A unique feature of cytokinesis in plant cells is the formation of a phragmoplast, which is involved in the deposition of material required to form the cell plate. The phragmoplast forms from the late anaphase to the late telophase stages of the cell cycle. Many of the structural and dynamic features of phragmoplast formation have been elucidated using electron microscopy and immunofluorescence methods (Schopfer and Hepler, 1991; Samuels et al., 1995). Cell plate formation starts in the cell centre and then progresses towards the cell periphery. The early stages are marked by the presence of two microtubular arrays with opposite polarity. These microtubules transport Golgi-derived vesicles to the newly forming cell plate, where they fuse to form a tubulo-vesicular network that later consolidates into a smooth tubular sheet in which callose is deposited. As the forming cell plate progresses towards the periphery of the cell, microtubules are displaced from the centre and follow the leading edges of the expanding phragmoplast.

Mitogen-activated protein (MAP) kinases have been demonstrated to have a role in meiosis but their involvement in mitotic events is less clear. Using a peptide antibody raised against the tobacco MAP kinase p43Ntf6 and extracts from synchronized tobacco cell suspension cultures, we show that this kinase is activated specifically during mitosis. Entry into mitosis appears to be necessary for the activation of the kinase, which occurs as a post-translational event. The activation of the kinase occurs in late anaphase/early telophase. The p43Ntf6 protein shows a transient localization to the cell plate in anaphase cells, in the middle of the two microtubule arrays characteristic of the phragmoplast, a plant-specific structure involved in laying down the new cell wall. The combined data support a role for the MAP kinase p43Ntf6 in cytokinesis in tobacco cells.

**Key words**: Cytokinesis, MAP kinase, Mitosis, Phragmoplast, Tobacco
Cells were synchronized by diluting a 7-day-old culture 1:5, and after 24 hours in Skoog medium according to the method of Nagata et al. (1992). Tobacco cells (cell line BY-2) were maintained in modified Linsmaier & Skoog medium, as described by Traas et al. (1992; Binarova et al., 1994; Smirnova et al., 1995).

Many MAP kinase cDNA clones have been isolated from plants (for a recent review, see Machida et al., 1997), and numerous signals leading to their activation have been described. Wounding (Seo et al., 1995; Usami et al., 1995; Bögè et al., 1997a), elicitors (Suzuki and Shinshi, 1995; Stratmann and Ryan, 1997; Zhang et al., 1998), drought (Jonak et al., 1996), pollen hydration (Wilson et al., 1997), and hormones (Zhang and Klessig, 1997), all result in the activation of MAP kinases or MAP kinase-like activities. However, little is known about the possible functions and the downstream targets of these kinases following stimulation. Treatment of parsley cells with a pathogen-derived oligopeptide elicitor leads to MAP kinase activation and translocation of the kinase to the nucleus (Ligterink et al., 1997), where it might function in the stimulation of expression of plant defense genes. A recombinant MAP kinase from alfalfa, MMK2, could phosphorylate a 39 kDa protein in detergent-resistant cytoskeletal preparations from carrot cells (Jonak et al., 1995).

We previously reported on the isolation of three MAP kinases from tobacco (Wilson et al., 1995). One of these, p45Ntf4, has been shown to be activated by hydration of dry pollen grains (Wilson et al., 1997). In the present study we have used a peptide antibody raised against one of the other MAP kinases, p43Ntf6, to investigate its role during the cell cycle in synchronized tobacco cultures, and show that it is activated in mitosis and possibly has a function in cytokinesis.

MATERIALS AND METHODS

Plant tissue culture, synchronization, and measurement of cell cycle parameters

Tobacco cells (cell line BY-2) were maintained in modified Linsmaier & Skoog medium according to the method of Nagata et al. (1992). Cells were synchronized by diluting a 7-day-old culture 1:5, and after incubation for 6 hours. 10 µg/ml aphidicolin was added to the medium. The cells were incubated for another 18 hours, and the drug was then removed by five washes with medium, before resuspension in the original volume. Flow cytometric analysis and measurement of the mitotic index were as described previously (Bögè et al., 1997b). Treatment of cells with roscovitine was performed by taking synchronized cultures 6 hours after the release from the aphidicolin block and adding 100 µM roscovitine. Washing out of the roscovitine was as described for aphidicolin.

Treatment of suspension cultures with microtubule drugs

The microtubule disrupting drugs APM (amiprophos-methyl) and propyzamide were added at the concentrations and for the times described in the text and figure legends. Taxol was added at a concentration of 50 µM. For the propyzamide blocking and release experiment, cells were synchronized with aphidicolin as described above, and 7 hours after the drug release propyzamide (10 µM) was added for a further 7 hours. Washing out of the propyzamide was as described for aphidicolin.

Antibodies and recombinant proteins

The anti-p43Ntf6 MAP kinase antibody (AbP6) was raised in rabbits against a synthetic peptide corresponding to the last 12 amino acids of the p43Ntf6 protein (Wilson et al., 1995). The AbP6 polyclonal serum was purified on Protein A-Sepharose (Pharmacia). The PSTAIRE antibody was raised against a synthetic peptide representing the conserved region of the cdc2 protein, and has been described previously (Bögè et al., 1997b). The secondary antibody used in immunoblotting was horseradish peroxidase-conjugated anti-rabbit IgG (Promega, Madison, WI, USA) used at a dilution of 1:9,000. As required, the AbP6 antibody was blocked by incubation with 100 µg peptide for 2 hours at 4°C, before immunoblotting or immunoprecipitation.

For immunofluorescence studies, the anti-tubulin antibody used was monoclonal anti-α-tubulin (mouse IgG1 isotype) (Sigma BioSciences, St Louis, MO, USA). Secondary antibodies were anti-mouse IgG FITC-conjugate for the anti-tubulin antibody, and anti-rabbit IgG CY3-conjugate for AbP6, both from Sigma BioSciences.

The tobacco MAP kinase proteins p43Ntf5, p45Ntf4 and p43Ntf6 used in this study have been described previously (Wilson et al., 1995). Approximately 40 ng of each protein was used in immunoblots.

Preparation of cell extracts

Frozen cells were ground in extraction buffer (25 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 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 Na3VO4, 1 mM NaF, 1 mM PMSF, 10 µg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, 5 µg/ml antipain, chymostatin, and pepstatin), and then centrifuged for 40 minutes at 20,000 g. The supernatants were taken and the protein concentration was determined by the Bio-Rad protein assay system.

Immunoblotting

Proteins were run on 12% (unless otherwise stated) SDS-polyacrylamide gels and blotted to polyvinylfluoride membranes (Immobilon-P, Millipore). The membrane was blocked in PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM disodium hydrogen phosphate, 1.4 mM potassium dihydrogen phosphate, and 0.05% Tween-20, pH 7.2) containing 3% non-fat dried milk overnight and then incubated in the same solution containing the primary antibody for 2 hours. The membranes were then washed in five changes of PBS-T, 10 minutes each wash, followed by incubation for 1 hour in the secondary antibody diluted in PBS-T containing 3% non-fat dried milk. After an additional five washes in PBS-T, the immunoreactivity was visualized using the SuperSignal Substrate western blotting detection system from Pierce (Rockford, IL, USA).

Measurements of protein kinase activities

p13Suc1 beads were prepared as described previously (Bögè et al., 1997b). For immunokinase assays to measure MAP kinase activity, the anti-p43Ntf6 antibody, AbP6, was bound to Protein A-Sepharose beads (Pharmacia) by incubation of the crude antibody with the beads for a further 7 hours. Washing out of the propyzamide was as described previously (Bögè et al., 1997b). The anti-p43Ntf6 MAP kinase antibody (AbP6) was raised in rabbits against a synthetic peptide corresponding to the last 12 amino acids of the p43Ntf6 protein (Wilson et al., 1995). The AbP6 polyclonal serum was purified on Protein A-Sepharose (Pharmacia). The PSTAIRE antibody was raised against a synthetic peptide representing the conserved region of the cdc2 protein, and has been described previously (Bögè et al., 1997b). The secondary antibody used in immunoblotting was horseradish peroxidase-conjugated anti-rabbit IgG (Promega, Madison, WI, USA) used at a dilution of 1:9,000. As required, the AbP6 antibody was blocked by incubation with 100 µg peptide for 2 hours at 4°C, before immunoblotting or immunoprecipitation.

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Activation of a MAP kinase during mitosis

Tobacco cell suspension cultures were synchronized by incubation of the cells with aphidicolin for 18 hours, which blocks cells in the G1/S transition. After washing out the drug, and incubation in drug-free medium, cells were taken at different time points and the cell cycle stages were analysed by flow cytometry. The measurement made immediately after washing out the drug showed that the cells were blocked at the G1/S boundary (Fig. 2A, 0 h). After 3 hours in drug-free medium the cells passed through S phase, and by 8 hours a single peak corresponding to G2 cells was seen. From 10 to 12 hours, the cells passed through mitosis. To determine the number of cells passing through mitosis, the mitotic index was measured at the same time points. The highest percentage of metaphase/anaphase/telophase cells were seen. From 10 to 12 hours, the cells passed through mitosis. The activity peaked at 12 hours and then declined to background levels at approximately 18 hours (Fig. 2C). Thus, the activation, and peak of activity, of the MAP kinase corresponded to the time when the cells were passing through mitosis.

Immunoblotting was performed using the protein extracts from the aphidicolin synchronized cells and the antibody AbP6. A single band with the expected molecular mass of 43 kDa was detected in all samples (Fig. 2D). No cell cycle-related changes occurred in the protein levels over the time course of the experiment, suggesting that the protein is expressed constitutively and that the changes seen in the kinase activity are due to post-translational events. The identification of a single band in immunoblots further demonstrates the specificity of the antibody.

For comparison, the same samples were incubated with p13suc1-Sepharose beads and the bound histone H1 kinase activity was measured. The p13suc1 protein is a yeast protein that is associated in a complex with the cyclin dependent kinase (CDK) cdc2 in Schizosaccharomyces pombe (Brizuela et al., 1987). The p13suc1-Sepharose beads bind several members of the plant CDK family, and can be used to measure CDK-associated kinase activity, with a major peak of activity during G2 and mitosis (Bögå et al., 1997b). Histone H1 kinase activity were observed at all stages from the synchronized tobacco cell extracts used in this study, since p13suc1 binds the different members of the CDK family which are expressed at different stages of the cell cycle (Magyar et al., 1997). However, a major peak of activity was observed at 10 hours, when the cells were passing through mitosis (Fig. 2C). The amount of material from each sample bound by the p13suc1 beads was controlled by western analysis of the complexes using a PSTAIRE
antibody which recognises CDKs (Bögre et al., 1997b). Approximately an equal amount of PSTAIRE-reactive protein was detectable in each sample (Fig. 2C).

To test whether entry into M-phase might be necessary for MAP kinase activation, synchronized cells were taken 6 hours after the release from the aphidicolin block, when they had accumulated in G2 (Fig. 2A), and incubated in the presence of the drug roscovitine for a further 13 hours. Roscovitine blocks cells in the G2 phase of the cell cycle through inhibition of CDKs (Meijer et al., 1997; Planchais et al., 1997). This was confirmed by flow cytometry and measuring the mitotic index of roscovitine-treated cells (Fig. 3A,B). Measurement of MAP kinase activity in protein extracts from cells taken during this time showed that no activation occurred over the entire 13 hours in roscovitine (Fig. 3C). Even though roscovitine acts as an inhibitor of CDK complexes, some p13suc1-associated kinase activity was detected in extracts from roscovitine-treated cells (Fig. 3C). This is most probably because the inhibitory action of roscovitine can be reversed after protein extraction and bead affinity purification (Planchais et al., 1997). Following 8 hours incubation in roscovitine, cell aliquots were washed and incubated in drug-free medium. Analysis of the cell cycle progression after the washout, by flow cytometry and measurement of the mitotic index, showed that the cells entered M-phase (Fig. 3A,B). Measurements of kinase activities showed that the entry into M phase was accompanied by an increase in MAP kinase activity and p13suc1-associated kinase activity (Fig. 3C), which was not seen in cells incubated in roscovitine for the same time. It cannot be excluded that the concentration of roscovitine used (100 μM) had an effect on protein kinases other than CDKs (Meijer et al., 1997). However, in tobacco BY-2 suspension cultures, 50 μM roscovitine was shown to be necessary to cause an efficient G2 arrest (Planchais et al., 1997), and roscovitine is a selective inhibitor of CDKs, requiring approximately 100 times higher concentrations to inhibit plant MAP kinases than plant CDKs, and is not very stable within plant cells (P. Binarova et al., unpublished). Therefore, it seems probable that the absence of MAP kinase activity in roscovitine treated cells resulted from a G2 arrest caused by inhibition of CDKs, and entry into mitosis is necessary for the activation of the MAP kinase.

MAP kinase activity is not present in metaphase arrested cells

Mitosis and cytokinesis in plant cells are marked by two microtubule arrays, the mitotic spindle which is involved in chromosome alignment and chromosome separation, and the phragmoplast which is involved in deposition of the cell plate (Samuels et al., 1995). We tested whether there might be some link between the mitotic MAP kinase activity which we detected in synchronized cultures and the organization of the microtubules. Cells which contained activated MAP kinase (12 hours after the release from the aphidicolin block) were treated with various concentrations of the microtubule destabilising drugs APM (amiprophos-methyl) and propyzamide for 2 hours. No MAP kinase activity was detected in the cells at higher drug concentrations (Fig. 4A). APM was more effective in the elimination of the kinase activity. A significant decrease in kinase activity occurred at 0.01 μM APM, and was completely absent at 0.1 μM (Fig. 4A). Instead, 1 μM propyzamide was required to eliminate the kinase activity (Fig. 4A). The mitotic index of each cell culture was determined, and showed that both microtubule destabilizing drugs led to
metaphase arrest of the cells (Fig. 4B). (Depolymerization of the microtubules results in condensed chromosomes which are not aligned on the cell equator because the mitotic spindle is not yet reformed (Samuels et al., 1998). Such prometaphase cells are herein referred to as metaphase arrested cells). Again, APM was more effective at arresting the cells than propyzamide. The decrease in kinase activity broadly mirrored the degree of metaphase arrest. Kinase assays after binding of the same samples to p13\textsuperscript{3uc1}-Sepharose beads showed that the histone HI activity increased in the drug-treated cells (Fig. 4A), as expected for cells blocked in metaphase. Either intact microtubules are required for MAP kinase activity, or the kinase is activated after metaphase when the cells pass to anaphase.

**Intact microtubules are not sufficient for MAP kinase activity**

To try and understand further the relationship between microtubules and MAP kinase activity, cells were taken 12 hours after the release from the aphidicolin block (when mitosis is occurring and the kinase is activated), treated with APM and propyzamide for shorter time intervals, and protein extracts were assayed for p43\textsuperscript{Ntf6} kinase activity. Within as little as 10 minutes, APM led to a significant reduction in MAP kinase activity, while both drugs eliminated MAP kinase activity from these cells by 30 minutes (Fig. 5A, left panels). Cells were also treated with the microtubule stabilizing drug taxol, and extracts were tested for kinase activity. The taxol-treated cells also showed a reduction in kinase activity, similar to that seen with the microtubule destabilizing drugs (Fig. 5A, left panels). The amount of p43\textsuperscript{Ntf6} protein bound by the antibody was controlled by immunoblot analysis of the immunoprecipitates of extracts from the taxol treated cells. A band of approximately equal intensity was observed in all samples, running below the IgG (Fig. 5B, left panel). The recombinant p43\textsuperscript{Ntf6} protein was also immunoprecipitated and run in parallel with these samples to verify the position of the p43\textsuperscript{Ntf6} protein (data not shown).

Assaying p13\textsuperscript{3uc1}-associated histone HI kinase activity showed a decrease in activity in the control samples, as cells progressed from G\textsubscript{2} into M phase (Fig. 5A, right panels). In the APM and propyzamide treated cells, there was an increase in histone HI kinase activity, as noted above due to the metaphase arrest. In taxol treated cells, an increase in histone HI kinase activity was observed, which could also be due to metaphase arrest of these cells (Fig. 5A, right panels). The amount of material bound by the p13\textsuperscript{3uc1} beads from extracts of taxol treated cells was controlled by western analysis of the complexes using a PSTAIRE antibody, and showed that the increase in activity was not due to differences in the amounts of CDKs complexed by the p13\textsuperscript{3uc1} beads (Fig. 5B, right panel). Mammalian cells arrested with taxol maintain high levels of p34\textsuperscript{cdc2} activity (Andreasen and Margolis, 1994), while treatment of cells with taxol can cause them to arrest in mitosis (Jordan et al., 1993).

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**Fig. 3.** Entry into mitosis is required for MAP kinase activation. Cells were taken 6 hours after the release from the aphidicolin block and incubated with roscovitine, and then sampled at the time points indicated. Roscovitine blocks cells in G\textsubscript{2}, as shown by flow cytometry (A, roscovitine) and measurement of the mitotic index (B, roscovitine), and prevents activation of the MAP kinase, as measured by kinase assays of the same samples (C, roscovitine) for MAP kinase activity (MBP) and p13\textsuperscript{3uc1}-associated kinase activity (HI). Cell aliquots were taken 8 hours after the addition of roscovitine, the roscovitine was washed out, and the cells were incubated in drug-free medium for a further 7 hours. Removal of the roscovitine releases cells from the G\textsubscript{2} block, resulting in entry into M phase (A, B, roscovitine wash out), and leads to activation of the MAP kinase (MBP) and p13\textsuperscript{3uc1}-associated kinase (HI) (C, roscovitine wash out).
Cells treated with APM, propyzamide and taxol were inspected microscopically by DAPI and anti-tubulin staining to investigate the state of the cells after the respective drug treatments. APM caused a rapid depolymerization of microtubules in as little as 10 minutes, and complete elimination of microtubules by 60 minutes (Fig. 5C, middle panels). Depolymerization of the microtubules by propyzamide occurred in approximately 30 minutes, and, as with APM, no tubulin staining was observed at 60 minutes (Fig. 5B, right panels). In taxol treated cells the microtubules were maintained, although there was some disordering of the chromatin.
MAP kinase in plant cytokinesis

spindles compared to the untreated control cells (Fig. 5C, left panels). After 60 minutes in taxol, all mitotic cells had the appearance shown in Fig. 5C, which appear to be blocked in metaphase, similarly to the APM and propyzamide treated cells. The loss of MAP kinase activity at earlier time points may be due to the exit of cells from the phase in which the kinase is active, while at the same time the entry of other cells into this phase is blocked by the drug. Since the loss of MAP kinase activity was observed within 10 minutes after APM-induced metaphase arrest, the active state of p43Ntf6 appears to be very transient. These data suggest that normal microtubule dynamics and/or progression into anaphase is necessary for MAP kinase activation.

MAP kinase activation occurs in late anaphase/early telophase

A more direct determination of the timing of MAP kinase activation was performed by taking cells at frequent time intervals after the release from a metaphase arrest and measuring cell cycle progression, spindle and phragmoplast formation, and kinase activities. Cells were synchronized by incubation with aphidicolin, as described above. After washing out the aphidicolin, the cells were incubated in drug-free medium for 7 hours, at which stage they are passing through the G2 stage. The cells were then incubated in the presence of propyzamide for 7 hours, which, as shown above, causes metaphase arrest. The propyzamide was washed out and the cells were cultured in drug-free medium and sampled at half hourly intervals.

To follow the cell cycle progression after the drug release, the number of metaphase and anaphase/telophase cells were counted at each time point. As shown in Fig. 6A, propyzamide induced a metaphase arrest. By 2 hours after the propyzamide release, the majority of metaphase cells had progressed to anaphase, and by 3 hours most of these latter cells were in late anaphase/early telophase (Fig. 6A). Staining with an anti-tubulin antibody showed that the microtubules were indeed depolymerized by the propyzamide treatment, but that mitotic microtubule arrays were fully recoverable after washing out the drug (Fig. 6B). This anti-tubulin staining also revealed a correlation between the number of anaphase/telophase cells

![Fig. 6.](image)

**Fig. 6.** Activation of the MAP kinase occurs in late anaphase/early telophase. (A) The number of metaphase cells and anaphase/telophase cells after release from a propyzamide-induced metaphase arrest was determined after staining with DAPI. (B) Cells were immunostained with an anti-tubulin antibody and the number of mitotic spindles and phragmoplasts were counted. (C) Kinase assays of cell extracts using p13\textsuperscript{3uc1} beads (HI) or the AbP6 antibody (MBP) show that the MAP kinase is activated after the inactivation of p13\textsuperscript{3uc1}-associated kinase, at a time when cells are in late anaphase/early telophase.

![Fig. 7.](image)

**Fig. 7.** Immunolocalization of p43\textsuperscript{Ntf6} by indirect immunofluorescence. (A) Immunostaining with AbP6. A distinct band is seen to traverse the width of the cell. This band is located in between the separating chromosomes in anaphase, as evidenced by DAPI staining (B) of the same cell. Immunostaining with the AbP6 antibody (C) and an anti-tubulin antibody (D) shows labelling by the AbP6 antibody of the cell plate in between the two microtubular arrays of the phragmoplast. (E) DAPI staining of the same cell. Bar, 10 \(\mu\)m.
and the formation of the phragmoplast (Fig. 6B), which forms from late anaphase to late telophase.

Cell extracts were measured for CDK-associated activity using p13suc1 beads and MAP kinase activity by immunokinase assays with the AbP6 antibody. As shown in Fig. 6C, the p13suc1-associated activity is high in the metaphase arrested cells, but then declines as cells pass through anaphase. CDK activity declines as mitotic cyclins are degraded at the metaphase to anaphase transition (Glotzer et al., 1991). Some histone HI kinase activity was observed at later time points, which may be due to the multiple CDKs in plants which can bind p13suc1, and some of which are activated in mitosis (Magyar et al., 1997). By contrast, the AbP6-precipitated kinase activity shows an increase 2.5 hours after the release from the propyzamide-induced metaphase arrest, when CDK activity had already decreased. Therefore, the MAP kinase appears to have a function later in the cell cycle than the mitotic cyclin dependent kinase. From the analysis of the cell cycle progression discussed above, this corresponds to late anaphase/early telophase.

**Intracellular localization of p43Ntf6**

Indirect immunofluorescence studies were undertaken to try and determine the cellular localization of the MAP kinase at different stages of the cell cycle. Cells were doubly labelled with the AbP6 antibody and DAPI. With AbP6, a generally diffuse staining pattern was observed in cells at different stages of the cell cycle, while the chromatin was not stained (data not shown). A distinct labelling pattern was found only in anaphase cells, where, as well as the diffuse staining, a band traversing the width of the cell was observed (Fig. 7A). This band appeared to be located in the middle of the separating chromosomes (Fig. 7B). This AbP6 labelling pattern was not observed in all anaphase cells, and may represent a transient accumulation of the protein in this location. Triple labelling with the AbP6 antibody (Fig. 7C), an anti-tubulin antibody (Fig. 7D) and DAPI (Fig. 7E) showed that the band is localized in the middle of the two tubulin arrays that are characteristic of the phragmoplast in anaphase/telophase.

**DISCUSSION**

**Activation of the tobacco p43Ntf6 MAP kinase in mitosis**

The involvement of MAP kinases in meiosis is well documented. MAP kinase activity is required for the progression of G2-arrested *Xenopus* oocytes into the first and second meiosis, and for the arrest of mature oocytes in metaphase II, possibly through the activation and stabilization of M-phase promoting factor, a complex of Cdc2 kinase and metaphase II, possibly through the activation and stabilization of second meiosis, and for the arrest of mature oocytes in metaphase II. The involvement of MAP kinases in meiosis is well documented. MAP kinase activity is required for the progression of G2-arrested *Xenopus* oocytes into the first and second meiosis, and for the arrest of mature oocytes in metaphase II, possibly through the activation and stabilization of M-phase promoting factor, a complex of Cdc2 kinase and cyclin B (Sagata, 1997). Release of clam oocytes from a G2/M cell cycle arrest by fertilization is accompanied by MAP kinase activation, but this MAP kinase is not active in succeeding mitotic cell cycles (Shibuya et al., 1992). In fact, a role for MAP kinases in mitosis is still unclear (Shibuya et al., 1992). MAP kinases (Tamemoto et al., 1992) and a MAP kinase-like molecule (Heider et al., 1994) have been reported to be activated during M phase in mammalian cells. Inactivation of MAP kinase is required for entry into M phase of the first mitotic cycle after fertilization of oocytes, but not in the subsequent rapid cell cycles (Abrieu et al., 1997), and has also been reported to be necessary for the G1/S-phase transition in starfish eggs (Tachibana et al., 1997). In *Xenopus* cell cycle extracts, which are thought to mimic mitosis, MAP kinase is required for the spindle assembly checkpoint, but not for normal M phase entry and exit (Minshull et al., 1994; Takenaka et al., 1997). The lack of kinase activity in metaphase arrested cells described in this study indicates that this MAP kinase is functionally distinct from the MAP kinase described in *Xenopus*, whose activity is required for metaphase arrest (Minshull et al., 1994; Takenaka et al., 1997). The absence of activity was not due solely to depolymerization of the metaphase microtubules, since a reduction in activity also occurred in cells treated with taxol, which stabilized the microtubules, but rather may depend on a transient cell cycle event after metaphase.

In the present study we provide clear evidence that a MAP kinase is activated during mitosis. Immunokinase assays of protein extracts from synchronized tobacco cells using a peptide antibody raised against the tobacco MAP kinase p43Ntf6 showed that the kinase is activated specifically as cells pass through mitosis. Further, immunokinase assays after blocking cells in the G2 phase of the cell cycle, and then releasing the block, allowing cells to enter into mitosis, indicated that entry into mitosis is necessary for the activation of the kinase. Immunoblotting and antibody blocking experiments showed that p43Ntf6 is responsible for the observed activity.

Four main groups of plant MAP kinases exist, and the so far identified functions of two of these groups suggest that they respond to different forms of stress. No functions have yet been ascribed to the other two groups, one of which includes p43Ntf6. The amino acid sequence of p43Ntf6 (Wilson et al., 1995) shows highest identity to a recently identified alfalfa MAP kinase which appears to perform a similar function (L. Bögre et al., unpublished). Together, these two MAP kinases may define a new functional group of plant MAP kinases.

**MAP kinase activation occurs in late anaphase/early telophase**

Phosphorylation/dephosphorylation events are important for a number of mitotic events. As in other organisms, plant cyclin-dependent kinases (CDKs) control the passage of cells through the G2/M transition (Doonan and Fobert, 1997). Multiple CDKs exist in plants, and are activated at different stages of the cell cycle (Bögre et al., 1997b). Immunoprecipitation of CDK activity using p13suc1-Sepharose beads has been shown to result in a major peak of activity during mitosis (Bögre et al., 1997b). The CDK activity decreases in the metaphase/anaphase transition due to the degradation of cyclins (Glotzer et al., 1991). In the synchronized tobacco cell suspension cultures used in this study, the p13suc1-associated histone HI kinase activity also showed a major peak of activity during mitosis. The kinetics of p43Ntf6 kinase activation showed a peak of activity overlapping histone HI kinase activity in cells released from a G1/S phase arrest. The timing of MAP kinase activation was further refined by following kinase activities in cells released from metaphase arrest due to propyzamide-induced microtubule depolymerization. The MAP kinase was activated later in the cell cycle than the p13suc1-associated CDK activity that, according to the analysis
A role for the p43Ntf6 MAP kinase in cytokinesis

In late anaphase/early telophase a plant-specific cytoskeletal structure, the phragmoplast, is found, that is required to build the cell wall between the daughter cells. An intimate relationship exists between microtubules and other molecules necessary for phragmoplast and cell plate formation. Motor proteins, such as kinesins (for review see Asada and Collings, 1997), and membranes (Schoepfer and Hepler, 1991; Samuels et al., 1995) are associated with the phragmoplast microtubular arrays, and the transport of vesicles along microtubules by motor proteins may be involved in the delivery of these vesicles to the nascent cell plate. Indirect immunofluorescence studies with the anti-p43Ntf6 antibody and double labelling with the anti-p43Ntf6 antibody and an anti-tubulin antibody identified a band in between the interdigitating phragmoplast arrays. Immunofluorescence studies have shown the co-localization of kinesin-like proteins (Liu et al., 1996; Asada et al., 1997; Bowser and Reddy, 1997) and MAPs (Chan et al., 1996) with the phragmoplast microtubules, while the cell plate in the middle of the two microtubular arrays was not labelled. By contrast, the anti-p43Ntf6 antibody showed labelling in the midzone between the microtubule arrays. Similar labelling patterns have been observed for the dynamin-like proteins phragmoplastin (Gu and Verma, 1996, 1997) and ADL1 (Lauber et al., 1997). Dynamins are a family of GTPases involved in endocytosis and Golgi membrane protein retention (Robinson et al., 1993; Wilsbach and Payne, 1993). The localization of plant dynamin to the cell plate is in accordance with a general role for dynamins in vesicular trafficking/fusion events, and phragmoplastin appears to be associated with small vesicles on the cell plate (Gu and Verma, 1997). Although mammalian dynamin can be phosphorylated by MAP kinases (Earnest et al., 1996), and phragmoplastin may be phosphorylated (Gu and Verma, 1997), the plant dynamins do not contain the consensus MAP kinase phosphorylation site(s). The KNOLLE protein in *Arabidopsis*, which is related to vesicle-docking syntaxins, also localizes to the cell plate in dividing cells, and appears to be necessary for vesicle fusion rather than vesicle transport (Lauber et al., 1997).

The detailed analysis of the timing of the activation of the tobacco MAP kinase p43Ntf6 presented in this study indicates that it is a late event in the cell cycle, with activation occurring later than the G2/M CDK, during late anaphase/early telophase. This together with the localization of the kinase to the cell plate suggests that p43Ntf6 may have a role in phragmoplast formation and synthesis of the new cell wall.

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REFERENCES


Machida, Y., Nishihama, R. and Kitakura, S.
Lukowitz, W., Mayer, U. and Jürgens, G.
Liu, B., Cyr, R. J. and Palevitz, B. A.
Sagata, N.
Nagata, T., Nemoto, Y. and Hasezawa, S.
Mitsui, H., Hasezawa, S., Nagata, T. and Takahashi, H.
Minshull, J., Sun, H., Tonks, N. K. and Murray, A. W.
Planchais, S., Glab, N., Tréhin, C., Perennes, C., Bureau, J.-M., Meijer, L.
Morishima-Kawashima, M. and Kosik, K. S.
Jordan, M. A., Toso, R. J., Thrower, D. and Wilson, L.
Kondorosi, É., Athanasiadis, A., Pongor, S., Bilgin, M., Bakó, L., Koncz, C. and Dudits, D.

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