

Differential Activation of Four Specific MAPK Pathways by Distinct Elicitors*

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Plant cells respond to elicitors by inducing a variety of defense responses. Some of these reactions are dependent on the activity of protein kinases. Recently, mitogen-activated protein kinases (MAPKs) have been identified to be activated by fungal and bacterial elicitors as well as by pathogen infection. In gel kinase assays of alfalfa cells treated with yeast cell wall-derived elicitor (YE) revealed that 44- and 46-kDa MAPKs are rapidly and transiently activated. Immunokinase assays with specific MAPK antibodies revealed that YE mainly activated the 46-kDa SIMK and the 44-kDa MMK3 and to a lesser extent the 44-kDa MMK2 and SAMK. When cells were treated with chemically defined elicitors potentially contained in the YE (chitin and *N*-acetylglucosamine oligomers, β -glucan, and ergosterol), the four MAPKs were found to be activated to different levels and with different kinetics. Whereas SIMK and SAMK have been found to be activated by a number of diverse stimuli, MMK3 is activated during mitosis and was therefore assumed to participate in cell division (22). No physiological process could be associated with MMK2 activity so far. This is the first report that MMK2 and MMK3 can be activated by external stimuli. Overall, our findings indicate that plant cells can sense different cues of a given microorganism through the activation of multiple MAPKs.

As sessile organisms, plants must cope with a number of adverse environmental conditions, including abiotic stresses, such as cold, drought, and UV irradiation, but also biotic challenges by pathogens. To withstand pathogen attack, plants have developed the ability to sense elicitors, signals that are derived from pathogens or degradation products of plant cell walls. Sensing of elicitors is followed by intracellular signal transduction resulting in defense reactions such as the generation of reactive oxygen species (oxidative burst), expression of genes encoding pathogen-related proteins, the synthesis of phytoalexins, and programmed cell death. Elicitor signaling was shown to involve protein phosphorylation (1–3), suggesting that protein kinase cascades are involved in the intracellular information transfer. The importance of protein kinases was

demonstrated by the ability of protein kinase inhibitors to block a variety of defense responses including the elicitor-induced medium alkalization, oxidative burst, and defense gene transcription (1, 3–5). A specific class of serine/threonine protein kinases, denoted as MAP kinases (MAPKs),¹ was shown to be activated by elicitors. In tobacco cells, MAP kinases are activated by elicitors derived from fungi (6, 7) and bacteria (8). An elicitor derived from the fungus *Phytophthora sojae* was shown to activate a MAP kinase in parsley cells (9). In intact tobacco, and in dependence of a functional product of the disease resistance *N* gene, tobacco mosaic virus infection leads to the activation of the MAP kinases SIPK and WIPK (10). Recently, it was shown that the *Cladosporium fulvum* avirulence gene product Avr9 can induce activation of SIPK and WIPK in tobacco cells that express a functional copy of the tomato disease resistance gene *Cf9* (11). These data indicate that MAP kinase cascades are involved in defense signaling and may constitute a major mechanism for activation of defense responses by various elicitors. MAP kinases have also been implicated in mediating other signals, such as hormones, touch stimuli, cold and drought, and osmotic stress (12–16). MAP kinases are also involved in wound signaling. Mechanical wounding of leaves induces the activation of MAPKs belonging to the SIMK and SAMK subfamilies in alfalfa² (17) and SIPK and WIPK in tobacco (18, 19).

In this study, we investigated the possibility that MAP kinases might also be involved in elicitor signaling in alfalfa. Treating suspension-cultured cells with yeast elicitor (YE) induces typical defense responses, such as the rapid alkalization of the medium and the production of active oxygen species (20). In-gel kinase analysis of extracts from YE-treated cells indicated that protein kinases with relative molecular masses of 46 and 44 kDa are rapidly and transiently activated after elicitor treatment. Using specific antibodies, we identified the YE-activated 46- and 44-kDa kinases as the SIMK, MMK2, MMK3, and SAMK MAP kinases. Multiple and differential activation of the four MAP kinases was observed with defined components of the YE. Although the MMK2 MAPK has been shown to specifically complement the yeast MPK1 MAPK (21), no physiological process could be associated with the activity of this MAPK so far. MMK3 has been shown to be activated in

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¹ The abbreviations used are: MAPK, mitogen-activated protein kinase; MAP, mitogen-activated protein; SIMK, salt-induced MAPK; SAMK, stress-activated MAPK; MMK2 and -3, *Medicago* MAPK2 and -3, respectively; SIPK, salicylate-induced protein kinase; WIPK, wound-induced protein kinase; YE, yeast elicitor; NAGA, *N*-acetylglucosamine; MBP, myelin basic protein; DP, degree of polymerization; PAGE, polyacrylamide gel electrophoresis.

² E. Baudouin, I. Meskiene, C. Jonak, A. Gross, S. Kiegerl, P. L. Rodriguez, and H. Hirt, submitted for publication.

mitosis (22), but no external stimulus has so far been defined that can activate these MAPKs. This study demonstrates that MMK2 and MMK3 activity can be induced by elicitors, suggesting their possible involvement in pathogen signaling and providing new tools for the investigation of plant-microbe interactions at the molecular level.

EXPERIMENTAL PROCEDURES

Cell Culture Handling and Treatment—Suspension cultures were prepared from callus derived from alfalfa roots (*Medicago sativa* L.) and maintained as described (20). Log phase cells were used 3–4 days after a weekly 1:3 dilution in fresh medium. For elicitor experiments, aliquots of cell cultures (10–15 ml) were prepared and grown in small Erlenmeyer flasks for several hours on a rotary shaker before treatment. Chemicals to be tested were delivered to the cells in water or Me₂SO (1 μ l per ml of cells; chitin, β -glucan, and the glycopeptide elicitor in 10 μ l of water per ml of cells). At given time points, 2-ml aliquots were removed from the shaking flasks, quickly harvested by filtration under slight vacuum, snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

Preparation and Sources of the Elicitors—The YE was prepared essentially as described (20). Briefly, bakers' yeast (250 g) was dissolved in 500 ml of citrate buffer (20 mM citrate, pH 7.5) by stirring at room temperature for 30 min. After autoclaving, the slurry was cleared by centrifugation (20 min, 7000 \times g); the supernatant was filtered and stirred overnight at 4 $^{\circ}\text{C}$ with 1 volume of ethanol. After centrifugation, the pellet was allowed to dissolve overnight in water and cleared again by centrifugation. The resulting supernatant was dialyzed overnight against water (Spectra-pore, molecular weight cut-off of 1000) and freeze-dried. 1 mg of the elicitor corresponded to a glucose equivalent of 185 μ g.

The glycopeptide was derived from yeast invertase (23). The *N*-acetylglucosamine (NAGA) oligomers of degree of polymerization (DP) 7–10 and >10 were prepared as described (24). The average molecular mass for the DP 7–10 and >10 fractions was considered to be 1.745 and 2.5 kDa, respectively.

All other compounds tested were chemically defined and obtained from Sigma: ergosterol, cholesterol, β -glucan, chitin, and NAGA oligomers of DP 1–5. The NAGA oligomer of DP 6 was obtained from Seikagaku, Japan.

Protein Extraction—Protein extracts were prepared by grinding frozen cells for 2 min in extraction buffer (25 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM NaVO₃, 15 mM *p*-nitrophenyl phosphate, 0.1% Tween 20, 15 mM β -glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 5 μ g/ml leupeptine, 5 μ g/ml aprotinin). After centrifugation at 20,000 \times g for 45 min, the cleared supernatant was immediately used for further analysis.

Antibody Production—The synthetic peptides FNPEYQQ, VRFNPD-PPIN, LNFCKEQILE, and LNPEYA, corresponding to the carboxyl-terminal parts of SIMK, MMK2, MMK3, and SAMK, respectively (14, 16, 21, 22), were conjugated to a purified derivative of tuberculin. Polyclonal antisera were raised in rabbits and finally purified by protein A column chromatography to produce the M23, M11, M14, and M24 antibodies.

Immunoblotting—Western analysis was essentially performed as described (17). Briefly, cell extracts containing 20 μ g of total protein were separated by SDS-PAGE, immunoblotted onto polyvinylidene difluoride membranes (Millipore Corp.), and probed with the M23, M11, M14, and M24 antibodies at a dilution of 1:10,000. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody, and the reaction was visualized by fluorography using CDP-Star (Amersham Pharmacia Biotech) as a substrate.

In-gel Kinase Activity Assays—For in-gel protein kinase reactions, cell extracts containing 20 μ g of total protein/lane were separated by SDS-PAGE. Alternatively, protein samples were used that had been immunoprecipitated with the different MAPK antibodies (see below). Myelin basic protein (MBP; 0.1 mg/ml) was polymerized in the gel and used as a substrate for the kinase reaction. Protein denaturation, renaturation, and kinase reactions were performed as described (26).

In Vitro Kinase Activity Assays—Cell extracts containing 100 μ g of total protein were immunoprecipitated overnight with 5 μ g of protein A-purified M23, M11, M14, and M24 antibodies for SIMK, MMK2, MMK3 and SAMK, respectively. The immunoprecipitated kinases were washed three times with wash buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% Tween 20, 5 mM NaF) and once with kinase buffer (20 mM HEPES, pH 7.4, 15 mM MgCl₂, 5 mM EGTA,

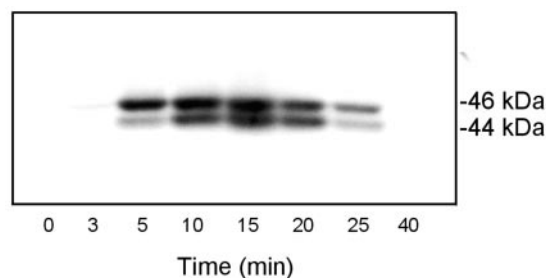


FIG. 1. The YE induces the activation of 46- and 44-kDa protein kinases in suspension-cultured cells. Alfalfa cells were treated with 500 μ g/ml of YE. At the indicated times, cell extracts were prepared. Extracts containing 20 μ g of total protein were separated by SDS-PAGE containing 0.1 mg/ml myelin basic protein. After protein renaturation, kinase reactions were performed in the gel and analyzed by autoradiography. The relative distance migrated by a 44- and 46-kDa marker protein is indicated.

1 mM dithiothreitol). Kinase reactions were performed as described (17). Briefly, the immunocomplexes were incubated for 30 min at room temperature in 15 μ l of kinase buffer containing 1 mg/ml MBP, 0.1 mM ATP, and 2 μ Ci of [γ -³²P]ATP. The reaction was stopped by adding SDS-PAGE loading buffer, and the phosphorylation of MBP was analyzed by autoradiography or quantified by PhosphorImager analysis after SDS-PAGE. For peptide competition analyses, 100 μ g of total proteins were subjected to a 2-h immunoprecipitation with 2 μ g of M23, M11, M14, or M24 protein A-purified antibodies that had been preincubated overnight with different amounts of the peptide against which the sera were raised; an *in vitro* kinase assay was then performed on the immunoprecipitated proteins, as described above.

RESULTS

YE Induces Protein Kinase Activation in Alfalfa Cells—Among the various responses to elicitor molecules in plants, the induction of MAPKs was reported in different systems (27, 28). As a first test for the activation of MAP kinases by elicitors in alfalfa, suspension-cultured cells were treated with YE. Aliquots from YE-treated cells were collected at different time points and analyzed by in-gel kinase assays using MBP as an artificial substrate. As shown in Fig. 1, protein kinases with relative molecular masses of 46 and 44 kDa are rapidly activated in elicitor-treated cells. The 46- and 44-kDa protein kinases revealed maximum activation levels at 15 min after elicitor addition before becoming inactivated by 40 min. These data show that the yeast elicitor rapidly induces the transient activation of protein kinase pathways in alfalfa cells.

The M23, M11, M14, and M24 Antibodies Specifically Recognize SIMK, MMK2, MMK3, and SAMK—To investigate whether the 46- and 44-kDa protein kinases correspond to known MAPKs from alfalfa, YE-treated cell extracts were immunoprecipitated with antibodies specifically recognizing SIMK, MMK2, MMK3, and SAMK. The specificity of the antibodies on the corresponding recombinant proteins was previously shown by Western blotting (16). Additional proof was obtained when elicitor-induced SIMK, MMK2, MMK3, and SAMK activity could be competed with the peptides against which the corresponding antibodies were raised (Fig. 2A). Equal amounts of total protein extracted from YE-treated cells were immunoprecipitated with antibodies that had been preincubated with increasing amounts of peptide. The immunoprecipitated MAPKs were subjected to *in vitro* kinase assay using MBP as a substrate, and the reaction products were analyzed by SDS-PAGE followed by autoradiography.

In order to discard the possibility of carry-over or nonspecific binding among the four MAPKs under study and their antibodies, *in vitro* translated ³⁵S-labeled SIMK, MMK2, MMK3, and SAMK were immunoprecipitated, separated by SDS-PAGE, and analyzed by autoradiography. Only the antibody that was raised against the respective C-terminal MAPK sequence could

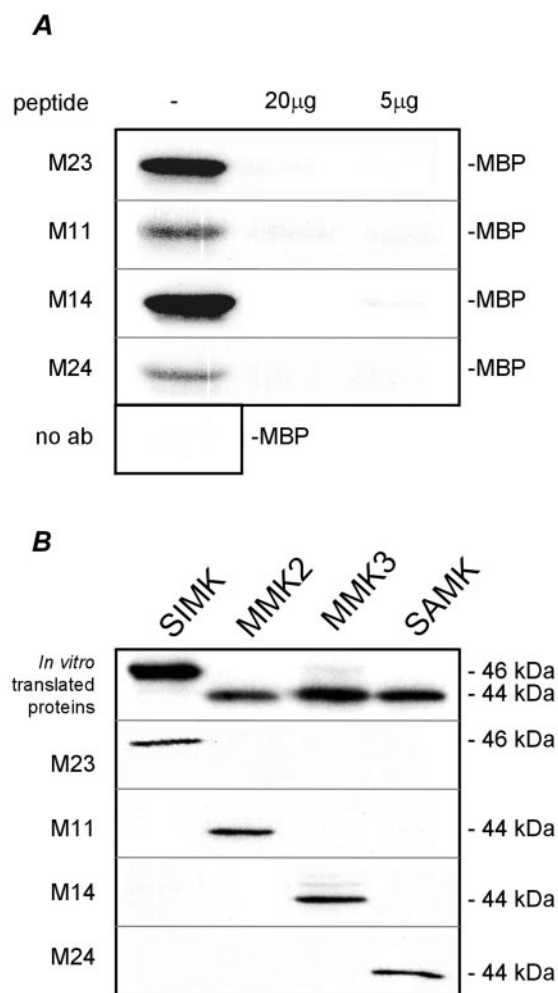


FIG. 2. Specificity of SIMK, MMK2, MMK3, and SAMK antibodies. M23, M11, M14, or M24 antibodies were raised against synthetic peptides encoding the C-terminal 7, 10, 10, and 6 amino acids of SIMK, MMK2, MMK3, and SAMK, respectively. Their specificity was previously tested on recombinant protein kinases (16). **A**, efficient peptide competition of the immunoprecipitation capacity of the antibodies. 100 µg of total protein extract from a YE-treated sample (15 min) were immunoprecipitated with 2 µg of protein A-purified M23, M11, M14, or M24 antibodies that had been preincubated alone or in the presence of different amounts of the peptides against which the antibodies had been raised. The immunoprecipitated MAPKs were analyzed by *in vitro* kinase assay using radiolabeled ATP and MBP as a substrate. After SDS-PAGE, the degree of MBP phosphorylation was visualized by autoradiography. The immunoprecipitation of YE-induced MAPK activity was efficiently competed by the appropriate peptides. **B**, specificity of the antibodies with respect to the immunoprecipitation of the respective MAPKs. SIMK, MMK2, MMK3, and SAMK were labeled with [³⁵S]methionine by *in vitro* translation and immediately analyzed by SDS-PAGE and autoradiography (*upper panel*; total proteins not immunoprecipitated). Alternatively, each of the kinases was separately immunoprecipitated with M23, M11, M14, or M24 antibodies (*lower panels*). Only the appropriate antibody could pull down the respective kinase.

precipitate the corresponding MAPK protein (Fig. 2B). These results indicate that M23, M11, M14, and M24 antibodies specifically immunoprecipitate SIMK, MMK2, MMK3, and SAMK, respectively.

SIMK, MMK2, MMK3, and SAMK Are Activated by the YE—A variety of bacterial and fungal elicitors as well as pathogen attack result in the activation of MAPKs belonging to the SIMK and/or SAMK subfamilies in tobacco, parsley, *Arabidopsis*, and tomato (7, 9–11, 29). This suggests that these two classes of MAP kinases might be general mediators of elicitor-induced responses in plants. To determine whether

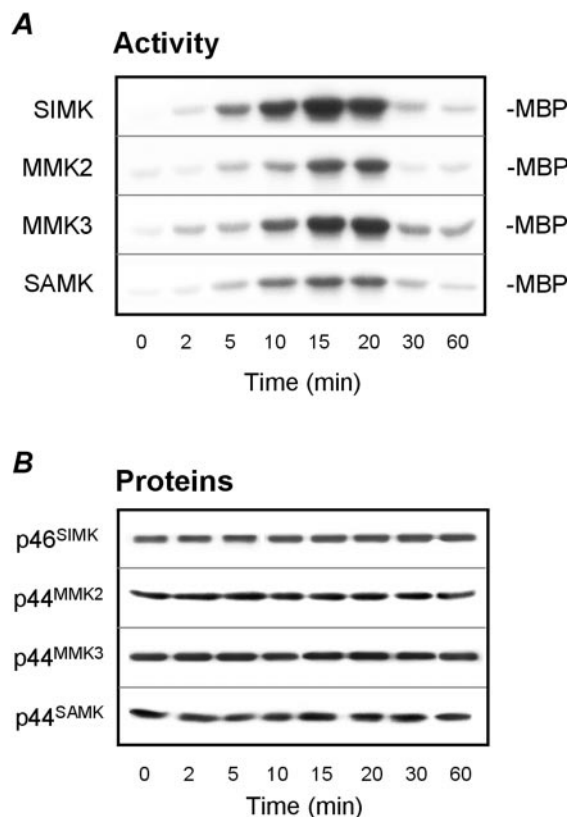


FIG. 3. The elicitor-activated 46- and 44-kDa protein kinases are MAP kinases encoded by the SIMK, MMK2, MMK3, and SAMK genes. **A**, immunokinase analysis of the elicitor-induced activation of SIMK, MMK2, MMK3 and SAMK. Extracts from elicitor-treated alfalfa cells, containing 100 µg of total protein, were immunoprecipitated with 5 µg of protein A-purified M23, M11, M14, or M24 antibody, respectively. Kinase reactions were performed with 1 mg/ml MBP as a substrate, 0.1 mM ATP, and 2 µCi of [³²P]ATP. Phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE. **B**, SIMK, MMK2, MMK3, and SAMK protein amounts do not change after elicitor treatment. YE-treated cell extracts (20 µg/lane) were separated by SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and decorated with M23, M11, M14, or M24 antibodies.

the YE-activated 46- and 44-kDa MAPK activities correlated with the activation of SIMK and SAMK, cell extracts were analyzed by immunokinase assays using the SIMK and SAMK-specific antibodies M23 and M24. SIMK was strongly activated by the YE, whereas SAMK revealed a comparatively weaker response (Fig. 3A). We also tested if activation of two other MAP kinases, MMK2 and MMK3, could be detected by kinase assays of MAPKs immunoprecipitated with the specific antibodies M11 and M14. As shown in Fig. 3A, both MMK3 and MMK2 were activated in response to the YE. The four MAP kinases showed similar activity profiles with a maximum of activity at about 15 min after YE addition (Fig. 3A). These results demonstrate that YE can activate several distinct MAP kinase pathways in alfalfa cells. They also show that the major MBP-phosphorylating activity associated with the 46- and 44-kDa MAPKs identified in the in-gel kinase assays of YE-treated cell extracts (see Fig. 1) is due to the activation of at least four distinct MAPKs.

To determine whether the protein amounts of the MAPKs change in response to YE, 20 µg of total protein from elicitor-treated cells were separated by SDS-PAGE and immunoblotted with the MAPK-specific antibodies. As shown in Fig. 3B, M23, M11, M14, and M24 antibodies detected protein bands of 46, 44, 44, and 44 kDa, respectively, corresponding to the SIMK, MMK2, MMK3, and SAMK proteins. In contrast

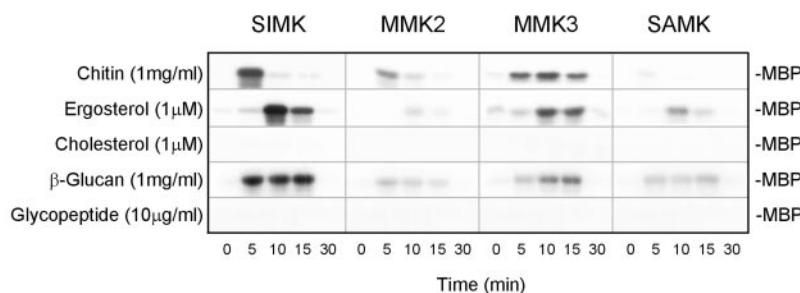


FIG. 4. **Differential activation of MAP kinase pathways by several components of the YE.** Time course analysis of the activation of SIMK, MMK2, MMK3, and SAMK by several components of the raw YE mixture was performed. Their activity was monitored at the indicated time points by immunokinase analysis; samples were electrophoresed, and MBP phosphorylation was shown by autoradiography.

to the changes in kinase activities, the amounts of the different MAPK proteins did not vary over the experimental period of 60 min. These data indicate that the transient activation of all four MAPK kinases occurs by a post-translational mechanism.

Differential Activation of Specific MAPK Pathways by YE Components—Given the complex mixture of chemical substances in the YE preparation, we investigated the possibility that specific MAPKs might respond to specific elicitors and that the complex activation pattern of the MAPKs could reflect the presence of distinct elicitors in the YE. For this purpose, several substances that are present in the YE and have been shown to possess elicitor activity were tested for their capacity of inducing the activation of the four distinct MAP kinase pathways. In addition, time course studies of MAPK activation were performed at different elicitor concentrations to define the threshold of perception and saturation.

Chitin Mainly Activates SIMK, MMK2, and MMK3—Chitin is a common constituent of fungal cell walls and has been shown to induce medium alkalization in tomato cells (24, 30). In alfalfa cells, chitin resulted in the transient activation of all four MAPK pathways, with a peak at 5 min for SIMK and MMK2. MMK3 activity showed a broader peak with a maximum at about 10 min (Fig. 4). SAMK was only activated to a minor degree. The results of dose-response studies confirmed the specific and highly sensitive perception of chitin, revealing a concentration threshold between 10 and 100 ng/ml (data not shown) as reported for tomato cells (24, 31).

Ergosterol Preferentially Activates SIMK, MMK3, and SAMK—Ergosterol is the main sterol of most fungi, and tomato cells can perceive it as a non-self-determinant in a highly sensitive and selective manner (32). The animal analogue cholesterol was taken as a negative control, since it was not perceived as an elicitor by tomato cells. Whereas cholesterol was unable to activate any MAPK, saturating concentrations of ergosterol were found to mainly activate SIMK, MMK3, and SAMK (Fig. 4). SIMK and SAMK activities peaked at about 10 min, and MMK3 activity peaked between 10 and 15 min. In agreement with a previous report (32), the sensitivity threshold of ergosterol was determined to be between 1 and 10 μ M (data not shown), which is in agreement with the assumption of a high affinity perception system in alfalfa cells.

β -(1,3)-Glucan Induces Activation of All Four MAPKs—The β -glucan elicitor is mainly composed of β -(1,3) glucose chains containing small amounts of β -(1,6)-linked glucose side branches. β -glucan is a component of the cell walls of many fungi and was shown to induce alkalization in tomato cells (24). In our system, β -glucan efficiently induced all four MAPKs, with broad activation profiles between 5 and 15 min for all MAPKs (Fig. 4). The dose-response threshold for this elicitor was compatible with previous observations that revealed the elicitation of several responses at concentrations between 10 and 100 ng/ml (data not shown; Ref. 24).

A Glycopeptide Elicitor Is Not Able to Induce the Activation of SIMK, MMK2, MMK3, and SAMK—A glycopeptide derived from yeast extracellular invertase was also tested as elicitor. This glycopeptide was shown to induce ethylene biosynthesis at very low doses in tomato cells, mimicking the response to a complex mixture of glycopeptides originally extracted from the YE (23, 33). The glycopeptide from yeast invertase was used at a maximal concentration of 10 μ g/ml but gave no induction of SIMK, MMK2, MMK3, or SAMK (Fig. 4). The elicitor concentration used was more than 100-fold higher than the saturation threshold for the ethylene response in tomato cells (23).

Chitin Fragments of DP Higher than 2 Are Able to Induce MAPK Activity—Chitin is an elicitor of defense responses and was able to activate SIMK, MMK2, MMK3, and to a lesser extent also SAMK. In some systems, chitin fragments can also act as elicitors, but short oligomers with a DP below 3 are unable to elicit a response (24). To investigate whether the chitin fragment length is important for the activation of specific MAPK pathways, we treated alfalfa cells with various NAGA oligomers spanning the whole range of DP 1–10. As shown in Fig. 5, *N*-acetylglucosamine and its dimer were unable to activate any of the four MAPK pathways. However, NAGA oligomers of DP 3 and higher induced the activation of the four MAPK pathways to levels and with kinetics similar to those induced by the chitin polymer. These results demonstrate that a minimum length of three glucosamine residues of the sugar backbone is required for the NAGA oligomers to be perceived. Moreover, our data suggest that at least four distinct MAP kinase pathways are involved in signaling this class of elicitors.

Differential Sensitivity of MAPKs to In-gel Kinase Conditions—In order to determine whether immunoprecipitated kinase activities corresponded to the MBP kinase activities detected by in-gel kinase assays, protein extracts of suspension-cultured cells that had been treated with chitin for 5 min (see Fig. 4) were immunoprecipitated with M23, M11, M14, and M24 antibodies. The immunoprecipitated MAPKs were subjected to SDS-PAGE and in-gel kinase activity assays. Under these conditions, single active protein kinases could be identified whose migration corresponded to that of the 46-kDa SIMK and to the 44-kDa MMK3 and SAMK (Fig. 6). The immunoprecipitated activities of SIMK, MMK3, and SAMK also corresponded to the relative activities observed in total extracts of chitin-treated cells (Fig. 6, *Total extract*). Surprisingly, no active MMK2 could be detected by in-gel kinase assays after immunoprecipitation with the MMK2-specific antibody (Fig. 6). Since MMK2 was found to be activated strongly by chitin in immunokinase assays (Fig. 4), the lack of detectable MMK2 activity in the in-gel kinase assay cannot be explained by inefficient immunoprecipitation of MMK2. The most likely explanation is that MMK2 is unable to renature into an active conformation under the conditions of the in-gel kinase assay.

FIG. 5. Time course analysis of MAPKs activated by NAGA oligomers. Cell suspension cultures were treated with NAGA oligomers of different DP at the final concentration of 1 μ M. Aliquots (2 ml) of treated cells were harvested and extracted at given time points. 100 μ g of them were immunoprecipitated with M23, M11, M14, or M24 antibodies and tested for specific kinase activity using MBP as a substrate.

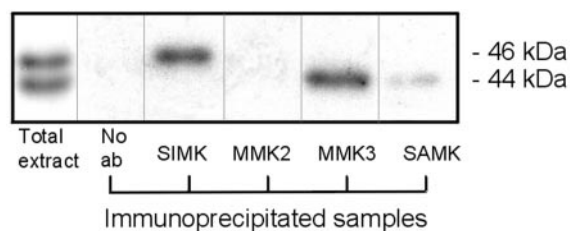
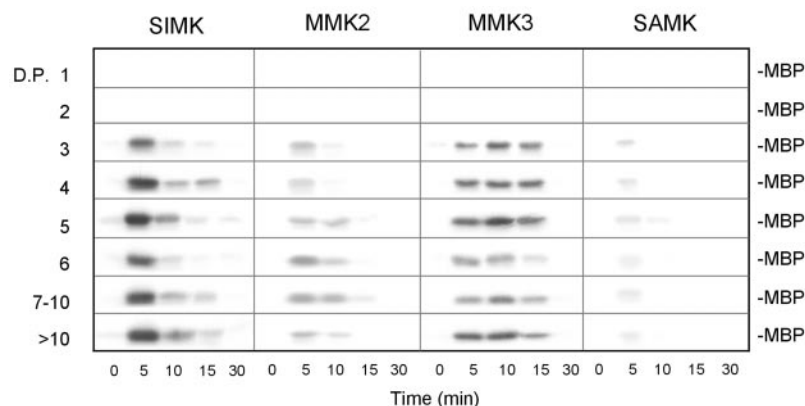


FIG. 6. Differential sensitivity of distinct MAPKs to in-gel kinase assay conditions. Total protein extracts from cells collected 5 min after chitin treatment (see Fig. 4) were immunoprecipitated with 5 μ g of M23, M11, M14, or M24 antibodies before SDS-PAGE and in-gel kinase activity assays. Single active bands of the appropriate molecular weight could be identified that corresponded to the 46- and 44-kDa active kinases shown in the positive control (20 μ g of total protein extract not immunoprecipitated; far left lane).

Furthermore, this experiment gives additional proof for the specificity of the antibodies showing no unspecific physical binding (as already proven in Fig. 2B and by Ref. 16) and no carry-over of foreign MAPK activity. Indeed, SIMK activity, which is visualized in this assay as a band migrating at 46 kDa, does not contaminate any of the three samples immunoprecipitated with MMK2, MMK3, and SAMK antibodies. *Vice versa*, the activity precipitated by the SIMK antibody is not contaminated by any activity for which a 44-kDa protein (such as MMK3 and SAMK) would be responsible. Samples immunoprecipitated with the MMK2 antibody are clearly devoid of MBP-phosphorylating activity from any of the three other MAPKs. Although this test would not allow us to exclude cross-reaction between MMK3 and SAMK antibodies, and *vice versa*, the activation of MMK3 but not of SAMK during cell cycle speaks against carry-over of MMK3 by the SAMK antibody (22). On the other hand, the wound-induced activation of SAMK, but not of MMK3, excludes the possibility of unspecific precipitation of SAMK by the MMK3 antibody (17).

DISCUSSION

Protein phosphorylation has been shown to be involved in mediating elicitor-induced defense responses in a number of systems. Although the responsible protein kinases and phosphatases are largely unknown, recent evidence indicates that MAP kinases are involved in these pathways (7, 9–11, 34–38). Previous reports identified SIMK and/or SAMK homologues as the MAP kinases activated upon elicitor treatment or pathogen infection in parsley (9), tobacco (7, 10, 11), and *Arabidopsis* (37). The 60-kDa BWMK1 from rice is so far the only MAP kinase that is induced by fungal infection and wounding (35) and does not belong to the SIMK or SAMK subfamilies (12).

Our results demonstrate that distinct elicitors not only activate SIMK and SAMK, but also MMK2 and MMK3, two MAP

kinases that have not been connected to stress signaling so far. In fact, MMK2 and MMK3 could not be activated in leaves upon wounding, cold, heat, and drought (14, 17), or in suspension-cultured cells upon osmotic stress (16). What could be the role of MMK2 and MMK3 in the response to biotic stresses? MMK2 was shown to be a functional kinase that can specifically complement yeast cells with a deficiency in the yeast MAPK Mpk1 (21). Similar to several cytoskeletal mutants, Mpk1-deficient cells show a cell wall defect and suffer from aberrant vesicular transport and cisternal membrane accumulation, suggesting that Mpk1 might regulate the cytoskeleton. In accordance with such a role, MMK2 was able to specifically phosphorylate a 39-kDa cytoskeletal protein (21). Dramatic changes and rearrangements of the cytoskeleton are observed upon pathogen attack (39). Although it would be highly interesting to identify a pathogen-responsive protein kinase that is involved in the regulation of the cytoskeleton, further studies are required to clarify whether MMK2 plays such a role.

MMK3 has been shown to be transiently activated during mitosis (22). During cytokinesis, MMK3 was found to be associated with the plane of cell division, a site where microtubule-based transport and fusion of vesicles takes place to form the phragmoplast. These data suggest a role for MMK3 in the regulation of cell cycle-dependent cell wall formation (22). Plant cells restructure and fortify their cell walls upon pathogen attack. It is therefore tempting to speculate that the elicitor-induced activation of the MMK3 pathway could serve a role in regulating certain aspects of this process, but more work is necessary to test this hypothesis.

Convergence of R/Avr-dependent signal transduction pathways, non-race-specific elicitors, and even abiotic stimuli at the level of MAP kinases has been shown in tobacco. In this system, the activation of SIPK is observed upon treatment by non-race-specific elicitors (7), as well as upon Avr9/Cf9-dependent recognition (11). Furthermore, SIPK was shown to be activated by pathogen infection (10) and abiotic stimuli like wounding and mechanical stress (19). We have reported that various abiotic stresses can activate SIMK (16) and SAMK (13, 14, 17), the alfalfa homologues of tobacco SIPK and WIPK, respectively. In this study, we show that different elicitors can also activate SIMK and SAMK, indicating that multiple signals must be integrated for stress activation of SIMK and SAMK pathways. In addition, we identified two elicitor-responsive MAPKs that were not found to be activated by abiotic stress and whose activation could be regarded as a landmark of cellular responses to biotic as compared with abiotic stress.

Given the heterogeneous chemical composition of the YE, it was unclear whether the activation of multiple MAP kinase pathways was due to a single component or to the combined effects of several factors present in the yeast elicitor preparation. To address this question, we treated cells with chemically

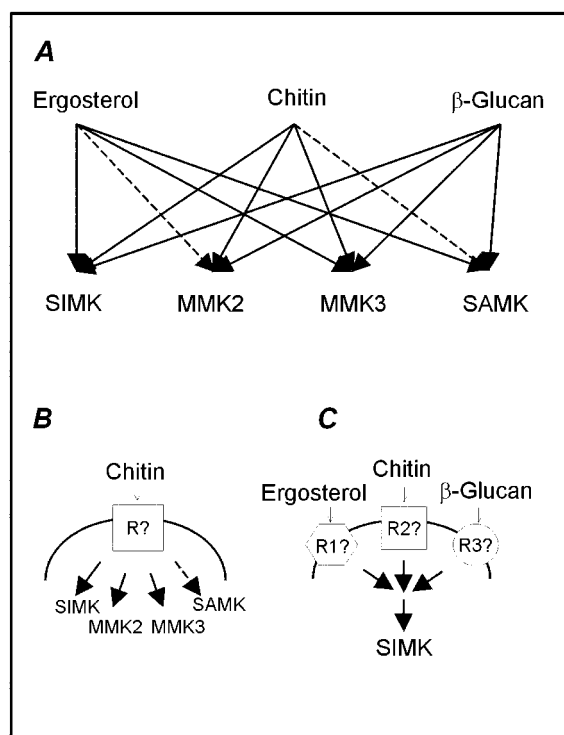


FIG. 7. Schematic representation of signal divergence and convergence in the alfalfa cell system. A, observed activation patterns of SIMK, MMK2, MMK3, and SAMK by different elicitors. The solid arrows indicate strong induction; dashed arrows show weaker activation. B, divergence of signal transduction downstream of a single receptor as shown for the activation of SIMK, MMK2, MMK3, and SAMK by chitin. C, convergence of signal transduction from distinct receptors into one MAPK cascade as shown for SIMK activation by ergosterol, chitin, and β -glucan.

defined substances that are potentially present in the YE preparation and for which elicitor activity has been demonstrated (24, 29, 30, 40). Immunokinase analysis confirmed that chitin, ergosterol, and β -glucan have the ability to activate MAPK pathways. It is noteworthy that the activation of the MAPK pathways occurred in a dose-dependent manner. To ensure receptor saturation, the experiments presented in Figs. 3–5 were all performed at concentrations at least 100-fold higher than the threshold level of saturation for the respective elicitor. It should also be noted that the absence of MAPK activation by the invertase glycopeptide does not necessarily mean that this substance is not perceived by alfalfa cells, but it could indicate that signaling is not coupled to MAPK cascades. In summary, for chitin fragments, ergosterol, and β -glucan, it is clear that these substances activate different sets of MAPK pathways (Fig. 7A). In at least these three cases, the specificity of the chemoperception system appears to be reflected in the combinatorial pattern as well as the kinetics and levels of the activated pathways. In analogy to proposed models in animal and yeast signaling (reviewed in Refs. 41 and 42), it is tempting to speculate that the response to a given elicitor might be specified not only by the particular mix of engaged pathways but also by the duration and level of their activities.

How can a plant cell integrate stimuli coming from the perception of substances that are so diverse in chemical composition and structure? The observation that a given elicitor can activate several distinct MAPKs suggests that soon after elicitor perception branching into several transduction pathways must occur (Fig. 7B). It is not yet clear how branching into multiple cascades occurs at the molecular level, but MAPK

kinase kinases are good candidates for such a function, because they can regulate diverse MAPK kinases that in turn are responsible for activation of distinct MAPKs. At the same time, we have also observed that in some cases elicitors of completely different chemical composition and structure can activate the same MAPK pathways (Fig. 7C). These findings indicate that convergence of distinct signaling pathways must occur upstream or at the level of MAPKs. At present, it is mostly unclear how branching and integration of signaling pathways work at the molecular level, but scaffold proteins might give an answer. A recently identified MAPK scaffold protein³ has binding sites for G-proteins and phospholipids and might therefore serve as integration point for multiple upstream signals. Whatever the exact mechanism(s), this discussion makes it clear that our present concept of signal transduction is still very immature. In our view, the identification of the upstream components and of the scaffold proteins of the elicitor-activated MAPK pathways will be essential to understand the interaction and activation of the different cascades in the context of different elicitors. This knowledge should also help to unravel how integration, branching, and cross-talk among different pathways occur at the molecular level.

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