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The MAP kinase kinase NtMEK2 is involved in tobacco pollen germination

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Abstract The tobacco *ntf4* mitogen-activated protein (MAP) kinase gene (and its encoded protein p45^{Ntf4}) is expressed at later stages of pollen maturation. We have found that the highly related MAP kinase SIPK is also expressed in pollen and, like p45^{Ntf4}, is activated upon pollen hydration. The MAP kinase kinase NtMEK2 activates SIPK, and here we show that it can also activate p45^{Ntf4}. In an attempt to inhibit the function of both MAP kinases simultaneously we constructed a loss-of-function mutant version of NtMEK2, which, in transient transformation assays, led to an inhibition of germination in the transformed pollen grains. These data indicate that NtMEK2, and by inference its substrates p45^{Ntf4} and/or SIPK, are involved in pollen germination.

Key words: Mitogen-activated protein kinase; Mitogen-activated protein kinase kinase; Pollen; Germination

1. Introduction

Pollen development and germination are fundamentally important processes in the life cycle of flowering plants as they are required for the fertilization of the ovule and subsequent seed set. In the majority of plants the mature pollen grain is highly dehydrated at dehiscence and essentially in a metabolically inactive state. Reactivation of the pollen grain occurs when it lands on a stigma, which leads to rehydration and swelling, reorganization of the cytoskeleton, and the rapid emergence of the pollen tube [1]. The maturation of pollen from microspores, and germination to produce a pollen tube, can be mimicked in vitro in defined media [2]. Pollen matured in vitro is similar to that in vivo, with the exception that the dehydration period is missing in the in vitro matured pollen [3]. We reported previously that the mitogen-activated protein (MAP) kinase p45^{Ntf4} was activated shortly after pollen hydration and that, given its early activation well before pollen tube emergence, it could have a role in the germination process of tobacco pollen [4]. MAP kinases are found in all eukaryotes and regulate diverse processes such as growth, stress responses, and development. They form part of signal transduction cascades that typically contain a three-component kinase module composed of a MAP kinase kinase kinase, a MAP kinase kinase (or MEK), and a MAP kinase, that are sequentially activated following cellular stimulation [5]. The activation of plant MAP kinases by various stresses has been described from different species and they have been implicated in cytokinesis, root hair tip growth, hormonal responses, and resistance to pathogen attack [6,7].

Three highly similar MAP kinases, SIPK, WIPK, and p45^{Ntf4}, are expressed in tobacco. SIPK has been shown to be involved in the hypersensitive response after pathogen infection [8] and responds to stresses such as osmotic shock [9] and the generation of reactive oxygen species [10]. WIPK was initially described as a wound-induced MAP kinase, but recent evidence suggests a role along with SIPK in defense signaling [11]. Interestingly the expression and activation of WIPK seems to be regulated by SIPK [10,11]. A single MEK, NtMEK2, can activate both SIPK and WIPK [12], and a complex interplay between the two MAP kinases and NtMEK2 may regulate the response of the plant to various types of stresses [11]. SIPK is approximately 93% identical to p45^{Ntf4} at the amino acid level. To date, SIPK expression has only been described in tobacco leaves and cell suspensions, while p45^{Ntf4} is expressed in pollen and seeds [13]. In the present work we show that SIPK and p45^{Ntf4} are co-expressed in tobacco pollen, that NtMEK2 can activate p45^{Ntf4}, and provide evidence that NtMEK2 may function in pollen germination and/or in pollen tube growth.

2. Materials and methods

2.1. Plasmid constructs

The loss-of-function NtMEK2 was constructed by changing lysine 111 to arginine (this lysine residue is conserved in all protein kinases and is required for kinase activity [14]). The NtMEK2 cDNA was amplified from the pBK-CMV/NtMEK2 clone (kindly provided by

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Abbreviations: GFP, green fluorescent protein; GST, glutathione *S*-transferase; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein

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Shuqun Zhang, University of Missouri) with oligonucleotides containing *Sma*I (forward primer) and *Hin*dIII (reverse primer) sites and cloned into pBluescript KS. The reverse primer removed the stop codon from NtMEK2 for subsequent fusion with green fluorescent protein (GFP). We took advantage of an *Eco*91I site near the lysine 111 residue to create a reverse primer containing an *Eco*91I site and the required change from lysine to arginine. This was then used with a forward primer containing a *Sma*I site to amplify the N-terminus of the cDNA. This fragment containing the mutated sites was then used to replace the corresponding fragment in the wild type sequence of the cDNA. Amplification of this clone with forward and reverse primers containing *Hin*dIII sites was used to clone the mutated construct into the vector pSP72-DC3GFP5ER, which contains GFP and a DC3 promoter [15]. The DC3 promoter drives expression during pollen development and germination, and during embryogenesis [15,16]. The vector was first modified by digesting with *Bam*HI, ligating a *Bam*HI-*Hin*dIII adapter, and then digesting with *Hin*dIII to generate an in-frame fusion between NtMEK2-K111R and GFP. Restriction analysis and DNA sequencing confirmed the correct orientation and sequence.

For recombinant protein expression, the NtMEK2 cDNA was amplified with oligonucleotides containing *Sma*I sites and cloned in frame with the glutathione *S*-transferase (GST) coding sequence in the vector pGEX-4T-1 (Amersham). The mutated GST-NtMEK2 loss-of-function version was created by replacing the *Sma*I-*Eco*91I fragment at the N-terminus with a polymerase chain reaction-amplified product containing the desired mutation as described above. Restriction analysis and sequencing were used to verify the identity and integrity of the clones.

2.2. Antibodies, preparation of fusion proteins, protein extracts, immunoblotting, and kinase assays

The anti-p45^{Ntf4} antibody [4] and the anti-SIPK antibody [9] were described previously. The anti-p45^{Ntf4} antibody was affinity-purified using purified p45^{Ntf4} protein bound to cyanogen bromide-activated Sepharose 4B beads according to the manufacturer's instructions (Amersham).

Expression of the GST fusion protein and protein purification were as previously described [17] except that the induction of the NtMEK2 fusion protein was done in Terrific Broth (1.2% bacto-tryptone, 2.4% bacto-yeast extract, 0.4% glycerol), with the addition of 1×phosphate buffer after autoclaving (10×phosphate buffer: 2.31 g KH_2PO_4, 12.54 g K_2HPO_4, per 100 ml).

Preparation of protein extracts, immunoblotting, and assays of kinase activity were performed essentially as described previously [4], except for the use of an alkaline phosphatase-coupled anti-rabbit IgG secondary antibody (Sigma; dilution 1:5000) for immunoblotting, and detection of the immunoreactivity using CDP Star (Amersham).

2.3. Pollen culture and particle bombardment

Isolation and germination of tobacco mature pollen in vitro was performed essentially as described previously [18]. The helium-driven PDS-1000/He particle delivery system (Bio-Rad, USA) was used for the biolistic transformation experiments. Plasmid DNA was precipitated onto gold particles that had an average diameter of 1.1 μm (ChemPur). For each bombardment, 1. 5μl (1 μg/μl) of plasmid was mixed with 30 μl of gold particles (30 mg/ml) and, under constant vortexing, 30 μl $CaCl_2$ (2.5 M) and 30 μl spermidine (0.1 M) were added. The complete mixture was placed on ice for 25 min, centrifuged and the pellet washed twice with 100% ethanol before being resuspended in 10 μl of 100% ethanol. The coated-particle suspension was loaded onto macrocarrier disks and left to dry completely. The parameters used were: 9 cm target distance, 1000 psi helium rupture pressure and a vacuum pressure of 27 inches Hg. Mature pollen of tobacco was bombarded immediately after isolation in the culture medium. The suspension (60 μl) containing 5×10^5 cells was dropped in the middle of a 3 cm wet Petri dish (Nunc, Denmark) to make a circle of evenly distributed cells on the bottom surface of the Petri dish without any support material. Immediately after bombardment, pollen was resuspended in medium T1 and cultured in the dark at 25°C. After 24 h mature pollen was germinated in medium GK [2] at 25°C for 3 h. Pollen germination frequencies were estimated by scoring duplicate samples for each bombardment, counting approximately 300 pollen grains per sample for the presence of a pollen tube. The expression of the GFP in the pollen was visualized with a fluorescence microscope (Leitz Diaplan) or an inverted microscope (Leitz Diavert).

3. Results

3.1. SIPK is expressed in pollen and activated upon pollen rehydration

The p45^{Ntf4} MAP kinase is expressed in pollen and seeds [13] and is rapidly activated after the rehydration of pollen grains [4]. We used an antisense approach to specifically target the *ntf4* gene in pollen, but no phenotypic alterations were observed in pollen in which p45^{Ntf4} expression was suppressed (unpublished results). We found subsequently that the SIPK MAP kinase is also expressed in pollen; Western analysis using an anti-SIPK antibody detected a band in wild type pollen protein extracts and in those from the *ntf4* antisense plants in which p45^{Ntf4} expression was suppressed (Fig. 1A). Although a full-length *ntf4* cDNA was used in the antisense plants, it clearly did not lead to the elimination of SIPK (Fig. 1A and data not shown). Immunokinase assays of protein extracts from germinating pollen using the anti-SIPK antibody showed that SIPK is activated shortly after the hydra-

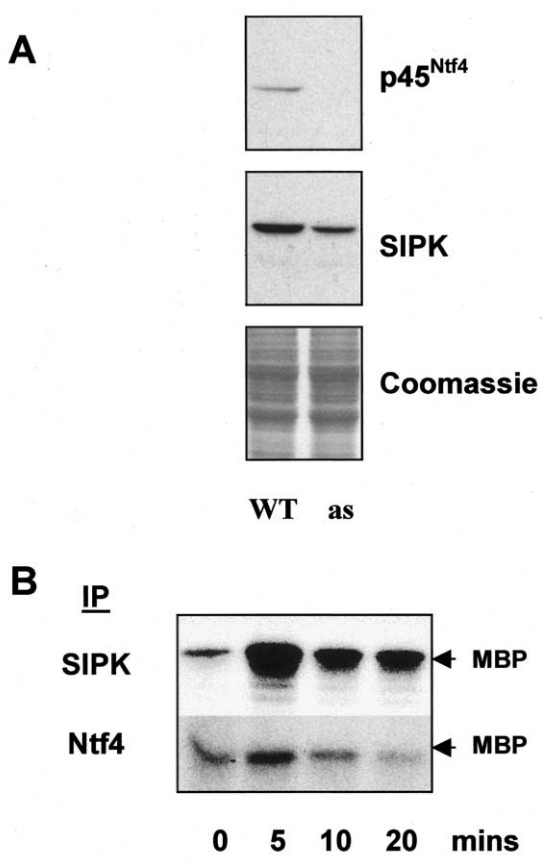

Fig. 1. Expression and activation of SIPK in pollen. A: Protein extracts from pollen of wild type (WT) or *ntf4* antisense plants (as) were immunodetected with the anti-p45^{Ntf4} or anti-SIPK antibody. Aliquots of the pollen extracts were stained with Coomassie brilliant blue as a loading control. B: Immunokinase assay of SIPK activity during pollen germination. Protein extracts from dry pollen (0) or from pollen after suspension in germination medium for the times shown (in minutes) were immunoprecipitated (IP) with the anti-SIPK antibody (SIPK) or the anti-p45^{Ntf4} antibody (Ntf4) and phosphorylation activity was assayed using MBP as a substrate.

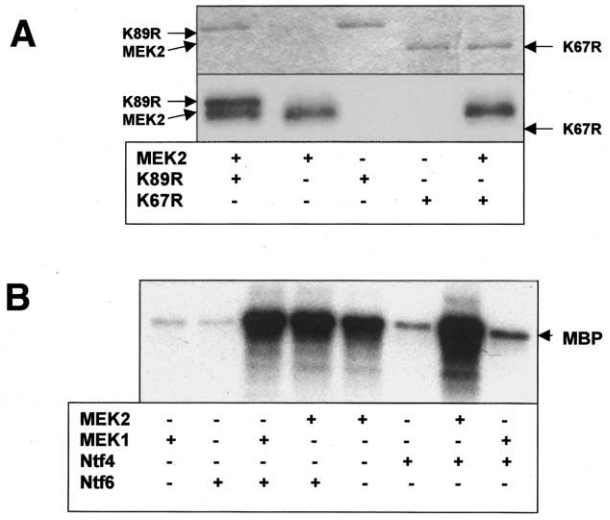

Fig. 2. Phosphorylation and activation of p45^{Ntf4} by NtMEK2. In vitro kinase assays with recombinant proteins. A: NtMEK2 phosphorylates a kinase-inactive version of p45^{Ntf4} (K89R) but not a kinase-inactive version of p43^{Ntf6} (K67R). The upper panel shows a Coomassie stain of the recombinant proteins. Because of the strong activity of NtMEK2, only a small amount of the protein was used in the assay, which is not visible by the Coomassie stain. The position of NtMEK2 (MEK2) is indicated in the upper panel, while the autophosphorylation of NtMEK2 is clearly visible in the kinase assays in the lower panel. B: NtMEK2 activates p45^{Ntf4}, while NtMEK1 activates p43^{Ntf6}. The recombinant proteins were combined in kinase assays as indicated in the presence of MBP as a substrate. The phosphorylation of MBP is shown.

tion of pollen grains in germination medium (Fig. 1B, top panel), similarly to p45^{Ntf4} (Fig. 1B, bottom panel) [4].

The anti-SIPK antibody does not recognize the recombinant p45^{Ntf4} protein (data not shown). We reported previously that the anti-p45^{Ntf4} antibody does recognize the recombinant SIPK protein, and we were surprised at not being able to detect SIPK in leaf protein extracts [13], where it is expressed, and now in the pollen extracts from the antisense plant (Fig. 1A). In addition, subsequent work has confirmed the inability of the anti-p45^{Ntf4} antibody to recognize SIPK in Western analysis or to pull down SIPK activity from pollen samples (paper in preparation). We can only speculate that the recognition of the recombinant SIPK protein by the anti-p45^{Ntf4} antibody is due to the high amounts of protein used in such analyses, while the affinity of the antibody for SIPK is too low to detect it efficiently in Western analysis.

3.2. The MAP kinase kinase NtMEK2 activates p45^{Ntf4}

The co-expression and co-activation of p45^{Ntf4} and SIPK might result in functional redundancy in pollen grains, which could account for the absence of any altered phenotype in the *ntf4* antisense pollen. We therefore adopted a strategy that might inhibit both kinases, by using the putative common activator of SIPK and p45^{Ntf4}, namely the MAP kinase kinase NtMEK2. MEKs are highly specific for their substrates, i.e. MAP kinases, and NtMEK2 has been shown to activate both SIPK and WIPK [12]. As p45^{Ntf4} is 93% identical to SIPK, it seems probable that it could also be activated by NtMEK2. Any particular MEK may activate a number of different MAP kinases; in *Arabidopsis* there are 20 MAP kinases but only 10 MEKs [19]. To test whether NtMEK2 also phosphorylates and activates p45^{Ntf4}, it was expressed as a GST fusion protein in bacteria, and the purified protein was tested in in vitro kinase assays using p45^{Ntf4} as a substrate. Although expressed poorly in bacteria, the recovered recombinant NtMEK2 protein exhibited strong autophosphorylation activity (Fig. 2A,B). We used a kinase-negative version of p45^{Ntf4} (K89R) or of another MAP kinase p43^{Ntf6} (K67R), which cannot autophosphorylate [20], as substrates in kinase assays with NtMEK2. Only K89R was phosphorylated by NtMEK2 (Fig. 2A). Therefore, NtMEK2 specifically phosphorylates p45^{Ntf4} in preference to p43^{Ntf6}.

To test whether phosphorylation of p45^{Ntf4} by NtMEK2 would also activate it, in vitro kinase assays were performed in the presence of the MAP kinase substrate myelin basic protein (MBP). When NtMEK2 and p45^{Ntf4} are present together in the reaction, stronger phosphorylation of MBP is observed than the sum of the activities of NtMEK2 or p45^{Ntf4} on their own (Fig. 2B; see also Fig. 3). By contrast, NtMEK2 did not activate p43^{Ntf6}, which was strongly activated by NtMEK1 (Fig. 2B), as previously reported [20]. We conclude that, at least in vitro, NtMEK2 phosphorylates and activates p45^{Ntf4}.

3.3. Loss-of-function NtMEK2 inhibits pollen germination

Since NtMEK2 is an upstream activator of SIPK [12] and it can also activate p45^{Ntf4}, inhibition of its function might lead to the simultaneous inhibition of SIPK and p45^{Ntf4}. We therefore generated a loss-of-function version of NtMEK2 by replacing a conserved lysine residue present in all protein kinases [14] with arginine (see Section 2). This lysine residue is present at position 111 in NtMEK2 and the mutated protein

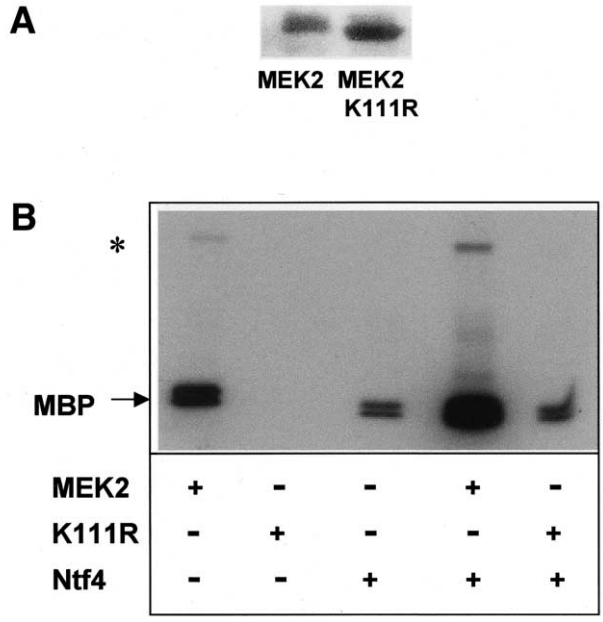

Fig. 3. Loss-of-function NtMEK2 is catalytically inactive. A: GST-NtMEK2 and the mutant version GST-NtMEK2-K111R used in the assay in B were blotted and probed with an anti-GST antibody as a loading control. B: The recombinant proteins were combined as indicated in kinase assays in the presence of MBP as a substrate. The NtMEK2-K111R mutant protein shows no autophosphorylation activity, nor phosphorylation or activation of p45^{Ntf4}. The asterisk indicates autophosphorylation.

Table 1
Percentage of germinating pollen grains after particle bombardment

Pollen bombarded with	GFP-positive (%)	GFP-positive germination (%)	Total pollen germination (%)
DC3-GFP	14.9 ± 1.8	28.9 ± 3.9	29.5 ± 4.2
DC3-NtMEK2-K111R-GFP	16 ± 0.1	12 ± 1.4	18.4 ± 5.5
No DNA	N.A.	N.A.	34.6
No bombardment	N.A.	N.A.	38.3 ± 0.4

Pollen was transformed with GFP alone or GFP-NtMEK2-K111R, both driven by the DC3 promoter. No DNA: pollen was bombarded with naked particles. No bombardment: pollen was treated as for the bombarded grains but without any bombardment. N.A., not applicable. ±, standard deviation.

was called NtMEK2-K111R, which was then expressed as a GST fusion protein in bacteria. Because of the low amounts of NtMEK2 protein used in the assays, the wild type and mutated GST fusion proteins were detected with an anti-GST antibody as a loading control (Fig. 3A). In vitro kinase assays of the purified NtMEK2-K111R protein showed that it had lost its kinase activity, being unable to autophosphorylate or to activate $p45^{Ntf4}$ (Fig. 3B). The mutant *NtMEK2* construct was then cloned as a fusion with GFP in a vector containing the DC3 promoter [15], which drives expression in developing pollen and during zygotic, somatic, and microspore embryogenesis.

Pollen grains were isolated 1 day before full maturation, bombarded with the *GFP-NtMEK2-K111R* construct, and then matured in vitro for 1 day. In vitro matured pollen were then transferred to germination medium and incubated for 3 h before scoring the germination frequency. In the control experiments, pollen grains were bombarded with the same vector containing only GFP, or with particles without any DNA. The germination frequency was reduced in both controls as a consequence of the damaging effect of the bombardment (Table 1). Comparison of the germination frequencies after bombardment with GFP alone or with GFP-NtMEK2-K111R showed that expression of the mutant MEK led to a significant reduction in the number of germinating pollen grains, when calculated from both the GFP-expressing and the total number of pollen grains (Fig. 4 and Table 1). This phenomenon was observed in five independent experiments. Therefore, the reduction in the number of visibly germinating pollen grains was due to the expression of the mutant NtMEK2.

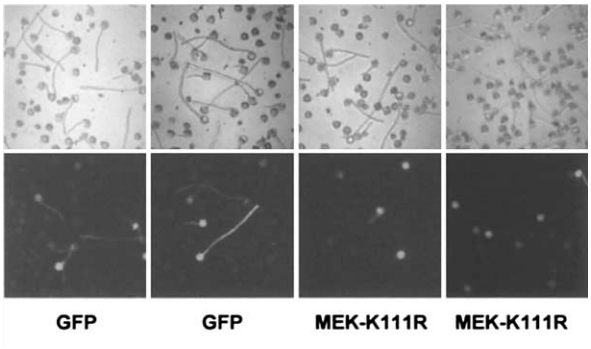

Fig. 4. Inhibition of pollen germination after bombardment with NtMEK2-K111R. Top and bottom panels show light and fluorescence microscopy views, respectively, 3 h after incubation of the bombarded pollen in germination medium. Pollen was transformed with GFP alone or with a GFP-NtMEK2-K111R (MEK2-K111R) construct. Quantification of the inhibitory effect is given in Table 1.

4. Discussion

We have shown here that the two highly similar MAP kinases $p45^{Ntf4}$ and SIPK are expressed in tobacco pollen, and both show similar activation kinetics upon pollen hydration. This raises the possibility that they are functionally redundant, such that the elimination of one can be compensated by the other, which might have resulted in the absence of any phenotype in the pollen of antisense *ntf4* plants (unpublished results). Functional redundancy may be a major problem in defining the role of specific gene products in all organisms, particularly in higher eukaryotes where extensive genome duplication has occurred, giving rise to multigene families. Despite their high level of similarity, *ntf4* and *SIPK* do not represent the ancestral orthologues from the two parental genomes of the amphidiploid *Nicotiana tabacum*, since SIPK was detected in both of the parents, *N. sylvestris* and *N. tomentosiformis*, of tobacco [21]. In addition, putative orthologues of both *ntf4* and SIPK are present in tomato (*Lycopersicon esculentum*, [22]) and potato (*Solanum tuberosum*, accession numbers BAB93529 and BAB93530), which are not amphidiploid. It seems probable that both gene products have at least partially divergent functions in some contexts, but where this is manifest remains to be determined. Such functional differentiation may occur through differential expression, different subcellular targets or the association with different molecules.

The MAP kinase kinase NtMEK2 has been shown to be an upstream activator of SIPK and WIPK in tobacco leaves [12]. Here, we have shown that NtMEK2 can phosphorylate and activate $p45^{Ntf4}$, at least in vitro. Pollen bombarded with a dominant-negative form of NtMEK2 (NtMEK-K111R) showed a significant reduction in the germination frequency compared to pollen bombarded with constructs lacking NtMEK2-K111R. Nevertheless, since MEKs are highly specific for MAP kinases [23], and NtMEK2 appears to be an activator of the SIPK, WIPK, $p45^{Ntf4}$ group of MAP kinases, we can infer that pollen germination requires the activity of one or more of these MAP kinases. Since expression of NtMEK2-K111R in transformed pollen co-exists with the expression of the wild type gene, a complete block of germination would not be anticipated. While it would be desirable to assay the activity of $p45^{Ntf4}$ and SIPK following transformation with NtMEK2-K111R, the experimental system employed here makes it very difficult technically to separate the activity in transformed from non-transformed pollen.

It is important to recognize that the reduced pollen germination frequency in the NtMEK2-K111R transformed pollen could be due to inhibition of pollen tube growth, and not inhibition of the germination (reactivation) of the pollen grain per se. At present it is not possible to distinguish between

these two possibilities. The MAP kinase pathway could conceivably have a role in processes common to both events, for example in cytoskeletal organization, which occurs both during activation of the rehydrated pollen grain and during tube growth. In this context it is interesting to note the parallel between the activation of $p45^{Ntf4}$ and SIPK in pollen and the association of the alfalfa SIMKK (a MEK) and SIMK (a MAP kinase) with both salt stress responses and cytoskeletal rearrangement during root hair tip growth [24]. SIMKK is a putative orthologue of NtMEK2, and SIMK is a putative orthologue of $p45^{Ntf4}$/SIPK. Therefore activation due to changes in osmoticum and polarized growth may be a common feature shared by the respective pairs of kinases, and both stimuli may alter the same underlying mechanism, the rearrangement of the cytoskeleton, via MAP kinase signaling pathways.

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