The Plant Cell, Vol. 11, 289–298, February 1999, www.plantcell.org © 1999 American Society of Plant Physiologists

Jasmonate-Based Wound Signal Transduction Requires Activation of WIPK, a Tobacco Mitogen-Activated Protein Kinase

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A gene encoding a tobacco mitogen-activated protein kinase (WIPK) is transcriptionally activated in response to wounding. Transgenic tobacco plants, in which expression of endogenous *wipk* was suppressed, did not accumulate jasmonic acid or its methyl ester when wounded, suggesting that WIPK is involved in jasmonate-mediated wound signal transduction. Here, we demonstrate that activation of WIPK is required for triggering the jasmonate-mediated signal transduction cascade that occurs when wild-type tobacco plants are wounded. We also show that when plants are wounded, WIPK is rapidly and transiently activated, whereas the quantity of WIPK protein is maintained at a constant level. A transgenic tobacco plant in which the *wipk* gene was constitutively expressed at a high level showed constitutive enzymatic activation of WIPK and exhibited three- to fourfold higher levels of jasmonate than did its wild-type counterpart. This plant also showed constitutive accumulation of jasmonate-inducible proteinase inhibitor II transcripts. These results show that WIPK is activated in response to wounding, which subsequently causes an increase in jasmonate synthesis.

INTRODUCTION

Plants quickly respond to wound stress, such as physical injury and insect attack, by activating diverse genes that encode proteins involved in healing injured tissues. Representative examples are the proteinase inhibitors (Ryan, 1990) whose genes are transcriptionally activated by jasmonic acid (JA) or its methyl ester (MeJA) (Farmer et al., 1992; Peña-Cortés et al., 1992). Further studies have revealed that synthesis of proteinase inhibitors is stimulated not only by jasmonates but also by their biosynthetic precursors, linolenic, 13(*S*)-hydroperoxylinolenic, and 12-oxo-phytodienoic acids (Farmer and Ryan, 1992). These studies suggest that plant wound signaling is mediated by octadecanoid pathways, which are analogous to inflammation signaling pathways in animals (Bergey et al., 1996). Jasmonates have been shown to accumulate in the plant body upon mechanical wounding (Albrecht et al., 1992; Creelman et al., 1992), a further indication that they are natural inducers of proteinase inhibitors. It has been proposed that the initial wound signal stimulates the phosphorylation of protein kinases and activates enzymes involved in jasmonate synthesis (Farmer and Ryan, 1992). This hypothesis was partly substantiated by our finding that a rapid, systemic increase in transcripts encoding WIPK, a mitogen-activated protein (MAP) kinase homolog in tobacco, was observed in response to mechanical wounding (Seo et al., 1995).

MAP kinases constitute a family of serine/threonine protein kinases that have been well conserved in all eukaryotes throughout evolution (Nishida and Gotoh, 1993; Jonak et al., 1994; Nishihama et al., 1995). They are enzymatically activated when threonine and tyrosine residues within the TXY motif in subdomain VIII are phosphorylated (Anderson et al., 1990). In animals and yeasts, MAP kinases function in the transduction of extracellular stimuli into intracellular signals to regulate expression of genes that are essential for a wide variety of cellular processes, including cell proliferation, differentiation, and stress responses (Blumer and Johnson, 1994; Davis, 1994; Cano and Mahadevan, 1995; Herskowitz, 1995).

Plant MAP kinases also have been implicated in defense and phytohormone responses (reviewed in Suzuki and Shinshi, 1996; Hirt, 1997; Machida et al., 1997; Mizoguchi et al., 1997). In tobacco, a 46-kD protein kinase with characteristics of a MAP kinase was found to be activated enzymatically by wound stress (Seo et al., 1995; Usami et al., 1995). However, the identity of the tobacco wounding-activated protein kinase has remained unclear. Zhang and Klessig (1998a) used SIPK-, a salicylic acid–activated tobacco MAP

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kinase, and WIPK-specific antibodies to show that wounding activates SIPK and does not activate WIPK (Zhang and Klessig, 1997). In this study, however, we report that under our experimental conditions, WIPK was found to be activated in response to wound stress.

Upon mechanical wounding of transgenic tobacco plants transformed with the *wipk* gene in which endogenous *wipk* gene expression was suppressed (Seo et al., 1995), the plants did not accumulate either JA or MeJA, and there was no expression of the JA-inducible *PI-II* gene, which encodes proteinase inhibitor II. Therefore, these plants show a loss of function of WIPK. These results suggest that MAP kinases are an essential component in the jasmonate-mediated wound signal transduction pathway. However, because loss-of-function transgenic plants were used, the evidence obtained in these experiments is indirect. In this study, active WIPK-overproducing transgenic plants were selected and used to assess whether constitutive enzymatic activation of WIPK results in constitutive activation of the JA signaling pathway.

RESULTS

WIPK Activation in Response to Wounding

When tobacco plants were wounded, rapid and transient activation of a 46-kD protein kinase, which phosphorylates a myelin basic protein (MBP), an artificial substrate, was seen (Seo et al., 1995; Usami et al., 1995). To examine whether MBP kinase activity correlates with activation of WIPK, an antibody specific to WIPK was raised against a synthetic peptide corresponding to the N-terminal 18–amino acid sequence (MADANMGAGGGQFPDFPS) of WIPK. This synthetic peptide does not show sequence identity with other known tobacco MAP kinases, including Ntf3 (Wilson et al., 1993), Ntf4 and Ntf6 (Wilson et al., 1995), and SIPK (Zhang and Klessig, 1997). To test the antibody's reactivity to the WIPK protein, the protein encoded by *wipk* was expressed in *Escherichia coli* as a fusion product with glutathione *S*-transferase (GST) by using the pGEX expression vector. The fusion protein that was purified using glutathione–Sepharose beads gave two major bands on an SDS–polyacrylamide gel (Figure 1, lane 1). The band of high molecular mass corresponds to the full-sized recombinant WIPK protein, whereas the band of low molecular mass corresponds to a truncated form of WIPK. The antibody reacted with the full-sized form (Figure 1, lane 3). The WIPK protein that separated from GST–WIPK upon digestion with thrombin was slightly larger on an SDS–polyacrylamide gel than was calculated from the predicted amino acid sequence of WIPK (42.8 kD; Figure 1, lane 2). This appears to be due to incorrect termination of translation in the expression vector. The antibody reacted with the WIPK protein but not with the GST protein (Figure 1, lane 4). In extracts of healthy tobacco leaves, the antibody reacted with a protein with an apparent molecular mass of ~43 kD (Figure 1, lane 5), suggesting that the 43-kD band is WIPK.

In a preliminary experiment, we discovered that when extracts from wounded tobacco leaves were immunoprecipitated with the WIPK antibody, the immune complex had MBP kinase activity. However, Zhang and Klessig (1998a) recently reported that when using an antibody raised against a peptide identical to our synthetic peptide, they detected no WIPK-mediated MBP kinase activity in immunoprecipitates from extracts of wounded leaves. Based on this result, the authors concluded that WIPK is not activated in response to wounding. To investigate these contradictory results, we carefully examined the experimental procedures used by Zhang and Klessig (1998a). Their assay of WIPK kinase activity was based on detection of phosphorylated MBP in the presence of γ-^{32}P-ATP. Although the extraction and immunoprecipitation procedures did not differ markedly in their kinase reaction procedure, a final concentration of 10 mM unlabeled ATP was used (Zhang and Klessig, 1998a) as compared with 100 μM in our protocol. An excess amount of unlabeled ATP prevents the transfer of γ-phosphate from γ-^{32}P-ATP to MBP; thus, apparent kinase activity represented by an increase in radioactivity would not be detected.

Therefore, we examined the effect of various concentrations of unlabeled ATP on the level of MBP kinase activity of WIPK. When concentrations of unlabeled ATP below 100

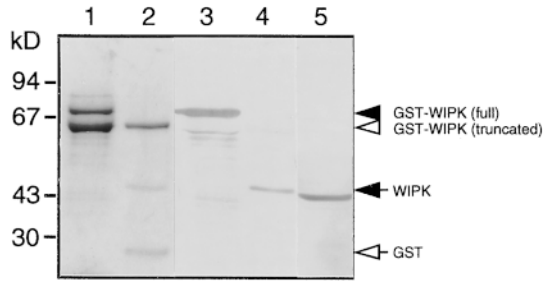

Figure 1. Immunoblot Analyses of the Recombinant GST–WIPK Fusion Protein and Tobacco Leaf Extracts.

Bacterially expressed GST–WIPK fusion proteins before (lanes 1 and 3) and after (lanes 2 and 4) cleavage with thrombin were separated by electrophoresis on 10% SDS–polyacrylamide gels. One of the duplicate gels was stained with Coomassie Brilliant Blue R 250 (lanes 1 and 2), and the other was transferred to a nitrocellulose membrane (lanes 3 and 4). The membrane was subjected to immunoblot analysis with an antibody raised against a synthetic peptide corresponding to the N-terminal 18–amino acid sequence of WIPK (see text). Fifty micrograms of protein from healthy tobacco leaf extracts also was probed with the anti-WIPK antibody (lane 5). The arrowhead, open arrowhead, arrow, and open arrow indicate the full-sized GST–WIPK, the truncated forms of GST–WIPK, WIPK, and GST separated from GST–WIPK by thrombin digestion, respectively. Size markers are indicated at left in kilodaltons.

μM were used, a strong signal from phosphorylated MBP was detected in immunoprecipitates from extracts of both healthy and wounded leaves (Figure 2A). Immunoprecipitates from the wounded leaves showed a higher level of MBP kinase activity than did those from healthy leaves. At 10 mM unlabeled ATP, kinase activity was not detected even in immunoprecipitates from extracts of wounded leaves. This result is in agreement with the results published by Zhang and Klessig (1998a). Antibody specificity is demonstrated by the lack of detectable MBP kinase activity in immunoprecipitates from the reaction of preimmune serum with extracts of wounded leaves (Figure 2B). From these results, we conclude that WIPK is actually activated in response to wounding. Hereafter in this study, we used a concentration of 100 μM unlabeled ATP.

We and others reported previously that in tobacco, activity of a 46-kD MBP kinase is induced by wounding (Seo et al., 1995; Usami et al., 1995). The observed 46-kD protein kinase is probably formed from both SIPK and WIPK because tobacco SIPK is activated in response to wound stress as well (Zhang and Klessig, 1998a).

Local and Systemic Activation of WIPK by Wounding

Time-course analyses of the level of MBP kinase activity and protein level of WIPK over a period of 60 min after wounding were studied using soluble cytoplasmic proteins. The kinase activity of WIPK increased by 3 min after wounding, reached a maximal level at 5 min, and declined to the basal level at 30 min (Figure 3A). Wounding induced an approximately twofold increase in kinase activity (Figure 3B). The immunoblot clearly shows that the level of WIPK protein in the cytoplasm during the wound response remains constant (Figure 3A).

Activation of WIPK was not limited to a wound in the leaves. When the stems of healthy wild-type plants were cut, rapid and transient activation of WIPK was observed in the adjacent upper leaves (Figures 4A and 4B). This suggests that the wound signal for activation of WIPK is transmitted locally and systemically.

Transgenic Tobacco Plants Constitutively Expressing *wipk* and *PI-II* Genes

The sense-oriented *wipk* cDNA was introduced into tobacco plants under the control of the 35S cauliflower mosaic virus (CaMV) promoter (Figure 5A). Twenty-nine independent 35S::*wipk* lines were obtained by kanamycin resistance. Selection of active WIPK-overproducing transgenic plants was accomplished by testing for constitutive accumulation of *PI-II* gene transcripts in healthy leaves at 3 months after transformation. RNA gel blot analysis revealed that six lines (21% of total transformants) constitutively accumulated the *PI-II* gene transcript (Figure 5B). At 1 hr after wounding, more 1.5-kb *wipk* transgene transcript had accumulated in five of these transgenic lines (except for 35S::*wipk*-6) than had the 1.8-kb endogenous transcript in control plants (Figure 5B). To study the constitutive activation of WIPK in constitutive *PI-II* gene expressers, we examined the kinase activity of WIPK in healthy plants. The level of MBP kinase activity in healthy leaves of the 35S::*wipk*-2, 35S::*wipk*-5, and 35S::*wipk*-8 lines, which contained high levels of *PI-II* transcript, was ~1.5-fold the level of MBP kinase activity in the control plants (Figure 6A). This indicates that the 35S::*wipk*-2, 35S::*wipk*-5, and 35S::*wipk*-8

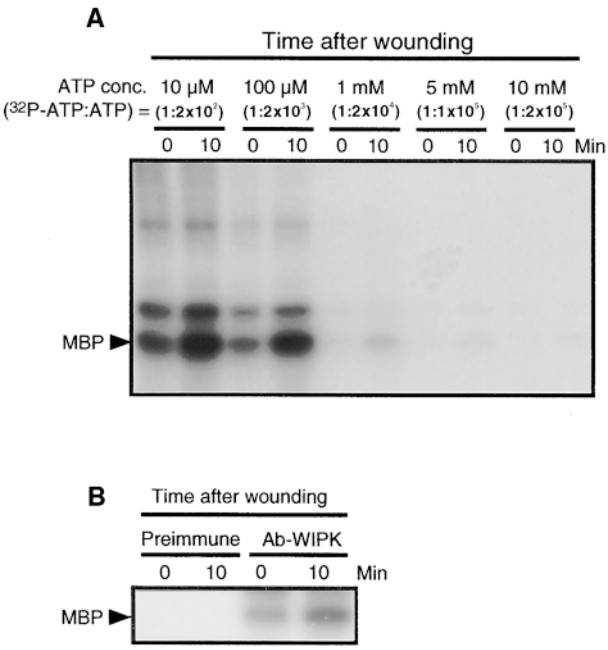

Figure 2. Detection of MBP Kinase Activity in Immunoprecipitates from Tobacco Extracts Using the Anti-WIPK Antibody.

Upper, fully expanded healthy leaves of 3-month-old wild-type tobacco plants were wounded, harvested at the indicated time points, and used for extraction of soluble cytoplasmic proteins.

(A) MBP kinase activity of immunoprecipitated WIPK in the presence of various concentrations of unlabeled ATP. Fifty micrograms of total protein was immunoprecipitated with 5 μg of the anti-WIPK antibody, and the immune complexes were subjected to an MBP kinase activity assay at 50 nM ^{32}P-labeled ATP and the indicated concentrations (conc.) of unlabeled ATP. The molar ratios of ^{32}P-labeled ATP (^{32}P-ATP) to unlabeled ATP (ATP) are shown. The arrowhead indicates the position of the phosphorylated MBP. An autoradiogram exposed for 18 hr at −80°C is shown. The experiment was repeated twice with similar results.

(B) MBP kinase activity in immunoprecipitates with the anti-WIPK antibody or preimmune serum. Fifty micrograms of total protein was immunoprecipitated with 5 μg of the anti-WIPK antibody (Ab-WIPK) or 5 μg of preimmune serum (Preimmune), and the immune complexes were subjected to MBP kinase activity assay in the presence of 100 μM unlabeled ATP. The arrowhead indicates the position of the phosphorylated MBP. An autoradiogram exposed for 12 hr at −80°C is shown. The experiment was repeated twice with similar results.

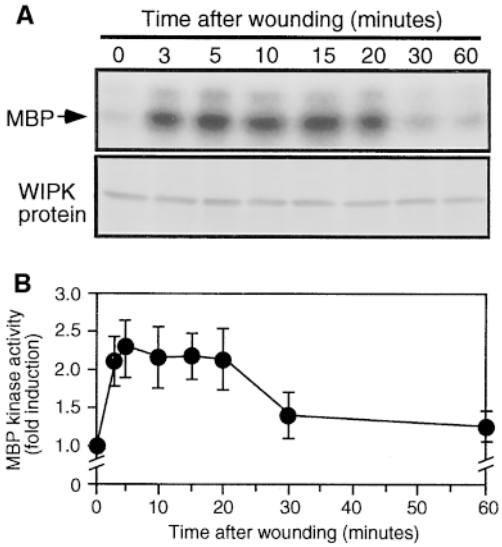

Figure 3. MBP Kinase Activity and WIPK Protein Level over the Course of 60 Min after Wounding.

Upper, fully expanded healthy leaves of 3-month-old wild-type tobacco plants were wounded and harvested at the indicated time points after wounding. Soluble cytoplasmic proteins were extracted for MBP kinase activity assay or immunoblot analysis.

(A) MBP kinase activity of WIPK (top). Cytoplasmic extracts containing 50 μg of total protein were immunoprecipitated with 5 μg of the anti-WIPK antibody, and the level of MBP kinase activity was measured in the immunoprecipitates. An autoradiogram exposed for 12 hr at −80°C is shown. The arrow indicates the position of the phosphorylated MBP. Protein levels of WIPK were determined (bottom). Cytoplasmic extracts containing 50 μg of total protein were immunoblotted with the anti-WIPK antibody.

(B) Relative MBP kinase activity of WIPK as shown in **(A)**. WIPK activity is shown as the fold induction of MBP kinase activity, with the level of MBP kinase activity at time zero set equal to 1. Values are the mean ±SD from four independent experiments.

lines have a higher constitutive level of MBP kinase activity. In wild-type plants, *wipk* transcripts accumulate in advance of JA (Seo et al., 1995), and the gene encoding PI-II is JA inducible. The levels of JA and MeJA were therefore examined in healthy leaves of both the control and transgenic plants (Figure 6B). The levels of JA and MeJA in the transgenic plants (lines 35S::*wipk*-2, 35S::*wipk*-5, and 35S::*wipk*-8) 3 months after transformation were three- to fourfold higher than those in the wild-type plants.

Loss of Constitutive Expression of the *PI-II* Gene and Accumulation of High Levels of Jasmonate in Aged 35S::*wipk* Lines

Five-month-old wild-type plants and plants of the 35S::*wipk*-2, 35S::*wipk*-5, and 35S::*wipk*-8 lines were wounded. At 1 hr after wounding, the quantity of the 1.5-kb *wipk* transgene transcript that had accumulated in the transgenic plants was less than half of the quantity of the 1.8-kb endogenous transcript in wild-type plants (Figure 7A). In healthy leaves of these three 35S::*wipk* lines, a higher constitutive level of MBP kinase activity of WIPK was not observed (Figure 7B). In 5-month-old 35S::*wipk* lines, constitutive accumulation of the *PI-II* transcript (Figure 7A) and accumulation of high levels of jasmonate (Figure 7C) were not seen. Similar phenomena were observed in other constitutive *PI-II* expressers shown in Figure 5B (lines 35S::*wipk*-6, 35S::*wipk*-7, and 35S::*wipk*-9; data not shown). Thus, all 35S::*wipk* lines constitutively expressing the *PI-II* gene lost the gain-of-function phenotype for WIPK at the adult stage.

DISCUSSION

In this study, we demonstrate that activation of WIPK is a prerequisite for jasmonate-based wound signal transduction.

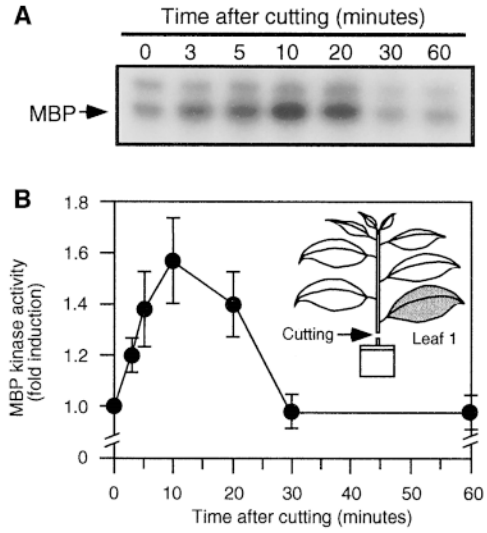

Figure 4. Systemic Activation of WIPK by Wound Stress.

(A) MBP kinase activity of WIPK over the course of 60 min after cutting. The stem of a 3-month-old healthy wild-type plant was cross-cut, and the leaf (Leaf 1 in **[B]**) adjacent to the cut position was harvested at the indicated time points after cutting. Soluble cytoplasmic proteins were extracted, and 50 μg of total protein was used for immunoprecipitation with 5 μg of the anti-WIPK antibody. The immunoprecipitates were then subjected to the MBP kinase activity assay. The arrow indicates the position of the phosphorylated MBP.

(B) Relative MBP kinase activity of WIPK as shown in **(A)**. WIPK activity is shown as the fold induction of MBP kinase activity, with the level of MBP kinase activity at time zero set equal to 1. Values are the mean ±SD from three independent experiments. A different plant was used for sampling at each time point. The leaf position is illustrated.

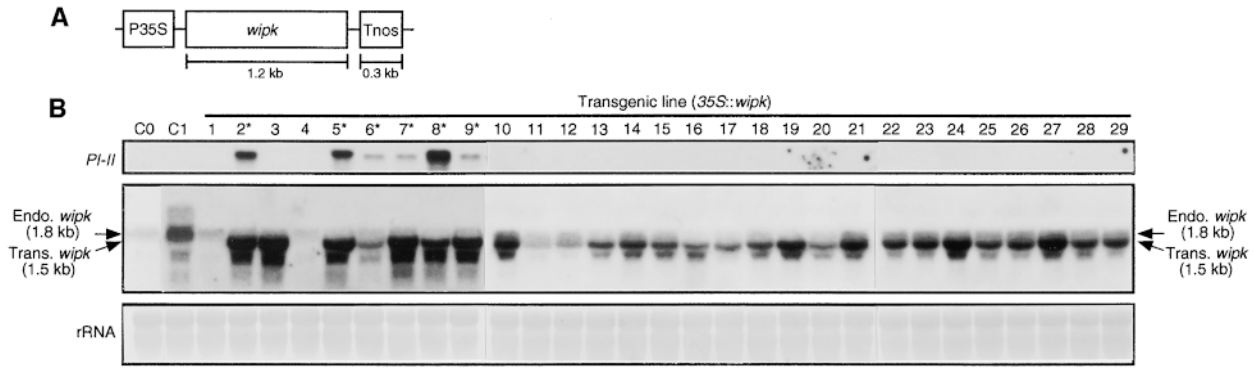

Figure 5. Analysis of Transgenic Tobacco Plants Transformed with the *wipk* Gene.

(A) Schematic representation of the *wipk* expression vector with CaMV 35S promoter. P35S, 5′ upstream sequence of the CaMV 35S promoter (−90 to −1); *wipk*, a 1.2-kb *wipk* cDNA in the binary vector pBI221; Tnos, polyadenylation signal of the gene for nopaline synthase in the Ti plasmid.
(B) RNA gel blot analysis of transgenic 35S::*wipk* lines. At 3 months after transformation, healthy leaves of 35S::*wipk* transgenic plants were detached, and total RNA was immediately extracted. Aliquots of 20 μg of RNA per lane were subjected to electrophoresis, transferred to a nylon membrane, and successively hybridized with ^{32}P-labeled cDNAs encoding PI-II and WIPK. For the control sample, healthy leaves of transgenic plants carrying the vector alone were harvested (C0) or wounded and then detached 1 hr later (C1), and total RNA was extracted. In the transgenic 35S::*wipk* lines, the 1.5-kb RNA (Trans. *wipk*) consists of a 1.2-kb *wipk* cDNA fragment and a 300-bp nopaline synthase terminator region originating from the vector. The 1.8-kb RNA (Endo. *wipk*) represents the length of the endogenous *wipk* gene. The transgenic plants marked with asterisks indicate that constitutive accumulation of the *PI-II* transcript was seen in that line. To standardize RNA loading, the blot was stained with methylene blue (rRNA; bottom). Autoradiograms exposed for 48 hr at −80°C are shown.

When wild-type tobacco plants are mechanically wounded, WIPK is rapidly and transiently activated. Transgenic tobacco plants that constitutively express active WIPK accumulate three- to fourfold higher levels of jasmonate than do wild-type plants.

Jasmonates have been proposed to be key signaling molecules in the transduction of wound stress events that cause the expression of defense-related genes (Koiwa et al., 1997; Wasternack and Parthier, 1997). Protein kinases have been proposed to play a role in the synthesis of jasmonates (Farmer and Ryan, 1992) and in the expression of jasmonate-inducible genes (Xu et al., 1994; León et al., 1998). It is highly probable that WIPK is one of these protein kinases, because this function would be consistent with the fact that MAP kinases have a wide and fundamental function in eukaryote signal transduction pathways.

Enzymatic activation of MAP kinases occurs post-translationally in animals and yeasts. This also happens in plants because WIPK is normally activated after wounding, despite maintaining steady protein levels. Consistent with our observation, alfalfa MMK4, which shows high sequence similarity to WIPK, is activated post-translationally in response to drought, cold, and wounding (Jonak et al., 1996; Bögre et al., 1997). MAP kinases generally may be activated at the post-translational level whenever they function.

In animals, transcript levels of MAP kinases are not affected by external stimuli. For example, no marked changes in transcript levels are observed during Xenopus oocyte maturation, despite a transient increase in MAP kinase activity (Gotoh et al., 1991). In plants, however, not only are MAP kinases activated but the gene expression of MAP kinases is induced at the transcriptional level with wounding, as evidenced by the rapid accumulation of the transcripts of WIPK (Seo et al., 1995) and MMK4 (Bögre et al., 1997). Transcript levels of Arabidopsis ATMPK3, which shows high sequence similarity to WIPK, increased when plants underwent cold, drought, or touch (Mizoguchi et al., 1996). The physiological significance of this transcriptional activation of plant MAP kinases remains to be clarified.

When WIPK is rapidly and transiently activated by phosphorylation, dephosphorylation can occur after the healing process is completed, and cellular levels of WIPK also may be decreased. Assuming that a steady state level of WIPK protein is necessary to allow rapid response to unpredictable wound stress, it could be expected that plants would produce *wipk* transcripts to compensate for the decreased protein levels of WIPK.

In tobacco, at least two MAP kinases, WIPK and SIPK, are activated in response to wound stress. These two kinases may play different roles in the wound response. SIPK seems to be a multistress-responsive MAP kinase because it is activated by various external stimuli, such as salicylic acid, elicitors, wounding, and tobacco mosaic virus infection (Zhang and Klessig, 1998a, 1998b; Zhang et al., 1998). However, the physiological function of SIPK is still unclear.

The mechanism underlying the constitutive activation of WIPK in transgenic 35S::*wipk* lines may involve autophosphorylation of the gene products by the introduced *wipk*

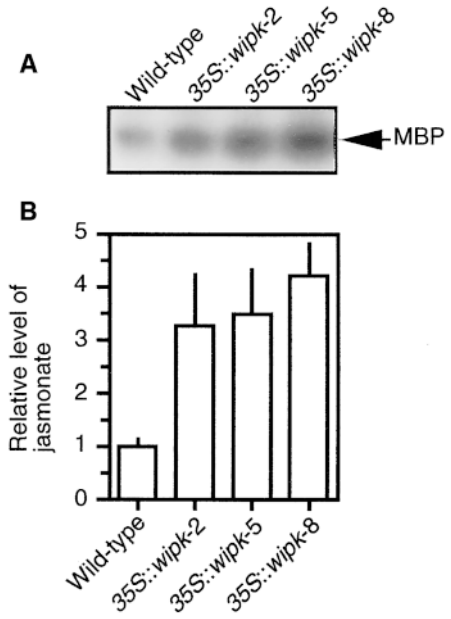

Figure 6. Levels of MBP Kinase Activity of WIPK and Jasmonate in Healthy Leaves of Wild-Type and 35S::*wipk* Transgenic Plants.

Healthy leaves of 3-month-old trangenic lines 35S::*wipk*-2, 35S::*wipk*-5, and 35S::*wipk*-8 were detached and used for immunoprecipitation with the anti-WIPK antibody or quantitation of JA and MeJA. For controls, healthy leaves of wild-type plants were used.

(A) MBP kinase activity of WIPK. The arrow indicates the position of the phosphorylated MBP.

(B) The quantity of JA and MeJA in the leaves of the transgenic lines expressed as a ratio of that present in the wild type. Values are the mean ±SD of three determinations from different leaves.

gene because recombinant WIPK protein is able to autophosphorylate in vitro (Seo et al., 1995). Another possibility is that an increase in *wipk* transcripts affects phosphorylation of WIPK, although such a mechanism is not known.

The results in Figure 5 suggest that to maintain constitutive accumulation of the transcript for the *PI-II* gene in transgenic plants, an equal or greater level of the *wipk* transgene transcript would be required when compared with the level of the endogenous *wipk* transcript accumulated 1 hr after wounding of wild-type plants. However, the 35S::*wipk*-3, 35S::*wipk*-24, and 35S::*wipk*-27 transgenic plants did not show constitutive expression of the *PI-II* gene, despite containing such high levels of *wipk* transcripts. Possible explanations are the failure of WIPK to be autophosphorylated or a disturbance of the pathway leading to phosphorylation by an increase in *wipk* transcripts. The 35S::*wipk*-6 transgenic line was a constitutive *PI-II* expresser, even though the level of *wipk* transgene transcripts in this plant was lower than was the level of endogenous *wipk* transcripts in wild-type plants 1 hr after wounding. The simplest possible explanation for the phenotype in the 35S::*wipk*-6 line is that autophosphorylation of WIPK occurred. Further studies on the relationship between an increase in *wipk* transcripts and phosphorylation of WIPK are required.

In the adult stage of transgenic 35S::*wipk* lines, constitutive activation of WIPK was lost (Figure 7B). If an increase in *wipk* transcripts affects phosphorylation of WIPK, the loss appears to be due to suppression of the *wipk* transgene. Developmental suppression of the *wipk* transgene also was observed in second-generation plants obtained by self-pollination of the transgenic lines (data not shown). One possible explanation for the mechanism of such developmental suppression is that the transcriptional activity of the CaMV 35S promoter is reduced during development of the plant. Indeed, there have been reports on developmental silencing of transgenes driven by the 35S promoter in transgenic tobacco plants (Kunz et al., 1996; Balandin and Castresana, 1997).

In animals and yeast, MAP kinase cascades are based on phosphorylation and dephosphorylation of the constituent kinases, and the dephosphorylation of MAP kinases is mediated by the kinase-specific serine/threonine or tyrosine phosphatases (Sun et al., 1993; Doi et al., 1994; Shiozaki and Russell, 1995; Wurgler-Murphy et al., 1997). Therefore, such phosphatases function as negative regulators of the MAP kinase cascade. In plants, an alfalfa homolog encoding protein phosphatase 2C is involved in inactivation of a specific MAP kinase activated by cold, drought, touch, and wounding (Meskiene et al., 1998). It is possible that the transient activation of WIPK after wounding also is due to a WIPK-specific phosphatase, although such a phosphatase has not been identified.

Wounding causes systemic activation of WIPK. It has been reported that the 18–amino acid polypeptide systemin, a systemic wound signal (Bergey et al., 1996), activates a 48-kD MAP kinase–like protein kinase in tomato (Stratmann and Ryan, 1997). By analogy, systemin or a similar systemic signal(s) could trigger the type of activation of WIPK at distant sites observed in our study, although a systemin analog has not been found in tobacco. A change in the electric current (Wildon et al., 1992) is another candidate for a signal for systemic activation of WIPK. Instantaneous changes of hydraulic pressure due to wounding (Malone, 1992) also may be involved in the systemic activation. Further studies on the signals inducing a systemic response to wounding are clearly warranted.

METHODS

Plant Material, Wound Stress Treatments, and Leaf Sampling

Wild-type tobacco (*Nicotiana tabacum* cv Samsun NN) plants were grown in a temperature-controlled greenhouse at 24°C under natural sunlight. Ten days before the wound stress treatments, wild-type

and transgenic tobacco plants were transferred to a temperature-controlled chamber maintained at 24°C with a 16-hr-light (120 μE m^{-2} sec^{-1})/8-hr-dark cycle. Our general wound stress treatment consisted of gently rubbing the upper epidermis of the whole leaves with wet carborundum (mesh 600; Kishida Chemical Co., Osaka, Japan) for 20 to 30 sec. For systemic induction experiments, stems of plants were cut basally using a pair of scissors. The cut plants were potted immediately, and at the specific time points, the upper leaves adjacent to the cut positions were harvested. All harvested leaves were frozen immediately in liquid nitrogen and stored at −80°C until processed. Because it has been reported that darkness induces a gene encoding a tobacco basic pathogenesis-related protein 1 (Eyal et al., 1992; Sessa et al., 1995), which is a member of pathogenesis-related proteins that also include proteinase inhibitors (Van Loon et al., 1994), wound stress treatments and leaf sampling for analysis of *PI-II* gene expression were performed at noon to rule out the possibility of the induction of the *PI-II* gene at night.

Production and Purification of Recombinant Glutathione *S*-Transferase–WIPK Protein

Bacterially expressed glutathione *S*-transferase (GST)–WIPK fusion protein was purified as described previously (Seo et al., 1995) and digested with thrombin (Smith and Johnson, 1988).

Antibody Production

A polyclonal antiserum was raised against synthetic peptide MADANMGAGGGQFPDFPS at the N terminus of WIPK (Seo et al., 1995). The antiserum was purified by affinity column chromatography (Quality Controlled Biochemicals, Inc., Hopkinton, MA).

Preparation of Protein Extracts

All the extraction procedures were performed on ice or at 4°C. For extraction of soluble cytoplasmic proteins, leaf material was ground in an ice-cold mortar with liquid nitrogen and extracted with an equal volume (1 mL per 1 g fresh weight of tissue) of extraction buffer (50 mM Hepes, 0.15 M NaCl, 15 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 60 mM β-glycerophosphate, 50 mM NaF, 10 mM Na_3VO_4, 5 mM DTT, and one tablet of complete protease inhibitors per 10 mL [complete, Mini; Boehringer Mannheim], pH 7.5, at 0°C). The extracted solution was subjected to centrifugation at 18,000*g* for 30 min at 4°C, and the supernatant was regarded as soluble cytoplasmic proteins and immediately used for analyses.

Measurement of Protein Concentration

The protein concentration was measured with a protein assay kit (Bio-Rad) with BSA as the standard.

Immunoprecipitation and Myelin Basic Protein Kinase Activity Assay

For immunoprecipitation, solutions containing proteins that had been made up to a total volume of 100 μL with immunoprecipitation buffer (20 mM Tris, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton

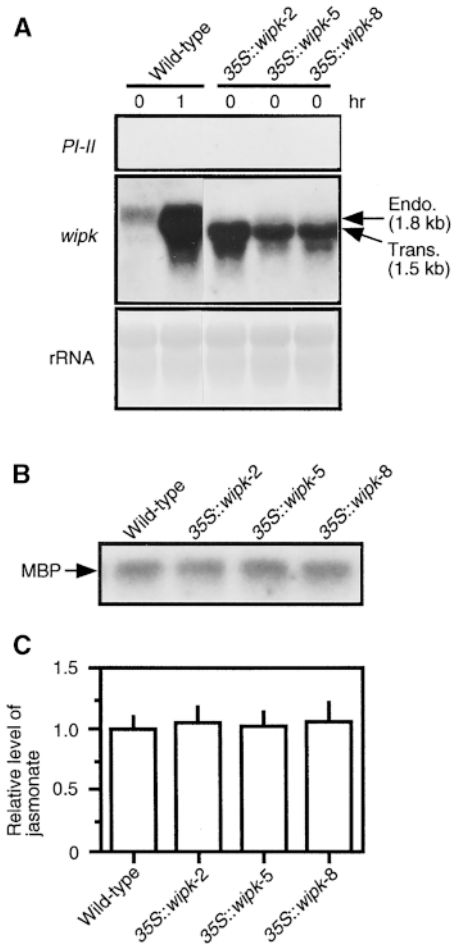

Figure 7. Analysis of 35S::*wipk* Transgenic Plants 5 Months after Transformation.

Five months after transformation, healthy upper leaves of transgenic 35S::*wipk* lines that showed the phenotype of a constitutive *PI-II* expresser were detached. Total RNA was extracted and subjected to immunoprecipitation with the anti-WIPK antibody or quantitation of JA and MeJA. For controls, healthy leaves of wild-type plants were used.

(A) Aliquots of RNA (20 μg per lane) were subjected to RNA blot analysis with the *PI-II* and *wipk* cDNA probes. As a control, healthy leaves of wild-type plants were harvested, or wounded and then harvested 1 hr later. In transgenic plants, the 1.5-kb RNA (Trans.) consists of a 1.2-kb *wipk* cDNA fragment and a 300-bp nopaline synthase terminator region originating from the vector. The 1.8-kb RNA (Endo.) represents the length of the endogenous *wipk* gene. To standardize RNA loading, the blot was stained with methylene blue (rRNA; bottom). Autoradiograms exposed for 60 hr at −80°C are shown.

(B) MBP kinase activity of WIPK. The arrow indicates the position of the phosphorylated MBP.

(C) The quantity of JA and MeJA is expressed as a ratio of that present in the control plants. Values are the mean ±SD of three determinations from different leaves.

X-100, 0.5% Nonidet P-40, 10 mM β-glycerophosphate, 50 mM NaF, 10 mM Na_3VO_4, and one tablet of complete protease inhibitors per 10 mL [Boehringer Mannheim], pH 7.5, at 0°C) were incubated in a 1.5-mL Eppendorf tube with anti-WIPK antibody or preimmune serum for 1 hr on ice. After binding for 1 hr to 50 μL of 50% protein A–Sepharose CL-4B (Pharmacia) on ice, the immunoprecipitates were washed three times with 1.3 mL of immunoprecipitation buffer and then six times with 1 mL of kinase reaction buffer (20 mM Hepes, pH 7.5, 5 mM EGTA, 15 mM $MgCl_2$, and 1 mM DTT). To detect kinase activity in the immunoprecipitates, Sepharose beads were suspended in 20 μL of kinase reaction buffer containing 0.375 mg/mL myelin basic protein (MBP), one of the following concentrations of cold ATP (10 μM, 100 μM, 1 mM, 5 mM, or 10 mM), and 50 nM γ-^{32}P-ATP (Amersham; ~4000 Ci/mmol) and incubated for 25 min at room temperature. The reaction was stopped by the addition of a half volume of 3 × SDS gel loading buffer (1 × SDS gel loading buffer is 65 mM Tris–HCl, pH 6.8, 2.95% SDS, 720 mM β-mercaptoethanol, 0.01% bromophenol blue, and 10% glycerol) and boiling for 2 min. Reaction products were separated by electrophoresis on a 15% SDS–polyacrylamide gel, and MBP phosphorylation was analyzed by autoradiography. Quantitation of relative kinase activities was determined with the NIH Image 1.61 (National Institutes of Health, Bethesda, MD) program.

Immunoblot Analysis

Protein samples were separated by electrophoresis on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schuell) in a solution containing 25 mM Tris and 92 mM glycine. After blocking with 5% BSA in TN (10 mM Tris–HCl, pH 7.4, and 0.15 M NaCl), membranes were incubated with the anti-WIPK antibody at a dilution of 1:2000 for 2 hr at room temperature and extensively washed first with TN twice, then twice with TN containing 0.05% Nonidet P-40, and finally twice with TN. The membranes were incubated with alkaline phosphatase–conjugated anti–rabbit IgG antibody (Organon Teknika Corporation, Durham, NC) at a dilution of 1:2000 for 1.5 hr at room temperature. After washing as descibed above, the antibody–antigen complexes were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Quantitation of the relative protein level was determined with the NIH Image 1.61 program.

Construction of *wipk* Transgene Vector and Tobacco Transformation

The *wipk* cDNA fragments without their 5′ and 3′ untranslated regions were synthesized by amplifying an appropriate region by using polymerase chain reaction (PCR) with primers 5′-ATGGCTGATGCAAATATGGGTGCCG-3′ (corresponding to positions 114 to 138; Seo et al., 1995) and 5′-GGAAAGTAGATACTCCAGATCA-3′ (corresponding to positions 1281 to 1302) containing BamHI and SacI sites in the 5′ flanking regions. The PCR product was verified by DNA sequencing, digested with BamHI and SacI, and then ligated, in the sense orientation relative to a promoter, to pBI221 (Clonetech, Palo Alto, CA), which had previously been digested with BamHI and SacI. The *wipk* expression construct was introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation (Wen-jun and Forde, 1989). Transformation of Samsun NN tobacco was performed by using the leaf disc cocultivation method (Horsch et al., 1985). Leaf discs were immersed in a bacterial solution for 5 min, placed on incubation medium (basal Murashige-Skoog [Nihon Seiyaku Co., Tokyo, Japan] medium with 3% sucrose and B5 vitamins) containing naphthaleneacetic acid (100 μg/L) and benzylaminopurine (1 mg/L) for 2 days at 25°C under continuous illumination by using a white fluorescence lamp at an intensity of 10 μE m^{-2} sec^{-1}, and transferred to the same medium but containing 250 μg/mL cefotaxime (Chugai Pharmaceutical Co., Tokyo, Japan). After 2 days, leaf discs were transferred to selection medium (incubation medium containing 250 μg/mL cefotaxime and 100 μg/mL kanamycin). Plates containing leaf discs on selection medium were incubated at 25°C with 16 hr of light at an intensity of 60 μE m^{-2} sec^{-1} for the first 10 days and then at 120 μE m^{-2} sec^{-1}. The transfer of leaf discs to new selection medium was repeated every 10 days until green shoots were generated from the cut surface of the discs. The shoots were transferred to hormoneless selection medium to allow rooting. Plantlets were transplanted to pots containing vermiculite and grown in an isolated greenhouse maintained at 25°C under sunlight.

RNA Gel Blot Analysis

Total RNA from leaf materials was extracted by the aurin tricarboxylic acid method described by Nagy et al. (1988). For RNA gel blot analysis, total RNA was denatured at 65°C, fractionated on a 1.2% formaldehyde–agarose gel, and blotted onto Hybond-N membrane (Amersham) in 20 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 16 hr. Blots were subjected to hybridization with the probe labeled with α-^{32}P-dCTP (ICN Biochemicals, Inc., Costa Mesa, CA) by the random priming method with a labeling kit (Amersham). Hybridization was performed at 42°C for 16 hr in a solution containing 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, pH 8.0, 50% (v/v) formaldehyde, 0.02% Ficoll 400 (Pharmacia), 0.02% BSA, 0.02% PVP, 0.5% SDS, 3 × SSC, 0.5% (w/v) blocking reagent (Boehringer Mannheim), and salmon sperm DNA (0.1 mg/mL). After hybridization, blots were washed twice for 10 min in 1 × SSC and 0.1% SDS at 25°C and three times for 15 min in 0.1 × SSC and 0.1% SDS at 65°C and exposed to X-OMAT AR films (Kodak) with an intensifying screen at −80°C. A cDNA probe of tobacco PI-II was synthesized by PCR using synthetic primers to give a 388-bp fragment (corresponding to positions 941 to 1328; Balandin et al., 1995). The *wipk* cDNA probe was used as a 1156-bp fragment (corresponding to positions 147 to 1302; Seo et al., 1995). Quantitation of relative transcript levels was determined with the NIH Image 1.61 program.

Quantitation of JA and MeJA

Quantitation of jasmonic acid (JA) and its methyl ester (MeJA) was performed as detailed previously (Seo et al., 1995), except that an HP6890 gas chromatograph fitted to a quadrupole mass spectrometer (Hewlett-Packard, Wilmington, DE) was used as the gas chromatography–mass spectrometry instrument.

ACKNOWLEDGMENTS

We thank Hideharu Seto (Institute of Physical and Chemical Research, Wako, Japan) for providing internal standards for JA analysis; Yoko Gotho, Hisako Ochiai, and Yumi Naito for mainte-

nance of the plant materials; Ichiro Mitsuhara, Norihiro Otsubo, and Shunichi Kosugi for helpful discussions; and Malcolm Moore for critical reading of the manuscript. This work was partially supported by Enhancement of Center of Excellence, Special Coordination Funds for Promoting Science and Technology, the Science and Technology Agency, Japan.

Received October 30, 1998; accepted December 15, 1998.

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