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Wounding Induces the Rapid and Transient Activation of a Specific MAP Kinase Pathway

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Mechanical injury in plants induces responses that are involved not only in healing but also in defense against a potential pathogen. To understand the intracellular signaling mechanism of wounding, we have investigated the involvement of protein kinases. Using specific antibodies, we showed that wounding alfalfa leaves specifically induces the transient activation of the p44^{MMK4} kinase, which belongs to the family of mitogen-activated protein kinases. Whereas activation of the MMK4 pathway is a post-translational process and was not blocked by α-amanitin and cycloheximide, inactivation depends on de novo transcription and translation of a protein factor(s). After wound-induced activation, the MMK4 pathway was subject to a refractory period of 25 min, during which time restimulation was not possible, indicating that the inactivation mechanism is only transiently active. After activation of the p44^{MMK4} kinase by wounding, transcript levels of the *MMK4* gene increased, suggesting that the *MMK4* gene may be a direct target of the MMK4 pathway. In contrast, transcripts of the wound-inducible *MsWIP* gene, encoding a putative proteinase inhibitor, were detected only several hours after wounding. Abscisic acid, methyl jasmonic acid, and electrical activity are known to mediate wound signaling in plants. However, none of these factors was able to activate the p44^{MMK4} kinase in the absence of wounding, suggesting that the MMK4 pathway acts independently of these signals.

INTRODUCTION

Mechanical injury (wounding) of plant organs results in the rapid activation of genes that play a role in two types of responses (reviewed in Bowles, 1993). A local response occurs in cells in the vicinity of the wound site and involves induction of genes involved in healing. Via a communication system(s) that may involve electrical signals and/or chemical factors, cells near the wound site trigger a systemic response in distal parts of the plant. Here, a set of genes that is responsible for the defense reaction against herbivorous pathogens becomes activated. The induction of the majority of defense proteins involves transcriptional activation and either can be restricted to the wound site or can occur systemically throughout the plant. The regulation of the expression of several of these genes is positively mediated by oligosaccharides, jasmonic acid, abscisic acid (ABA), and a peptide called systemin but is negatively affected by auxin. Although all of these compounds are involved in the wound response, the sequence of events and the causal relationship between these factors and the transcriptional activation of wound-induced genes are as yet unclear.

Protein phosphorylation is one of the major mechanisms for controlling cellular functions in response to external signals in animals and yeast. A specific class of serine/threonine protein kinases, designated mitogen-activated protein (MAP) kinases, is involved in many of these processes. In animals, these kinases are activated in response to mitogenic stimuli (Ray and Sturgill, 1987; Hoshi et al., 1988; Rossomando et al., 1992), meiosis (Gotoh et al., 1991; Posada et al., 1991), differentiation (Gotoh et al., 1990; Boulton et al., 1991), or various stresses (Ely et al., 1990; Stratton et al., 1991; Galcheva-Gargova et al., 1994; Han et al., 1994). MAP kinase activation requires phosphorylation on tyrosine and threonine residues (Anderson et al., 1990; Posada et al., 1991) and is mediated by a single dual-specific activator protein kinase, MAP kinase kinase (Alessandrini et al., 1992; Crews and Erikson, 1992; Matsuda et al., 1992). Activation of the MAP kinase activator occurs by phosphorylation on serine residues by other protein kinases, Raf-1, mos, and MAP kinase kinase kinases (Hattori et al., 1992; Kyriakis et al., 1992; Lange-Carter et al., 1993). This set of three functionally interlinked protein kinases has been identified in yeast, animals, and plants and appears to be conserved in modular form in all eukaryotes (reviewed in Hirt, 1997).

MAP kinases have also been implicated in signal transduction in plants. Touch stimuli induce the activation of a MAP kinase (Bögre et al., 1996) as well as the accumulation of transcripts encoding different protein kinases, including a MAP kinase and a MAP kinase kinase kinase (Mizoguchi et

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al., 1996). Cold and drought have also been shown to lead to the accumulation of steady state transcripts of a MAP kinase gene (Jonak et al., 1996; Mizoguchi et al., 1996) as well as to the activation of the MAP kinase enzyme (Jonak et al., 1996). Several hormones have been reported to positively or negatively affect the enzymatic activity of MAP kinases. ABA induces the activation of a MAP kinase in barley aleurone cells (Knetsch et al., 1996) but not in alfalfa leaves (Jonak et al., 1996). Gibberellic acid might affect the aleurone MAP kinase pathway in a negative way, because transcript levels of a MAP kinase gene in oat aleurone cells decreased when treated with gibberellic acid (Huttly and Phillips, 1995). Because refeeding auxin to auxin-starved cells leads to MAP kinase activation (Mizoguchi et al., 1994), it has been suggested that a MAP kinase might be involved in auxin signal transduction. Finally, it is possible that ethylene signaling might be transmitted by a MAP kinase cascade. The evidence rests on the isolation of the constitutive triple response mutant *ctr1*. *CTR1* encodes a Raf homolog and potentially acts as a MAP kinase kinase activator (Kieber et al., 1993).

Recently, mounting evidence has shown that MAP kinases might also be involved in signaling pathogens. After treating tobacco cells with a fungal elicitor, Suzuki and Shinshi (1995) activated a 47-kD MAP kinase-like protein. Cutting leaves induced the activation of a 46-kD protein kinase in tobacco and a variety of other plant species (Seo et al., 1995; Usami et al., 1995). Although circumstantial evidence highly suggests a role for a MAP kinase in pathogen signal transduction, so far no direct evidence shows the direct activation of a MAP kinase protein in response to a pathogen.

In this article, we report the involvement of a particular MAP kinase in mediating wound signal transduction in alfalfa. By using specific antibodies, we showed that wounding activates the p44^{MMK4} kinase (where *MMK4* stands for *Medicago* MAP kinase gene 4). After wounding, the activity of the p44^{MMK4} kinase rose within 1 min but decreased to basal levels within 30 min. Inhibitor studies indicated that two distinct mechanisms control the p44^{MMK4} kinase: an activating and an inactivating mechanism. Restimulation of the MMK4 pathway was not possible over a refractory period of 30 min. Finally, we tested several upstream candidates that are known to mediate wound signaling in plants and might be responsible for activation of the MMK4 pathway. However, ABA, methyl jasmonic acid (MeJA), and electrical activity were unable to stimulate p44^{MMK4} kinase activation in the absence of wounding.

RESULTS

Wounding Transiently Activates a MAP Kinase Pathway

In an attempt to investigate whether protein phosphorylation events are part of the wound signal transduction pathway, we investigated protein kinase activation in extracts from wounded leaves. For this purpose, alfalfa leaf protein extracts were analyzed at different times after wounding by in-gel protein kinase assays with the myelin basic protein

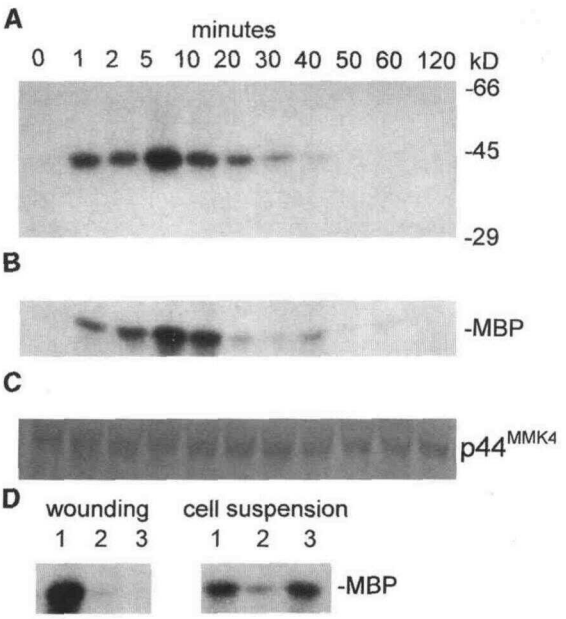

Figure 1. MMK4 MAP Kinase Is Induced by Wounding.

Alfalfa leaves were mechanically wounded by cutting the lamina with a razor blade. Leaf extracts were prepared at 0, 1, 2, 5, 10, 20, 30, 40, 50, 60, and 120 min after wounding.

(A) An MBP kinase is activated by wounding. Each lane contains 20 μg of total protein from leaf extracts. The protein was separated on an SDS–polyacrylamide gel containing 0.5 mg/mL MBP. After protein renaturation, kinase reactions were performed in the gel and analyzed by autoradiography. Numbers at right indicate the molecular masses of the marker proteins.

(B) The p44^{MMK4} kinase is activated by wounding. Leaf extracts containing 100 μg of total protein were immunoprecipitated with 5 μg of the protein A–purified M7 antibody. This antibody was raised against a synthetic peptide encoding the C-terminal 10 amino acids of the alfalfa MMK4 kinase. Kinase reactions with the immunoprecipitated protein were performed with 0.5 mg/mL MBP, 0.1 mM ATP, and 2 μCi of γ-^{32}P-ATP. The phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE.

(C) p44^{MMK4} protein amounts remain constant after wounding. Leaf extracts containing 20 μg of total protein were separated by SDS-PAGE, blotted, and probed with the M7 antibody.

(D) The p44^{MMK4} kinase, but not the MMK2 or MMK3 kinase, is specifically activated by wounding. Leaf extracts containing 100 μg of total protein at 5 min after wounding (left) or from nonwounded suspension-cultured cells (right) were immunoprecipitated with M7 (lanes 1), M11 (lanes 2), and M14 (lanes 3) antibodies. These antibodies were raised against peptides encoding the C-terminal 10 amino acids of MMK4, MMK2, and MMK3, respectively. The kinase activity of the immunoprecipitated proteins was determined as described in **(B)**.

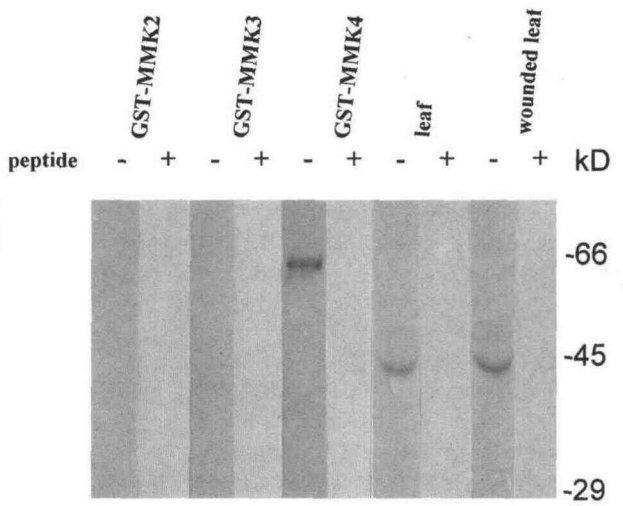

Figure 2. Specificity of the M7 Antibody.

Glutathione S-transferase (GST) fusion constructs of the *MMK2*, *MMK3*, and *MMK4* gene products were expressed in bacteria and affinity purified. The GST–MMK2, GST–MMK3, and GST–MMK4 proteins as well as crude protein extracts of alfalfa leaves and wounded leaves were separated by SDS-PAGE, blotted, and probed with the M7 antibody, which was preincubated without (−) or with (+) an excess of the peptide that was used to raise the M7 antibody. Numbers at right indicate the molecular masses of the marker proteins in kilodaltons.

(MBP) as an artificial substrate. Comparing unwounded leaves (Figure 1A, at 0 min) with wounded ones, we found that wounding induced rapid activation of an MBP kinase (Figure 1A, at 1 min). Within 60 min after wounding, MBP kinase activity had decreased to noninduced levels (Figure 1A, at 60 min). In comparison to marker proteins, the relative mobility of the protein with MBP kinase activity after being electrophoresed on an SDS–polyacrylamide gel was determined to be ~45 kD, reminiscent of the molecular mass of many MAP kinases.

To determine whether the MBP kinase activity correlated with the activation of a MAP kinase, we immunoprecipitated the same leaf extracts with the polyclonal antibody M7. The M7 antibody was raised against a synthetic peptide encoding the C-terminal 10 amino acids of the alfalfa MMK4 kinase (Jonak et al., 1996). The MBP kinase activity of the immunoprecipitated $p44^{MMK4}$ kinase was analyzed by SDS-PAGE and subsequent autoradiography (Figure 1B). The $p44^{MMK4}$ kinase was activated at 1 min, and kinase activity decreased to nondetectable levels at 30 min after wounding. Protein gel blotting of these leaf extracts with the M7 antibody detected a single band of 44 kD in all extracts (Figures 1C and 2). In contrast to the changes in immunoprecipitated MBP kinase activities, the 44-kD MMK4 protein amounts stayed constant over the experimental period of 120 min and did not parallel the changes in $p44^{MMK4}$ kinase activity. The similar kinetics of the changes in activity detected for the in-gel MBP kinase and the immunoprecipitated $p44^{MMK4}$ kinase indicate that wounding transiently activates a MAP kinase. These results agree with a recent report on the rapid activation of a 46-kD MBP kinase in leaves of a variety of plants after cutting (Seo et al., 1995; Usami et al., 1995) and identify a MAP kinase pathway as a general mechanism in wound-induced signal transduction in plants.

Wound-Specific Activation of the MMK4 Pathway

To date, several MAP kinase genes have been identified from alfalfa (Jonak et al., 1993, 1995, 1996). Because the predicted molecular masses of the alfalfa MAP kinases do not differ by >5 kD, we investigated the possibility that only a particular alfalfa MAP kinase is activated in the wounding process. For this purpose, cell extracts of wounded leaves were immunoprecipitated with three different antibodies, M7, M11, and M14, that were raised against synthetic peptides encoding the C-terminal 10 amino acids of the alfalfa MMK4, MMK2, and MMK3 MAP kinases, respectively. Immune kinase assays of protein extracts of leaves at 5 min after wounding (Figure 1D, left) showed that only the M7 antibody that was raised against the MMK4 C terminus had immunoprecipitated active kinase complexes (Figure 1D, lanes 1). To prove that immunoprecipitation with the different antibodies did not inactivate the kinases, protein extracts of suspension-cultured alfalfa cells expressing the MMK2, MMK3, and MMK4 kinases (L. Bögre, I. Meskiene, and H. Hirt, unpublished results) were also immunoprecipitated with the M7, M11, and M14 antibodies. Immune complex kinase assays (Figure 1D, right) showed that all kinases had retained activity after immunoprecipitation. These data indicate that wounding activates a particular MAP kinase pathway.

Specificity of the M7 Antibody

The specificity of the M7 antibody was tested by immunoblotting different alfalfa MAP kinases. For this purpose, the *MMK2*, *MMK3*, and *MMK4* gene products were expressed as glutathione *S*-transferase (GST) fusion proteins in bacteria. After affinity purification, we separated equal amounts of proteins by SDS-PAGE and immunoblotted these with the M7 antibody. As shown in Figure 2, only GST–MMK4 was decorated with the M7 antibody. Preincubation of the M7 antibody with the M7 peptide completely blocked the reaction (Figure 2, GST–MMK4 plus the peptide). Protein gel blots of crude protein extracts from nonwounded leaves showed a single band with a molecular mass of 44 kD that disappeared when the M7 antibody was preincubated with the M7 peptide (Figure 2, leaf without and with the peptide, respectively). M7 antibody immunoblots of crude protein extracts of leaves at 10 min after wounding revealed the same

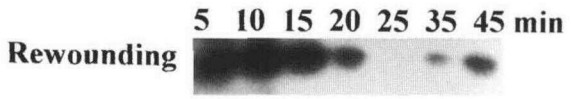

Figure 3. Activation of the MMK4 Pathway Is Transiently Refractory to Rewounding.

Alfalfa leaves were mechanically wounded by cutting the lamina with a razor blade. At 5, 10, 15, 20, 25, 35, and 45 min after wounding, leaves were rewounded. At 5 min after the rewounding treatment, leaf extracts were prepared. Leaf extracts containing 100 μg of total protein were immunoprecipitated with 5 μg of the protein A–purified M7 antibody. p44^{MMK4} kinase activity was assayed by in vitro kinase reactions with MBP and γ-^{32}P-ATP, as given in Figure 1.

amount of the 44-kD band, and the antibody reaction fully competed with the M7 peptide (Figure 2, wounded leaf, without and with the peptide, respectively).

These results show that the M7 peptide specifically recognizes the product of the *MMK4* gene. To test whether the M7 antibody was able to immunoprecipitate active MAP kinases, we immunoprecipitated the affinity purified GST–MMK2, GST–MMK3, and GST–MMK4 kinases with the M7 antibody. Subsequently, the activity of the immunoprecipitated proteins was determined by in vitro kinase reactions using MBP as a substrate. In contrast to immunoprecipitated GST–MMK4 kinase, no kinase activity was detected in the other alfalfa MAP kinases (data not shown). These results show that the M7 antibody specifically immunoprecipitates GST–MMK4 but not GST–MMK2 and GST–MMK3 kinases.

Rewounding Does Not Stimulate the MMK4 Pathway during the Refractory Period

The transient nature of the activation of the p44^{MMK4} kinase was investigated further by rewounding leaves at different times after the initial mechanical injury. Because the p44^{MMK4} kinase activity is maximal at 5 min after wounding, the rewounded leaves were collected at 5 min after the rewounding treatment and analyzed for p44^{MMK4} kinase activity by immune kinase assays. As shown in Figure 3, rewounding leaves over the first 25 min after the first wounding treatment did not induce p44^{MMK4} kinase activation above the levels induced by the initial stimulation. At 25 min after the first wounding, p44^{MMK4} kinase activity had decreased to nondetectable levels and could not be restimulated by a second wounding treatment. At 35 min and later, however, rewounding induced increasing levels of p44^{MMK4} activation. These results indicate that after initial stimulation, the MMK4 pathway is refractory to restimulation for ~25 min, suggesting that wounding also induces a mechanism to inactivate the MMK4 pathway (see below) that is active for 25 min.

Wounding Specifically Induces *MMK4* Transcript Accumulation

Wounding has been shown to induce the transcription of a variety of genes. To study the transcriptional events after wounding, we extracted RNA from alfalfa leaves at different times. After blotting to nylon filters, the RNA was hybridized with radiolabeled fragments of *MsWIP*, an alfalfa homolog of the maize *WIP1* gene that encodes a Bowman-Birk–type proteinase inhibitor and becomes transcriptionally induced within 30 min after wounding in maize (Rohrmeier and Lehle, 1993). No *MsWIP* transcript was detected in nonwounded alfalfa leaves (Figure 4). One hour after wounding, *MsWIP* transcripts became detectable and strongly increased for at least 12 hr (Figure 4). To test whether MAP kinase genes are transcriptionally induced by wounding, we subsequently blotted the same blot with radiolabeled probes of the four alfalfa MAP kinase genes. Nonwounded leaves contained low levels of *MMK4* mRNA but not of the other MAP kinases (Figure 4). In contrast to the *MMK1*, *MMK2*, and *MMK3* mRNAs, *MMK4* transcript amounts increased strongly at 20 min after wounding and decreased to the basal levels of the nonwounded leaves by 4 hr. *MMK4* transcripts accumulated after p44^{MMK4} kinase activation (Figure 1B), suggesting that transcriptional induction of *MMK4* gene expression is not necessary for the activation of the MMK4 pathway but might be a downstream target of the pathway.

Interestingly, at the time that *MMK4* transcripts accumulated, p44^{MMK4} kinase activity and the protein did not show an increase (Figures 1B and 1C, respectively). At first

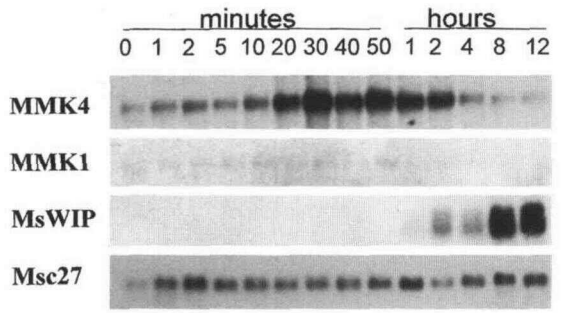

Figure 4. Transcriptional Induction of the *MMK4* and the *MsWIP* Genes by Wounding.

RNA was extracted from leaves at 0, 1, 2, 5, 10, 20, 30, 40, and 50 min and at 1, 2, 4, 8, and 12 hr after wounding. Two micrograms of poly(A)$^+$ RNA was separated per lane on a denaturing formaldehyde gel. After blotting to nylon membranes, the blot was sequentially hybridized with fragments containing the 3′ nontranslated regions of the *MMK4* and the *MMK1* genes, respectively, and the coding region of the *MsWIP* gene. As a control, the blot was hybridized with a radiolabeled fragment of the constitutively expressed *Msc27* gene.

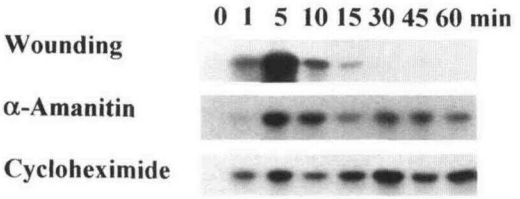

Figure 5. Inactivation of the Wound-Induced MMK4 Kinase Requires de Novo Synthesis of a Protein(s).

Before laminar wounding, we detached alfalfa leaves at the petioles and preincubated them for 2 hr in medium containing 100 μM translation inhibitor cycloheximide and 100 μM transcription inhibitor α-amanitin. Leaf extracts were prepared at 0, 1, 5, 10, 15, 30, 45, and 60 min after wounding. Extracts containing 100 μg of total protein were immunoprecipitated with the M7 antibody. Kinase reactions of immunoprecipitated MMK4 proteins were performed with γ-^{32}P-ATP and MBP as a substrate and analyzed by autoradiography after separation by SDS-PAGE.

glance, these results are puzzling but could be explained by the different turnover rates in various cellular compartments or by post-transcriptional regulation events that require further study.

Activation of the MMK4 Pathway Is Independent of de Novo Transcription and Translation

Because plant MAP kinases appear to be induced transcriptionally by wounding (Seo et al., 1995), we also investigated whether wound-induced MMK4 activation depends on de novo transcription or translation of the *MMK4* gene. Under conditions in which transcription of wound-induced genes was completely inhibited after preincubation of detached leaves with α-amanitin, the timing of activation and activity levels of p44^{MMK4} kinase was similar for leaves containing no inhibitor (Figure 5), indicating that wound-induced activation of the MMK4 pathway is not dependent on de novo transcription of genes. To investigate whether translation is necessary for the activation of the MMK4 pathway, we incubated detached leaves with cycloheximide before wounding. In contrast to the work reported by Usami et al. (1995), neither α-amanitin nor cycloheximide induced activation of the p44^{MMK4} kinase in the absence of wounding. Under conditions that inhibited incorporation of ^{35}S-methionine into proteins by >95%, the p44^{MMK4} kinase remained fully activable by wounding (Figure 5). Although a dose-dependent inhibition of transcription and translation was obtained when different concentrations of α-amanitin and/or cycloheximide were used (data not shown), the wound-induced activation of the p44^{MMK4} kinase was not affected. These results support the idea that wounding activates the MMK4 pathway by a post-translational mechanism.

Inactivation of the MMK4 Pathway Depends on de Novo Synthesis of a Protein Factor(s)

Although α-amanitin treatment of detached leaves before wounding did not affect the activation of the p44^{MMK4} kinase by wounding, inactivation of the wound-induced p44^{MMK4} kinase was completely inhibited (Figure 5), indicating that inactivation depends on the transcription of a specific gene(s). Gene transcription alone is not sufficient to inactivate the wound-induced p44^{MMK4} kinase, however, because pretreating detached leaves with cycloheximide led to the sustained activation of the p44^{MMK4} kinase. These results show that inactivation of the wound-induced MMK4 pathway requires the transcription and the translation of a protein factor(s). The inactivation mechanism appears to be active for a limited period of time, as indicated by the inability to restimulate the activated p44^{MMK4} pathway over a refractory period of 25 min.

ABA and MeJA Do Not Activate the MMK4 Pathway

ABA and MeJA are known to be involved in mediating the wound response and can induce several wound-inducible genes in the absence of a wound signal (Bowles, 1993). To test the idea that ABA or MeJA could activate the p44^{MMK4} kinase pathway, we treated intact leaves or leaf pieces with ABA and MeJA and determined p44^{MMK4} kinase activity by immune complex kinase assays. No activation was observed over a range of concentrations or time periods (data not shown). To exclude the possibility that the lack of p44^{MMK4} kinase activation was a result of the fact that ABA and MeJA were not taken up by leaf tissues, we analyzed ABA- and MeJA-treated leaves for the induction of an ABA-inducible alfalfa gene, *MsABA1*, encoding a homolog of the ABA-inducible maize pMAH9 gene (Gomez et al., 1988). ABA as well as MeJA induced activation of the alfalfa *MsABA1* gene within 10 min (data not shown; Jonak et al., 1996), indicating that the failure to activate the p44^{MMK4} kinase was not caused by uptake problems of the plant hormones.

DISCUSSION

Wounding triggers the rapid activation of a specific MAP kinase in alfalfa, suggesting that a MAP kinase pathway is involved in the intracellular signal transduction of the wound stimulus. This wound-signaling mechanism appears to be highly conserved in plants, because protein kinases with all of the properties of MAP kinases were shown to be activated by wounding in a variety of plants (Seo et al., 1995; Usami et al., 1995).

Wounding has been shown to stimulate transcription of specific genes that are thought to play a role in healing and

defense responses to pathogens. We show that the *MMK4* and the *MsWIP1* genes qualify as wound-inducible genes, because transcripts of these genes specifically accumulate upon wounding. The accumulation of *MMK4* transcripts after wounding agrees with the report on the wound-induced transcription of the tobacco *WIPK* gene encoding a MAP kinase (Seo et al., 1995). Although these authors found no basal transcript levels of the *WIPK* gene in unwounded leaves, *WIPK* mRNA accumulation was observed almost immediately. Accumulation began at 1 min after wounding, with maximal accumulation between 30 and 60 min after wounding. Because wounding activated an MBP kinase with the same kinetics, these results might be taken as evidence that the WIPK protein is produced de novo before activation of the MAP kinase pathway occurs. In alfalfa, the wound-induced activation of the MMK4 kinase clearly precedes the accumulation of transcripts of the *MMK4* gene. Whereas the highest p44^{MMK4} kinase activation was observed at 5 min after wounding of alfalfa leaves, *MMK4* transcript levels did not increase before 20 min after wounding. At this time, p44^{MMK4} kinase activity had almost decreased to basal levels. Furthermore, we showed that the p44^{MMK4} protein is already present in unwounded leaves and that the activity but not the steady state protein levels of p44^{MMK4} changed after wounding.

To date, MAP kinases in all eukaryotes are activated by a post-translational mechanism, culminating in the phosphorylation of a threonine and tyrosine residue in kinase domain VIII of the MAP kinases. The inhibition of transcription or protein synthesis did not prevent p44^{MMK4} kinase activation by wounding. These results are consistent with the idea that wounding activates the p44^{MMK4} MAP kinase by a post-translational mechanism and that de novo transcription and translation are not required.

The inactivation of MAP kinase pathways is less well understood but involves serine/threonine protein phosphatases such as PP2A as well as dual-specificity phosphatases that dephosphorylate tyrosine and threonine residues. Wounding induces a transient activation of the p44^{MMK4} kinase, indicating that two specific mechanisms must operate: one that activates the kinase and one that inactivates it. The action of the two distinct mechanisms was shown by experiments in which leaves were preincubated with either α-amanitin or cycloheximide before wounding. Although the p44^{MMK4} kinase was fully activable by wounding, sustained activation of the kinase was obtained under these conditions. These data show that wound-induced activation of the MMK4 pathway does not depend on transcription and translation but that inactivation of the p44^{MMK4} kinase is completely dependent on de novo synthesis of a protein factor(s).

A second wounding did not lead to further activation of the p44^{MMK4} kinase over a time period of ~25 min. This indicates that once stimulated, the inactivation mechanism remains fully operational over the refractory period, probably by immediately inactivating any newly stimulated p44^{MMK4} kinase. Obviously, the inactivation mechanism has to be switched off as well, otherwise the plant would be unable to respond to a second wound stimulus. This predicts that the inactivating protein factor might only be produced over a short period of time after activation of the MMK4 pathway and/or that the de novo–synthesized protein(s) must be highly unstable. Both predictions appear to be correct, because a recently isolated protein phosphatase meets all of these criteria (I. Meskiene, L. Bögre, W. Glaser, M. Brandstötter, G. Ammerer, and H. Hirt, manuscript in preparation).

The fast activation of the MMK4 kinase after wounding suggests that the stimulation of the MMK4 pathway must be one of the cells' immediate responses to the wound stimulus. In the search for possible candidates that may act upstream of this pathway, it became clear that only those factors that have faster or similar kinetics could qualify. ABA and MeJA are considered to play a role in wound signaling and rapidly accumulate after wounding. Although treating leaves with these substances induced gene transcription of an ABA- and MeJA-inducible gene, neither substance was able to activate the p44^{MMK4} kinase in the absence of wounding. These data suggest that the activation of the p44^{MMK4} kinase is upstream of ABA and/or MeJA or that the MMK4 and the ABA and/or MeJA pathways act independently of each other. A genetic analysis on the *WIPK* gene from tobacco (Seo et al., 1995) convincingly demonstrated that the wound-induced MAP kinase acts upstream of the MeJA pathway.

The activation of the MAP kinase pathway by electrical signals, which are one of the earliest responses of plants to tissue damage, is another possible factor that was considered. Electrical signals can propagate systemically and have been shown to induce proteinase inhibitor genes at distant sites (Wildon et al., 1992). Alfalfa leaves were electrically stimulated over a range of conditions, but no activation of the p44^{MMK4} kinase was observed (data not shown). Although these data might indicate that the wound induction of the MMK4 pathway is not mediated by changes in electrical potentials, more rigorous tests are necessary to prove this point unequivocally.

Taken together, we have presented evidence that a specific MAP kinase pathway is rapidly and transiently activated in alfalfa leaves by wounding. Evidence from other groups suggests that this is a highly conserved and general mechanism by which plants sense and respond to wounding (Seo et al., 1995; Usami et al., 1995). Surprisingly, the same MAP kinase pathway is activated by other seemingly unrelated signals. Drought and cold were found to activate the p44^{MMK4} kinase in alfalfa (Jonak et al., 1996). Touch and/or mechanical manipulation also activate the MMK4 pathway (Bögre et al., 1996). The common denominator of these inducers is that they are different forms of stress, suggesting that the p44^{MMK4} kinase may be part of a general stress-induced signaling pathway. Therefore, we propose to rename the p44^{MMK4} kinase as SAM, for stress-activated MAP kinase.

Stress-induced MAP kinase pathways are also present in metazoans. The SAPK (stress-activated protein kinase) or

JNK (Jun N-terminal kinase) and the p38 kinase are MAP kinases that become activated by a variety of different stresses, including heat stress, osmotic stress, pathogen components, and proinflammatory signals (Galcheva-Gargova et al., 1994; Han et al., 1994; Kyriakis et al., 1994). One obvious question is how a given MAP kinase pathway can be activated by different signals. In metazoans, it appears that different receptors can feed into the same MAP kinase pathway. A major convergence point appears to occur at the level of the Raf, mos, and MAP kinase kinase kinases. Another question is how a given MAP kinase pathway can give rise to different responses. Convincing evidence from work with mammalian cells indicates that the activation level and the total time of activation of a MAP kinase are critical factors influencing the outcome of the signal transduction process. In PC12 cells, the epidermal growth factor produces a short transient activation of the ERK MAP kinase, resulting in cell proliferation. In contrast, the nerve growth factor induces a prolonged activation of the ERK kinase, leading to differentiation (Traverse et al., 1992). These and other results, including the expression of mutant components of the ERK pathway (Cowley et al., 1994), suggest that the time and activation levels of ERK are crucial factors determining the direction of development in these cells. Cold, drought, touch, and wounding also induce different activation levels and kinetics of the $p44^{MMK4}$ kinase, making it plausible that similar mechanisms may also operate in plants. Definitive answers to these speculations require further investigations and the identification and study of the factors that regulate the SAM kinase pathway. The multiple involvement of the SAM kinase pathway in mediating different signals predicts that understanding signal transduction is not a linear matter but probably requires the study of interactions (cross-talk) between different pathways that previously have been considered to act independently of one another. In this respect, we may compare signal transduction processes with neuronal networks that also have linear and interlinked components. The final goal in both systems is the same: to respond to changes in the environment in an optimal way. To define what is optimal requires the integration of different signals and the potential to respond at mulitple levels. Seen from this point of view, studying signal transduction should help us to understand plant physiology.

METHODS

Plant Culture Conditions and Treatments

Alfalfa (*Medicago sativa* subsp *varia* cv Rambler, line A2) plants were grown in soil or under sterile conditions on hormone-free MS (Murashige and Skoog, 1962) medium. Suspension-cultured alfalfa cells were cultivated in MS medium containing 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.1 mg/L kinetin.

Alfalfa leaves were wounded mechanically by cutting the lamina with a razor blade. Leaf extracts were prepared at 0, 1, 2, 5, 10, 20, 30, 40, 50, 60, and 120 min after wounding in extraction buffer (25 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM DTT, 0.1% Nonidet P-40, 15 mM *p*-nitrophenyl phosphate, 60 mM β-glycerophosphate, 0.1 mM $NaVO_3$, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL each of leupeptin, aprotinin, soybean trypsin inhibitor, and 5 μg/mL each of antipain, chymostatin, and pepstatin. The cleared supernatant, after centifugation at 100,000*g* for 1 hr, was used.

For activation studies of $p44^{MMK4}$ kinase in the absence of wounding, leaves were detached at the petioles and preincubated for 2 hr in MS medium containing 1, 10, and 100 μM abscisic acid (ABA) (Sigma) or methyl jasmonic acid (MeJA). Electrical stimulation was performed as described by Wildon et al. (1992).

Inhibitor Studies

Before laminar wounding, we detached alfalfa leaves at the petioles and preincubated them for 2 hr in MS medium containing 1, 10, or 100 μM of the translation inhibitor cycloheximide or 1, 10, or 100 μM of the transcription inhibitor α-amanitin. To study the effect of cycloheximide on protein synthesis, leaf pieces were pulse labeled for 1 hr with 100 μCi of ^{35}S-methionine. The degree of inhibition of protein synthesis was quantified by scintillation counting of trichloroacetic acid–precipitated total protein from untreated and cycloheximide-treated samples. For qualitative analysis, autoradiography was performed with protein extracts that were separated by SDS-PAGE. A linear relationship between the amount of cycloheximide and the amount of incorporated label was obtained whereby 100 μM cycloheximide inhibited protein synthesis for >97% and was therefore used for further studies.

For analysis of the effect of α-amanitin on transcription, RNA gel blot analysis was performed with untreated and α-amanitin–treated samples containing 20 μg of total RNA. After blotting the RNA to nylon membranes, the filters were hybridized with a radiolabeled EcoRI-XhoI fragment of the constitutively expressed *Msc27* gene (Pay et al., 1992). Imaging was with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) to quantify the degree of transcription inhibition by α-amanitin. A more or less linear relationship between the concentration of α-amanitin and the degree of transcriptional inhibition was observed. A 100-μM concentration of α-amanitin showed >90% inhibition and was used for additional experiments.

In-Gel Protein Kinase Assays

For in-gel protein kinase reactions, leaf extracts containing 20 μg of total protein per lane were separated by SDS-PAGE. Myelin basic protein (MBP; 0.5 mg/mL) was used as a substrate that was polymerized in the gel. After protein renaturation, we performed the kinase reactions in the gel as described by Usami et al. (1995).

Antibody Production

The peptides VRFNPDPPIN, LNFCKEQILE, and EALALNPEYA, corresponding to the C termini of the MMK2, MMK3, and MMK4 kinases (Jonak et al., 1995, 1996), respectively, were produced synthetically and conjugated to a purified derivative protein of tuberculin. A polyclonal antiserum was raised in rabbits and purified by protein A column chromatography.

Immune Complex Kinase Assays

Leaf extracts containing 100 μg of total protein were immunoprecipitated with 5 μg of protein A–purified M11, M14, and M7 antibodies that were raised against synthetic peptides encoding the C-terminal 10 amino acids of the alfalfa MMK2, MMK3, and MMK4 mitogen-activated protein (MAP) kinases, respectively (Jonak et al., 1995, 1996). The immunoprecipitated kinases were washed three times with wash buffer I (20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, and 1% Triton X-100), once with the same buffer but containing 1 M NaCl, and once with kinase buffer (20 mM Hepes, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, and 1 mM DTT). Kinase reactions of the immunoprecipitated protein were performed in 15 μL of kinase buffer containing 0.5 mg/mL MBP, 0.1 mM ATP, and 2 μCi of γ-^{32}P-ATP. The protein kinase reactions were performed at room temperature for 30 min. The reaction was stopped by the addition of SDS sample buffer. The phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE.

Immunoblotting

Immunoblotting was performed as described by Bögre et al. (1995). Briefly, leaf extracts containing 20 μg of total protein were separated by SDS-PAGE, immunoblotted to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and probed with the M7 antibody at a 1:1000 (v/v) dilution. Alkaline phosphatase–conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody, and the reaction was visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as the substrate.

RNA Gel Blot Analysis

RNA was extracted from leaves as described by Meskiene et al. (1995). Two micrograms of poly(A)$^+$ RNA was separated per lane on a denaturing formaldehyde gel. After blotting to nylon membranes, the blot was sequentially hybridized with radiolabeled XhoI-SphI and EcoRI fragments containing the 3′ nontranslated regions of the *MMK4* and the *MMK1* genes, respectively, and the coding region of the *MsWIP* gene. As a control, the blot was hybridized with a radiolabeled EcoRI-XhoI fragment of the constitutively expressed *Msc27* gene (Pay et al., 1992).

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