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Biochemistry

Cutting activates a 46-kilodalton protein kinase in plants

(wounding/protein kinase/tyrosine phosphorylation)

SHOJI USAMI, HIROHARU BANNO, YUKIHIRO ITO, RYUICHI NISHIHAMA, AND YASUNORI MACHIDA*

Department of Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

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ABSTRACT **Using SDS/polyacrylamide gels that contained myelin basic protein, we identified a 46-kDa protein kinase in tobacco that is transiently activated by cutting. Although the activity of the kinase was rarely detectable in mature leaves, marked activity became apparent within several minutes after isolation of leaf discs and subsided within 30 min. In the presence of cycloheximide (CHX), the kinase activity did not diminish after the isolation over the course of 2 hr, suggesting that protein synthesis was not required for the activation of the kinase. A second cutting of leaf discs between 30 min and 60 min after the isolation failed to activate the kinase, whereas a second cutting given 3 hr after isolation apparently activated the kinase. These results suggest that the 46-kDa protein kinase is desensitized immediately after the first activation, which can be blocked by CHX, but the response ability recovers with time. When protein extracts containing the active kinase were treated with serine/threonine-specific or tyrosine-specific protein phosphatase, the kinase activity was abolished. After immunoprecipitation with antibody against phosphotyrosine, activity of the kinase was recovered in the immunoprecipitate. These results suggest that the active form of the kinase is phosphorylated at both serine/threonine and tyrosine residues. It seems likely that the 46-kDa protein kinase can be activated by dual phosphorylation. The activity of a 46-kDa protein kinase was also detected in leaves of a wide variety of plant species including dicotyledonous and monocotyledonous plants. We propose the name PMSAP (plant multisignal-activated protein) kinase for this kinase because the kinase was also activated by various signals other than cutting.**

Protein kinases play a key role in many organisms both in transduction of external signals into cells and in the intracellular transmission of such signals. Signal pathways known as mitogen-activated protein (MAP) kinase cascades have been established recently in yeasts and animals and shown to be involved in various physiological processes (1–4). Furthermore, several groups including our own have shown that plants also have homologs of the components of the MAP kinase cascade (5–12). Thus, it would seem likely that MAP kinase cascades occur in plants even though the relationships among these plant homologs have yet to be determined (13). Recent genetic and molecular analyses of plant genes such as *CTR1* (14), *ETR1* (15), and *Pto* (16) suggested that a number of protein kinases in plants exhibit structural and functional similarities to protein kinases identified in other organisms.

For the detection of enzymatic activities of protein kinases, several proteins such as myelin basic protein (MBP) and caseins have been used as substrates even though these substrates are not physiologically relevant (17–20). Using these proteins, we have examined protein kinases in tobacco cells, looking for those that might be activated in response to infection by *Agrobacterium tumefaciens*, a causative agent for the formation of crown gall tumor (21–24). During this experiment, we found a protein kinase with a molecular mass of 46 kDa that phosphorylated MBP. Although the kinase was initially detected as an activity that became rapidly apparent upon infection of tobacco leaf discs with agrobacteria, further investigations showed that this protein kinase could be activated just by cutting mature leaves of tobacco. In the present report, we describe the physiological responses of leaf tissue and the biochemical and immunological characteristics of the 46-kDa protein kinase from tobacco. To our knowledge, a protein kinase in plants that is activated by cutting has not been reported before.

MATERIALS AND METHODS

Plants. Plants of *Nicotiana tabacum* L. cv. SR1 and Xanthi nc, *Nicotiana glauca, Arabidopsis thaliana, Petunia hybrida, Medicago sativa, Lycopersicon esculentum, Zea mays*, and *Oryza sativa* were grown at 28°C with 16-h/8-h light/dark cycles in a greenhouse or a growth chamber.

Isolation of Tobacco Leaf Discs and Preparation of Protein Extracts. Five- to 7-week-old tobacco plants (SR1) were allowed to rest for at least 3 hr in the laboratory before use to prevent accidental activation of the 46-kDa protein kinase. Discs (≈0.5 cm^2) were isolated from mature leaves with the cap of an Eppendorf tube and were allowed to stand at 26°C in incubation buffer (20 mM Hepes·KOH, pH 7.0/0.1% Tween 20). In some experiments, the leaf discs were isolated, vacuum-infiltrated, and allowed to stand in the incubation buffer supplemented with 0.3 M mannitol. Then the leaf discs were frozen in liquid nitrogen to stop cellular reactions. In the rapid method, the leaf discs were transferred to extraction buffer (buffer A) that contained 50 mM Tris·HCl (pH 6.8), 2% SDS, 10% (vol/vol) glycerol, and 5% (vol/vol) 2-mercaptoethanol; homogenized with a microhomogenizer (Niti-On, Tokyo) for 10 sec; and heated in boiling water for 5 min. To obtain extracts that contained native proteins, the leaf discs were transferred to extraction buffer (buffer B) that contained 50 mM Hepes·KOH (pH 7.6), 1 mM EDTA, 1 mM EGTA, 1 mM *o*-vanadate, 20 mM β-glycerophosphate, 20% (vol/vol) glycerol, 2 μg of leupeptin and 2 μg of pepstatin A per ml, 2 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride and were homogenized as described above. Homogenates were centrifuged at 15,000 × *g*, and supernatants were recovered. In the latter method, all of the procedures were carried out at 4°C. Protein was quantitated with a protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

Detection of Activity of the 46-kDa Protein Kinase. The kinase activity was assayed as described (17, 25). In brief, 10 μg of protein per lane was subjected to electrophoresis on a 10% polyacrylamide gel that contained SDS and 0.5 mg of bovine brain MBP (Sigma) per ml. After electrophoresis, the SDS was

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Abbreviations: PMSAP kinase, plant multisignal-activated protein kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; CHX, cycloheximide; PP2A, protein phosphatase 2A; 2-D, two dimensional.

*To whom reprint requests should be addressed.

removed by successive washing of the gel with a solution that contained 20% (vol/vol) 2-propanol and 50 mM Tris·HCl (pH 8.0) and a solution that contained 50 mM Tris·HCl (pH 8.0) and 5 mM 2-mercaptoethanol (buffer C). After denaturation of the gel with buffer C that contained 6 M guanidine hydrochloride for 1 hr and renaturation with buffer C that contained 0.04% Tween 40 for 16 hr at 4°C, the gel was incubated at room temperature for 1 hr in the kinase reaction buffer [40 mM Hepes·KOH (pH 7.6)/2 mM dithiothreitol/0.1 mM EGTA/15 mM $MgCl_2$/25 μM [γ-^{32}P]ATP (1.85 GBq/mmol)]. The gel was washed extensively with the stop solution (5% trichloroacetic acid/1% pyrophosphoric acid) and dried. Autoradiography was performed with XAR-5 film (Kodak).

Two-Dimensional (2-D) Gel Electrophoresis. 2-D gel electrophoresis was performed essentially as described by O'Farrell (26). Aliquots of soluble protein extracted in buffer B were precipitated with 50% acetone, and the precipitates were dissolved in loading buffer that contained 9.5 M urea, 2% Nonidet P-40, 2% Ampholine (preblended, pH 3.5–9.5; Pharmacia LKB), and 5% 2-mercaptoethanol. Five micrograms of protein was loaded on an isoelectric focusing gel (90 mm × 1.13 mm) that contained 8.5 M urea, 4% acrylamide, 0.2% *N,N'*-methylenebisacrylamide, 2% Ampholine, and 2% Nonidet P-40 and was subjected to electrophoresis at 200 V for 30 min and then at 700 V for 2 hr in a cold room (4°C). The gel was then washed with buffer A and loaded on a SDS/polyacrylamide gel that contained MBP. Then proteins were subjected to electrophoresis at 20 mA for 20 min.

Treatment with Phosphatases. Leaf discs were allowed to stand in the incubation buffer supplemented with 0.3 mM cycloheximide (CHX; Wako Pure Chemical, Osaka) for 3 hr, and soluble proteins were extracted with buffer B lacking *o*-vanadate and β-glycerophosphate. For treatment with serine/threonine-specific protein phosphatase, aliquots of the protein extracts were added to buffer B supplemented with 50 mM NaCl, 2 mM $MgCl_2$, and 0.02 units of the catalytic subunit of protein phosphatase 2A (PP2A; GIBCO/BRL) (27–29) and incubated at 30°C in the presence or the absence of 1 μM okadaic acid (Wako Pure Chemical). For treatment with tyrosine-specific protein phosphatase, aliquots of the protein extracts were added to the phosphatase buffer (25 mM imidazole, pH 7.0/50 mM NaCl/2.5 mM EDTA/5 mM dithiothreitol/0.1 mg of deacetylated bovine serum albumin per ml) containing 5 units of LAR_R (30, 31) or T-$Cell_R$ protein-tyrosine phosphatase (32, 33) (New England BioLabs) and incubated at 30°C in the presence or the absence of 1 mM vanadate for LAR_R phosphatase or 1 mM vanadate and 0.1 mM molybdate for T-$Cell_R$ phosphatase.

Immunoprecipitation. Soluble protein extracts were prepared as described above. NaCl (150 mM) and 1% (vol/vol) Nonidet P-40 were added to the protein extracts. One microgram of mouse monoclonal antibody 4G10 against phosphotyrosine (Upstate Biotechnology, Lake Placid, NY) or 9E10 against human c-myc (Cambridge Research Biochemicals) was added, and the mixture was incubated at 4°C for 1 hr. After addition of 10 μl of protein A-Sepharose CL-4B (Pharmacia LKB) (10% vol/vol), the mixture was slowly shaken at 4°C for 1 hr. The Sepharose beads were collected by brief centrifugation and washed twice with buffer B that contained 150 mM NaCl and 1% Nonidet P-40. Proteins were eluted from the Sepharose beads with buffer A, and activity of the kinase in the eluate was examined.

RESULTS

Cutting Activates a 46-kDa Protein Kinase. We examined the activities of protein kinases in isolated leaf discs of tobacco in SDS/polyacrylamide gels that contained MBP as a model substrate (17, 25). We isolated leaf discs from mature leaves of tobacco and placed them in the incubation buffer. After various periods, a protein extract was prepared in the extraction buffer that contained SDS; the extract was heated, and proteins were subjected to electrophoresis in a SDS/polyacrylamide gel that contained MBP. After renaturation of proteins in the gel, the kinase activity was determined. The activity of a single protein kinase with a molecular mass of 46 kDa appeared as early as 1 min after isolation of the leaf discs (Fig. 1 *Top*). Maximum activity was observed 2–5 min after isolation, and the activity then fell to the basal level within 30 min. Essentially the same pattern of activation was observed when the leaf on a living plant was cut (data not shown).

The activity of the kinase also appeared in the presence of CHX, an inhibitor of protein synthesis, but in this case the activity did not diminish over the course of 2 hr (Fig. 1 *Middle*). The rate of protein synthesis in the leaf discs was reduced by about 90% within 30 min in the presence of CHX under our conditions (data not shown), a result that is consistent with previous observations (34).

Leaf Discs Are Unable to Respond to a Second Cutting Immediately After the First Activation. We examined whether,

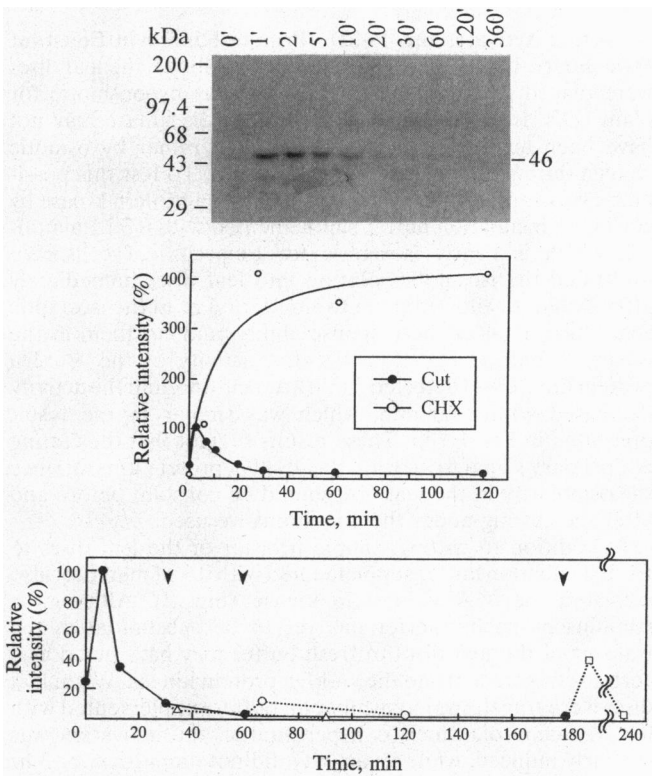

FIG. 1. (*Top*) Detection of the kinase activity of a 46-kDa protein on a SDS/polyacrylamide gel that contained MBP. Leaf discs were isolated from mature leaves of tobacco and incubated for the minutes indicated at the top of each lane. Total protein was extracted in buffer A, and the activity of a 46-kDa protein kinase was determined. Molecular size markers, and the band of the 46-kDa protein kinase are indicated. (*Middle*) Effects of CHX on activation of the 46-kDa protein kinase by cutting. Leaf discs were incubated for various times in incubation buffer with (○) or without (●) 0.3 mM CHX, and the activity of the 46-kDa protein kinase was determined. The radioactivity of the band of the 46-kDa protein kinase was measured with a Bio Image Analyzer (BAS2000; Fuji), and the values were normalized to the maximum intensity obtained without CHX. (*Bottom*) Reinduction of the activity of the 46-kDa protein kinase by a second cutting. Leaf discs were isolated and incubated for the times indicated, and the activity of the 46-kDa protein kinase was determined (●—●). To examine reinduction by a second cutting, leaf discs which had been isolated and incubated for 30 min, 60 min, or 180 min were cut in half, and the leaves were further incubated for 5 min and 60 min. Arrowheads indicate the time of the second cutting. The graphs show the activity at 5 min and 60 min after a second cutting at 30 min (△), 60 min (○), or 180 min (□) after the cutting of the leaf discs.

after the first activation due to isolation of leaf discs had subsided, a second cutting could activate the 46-kDa protein kinase once again. Leaf discs were isolated, placed on the incubation buffer for 30 min, 60 min, or 180 min and then cut again. Proteins were extracted 5 min and 60 min after each second cutting, and the activity was examined. Figure 1 *Bottom* shows that, when the second cutting was performed at 30 min, no activation occurred. However, significant activity was induced again by a second cutting as little as 60 min after isolation of leaf discs. This result suggests that the 46-kDa protein kinase is desensitized immediately after the first activation, but the ability to respond to cutting recovers gradually with time.

After the isolated leaf discs were left for 3 hr in the incubation buffer to quench the first activation and to recover the response ability, addition of CHX to the incubation buffer activated the kinase, and the activity was maintained over the course of 24 hr (data not shown). This result suggests that CHX not only inhibits the decrease of the activity of the 46-kDa protein kinase activated by cutting (Fig. 1 *Middle*) but also itself activates the kinase.

Cutting Activates the 46-kDa Protein Kinase in Constant Osmolarity. In the experiments described above, the leaf discs were placed in incubation buffer that was hypoosmotic for plant cells. It is, therefore, possible that the kinase may not have been activated by cutting itself but rather by osmotic change during incubation of the leaf discs. To test this possibility, we examined activation of the 46-kDa protein kinase by cutting in incubation buffer supplemented with 0.3 M mannitol, which is nearly isosmotic for tobacco leaf cells. We infiltrated the isosmotic solution into leaf discs immediately after isolation, allowed them to stand for 6 hr in the isosmotic solution to recover the response ability, and cut them in the isosmotic buffer. Fig. 2 shows that activity of the 46-kDa protein kinase was detected 5 min after cutting, and the activity decreased within 60 min, which was similar to the result presented in Fig. 1 *Top*. These results suggest that the cutting is a primary signal to activate the 46-kDa protein kinase, since the osmolarity in the leaf disc should be constant before and after the cutting under the conditions we used.

In addition to cutting, simple transfer of the leaf discs to fresh incubation buffer supplemented with 0.3 M mannitol also activated the 46-kDa protein kinase (Fig. 2). Although a stimulus by such transfer has yet to be substantiated, the transfer of the leaf discs to fresh buffer may have acted as a certain stress to activate the 46-kDa protein kinase. When leaf discs were transferred to incubation buffer supplemented with 0.8 M mannitol, which is hyperosmotic, the activation was similarly induced, while its activity did not diminish over 3 hr (Fig. 2). Thus, the activity seems to be stable in the hyperosmotic solution. In the latter experiment, a second band with a lower molecular mass appeared at 60 min, but the appearance of the second band was not reproducible.

We also examined whether drying (drought) can activate the 46-kDa protein kinase. Leaf discs were isolated, allowed to stand for 3 hr in the incubation buffer, and then dried until the fresh weight of the leaf discs decreased to 50%. Activity was not detected in the dried leaf discs, but when the dried leaf discs were cut, the 46-kDa protein kinase was transiently activated (data not shown).

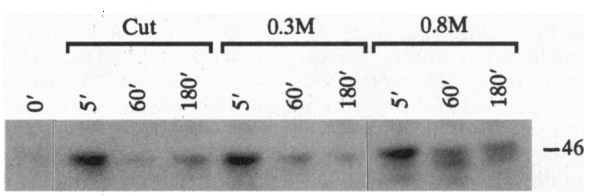

FIG. 2. Activation of the 46-kDa protein kinase in constant osmolarity. Leaf discs were isolated and vacuum-infiltrated with incubation buffer supplemented with 0.3 M mannitol and incubated for 6 hr. The leaf discs were cut again (lanes Cut) or transferred to fresh incubation buffer supplemented with 0.3 M (lanes 0.3 M) or 0.8 M mannitol (lanes 0.8 M). After incubation for the minutes indicated above each lane, the activity of the 46-kDa protein kinase was determined.

Active Form of the 46-kDa Protein Kinase Has Both Phosphorylated Serine/Threonine and Tyrosine Residues. We next examined whether the active form of the 46-kDa protein kinase might be phosphorylated. Soluble proteins were extracted from CHX-treated leaf discs; the extract contained >90% of the total activity of the 46-kDa protein kinase (data not shown), indicating that the 46-kDa protein kinase is a cytoplasmic protein. We treated the extract with PP2A, one of the major serine/threonine-specific protein phosphatases (27–29), in the presence and the absence of okadaic acid, an inhibitor of PP2A. The treatment with PP2A without okadaic acid abolished the activity of the 46-kDa protein kinase, while such inactivation was not observed in the presence of okadaic acid (Fig. 3 *Upper Left*). These results suggest that the active form of the 46-kDa protein kinase has a serine and/or threonine residue(s) that is phosphorylated and that dephosphorylation renders it inactive.

Among cytoplasmic protein kinases of yeasts and animals, only a very few kinases, such as the MAP kinase (2) and the CDC2 kinase (35), undergo tyrosine phosphorylation that is crucial for their respective activities and physiological functions. We examined the possibility of tyrosine phosphorylation of the 46-kDa protein kinase by treatment of the extracts with tyrosine-specific protein phosphatases [LAR_R (30, 31) and T-$Cell_R$ phosphatases (32, 33)] in the presence and the absence of inhibitors. Fig. 3 *Upper Right* and *Lower Left* show that both LAR_R and T-$Cell_R$ phosphatases without inhibitors abolished the activity of the 46-kDa protein kinase, while such inactivations were not observed in the presence of inhibitors. We also assayed activity in an immunoprecipitate prepared with mouse monoclonal antibody against phosphotyrosine. Fig. 3 *Lower Right* shows that activity was recovered in the immunoprecipi-

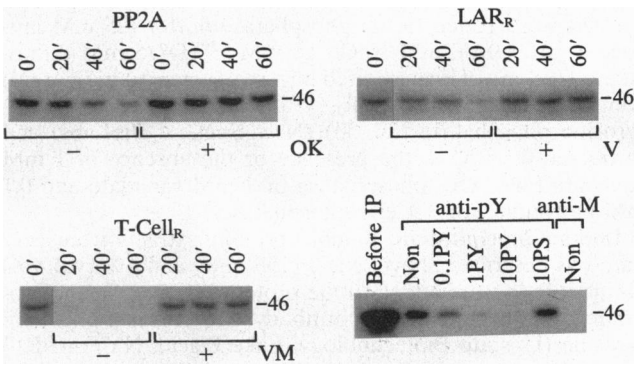

FIG. 3. (*Upper* and *Lower Left*) Effects of serine/threonine-specific (*Upper Left*) and tyrosine-specific (*Upper Right* and *Lower Left*) phosphatases on the activity of the 46-kDa protein kinase. Leaf discs were treated for 3 hr with 0.3 mM CHX, and soluble proteins were prepared in buffer B as described in text. The protein extracts were treated with 0.02 unit of PP2A (*Upper Left*), 5 units of LAR_R (*Upper Right*), or T-$Cell_R$ phosphatases (*Lower Left*) in the absence (−) or the presence (+) of each inhibitor and were incubated for the minutes indicated above each lane. The activity of the 46-kDa protein kinase was determined as described in text. OK, 1 μM okadaic acid; V, 1 mM vanadate; VM, 1 mM vanadate/0.1 mM molybdate. (*Lower Right*) Activity of the 46-kDa protein kinase in immunoprecipitates. Aliquots of protein extracts prepared as described above were incubated with 1 μg of mouse monoclonal antibody in the absence (lane Non) or the presence of 0.1 mM, 1 mM, and 10 mM phosphotyrosine (0.1PY, 1PY, 10PY, respectively) or 10 mM phosphoserine (10PS), and the activity of the 46-kDa protein kinase was determined as described in text. IP, immunoprecipitation; anti-pY, phosphotyrosine-specific antibody; anti-M, antibody against human c-myc protein as a negative control.

tate only with the phosphotyrosine-specific antibody but not with monoclonal antibody against human c-myc protein as a negative control. The immunoprecipitation was inhibited by addition of phosphotyrosine as a competitor but not of phosphoserine (Fig. 3 *Lower Right*). These results suggest that the active form of the 46-kDa protein kinase contains a phosphorylated tyrosine residue(s).

Activation of the 46-kDa protein kinase by cutting was inhibited by retreatment of the leaf discs with K252a, a potent inhibitor of protein kinases (data not shown), a result that suggests that the 46-kDa protein kinase is activated as a consequence of phosphorylation and is consistent with the results as described above.

Molecular Species of the Active Form of the 46-kDa Protein Kinase. We examined whether the activity that we detected as the band of a protein of 46 kDa was due to a single species of protein kinase. A protein extract prepared from leaf discs 5 min after isolation was fractionated by 2-D gel electrophoresis. To detect the activity of the kinase, a SDS/polyacrylamide gel containing MBP was used as the second gel. Four activities associated with a protein with a molecular mass of 46 kDa were detected, but they had different isoelectric points (Fig. 4). When the leaf discs were treated with CHX, the similar pattern of distribution of activities was seen (Fig. 4). Thus, molecular species of the protein kinase that can be activated by cutting were also activated with CHX.

Cutting Activates the 46-kDa Protein Kinase in Other Plant Species. Activities of protein kinases, all of which migrated as proteins with a molecular mass of 46 kDa, were detected in leaf discs 5 min after isolation from mature leaves of all the dicotyledonous and monocotyledonous plants that we examined (Fig. 5).

DISCUSSION

We have demonstrated that a 46-kDa protein kinase, which is rapidly activated by cutting the leaves, can be found in a variety of plant species (Fig. 1 *Top* and Fig. 5). The activation by cutting was transient; after the activity had subsided, the leaves did not respond to a second cutting for about 1 hr, during which time the ability to respond to cutting was gradually restored (Fig. 1 *Bottom*). When a leaf on a living tobacco plant was wounded with Carborundum, the 46-kDa protein kinase was similarly activated around the wounded area (our unpublished data). A similar pattern of activation was also observed after application of water-soluble chitosan, a digest of tobacco cell walls, and salicylic acid to leaf discs (our unpublished data). Activities of the 46-kDa protein kinase were found in roots and tissues containing the shoot apical meristem and flower buds without cutting (our unpublished data). Therefore, the activation of the protein kinase seems to be related to plant responses to various signals including wounding. We have chosen to designate this kinase plant multisignal-activated protein (PMSAP) kinase.

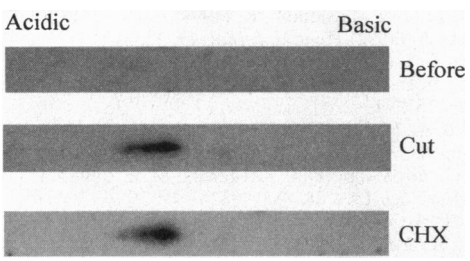

FIG. 4. Analysis of the 46-kDa protein kinase molecules by 2-D gel electrophoresis. Leaf discs were isolated (Before) and incubated for 5 min in the incubation buffer (Cut) or incubated for 2 hr in the incubation buffer containing 0.3 mM CHX. Soluble proteins were extracted in buffer B and concentrated with 50% acetone. The proteins were successively fractionated by electrophoresis on an isoelectric focusing gel and a SDS/polyacrylamide gel that contained MBP, and the activity of the 46-kDa protein kinase was determined as described in text.

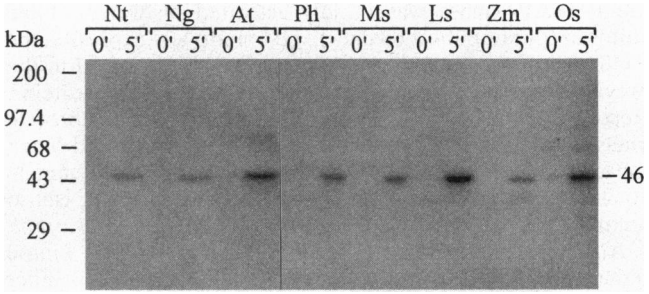

FIG. 5. Activation of the 46-kDa protein kinase by cutting in various plant species. Leaf discs were isolated from various plants and incubated for the minutes indicated above each lane. Proteins were extracted in buffer A, and the activity of protein kinase was examined. Nt, *Nicotiana tabacum* L. cv. Xanthi nc; Ng, *Nicotiana glauca*; At, *Arabidopsis thaliana*; Ph, *Petunia hybrida*; Ms, *Medicago sativa*; Ls, *Lycopersicon esculentum*; Zm, *Zea mays*; Os, *Oryza sativa*.

Even in the presence of CHX, the PMSAP kinase was activated by cutting (Fig. 1 *Middle*). This result together with the observation that treatment with serine/threonine-specific (Fig. 3 *Upper Left*) and tyrosine-specific (Fig. 3 *Upper Right* and *Lower Left*) protein phosphatases abolished the activity of PMSAP kinase indicates that the kinase can be activated either by phosphorylation by another protein kinase or by autophosphorylation that might be also stimulated by an unidentified component. Thus, the pathway for activation of the PMSAP kinase may include at least one other component, the function of which, in this context, may also be regulated by cutting. In addition to such a putative activator of the PMSAP kinase, we postulate the existence of a negative regulator because the activity of the PMSAP kinase decreased rapidly and the isolated leaf disc was temporarily unable to respond to a second cutting (Fig. 1 *Bottom*). The rapid inactivation was blocked in the presence of CHX (Fig. 1 *Middle*), suggesting that synthesis of a new protein, presumably induced after cutting, is required for the inactivation and that the protein must also be rapidly turning over or inactivated. The simplest explanation for the inactivation and the refractory state that follow the first cutting is that a protein phosphatase that can inactivate the PMSAP kinase is synthesized *de novo* in response to cutting. Similar inactivation mechanisms have recently been reported for the regulation of MAP kinase cascades in animal and yeast cells (36–39). Only after the protein phosphatase molecule has disappeared from cells or has been inactivated, or both, can the PMSAP kinase be effectively activated by a subsequent cutting.

Although cutting or wounding has been shown to induce various molecular events, which include production of signaling chemicals such as systemin and abscisic acid, the biosynthesis of ethylene, oxidative cross-linking of proteins in the cell walls, changes in electrical potential, and expression of specific plant genes (40, 41), to our knowledge there are no previous reports indicating the activation of a protein kinase or induction of protein phosphorylation by cutting or wounding. The physiological and biochemical processes that are stimulated by cutting or wounding and can be regulated by the PMSAP kinase remain to be identified.

Three groups have reported that elicitor molecules can induce phosphorylation of unknown plant proteins *in vitro* (42) and *in vivo* (43–45). In particular, it is worth noting here that when suspension-cultured tomato cells are treated with chitin fragments, alkalinization of the culture medium is induced within several minutes, with accompanying rapid changes in the patterns of phosphorylation of several proteins (45). Since

the PMSAP kinase was rapidly activated by water-soluble chitosan (our unpublished data) and tomato leaf expressed the PMSAP kinase (Fig. 5), this kinase could be involved in the previously reported phosphorylation of these plant proteins. Recently, other workers reported that the activity of a protein kinase with a molecular mass of 47 kDa is induced when tobacco cells in suspension culture are treated with fungal elicitors (46). The physiological function of this kinase is also unknown.

Although the molecular features of the PMSAP kinase remain to be elucidated, the kinase might be a plant member of the MAP kinase family because several of its biochemical properties are similar to those of MAP kinases from animals and yeasts. (*i*) A tyrosine residue(s) of the PMSAP kinase was phosphorylated (Fig. 3 *Upper Right* and *Lower*) and tyrosine phosphorylation is known to be essential for activation of animal and yeast MAP kinases (2). (*ii*) The observed molecular mass of the PMSAP kinase is close to those of known members of the MAP kinase family from many organisms. (*iii*) The PMSAP kinase efficiently phosphorylated MBP, as do the active forms of yeast and animal MAP kinases. Although the tyrosine phosphorylation in the PMSAP kinase tends to support our idea, other observations tend not to do so. For example, other plant protein kinases, such as tobacco protein kinases NPK5 and calcium-dependent protein kinase (CDPK), that apparently do not belong to the MAP kinase family are known to phosphorylate MBP and/or synthetic peptides derived from MBP (47, 48). Although many cDNAs that encode putative members of the MAP kinase family have been isolated from various plants (5–10), the characteristics of the active forms of the corresponding proteins have not yet been examined. Recently, novel kinases called SAPKs or JNKs, which have been grouped in a MAP kinase subfamily on the basis of their amino acid sequences, have been isolated from mammalian cells, but they failed to phosphorylate MBP (49, 50). The molecular nature of the PMSAP kinase should now be elucidated by molecular cloning of its gene and cDNA.

Analysis by 2-D gel electrophoresis showed that protein extracts from activated leaf discs contained four types of protein molecules that differed in isoelectric point but had the same molecular mass of 46 kDa (Fig. 4). Two possible conclusions about the active molecules of the PMSAP kinase can be drawn from this result: (*i*) the PMSAP kinase molecule may be a single species of a protein, but in stimulated cells it may be phosphorylated to various extents to yield four forms of active molecule with different numbers of phosphate groups and different isoelectric points; and (*ii*) the PMSAP kinase detected here might consist of four different protein species, each of which has a different isoelectric point. Analyses of purified protein kinases and molecular cloning of the corresponding genes should help to resolve this issue.

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