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A Developmentally Regulated MAP Kinase Activated by Hydration in Tobacco Pollen

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A novel mitogen-activated protein (MAP) kinase signaling pathway has been identified in tobacco. This pathway is developmentally regulated during pollen maturation and is activated by hydration during pollen germination. Analysis of different stages of pollen development showed that transcriptional and translational induction of MAP kinase synthesis occurs at the mid-bicellular stage of pollen maturation. However, the MAP kinase is stored in an inactive form in the mature, dry pollen grain. Kinase activation is very rapid after hydration of the dry pollen, peaking at ~5 min and decreasing thereafter. Immunoprecipitation of the kinase activity by an anti-phosphotyrosine antibody is consistent with the activation of a MAP kinase. The kinetics of activation suggest that the MAP kinase plays a role in the activation of the pollen grain after hydration rather than in pollen tube growth.

INTRODUCTION

The development of the male gametophyte of angiosperms is a tightly controlled event that can be divided into two phases: microsporogenesis and microgametogenesis. Microsporogenesis includes the stages from the formation of the microspore mother cell until the first haploid pollen mitosis. Microgametogenesis involves the further maturation of these pollen to form bicellular or tricellular pollen grains. Approximately 25% of plant species produce tricellular pollen grains that contain a vegetative cell and two sperm cells. The other 75% of plant species, which includes tobacco, produce bicellular pollen grains in which the microspores undergo a single mitosis during pollen maturation, resulting in a vegetative cell and a generative cell. The latter cell divides again during pollen tube growth to produce the two sperm cells (Mascarenhas, 1989; McCormick, 1993). Mature pollen is highly dehydrated, with a reduction in water content from 90% to 40 to 60%, depending on the species (Bedinger et al., 1994). After anthesis, pollen landing on a receptive stigma is hydrated, which is essential for pollen germination (Dickinson, 1995), and subsequent pollen tube growth through the transmitting tissue of the style results in delivery of the two sperm cells to the ovary and double fertilization.

Despite the extensive overlap of sporophytic and gametophytic gene expression, 10% of the estimated 20,000 individual mRNAs present in the mature pollen grain at anthesis are considered to be pollen specific. Although some genes are expressed throughout pollen development, others are expressed exclusively before the first pollen mitosis (early genes) or only after mitosis (late genes) (Mascarenhas, 1993; Astwood and Hill, 1996). The majority of the mRNAs and some or all of the proteins required for germination and early pollen tube growth are present in the pollen grain at anthesis (Mascarenhas, 1993). Some of these genes should encode protein kinases, as shown by the de novo phosphorylation of proteins in Brassica and rye after pollination and germination (Wehling et al., 1994; Hiscock et al., 1995); however, relatively little is known about specific protein kinases and their activities during pollen development, germination, and tube growth. A calcium-dependent protein kinase activity, which phosphorylates self-incompatibility RNases, has been isolated from pollen tubes of *Nicotiana alata* (Kunz et al., 1996), and a pollen-specific calcium-dependent calmodulin-independent protein kinase, which is involved in germination, has been identified in maize (Estruch et al., 1994). A pollen-specific protein kinase gene, *PRK1*, which encodes a receptor-like kinase, is expressed as a late gene in petunia pollen and is essential for normal pollen development (Lee et al., 1996).

We previously reported on the isolation and characterization of mitogen-activated protein (MAP) kinases from tobacco (Wilson et al., 1995). In this study, we investigate their possible involvement in tobacco pollen development and germination. Despite the large number of MAP kinase cDNAs isolated from plants (Mizoguchi et al., 1993; Decroocq-Ferrant et al., 1995; Jonak et al., 1995; Wilson et al., 1995), relatively little is known about the activation and functions of these kinases compared with those from yeast and mammals (Hirt, 1997). Strong evidence has been obtained for the

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involvement of MAP kinases in response to touching and wounding (Bögre et al., 1996, 1997) or cold and drought (Jonak et al., 1996; Mizoguchi et al., 1996), whereas a salicylic acid–inducible MAP kinase has recently been reported in tobacco (Zhang and Klessig, 1997). A mammalian anti-ERK1 antibody precipitated a kinase activity from abscisic acid–stimulated barley aleurone protoplasts (Knetsch et al., 1996). MAP kinaselike activities have been shown to respond to a number of stimuli, such as wounding (Seo et al., 1995; Usami et al., 1995) and fungal elicitor (Suzuki and Shinshi, 1995). In this study, we describe the developmental regulation of a MAP kinase and a novel stimulus, hydration, leading to MAP kinase activation in plants.

RESULTS

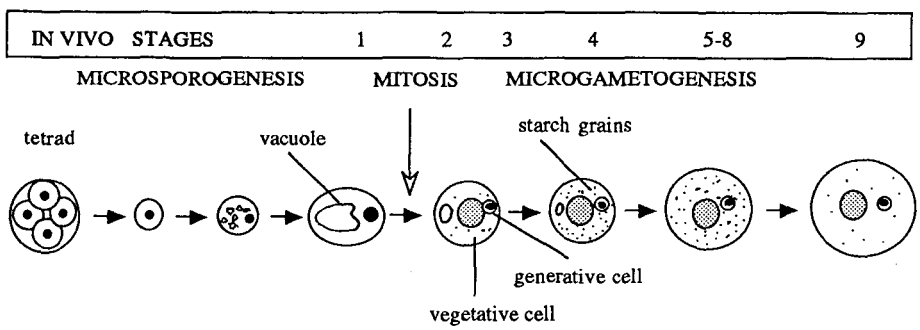
Stages of Pollen Development

A schematic representation of pollen development is shown in Figure 1A. Pollen was isolated from flower buds at different developmental stages (Figure 1B). The mature pollen at stage 9 was collected from anthers just before dehiscence when studying pollen development or during dehiscence for pollen germination. The sequence of events during pollen development shown in Figure 1A was confirmed by microscopical analysis and 4′,6-diamidino-2-phenylindole staining (data not shown) and has been described elsewhere (Touraev et al., 1995a, 1995b). When transferred to GK germination medium, the pollen grains swelled rapidly, and pollen tube emergence was observed after ~1 hr (data not shown).

Induction of MAP Kinase Synthesis during Pollen Development

The isolation and characterization of the tobacco MAP kinase genes *ntf3*, *ntf4*, and *ntf6* and their corresponding bacterially expressed proteins $p43^{Ntf3}$, $p45^{Ntf4}$, and $p43^{Ntf6}$ have been described previously (Wilson et al., 1995). To investigate a possible involvement of these MAP kinases in pollen development, RNA was isolated at different developmental stages from in vivo–matured pollen, and RNA gel blot analysis was performed. Using an *ntf4* probe, we found a signal from the mid-bicellular stage of development up to mature pollen (stages 4 to 9; see Figure 1), indicating that transcrip-

A



B

Stage	In vivo maturation	
1	microspores	(11 mm)
2	early bicellular	(13 mm)
3	early bicellular	(15-16 mm)
4	mid-bicellular	(18-20 mm)
5	late bicellular	(25-26 mm)
6	late bicellular	(28-30 mm)
7	late bicellular	(32-35 mm)
8	late bicellular	(36-38 mm)
9	mature	(40-42 mm)

Figure 1. Pollen Stages.

(A) Schematic representation of pollen development and the stages (1 to 9) used in this study.
(B) Bud length (in parentheses) and the corresponding developmental stage of pollen.

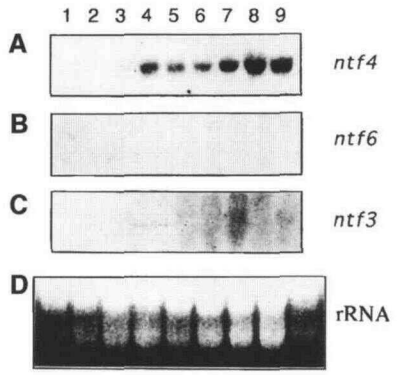


Figure 2. The *ntf4* Transcript Is Induced at the Mid-Bicellular Stage of Pollen Development.

(A) to **(C)** RNA gel blot analysis of total RNA from pollen hybridized with probes from *ntf4*, *ntf6*, and *ntf3*, respectively.
(D) Before blotting, the gel was stained with ethidium bromide to visualize the rRNA bands to demonstrate the amount of RNA loaded per lane.
Stages 1 to 9 are as described in Figures 1A and 1B.

tional induction occurs at the mid-bicellular stage of development (Figure 2A). No signals were found when probes from the two other tobacco MAP kinase genes, *ntf6* and *ntf3*, were used (Figures 2B and 2C, respectively). To analyze further the regulation of $p45^{Ntf4}$ during pollen development, we isolated protein from pollen at different developmental stages. Only two of the four late-bicellular pollen stages were used in this analysis because all of these cells are morphologically similar and showed the same *ntf4* transcript levels.

The protein extracts from the maturing pollen were analyzed by protein gel blot analysis, using the anti-$p45^{Ntf4}$ antibody AbC4 (see Methods). This antibody is specific for $p45^{Ntf4}$ and does not cross-react with the other two tobacco MAP kinases (Figure 3A). A band of ~45 kD was detected in extracts from the maturing pollen, beginning at the mid-bicellular stage of development and continuing up to maturity (Figure 3B, bottom), which is in agreement with the increase in transcript levels seen at the same stages. Immunokinase assays of the same extracts showed that AbC4 immunoprecipitated myelin basic protein phosphorylating activity at all stages from mid-bicellular to mature pollen (Figure 3B, top) but not at the earlier stages of pollen development. Although protein levels appeared to increase from mid-bicellular pollen to mature pollen, the level of activity appeared to be the same at all stages, possibly because of post-translational events. In protein gel blot analysis, antibody binding could be blocked by preincubation of the antibody with the $p45^{Ntf4}$ protein (Figure 3C), demonstrating the specificity of the binding. This analysis of pollen maturation clearly shows that MAP kinase synthesis is under developmental control, being induced at the mid-bicellular stage of pollen development.

MAP Kinase Activity during Pollen Germination

To study pollen germination, we isolated dry pollen from dehiscing anthers and ground one portion in liquid nitrogen as the 0 time point, whereas we suspended the rest of the pollen in germination medium and subsequently assayed it at different time points. Protein was extracted and tested by

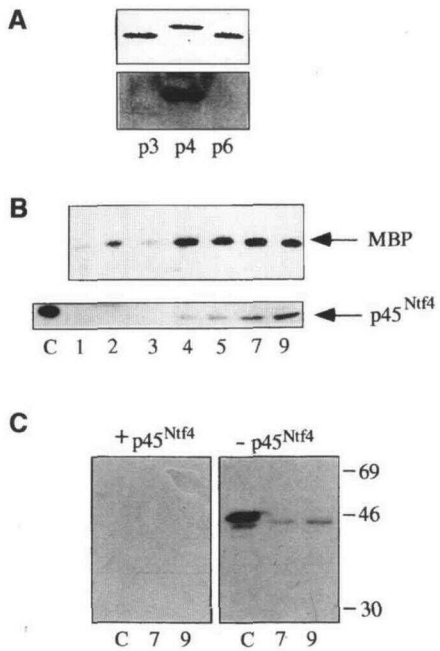


Figure 3. MAP Kinase Activity and Induction of $p45^{Ntf4}$ Protein Synthesis during Pollen Development.

(A) Specificity of the anti-$p45^{Ntf4}$ antibody AbC4. The tobacco MAP kinase recombinant proteins $p43^{Ntf3}$ (p3), $p45^{Ntf4}$ (p4), and $p43^{Ntf6}$ (p6) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R 250 (top) or blotted to membranes for protein gel blot analysis with AbC4 (bottom).
(B) MAP kinase activity in pollen at different developmental stages. Pollen protein extracts were immunoprecipitated with the anti-$p45^{Ntf4}$ antibody AbC4, and the immunoprecipitates were tested for kinase activity by using the myelin basic protein (MBP) as a substrate in the presence of γ-^{32}P-ATP (top). The immunokinase reactions were separated by SDS-PAGE and autoradiographed. The phosphorylation state of the myelin basic protein (arrow) at each stage tested is shown. Protein gel blot analysis of the same samples using the antibody AbC4 is shown at the bottom. Lane C contains the recombinant protein $p45^{Ntf4}$ as a marker. The arrow to the right indicates the 45-kD band representing the $p45^{Ntf4}$ MAP kinase. The numbers refer to the stages shown in Figure 1.
(C) Specificity of the AbC4 antibody using protein extracts from pollen from the last two developmental stages (7 and 9) used in **(B)**. The antibody was preincubated with (+$p45^{Ntf4}$) or without (−$p45^{Ntf4}$) the recombinant $p45^{Ntf4}$ protein before being used in protein gel blot analysis. Lane C contains the recombinant protein $p45^{Ntf4}$ as a marker. The numbers to the right indicate the molecular masses of the marker proteins in kilodaltons.

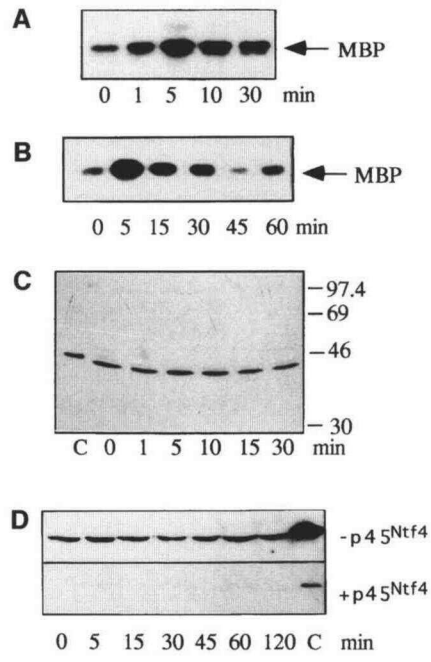


Figure 4. p45^{Ntf4} Kinase Activation during Pollen Germination.

(A) and **(B)** Mature pollen was germinated in vitro and collected at short **(A)** or long **(B)** time intervals (in minutes) after suspension in the germination medium. Proteins were extracted from the pollen and were tested for myelin basic protein (MBP) phosphorylating activity by using immunokinase assays, as described in Figure 3B. Shown is the phosphorylation state of myelin basic protein (arrows) at each time point tested.

(C) Protein gel blot analysis of proteins from germinating pollen at different time points (in minutes) after suspension in germination medium, using the antibody AbC4. Lane C contains the recombinant protein p45^{Ntf4}. The numbers to the right indicate the molecular masses of the marker proteins in kilodaltons.

(D) Specificity of the AbC4 antibody, using protein extracts from pollen after in vitro germination for the indicated time (in minutes). The AbC4 antibody was preincubated with (+p45^{Ntf4}) or without (−p45^{Ntf4}) the recombinant p45^{Ntf4} protein before being used in protein gel blot analysis. Lane C contains the recombinant protein p45^{Ntf4}.

protein gel blot analysis and immunokinase assays using the antibody AbC4. There was a low level of kinase activity in the dry pollen (time 0; Figures 4A and 4B). However, the kinase was activated rapidly after suspension of the pollen in germination medium. By using short time intervals, we could show the kinase to be activated almost immediately after suspension in the germination medium, there being an increase in myelin basic protein phosphorylation after as little as 1 min (Figure 4A). The kinase activity reached a peak at 5 min and decreased thereafter (Figure 4A). To determine the kinetics of inactivation of the kinase, longer time intervals were taken. Again, maximal activity occurred after 5 min, returning to basal levels after ~45 min (Figure 4B). Protein gel blot analysis showed that the steady state protein levels did not vary over the time course of the experiments (Figures 4C and 4D). In protein gel blot analysis of extracts from germinating pollen, antibody binding could be blocked by preincubation of the antibody with the p45^{Ntf4} protein (Figure 4D), demonstrating the specificity of the binding.

Hydration Leads to MAP Kinase Activation

The measurements of the kinase activity during pollen development indicated that the kinase is active from the mid-bicellular stage to maturity (Figure 3B). By contrast, the kinase appeared to have low activity when dry pollen was analyzed in the in vitro germination experiments (Figures 4A and 4B). Yet, the pollen from the last stage of pollen maturation and the dry pollen used for germination are functionally similar mature pollen grains.

To gain better insight into the relative levels of p45^{Ntf4} activity during maturation and germination, we compared immunokinase assays from the last stages of pollen maturation directly with the early stages of pollen germination. As shown in Figure 5A, the apparent kinase activity level in the mature pollen isolated from the anthers (stage 9; see Figure 1 and 3B) is much higher than that in the dry pollen used for pollen germination (time 0). An explanation for the difference in kinase activity in these two types of mature pollen might

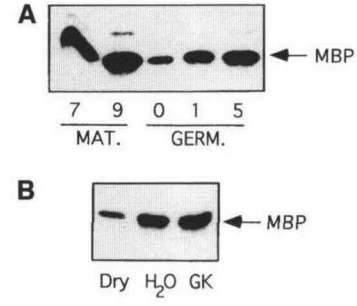


Figure 5. Hydration Leads to MAP Kinase Activation.

(A) Comparison of MAP kinase activity, measured as the phosphorylation of myelin basic protein (MBP; arrow), during the last stages of pollen maturation (MAT.) and the early stages of pollen germination (GERM.). Immunokinase assays were perfomed as described in Figure 3B. The numbers 7 and 9 refer to the last two stages of pollen maturation shown in Figure 3B; 0, 1, and 5 refer to the first three time points (in minutes) shown in Figure 4A. The pollen from stage 9 and the dry pollen at time point 0 are functionally and morphologically similar, yet they show different levels of kinase activity. This is most probably due to hydration of the pollen during isolation from the anthers (see text).

(B) Water alone is sufficient to activate the MAP kinase. Protein from dry pollen (Dry) or from pollen incubated for 5 min in water (H_2O) or germination medium (GK) was tested in immunokinase assays, as described in Figure 3B, with myelin basic protein (MBP; arrow) as a substrate.

be that the pollen became rehydrated during the procedure of isolating pollen from anthers. The anthers had to be squeezed in buffer, and the pollen grains were subsequently isolated. These pollen grains would then become hydrated, whereas the mature pollen used in the germination experiment at time 0 would remain dry. This implied that a cause of the high level of activity in the stage 9 mature pollen could have been due to hydration during pollen isolation from the anther. To test this empirically, dry pollen was suspended in water or in germination medium, and the level of activity was compared with that of dry pollen. As shown in Figure 5B, water alone was sufficient to activate the kinase, confirming that hydration is the signal that leads to MAP kinase activation.

Phosphorylation on Tyrosine Correlates with p45^{Ntf4} Activation

Because MAP kinases are activated by dual phosphorylation on threonine and tyrosine residues (Payne et al., 1991; Gartner et al., 1992), we tested whether such a situation might hold for the kinase activity found in germinating pollen. Extracts from dry pollen and from pollen incubated in germination medium for 5 min were immunoprecipitated with the anti-p45^{Ntf4} antibody or with an antiphosphotyrosine antibody, and the immunoprecipitates were tested for myelin basic protein phosphorylating activity. An increase in activity was seen with both antibodies after 5 min in the germination medium (Figure 6A). Preincubation of the antiphosphotyrosine antibody with phosphotyrosine reduced the activity in the immunoprecipitate, whereas preincubation with phosphothreonine or phosphoserine had no effect, demonstrating the specificity of the antibody (Figure 6B). Correlation of an immunoprecipitatable myelin basic protein phosphorylating activity with both the antiphosphotyrosine antibody and AbC4 is consistent with the activation of a MAP kinase during pollen germination.

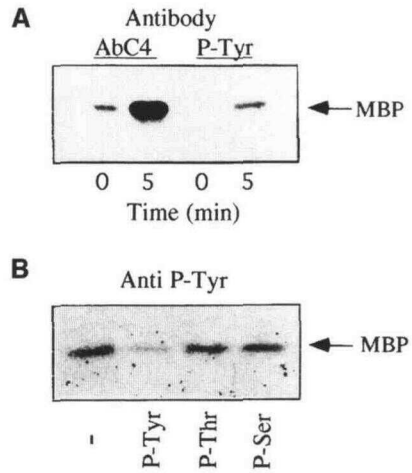


Figure 6. Phosphorylation on Tyrosine Correlates with MAP Kinase Activation.

(A) MAP kinase activity, measured as the degree of phosphorylation of myelin basic protein (MBP; arrow), in protein extracts from dry pollen (0) or after 5 min of incubation in germination medium (5). Immunokinase assays were performed as described in Figure 3B. Immunoprecipitation of the pollen protein extracts was with the anti-p45^{Ntf4} antibody (AbC4) or an antiphosphotyrosine antibody (P-Tyr) .
(B) The antiphosphotyrosine (Anti P-Tyr) antibody was preincubated with phosphotyrosine (P-Tyr), phosphothreonine (P-Thr), or phosphoserine (P-Ser) or without any addition (−) before immunoprecipitation and kinase assays, with myelin basic protein (MBP) as a substrate (arrow), using an extract from pollen that had been incubated for 5 min in germination medium.

DISCUSSION

We provide evidence for a novel signal transduction pathway in plants that involves regulation of MAP kinase synthesis during pollen development and activation of the MAP kinase during pollen germination. To date, activation of MAP kinase pathways in plants has involved specific treatments, such as wounding, the application of an elicitor, or salt, cold, or drought (Hirt, 1997). In these cases, transcriptional activation of the MAP kinase gene depended on the application of an external stimulus. MAP kinase synthesis in pollen occurs at the mid-bicellular stage of maturation, as shown by an increase in RNA synthesis that is accompanied by an increase in protein levels. Therefore, the system described here appears to be part of a developmental program involved in plant reproduction. The expression pattern of the MAP kinase is typical of a late-expressed gene in pollen. To our knowledge, only one other instance of MAP kinase synthesis being subject to developmental regulation has been described to date, namely, that of SMK1, which is required for spore wall assembly in yeast (Krisak et al., 1994).

Analysis of myelin basic protein phosphorylating activity by immunokinase assays during pollen development indicated that the kinase was active in all stages of pollen development, from mid-bicellular to maturity. However, the kinase is clearly in an inactive state in mature dry pollen, as shown in the pollen germination experiments. Therefore, the active state of the kinase, which was seen in mature pollen grains when we studied pollen development, was due to hydration while isolating pollen from the anthers. It is difficult to determine the level of kinase activation at developmental stages before dehiscence because sufficient pollen required for biochemical analysis cannot be isolated from the anthers without hydration. When pollen was isolated in nonaqueous fluid, the water released from the anther was enough to hydrate the pollen (C. Wilson, unpublished observations).

Two possibilities remain. Either the kinase is inactive throughout pollen maturation, or it is active at the mid-bicellular

stage and is subsequently inactivated before full maturity. We favor the first of these two possibilities for the following reasons. The steady state protein levels of $p45^{Ntf4}$ are more or less constant or may even increase during maturation from the mid-bicellular stage to mature pollen. If the kinase is activated at the mid-bicellular stage of development, one would predict the involvement of a phosphatase in the inactivation of the kinase before full maturation of the pollen. Yet, activity levels are similar at all stages of maturation from mid-bicellular to mature pollen. In addition, it seems contradictory that a kinase that is activated by hydration during germination should be activated by drying during pollen development. Indeed, pollen seems to begin to dry out at the mid-bicellular stage of development, as shown, for example, by the analysis of a promoter that responds to dryness from the resurrection plant *Craterostigma plantagineum* (Michel et al., 1993). Fused to a β-glucuronidase reporter gene in transgenic tobacco plants, this promoter became active at the mid-bicellular stage of pollen maturation.

The signal for the activation of the MAP kinase is clearly hydration, because it could be activated by water alone. Because hydration of the pollen grains after landing on the stigma is essential for pollen germination (Dickinson, 1995), it is likely that the activation of the MAP kinase, which we observed in in vitro germination experiments, also occurs in vivo when pollen grains land on the stigma and thus represents a physiologically relevant phenomenon. The rapid and transient activation of the MAP kinase occurs before pollen tube emergence, making it likely that it has a role in early events of pollen activation. An immediate consequence of hydration is swelling of the pollen grain, and MAP kinases have been shown to be activated in mammals by cell swelling in hypotonic media (Tilly et al., 1993; Schliess et al., 1995; Sadoshima et al., 1996) and in reponse to hypotonicity in yeast (Davenport et al., 1995). One of the early events that follows pollen hydration is a rapid reorganization of the cytoskeleton (Tiwari and Polito, 1988; Heslop-Harrison and Heslop-Harrison, 1992). MAP kinases are also involved in cytoskeletal dynamics associating with the microtubule cytoskeleton and phosphorylating microtubule-associated proteins (Reszka et al., 1995; Morishima-Kawashima and Kosik, 1996). However, cytoskeletal reorganization after pollen hydration largely involves the actomyosin system; the role of microtubules during the germination process remains unclear (Mascarenhas, 1993; Tirlapur et al., 1995; Cai et al., 1996).

The MAP kinase identified here as being involved in pollen maturation and germination would appear to be $p45^{Ntf4}$ because no transcripts corresponding to the other tobacco MAP kinases were detected, the antibody did not cross-react with $p43^{Ntf3}$ or $p43^{Ntf6}$, and the antibody could be blocked by preincubation with the $p45^{Ntf4}$ protein. The $p45^{Ntf4}$ amino acid sequence has >90% identity with the pea MAP kinase PsMAPK. This high level of sequence conservation between species might suggest that a functional conservation also exists. The pea MAP kinase PsMAPK was shown to partially complement a *Saccharomyces cerevisiae hog1* deletion mutant (Pöpping et al., 1996). The HOG1 MAP kinase signaling pathway is involved in osmoregulation under hyperosmotic stress. However, the stimulus leading to PsMAPK activation in plant cells has not yet been identified.

METHODS

Pollen Isolation and Culture Techniques

Pollen from *Nicotiana tabacum* anthers was isolated at different developmental stages essentially as described by Benito Moreno et al. (1988) and Touraev et al. (1995b). Briefly, the anthers were squeezed gently into medium B (300 mM mannitol, 20 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 1 mM potassium phosphate, pH 6.8) by using a mortar and pestle, and the pollen was collected by centrifugation. The pollen pellets were washed, frozen in liquid nitrogen, and stored at −80°C until further processing. For germination of matured pollen, dry pollen grains were suspended in GK medium (3.2 mM H_3BO_4, 0.8 mM $MgSO_4$, 1 mM KNO_3, 1.3 mM $Ca[NO_3]_2$, and 0.29 M sucrose) for the required time and centrifuged, and the pollen pellet was frozen in liquid nitrogen until processing. Dry pollen was also frozen immediately in liquid nitrogen, and protein was extracted as described below.

Isolation of Total Proteins from Plant Tissues

Frozen pollen was ground in liquid nitrogen, and the resulting powder was suspended in protein extraction buffer (30 mM Hepes, pH 7.4, 150 mM NaCl, and 3 mM $MgCl_2$) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.7 μg/mL pepstatin A, and 1 μg/mL antipain) and phosphatase inhibitors (15 mM β-glycerophosphate and 5 mM sodium fluoride). It was then centrifuged for 10 min at 10,000 rpm at 4°C, and the supernatant was centrifuged again at 80,000 rpm for 1 hr at 4°C. Protein determination was performed using the BioRad protein assay system.

Antibodies

The anti-$p45^{Ntf4}$ MAP kinase antibody (AbC4) was raised in rabbits against a protein that was a fusion between glutathione *S*-transferase and the C-terminal 68 amino acids of the $p45^{Ntf4}$ protein that was expressed in bacteria and purified by affinity purification on glutathione–Sepharose 4B (Pharmacia). The AbC4 polyclonal serum was purified on protein A–Sepharose (Pharmacia). The antiphosphotyrosine antibody was 4G10 (1 μg/μL) from Upstate Biotechnology, Inc. (Lake Placid, NY). The secondary antibody was horseradish peroxidase–conjugated anti–rabbit IgG (Amersham) used at a dilution of 1:9000. The tobacco MAP kinase proteins $p43^{Ntf3}$, $p45^{Ntf4}$, and $p43^{Ntf6}$ used in this study have been described previously (Wilson et al., 1995).

Immunokinase Assays

Immunoprecipitation was performed by adding 10 μL of antibody AbC4 or 5 μL of antibody 4G10 to 30 μg of total proteins from each sample in immunoprecipitation buffer (10 mM Tris, pH 7.4, 150 mM

NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 0.5% Nonidet P-40, final concentrations), and the resulting mixture was left on ice for 2 hr. After binding for 1 hr to 30 μL of a 50% slurry of protein A–Sepharose (Pharmacia), the immunoprecipitates were washed three times with immunoprecipitation buffer and once in kinase buffer (30 mM Hepes, pH 7.4, 50 mM KCl, and 4 mM $MgCl_2$). Kinase reactions were performed in 10 μL of kinase buffer containing 20 μM cold ATP, 2 μCi γ-^{32}P-ATP (3000 Ci/mmol), and 2 μg of myelin basic protein as substrate at 30°C for 15 min and then stopped by the addition of Laemmli sample buffer (60 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol, 0.0001% bromophenol blue). The reactions were run on 12% SDS–polyacrylamide gels, dried, and exposed to Kodak X-OMAT AR films.

Preincubation of the antiphosphotyrosine antibody with phosphotyrosine, phosphothreonine, or phosphoserine (all at 10 mM) was performed in immunoprecipitation buffer for 2 hr at 4°C before being used for immunoprecipitation.

Protein Gel Blot Analysis

Proteins were run on 12% SDS–polyacrylamide gels and blotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The membranes were blocked overnight 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% nonfat dried milk and then incubated in the same solution containing the primary antibody at a dilution of 1:2000 for 2 hr. The membranes were then washed in five changes of PBS-T, 10 min each wash, followed by incubation for 1 hr in the secondary antibody diluted in PBS-T containing 3% nonfat dried milk. After an additional five washes in PBS-T, the immunoreactivity was visualized using the enhanced chemiluminescence detection system from Amersham.

Approximately 40 ng of recombinant p45^{Ntf4} (Wilson et al., 1995) was run on the gels as a size control. Blocking of the AbC4 antibody (2 μL) by the recombinant protein (~8 μg) was performed in immunoprecipitation buffer for 2 hr at 4°C before protein blot analysis, as described above.

RNA Extraction and RNA Gel Blot Analysis

RNA was extracted by grinding tissues in extraction buffer (5 M guanidium monothiocyanate, 10 mM EDTA, 50 mM Tris, pH 7.5, and 8% β-mercaptoethanol) followed by centrifugation at 10,000 rpm for 20 min. To the supernatant, 7 volumes of 4 M $LiCl_2$ was added and incubated at 4°C overnight. After centrifugation at 10,000 rpm for 60 min at 4°C, the pellet was resuspended in 5 mL of 3 M $LiCl_2$ and then centrifuged again for 60 min at 10,000 rpm at 4°C. The pellet was resuspended in 3 mL of solubilization buffer (10 mM Tris, pH 7.5, 1 mM EDTA, and 0.1% SDS) and then vortexed and freeze/thawed in liquid nitrogen twice. After phenol and phenol–chlorofrom extraction, 0.1 volume of 3 M sodium acetate, pH 4.9, was added, followed by chloroform extraction. The RNA was precipitated with 2.5 volumes of ethanol and left at −20°C overnight. The pellet was collected by centrifugation at 10,000 rpm for 20 min at 4°C and washed with 70% ethanol. After drying under vacuum, the RNA was dissolved in solubilization buffer. RNA (15 μg, each sample) was run on denaturing formaldehyde gels and blotted to Hybond N membranes (Amersham), which were incubated with a γ-^{32}P–labeled probe from *ntf3*, *ntf4*, or *ntf6* in hybridization solution (50% formamide, 50 mM sodium phosphate, pH 7.0, 5 mM EDTA, 0.9 M NaCl, 10 × Denhardt's solution [1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA], and 0.1% SDS) containing 250 μg/mL denatured sonicated salmon sperm DNA. After hybridization at 42°C, the membranes were washed (in 50 mM sodium phosphate, pH 7.0, 5 mM EDTA, 50 mM NaCl, and 0.1% SDS) sequentially at 22, 42, and 65°C before exposure to Kodak X-OMAT AR films at −70°C.

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REFERENCES

Astwood, J.D., and Hill, R.D. (1996). Molecular biology of male gamete development in plants—An overview. In Pollen Biotechnology, S.S. Mohapatra and R.B. Knox, eds (New York: Chapman and Hall), pp. 3–37.

Bedinger, P.A., Hardeman, K.J., and Loukides, C.A. (1994). Travelling in style: The cell biology of pollen. Trends Cell Biol. **4,** 132–138.

Benito Moreno, R.M., Macke, F., Alwen, A., and Heberle-Bors, E. (1988). In situ seed production after pollination with in-vitro matured, isolated pollen. Planta **176,** 145–148.

Bögre, L., Ligterink, W., Heberle-Bors, E., and Hirt, H. (1996). Mechanosensors in plants. Nature **383,** 489–490.

Bögre, L., Ligterink, W., Meskiene, I., Barker, P.J., Heberle-Bors, E., Huskisson, N.S., and Hirt, H. (1997). Wounding induces the rapid and transient activation of a specific MAP kinase pathway. Plant Cell **9,** 75–83.

Cai, G., Moscatelli, A., Del Casino, C., and Cresti, M. (1996). Cytoplasmic motors and pollen tube growth. Sex. Plant Reprod. **9,** 59–64.

Davenport, K.R., Sohaskey, M., Kamada, Y., Levin, D.E., and Gustin, M.C. (1995). A second osmosensing signal transduction pathway in yeast. J. Biol. Chem. **270,** 30157–30161.

Decroocq-Ferrant, V., Decroocq, S., VanWent, J., Schmidt, E., and Kreis, M. (1995). A homologue of the MAP/ERK family of protein kinases is expressed in vegetative and in female reproductive organs of *Petunia hybrida*. Plant Mol. Biol. **27,** 339–350.

Dickinson, H. (1995). Dry stigmas, water and self-incompatibility in *Brassica*. Sex. Plant Reprod. **8,** 1–10.

Estruch, J., Kadwell, S., Merlin, E., and Crossland, L. (1994). Cloning and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase. Proc. Natl. Acad. Sci. USA **91,** 8837–8841.

Gartner, A., Nasmyth, K., and Ammerer, G. (1992). Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. Genes Dev. **6,** 1280–1292.

Heslop-Harrison, J., and Heslop-Harrison, Y. (1992). Intracellular motility, the actin cytoskeleton and germinability in the pollen of wheat (*Triticum aestivum* L.). Sex. Plant Reprod. **5,** 247–255.

Hirt, H. (1997). Multiple roles of MAP kinases in plant signal transduction. Trends Plant Sci. **2,** 11–15.

Hiscock, S.J., Doughty, J., and Dickinson, H.G. (1995). Synthesis and phosphorylation of pollen proteins during the pollen–stigma interaction in self-compatible *Brassica napus* L. and self-incompatible *Brassica oleracea* L. Sex. Plant Reprod. **8,** 345–353.

Jonak, C., Kiegerl, S., Lloyd, C., Chan, J., and Hirt, H. (1995). MMK2, a novel alfalfa MAP kinase, specifically complements the yeast MPK1 function. Mol. Gen. Genet. **248,** 686–694.

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N.S., and Hirt, H. (1996). Stress signalling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought. Proc. Natl. Acad. Sci. USA **93,** 11274–11279.

Knetsch, M.L., Wang, M., Snaar-Jagalsk, B.E., and Heimovaara-Dijkstra, S. (1996). Abscisic acid induces mitogen-activated protein kinase activation in barley aleurone protoplasts. Plant Cell **8,** 1061–1067.

Krisak, L., Strich, R., Scott-Winters, R., Perry Hall, J., Mallory, M.J., Kreitzer, D., Tuan, R.S., and Winter, E. (1994). SMK1, a developmentally regulated MAP kinase, is required for spore wall assembly in *Saccharomyces cerevisiae.* Genes Dev. **8,** 2151–2161.

Kunz, C., Chang, A., Faure, J.-D., Clarke, A.E., Polya, G.M., and Anderson, M.A. (1996). Phosphorylation of style S-RNases by Ca^{2+}-dependent protein kinases from pollen tubes. Sex. Plant Reprod. **9,** 25–34.

Lee, H.-S., Karunanandaa, B., McCubbin, A., Gilroy, S., and Kao, T.H. (1996). *PRK1*, a receptor-like kinase of *Petunia inflata*, is essential for postmeiotic development of pollen. Plant J. **9,** 613–624.

Mascarenhas, J.P. (1989). The male gametophyte of flowering plants. Plant Cell **1,** 657–664.

Mascarenhas, J.P. (1993). Molecular mechanisms of pollen tube growth and differentiation. Plant Cell **5,** 1303–1314.

McCormick, S. (1993). Male gametophyte development. Plant Cell **5,** 1265–1275.

Michel, D., Salamini, F., Bartels, D., Dale, P., Baga, M., and Szalay, A. (1993). Analysis of a desiccation and ABA-responsive promoter isolated from the resurrection plant *Craterostigma plantagineum.* Plant J. **4,** 29–40.

Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K.T., Kamada, H., and Shinozaki, K. (1993). ATMPKs: A family of plant MAP kinases in *Arabidopsis thaliana.* FEBS Lett. **336,** 440–444.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K., and Shinozaki, K. (1996). A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold and water stress in *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA **93,** 765–769.

Morishima-Kawashima, M., and Kosik, K.S. (1996). The pool of MAP kinase associated with microtubules is small but constitutively active. Mol. Biol. Cell **7,** 893–905.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J., and Sturgill, T.W. (1991). Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J. **10,** 885–892.

Pöpping, B., Gibbons, T., and Watson, M.D. (1996). The *Pisum sativum* MAP kinase homologue (PsMAPK) rescues the *Saccharomyces cerevisiae hog1* mutant under conditions of high osmotic stress. Plant Mol. Biol. **31,** 355–363.

Reszka, A.A., Seger, R., Diltz, C.D., Krebs, E.G., and Fischer, E.H. (1995). Association of mitogen-activated protein kinase with the microtubule cytoskeleton. Proc. Natl. Acad. Sci. USA **92,** 8881–8885.

Sadoshima, J., Qiu, Z., Morgen, J.P., and Izumo, S. (1996). Tyrosine kinase activation is an immediate and essential step in hypotonic cell swelling-induced ERK activation and c-*fos* gene expression in cardiac myocytes. EMBO J. **15,** 5535–5546.

Schliess, F., Schreiber, R., and Häussinger, D. (1995). Activation of extracellular signal-regulated kinases Erk-1 and Erk-2 by cell swelling in H4IIE hepatoma cells. Biochem. J. **309,** 13–17.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. (1995). Tobacco MAP kinase: A possible mediator in wound signal transduction pathways. Science **270,** 1988–1992.

Suzuki, K., and Shinshi, H. (1995). Transient activation and tyrosine phosphorylation of a protein kinase in tobacco cells treated with a fungal elicitor. Plant Cell **7,** 639–647.

Tilly, B.C., Van der Berghe, N., Tertoolen, L.G.J., Edixhoven, M.J., and de Jonge, H.R. (1993). Protein tyrosine phosphorylation is involved in osmoregulation of ionic conductances. J. Biol. Chem. **268,** 19919–19922.

Tirlapur, U.K., Cai, G., Faleri, C., Moscatelli, A., Scali, M., Del Casino, C., Tiezzi, A., and Cresti, M. (1995). Confocal imaging and immunogold electron microscopy of changes in distribution of myosin during pollen hydration, germination and pollen tube growth in *Nicotiana tabacum* L. Eur. J. Cell Biol. **67,** 209–217.

Tiwari, S.C., and Polito, V.S. (1988). Spatial and temporal organization of actin during hydration, activation, and germination of pollen in *Pyrus communis* L.: A population study. Protoplasma **147,** 5–15.

Touraev, A., Fink, C.S., Stöger, E., and Heberle-Bors, E. (1995a). Pollen selection: A transgenic reconstruction approach. Proc. Natl. Acad. Sci. USA **92,** 12165–12169.

Touraev, A., Lezin, F., Heberle-Bors, E., and Vicente, O. (1995b). Maintenance of gametophytic development after symmetrical division in tobacco microspore culture. Sex. Plant Reprod. **8,** 70–76.

Usami, S., Banno, H., Ito, Y., Nishihama, R., and Machida, Y. (1995). Cutting activates a 46-kilodalton protein kinase in plants. Proc. Natl. Acad. Sci. USA **92,** 8660–8664.

Wehling, P., Hackauf, B., and Wricke, G. (1994). Phosphorylation of pollen proteins in relation to self-incompatibility in rye (*Secale cereale* L.). Sex. Plant Reprod. **7,** 67–75.

Wilson, C., Anglmayer, R., Vicente, O., and Heberle-Bors, E. (1995). Molecular cloning, functional expression in *Escherichia coli*, and characterization of multiple mitogen-activated-protein kinases from tobacco. Eur. J. Biochem. **233,** 249–257.

Zhang, S., and Klessig, D.F. (1997). Salicylic acid activates a 48-kD MAP kinase in tobacco. Plant Cell **9,** 809–824.