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Transient Activation and Tyrosine Phosphorylation of a Protein Kinase in Tobacco Cells Treated with a Fungal Elicitor

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Suspension-cultured tobacco cells respond to fungal elicitor by activating the transcription of so-called defense genes. This response has been shown to be blocked by staurosporine, an inhibitor of protein kinases, and by Gd^{3+}, which blocks Ca^{2+} channels. We report here that treating tobacco cells with the elicitor triggers the rapid and transient activation of a 47-kD protein kinase that phosphorylates serine and/or threonine residues of the myelin basic protein (MBP). Staurosporine and Gd^{3+} inhibited the elicitor-induced activation of the 47-kD MBP kinase, and staurosporine inhibited the activity of the MBP kinase itself. In the presence of either cycloheximide or calyculin A, the elicitor induced sustained activation of the MBP kinase. Immunoblot and immunoprecipitation analysis using a phosphotyrosine-specific antibody showed that tyrosine phosphorylation of the 47-kD MBP kinase was induced in tobacco cells that had been treated with the elicitor. The results suggest that the 47-kD MBP kinase is a component of the pathway for transduction of the elicitor signal in tobacco cells and that the activity of the MBP kinase is regulated by the post-translational phosphorylation of tyrosine residues.

INTRODUCTION

Higher plants are able to survive exposure to severe physical, chemical, and biological stress. One source of severe stress is infection by pathogens, such as fungi, bacteria, and viruses. When a pathogen penetrates plant tissue by degrading the walls of plant cells, plant cells perceive signals from pathogens. These signals, known as elicitors, are transmitted to the so-called defense genes via intracellular signaling cascades (Dixon and Lamb, 1990).

Evidence has been presented that extracellular signals are transduced via protein phosphorylation, with the eventual induction of expression of specific genes in animal and yeast cells, that various kinds of protein kinases play important roles in such signal transduction systems (Hunter, 1987), and that protein phosphorylation/dephosphorylation cascades alter the phosphorylation status of transcription factors regulating gene expression (Hunter and Karin, 1992). In plant cells, protein kinases have been identified by biochemical methods and by probing for homologies among gene products; it has been proposed that protein kinases may also be important mediators in signal transduction pathways in plant cells (Ranjeva and Boudet, 1987; Trewavas and Gilroy, 1991). In elicitor-treated cells, changes in the phosphorylation status of proteins have been observed (Grab et al., 1989; Dietrich et al., 1990; Grosskopf et al., 1990; Felix et al., 1991), and inhibition of such changes by inhibitors of protein kinases has been demonstrated (Grosskopf et al., 1990; Conrath et al., 1991; Felix et al., 1991). Therefore, it has been postulated that elicitor signals are transduced via a protein kinase cascade in plant cells. However, the identity of the protein kinases and the function of the phosphorylated proteins remain to be determined.

It has been demonstrated in animal and yeast cells that the mitogen-activated protein (MAP) kinases, also known as extracellular signal–regulated protein kinases, are important intermediates in a variety of signal transduction pathways (Thomas, 1992). These kinases form a group of serine/threonine protein kinases, and each is activated as a result of the simultaneous phosphorylation of threonine and tyrosine residues by upstream protein kinases (Payne et al., 1991; Gartner et al., 1992; Posada and Cooper, 1992). The members of the family of genes encoding MAP kinases have been highly conserved during evolution, and they are found in such diverse organisms as mammals, Xenopus, Drosophila, nematodes, and yeasts as well as plants.

In several plant species, the existence of the MAP kinase/extracellular signal–regulated protein kinases family has been demonstrated by the cloning of homologs, and it has been postulated that these kinases are involved in cell proliferation (Duerr et al., 1993; Jonak et al., 1993; Stafstrom et al., 1993; Wilson et al., 1993; Mizoguchi et al., 1994). Mutants of Arabidopsis are proving useful for investigations of signal

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transduction systems in plants, and genes for possible components of the ethylene signal transduction pathway have been identified in several recent studies. These genes encode putative protein kinases related to a histidine kinase, the first component of the so-called two-component system in prokaryotic signal transduction (Chang et al., 1993), and to the Raf family of protein kinases (Kieber et al., 1993). In yeast cells, it has been reported that a two-component system mediates transduction of osmotic signals and that a MAP kinase cascade acts as signal mediator downstream of the two-component system (Maeda et al., 1994). In addition, Raf-1 has been identified as a kinase that phosphorylates MAP kinase kinase in human cells (Kyriakis et al., 1992). Therefore, the MAP kinase is a strong candidate as a component of a signal transduction pathway from extracellular signals to induction of expression of specific genes in plant cells.

To elucidate the regulatory mechanism of expression of defense genes, we are investigating the signal transduction pathway that leads to the expression of defense genes in elicitor-treated tobacco cells. We recently reported that protein phosphorylation appears necessary for activation of the transcription of defense genes in tobacco cells (Suzuki et al., 1995). In this study, we examined protein kinase activity using an in-gel kinase assay with the myelin basic protein (MBP) as the substrate protein, and we examined tyrosine phosphorylation of proteins by using an immunoblotting method with a phosphotyrosine-specific antibody in an attempt to investigate the possible involvement of MAP kinase in transduction of the elicitor signal.

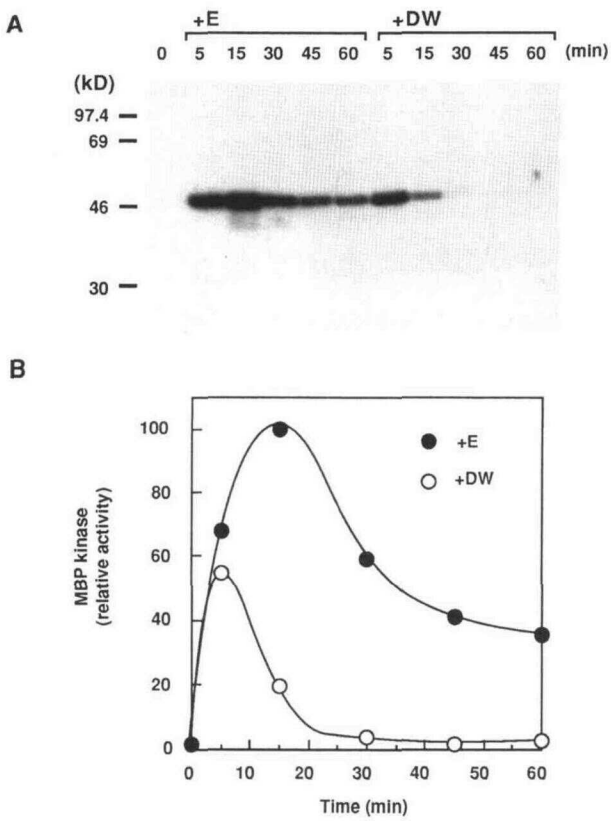

Figure 1. Detection of Protein Kinase Activity by the In-Gel Kinase Assay.

Tobacco cells were transferred to plastic dishes that contained 25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.8, plus elicitor (+E) or distilled water (+DW). After incubation for various periods of time, cells were harvested and crude extracts were prepared for in-gel kinase assays, as described in Methods.

(A) Autoradiogram of a gel after an in-gel kinase assay. The positions of migration of marker proteins are shown at left.

(B) MBP kinase activity quantitated in terms of the intensity of the major band of the phosphorylated MBP shown in **(A)** with a laser densitometer. The data are presented in relative activity.

RESULTS

Detection of a 47-kD MBP Kinase by an In-Gel Kinase Assay

Plants recognize extracellular signals and respond to such signals by activating transcription of specific genes whose expression helps the plant cope with the changing environment. Previous studies have demonstrated that tobacco cells (line XD6S) respond to fungal elicitor by expressing defense genes, and this response has been shown to be blocked by staurosporine, an inhibitor of protein kinases (Suzuki et al., 1995). To establish a functional link between protein phosphorylation and transduction of the elicitor signal that leads to activation of the transcription of defense genes, we have chosen a biochemical approach and have attempted to analyze the protein kinase activated in cells treated with elicitor. In animal and yeast cells, MAP kinases reportedly have been activated rapidly and transiently by extracellular signals and have phosphorylated MBP. We examined whether MAP kinase–like activity could be stimulated by fungal elicitor in tobacco cells using an in-gel kinase assay with MBP as the protein substrate.

Figure 1A shows that after SDS-PAGE of extracts prepared from tobacco cells treated with the elicitor, the activity of one major protein kinase could be detected in the polyacrylamide gel containing the MBP. The activity of the MBP kinase detected by the in-gel assay increased in proportion to protein content per lane and to the duration of incubation in the reaction mixture (data not shown). From the mobility of the MBP kinase activity during SDS-PAGE, the apparent molecular mass of the enzyme was estimated to be ~47 kD. Other faint bands with different mobilities were also detected by prolonged autoradiography, but their activities were unchanged by treatment with the elicitor (data not shown). In a gel without the MBP, much lower phosphorylating activity, which probably represents autophosphorylation, was detected, and the relative position of migration and the activation kinetics were similar to those of MBP kinase activity (data not shown).

Rapid and transient activation of the MBP kinase in elicitor-treated and untreated cells, after their transfer to plastic dishes, was detected (Figure 1B). The level of MBP kinase activity in elicitor-treated cells was much higher than that in untreated cells. The time course of activation of the MBP kinase was also different in each case. In the presence of the elicitor, MBP kinase activity was found to increase within 5 min after the addition of the elicitor, reaching a maximum level after 15 min and rapidly decreasing to half the maximum level after 30 min. Activity then continued to decrease gradually, and the kinase still exhibited some activity after 60 min. In the case of untreated cells, the activity reached a maximum after 5 min and then rapidly decreased to the control level within 30 min after transfer.

Elicitor-Responsive Activation of a 47-kD MBP Kinase

The results of the aforementioned experiment suggest that mechanical stress might be sufficient to stimulate MBP kinase activity in the cells, although the stress-induced activity was transient and activity returned to the basal level within 30 min (Figure 1B). To demonstrate the elicitor-dependent activation of the MBP kinase, the elicitor was added to the medium after a 60-min preincubation that followed transfer to Petri dishes. As shown in Figure 2, the addition of the elicitor to the medium resulted in a second stimulation of MBP kinase activity, whereas the addition of water to the medium had no effect. Therefore, in subsequent experiments, the elicitor was added

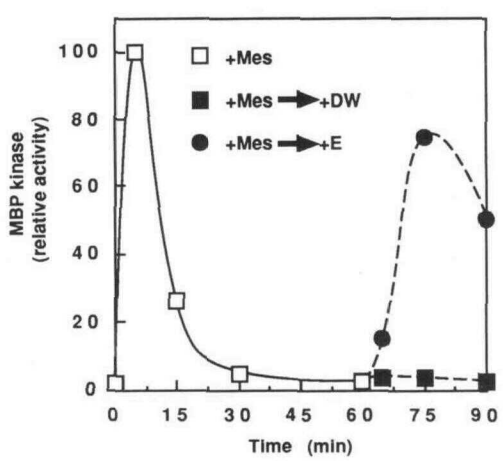

Figure 2. Activation of the MBP Kinase in Response to the Elicitor.

Tobacco cells were transferred to plastic dishes that contained 25 mM 2-(*N*-morpholino)ethanesulfonic acid (+Mes), pH 5.8. After incubation for 60 min, elicitor (+E) or distilled water (+DW) was added to the cell suspension, and incubation was continued for various periods of time. Cells were harvested and crude extracts were prepared for the in-gel kinase assay, as described in Methods. The intensity of the major band of phosphorylated MBP on the autoradiogram of the gel after the in-gel kinase assay was determined by laser densitometry. The data are presented in relative activity.

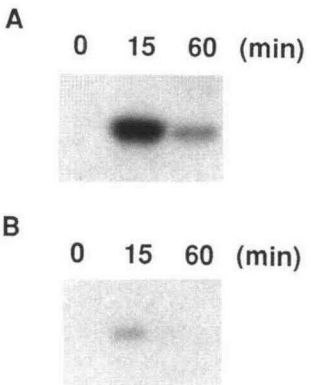

Figure 3. Susceptibility of Phosphorylated Residues in the MBP to Alkali.

Tobacco cells were transferred to plastic dishes that contained 25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.8. After incubation for 60 min, the elicitor was added to the cell suspension, which was then incubated for an additional 0, 15, or 60 min. Cells were harvested and crude extracts were prepared for the in-gel kinase assay, as described in Methods.

(A) Autoradiogram after the in-gel kinase assay.

(B) The same gel as shown in **(A)** after treatment for 2 hr with 1 M KOH.

to cell suspensions that had been preincubated for 60 min after transfer to Petri dishes. To determine whether the phosphorylation of the MBP occurs at serine/threonine or tyrosine residues, the gel was treated with alkali. This treatment selectively removes phosphate groups from phosphorylated serine/threonine residues but not from phosphorylated tyrosine residues (Cooper et al., 1983). Alkali treatment eliminated the radioactivity of the 47-kD band (Figure 3B), an indication that MBP is phosphorylated on serine/threonine residues. The effect of a protein kinase inhibitor on MBP phosphorylation by the 47-kD kinase was examined. The activity of the 47-kD kinase was inhibited by 74 or 86% in the presence of 0.1 or 1.0 μM staurosporine, respectively (data not shown).

Effects of Inhibitors on the Activation of the Elicitor-Responsive MBP Kinase

We reported previously that inhibitors related to the action of intracellular signal mediators block the induction of transcription and the accumulation of mRNAs that correspond to defense genes in cells of the XD6S line (Suzuki et al., 1995). Staurosporine, an inhibitor of protein kinases, and Gd^{3+}, which blocks Ca^{2+} channels in the plasma membrane, were shown to inhibit the elicitor-inducible accumulation of mRNA transcribed from defense genes in tobacco cells. The effects of these inhibitors on the elicitor-activated MBP kinase were examined in this study. Gd^{3+} (0.5 mM) and staurosporine (1 μM) inhibited the activation of the elicitor-activated MBP kinase (Figures 4A and 4B).

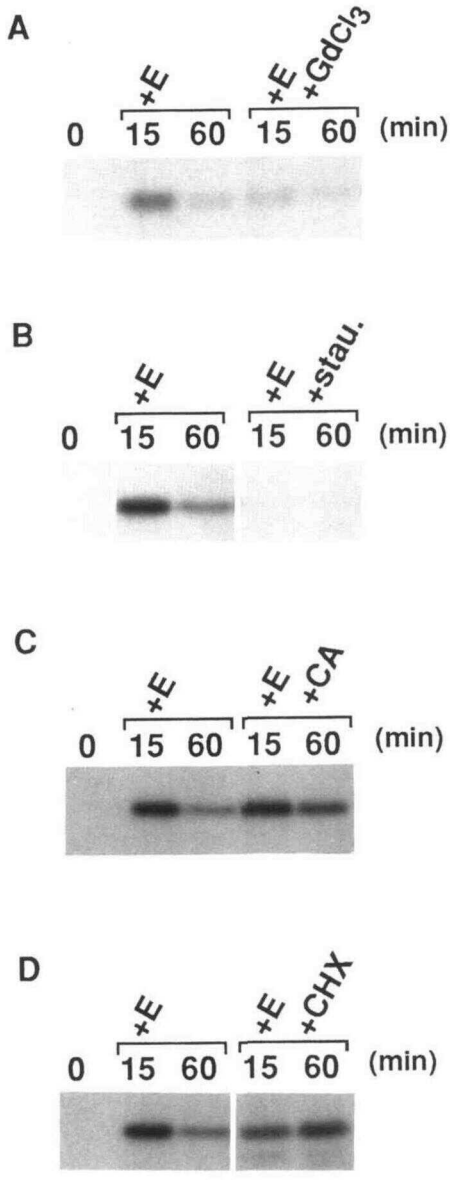

Figure 4. Effects of Inhibitors on the Elicitor-Responsive Activation of the MBP Kinase.

Tobacco cells were transferred to plastic dishes that contained 25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.8. After incubation for 60 min, inhibitors were added to the medium with thorough mixing, the elicitor (+E) was then added, and incubation was continued for an additional 15 or 60 min.

(A) $GdCl_3$ (+$GdCl_3$; 0.5 mM).

(B) Staurosporine (+stau.; 1 μM).

(C) Calyculin A (+CA; 1 μM).

(D) Cycloheximide (+CHX; 1 μg/mL).

As controls, distilled water was added rather than a solution of $GdCl_3$, and DMSO was added rather than a solution of staurosporine, calyculin A, or cycloheximide. Cells were harvested and crude extracts were prepared for the in-gel kinase assay, which was performed as described in Methods.

Protein phosphorylation is a reversible process. Therefore, additional regulatory control may be exerted by the action of protein phosphatases. To study the possible role of protein phosphatases in regulating activity of the MBP kinase, we examined the effects of calyculin A, an inhibitor of protein phosphatases 1 and 2A in plant and animal cells (Ishihara et al., 1989; Felix et al., 1994). The elicitor-induced activation of the MBP kinase was sustained for at least 60 min after administering the elicitor in the presence of 1 μM calyculin A (Figure 4C). We also observed the activation of the MBP kinase in tobacco cells that had been treated with calyculin A in the absence of the elicitor (data not shown).

To confirm that MBP kinase activity is regulated post-translationally, we examined the effect of cycloheximide, an inhibitor of protein synthesis, on the kinetics of activation of the MBP kinase in elicitor-treated cells. As shown in Figure 4D, in the presence of cycloheximide, the elicitor induced the sustained activation of MBP kinase for at least 60 min after addition of the elicitor. This result suggests that the synthesis of new protein is not required for activation of the MBP kinase but is required for down-regulation of MBP kinase activity.

Elicitor-Induced Phosphorylation of Tyrosine Correlated with the Activation of the Elicitor-Responsive MBP Kinase

To examine whether the activation of the elicitor-responsive MBP kinase might be correlated with tyrosine phosphorylation, immunoblot analysis with a phosphotyrosine-specific monoclonal antibody was performed after SDS-PAGE of extracts of elicitor-treated tobacco cells. The results are shown in Figure 5. Several bands were detected on a gel blot, but most bands except that of a 47-kD protein were unchanged by elicitation and could be detected when the gel blot was incubated with a secondary antibody without treatment with the phosphotyrosine-specific monoclonal antibody (data not shown). Elicitor-induced tyrosine phosphorylation of the protein was detected, and its relative position of migration (corresponding to that of a protein of ~47 kD) and kinetics of phosphorylation were similar to that of the elicitor-activated MBP kinase detected using the in-gel kinase assay (Figure 5A). We confirmed that the signal detected by the antibody was attributable to specific binding to phosphotyrosine by incubating the gel blots with the anti-phosphotyrosine antibody in the presence of phosphoserine, phosphothreonine, or phosphotyrosine. Figure 5B shows the complete elimination of the signal from the 47-kD protein by incubation of the gel blots and antibody with phosphotyrosine but not with phosphoserine or phosphothreonine. A preliminary experiment indicated that the phosphotyrosine signal from the 47-kD protein was no longer visible on the immunoblot when the extract of elicited tobacco cells was treated with protein tyrosine phosphatase prior to SDS-PAGE (data not shown). An extract of elicitor-treated tobacco cells was incubated with the anti-phosphotyrosine antibody coupled with protein G plus agarose or protein G plus agarose alone. The resultant immunoprecipitates were

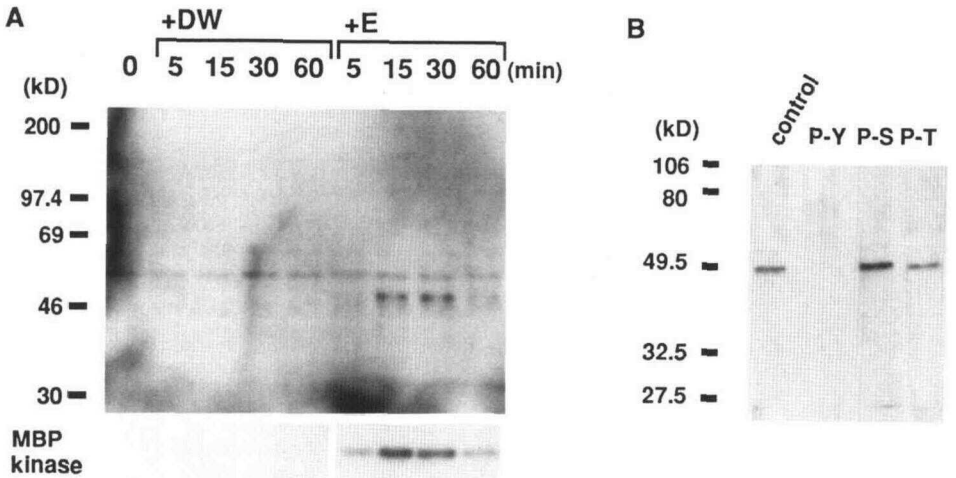

Figure 5. Elicitor-Induced Phosphorylation of Tyrosine.

Tobacco cells were transferred to plastic dishes that contained 25 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.8. After incubation for 60 min, the elicitor (+E) was added, and incubation was continued for an additional 5, 15, 30, or 60 min. Distilled water (+DW) was added as a control. Cells were harvested and crude extracts were prepared and electrophoresed on SDS–polyacrylamide gels; immunoblotting was with the phosphotyrosine-specific monoclonal antibody. The positions of migration of marker proteins are shown at left.

(A) *Elicitor-induced phosphorylation of tyrosine (top) and elicitor-induced activation of MBP kinase (bottom).*

(B) *To examine the specificity of the antibody for phosphotyrosine, membrane strips, which had been blotted with the same amount of protein from cells that had been treated with the elicitor for 15 min, were incubated with the primary antibody in the absence (control) and in the presence of 1 mM phosphotyrosine (P-Y), 1 mM phosphoserine (P-S), or 1 mM phosphothreonine (P-T).*

analyzed by the in-gel kinase assay, which showed that the 47-kD MBP kinase was immunoprecipitated by the phosphotyrosine-specific monoclonal antibody, as shown in Figure 6. These results indicate that tyrosine residue(s) on the 47-kD protein kinase activated by elicitor treatment is phosphorylated.

Effects of Inhibitors on Elicitor-Responsive Phosphorylation of Tyrosine

The induction of tyrosine phosphorylation of the 47-kD protein by the elicitor was inhibited by staurosporine and Gd^{3+}, as shown in Figures 7A and 7C. In the presence of calyculin A or cycloheximide, the elicitor induced a sustained state of tyrosine phosphorylation of the 47-kD protein (Figures 7A and 7B). The level of phosphotyrosine in the protein changed in proportion to the activity of MBP kinase, and effects of inhibitors on the elicitor-responsive activation of the MBP kinase and on tyrosine phosphorylation of the protein are quite similar. These results strongly suggest that the 47-kD MBP kinase is phosphorylated on tyrosine residues, in conjunction with the elicitor-induced activation of kinase activity.

DISCUSSION

In this study, MBP phosphorylation activity and tyrosine phosphorylation of the protein in elicitor-treated tobacco cells were investigated. We observed the rapid and transient activation of MBP kinase activity and tyrosine phosphorylation of this kinase when treated with the elicitor. In a number of plants, induction of protein phosphorylation in vitro and in vivo and in conjunction with the activation of defined defense responses of cells to elicitor treatment has been demonstrated (Dietrich et al., 1990; Felix et al., 1991), but information about the specific kinase that might be involved has been limited. Because protein

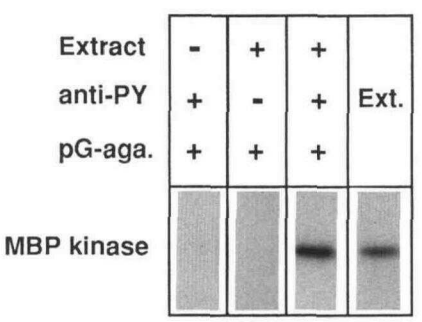

Figure 6. Immunoprecipitation of the MBP Kinase with the Phosphotyrosine-Specific Monoclonal Antibody.

The extract from elicitor-treated tobacco cells was incubated with the anti-phosphotyrosine antibody (anti-PY) and/or protein G plus agarose (pG-aga.) and precipitated. MBP kinase activity was then determined by the in-gel kinase assay. MBP kinase activity in the supernatant incubated without the antibody was also assayed (Ext.). (+), presence; (–), absence.

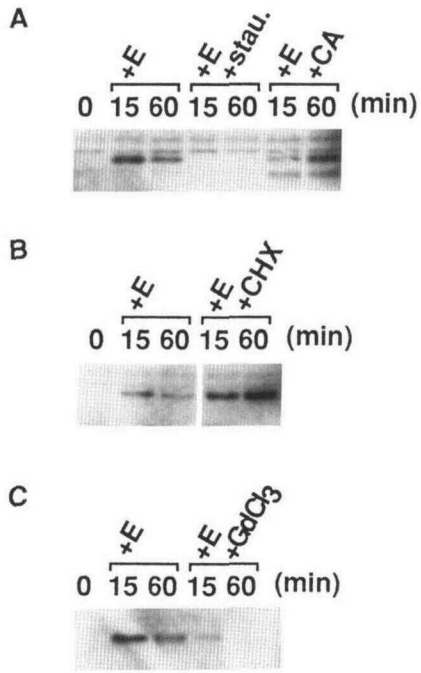

Figure 7. Effects of Inhibitors on Elicitor-Induced Phosphorylation of Tyrosine.

Tobacco cells were transferred to plastic dishes that contained 25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.8. After incubation for 60 min, inhibitors were added to the medium with thorough mixing, the elicitor (+E) was then added, and incubation was continued for an additional 15 or 60 min.

(A) Staurosporine (+stau.; 1 μM) and calyculin A (+CA; 1 μM).

(B) Cycloheximide (+CHX; 1 μg/mL).

(C) $GdCl_3$ (+$GdCl_3$; 0.5 mM).

As controls, distilled water was added rather than a solution of $GdCl_3$, and DMSO was added rather than a solution of staurosporine, calyculin A, or cycloheximide. Cells were harvested and crude extracts were prepared and electrophoresed on SDS–polyacrylamide gels; immunoblotting was with the phosphotyrosine-specific monoclonal antibody.

kinase activities can be distinguished on the basis of molecular mass by an in-gel kinase assay using MBP-containing polyacrylamide gels after SDS-PAGE, we were able to estimate the apparent molecular mass of the relevant kinase in our system. The protein kinase that was activated by transfer stress and treatment with the elicitor migrated as a protein with an apparent molecular mass of ~47 kD, as shown in Figure 1. This protein kinase seemed to phosphorylate serine/threonine residues of the MBP.

MAP kinases, members of a group of serine/threonine protein kinases, are important transducers of intracellular signals via protein phosphorylation that is initiated by various extracellular stimuli, and they are involved in proliferation, differentiation, and responses to stress in animal and yeast cells. Their apparent molecular masses, as determined by SDS-PAGE, range from 40 to 50 kD (for recent reviews, see Ahn, 1993; Davis, 1993; Pelech et al., 1993; Jonak et al., 1994). A unique characteristic of MAP kinases is reflected in the fact that their activities are regulated by the simultaneous phosphorylation of tyrosine and threonine residues by upstream protein kinases in a conserved sequence (Payne et al., 1991; Gartner et al., 1992; Posada and Cooper, 1992). This conserved sequence has been found in all of the amino acid sequences deduced from cDNA clones for MAP kinases of plants (Duerr et al., 1993; Jonak et al., 1993; Stafstrom et al., 1993; Wilson et al., 1993; Mizoguchi et al., 1994). Wilson et al. (1993) reported that a homolog of the MAP kinase in tobacco is expressed in suspension-cultured tobacco cells. Duerr et al. (1993) demonstrated that the tyrosine residue in the conserved sequence is crucial for the activity of a recombinant MAP kinase of alfalfa expressed in *Escherichia coli*, and the phosphorylated tyrosine residues in the protein are recognized by a phosphotyrosine-specific antibody. However, tyrosine phosphorylation of a regulatory protein in an extract of a higher plant has rarely been detected by immunoblotting with phosphotyrosine-specific antibodies.

In our study, we detected tyrosine phosphorylation of protein in an extract of elicitor-treated tobacco cells by immunoblotting with a phosphotyrosine-specific monoclonal antibody, and we demonstrated that activation of the MBP kinase in tobacco cells is correlated with tyrosine phosphorylation. It is reasonable to postulate that the 47-kD MBP kinase is regulated by post-translational phosphorylation of tyrosine (Figure 8). The improvements that led to our success seem to be our selection of a suitable experimental system and our focus on a distinct candidate protein. It has been demonstrated that the MBP is phosphorylated in vitro by several types of protein

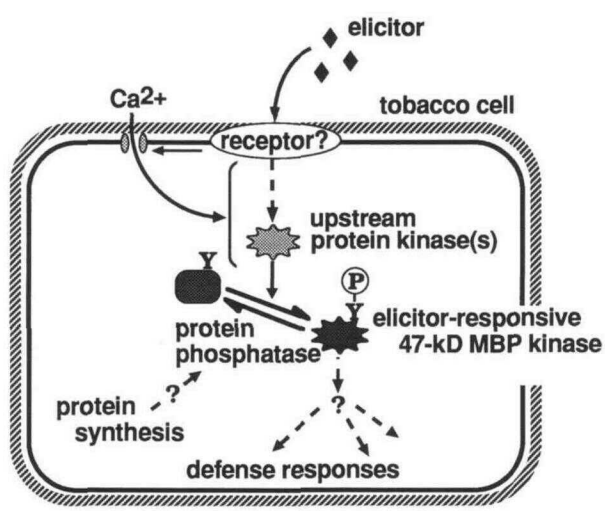

Figure 8. Proposed Model for Elicitor-Responsive Activation of the 47-kD MBP Kinase in Tobacco Cells.

P, phosphate; Y, tyrosine; ?, uncertain event and component.

kinases. MAP kinases phosphorylate the MBP very effectively under our chosen conditions, but they do not phosphorylate casein or histone in vitro (Ray and Sturgill, 1988; Gotoh et al., 1990). The elicitor-activated protein kinase was not detected by an in-gel kinase assay when the MBP was replaced by casein or histone as the protein substrate under identical conditions (data not shown). The characteristics of the elicitor-activated MBP kinase in tobacco cells resemble those of other MAP kinases.

Staurosporine has been used as an effective inhibitor of various protein kinases, and our previous work has demonstrated that staurosporine prevents the elicitor-inducible accumulation of mRNA transcribed from defense genes in tobacco cells (Suzuki et al., 1995). In this study, we observed the inhibitory effects of staurosporine on both the kinase activity and the stimulation of protein kinase activity in response to an elicitor. These results suggest that the elicitor signal leads to the activation of a staurosporine-sensitive upstream protein kinase(s) and that the activated kinase(s) phosphorylates and activates the 47-kD MBP kinase in tobacco cells (Figure 8).

The effect of cycloheximide in our system suggests that continuous synthesis of protein might be necessary for down-regulation of MBP kinase activity and that up-regulation of MBP kinase activity is controlled post-translationally. The effects of calyculin A, a potent inhibitor of protein phosphatases 1 and 2A, on the elicitor-responsive activation of the MBP kinase suggest that the 47-kD protein kinase activity in tobacco cells may be down-regulated by such protein phosphatases (Figure 8). Calyculin A has been shown to inhibit effectively the dephosphorylation of proteins from a plant extract in vitro (Felix et al., 1994) and to induce the phosphorylation of polypeptides as well as to mimic the action of the elicitor in plant cells (Felix et al., 1994; MacKintosh et al., 1994).

The important role of Ca^{2+}-dependent phosphorylation of proteins in signal transduction pathways has been well characterized in animal cells (Hunter, 1987). Such a role in plant cells is also likely (Ranjeva and Boudet, 1987), and the involvement of Ca^{2+} in the elicitor-induced phosphorylation of proteins in plant systems has also been suggested (Ebel and Scheel, 1992). In elicitor-treated cells of parsley, several proteins were transiently labeled by phosphorylation in vivo, and the extent of this elicitor-mediated phosphorylation was greatly reduced in the absence of extracellular Ca^{2+} (Dietrich et al., 1990). From these results, involvement of the Ca^{2+}-dependent protein kinase(s) in the signal transduction chain from elicitor to activation of plant defense genes has been proposed. As shown in Figure 3, the elicitor-responsive activation of the MBP kinase was prevented by a blocker of Ca^{2+} channels in the plasma membrane. In addition, the MBP kinase of tobacco cells was not activated in the presence of a high concentration of Ca^{2+} and the Ca^{2+} ionophore A23187 without the elicitor (data not shown). These results suggest that entry of extracellular Ca^{2+} into the cytosol is necessary but not sufficient to activate the elicitor-responsive 47-kD MBP kinase and that the Ca^{2+}-dependent step(s) functions upstream of the MBP kinase in the elicitor–signal transduction cascade in tobacco cells (Figure 8).

In wounded leaf discs of tobacco, transient activation of the MBP kinase was detected by an in-gel kinase assay, but activity was not detected when casein or histone was used as the protein substrate under identical conditions. The apparent molecular mass of the MBP kinase was estimated to be ~46 kD (S. Usami and Y. Machida, personal communication). The activation kinetics of the 46-kD kinase in leaf discs resembles that of the 47-kD MBP kinase in cells of the XD6S tobacco line induced by transfer stress. We also detected such wound-activated and transient activity of a protein kinase in a crude extract of tobacco leaf discs. During SDS-PAGE, the activity had a mobility similar to that of MBP kinase activity from an extract of cultured tobacco cells (line XD6S) that had been treated with elicitor (K. Suzuki and H. Shinshi, unpublished data). In addition, Mizoguchi et al. (1994) recently reported that the activity of an MBP kinase of ~46 kD could be detected by an in-gel kinase assay and is activated by the addition of 2,4-D to 2,4-D–starved suspension-cultured tobacco cells (line BY-2). These researchers also indicated that this protein kinase fails to phosphorylate casein or histone. Thus, MBP kinases that are rapidly and transiently activated by fungal elicitor, mechanical stress, and 2,4-D seem to be present in tobacco cells.

In cultured tobacco cells (line XD6S) transferred to plastic dishes, the 47-kD protein kinase was rapidly and transiently activated, but the levels of mRNAs for defense genes, such as class I basic chitinase, class II acidic chitinase, basic β-1,3-glucanase, and 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase, were unchanged (K. Suzuki and H. Shinshi, unpublished data). Such very short term activation of the 47-kD protein kinase seems insufficient to induce expression of defense genes. The rapid and transient elevation of active extracellular oxygen was induced by elicitor treatment of tobacco cells, and induction was blocked in the presence of a protein kinase inhibitor (K. Suzuki and H. Shinshi, unpublished data). It has been reported that a protein kinase inhibitor blocks the elicitor-inducible extracellular alkalinization of tomato suspension cultures (Felix et al., 1991, 1994). Thus, elicitor treatment of cells that activate the 47-kD protein kinase also stimulates the transcription of defense genes, oxidative burst, and alkalinization.

The phosphorylation and dephosphorylation of proteins have been thought to play a key role in the transduction of elicitor signals in plant cells. However, only recently has it been shown that modification of proteins by phosphorylation in plant cells occurs in conjunction with the activation of defined defense responses of cells to treatment with elicitors (Dietrich et al., 1990; Felix et al., 1991). In this report, we show that a specific protein kinase was activated by treatment with an elicitor and that activation of this kinase is correlated with phosphorylation of tyrosine residue(s) (Figure 8). It would be interesting to determine whether the 47-kD protein kinase is a component of the signal transduction pathway linking the elicitor signal and the defense responses of the plant cells. The identification of a protein kinase that can

be assayed by a biochemical method provides us with opportunities to isolate the protein kinase and corresponding gene to investigate the elicitor signal transduction pathway.

METHODS

Cell Culture and Treatment of Cells with an Elicitor

Suspension-cultured tobacco cells (*Nicotiana tabacum* line XD6S) were treated with an elicitor derived from the cell walls of *Phytophthora infestans* (Suzuki et al., 1995). The conditions for cell culture and treatment of the cells with an elicitor were described previously (Suzuki et al., 1995). Suspension cultures of tobacco cells were subcultured at weekly intervals in Murashige and Skoog medium (Wako Pure Chemical, Osaka, Japan), pH 5.8, that contained 3% sucrose and 5 μM 2,4-D. The activation of an MBP kinase was induced by administering the elicitor (50 μg/mL) to a 3-day-old suspension of XD6S cells. 2-(*N*-Morpholino) ethanesulfonic acid (pH 5.8; final concentration, 25 mM) was also added to the suspension of cells to stabilize the pH of the culture medium.

Preparation of the Crude Extract

Elicitor-treated cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80°C. The cells were thawed in homogenization buffer (50 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 10 mM Na_3VO_4, 10 mM sodium fluoride (NaF), 50 mM glycerophosphate, 5 μg/mL leupeptin, 5 μg/mL antipain), homogenized in the same buffer, and then centrifuged at 14,000 rpm for 20 min in a microcentrifuge. The supernatant was desalted on a NAP-5 column (Pharmacia Biotech, Tokyo, Japan) that had been equilibrated with elution buffer (20 mM Hepes, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 1 mM Na_3VO_4, 5 mM NaF, 10 mM glycerophosphate, 5 μg/mL leupeptin, 5 μg/mL antipain), and the eluent is referred to herein as the crude extract. The concentration of protein in the extract was estimated with a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan) with γ-globulin as the standard.

In-Gel Kinase Assay

The in-gel kinase assay was performed using the method of Gotoh et al. (1990). The aliquots (20 μg of protein) of crude extract were subjected to SDS-PAGE as described by Laemmli (1970), except that the 10% polyacrylamide gel was polymerized with 0.5 mg/mL myelin basic protein (MBP). After electrophoresis, SDS was removed by washing the gel with buffer A (50 mM Tris-HCl, pH 8.0, 20% 2-propanol) and then with buffer B (50 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol). Proteins in the gel were then denatured in buffer C (50 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, 6 M guanidine-HCl) at room temperature and were renatured in buffer D (50 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, 0.04% Tween 40) at 4°C. Next, the gel was incubated with buffer E (40 mM Hepes, pH 7.5, 0.1 mM EGTA, 20 mM $MgCl_2$, 2 mM DDT) and then with buffer E that contained 25 μM ATP (1.85–3.7 MBq γ-^{32}P-ATP; Amersham K.K., Tokyo, Japan). The gel was washed extensively with a solution of 5% trichloroacetic acid and 1% pyrophosphate. The dried gel was subject to autoradiography, and MBP kinase activity was quantified by scanning densitometry (personal densitometer; Molecular Dynamics, Sunnyvale, CA). The apparent molecular mass of the protein kinase detected on SDS–polyacrylamide gels was estimated with a prestained SDS-PAGE standard marker (Nippon Bio-Rad Laboratories) or Rainbow Marker (Amersham K.K.).

To determine whether phosphorylated residues of MBP were susceptible to alkali, the gel was treated with alkali after an in-gel kinase assay according to a slightly modified version of the method of Cooper et al. (1983). The gel was treated with 1 M KOH at 60°C for 2 hr and then neutralized with 10% acetic acid prior to drying and autoradiography.

Immunoblot and Immunoprecipitation

Crude extracts of tobacco cells were prepared and fractionated by SDS-PAGE as described previously, and proteins were then transferred to a polyvinylidine difluoride membrane (Immobilon-P; Nippon Millipore Ltd., Tokyo, Japan) by a semidry electroblotting method using Trans-Blot SD (Nippon Bio-Rad Laboratories). The blots were blocked overnight in Tris buffer (10 mM Tris-HCl, pH 7.5, 0.1% Tween 20, 100 mM NaCl) that contained 10% BSA at 4°C and then incubated with the phosphotyrosine-specific monoclonal antibody (Upstate Biotechnology Incorporated, Lake Placid, NY) in the same Tris buffer plus 5% BSA at room temperature for 1 hr. After five washes in Tris buffer, the blots were incubated with horseradish peroxidase–conjugated anti-mouse IgG (Amersham K.K.) in Tris buffer plus 5% BSA and washed again five times in Tris buffer. The antibody–antigen complexes were visualized using an enhanced chemiluminescence system (ECL kit; Amersham K.K.). The blots were exposed to x-ray film (X-OMAT; Nippon Kodak, Tokyo, Japan).

The extract of elicited tobacco cells was incubated with the phosphotyrosine-specific monoclonal antibody in an immunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 5 μg/mL leupeptin, 5 μg/mL antipain, 10 mM β-glycerophosphate, 1% Triton X-100, 0.5% Nonidet P-40) at 4°C for 1 hr. Protein G plus agarose (Oncogene Science, Uniondale, NY) was added to this mixture, and the mixture was gently agitated at 4°C overnight. The immunoprecipitate was washed three times with immunoprecipitation buffer. The SDS sample buffer was then added, and the sample was boiled for about 5 min to release immunoprecipitated proteins. After centrifugation, the supernatant fraction was electrophoresed on SDS–polyacrylamide gels, and the in-gel kinase assay was performed as described previously.

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