Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11274–11279, October 1996
Plant Biology

Stress signaling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought

(signal transduction/cold stress/salt stress/heat stress)

CLAUDIA JONAK*†, STEFAN KIEGERL*, WILCO LIGTERINK*, PATRICK J. BARKER‡, NEVILLE S. HUSKISSON‡, AND HERIBERT HIRT*§

*Institute of Microbiology and Genetics, Vienna Biocenter, University of Vienna, Dr. Bohrgasse 9, 1030 Vienna, Austria; and ‡Microchemical Facility, The Babraham Institute, Cambridge CB2 4AT, United Kingdom

Communicated by Winslow R. Briggs, Carnegie Institution of Washington, Stanford, CA, July 22, 1996 (received for review March 12, 1996)

ABSTRACT **Yeast and animals use mitogen-activated protein (MAP) kinase cascades to mediate stress and extracellular signals. We have tested whether MAP kinases are involved in mediating environmental stress responses in plants. Using specific peptide antibodies that were raised against different alfalfa MAP kinases, we found exclusive activation of p44^{MMK4} kinase in drought- and cold-treated plants. p44^{MMK4} kinase was transiently activated by these treatments and was correlated with a shift in the electrophoretic mobility of the p44^{MMK4} protein. Although transcript levels of the *MMK4* gene accumulated after drought and cold treatment, no changes in p44^{MMK4} steady state protein levels were observed, indicating a posttranslational activation mechanism. Extreme temperatures, drought, and salt stress are considered to be different forms of osmotic stress. However, high salt concentrations or heat shock did not induce activation of p44^{MMK4}, indicating the existence of distinct mechanisms to mediate different stresses in alfalfa. Stress adaptation in plants is mediated by abscisic acid (ABA)-dependent and ABA-independent processes. Although ABA rapidly induced the transcription of an ABA-inducible marker gene, *MMK4* transcript levels did not increase and p44^{MMK4} kinase was not activated. These data indicate that the MMK4 kinase pathway mediates drought and cold signaling independently of ABA.**

Plants respond to a variety of biotic and abiotic signals that influence growth and development. Although the responses of plants to these signals have been extensively studied at the physiological and the biochemical levels, the perception and the intracellular transmission mechanisms are largely unknown. Progress in understanding signal transduction in animals and yeast showed that reversible protein phosphorylation plays a pivotal role in many signaling cascades. Increasing evidence indicates that protein kinase pathways are also involved in signal transduction in plants. Changes in protein phosphorylation patterns were observed after exposure of plant cells to fungal elictors (1–3) and to ethylene (4), and during establishment of freezing tolerance (5). Furthermore, a continuously growing number of genes coding for protein kinases in plants have been reported. Recently, a highly conserved group of serine/threonine protein kinases, which show sequence homology to MAP kinases, have been isolated from different plant species (6–12). In animals and yeast, mitogen-activated protein (MAP) kinases are involved in differentiation, cell division, and stress response. Animal MAP kinases, also known as extracellular signal-regulated kinases (ERKs), were originally found to be involved in differentiation and reentry into the cell cycle. Later, mammalian osmoregulated and stress-activated MAP kinases were discovered. The family of SAPK (stress-activated protein kinase)/JNK (Jun N-terminal kinase)/p38 is activated by various types of stress (13–15). JNK and p38 can functionally replace the HOG1 yeast MAP kinase that is necessary for adaptation to high extracellular osmolarity. Genetic and biochemical studies in yeast revealed several distinct MAP kinase cascades in signaling different extracellular stimuli. These kinase cascades are important regulators in pheromone response, pseudohyphal differentiation, and osmolarity responses (16, 17).

Activation of MAP kinases requires tyrosine and threonine phosphorylation (4). The highly conserved threonine and tyrosine residues are located close to kinase domain VIII and seem to be also important for activation of plant MAP kinases (7). Phosphorylation of these crucial residues is performed by a dual specific MAP kinase kinase that in turn has to be activated by a serine/threonine MAP kinase kinase kinase (18, 19). These kinase cascades seem to be conserved in modular form throughout evolution, mediating distinct signal transduction pathways. Isolation of homologous upstream kinases from plants (20–23) indicate the presence of similar biochemical modules for extracellular signal transmission.

Despite the fact that all of the components of MAP kinase modules have been identified in plants, little is known about their functions. An *Arabidopsis* MAP kinase was proposed to be involved in auxin signal transduction (24). Genetic studies and the isolation of the *CTR1* gene suggest that a MAP kinase cascade may also be involved in mediating ethylene responses (21). Recently, MAP kinases have been demonstrated to be activated upon cutting of leaves (22, 28) and exposure of cells to fungal elicitor (25). Increased transcript levels of genes encoding a MAP kinase module have been taken as evidence for the involvement of a MAP kinase pathway in signaling touch, cold, salt, and water stress (26).

In this article, we present evidence that environmental stresses are mediated by posttranslational activation of a specific MAP kinase in alfalfa. The MAP kinase pathway appears to mediate only specific forms of stress, because cold and drought, but not high temperature and osmotic stress, induce the activation of this pathway.

MATERIALS AND METHODS

Isolation, Sequence Analysis, and Cloning of MMK4. Two redundant oligonucleotides, corresponding to the GNFDN and TEYVV motifs of MAP kinases, were synthesized to isolate genes coding for new MAP kinases by polymerase chain

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MAP, mitogen-activated protein; GST, glutathione *S*-transferase; MBP, myelin basic protein; ABA, abscisic acid.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. X82270).

†Present address: Laboratoire de Biologie Cellulaire, INRA, Route de St. Cyr, F-78026 Versailles Cedex, France.
§To whom reprint requests should be addressed. e-mail: HEHI@gem.univie.ac.at.

reaction (PCR) (9). The PCR fragment obtained was cloned into pBluescript SK(+) vector (Stratagene). Sequence analysis (T7 Sequencing kit; Pharmacia) revealed a new type of alfalfa MAP kinase that was thereafter denoted as *MMK4*. A radiolabeled fragment of the *MMK4* gene was used to screen alfalfa cDNA libraries prepared from somatic embryos and suspension cultured cells (9). Sequences of the isolated cDNAs were identical, but all lacked the 5′ coding region. The N-terminal part of the *MMK4* gene was obtained by primer extension PCR using the 5′-AmpliFINDER RACE Kit (Clontech).

Expression of Alfalfa MMK4 in Bacteria. The *MMK4* gene was cloned in the pGEX-3x vector (Pharmacia) by introducing *Bam*HI sites through PCR amplification with the following primers: MMK4x1, 5′-TTTTGGATCCCAATGGCCAGAGTTAACC-3′; MMK4x2, 5′-TATGGATCCTTAAGCATACTCAGGATTG-3′. After transforming *Escherichia coli* with these constructs, positive clones containing the fragments in the correct orientation were isolated and sequenced to verify that no mutations had occurred in the open reading frames. Preparation of the glutathione *S*-transferase (GST) fusion proteins and affinity purification was done according to the manufacturer's instructions (Pharmacia). Protein concentration was determined with a Bio-Rad detection system. Proteins were collected in 50 μl aliquots, frozen in liquid nitrogen, and stored at −80°C. The quality of the purification was checked on denaturing 10% polyacrylamide gels.

***In Vitro* Phosphorylation Assays.** Phosphorylation assays were carried out as described (9). Myelin basic protein (MBP) (1 μg) (Sigma) or purified bovine MAP2 protein (1 μg) (kind gift of L. Ballou, Institute of Molecular Pathology, Vienna) was used as substrate. Samples were either frozen at −20°C or directly analyzed on denaturing 15% polyacrylamide gels before autoradiography.

Plant Material and Stress Treatment. Alfalfa (*Medicago sativa* ssp. varia cv Rambler, line A2) was grown in soil or *in vitro* on hormone-free Murashige and Skoog medium (27). Cold and heat treatment were performed by shifting soil-grown plants to 4°C or 37°C, respectively. *In vitro* grown plants were exposed to severe drought stress by removing the lid of the containers. Leaves were collected at the indicated time points and immediately shock-frozen in liquid nitrogen. Salt stress treatment was performed on leaf pieces from soil-grown alfalfa plants as described (28).

RNA Gel Blot Analysis. Total RNA of plant tissues was isolated using the acid guanidium thiocyanate-phenol-chloroform extraction method (29). Poly(A)$^+$ RNA was purified using Dynabeads (Dynal, Great Neck, NY) according to the manufacturer's instructions. From each sample, 1 μg of poly(A)$^+$ RNA was separated on denaturating agarose gels and blotted to nylon filters. Hybridizations were performed according to standard procedures with radiolabeled fragments of the 3′ nontranslated regions of the *MMK1, MMK2, MMK3,* and *MMK4* genes or of the entire coding region of the *Msc27* gene as a control.

Antibody Production. The following peptides (VRFNPDPPIN, LNFCKEQILE, and EALALNPEYA), corresponding to the C termini of the MMK2, MMK3, and MMK4 kinases, respectively, were synthesized synthetically and conjugated to a purified derivative protein of tuberculin. Polyclonal antiserum was raised in rabbits and finally purified by protein A column chromatography.

Immunokinase Assays. Plant material was extracted in 25 mM Tris·HCl (pH 7.8), 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM DTT, 1 mM NaF, 0.5 mM $NaVO_3$, 15 mM β-glycerophosphate, 15 mM paranitrophenylphosphate, 0.1% Tween 20, and 0.5 mM phenylmethylsulfonyl fluoride and was centrifuged at 15 krpm. The cytoplasmatic protein extract was further purified from nonsoluble particles by centrifugation at 80 krpm. Protein concentration was determined with a Bio-Rad detection system. Ten microliters of antibody were added to 100 μg of crude alfalfa protein extract and the mixture was gently agitated overnight at 4°C. After addition of 50 μl of 50% protein A–Sepharose beads, samples were incubated for 2 hr at 4°C. The Sepharose beads were washed three times in 20 mM Tris·HCl (pH 7.5), 5 mM EDTA, 100 mM NaCl, 1 mM Triton X-100, once in 20 mM Tris·HCl (pH 7.5), 5 mM EDTA, 1 M NaCl, 1 mM Triton X-100, and twice in 20 mM Hepes (pH 7.5), 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT. Kinase reactions were performed in 20 mM Hepes (pH 7.5), 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 0.2 mg/ml BSA, 0.2 mM ATP, 0.5 mg/ml MBP, and 2.5 μCi (1 Ci = 37 GBq) [γ-^{32}P]ATP for 30 min at room temperature. Reactions were stopped by adding sample buffer before loading on denaturing 15% polyacrylamide gels. GST–MMK fusion proteins were prepared as described (9). Each fusion protein (100 ng and 20 ng) was immunoprecipitated, washed, and used for kinase assays as described above.

Immunoblots. Samples of 20 μg of total cytoplasmic protein extract were separated by denaturing polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) by electroblotting. MAP kinase was visualized by Enhanced Chemiluminescence according to the manufacturer's directions (Amersham).

RESULTS

Isolation of a New Functional MAP Kinase from Alfalfa. Degenerate oligonucleotides corresponding to two highly conserved regions of eukaryotic MAP kinases were used to amplify a 300-bp fragment from an alfalfa cDNA library by PCR. Sequence analysis showed clear similarity to MAP kinases, but also revealed distinct differences to the other alfalfa MAP kinases isolated so far (8, 9). Screening of different alfalfa cDNA libraries with the radiolabeled PCR fragment resulted in the isolation of several cDNA clones. Sequencing of the clones showed that they all contained the same coding region but were truncated at the 5′ end. The N-terminal region of the gene was obtained by primer extension PCR. The amplified fragment was found to contain the missing 5′ end. The nucleotide and predicted protein sequence of the full-length cDNA, denoted as *MMK4* for *M*edicago *MAP kinase 4*, is available in the GenBank Sequence Data Bank, DNA Data Base in Japan, European Molecular Biology Laboratory, and National Center for Biotechnology Information data bases under the accession number X82270. The deduced MMK4 protein sequence is 371 amino acids long and is predicted to encode a protein of 43 kDa that shows highest similarity (81% identity) to the *Arabidopsis* ATMPK3 (10), but only 73% and 65% identity to the previously identified alfalfa MAP kinases MMK1 and MMK2, respectively (8, 9). Considerably less homology (41–48%) was found when MMK4 was compared with the available yeast and animal MAP kinase protein sequences. The main differences in the amino acid sequences were found in the N and C termini of the respective proteins.

To test whether *MMK4* encodes a functional protein kinase, the full-length *MMK4* cDNA was expressed in *E. coli* as a fusion protein with the GST gene. As shown in Fig. 1, the GST–MMK4 protein (lane 2), but not the GST protein (lane 1), showed autophosphorylation after incubation with [γ-^{32}P]ATP. GST–MMK4 kinase (Fig. 1, lane 4), but not GST (Fig. 1, lane 3), was also able to phosphorylate MBP and bovine microtubule-associated protein MAP2 (Fig. 1, lane 6), indicating that the *MMK4* gene encodes a functional protein kinase.

p44^{MMK4} Kinase Is Transiently Activated by Cold Stress. In animals and yeast, MAP kinases are involved in transmitting different signals including stress. Plants have to cope with extreme environmental conditions, including changes in temperature and water availability. To test whether a MAP kinase

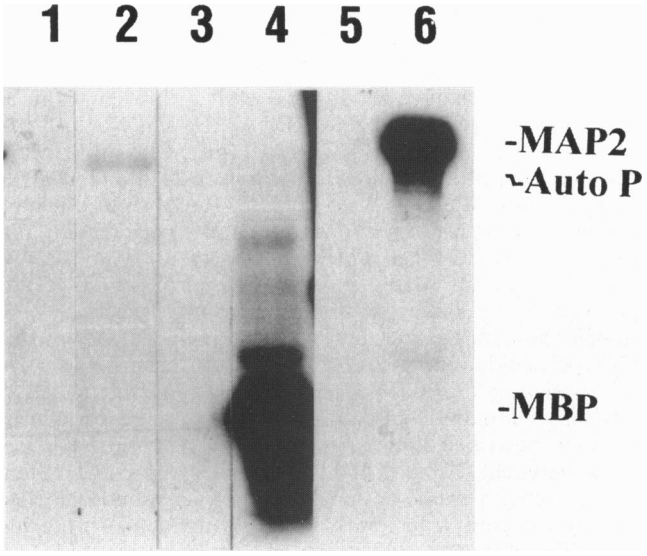

FIG. 1. The *MMK4* gene encodes a functional protein kinase. The *MMK4* gene was expressed in bacteria as a GST fusion protein and affinity purified. Affinity-purified GST protein was used as a control. Autophosphorylation activity was observed with GST–MMK4 (lane 2) but not with GST (lane 1). MBP was phosphorylated by GST–MMK4 (lane 4) but not by GST (lane 3). Bovine microtubule-associated protein MAP2 is phosphorylated by GST–MMK4 (lane 6) but not by GST (lane 5). The proteins in lanes 1–4 were separated on a 15% denaturing polyacrylamide gels, whereas the proteins in lanes 5 and 6 were separated on an 8% gel. MAP2 and MBP denote the positions of the bovine microtubule-associated protein MAP2 and the MBP on the gels. Auto P, the autophosphorylated GST–MMK4 band on the gel.

could be involved in the signal transduction of these stresses, we raised a polyclonal antibody against the C-terminal 10 amino acids of MMK4. The antibody, but not the preimmune serum, recognized the bacterially expressed GST–MMK4 protein and decorated a 44-kDa protein in crude extracts of suspension-cultured alfalfa cells. Preincubation of the antibody with the C-terminal peptide completely inhibited the reactions, indicating that the antibody specifically recognizes the MMK4 protein. The antibody was also found to immunoprecipitate autoactivated GST–MMK4 kinase protein. These data indicated that the antibody can be used to determine $p44^{MMK4}$ kinase activity and protein levels.

To analyze whether $p44^{MMK4}$ kinase is activated by low temperature, alfalfa plants were exposed to cold stress by shifting soil-grown alfalfa plants from room temperature to 4°C. At different time points after the stress treatment, leaf protein extracts were immunoprecipitated with MMK4 antibody and assayed for their ability to phosphorylate MBP. Although intact leaves from alfalfa plants grown at 22°C had no detectable activity of $p44^{MMK4}$ kinase (Fig. 2*A*, 0 min), at 10 min after cold treatment, activation of $p44^{MMK4}$ kinase became detectable. Maximal activation of the $p44^{MMK4}$ kinase was observed at 60 min, but decreased to basal levels within 120 min. As a control, the same samples were also immunoprecipitated with antibodies that were raised against the C-terminal domains of the MMK2 and MMK3 kinases, respectively. No activation of immunoprecipitated kinase complexes was observed at any time (Fig. 2*A*).

Drought, but Not Heat Shock or Osmotic Stress, Induces $p44^{MMK4}$ Kinase Activation. To investigate whether the activation of the MMK4 kinase pathway is a general response to environmental stresses, we also shifted alfalfa plants from moderate (22°C) to high temperatures (37°C), conditions that were shown to induce transcription of heat shock proteins (30). Kinase assays of immunoprecipitated $p44^{MMK4}$ protein from leaf extracts at different times after the temperature shift

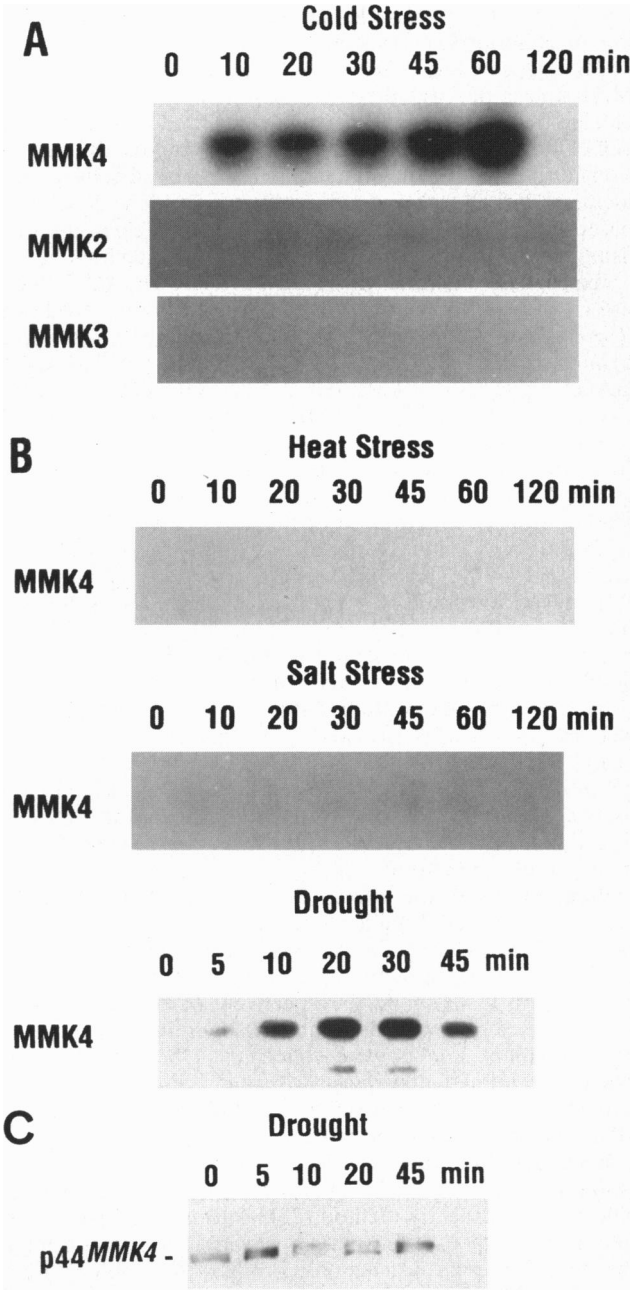

FIG. 2. $p44^{MMK4}$ kinase is activated by cold and drought, but not by heat and osmotic stress. Soil grown alfalfa plants were treated with cold (4°C), heat (37°C), drought, or salt stress. Leaves were collected and frozen in liquid nitrogen at the indicated time points. After protein extraction, leaf extracts, containing 100 μg of total protein, were immunoprecipitated or immunoblotted with antibodies directed against the C-terminal 10 amino acids of MMK4, MMK2, and MMK3. The kinase activity of the immunoprecipitated kinases was determined by kinase assays with [γ-^{32}P]ATP and MBP as substrate and subsequent autoradiography. (*A*) MMK4, MMK2, and MMK3 immunokinase assays of alfalfa leaf extracts from cold-treated plants. (*B*) MMK4 immunokinase assays of alfalfa leaf extracts from heat-, salt-, and drought-stressed plants. (*C*) $p44^{MMK4}$ immunoblot of leaf extracts from drought-stressed alfalfa plants.

showed no activation of the MAP kinase after heat shock (Fig. 2*B*). In contrast, immunokinase assays of leaf extracts from drought-stressed alfalfa plants showed activation of the MMK4 kinase within 5 min (Fig. 2*B*). Drought-induced MMK4 kinase activation was transient, because after maximal activation at 20–30 min, MMK4 kinase activity decreased again (Fig. 2*B*).

Cold, drought, and salt stress induce a partially overlapping set of genes, and are considered to be different forms of osmotic stress. Furthermore, it was recently shown that incubation of leaf pieces in high salt rapidly induced the activation of a 46-kDa MBP kinase that has very similar properties to MAP kinases (28). To investigate whether p44^{MMK4} kinase also becomes activated by salt stress, we performed similar experiments with alfalfa leaf pieces. After preincubation for 2 hr in isotonic medium, the leaf pieces were transferred to high salt medium. At different time points, leaf pieces were collected and frozen in liquid nitrogen. Immunokinase assays of leaf extracts from these samples showed no activation of p44^{MMK4} kinase at any time (Fig. 2*B*), indicating that the MMK4 kinase is not activated by high salt. No activation of p44^{MMK4} kinase was also obtained after salt stress of hydroponically grown alfalfa plants or suspension-cultured cells (data not shown).

Drought and Cold Induce a Shift in the Electrophoretic Mobility of the p44^{MMK4} MAP Kinase. Activation of MAP kinases is mediated by phosphorylation of a threonine and tyrosine residue close to kinase domain VIII, leading to a shift in the electrophoretic mobility on denaturing polyacrylamide gel electrophoresis (4). To test whether drought or cold stress induce a similar shift in the mobility of the alfalfa p44^{MMK4} protein, the same leaf extracts of drought-treated plants that were used for immunokinase assays (Fig. 2*B*) were separated by denaturing polyacrylamide gel electrophoresis and analyzed by protein gel blotting with the MMK4 antibody. A single 44-kDa protein was detected in untreated leaf extracts (Fig. 2*C*). The appearance of two slightly slower migrating bands at 10 min after drought stress (Fig. 2*C*) was correlated with the activation of the MMK4 kinase (Fig. 2*B*). At 45 min after dehydration, the two bands disappeared in parallel to the decrease in activity of the p44^{MMK4} kinase. An analysis with the protein extracts of cold-stressed leaves also showed a transient shift in the electrophoretic mobility of the p44^{MMK4} protein when the MMK4 kinase was activated (data not shown), strongly suggesting that the drought- and cold-induced activation of the p44^{MMK4} kinase occurs by posttranslational modification of the p44^{MMK4} protein through upstream factors.

***MMK4* Transcripts Selectively Accumulate upon Cold and Drought Stress.** Although it is generally agreed that MAP kinases become activated by posttranslational phosphorylation of a highly conserved threonine and tyrosine residue through upstream kinases, the possibility that transcriptional control is also involved in the regulation was investigated by examining the expression of the alfalfa *MMK* gene family. Leaves of plants were collected at different time points after applying cold, heat, and dehydration stress. RNA gel blot analysis of these samples was performed with radiolabeled fragments of the *MMK1* (8), *MMK2* (9), *MMK3* (unpublished results), and *MMK4* genes. After exposure to cold stress, transcript levels of *MMK4* increased within 20 min, reaching maximal levels at 2 hr after cold treatment (Fig. 3*A*). No increase in transcript levels was observed in heat stressed plants (data not shown).

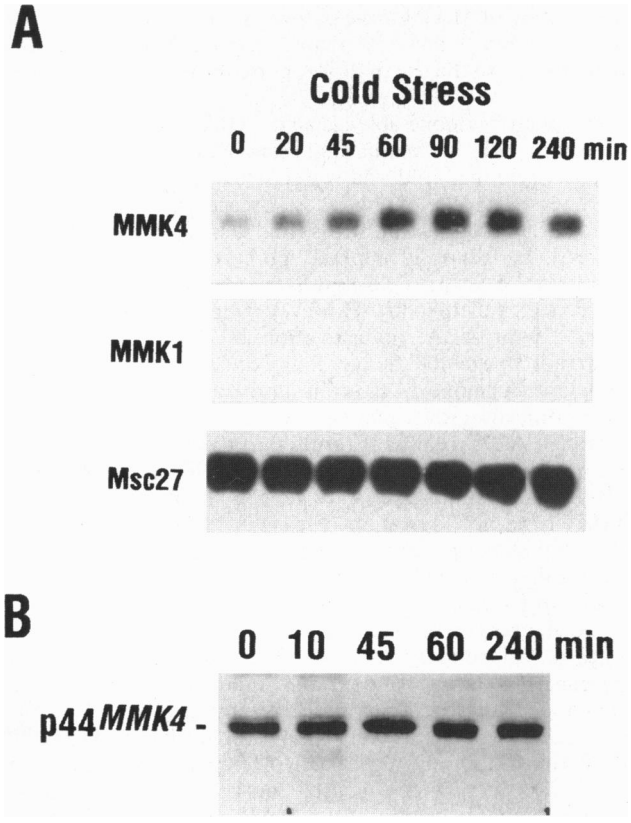

FIG. 3. MMK4 transcript but not p44^{MMK4} protein levels accumulate after cold stress. After cold stress treatment, leaves were detached and frozen in liquid nitrogen at the indicated time points and extracted for RNA or protein. After extraction of total RNA from the samples, poly(A)$^+$ RNA was selected. One microgram of poly(A)$^+$ RNA was applied per lane of a denaturing agarose gel. After blotting to nylon membranes, the filter was hybridized with radiolabeled fragments of the *MMK4, MMK1*, and *Msc27* (control) genes. After protein extraction, leaf extracts, containing 20 μg of total protein, were immunoblotted with antibody directed against the C-terminal 10 amino acids of MMK4. (*A*) Transcript analysis of alfalfa leaves after cold treatment. (*B*) p44^{MMK4} immunoblot analysis of alfalfa leaves after cold treatment.

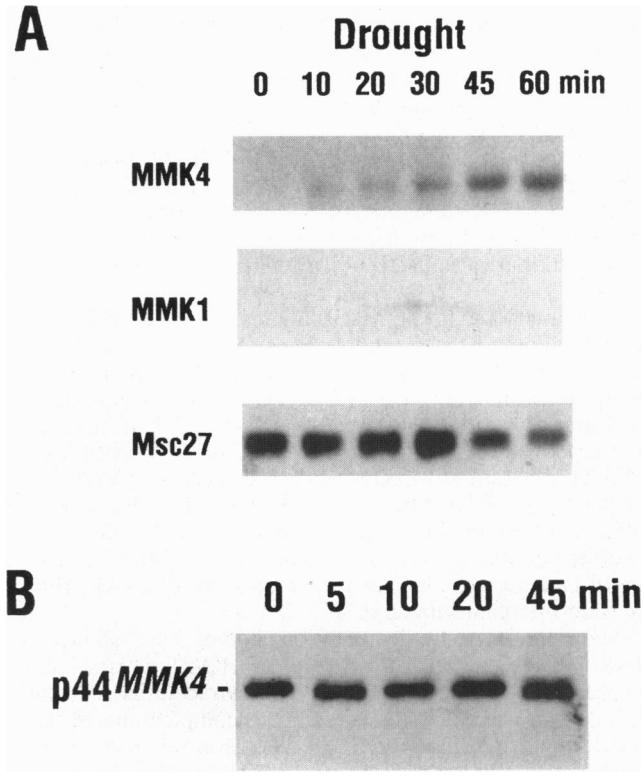

FIG. 4. MMK4 transcript but not p44^{MMK4} protein levels accumulate after drought stress. After dehydration, leaves were detached and frozen in liquid nitrogen at the indicated time points and extracted for RNA or protein. After extraction of total RNA from the samples, poly(A)$^+$ RNA was selected. One microgram of poly(A)$^+$ RNA was applied per lane of a denaturing agarose gel. After blotting to nylon membranes, the filter was hybridized with radiolabeled fragments of the *MMK4, MMK1*, and *Msc27* (control) genes. After protein extraction, leaf extracts, containing 20 μg of total protein, were immunoblotted with antibody directed against the C-terminal 10 amino acids of MMK4. (*A*) Transcript analysis of alfalfa leaves after cold treatment. (*B*) p44^{MMK4} immunoblot analysis of alfalfa leaves after cold treatment.

Hybridization of the same RNA gel blots with radiolabeled fragments either of the *MMK1* gene (Fig. 3*A*) or of the *MMK2* and *MMK3* genes (data not shown) showed no induction of any of these alfalfa MAP kinase genes under these conditions. Within 10 min after drought stress treatment, *MMK4* transcripts increased continuously up to 60 min (Fig. 4*A*). Neither *MMK1* (Fig. 4*A*) nor any of the other alfalfa MAP kinase genes (data not shown) were induced under these conditions. As a control for loading equal amounts of RNA, the same filters were hybridized with a radiolabeled fragment of the *Msc27* gene (31) (Figs. 3*A* and 4*A*). These data indicate that transcripts of the *MMK4* gene accumulate upon cold and drought stress.

p44^{MMK4} Protein Amounts Do Not Increase upon Cold and Drought Stress. When aliquots of the same leaves that were used for RNA extraction and MMK4 immunokinase assays from cold- and drought-stressed alfalfa plants were immunoblotted with MMK4 antibody, a 44-kDa protein was specifically decorated (Figs. 3*B* and 4*B*, respectively). In contrast to the cold- and drought-induced fluctuation of kinase activity levels (Fig. 2 *A* and *B*, respectively) and the increases in transcript levels (Figs. 3*A* and 4*A*, respectively), no change in p44^{MMK4} protein levels was observed after these stresses (Figs. 3*B* and 4*B*, respectively).

DISCUSSION

Because in animals and yeast, distinct MAP kinase pathways have been identified to mediate a diverse range of biotic and abiotic factors, including stress, we analyzed the involvement of a MAP kinase pathway in stress signaling in alfalfa. In this report, we present several lines of evidence that suggest that a specific MAP kinase pathway is involved in signaling cold and drought stress in alfalfa plants. First, p44^{MMK4} kinase is rapidly activated by low temperature and dehydration. Second, the cold- and drought-induced activation of the MMK4 kinase is a specific response because two other alfalfa MAP kinases are not activated by these stresses. Third, only specific environmental stresses are mediated by the MMK4 pathway, because p44^{MMK4} kinase is not activated by high temperature or osmotic stress.

In animals and yeast, MAP kinases are involved in differentiation, cell division, and response to stress. The family of mammalian MAP kinases, including the SAPK (stress-activated protein kinase)/JNK (Jun N-terminal kinase)/p38, is activated by various types of stress (13–15). A feature of these stress-activated MAP kinases is a TGY or TPY motif instead of a TEY between the catalytic domains VII and VIII. It was suggested to distinguish stress signaling MAP kinases from members that are involved in mediating other signals. However, the cold and drought stress-activated MMK4 has a TEY motif and has similar identity scores to all MAP kinases outside the plant kingdom.

Heterologous complementation studies have shown that JNK and p38 can functionally replace HOG1, which is necessary for adaptation of yeast to high extracellular osmolarity (32, 33), and alfalfa MMK2 specifically complements MPK1 in mediating hypoosmotic stress (9). However, expression of the *MMK4* gene did not rescue yeast cells deficient in the HOG1, MPK1, and FUS3/KSS1 MAP kinases (data not shown).

After dehydration and cold stress, increased *MMK4* transcript levels were observed. In the majority of experiments, the activation of p44^{MMK4} kinase preceded the increase in mRNA levels, suggesting that the *MMK4* gene might be a target of the MMK4 kinase pathway. This pattern is reminiscent of a positive feedback loop that occurs in the activation of several protein kinase cascades. However, an increase of *MMK4* transcripts was not correlated with an increase in p44^{MMK4} protein levels and kinase activity. Several possibilities exist to explain these data. It might be possible that the transcripts accumulate but are not translated. Alternatively, assuming translation of *MMK4* transcripts, steady state p44^{MMK4} protein levels might stay constant, if different pools of p44^{MMK4} protein had different rates of turnover. This could provide a mechanism to get rid of active p44^{MMK4} protein. Too little is yet known to evaluate the significance of these observations, but work is in progress to investigate the different possibilities.

Extreme temperatures, drought, and salt stress induce a partially overlapping set of genes in different organisms. This and other evidence led to the suggestion that these stresses all affect the water potential of the cell. Incubation of tobacco leaf pieces in high salt medium induces a protein kinase with very similar properties to a MAP kinase (28). Therefore, we investigated the possibility that high salt concentrations might also activate this pathway. However, no activation of p44^{MMK4} kinase was observed by applying hyperosmotic stress to intact plants, leaf pieces, or isolated cells. Our failure to detect activation of the MMK4 kinase under different conditions of salt stress clearly indicates that hyperosmotic stress is not mediated by the MMK4 pathway in alfalfa. Although salt stress did not induce activation of the MMK2 and MMK3 kinases, we cannot exclude the possibility that yet another MAP kinase might be involved in mediating hyperosmotic stress. In this respect, it is surprising that transcript levels of several kinase genes, including the *ATMPK3* gene, which encodes an *Arabidopsis* MAP kinase that is 81% identical to the alfalfa MMK4 protein, accumulate after cold, drought, salt, and touch stress (26). Although the significance of increased mRNA levels for the function of MAP kinase pathways is presently unclear, our failure to detect p44^{MMK4} kinase activation after salt stress might be due to the use of different pathways in different plant species.

The plant hormone abscisic acid (ABA) appears to have an important role in mediating responses to environmental stresses including cold and water stress. Consistent with this hypothesis, levels of endogenous abscisic acid increase during periods of drought, salt stress, and during cold acclimation (34–36). Exogenously applied ABA is also able to induce expression of several cold-, salt stress-, and drought-regulated genes (e.g., refs. 37–40). However, when alfalfa plants were sprayed with ABA, no activation of p44^{MMK4} kinase was observed. To exclude the possibility of an inefficient uptake of the plant hormone, ABA was also added to the water of hydroponically grown plants or to suspension-cultured cells. Although ABA treatment resulted in the rapid accumulation of transcripts of the *ABAMs1* gene, encoding a homolog of the ABA-inducible maize *pMAH9* gene (37), no induction of the *MMK4* gene nor activation of p44^{MMK4} kinase was observed (data not shown). These experiments indicate that the cold- and drought-induced activation of p44^{MMK4} kinase are independent of ABA.

The expression of several drought- and/or cold-induced genes is independent of ABA (38, 39, 41), indicating the presence of ABA-dependent and -independent pathways for low temperature and dehydration responses. The activation of an ABA-independent cold- and drought-induced p44^{MMK4} MAP kinase pathway might be involved in the induction of this set of ABA-independent genes. Consistent with this role, the kinetics of the activation of the MAP kinase shortly precedes the rapid induction of the majority of the ABA-independent cold- or drought-induced genes (38–41). In contrast, ABA levels rise with a much slower kinetics correlating with the relatively slow induction of many ABA-dependent stress genes. Our data are consistent with two possible hypotheses. In one case, the MMK4 and the ABA pathways might act independently of one another. Activation of each pathway would give rise to the expression of distinct sets of genes. The other scenario takes into account the rapid activation of the MMK4 kinase and the slower increase of ABA concentrations, and is compatible with the notion that the activation of the

MMK4 kinase after cold and drought stress is a necessary step to induce the synthesis of ABA itself, either directly by influencing the activity of the relevant enzymes or indirectly by the induction of the genes encoding the biosynthesis machinery of ABA. The production of transgenic plants expressing dominant gain-of-function and loss-of-function *MMK4* mutants is under way and should help to understand the causal relationship between this MAP kinase and certain ABA-independent stress-induced genes.

We thank Laszlo Bögre and Claudia Petritsch for technical assistance and Heather Macdonald for critical reading of the manuscript. The work was supported by the Austrian Science Foundation Grants P10020-MOB and P10394-MOB.

1. Dietrich, A., Mayer, J. E. & Hahlbrock, K. (1990) *J. Biol. Chem.* **265,** 6360–6368.
2. Felix, G., Grosskopf, D. G., Regenass, M. & Boller, T. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8831–8834.
3. Grab, D., Feger, M. & Ebel, J. (1989) *Planta* **179,** 340–348.
4. Raz, V. & Fluhr, R. (1995) *Plant Cell* **5,** 523–530.
5. Monroy, A. F., Sarhan, F. & Dhindsa, R. S. (1993) *Plant Physiol.* **102,** 1227–1235.
6. Decroocq-Ferrant, V., Decroocq, S., Van Went, J., Schmidt, E. & Kreis, M. (1995) *Plant Mol. Biol.* **27,** 339–350.
7. Duerr, B., Gawienowski, M., Ropp, T. & Jacobs, T. (1993) *Plant Cell* **5,** 87–96.
8. Jonak, C., Pay, A., Boegre, L., Hirt, H. & Heberle-Bors, E. (1993) *Plant J.* **3,** 611–617.
9. Jonak, C., Kiegerl, S., Lloyd, C., Chan, J. & Hirt, H. (1995) *Mol. Gen. Genet.* **248,** 686–694.
10. Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K., Kamada, H. & Shinozaki, K. (1993) *FEBS Lett.* **336,** 440–444.
11. Stafstrom, J. P., Altschuler, M. & Anderson, D. H. (1993) *Plant Mol. Biol.* **22,** 83–90.
12. Wilson, C., Eller, N., Gartner, T., Vicente, O. & Heberle-Bors, E. (1993) *Plant Mol. Biol.* **23,** 543–551.
13. Galcheva-Gargova, Z., Derijard, B., Wu, I.-H. & Davis, R. J. (1994) *Science* **265,** 806–808.
14. Han, J., Le, J.-D., Bibbs, L. & Ulevitch, R. J. (1994) *Science* **265,** 808–811.
15. Kyriakis, J. M., Banerjee, P., Nikolakai, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. & Woodgett, J. R. (1994) *Nature (London)* **369,** 156–160.
16. Herskowitz, I. (1995) *Cell* **80,** 187–197.
17. Schultz, J., Ferguson, B. & Sprague, G. F. (1995) *Curr. Opin. Genet. Dev.* **3,** 31–37.
18. Posada, J. & Cooper, J. A. (1992) *Science* **255,** 212–215.
19. Marshall, C. J. (1994) *Curr. Opin. Genet. Dev.* **4,** 82–89.
20. Banno, H., Hirano, K., Nakamura, T., Irie, K., Nomoto, S., Matsumoto, K. & Machida, Y. (1993) *Mol. Cell. Biol.* **13,** 4745–4752.
21. Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A. & Ecker, J. R. (1993) *Cell* **72,** 427–441.
22. Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H. & Ohahshi, Y. (1995) *Science* **270,** 1988–1992.
23. Shibata, W., Banno, H., Ito, Y., Hirano, K., Irie, K., Usami, S., Machida, C. & Machida, Y. (1995) *Mol. Gen. Genet.* **246,** 401–410.
24. Mizoguchi, T., Gotoh, Y., Nishida, E., Yamaguchi-Shinozaki, K., Hayashida, N., Iwasaki, T., Kamada, H. & Shinozaki, K. (1994) *Plant J.* **5,** 111–122.
25. Suzuki, K. & Shinshi, H. (1995) *Plant Cell* **7,** 639–647.
26. Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K. & Shinozaki, K. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 765–769.
27. Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15,** 473–479.
28. Usami, S., Banno, H., Ito, Y., Nishihama, R. & Machida, Y. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 86660-8664.
29. Chomzynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
30. Györgyey, J., Gartner, A., Nemeth, K., Magyar, Z., Hirt, H., Heberle-Bors, E. & Dudits, D. (1991) *Plant Mol. Biol.* **16,** 999-1007.
31. Pay, A., Heberle-Bors, E. & Hirt, H. (1992) *Plant Mol. Biol.* **19,** 501–503.
32. Cooper, J. A. (1994) *Curr. Biol.* **4,** 1118–1121.
33. Marshall, C. J. (1995) *Cell* **80,** 179–185.
34. Chen, T. H. H., Li, P. H. H. & Brenner, M. L. (1983) *Plant Physiol.* **71,** 362–365.
35. Guerrero, F. & Mullet, J. E. (1986) *Plant Physiol.* **80,** 588–591.
36. Lang, V., Mäntylä, E., Welin, B., Sundberg, B. & Palva, E. T. (1994) *Plant Physiol.* **104,** 1341–1349.
37. Gomez, J., Sanches-Martinez, D., Stiefel, V., Rigau, J., Puidomènech, P. & Pagès, M. (1988) *Nature (London)* **334,** 262–264.
38. Gosti, F., Bertauche, N., Vatanian, N. & Girodat, J. (1995) *Mol. Gen Genet.* **246,** 10–18.
39. Nordin, K., Heino, P. & Palva, E. T. (1991) *Plant Mol. Biol.* **16,** 1061–1017.
40. Yamaguchi-Shinozaki, K. & Shinozaki, K. (1994) *Plant Cell* **6,** 251–264.
41. Mohapatra, S. S., Wolfraim, L., Poole, R. J. & Dhindsa, R. S. (1989) *Plant Physiol.* **89,** 375–380.