A MAP Kinase Is Activated Late in Plant Mitosis and Becomes Localized to the Plane of Cell Division

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In eukaryotes, mitogen-activated protein kinases (MAPKs) are part of signaling modules that transmit diverse stimuli, such as mitogens, developmental cues, or various stresses. Here, we report a novel alfalfa MAPK, Medicago MAP kinase 3 (MMK3). Using an MMK3-specific antibody, we detected the MMK3 protein and its associated activity only in dividing cells. The MMK3 protein could be found during all stages of the cell cycle, but its protein kinase activity was transient in mitosis and correlated with the timing of phragmoplast formation. Depolymerization of microtubules by short treatments with the drug amiprophosmethyl during anaphase and telophase abolished MMK3 activity, indicating that intact microtubules are required for MMK3 activation. During anaphase, MMK3 was found to be concentrated in between the segregating chromosomes; later, it localized at the midplane of cell division in the phragmoplast. As the phragmoplast microtubules were redistributed from the center to the periphery during telophase, MMK3 still localized to the whole plane of division; thus, phragmoplast microtubules are not required to keep MMK3 at this location. Together, these data strongly support a role for MMK3 in the regulation of plant cytokinesis.

INTRODUCTION

Plant shape is elaborated by developmental signals regulating the timing and orientation of cell division and cell enlargement. Many components of cell cycle control are conserved evolutionarily among yeasts, animals, and plants, including the cyclin-dependent protein kinases, which are key regulators of cell cycle progression (Hirt and Heberle-Bors, 1994). The spatial control of cell division, on the other hand, is largely dependent on plant-specific cytoskeletal structures, such as the prepophase band, which marks the division plane before mitosis, and the phragmoplast, which is required to lay down the new cell wall during cytokinesis (Wick, 1991).

Little is known about how these cytoskeletal arrays are constructed and function during cell division, but evidence is accumulating that protein phosphorylation plays an important role (Katsuta and Shiboka, 1992; Wolniak and Larsen, 1995).

The mitogen-activated protein kinases (MAPKs) are utilized in eukaryotes to transmit diverse stimuli, such as mitogens, developmental cues, or various stresses (Robinson and Cobb, 1997). They are integrated into a signaling module composed of three linked protein kinases: the MAPK, the MAPK-activating kinase (MEK), and the MEK-activating kinase (MEKK). The functions of MAPKs are best understood in yeast, for which there are six different genes encoding MAPKs. Five of these have been assigned to specific signaling pathways, such as pheromone response, filamentation, response to hypotonic and hypertonic shock, and sporulation (Madhani and Fink, 1998). The genetic dissection of these pathways is not always straightforward, because a MAPK normally assigned to one pathway can overtake the function in another if the relevant MAPK is knocked out by a loss-of-function mutation (Madhani and Fink, 1998). In plants, the situation might be even more complex because up to nine different MAPK genes have been reported for Arabidopsis, but the list is probably incomplete (Mizoguchi et al., 1997). Most of the data obtained on the possible functions of MAPKs in plant and animal cells are from studies on the activation of these enzymes (Robinson and Cobb, 1997).

MAPKs are required to reenter the cell cycle from both the G_1 and G_2 phases. MAPKs stimulated by growth factors exert their effects on the cell cycle by influencing G_1-specific cyclin expression (Lavoie et al., 1996). A MAPK pathway is utilized at two points during meiotic cell divisions in Xenopus: to start oocyte maturation and meiosis I from G_2-arrested oocytes and to arrest cell cycle progression in a metaphase-like state at the end of meiosis II (Sagata, 1997).

There is accumulating evidence for the mitosis-specific activation of MAPKs in somatic animal cells (Tamemoto et al., 1992; Heider et al., 1994; Chiri et al., 1998; Philipova and Whitaker, 1998). However, the MAPK seems to be dispensable for normal mitosis in Xenopus cell extracts but is required for the microtubule integrity checkpoint during mitosis (Minshull et al., 1994; Takenaka et al., 1997). MAPKs regulate multiple aspects of microtubule functions both in meiotic and mitotic cell divisions of animal cells. MAPKs were found to be associated with microtubules (Verlhac et al., 1994; Reszka et al., 1995; Morishima-Kawashima and Kosik, 1996), to phosphorylate microtubule-associated proteins (Hoshi et al., 1992; Shiina et al., 1992), and to regulate microtubule dynamics (Gotoh et al., 1991). MAPKs also regulate other motility functions, such as vesicle transport (Earnest et al., 1996). Using antibodies specific for activated MAPKs, researchers detected ERK1 and ERK2 on various mitotic structures, that is, at kinetochores when chromosomes were in the process of alignment, in centrosomes during the entire period of mitosis, and in the midbody during cytokinesis (Shapiro et al., 1998; Zecevic et al., 1998). Therefore, ERK1 and ERK2 have been implicated as playing roles at multiple steps during mitosis.

More than 20 plant MAPK cDNAs have been isolated from various plant species, and by sequence similarity analyses, these cDNAs have been classified into four major groups (Machida et al., 1997). Members of the first two groups appear to transmit various forms of biotic and abiotic stress signals (Hirt, 1997). The elevated mRNA levels of Medicago MAP kinase 1 (*MMK1*) in alfalfa cells in the G_2 phase of the cell cycle indicate that this MAPK might be involved in cell cycle control as well, but it is not known whether this MAPK is also active in these cells (Jonak et al., 1993). The high levels of expression of a tobacco MEKK, *NPK1*, in proliferating cells also point to a possible involvement in cell cycle control (Banno et al., 1993). *NPK1* also was found to be involved in a signaling pathway that inhibits auxin-induced gene expression (Kovtun et al., 1998).

Here, we report on the isolation of a novel alfalfa MAPK, designated Medicago MAP kinase 3 (MMK3). Our data indicate that MMK3 is involved in cell cycle regulation of mitosis. Although the MMK3 protein is present during all stages of the cell cycle, its protein kinase activity is activated only after metaphase. These results and the localization of MMK3 to the midplane of cell division suggest that MMK3 plays a role in plant cytokinesis.

RESULTS

Isolation of a Novel MAPK from Alfalfa

In a search for alfalfa MAPKs, we used degenerate oligonucleotides corresponding to two highly conserved regions of genes encoding MAPKs to amplify a 300-bp fragment from

a cDNA library by using the polymerase chain reaction (PCR). Previously, we reported on three alfalfa MAPK genes, *MMK1*, *MMK2*, and *MMK4* (Jonak et al., 1993, 1995, 1996). Restriction analysis and sequencing of additional PCR products revealed another clone with high sequence similarities to MAPKs but distinct from the previously isolated alfalfa genes encoding MAPKs. We screened an alfalfa cDNA library with this PCR fragment and identified five cDNA clones of the same length. All five were sequenced at their 5′ and 3′ ends and were found to be identical. One of the clones was sequenced on both strands and was named MMK3, for Medicago MAP kinase 3 (EMBL accession number AJ224336). This cDNA clone contains an open reading frame with 41 to 46% sequence identity to known yeast and animal MAPKs and is 63, 70, and 60% identical to the previously isolated alfalfa MAPKs MMK1, MMK2, and MMK4, respectively (Figure 1). MMK3 is related most closely to the tobacco MAPK, NTF6 (Wilson et al., 1995). Although there is no stop codon before the first ATG on our cDNA clone, the close sequence similarity of the putative N terminus of MMK3 to NTF6 indicates that protein translation starts at the first ATG on the currently available cDNA. This was corroborated further by the finding that the size of the protein obtained by in vitro translation of the cDNA template is identical to the size of the protein detected by a specific antibody (Figures 2A and 2C).

Sequence similarity of plant MAPKs is high in the protein kinase domains and variable on a relatively short stretch at the C and N termini (Figure 1A). Although MMK3 and NTF6 are only 79% identical, the sequence similarity extends over the entire sequence, indicating that these two genes might have similar functions. By using sequence similarity analysis with Clustal W software (Thomson et al., 1994), we were able to group the known plant MAPKs into four major classes (Figure 1B). MMK3 is most similar to NTF6 but shares similarity with other plant MAPKs as well, including MMK2, which was shown to complement the *mpk1* mutation in yeast (Jonak et al., 1995).

MMK3 Is a 44-kD Protein Kinase

To determine the possible function of the MMK3 kinase, we produced a polyclonal antibody against a synthetic peptide encoding the C-terminal 10 amino acids of MMK3. The antibody recognized a single 44-kD protein in extracts from cultured alfalfa cells. This immunoreaction was specific because the binding of the antibody could be competed with the peptide used for immunization (Figure 2A). This antibody specifically recognized the bacterially produced MMK3 as a glutathione *S*-transferase fusion product but not the other three currently known alfalfa MAPKs (Figures 2A and 2B). The MMK3–glutathione *S*-transferase fusion protein was resolved as a doublet of ∼66 kD by SDS-PAGE, but only the higher, probably intact form was recognized by the antibody. The bacterially produced MMK3 protein was active as
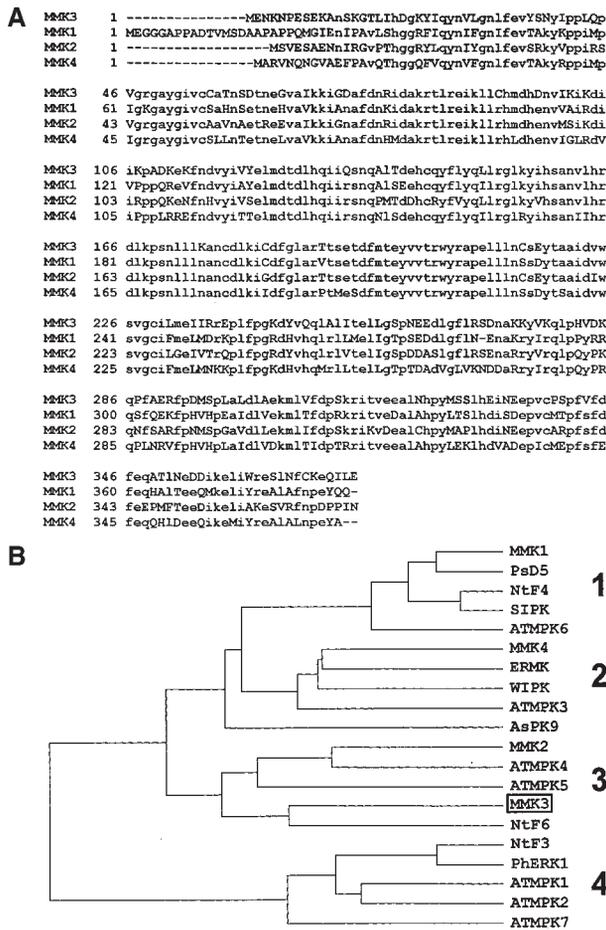
A

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MMK3    1  ---------------MENKNPESEKAnSKGTLIhDgKYIqynVLgnlfevYSNyIppLQp
MMK1    1  MEGGGAPPADTVMSDAAPAPPQMGIEnIPAvLShggRFIqynIFgnIfevTAkyKppiMp
MMK2    1  ---------------MSVESAENnIRGvPThggRYLqynIYgnlfevSRkyVppiRS
MMK4    1  ---------------MARVNQNGVAEFPAvQThggQFVqynVFgnlfevTAkyRppiMp

MMK3   46  VgrgaygivcCaTnSDtneGvaIkkiGDafdnRidakrtlreikllChmdhDnvIKiKdi
MMK1   61  IgKgaygivcSaHnSetneHvaVkkiAnafdnKidakrtlreikllrhmdhenvVAiRdi
MMK2   43  VgrgaygivcAaVnAetReEvaIkkiGnafdnRidakrtlreikllrhmdhenvMSiKdi
MMK4   45  IgrgaygivcSLLnTetneLvaVkkiAnafdnHMdakrtlreikllrhLdhenvIGLRdV

MMK3  106  iKpADKeKfndvyiVYelmdtdlhqiiQsnqAlTdehcqyflyqLlrglkyihsanvlhr
MMK1  121  VPppQReVfndvyiAYelmdtdlhqiirsnqAlSEehcqyflyqYlrglkyihsanvlhr
MMK2  103  iRppQKeNfnHvyiVSelmdtdlhqiirsnqPMTdDhcRyfVyqLlrglkyVhsanvlhr
MMK4  105  iPppLRREfndvyiTTelmdtdlhqiirsnqNlSdehcqyflyqIlrglRyihsanIIhr

MMK3  166  dlkpsnlllKancdlkiCdfglarTtsetdfmteyvvtrwyrapelllnCsEytaaidvw
MMK1  181  dlkpsnlllnancdlkiCdfglarVtsetdfmteyvvtrwyrapelllnSsDytaaidvw
MMK2  163  dlkpsnlllnancdlkiGnancdlkiGdfglarTtsetdfmteyvvtrwyrapelllnCsEytaaidIw
MMK4  165  dlkpsnlllnancdlkiIdfglarPtMeSdfmteyvvtrwyrapelllnSsDytSaidvw

MMK3  226  svgciLmeIIRrEplfpgKdYvQqlAlItelLgSpNEEdlgflRSDnaKKyVKqlpHVDK
MMK1  241  svgciFmeLMDrKplfpgRdHvhqlrlLMelIgTpSEDdlgflN-EnaKryIrqlpPyRR
MMK2  223  svgciLGeIVTrQplfpgRdYvhqlrlVtelIgSpDDASlgflRSEnaRryVrqlpQyPK
MMK4  225  svgciFmeLMNKKplfpgKdHvhqMrlLtelLgTpTDAdVgLVKNDDaRryIrqlpQyPR

MMK3  286  qPfAERfpDMSpLaLdlAekmlVfdpSkritveealNhpyMSSlhEiNEepvcPSpfVfd
MMK1  300  qSfQEKfpHVHpEaIdlVekmlTfdpRkritveDalAhpyLTSlhdiSDepvcMTpfsfd
MMK2  283  qNfSARfpNMSpGaVdlLekmlIfdpSkriKvDealChpyMAPlhdiNEepvcARpfsfd
MMK4  285  qPLNRVfpHVHpLaIdlVDkmlTIdpTRritveealAhpyLEKlhdVADepIcMEpfsfE

MMK3  346  feqATlNeDDikeliWreSlNfCKeQILE
MMK1  360  feqHAlTeeQMkeliYreAlAfnpeYQQ-
MMK2  343  feEPMFTeeDikeliAKeSVRfnpDPPIN
MMK4  345  feqQHlDeeQikeMiYreAlALnpeYA--
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B

Figure 1. Sequence Comparison of MMK3 with Other Alfalfa and Plant MAPKs.

(A) Alignment of amino acid sequences of alfalfa MAPKs: MMK3 (EMBL accession number AJ224336), MMK1, MMK2, and MMK4 (Jonak et al., 1993, 1995, 1996). Identical amino acids are indicated by lowercase letters; dashes denote gaps.
(B) Grouping of plant MAPKs according to sequence similarity with the Clustal W computer program. The numbers 1 to 4 on the right indicate the four groups of plant MAPKs. MMK3 is highlighted by a box.

kinase activity was immunoprecipitated when the antibody was preincubated with an excess of the peptide that had been used for immunization (Figure 2D, lane 2), and no activity was found when the antibody was omitted from the immunoprecipitation (Figure 2D, lane 3).

MMK3 Is Most Abundant and Active in Meristematic Parts of the Plant

In initial experiments, neither the *MMK3* mRNA nor MMK3 protein was found in any organ of mature alfalfa plants, but both were present in young leaves and in cultured alfalfa cells (data not shown). To test whether the *MMK3* gene is expressed only in dividing cells, we analyzed leaves and flowers at different developmental stages for MMK3 protein levels and protein kinase activity (Figure 3). The activity of cyclin-dependent kinases (CDKs) was used as a biochemical marker for the presence of dividing cells. CDK activity purified from extracts by binding to the yeast regulatory protein p13[suc1] or by immunoprecipitation with a specific antibody directed against the cell division cycle 2 (Cdc2) protein from *Medicago sativa* (Cdc2-Ms) correlated with cell proliferation during these developmental stages (Figure 3B). Closely paralleling CDK activity were MMK3 protein levels and activity, with the highest being in young organs and the lowest during leaf and flower development (Figures 3A and 3B). However, protein levels of MMK1, another alfalfa MAPK, were similar at all developmental stages (Figure 3A). These data suggested that MMK3 might play a role in cell cycle regulation.

MMK3 Is Active in Mitosis

To determine whether MMK3 is activated during a specific phase of the cell cycle, we synchronized cultured alfalfa cells by transiently arresting them at the G_1-to-S transition with the DNA polymerase inhibitor aphidicolin. Synchrony of cell cycle progression was followed by flow cytometric measurements of the DNA content of cells, reflecting the G_1, S, and G_2 stages (Figure 4D), and by counting the number of cells in various stages of mitosis (Figure 4E). Approximately 60% of the cells had intermediate DNA values between the G_1 and G_2 phases, which is indicative of the S phase, 3 hr after aphidicolin release; ~70% of the cells had a G_2 phase DNA content at 6 to 8 hr. The reappearance of a high proportion of cells in G_1 at 12 hr indicated that the cells were passing through mitosis at this time (Figure 4D).

Mitotic nuclei cannot be measured by flow cytometry; therefore, passage through mitosis was confirmed by counting the number of cells in various phases of mitosis after staining the chromatin with the DNA stain 4′,6-diamidino-2-phenylindole (DAPI). The highest proportion of cells in mitosis was at 10 to 12 hr (Figure 4E). CDKs, purified by binding to p13[suc1], were active in alfalfa cells from 8 to 12 hr, which
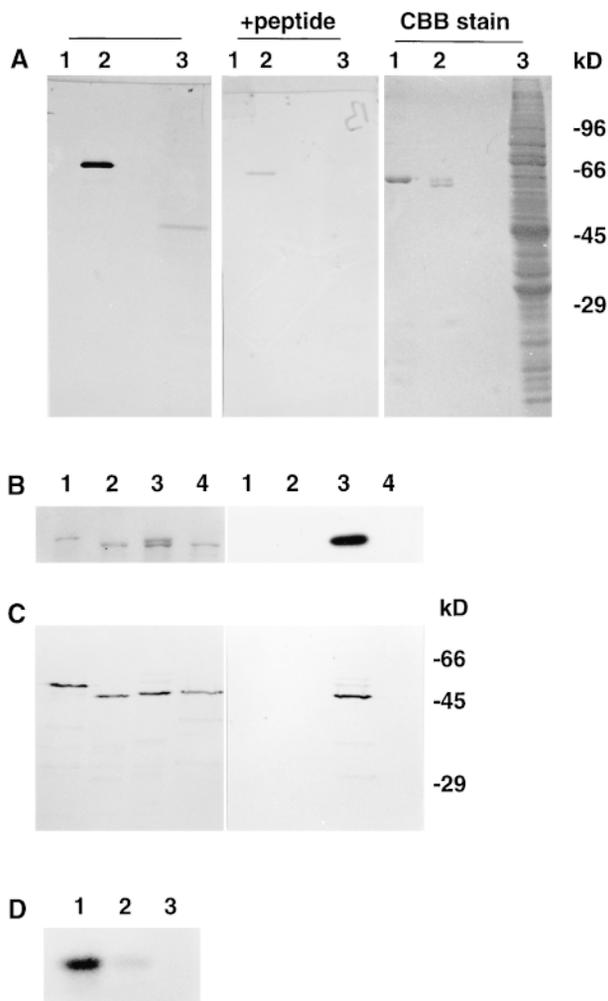
a protein kinase and phosphorylated the myelin basic protein (MBP; data not shown). In vitro translation of the four known alfalfa MAPKs all resulted in protein products of ~45 kD, which correlated well with the expected sizes deduced from the open reading frames of the cDNA clones (Figure 2C).

The MMK3-specific antibody specifically immunoprecipitated the MMK3 protein with almost 100% efficiency but not the other in vitro–translated alfalfa MAPKs (Figure 2C). Similar to the recombinant protein, immunopurified MMK3 from alfalfa cell extracts was active as a protein kinase and phosphorylated MBP as a substrate (Figure 2D). The immunopurification was specific because strongly reduced MBP–

Figure 2. Specificity of the Anti-MMK3 Antibody.

(A) Protein gel blot analysis of the bacterially produced MMK1–glutathione *S*-transferase (lanes 1) and the MMK3–glutathione *S*-transferase (lanes 2) proteins and an extract prepared from cultured alfalfa cells (lanes 3) with the MMK3-specific antibody (left), the MMK3-specific antibody preincubated with an excess of the peptide (+ peptide) that had been used for immunization (middle), and the Coomassie Brilliant Blue R 250 (CBB) staining of a gel containing the same samples (right). One microgram of the recombinant proteins and 25 μg of the cell extract were used. The apparent molecular masses in kilodaltons are indicated at right.
(B) Protein gel blot analysis of bacterially expressed MMK1–glutathione *S*-transferase (lanes 1), MMK2–glutathione *S*-transferase (lanes 2), MMK3–glutathione *S*-transferase (lanes 3), and MMK4–glutathione *S*-transferase (lanes 4) proteins. Shown are Coomassie blue staining of a gel with these samples (left) and a protein gel blot with the MMK3-specific antibody (right).
(C) In vitro translation of MMK1 (lanes 1), MMK2 (lanes 2), MMK3 (lanes 3), and MMK4 (lanes 4) proteins. Shown is an autoradiogram of the in vitro–translated and ^{35}S-methionine–labeled proteins (left) and an autoradiogram of the immunoprecipitated labeled proteins with the MMK3-specific antibody (right). The apparent molecular masses in kilodaltons are indicated at right.

corresponds to late G_2 to M phases, and became inactive at the metaphase-to-anaphase transition (Figure 4A). mRNA levels of the alfalfa mitotic cyclin gene, *cycMs2*, were analyzed in the same cells (Figure 4B). The highest *cycMs2* mRNA amounts were present at 12 hr. The expression of this mitotic cyclin gene is confined to the transition from the G_2 to M phases (Hirt et al., 1992). As a control for the mRNA amounts loaded, the level of a constitutively present mRNA, corresponding to the c27 cDNA (Pay et al., 1992), was also analyzed. These results established that mitosis takes place at ~12 hr after the release from aphidicolin arrest.

Measurement of protein kinase activity of MMK3 after immunopurification with a specific antibody taken from samples at different times after the removal of aphidicolin showed that MMK3 activity appeared shortly before mitosis at 8 hr and peaked when cells were in mitosis, which was between 12 and 14 hr (Figure 4A). These results suggest that MMK3 is active in mitosis. The amount of MMK3 protein decreased slightly and gradually from the point of aphidicolin removal; however, the protein was present at all stages of the cell cycle, indicating that activation of the MMK3 kinase in mitosis is a post-translational event (Figure 4C).

MMK3 Is Not Activated in G_2-Arrested Cells

To obtain further, independent evidence for the involvement of MMK3 in mitosis, we used roscovitine, a drug that specifically inhibits CDKs, to block entry into mitosis (Planchais et al., 1997). Cells were released from an aphidicolin block, and roscovitine was added to the culture 8 hr later when cells were in G_2 phase. Although cells without roscovitine progressed normally through the cell cycle (Figure 4D), roscovitine-treated cells with a G_2 DNA content were arrested (Figure 5C). This block was at least partially reversible because washing out roscovitine at 14 hr (after the aphidicolin release) allowed a fraction of the cells to progress through mitosis, as indicated by an increase in the number of cells with a G_1 DNA content 7 hr after roscovitine removal (Figure 5C, 21-hr wash). MMK3 was not active in cells that were arrested by roscovitine in the G_2 phase but was activated as cells entered mitosis after the removal of the drug (Figure 5A). The amount of MMK3 protein did not change in roscovitine-treated cells (Figure 5B). In contrast, CDKs, when isolated as a complex with p13^{suc1} and assayed for kinase activity, were active in roscovitine-treated cells (Figure 5A).

(D) MMK3 activity after immunoprecipitation with the MMK3-specific antibody from alfalfa cell extracts (lane 1), immunoprecipitation with the antibody preincubated with the peptide used for immunization (lane 2), and immunoprecipitation without the addition of the antibody (lane 3). In vitro kinase assays of immunopurified proteins were performed with MBP as a substrate. An autoradiogram of ^{32}P-labeled MBP on an SDS–polyacrylamide gel is shown.

Roscovitine specifically inhibited CDK activity in treated cells, but apparently it did not influence the activated state of CDK. Therefore, CDK activity, purified and assayed in the absence of the drug, did not change significantly. Similar results have been published previously (Planchais et al., 1997).

That roscovitine directly inhibits MMK3 activity is unlikely. A 100 times higher concentration of roscovitine than that needed for CDK inhibition is required to block MMK3 activity (data not shown). Therefore, the effect of roscovitine on cell cycle progression is probably due to inhibition of CDK activity. These data are consistent with the idea that entry into mitosis is required for the activation of MMK3 or that a mitosis-specific CDK complex might be directly responsible for the activation of MMK3.

MMK3 Is Activated after Metaphase Arrest

To refine the time window in which MMK3 is active during mitosis, alfalfa cells that had passed synchronously through the cell cycle after release from aphidicolin arrest were blocked again in metaphase with the microtubule-depoly-
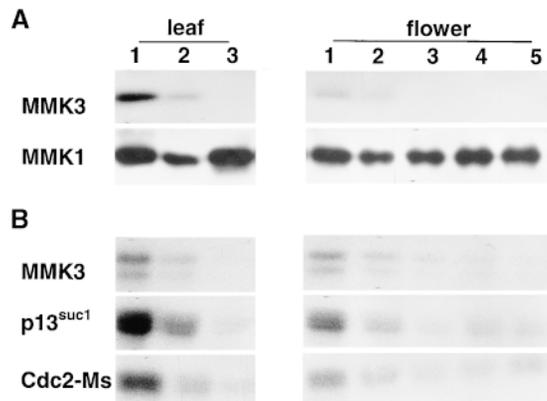
Figure 3. Amount of MMK3 Protein and Protein Kinase Activities in Plant Organs at Various Developmental Stages.

(A) Protein gel blot analysis of MMK3 and MMK1 protein levels in leaves (young folded leaf, young unfolded leaf, and mature leaf; lanes 1 to 3, respectively) and in flowers (floral meristem, 1-mm flower bud, 2-mm closed flower, 4-mm closed flower, and opened flower; lanes 1 to 5, respectively).
(B) Protein kinase activities determined by autoradiography of the ^{32}P-labeled substrate proteins MBP and histone H1 phosphorylated by immunopurified MMK3, the Cdc2-Ms kinase, or by CDKs isolated by binding to p13^{suc1}–Sepharose from leaves and flowers at the same developmental stages as given in **(A)**. MMK3 was immunopurified from extracts; CDKs were purified by binding to p13^{suc1} beads or immunopurified. The protein kinase activities of MMK3 or CDKs were determined by in vitro phosphorylation of MBP or histone H1 proteins, respectively. The labeled substrate proteins were separated by SDS-PAGE and detected by autoradiography.

merizing drug propyzamide. MMK3 was not active in propyzamide-arrested metaphase cells (Figure 6A, 0 hr), or its activity was low (Figure 7A, 0 hr). In propyzamide-treated samples with no MMK3 activity, all microtubules were depolymerized completely, and no anaphase cells could be found (Figure 6B, 0 hr), whereas remnants of phragmoplast microtubules could be observed in a low number of anaphase cells derived from cells with detectable MMK3 activity (Figure 7C, 0 hr). The p13^{suc1}-bound CDKs were fully active in these metaphase-arrested cells at 0 hr (Figure 7C, 0 hr).

Removal of propyzamide allowed a portion of these cells to reconstitute microtubule structures, such as the spindle and phragmoplast, and to progress through anaphase and telophase, which was determined by counting the number of phragmoplasts after visualizing tubulin by indirect immunofluorescence. The timing of the progress through anaphase and telophase was somewhat variable in five independent experiments, of which two are presented here (Figures 6 and 7, control). In the first experiment, 15% of the cells contained a phragmoplast 0.5 hr after removal of propyzamide from the culture, and a similar percentage of cells with a phragmoplast could still be found at 1 and 1.5 hr (Figure 6B). In the second experiment, cells entered anaphase more synchronously, and within 0.5 hr, 30% of the cells contained a phragmoplast (Figure 7C, control).

A decrease in the activity of p13^{suc1}-bound CDK activities in cells released from propyzamide-induced metaphase arrest also indicated that cells exited from the metaphase arrest and passed through anaphase and telophase (Figure 7A). In plants, some of the CDKs present in the p13^{suc1}-bound complexes seem to persist until anaphase and associate with the phragmoplast (Mews et al., 1997), which could explain the relatively high p13^{suc1}-bound CDK activity 0.5 hr after the removal of propyzamide. Contrary to this, MMK3 was activated as cells entered anaphase, and its activity paralleled the percentage of phragmoplasts determined in these synchronization experiments (Figures 6A and 7A). These data indicate that MMK3 activation occurs after metaphase and correlates with phragmoplast formation.

MMK3 Activity Is Lost in Anaphase Cells with Destabilized Microtubules

Because MMK3 activity was absent when cells were arrested in metaphase with depolymerized microtubules, and its activity was recovered when microtubules repolymerized after the removal of the drug and cells passed through anaphase and telophase, we investigated whether intact microtubules are required for MMK3 activity at these phases. Cells were again synchronized by arresting them during metaphase by using propyzamide; as cells passed through anaphase and telophase after the removal of propyzamide, 30-min pulse treatments of amiprophosmethyl (APM), a potent microtubule-destabilizing agent, were used (Figure 7). No phragmoplast or any other microtubule structures could
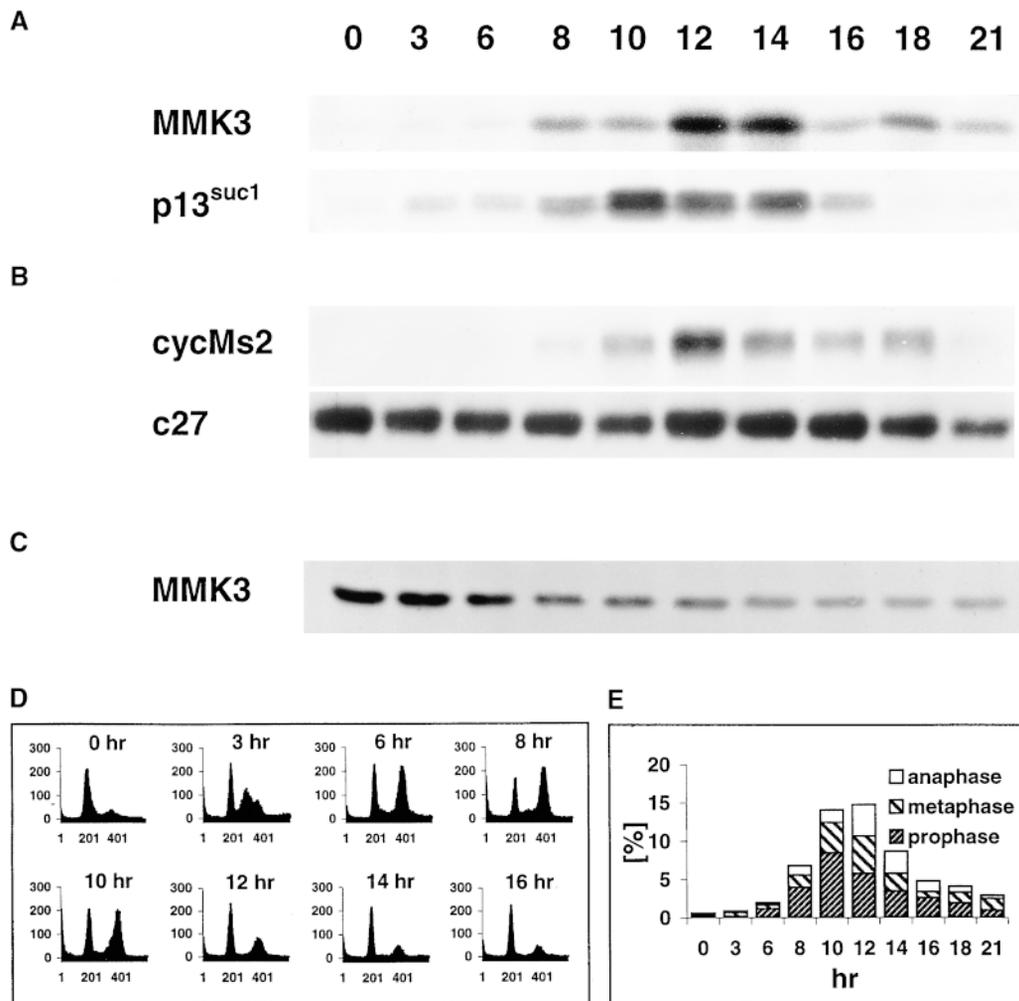
Figure 4. MMK3 Is Active during Mitosis.

(A) Cultured alfalfa cells were arrested at the G_1-to-S phase transition by treatment with aphidicolin (0 hr). After removal of the drug, extracts were prepared at the indicated time points (at top; hours) and analyzed for protein kinase activities of MMK3 or p13^{suc1}-bound CDKs, as described in the legend to Figure 3B. An autoradiogram of ^{32}P-labeled MBP on an SDS–polyacrylamide gel is shown.

(B) RNA gel blot analysis showing *cycMs2* mRNA transcript levels, with *c27* as a control for equal loading.

(C) Protein gel blot analysis of MMK3 protein levels.

(D) Flow cytometric analysis of cell cycle stages derived by measuring the DNA content in cells taken at various time points. Abscissa, relative fluorescence intensity; ordinate, cell number. The fluorescence intensity of 200 represents the 2N DNA content of G_1 cells, whereas 400 is the 4N DNA content of G_2 cells.

(E) Percentage of mitotic stages in samples at the indicated time points.

be observed in cells treated with APM for half an hour (Figure 7C). That cells exited metaphase and passed through anaphase and telophase, similar to control cells, was indicated by the decrease in p13^{suc1}-bound CDK activity. However, in anaphase and telophase cells with depolymerized microtubules, no MMK3 activity could be detected (Figure 7B), whereas the MMK3 protein was found at similar levels in control and APM-treated cells (Figures 7A and 7B). From this experiment, we concluded that intact microtu-

bules are required for MMK3 activity in anaphase and telophase cells.

MMK3 Localizes to the Midplane of Cell Division in Late Anaphase and Telophase Cells

To determine where in the cell the MMK3 protein is located, we conducted indirect immunofluorescence microscopy of

alfalfa and fava bean root tip cells. The MMK3-specific antibody recognized a single band of the same size on protein gel blots of fava bean and alfalfa extracts, and in both cases similar localization was obtained (data not shown).

During interphase, we found MMK3 to be located diffusely in the cytoplasm of fava bean root tip cells (data not shown), but it became concentrated around the nucleus and was also found to invade the nucleus as cells progressed to prophase (Figures 8A and 8B). During metaphase, MMK3 was dispersed throughout the whole cell but was excluded from the chromosomes (Figures 8C and 8D). During early anaphase, MMK3 became gradually localized between the segregating chromosomes (Figures 8E and 8F). During late anaphase and early telophase, when chromosomes began to decondense (Figure 8H), MMK3 was detected at the midplane of division in a portion of the cells (~10 to 50% of the late anaphase and telophase cells in different preparations; Figure 8G).

Because MMK3 activity was found to correlate with the number of phragmoplasts as cells progressed through anaphase and telophase, and intact microtubules were required for its activity, we determined the localization of MMK3 in relation to mitotic stages and microtubule structures by triple labeling with anti-MMK3 and anti–α-tubulin
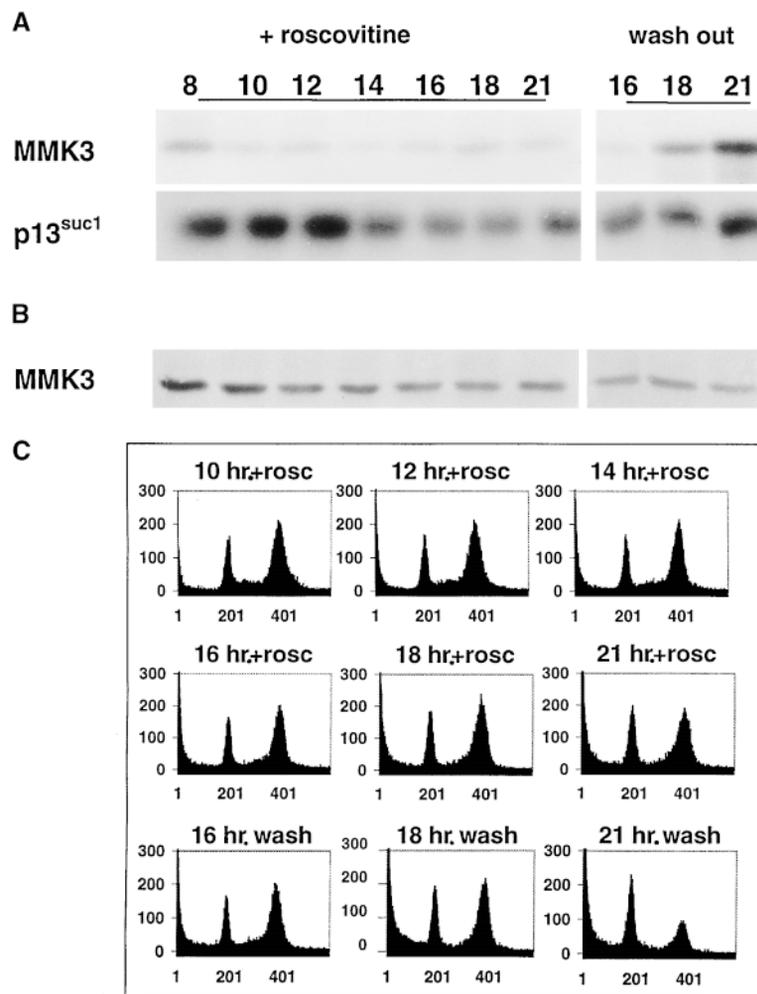

Figure 5. MMK3 Is Not Active When Cells Are Blocked at the G_2 Phase with Roscovitine.

(A) Protein kinase activities of MMK3 and p13^{suc1}–bound CDKs isolated from roscovitine-treated cells (+ roscovitine) or after the removal of roscovitine (wash out). Time points in hours refer to release after aphidicolin treatment.

(B) Protein gel blot analysis of the same samples with the MMK3-specific antibody.

(C) Flow cytometric analysis of DNA content in cells taken at the indicated time points (hours) with roscovitine (+ rosc) or after the removal of roscovitine (wash). The values on the abscissas and ordinates are as given for Figure 4D.
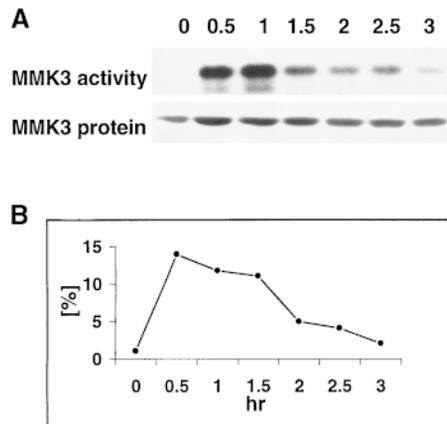
Figure 6. MMK3 Is Active after Metaphase.

(A) Cells were arrested during metaphase by treating synchronously dividing cells with the microtubule-depolymerizing drug propyzamide (0 hr). After removal of the drug, extracts were prepared at the indicated time points (in hours), and the activities of MMK3 and MMK3 protein levels were determined as described for Figure 3B.
(B) The percentage of phragmoplast microtubules counted from slides stained to detect α-tubulin and observed by indirect immunofluorescence by using samples of the cells described in **(A)**.

antibodies as well as DAPI (Figure 9). In this experiment, alfalfa root tip cells were used. In a cell with an anaphase spindle (Figure 9B), MMK3 was diffusely located in between the separating chromosomes (Figures 9A and 9C). No clear association with microtubules was seen. In late anaphase (Figure 9F), when phragmoplast microtubules began to form in the midplane between the two nuclei (Figures 9E and 9F), MMK3 became more concentrated at the phragmoplast (Figure 9D). As the phragmoplast grew outward, MMK3 was detected as a disc on the cell plate (Figures 9G to 9I). When the phragmoplast microtubules were redistributed to the edges of the growing cell plate toward the parental cell walls in late telophase, MMK3 still was localized across the whole area of the cell plate (Figures 9J to 9L). MMK1 and MMK2, the two other alfalfa MAPKs, were not found to localize to the cell plate (data not shown).

Although microtubules or other cytoskeletal elements seem to be necessary to locate MMK3 to the cell plate during anaphase and early telophase, they clearly are not required to keep MMK3 at this location in later stages, when phragmoplast microtubules are redistributed toward the periphery of the cell.

DISCUSSION

Here, we report on the isolation of a novel alfalfa MAPK gene, *MMK3.* The deduced protein sequence of MMK3 is

most similar to a previously isolated tobacco MAPK, NTF6 (Wilson et al., 1995). With a specific antibody, we found that NTF6 also is active during mitosis (Calderini et al., 1998). However, it is not clear whether *NTF6* is the ortholog of the alfalfa *MMK3* gene.

MMK3 shares high sequence similarity with another alfalfa MAPK, MMK2. Previously, we showed that only *MMK2* among the alfalfa MAPKs could complement a yeast MAPK mutation, *mpk1*, which is implicated in cell wall biosynthesis and actin organization in yeast (Jonak et al., 1995). However, *MMK3* did not complement *mpk1* (S. Kiegerl, unpublished results), and it is constitutively active during the cell
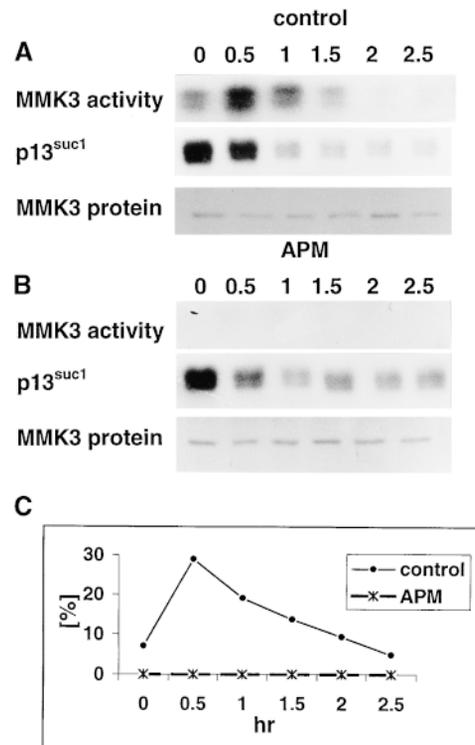

Figure 7. MMK3 Is Not Active in Anaphase and Telophase Cells Treated with the Microtubule Drug APM.

(A) Activity of MMK3 and CDKs in control cells after propyzamide release. Extracts were prepared at the indicated time points (in hours), and the activities of MMK3 and p13^{suc1}-bound CDKs and MMK3 protein levels were determined as described for Figure 3B.
(B) Activity of MMK3 and CDKs in APM-treated cells. After removal of propyzamide, a portion of the cells synchronously passing through anaphase and telophase was pulse treated at the indicated time points for half an hour with 10 μM of the microtubule destabilizing drug APM.
(C) The percentage of phragmoplast microtubules counted from slides stained to detect α-tubulin and observed by indirect immunofluorescence by using samples of the cells described in **(A)** and **(B)**.
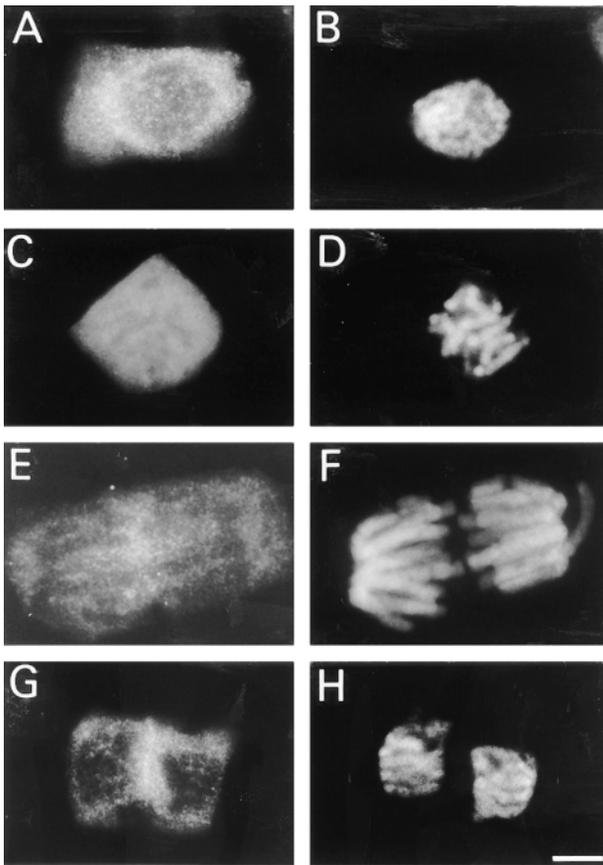
Figure 8. Localization of MMK3 within Fava Bean Root Tip Cells at Various Mitotic Stages.

(A) and **(B)** Prophase.
(C) and **(D)** Metaphase.
(E) and **(F)** Anaphase.
(G) and **(H)** Telophase.
(A), **(C)**, **(E)**, and **(G)** show indirect immunofluorescence of cells using the MMK3-specific antibody; **(B)**, **(D)**, **(F)**, and **(H)** show DAPI staining of nuclei in the same cells. Bar in **(H)** = 10 μm for **(A)** to **(H)**.

cycle (L. Bögre, unpublished results). Possibly, MMK2 is required for cell wall synthesis during growth, whereas MMK3 is specialized for cross-wall synthesis during mitosis.

Substantial evidence indicates that the ERK1 and ERK2 MAPKs play a role in the spindle checkpoint in Xenopus egg extracts (Minshull et al., 1994; Takenaka et al., 1997). Another mammalian MAPK, p38, was also implicated in the spindle checkpoint (Takenaka et al., 1998). We found that MMK3 is unlikely to be involved in this process, because MMK3 is not active in metaphase-arrested cells. MMK3 is activated only after metaphase when cells enter late anaphase.

The functions of animal MAPKs in somatic cell mitosis are less clear. Similar to MMK3, ERK1 and ERK2 are inactive in

extracts prepared from somatic cells arrested in mitosis by depolymerization of microtubules, but they are activated as cells pass through mitosis (Tamemoto et al., 1992). The in situ localization of active ERK1 and ERK2 in somatic cells implies a function for these MAPKs at multiple steps during mitosis (Shapiro et al., 1998; Zecevic et al., 1998). Although we cannot distinguish at present between the localization of
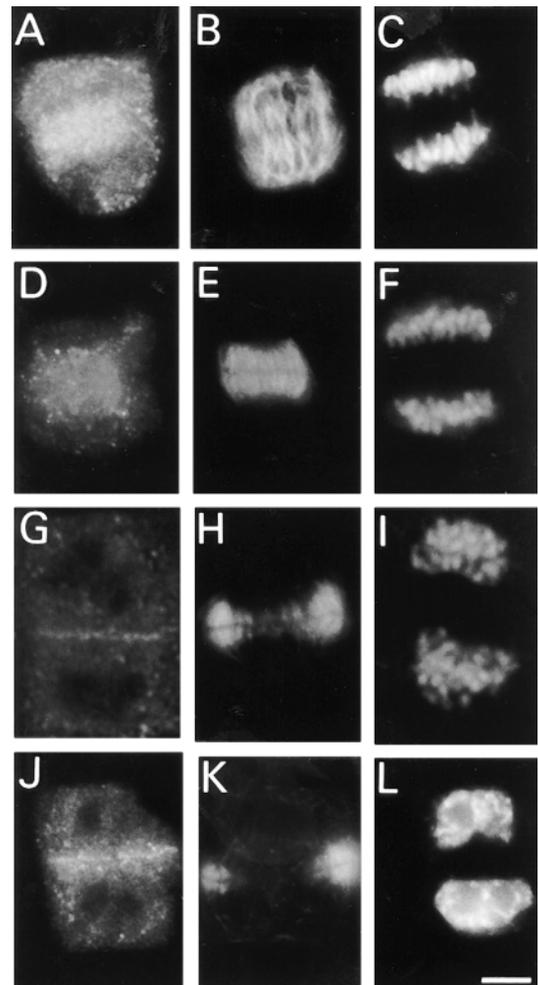

Figure 9. Temporal Profile of the Localization of MMK3 and Microtubule Structures during Anaphase and Telophase in Alfalfa Root Tip Cells.

(A) to **(C)** Anaphase.
(D) to **(F)** Late anaphase.
(G) to **(I)** Telophase.
(J) to **(L)** Late telophase.
Triple labeling of a telophase cell with the MMK3-specific antibody is shown in **(A)**, **(D)**, **(G)**, and **(J)**; triple labeling with the α-tubulin antibody is shown in **(B)**, **(E)**, **(H)**, and **(K)**; triple labeling with DAPI is shown in **(C)**, **(F)**, **(I)**, and **(L)**. Bar in **(L)** = 2 μm for **(A)** to **(L)**.

active and inactive MMK3 forms, MMK3 localization is reminiscent of active ERK1 and ERK2 localization. Both ERKs as well as MMK3 are found in the cytoplasm in interphase cells and invade the nucleus as cells enter prophase. All three MAPKs also are active at this phase. All three MAPKs again are found in comparable locations late in mitosis. Whereas MMK3 was found associated with the plant-specific phragmoplast during late anaphase and at the midplane of cell division in telophase cells, active ERK1 and ERK2 were detected at the midzone regions during late anaphase and at the midbody during telophase and cytokinesis in animal cells (Shapiro et al., 1998; Zecevic et al., 1998). The fact that both ERKs as well as MMK3 behave similarly throughout mitosis with respect to activity and location strongly suggests that these MAPKs could play similar roles in animal and plant mitosis, respectively.

The tobacco MEKK, NPK1, has also been implicated in mitosis, based on the findings that its protein levels increase as cells progress to mitosis and, similar to MMK3, that NPK1 has been localized to the cell plate (Machida et al., 1998). A search for activators of NPK1 in a yeast screen led to the identification of kinesin-like proteins (Machida et al., 1998). Similar to NPK1, ERK1 and ERK2 also are complexed with a microtubule motor protein, CENP-E (Zecevic et al., 1998). The kinesin-like tobacco proteins as well as CENP-E specifically accumulate during mitosis. These findings suggest that NPK1 might be an upstream activator of MMK3.

Another regulator of MMK3 could be the putative plant homolog of the *Schizosaccharomyces pombe cdc7* gene, a critical regulator of cytokinesis. *cdc7* encodes a protein kinase similar to *Saccharomyces cerevisiae* STE11, a signaling component upstream of a MAPK module (Sohrmann et al., 1998). The Cdc7 protein is associated with one of the spindle pole bodies during anaphase, which ensures that the division plane is restricted to a single location. Plant cells do not have a well-defined microtubule-organizing center, but the focusing of MMK3 from a diffuse distribution to a flat disc at the midplane of cell division might serve a similar function.

Rather than dividing up the cytoplasm by constriction from the plasma membrane as in animals (Glotzer, 1997), plant cells construct a new cell plate from the inside out (Staehelin and Hepler, 1996). The phragmoplast, consisting of microtubules and microfilaments that are organized as two parallel interdigitating strands in the plane of cell division, emerges between the daughter nuclei during late anaphase. Golgi-derived vesicles and elements of the endoplasmatic reticulum are transported along these cytoskeletal arrays; later, the vesicles fuse to form first a continuous tubular network and then gradually consolidate into a flattened disc in which callose is deposited (Samuels et al., 1995). A dynamin-like protein, phragmoplastin, has been isolated from soybean and shown to be associated with cell plate formation (Gu and Verma, 1996, 1997). Phragmoplastin was found to appear first in the center of the forming cell plate, and, as the cell plate grew outward, it redistributed to the growing margins of the cell plate. Contrary to this, we have not found a redistribution of MMK3 from the center to the periphery, indicating that MMK3 is not associated with phragmoplastin during this process.

Recently, embryo-patterning genes in Arabidopsis, such as *KNOLLE* (Lukowitz et al., 1996) and *KEULE* (Assaad et al., 1996), have been identified as being involved in cytokinesis, and the syntaxin-related KNOLLE protein was localized at the cell plate (Lauber et al., 1997). Similar to MMK3 during late telophase, when the phragmolast reached the lateral cortex of the cell, KNOLLE appeared to be present across the entire plane of division. Phragmoplast microtubules might be required to bring these proteins to the cell plate, but apparently the microtubules are not required to keep KNOLLE and MMK3 at this location. Fusion of MMK3 with the green fluorescent protein will provide a visual tag to determine to what extent immunofluorescence labeling on fixed cells reflects the in vivo localization of the MMK3 protein.

Which events of cytokinesis might be regulated by MMK3 is not known at present. These may include the construction of the phragmoplast by regulating microtubule stability or microtubule-based motor proteins (Chan et al., 1996; Liu et al., 1996; Asada et al., 1997; Bowser and Reddy, 1997), vesicle transport along the phragmoplast by plus-end-directed motor proteins (Asada and Collins, 1997), or the fusion of these vesicles at the cell plate (Verma and Gu, 1996).

METHODS

Isolation, Sequence Analysis, and Cloning of the *MMK3* Gene

Two redundant oligonucleotides, MAP1 (5′-TTGAATTCGGNAAC/ TGCNTTC/TGAC/TAA-3′) and MAP2 (5′-TTTCTAGANGTNACNACA/ GTAC/TTCNGT-3′), were prepared (N stands for all four nucleotides). Their GNFDN and TEYVV motifs, which are characteristic of mitogen-activated protein kinases (MAPKs), were synthesized to isolate genes encoding new MAPKs by using the polymerase chain reaction (PCR; Jonak et al., 1995). Forty cycles of PCR were performed at conditions of 95°C denaturation for 1.5 min, 45°C annealing for 2 min, and 72°C extension for 3 min. Sequence analysis of the cloned PCR fragments with the T7 sequencing kit (Pharmacia, Uppsala, Sweden) revealed a new alfalfa MAPK. A radiolabeled fragment of this PCR fragment was used to screen alfalfa cDNA libraries prepared from somatic embryos and from suspension-cultured cells (Jonak et al., 1995). Five isolated clones contained identical inserts after in vivo excision in a pBluescript SK− (Stratagene, La Jolla, CA) plasmid, as determined by sequencing the 5′ and 3′ ends. One of these cDNA inserts of 1442 bp was sequenced completely on both strands and designated *MMK3*.

Immunochemical Procedures

The MMK3- and MMK1-specific antibodies were produced against synthetic peptides encoding the C terminus of MMK3 (LNFCKEQILE)

or MMK1 (EALAFNPEYQQ). The Cdc2-Ms–specific antibody was described previously (Bögre et al., 1997). For protein gel blotting and immunofluorescence, the antibody was affinity-purified on the peptide (Bögre et al., 1997). Protein gel blotting was done as described previously (Bögre et al., 1997) and visualized with enhanced chemiluminescence (Amersham International; Figures 2B and 3A) and by observing the color reaction of alkaline phosphatase (Figures 2A, 4C, 5C, 6A, 7A, and 7B). The specificity of the antibody was tested by probing MMK1, MMK2, MMK3, and MMK4 alfalfa MAPKs produced in *Escherichia coli* as glutathione *S*-transferase fusion proteins (Jonak et al., 1995). Only MMK3 was recognized by the MMK3-specific antibody, and both MMK1 and MMK4 were recognized by the MMK1-specific antibody, but this antibody had ~10 times higher affinity for MMK1. In alfalfa and *Vicia faba* cell extracts, the purified antisera recognized a single band of 44 kD, and this reaction was stopped if 2 μg of purified antibody was preincubated for 1 hr with 5 μg of the corresponding peptide. For in vitro translation of alfalfa MAPKs, we used a kit from Amersham to perform a coupled in vitro transcription and translation reaction on the linearized pBluescript SK− plasmids containing the MAPK cDNAs.

Measurement of Protein Kinase Activities

Samples with 0.1 to 0.2 g of cells were homogenized in 3 volumes of homogenization buffer containing 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, 5 mM p-nitrophenylphosphate, 60 mM β-glycerophosphate, 0.1 mM Na_3VO_3, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg mL^{-1} of leupeptin, aprotinin, and soybean trypsin inhibitor, and 5 μg mL^{-1} of antipain, chymostatin, and pepstatin. The crude extract was centrifuged at 40,000g for 40 min. The cleared supernatant containing 0.1 mg of protein in 0.1 mL of extraction buffer was used. For immunoprecipitation, 10 μg of protein A–purified IgG was added to the extract. After 1 hr, 50 μL of protein A beads was added from a 50% protein A–Sepharose suspension. The tubes were rotated for an additional hour, and the beads were then washed four times with 1 mL of wash I buffer (50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1% Nonidet P-40, and 0.5 mM PMSF) and once with kinase buffer (20 mM Hepes, pH 7.4, 15 mM EGTA, and 1 mM DTT). Finally, all of the buffer was aspirated using a needle, and the kinase assay was performed on the beads. p13^{suc1} binding was done in essentially the same way, with 50 μL of suc1 beads being added to the samples and allowed to bind for 2 hr. The kinase assays were performed with proteins immobilized on p13^{suc1}–Sepharose or protein A–Sepharose in a final volume of 15 μL. The reaction was started by adding the assay buffer (20 mM Hepes, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 0.1 mM ATP, 2 μCi γ-^{32}P-ATP, and 0.5 mg mL^{-1} histone H1 type III [Sigma]) in the case of cyclin-dependent kinase (CDK) measurements or myelin basic protein (MBP) in the case of MAPK activity measurements. The reaction was incubated at room temperature for 30 min and terminated by the addition of 5 μL of 4 × SDS sample buffer. The samples were analyzed by SDS-PAGE and subsequent autoradiography.

Synchronization of Cultured Alfalfa Cells

Alfalfa (*Medicago sativa* A2) cells were maintained in Murashige and Skoog liquid medium (Murashige and Skoog, 1962) supplemented

with 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 0.2 mg L^{-1} kinetin. Synchronization started with a 1:5 dilution of a 7-day-old culture. After 8 hr, 10 μg L^{-1} aphidicolin (Sigma) was added to the medium. After incubation for 16 hr, the drug was removed by washing the cells five times with the medium before resuspending them in the original volume of medium. Samples were collected at various intervals, frozen in liquid N_2, and kept at −70°C until analysis. For metaphase arrest, 10 μM propyzamide was added 8 hr after the removal of aphidicolin. After 6 hr, the drug was removed, and samples were collected at 30-min intervals. G_2 arrest by roscovitine was done by adding 100 μM roscovitine (a gift from M. Strnad, Palacky University, Olomouc, Czech Republic) to the cultured cells 6 hr after the removal of aphidicolin.

Measurement of Cell Cycle Stages

Flow cytometric analysis was done by incubating aliquots of cells sedimented from 1 mL of culture with 0.2 mL of 2% cellulase Onozuka R10 (Yakult, Tokyo, Japan) and 1% pectinase dissolved in 0.6 M mannitol, 5 mM $CaCl_2$, and 3 mM Mes, pH 5.7. After incubation at 37°C for 30 min, nuclei were released in 0.4 mL of staining solution (10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, and 4 μg mL^{-1} 4′,6-diamidino-2-phenylindole [DAPI]). The released nuclei were measured directly in a PAS2 flow cytometer (Partec, Münster, Germany). For determination of the mitotic index, cells were fixed in three parts ethanol and one part acetic acid and then washed with 70% ethanol. The DNA was stained with DAPI and observed by using epifluorescence microscopy.

Immunofluorescence Microscopy

For indirect immunofluorescence, *Vicia faba* root tip cells were fixed in 3.7% formaldehyde in microtubule-stabilizing buffer (MTSB; 100 mM Pipes, 1 mM $MgSO_4$, and 2 mM EGTA, pH 6.9) for 1 hr. After washing in MTSB, the cell wall was removed by digestion for 30 min in 0.5% cellulase R-10, 0.25% macerozyme (Yakult), 0.25% pectinase (Serva, Heidelberg, Germany), and 0.25% Driselase (Sigma) dissolved in MTSB with 5 mM $CaCl_2$, 0.5 M manitol, 300 μM PMSF, 5 μg mL^{-1} leupeptin, and 1 μg mL^{-1} pepstatin. After washing in MTSB, cells were attached to slides coated with poly-L-lysine (Sigma) and extracted with 100% methanol (−20°C) for 10 min and 0.5% Triton X-100 for 10 min. After washing with PBS and BPBS (PBS plus 1% [w/v] BSA), samples were processed for immunostaining. The affinity-purified MMK3-specific antibody was used at a concentration of 1 μg mL^{-1} IgG overnight at 4°C. After washing with PBS and BPBS, samples were incubated with secondary anti–rabbit Cy3-conjugated antibody (Sigma) at a dilution of 1:200 for 45 min at room temperature. Samples were washed with PBS, and DNA was stained with 1 μg mL^{-1} DAPI in PBS. Slides were mounted in antifade mounting medium (Dako, Glostrup, Denmark). Preparations were examined using an epifluorescence microscope (Olympus, Tokyo, Japan) equipped with standard fluorescence filter sets. Photographs were taken on Kodak T-MAX 400 film. A control experiment was also performed in which 1 μg of antibody was preadsorbed with 1 μg of the peptide used for immunization; however, no immunofluorescence was observed. Staining of microtubules was performed with the mouse monoclonal antibody DM1A (Sigma) and with a secondary anti–mouse fluorescein isothiocyanate–conjugated antibody (Sigma), as described previously (Binarova et al., 1993).

RNA Gel Blot Analysis

Poly(A)[+] RNA was isolated directly from 100 mg of cells by binding to oligo(dT)–Dynabeads, according to the manufacturer's instructions (Dyanal, Oslo, Norway). One microgram of poly(A)[+] RNA was loaded on a gel, and the filter was hybridized with a fragment derived from *cycMs2* and *c27*, as described previously (Hirt et al., 1992).

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