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

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

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

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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. The mitogen-activated protein (MAP) kinase pathway is a ubiquitous and highly conserved signaling module that is involved in diverse stress responses and participates in the activation of transcription factors. The MAP kinase cascade is composed of three enzymes: a MAP kinase, a MAP kinase kinase, and a MAP kinase kinase kinase. The MAP kinase cascade is activated by phosphorylation of the MAP kinase by the MAP kinase kinase, which is in turn activated by phosphorylation of the MAP kinase kinase by the MAP kinase kinase kinase. The MAP kinase cascade is an important signaling pathway that is involved in the regulation of a wide range of cellular processes, including stress response, growth, and development.
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 BamHI sites through PCR amplification with the following primers: MMK4x1, 5'-TTTTGGATCCCAATGGCCA-GAGTTAACC-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 from 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

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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 Trition 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 MgCl2, 5 mM EGTA, 1 mM DTT. Kinase reactions were performed in 20 mM Hepes (pH 7.5), 15 mM MgCl2, 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-32P]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

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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 p44MMK4 kinase activity and protein levels.

To analyze whether p44MMK4 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 p44MMK4 kinase (Fig. 2A, 0 min), at 10 min after cold treatment, activation of p44MMK4 kinase became detectable. Maximal activation of the p44MMK4 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. 2A).

Drought, but Not Heat Shock or Osmotic Stress, Induces p44MMK4 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).
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 p44MMK4 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 p44MMK4 kinase at any time (Fig. 2B), indicating that the MMK4 kinase is not activated by high salt. No activation of p44MMK4 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 p44MMK4 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 p44MMK4 protein, the same leaf extracts of drought-treated plants that were used for immunokinase assays (Fig. 2B) were separated by denaturating 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. 2C). The appearance of two slightly slower migrating bands at 10 min after drought stress (Fig. 2C) was correlated with the activation of the MMK4 kinase (Fig. 2B). At 45 min after dehydration, the two bands disappeared in parallel to the decrease in activity of the p44MMK4 kinase. An analysis with the protein extracts of cold-stressed leaves also showed a transient shift in the electrophoretic mobility of the p44MMK4 protein when the MMK4 kinase was activated (data not shown), strongly suggesting that the drought- and cold-induced activation of the p44MMK4 kinase occurs by posttranslational modification of the p44MMK4 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. 3A). No increase in transcript levels was observed in heat stressed plants (data not shown).

**FIG. 3.** MMK4 transcript but not p44MMK4 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) p44MMK4 immunoblot analysis of alfalfa leaves after cold treatment.

**FIG. 4.** MMK4 transcript but not p44MMK4 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) p44MMK4 immunoblot analysis of alfalfa leaves after cold treatment.
Hybridization of the same RNA gel blots with radiolabeled fragments either of the MMK1 gene (Fig. 3A) 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. 4A). Neither MMK1 (Fig. 4A) 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. 3A and 4A). These data indicate that transcripts of the MMK4 gene accumulate upon cold and drought stress.

p44MMK4 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. 3B and 4B, 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. 3A and 4A, respectively), no change in p44MMK4 protein levels was observed after these stresses (Figs. 3B and 4B, 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, p44MMK4 kinase is rapidly activated by low temperature and dehydration. Second, the protein accumulate but are not translated. Alternatively, assuming translation of MMK4 transcripts, steady state p44MMK4 protein levels might stay constant, if different pools of p44MMK4 protein had different rates of turnover. This could provide a mechanism to get rid of active p44MMK4 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 p44MMK4 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 p44MMK4 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 drought stress. Consistent with this.
MMK4 kinase after cold and drought stress is a necessary step to induce the synthesis of ARA itself either directly by