 20,000  $\times g$  for 10 min, and the supernatant was lyophilized and redissolved to yield a stock solution of 50 mg/ml.

**Protein Kinase Activity Assays**—Suspension-cultured cells were transferred to a beaker on a rotary shaker at 120 cycles/min. The pH of the culture medium was measured continuously with a small combined glass electrode (Metrohm) and registered with a pen recorder.

At the indicated times after the addition of an elicitor, samples of 0.5 ml were taken, the medium was removed by filtration, and the cells were frozen in liquid nitrogen. Further sample processing was done according to Ref. 18 with minor modifications. Briefly, cells were ground in liquid nitrogen after the addition of 2 ml/(g of plant material) homogenization buffer (50 mM Tris-HCl pH 7.5, 15 mM MgCl<sub>2</sub>, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM NaVO<sub>3</sub>, 15 mM  $\beta$ -glycerophosphate, 15 mM *para*-nitrophenylphosphate, 0.1% Tween 20, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 20,000  $\times g$  for 5 min to yield a crude, soluble protein extract. For in-gel kinase assays, 50  $\mu$ g of protein (assayed with the D<sub>C</sub> system; Bio-Rad) were precipitated in 80% acetone, resuspended in SDS sample buffer, and separated on a 10% SDS-polyacrylamide gel containing 0.2 mg/ml myelin basic protein (MBP) (Sigma). Renaturation and kinase assay were performed according to Ref. 36. Radioactive bands were visualized by exposure of the dried gels to a PhosphorImager and quantified with ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA). For immunoprecipitations, 0.5–2  $\mu$ l of the M23 antibody (raised against the C-terminal sequence FNPEYQQ of the alfalfa MAP kinase MMK1)<sup>2</sup> were incubated with 100  $\mu$ l of protein A-Sepharose slurry (Amersham Pharmacia Biotech) in Sucl buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1% Tween 20) for 2 h at 4 °C with gentle rocking. After four washes with Sucl buffer, 200  $\mu$ g of crude protein extract was added and incubated with the antibody for 4 h at 4 °C. Washing steps were performed as in Ref. 18. Kinase assays were performed in 25  $\mu$ l of 20 mM HEPES/KOH, pH 7.5, 15 mM MgCl<sub>2</sub>, 5 mM EGTA, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 0.5 mg/ml MBP, 0.2 mM ATP, and 37 kBq of [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Pharmacia Biotech) for 15 min at room temperature. Phosphate incorporation into MBP was quantified after SDS-polyacrylamide gel electrophoresis on a 15% gel.

**Leaf Strip Assay**—*A. thaliana* (ecotype "Landsberg erecta") was grown in soil in a growth chamber for 5 weeks. Fully expanded leaves were cut into 1–2-mm strips and floated overnight on water with gentle shaking. Ten strips were submerged in a tube with 1 ml of water or dilute elicitor solutions; vacuum-infiltrated for 20 s; and incubated for another 3 or 10 min before drainage, freezing in liquid nitrogen, and sample processing as described for suspension-cultured cells.

**In Vivo Labeling, Phosphopeptide, and Phosphoamino Acid Analysis**—Suspension-cultured cells were equilibrated on a rotary shaker as described and buffered 30 min before labeling by the addition of 20 mM



**FIG. 1. Induction of MBP kinase activities in *Arabidopsis* cell culture by flg22.** Samples were taken at different times before and after treatment of an *Arabidopsis* suspension culture with 100 nM flagellin peptide (flg22). 50  $\mu$ g of crude cell extracts were separated by SDS-PAGE on a gel containing MBP as substrate. After renaturation, an in-gel kinase assay was performed (see "Experimental Methods"), and kinase-active bands were visualized by autoradiography. Molecular mass markers (in kDa) migrated as indicated on the left of the gel.

MES/KOH, pH 6. One-ml aliquots were taken before and 2 min after elicitation with 100 nM flg22 and added to 300 kBq [<sup>32</sup>P]orthophosphate (Amersham Pharmacia Biotech) in a small glass potter. After 30 s, the medium with the excess radioactivity was removed. One ml of homogenization buffer was added, and the cells were homogenized on ice. Immunoprecipitation was done as described, and the precipitate was analyzed on a 12% SDS-polyacrylamide gel. The gel was stained and dried, and the labeled 49-kDa band was localized by autoradiography. The excised band was washed with 50% acetonitrile, 0.1 M ammonium bicarbonate, dried, and digested with 1  $\mu$ g of modified trypsin (Promega) in 0.1 M ammonium bicarbonate overnight at 37 °C. Phosphopeptides were recovered by extraction of the gel piece with 50% acetonitrile, dried, and analyzed by two-dimensional thin layer chromatography/electrophoresis on cellulose plates as described by Boyle *et al.* (37). Chromatographic separation in *n*-butanol/H<sub>2</sub>O/pyridine/glacial acetic acid (7.5:6:5:1.5) in the first dimension was followed by electrophoresis in pH 8.9 buffer (1% ammonium bicarbonate) for 1 h at 1000 V in the second dimension. Phosphoamino acid analysis was performed with protein blotted to Immobilon P (Millipore Corp.) for 1 h at 2.4 mA/cm<sup>2</sup> gel area in a Milliblot-SDE electroblotting apparatus (Millipore). The identified band was excised and hydrolyzed with 6 N HCl for 1 h at 110 °C. The hydrolysate was cleared by centrifugation (5 min at 20,000  $\times g$ ), dried in a vacuum concentrator, redissolved in H<sub>2</sub>O, and analyzed by one-dimensional thin layer electrophoresis at pH 3.5 (5% glacial acetic acid, 0.5% pyridine) as described by Boyle *et al.* (37). Alternatively, phosphopeptides were recovered from TLC plates, eluted with 50% acetonitrile, dried, and hydrolyzed as described above.

#### RESULTS

**Microbial Elicitors Lead to a Transient Activation of MBP Kinase Activities**—Initial experiments were performed to test whether flg22, a peptide with potent elicitor activity derived from the most conserved domain of bacterial flagellin (33), would induce MAP kinase-like activity in *Arabidopsis* cell suspension cultures. After the addition of flg22 to the culture, cells were harvested at various times, and cell extracts were analyzed by an in-gel kinase assay with MBP as substrate. Within 2 min after the addition of 100 nM flg22, MAP kinase-like activity of two protein classes with apparent molecular masses of 45 and 49 kDa began to increase (Fig. 1). These MBP kinase activities peaked at 5–10 min after elicitation and returned to nearly basal levels within 60 min. A weak band of approximately 40 kDa was sometimes observed that showed a pattern of activation opposite to the 45- and 49-kDa bands. It was detectable in control samples, disappeared when the 45- and 49-kDa bands displayed maximal activity, and reappeared 15–30 min after elicitation.

The kinase activities detected in the in-gel kinase assays showed many of the characteristics that have been reported in other systems (21, 38). They could be blocked by the kinase inhibitor K-252a and activated in the absence of elicitors by the addition of the phosphatase inhibitor calyculin A (data not shown). One difference, however, was that valinomycin, an ionophore that evokes alkalinization of the culture medium to a similar degree as elicitors, does not induce MBP kinase ac-

<sup>2</sup> Munnik, T., Ligterink, W., Meskiene, I., Calderini, O., Beyerly, J., Musgrave, A., and Hirt, H. (1999) *Plant J.* 20, 381–388

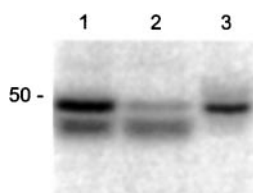


FIG. 2. **Immunodepletion of AtMPK6 from cell extracts.** A sample was taken 4 min after treatment of a culture with 100 nM flg22, and AtMPK6 was immunoprecipitated from 10  $\mu$ g of protein extract. Lane 1, 10  $\mu$ g of total protein; lane 2, 10  $\mu$ g of protein depleted of AtMPK6; lane 3, AtMPK6 immunoprecipitate.

tivities in the in-gel assay (data not shown). This result indicates that ion fluxes alone are not sufficient to induce the kinase response, in contrast to what has been reported in other systems (26). The alkalization of the medium is not necessary for AtMPK6 activation, since buffering, as performed in the *in vivo* labeling experiment (see below), has no effect on AtMPK6 phosphorylation and activation.

**Immunoprecipitation of an Elicitor-induced 49-kDa Kinase**—In tobacco, SIPK was identified as a MAP kinase that is activated after treatment with salicylic acid and elicitors (22, 24). Although *Arabidopsis* contains at least nine genes encoding MAP kinases, the SIPK sequence is most similar to *Arabidopsis* AtMPK6 (31). Therefore, we tested whether AtMPK6 was one of the proteins with elicitor-induced kinase activity in *Arabidopsis* cells using immunoprecipitation followed by an in-gel assay for MBP kinase activity. The antibody employed, M23, was raised against the C-terminal seven amino acids of MMK1, an elicitor-induced MAP kinase in alfalfa.<sup>2</sup> These seven amino acids are identical to the C terminus of AtMPK6 but not to any of the other known *Arabidopsis* MAP kinases. M23 immunoprecipitates of cell extracts taken 4 min after elicitation contain a kinase activity with an apparent molecular mass of 49 kDa, as seen in the in-gel kinase assay (Fig. 2). In the extract that has been immunodepleted of AtMPK6, the 49-kDa band is strongly reduced compared with an untreated sample, while the 45-kDa band remains unchanged. These results indicate that activated AtMPK6 is at least partially, if not entirely, responsible for the activity in the 49-kDa band of the in-gel assay.

In tobacco, the activation of another kinase, WIPK, was reported after wounding (28) and in Avr9-treated tobacco cells expressing the *Cf9* gene (26). Our attempts to demonstrate activation of AtMPK3, the closest homologue of WIPK in *Arabidopsis*, were unsuccessful. Using assay conditions that allow detection of basal MBP kinase activity (28), neither an antibody directed against the six C-terminal amino acids of MMK4, the alfalfa homologue of WIPK, nor an antibody against the 11 C-terminal amino acids of AtMPK3 precipitated any elicitor-induced kinase activity (data not shown). Therefore, our study focused on AtMPK6.

**AtMPK6 Is Activated Concomitantly with Other Early Responses in Arabidopsis Cell Cultures**—Alkalization of the culture medium represents one of the earliest responses observable in suspension cultures treated with microbial elicitors (40). We examined the relative timing of changes in ion fluxes and MAPK activation by immunoprecipitating AtMPK6 from samples taken at intervals from cultured cells while the pH of the culture medium was continuously measured. Activity in these samples was measured as MBP-kinase activity using an in-solution assay. The time course of activation of AtMPK6 kinase activity (Fig. 3A) was very similar to that of the in-gel activity bands shown in Fig. 1. A comparison of the phosphorylation of MBP with the change in the medium pH (Fig. 3B) showed that both responses initiated after a lag phase of ~2

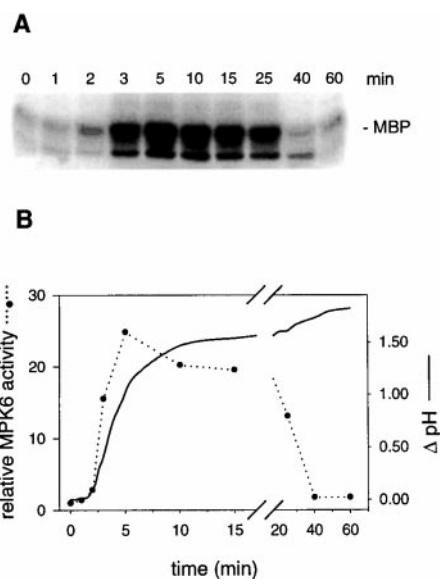


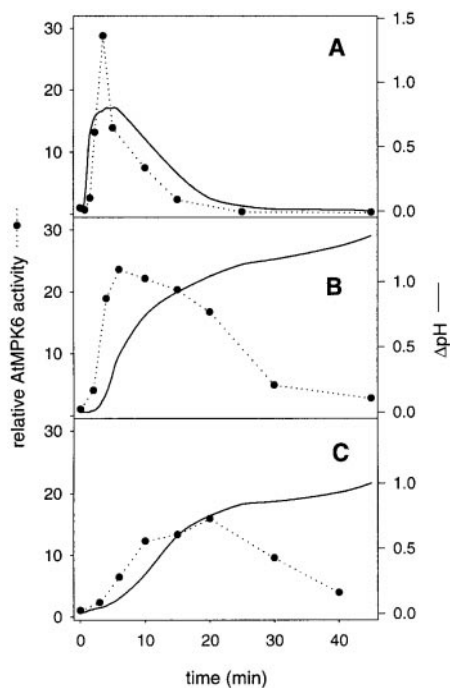
FIG. 3. **Time course of AtMPK6 activity and extracellular alkalization after treatment with flg22.** A, immunokinase assay of AtMPK6 after treatment of cells with 100 nM flg22. AtMPK6 was immunoprecipitated from 200  $\mu$ g of the crude cell extracts analyzed in the in gel assay in Fig. 1. MBP kinase activity was assayed as described under "Experimental Procedures." The assay mix was separated by SDS-PAGE, and MBP phosphorylation was visualized by autoradiography. B, comparison of AtMPK6 activity and extracellular medium pH. Kinase activity was quantified with ImageQuant from the data shown in A and shown as activity relative to the control sample. The pH of the extracellular medium was continuously monitored with a pH electrode in the same culture from which the samples for kinase assays were taken.

min and increased almost simultaneously during the first 5 min after elicitation. After the first 5 min, AtMPK6 activity remained constant for 10 min and then slowly returned to its basal level, while the medium pH remained at a high level and did not decrease until several hours after elicitation (data not shown).

**Elicitors of Bacterial, Fungal, and Plant Origin Activate AtMPK6 with Characteristic Time Courses**—In addition to the flg22 peptide of bacterial origin, at least three more elicitors derived from fungi and plants acted as elicitors of AtMPK6 activity and medium alkalization (Fig. 4) in *Arabidopsis* suspension-cultured cells. Pectic fragments (oligogalacturonides) elicited a particularly rapid and only transient activation of the MAP kinase, with a maximal activity around 3.5 min and a nearly complete return to basal activity after 15 min (Fig. 4A). Hexameric chitin fragments elicited AtMPK6 activity to a similar degree and with nearly identical kinetics as the flg22 peptide (Fig. 4B). Xylanase from the fungus *Trichoderma viride* induced AtMPK6 activity more slowly than other elicitors (Fig. 4C), with a maximum at 20 min. The activity decreased slowly thereafter but did not reach the basal level within 40 min.

As was observed in the experiments with flg22, no increase in AtMPK6 activity was measured before the onset of the pH response. With xylanase and chitin, the increase in kinase activity typically paralleled the pH curve. During the oligogalacturonide treatment, however, kinase activation lagged slightly behind the pH response.

**AtMPK6 Is Induced in Elicitor-treated Leaf Tissue**—To test whether responses in suspension-cultured cells are similar to those of cells within differentiated tissue, we examined if flg22 and chitin activated AtMPK6 in leaf tissue. To achieve rapid and even exposure, leaves of 5-week-old *Arabidopsis* plants were cut into 1–2-mm strips, incubated on water to allow the wound response to subside, and vacuum-infiltrated with elici-

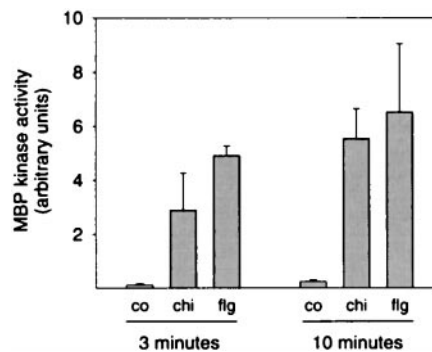


**FIG. 4. Time course of AtMPK6 activity and medium alkalization after treatment with plant and fungal elicitor.** Aliquots of cell cultures were treated with 50  $\mu\text{g/ml}$  pectic fragments (A), 500 nM hexameric chitin fragments (*panel B*), or 10  $\mu\text{g/ml}$  *T. viride* xylanase (C), respectively. AtMPK6 immunokinase assays were performed as described under "Experimental Procedures," and pH was measured as described in the legend to Fig. 3.

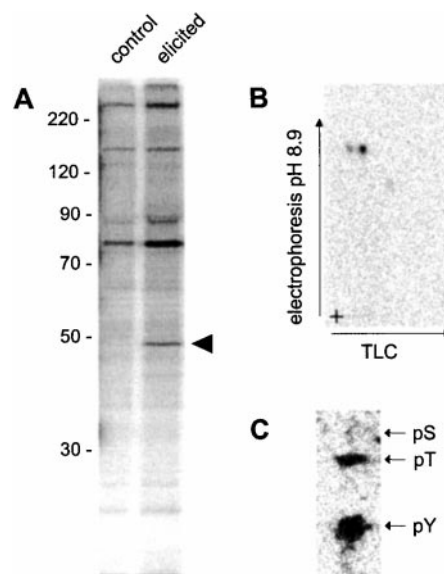
tor solutions or water. After immunoprecipitation, a low basal level of AtMPK6 activity was detected in water-infiltrated tissue after 3 min, with a slight increase after 10 min. In similar cases, this has been attributed to physical stress (26, 36). After infiltration with flg22 or chitin fragments, however, AtMPK6 activity increased strongly within 3 min and further increased within 10 min (Fig. 5). These data demonstrate that differentiated plant tissue of *A. thaliana* responds to general elicitors by activation of AtMPK6 in a similar way as suspension-cultured cells.

**Dual Phosphorylation of AtMPK6 in Elicitor-treated Cells**—In mammalian cells, activation of MAP kinases is correlated with their dual phosphorylation on threonine and tyrosine residues within a TXY motif (2). Plant MAP kinases also contain this conserved phosphorylation motif as TEY or TDY. Therefore, a similar mode of activation has been inferred from Western blots and immunoprecipitations with antibodies against phosphotyrosine and/or active mammalian MAP kinases (26, 36, 38) and from inactivation of MBP kinase activity with tyrosine- and serine/threonine phosphatases (20, 27). In an *in vitro* assay, the *Arabidopsis* MAP kinases AtMPK1 and AtMPK2 can be phosphorylated by a *Xenopus* MAPKK on threonine and tyrosine residues (39). In our laboratory, *in vivo* pulse labeling of suspension-cultured cells has been established as a method to detect rapid changes in protein phosphorylation (40). This technique provides a more direct method to study the phosphorylation of threonine and tyrosine in AtMPK6.

An aliquot of suspension culture was pulse-labeled with [ $^{32}\text{P}$ ]orthophosphate for 30 s followed by immunoprecipitation of AtMPK6. Autoradiography of the proteins separated by SDS-PAGE showed a radioactive band of 49 kDa in the elicited but not the control sample (Fig. 6A). Several phosphorylated proteins of higher molecular weight were also present in the immunoprecipitation material, but none was differentially phosphorylated in response to elicitor treatment as is the 49-kDa



**FIG. 5. Induction of AtMPK6 activity in leaf tissue.** Immunokinase assays were performed with cell extracts from elicitor-treated leaf tissue. Leaf strips (1–2 mm) were vacuum-infiltrated for 15 s with water (co), 10  $\mu\text{M}$  chitin hexamers (chi), or 100 nM flg22 (flg), respectively, and incubated another 3 or 10 min before sample extraction. Data are given as mean values  $\pm$  S.D. of triplicate samples.



**FIG. 6. *In vivo* phosphorylation of AtMPK6.** A, SDS-polyacrylamide gel of AtMPK6 immunoprecipitates from *in vivo* labeled cells. Labeling was performed for 30 s before (control) and 2.5 min after the addition of 100 nM flg22 to the cell culture. Phosphorylated proteins were visualized by autoradiography. The 49-kDa protein analyzed for phosphopeptides and phosphoamino acids is indicated with an arrow. B, autoradiograph of a map of the tryptic peptides of the 49-kDa band indicated in A. Peptides were separated by chromatography in the first dimension and by electrophoresis at pH 8.9 in the second dimension, as indicated by the arrows. The origin is marked with a cross. C, phosphoamino acid analysis of the 49-kDa band. The protein was blotted to Immobilon P, and the indicated band was excised and hydrolyzed with HCl. The hydrolysate was electrophoretically separated at pH 3.5.

protein. After tryptic digestion of the 49-kDa band, two-dimensional phosphopeptide analysis revealed a single major and one minor phosphopeptide of identical high electromobility (Fig. 6B). AtMPK6 phosphorylated on the amino acids Thr<sup>221</sup> and Tyr<sup>223</sup> should give rise to a single radioactive peptide ( $^{213}\text{VT-SESDFMpTEpYVVTR}^{227}$ , where pT and pY represent phosphothreonine and phosphotyrosine, respectively), with an electrophoretic mobility consistent with our data. In a parallel experiment, the phosphorylated 49-kDa protein was blotted to Immobilon P and hydrolyzed for one-dimensional phosphoamino acid analysis (Fig. 6C). Radioactive phosphothreonine and phosphotyrosine, but not phosphoserine, were found in the hydrolysate. The same results were obtained when the peptide spots from Fig. 6B were hydrolyzed, although the signal intensities were considerably weaker. We infer that both spots are identical with regard to the amino acid sequence and

phosphorylation, and we hypothesize that one may have arisen from methionine oxidation of the original peptide. Our data provide direct evidence that activation of the *Arabidopsis* MAP kinase AtMPK6 correlates with its *de novo* phosphorylation on tyrosine and threonine, most likely on the conserved motif <sup>221</sup>TEY<sup>223</sup>.

#### DISCUSSION

In this study, we demonstrate that a member of the MAP kinase family of *A. thaliana*, AtMPK6, is rapidly and transiently activated in suspension-cultured cells and leaf tissue after treatment with a variety of elicitors. Although *A. thaliana* is a well established model for plant-pathogen interactions (29), the perception of general (as opposed to race-specific) elicitors by this species has rarely been addressed (33, 34, 41). In suspension-cultured cells of *Arabidopsis*, the bacterial flagellin peptide as well as the fungal elicitors xylanase and chito-hexaose lead to an alkalization of the culture medium and rapid changes of the protein phosphorylation pattern.<sup>3</sup> These results prompted us to search for elicitor-induced kinases. An in-gel kinase assay with MBP as substrate has been used in several studies to demonstrate the activation of MAP kinase-like activity in various plant species. Employing this assay, we demonstrate that the rapid induction of two major bands of 45 and 49 kDa is visible in elicitor-treated cells of *A. thaliana*. Similar results have been reported for elicitor-treated tobacco cell cultures (22, 26, 38), although the 45-kDa band appeared not always as rapidly and strongly as the 49-kDa band in these studies. In tobacco, there is substantial evidence that the MAP kinases SIPK and WIPK give rise to the two in-gel bands of ~46 and ~49 kDa (22, 24, 26). With the high degree of sequence conservation among plant MAPKs, it was reasonable to hypothesize that the bands of the same size, activated by comparable stimuli in different plant species, correspond to kinases of the same family (9, 10). SIPK and WIPK have very close homologues in *Arabidopsis* (AtMPK6 with 90% similarity to SIPK and AtMPK3 with 91% similarity to WIPK).

Immunoprecipitation experiments with antibodies against the C termini of AtMPK6 and AtMPK3 showed a rapid and strong activation of AtMPK6 by four elicitors of bacterial, fungal, and plant origin. However, no activation of AtMPK3 was detected. In analogy to the situation in tobacco, it would be expected that AtMPK3, the WIPK orthologue, gives rise to the 45-kDa band. It is possible that activated AtMPK3 has escaped detection in our immunoprecipitation experiments. The kinase activity of WIPK has been shown to be particularly sensitive to prolonged sample handling (26) and assay conditions (28). On account of the unambiguous data that we obtained with AtMPK6, we focused exclusively on the latter kinase.

Suspension-cultured cells serve as an excellent model system for early cellular responses to biological and physical stimuli, but it is uncertain whether midterm and long term responses occur. In order to corroborate our data *in planta*, we tested if the flagellin-derived peptide flg22 and chitin acted as elicitors of AtMPK6 activity in leaf tissue. Both compounds lead to a severalfold increase in immunoprecipitated MPK6 activity, and the transduction of the elicitor signal appears to proceed *in planta* at a similar speed as in suspension cultured cells.

There is ample evidence for the involvement of protein phosphorylation as an early, critical step in elicitor signal transduction. Many studies have made use of kinase inhibitors to block elicitor-induced responses (40, 42), and in some cases, rapid changes in the protein phosphorylation pattern have been revealed by *in vivo* labeling (40, 43). With this technique, direct evidence for the elicitor-induced phosphorylation of a particu-

lar protein was presented only in very few cases, among them two components of the plasma membrane NADPH oxidase complex, p47 and p67 (44), and the transcription factor G/HBF1 (45). This has not been shown for MAPKs, although elicitor-induced phosphorylation on tyrosine was inferred from immunoblots (26, 36, 38).

Here, we present direct evidence that AtMPK6 is phosphorylated *in vivo* on threonine and tyrosine after treatment of cells with an elicitor. According to the established mechanism of MAPK activation, the amino acids Thr<sup>221</sup> and Tyr<sup>223</sup> residing in the activation loop (46) are the expected sites of phosphorylation. Indeed, only a single major phosphopeptide containing phosphothreonine and phosphotyrosine was found in the analysis of a 49-kDa phosphoprotein in AtMPK6 immunoprecipitates. To our knowledge, this is the first report presenting direct evidence for tyrosine phosphorylation in plant cells on the basis of *in vivo* labeling and phosphoamino acid analysis. The opportunity to analyze the phosphorylation status of AtMPK6 will allow us to study in detail the mode and kinetics of its activation as well as deactivation.

There is a characteristic lag phase between 30 s and several minutes in the earliest responses of the cells to all elicitors used here, in particular with respect to H<sup>+</sup> and Ca<sup>2+</sup> influx, K<sup>+</sup> efflux, oxidative burst, and protein phosphorylation (32). A comparison of the time courses of induction of kinase activity with changes in the pH of the extracellular medium clearly shows that both are initiated with a similar lag phase between one and several minutes, placing the MAPK in the first tier of elicitor responses. Because of the time needed for sample handling, in most cases it was not possible to say if one response preceded the other or whether both occur in parallel. Although after treatment with pectic fragments the pH increased prior to MAPK activation, the changes in ion fluxes are probably not functionally upstream and probably not the cause of activation, since AtMPK6 is not activated in ionophore-treated cells. The extremely transient activation of AtMPK6 after treatment with pectic fragments is interesting, since it raises the question of how the rapid down-regulation is accomplished. There is an indication of a possible mode of plant MAPK deactivation in the observation of an increase in transcript levels of phosphatase 2C, as observed 5 minutes after wounding of alfalfa plants (47). The relatively slow decrease in MAPK activity after stimulation with flagellin, chitin, and xylanase is compatible with such a mechanism. The transcriptional activation of a MAPK phosphatase within 3 min after perception of oligogalacturonides, however, seems unlikely. In other eukaryotes, both transcriptional (48) and posttranslational (49, 50) activation of phosphatases was reported following MAPK activation. It is possible that the pectic fragments, being derived from plant cell walls (51), activate a different subset of cellular responses than elicitors of fungal or bacterial origin, including the recruitment of a phosphatase that rapidly down-regulates MAPK activity. Further studies on the balance between MAPK activities and MAPK phosphatase activities will be necessary to address this question.

Classic genetic analyses of disease resistance in *Arabidopsis* has proven to be a potent method for identification of genes involved in the recognition of specific avirulence gene products, namely the R genes (29). It has been argued that the failure to identify many downstream signal components may be due to lethality of mutations as a number of R genes converge into relatively few pathways (52). Indeed, it has been demonstrated that multiple R genes require NDR1 for signaling, while another subset of R genes require EDS1, defining at least two disease resistance pathways (53). Our results as well as the work of other laboratories studying the regulation of MAP

<sup>3</sup> S. Peck and T. Nühse, unpublished data.

kinases support the concept of a convergence of signaling pathways put forth by these genetic studies. Here, we have shown that pathways involved in the recognition of general elicitors from plants, bacteria, and fungi converge to activate AtMPK6. In tobacco, signals from general (22, 38) and race-specific (23, 26) elicitors as well as wound signals (25) also converge at the level of SIPK or earlier. In the future, it will be interesting to integrate genetics and biochemistry by studying the regulation of AtMPK6 in the available mutants, particularly in *NDR1* and *EDS1*, to determine which of the known pathways utilize this MAP kinase.

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