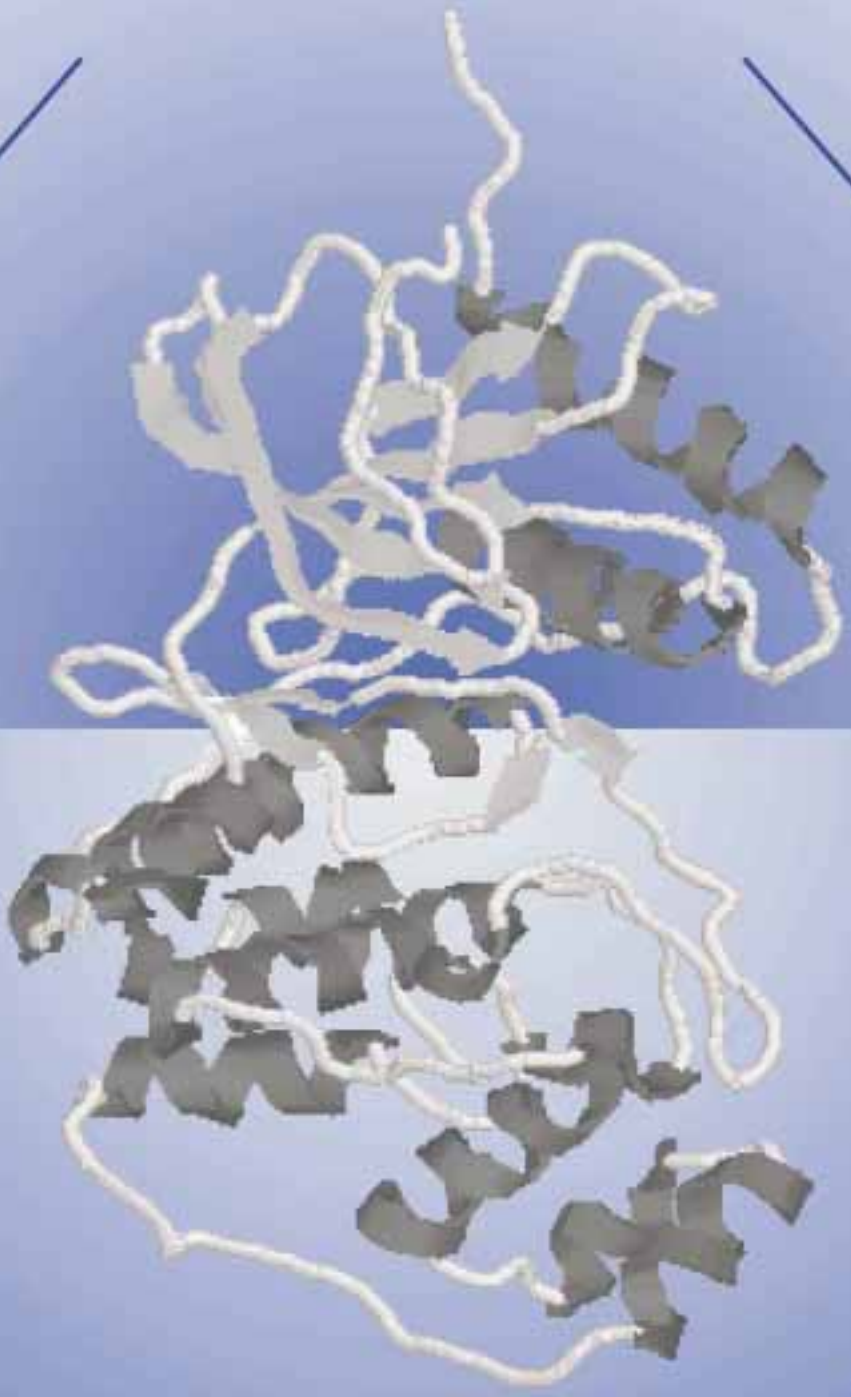
Stress activated MAPKs in plants



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The front cover shows a 3-D structure of the ERK2 MAPK (see chapter 2)

Aan mijn ouders

Voor Caroline

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Outline

Plants are exposed to a wide variety of extracellular stimuli and employ a broad set of signaling pathways to give the appropriate response. Mitogen activated protein kinases (MAPKs) play an important role in the signal transduction of yeast and animals and increasing evidence suggests a similar role of MAPKs in the signal transduction of plants. MAPKs employ their function as part of protein kinase cascades, composed of a MAPK, a MAPK kinase (MKK), and a MAPK kinase kinase (MKKK). MKKKs activate MKKs by phosphorylation of conserved threonine or serine residues, and subsequently MKKs activate the MAPKs by phosphorylation of highly conserved tyrosine and threonine residues. The introduction gives a brief overview of the MAPK cascades employed by yeast and animals and a more extensive overview of our current knowledge about the function of MAPK cascades in plants. To get an overview of the plant MAPKs and their functions the known plant MAPKs were classified and the analyses of all isolated full-length plant MAPK sequences reveals that they can be divided into at least five distinct subfamilies (Chapter 2). For some of these groups it could be shown that MAPKs with similar sequences also perform similar functions. In addition, analysis of expressed sequence tags (ESTs) and partial cDNAs coding for MAPKs revealed the existence of a new plant MAPK subfamily.

The goal of the research described in this thesis was to provide insight in the role of several plant MAPKs in stress responses of plants. One of the most severe environmental stresses to which plants can be exposed is wounding. It can be the result of physical injury, herbivore or pathogen attack and induces a wide range of responses, in general involving the induction of genes active in healing and defense processes. In chapter 3 the involvement of a MAPK in the wound response of alfalfa is discussed. It could be shown that wounding activates the alfalfa stress activated MAPK (SAMK) both at the post-translational and the transcriptional level. The inactivation but not the activation of SAMK was dependent on *de novo* transcription and translation.

Another important stress to which plants are exposed is pathogen attack. The various defense responses of plants to pathogens can normally also be activated by specific pathogen derived factors (elicitors). A 13 amino acid oligopeptide fragment from a 42 kDa extracellular glycoprotein of the pathogenic fungus *Phytophthora sojae* was used. Treatment of parsley cells with this elicitor results in the induction of a broad set of defense responses. The signaling pathways leading to these responses include the activation of several ion channels and the production of reactive oxygen species. A MAPK was identified that is also activated upon elicitor treatment (Chapter 4). This MAPK was shown to act downstream of the ion channel activation and upstream or independently of the oxidative burst. The MAPK is translocated to the nucleus after activation by the elicitor, where it might activate transcription factors responsible for the induction of expression of plant defense genes.

To extend these results to other plant species, the responses of alfalfa cells upon yeast elicitor treatment were analyzed. Two protein kinases with relative molecular masses of 44-kD and 46-kD were found to be rapidly and transiently activated upon elicitor treatment (Chapter 5). These kinases were identified as SAMK and SIMK (stress-inducible MAP kinase), respectively. Yeast elicitor-induced medium alkalinisation, oxidative burst, and MAPK activation could be blocked by the protein kinase inhibitor K252a, demonstrating that protein kinase pathways are responsible for mediating these elicitor responses. However, SAMK and SIMK pathways are not involved in elicitor-induced medium alkalinisation or

oxidative burst, because staurosporine, another protein kinase inhibitor, did not affect elicitor-induced activation of SAMK and SIMK pathways but totally inhibited medium alkalinisation and production of reactive oxygen species. These data show that whereas elicitor-induced medium alkalinisation and oxidative burst depend on protein kinase pathways, these protein kinases lie on separate pathways from elicitor-activated SAMK and SIMK cascades.

In summary the data provided in this thesis give proof for the involvement of various MAPKs in stress responses of plants, but further research will be needed to elucidate the exact role of these MAPKs in their respective pathways.

Chapter 1

Introduction:
MAP Kinase pathways in plants:
Versatile signaling tools

Wilco Ligterink and Heribert Hirt

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Contents

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
CDPK	calcium-dependent protein kinase
CKI	cytokinin insensitive
CTR	constitutive triple response
EIN	ethylene insensitive
ERK	extracellular signal regulated kinase
EST	expressed sequence tag
ETR	ethylene resistant
GA	Gibberellin
hcd	hypersensitive cell death
HOG1	high osmolarity glycerol 1
HR	hypersensitive response
JA	jasmonic acid
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MeJA	methyl jasmonate
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MKK	MAPK kinase
MKKK	MKK kinase
NPK	*Nicotiana tabacum* protein kinase
NtF	*Nicotiana tabacum* FUS3-like kinase
PiE	*Phytophthora infestans* elicitor
PKC	protein kinase C
PLA	phospholipase A
PP2C	protein phosphatase 2C
PR	pathogen response
PTP	protein tyrosine phosphatase
SA	salicylic acid
SAMK	stress-activated MAPK
SAPK	stress-activated protein kinase
SIPK	SA-induced protein kinase
SMK1	sporulation MAP kinase 1
TMV	tobacco mosaic virus
TvX	xylanase from *Trichoderma viride*
WIPK	wound-induced protein kinase

Abstract

Mitogen-activated protein kinases (MAPK) are important signaling tools in all eukaryotes, and function in mediating an enormous variety of external signals to appropriate cellular responses. MAPK pathways have been studied extensively in yeast and mammalian cells and a large body of knowledge on their functioning has accumulated that is summarized briefly. In recent years, plant MAPK pathways have attracted increasing interest resulting in the isolation of a large number of different components of MAPK cascades. Studies on the functions of these components have revealed that MAPKs play important roles in the response to a broad variety of stresses, but also in the signaling of most plant hormones and in developmental processes. Finally the involvement of various plant phosphatases in the inactivation of MAPKs is discussed.

I. Introduction

Like all living organisms, plants must be able to sense many external stimuli and signal them to cellular targets to give appropriate responses. MAPKs are important components for some of these signaling pathways. MAPKs are serine/threonine protein kinases that can be found in all eukaryotes, and perform their function as part of protein kinase cascades. Besides MAPKs, these cascades are composed of MAPK kinases (MKKs) and MAPK kinase kinases (MKKKs).

MAPKs were first discovered by their ability to phosphorylate microtubule-associated protein 2 (MAP2) and were therefore named MAP2 kinases (Ray and Sturgill, 1987). MAP2 kinase turned out to be identical to a 42 kD protein that was previously identified to become tyrosine phosphorylated upon mitogenic stimulation of cells (Cooper *et al.*, 1982; Rossomando *et al.*, 1989). MAP2 kinase was therefore renamed mitogen-activated protein kinase (Rossomando *et al.*, 1989). The cloned gene had high homology to the previously isolated yeast kinases FUS3 and KSS1 (Elion *et al.*, 1990; Courchesne *et al.*, 1989) and since this family of kinases was not only activated by mitogens but also by other stimuli, it was named ERK1 for extracellular signal-regulated kinase 1 (Boulton *et al.*, 1990). MAPK and ERK are often used interchangeably, but it was suggested that ERK should refer to a particular group of isoforms within the MAPK family (Seger and Krebs, 1995).

Soon after the identification of MAPKs, a MAPK activator was identified (Ahn *et al.*, 1991) that was able to phosphorylate MAPKs (Seger *et al.*, 1992). This activator was cloned in 1992 by Crews and co-workers, and was named MEK (for MAPK / ERK kinase). The MEKs also turned out to be activated by phosphorylation (Gomez and Cohen, 1991; Ahn *et al.*, 1993). The proto-oncogene Raf-1 was the first identified MEK activator (Kyriakis *et al.*, 1992), but also other MEK activators, like MEKK1 and c-Mos, were isolated soon after (Lange-Carter *et al.*, 1993; Posada *et al.*, 1993). The high degree of homology of MEK and MEKK1 to yeast protein kinases upstream of FUS3 and KSS1, were the first signs of an evolutionarily conserved structure for MAPK cascades.

Over the past few years several plant MAPK pathway components have been isolated and this review will focus on what is known about the role of these components in signal transduction in plants.

II. Structure of MAPK cascades

II.A. MAPKs

MAPKs belong to the superfamily of protein kinases and are classified into the CMGC group, further consisting of cyclin-dependent kinases (Cdks), glycogen synthase kinase-3 isoforms, and the Cdk-like kinases (Hanks and Hunter, 1995). All MAPKs exhibit eleven subdomains that are characteristic for all serine/threonine protein kinases (Hanks *et al.*, 1988). The different MAPKs can be divided into three major groups: the ERKs (extracellular regulated kinases), the SAPKs/JNKs (stress-activated protein kinases / c-Jun N-terminal kinases) and the p38 kinases. Crystallographic analysis of members of all 3 groups, ERK2, p38, and JNK3, revealed a highly similar 3-D architecture (Zhang *et al.*, 1994; Wilson *et al.*, 1996; Wang *et al.*, 1997; Xie *et al.*, 1998), having a two lobed structure, where the active site is found at the domain interface (Goldsmith and Cobb, 1994). The N-terminal domains are mainly composed of β-strands whereas the C-terminal domains are predominantly α-helical. This overall topology is similar to that of cAMP-dependent protein kinase and CDK2 (Taylor and Radzio-Andzelm, 1994).

One of the most important and least stable secondary structure elements of MAPKs is the activation loop that forms the mouth of the active site (Zhang *et al.*, 1995) and contains the MAPK specific TXY (threonine-X-tyrosine) dual-phosphorylation motif (Payne *et al.*, 1991; Gartner *et al.*, 1992). Phosphorylation of both, threonine and tyrosine in this motif, is required for full activation of the MAPKs. In the inactive form some of the catalytic residues are misaligned and the substrate binding pocket of the kinase is blocked. Both kinase-inhibiting features are corrected by conformational changes upon MAPK activation as determined by comparing the crystal structures of inactive and active, dual phosphorylated ERK2 (Canagarajah *et al.*, 1997).

Distinct MAPKs have specific lengths of the activation loop and sequences of the dual phosphorylation motif. ERK, JNK and p38 kinases have mostly activation loop lengths of 25, 21, or 19 residues, and TEY, TPY, or TGY dual phosphorylation motifs, respectively. By site directed mutagenesis, it was found that the particular amino acid residues of the dual phosphorylation motif and the activation loop influence substrate specificity but are not crucial for the specificity of activation by upstream signals, whereas the length of the activation loop plays a role in controlling autophosphorylation (Robinson *et al.*, 1996; Jiang *et al.*, 1997).

Several groups have tried to elucidate the requirements of MAPKs for the specific recognition by MKKs. In chimeras, constructed from p38 and ERK2, an amino-terminal region of 40 residues was identified to be involved in specifying the response of the kinase to different external signals, whereas the carboxyl-terminal half of the molecule specified

substrate recognition (Brunet and Pouysségur, 1996). Recent work suggests that multiple MAPK regions in the amino- and carboxyl-terminal domain are responsible for the specific interaction with MKKs (Wilsbacher *et al.*, 1999).

Downstream of the MAPK cascade the signal is translated into different cellular responses. MAPKs may be translocated to the nucleus to phosphorylate and thereby activate specific transcription factors or stay in the cytoplasm to phosphorylate cytoskeleton-associated proteins, or certain enzymes such as protein kinases, phosphatases, and phospholipases. Phosphorylation of these substrates occurs only at serine or threonine residues that are immediately followed by a proline (Gonzalez *et al.*, 1991). This substrate specificity is regulated by the so-called P+1 loop in the substrate binding pocket of MAPKs. In ERK2, specific binding of substrate proline by this loop is only possible after activation of the kinase (Canagarajah *et al.*, 1997).

II.B. MKKs

MKKs are dual-specificity protein kinases which activate MAPKs by phosphorylation of both the threonine and tyrosine residue of the TXY motif of MAPKs. MKKs are activated themselves by phosphorylation of two conserved serine or threonine residues between kinase subdomains VII and VIII (Alessi *et al.*, 1994; Zheng and Guan, 1994). In most MKKs these conserved amino acids have the motif $^{S}/_{T}XXX^{S}/_{T}$.

Besides phosphorylation-mediated activation of MKKs, it has also been demonstrated that phosphorylation on other residues can negatively regulate MKK activity (Brunet *et al.*, 1994; Rossomando *et al.*, 1994). This phosphorylation can be mediated by MAPKs (Wu *et al.*, 1993; Brunet *et al.*, 1994), although phosphorylation of MKKs by MAPKs is also thought to facilitate the binding of MKK to Raf (Catling *et al.*, 1995). MKK function cannot only be regulated at the posttranslational level by phosphorylation, but also posttranscriptionally by differential splicing. There are indications that different splicing forms have different cellular localizations (English *et al.*, 1995).

MKKs have restricted substrate specificity in that they have no other known substrates beyond MAPKs (Seger *et al.*, 1992). Furthermore, MKKs normally only function in 1 or 2 distinct MAPK cascades (Robinson and Cobb, 1997), and therefore MKKs are thought to be convergence points of MAPK cascades by intergrating different input signals into a given pathway. As mentioned before, the substrate specificity of MKKs is thought to be determined by multiple MAPK domains, and MKKs are known to recognize the tertiary structure of their substrates, effectively restricting the interaction of different MKKs and MAPKs (Seger *et al.*, 1992). In MKKs, functioning in cell proliferation and differentiation, a conserved N-terminal sequence can be found that is suggested to function as a MAPK

docking site (Bardwell and Thorner, 1996). This region could determine binding specificity between these MKKs and MAPKs. It is not known if a site with a similar function exists in other MKKs.

The lack of cross-talk between different MAPK cascades at the MKK level implicates that the activation of MAPKs by MKKs has mainly a role in signal amplification (Brown *et al.*, 1997). However , since MKKs and MAPKs are present in roughly equal concentrations (Ferrell, 1996), this hypothesis seems unlikely, and MKKs could function in kinetic regulation (Huang and Ferrell, 1996). Since MKKs are normally present in considerable excess of MKKKs, amplification is more likely to occur at this step of the cascade (Ferrell, 1996).

II.C. MKKKs

MKKKs can be classified into four major families: the MEKK/STE11, the Raf, the MLK, and Mos kinases (Widmann *et al.*, 1999). All yeast MKKKs belong to the MEKK/STE11 family, whereas MKKKs of higher eukaryotes can be found in all four groups.

In contrast to the MAPKs and MKKs, the structure of the different MKKKs is rather diverse, and different regulatory motifs can be found in MKKKs. Among them are Pleckstrin Homology (PH) domains, proline-rich sequences involved in SH3 binding, zinc finger motifs, leucine zippers, binding sites for G proteins and several tyrosine and serine/threonine phosphorylation sites (Garrington and Johnson, 1999). In accordance with their diverse structures MKKKs can be activated by different mechanisms, such as phosphorylation through MKKK kinases and PKCs, by interaction with G proteins of the Ras and Rho family or by direct activation through two-component receptor systems (Whitmarsh and Davis, 1996; Fanger *et al.*, 1997; Wurgler-Murphy and Saito, 1997; Rommel and Hafen; 1998). Thus the heterogeneity in the structure and the diversity of regulatory domains of the different MKKKs confers to MAPK cascades the flexibility to respond to a wide range of stimuli. Furthermore, whereas MKKs have a very restricted substrate specificity, functioning mainly in one single MAPK cascade, MKKKs can feed into multiple MAPK pathways (Fanger *et al.*, 1997; Gustin *et al.*, 1998), and there are examples of the involvement of MKKKs in MAPK-independent pathways (Lee *et al.*, 1997a).

The best studied MKKK is Raf and it gives a good example for the complexity of MKKK activation. Raf activation is initiated by Ras-GTP-mediated translocation to the plasma membrane (Vojtek *et al.*, 1993; Rommel and Hafen, 1998), whereby Raf binds to activated Ras with two amino-terminal domains (Brtva *et al.*, 1995). The exact mechanism of Raf activation is not known, but it is thought that binding of Raf to activated Ras and

possibly other unknown membrane components will relieve the repression of Raf activity by its amino-terminal non-catalytic region (Cutler *et al.*, 1998). Repression of MKKK activity by their kinase-unrelated domains is also observed for other MKKKs like the yeast STE11 kinase (Cairns *et al.*, 1992).

Activated Ras is not sufficient for full Raf activation and other components contribute to Raf activation, including 14-3-3 proteins, phospholipids, serine/threonine kinases, like PKC, and tyrosine kinases, like Src (Morrison and Cutler, 1997).

The amino terminus of Raf seems to be important for its activation and many binding events with both upstream and downstream kinases as well as proteins outside the cascade, but also tyrosine and serine phosphorylation of its carboxyl-terminal region is important for Raf activation (Chow *et al.*, 1995; Mason *et al.*, 1999). Furthermore, autophosphorylation is also thought to play a role in the complex mechanism of Raf regulation (Morrison *et al.*, 1993).

MAPK cascade components are not only linked in a linear manner in an activation cascade. MAPKs and MKKs can also phosphorylate Raf or upstream activators of Raf as part of a negative feedback loop (Ueki *et al.*, 1994; Waters *et al.*, 1995; Holt *et al.*, 1996), and MAPKs can also mediate activation of Raf via a positive feedback mechanism (Zimmermann *et al.*, 1996).

Much less is known about the activation mechanisms of other MKKKs. MEKK1 can be activated by Ras, but also by Ras-independent pathways involving MKKK kinases and other small G proteins (Fanger *et al.*, 1997; Widmann *et al.*, 1999). It is also thought that autophosphorylation of its activation loop can activate MEKK1 (Deak and Templeton, 1997). In contrast, the Mos MKKK is thought to be mainly regulated by its synthesis and degradation, whereby phosphorylation of Mos is probably important for its stability (Nishizawa *et al.*, 1992).

The physiological significance of the modular arrangement of MAPK cascades is not entirely understood but it will enable an amplification of a certain input signal and branching or crosstalk between signaling pathways. Furthermore, together with the possibility for positive feedback loops between MAPKs and MKKs or MKKKs, it makes the MAPK cascade also suitable for regulating developmental decisions by converting graded inputs into an all-or-none switch (Ferrell and Machleder, 1998). Switch-like responses are also thought to be enhanced by the dual-phosphorylation activating event of MAPKs and translocation of MAPK cascade components (Ferrell and Bhatt, 1997; Ferrell, 1998).

III. MAPK pathways exists in all eukaryotes

III.A. MAPK cascades in yeast

The yeast genome encodes for six MAPKs (Hunter and Plowman, 1997). Only five of them have been assigned to a specific pathway (Fig. 1) (for reviews see Herskowitz, 1995, Gustin *et al.*, 1998). From the upstream MKKs and MKKKs, four members of each class of kinases can be found in yeast.

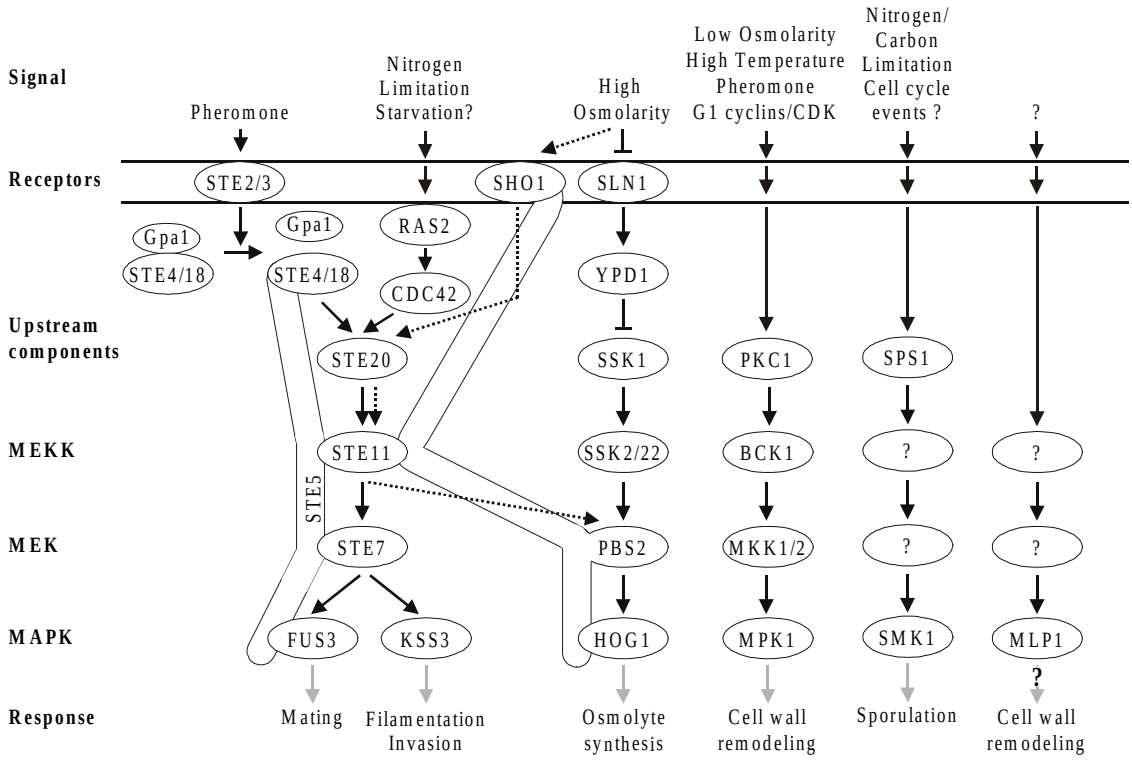

Fig. 1. **MAPK cascades in *S. cerevisiae*.** Question marks indicate uncertainties or unknown components. Black arrows indicate direct interaction / activation, whereas grey arrows indicate an indirect connection. The dotted arrows show the SHO1-mediated hyperosmo-sensing pathway that is thought to be complexed by PBS2.

The first yeast MAPKs isolated were FUS3 (for cell fusion defective 3) and KSS1 (for kinase suppression of SST2 1) and they initially were thought to have a redundant function in the pheromone response pathway. New data, however, suggest FUS3 to have a direct function in the mating pathway and KSS1 to function in the signaling pathway leading to pseudohyphal and invasive growth. Both kinases have the same upstream activator. STE7 is the MKK that activates both MAPKs and STE7 gets activated by STE11.

The high osmolarity glycerol (HOG1) pathway has a role in signaling hyperosmotic stress. Activation of this pathway upon hyperosmotic stress leads to an increased production of the osmotic stabilizer glycerol, and HOG1-deficient yeast mutants have reduced salt-tolerance. The upstream MKK of HOG1 is PBS2. Two pathways have been discovered that feed into the HOG1 pathway. One pathway includes the MKKKs SSK2 and SSK22. The other pathway includes the osmosensor SHO1 that activates the MKKK STE11, which in turn activates PBS2 (Ruis and Schüller, 1995).

MPK1 (for MAP kinase 1), also known as SLT2 (for suppression of low temperature 2), has a function in regulating cell wall integrity. MPK1 gets activated in response to high temperature, hypoosmolar stress, mating pheromone, and in a cell cycle-dependent manner. The MPK1 pathway further consists of the MKKs, MKK1 and MKK2, and the MKKK, BCK1. Input into the pathway occurs via protein kinase C (PKC1). Cells lacking MPK1 or one of the other members of the pathway are unable to grow at elevated temperatures and under low osmotic conditions.

The SMK1 (for sporulation MAP kinase 1) pathway gets activated upon growth of yeast on a nitrogen-deficient medium that lacks a fermentable carbon source. Under these conditions, yeast starts to form spores. As part of this process, the SMK1 pathway is responsible for the assembly of spore walls. At the moment no MKK or MKKK has been identified for this pathway, and it remains open if SMK1 is part of a classical MAPK module. However, the protein kinase SPS1, that is similar to MKKK kinases, has a role as an upstream activator of the SMK1 pathway.

The putative protein kinase MLP1 (for MPK1-like protein kinase 1) was discovered by the yeast genome project and clusters together with the MAPKs. Because of this fact MLP1 is believed to be a MAPK, although it has a KXY motif instead of the TXY at its activation site (Hunter and Plowman, 1997). No clear function is known for this kinase at the moment, but there are some indications that MLP1 has a partially complementary function to MPK1 (Watanabe, 1997).

Besides the three core elements of the MAPK cascade, it has been shown that so-called scaffold proteins also play an important role in MAPK pathway activation and regulation. At the moment, two scaffold proteins are known in yeast. STE5 functions in the pheromone pathway and is known to bind STE11, STE7 and FUS3 (Printen and Sprague, 1994), and also the upstream G-protein STE4 (Gustin *et al.*, 1998). Besides STE5, PBS2 also functions as a scaffold protein and, via its N-terminal SH3-binding domains, binds to both STE11 and HOG1 to form a signaling complex (Posas and Saito, 1997). Scaffold proteins are thought to be important for preventing cross-talk between different cascades allowing single kinases to function in more than one MAPK module without affecting the specificity of their responses.

III.B. MAPK cascades in animals

To date, 13 different MAPKs have been identified in mammalian cells: 6 different ERKs, 3 JNKs and 4 p38 kinases (Widmann *et al.*, 1999; Abe *et al.*, 1999). It should be mentioned that many MAPKs exist in several isoforms and splicing variants. The 3 JNK genes are known to code for at least 10 different isoforms (Gupta *et al.*, 1996). The relevance of this complexity is poorly understood. In addition, the human genome codes for at least 7 MKKs and 14 MKKKs, of which also different isoforms and splicing variants exist (Tournier *et al.*, 1999; Widmann *et al.*, 1999). Figure 2 gives a summary of the mammalian MAPK pathways.

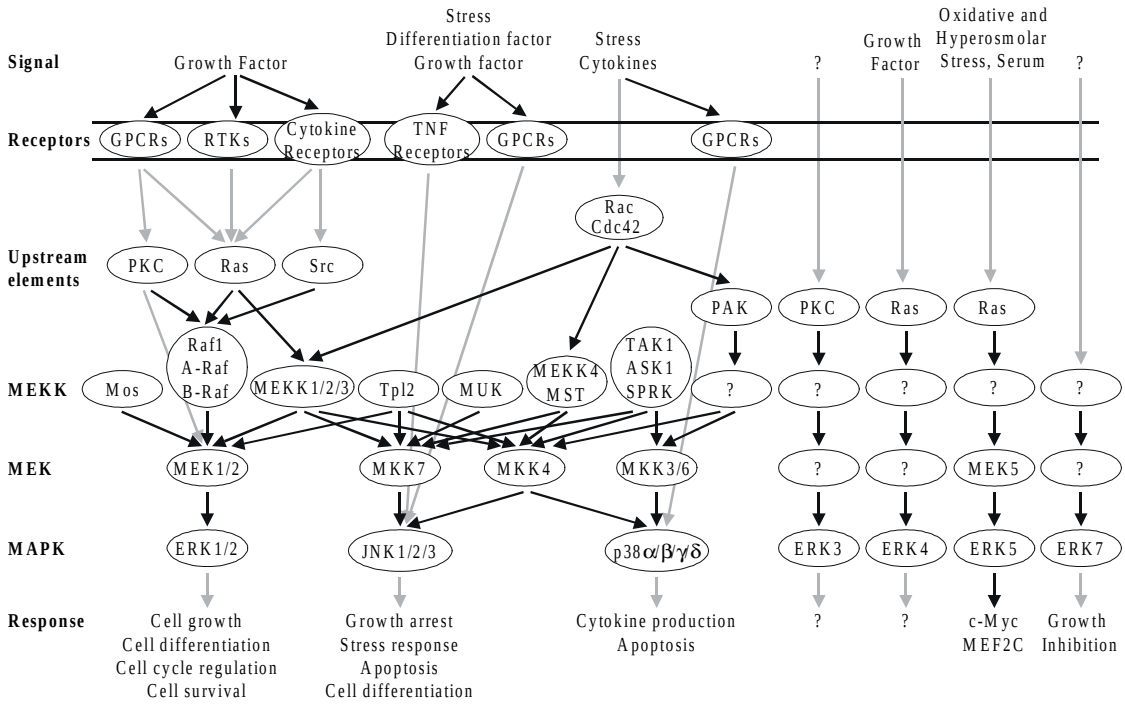

Fig. 2. Overview of mammalian MAPK pathways. Question marks indicate unknown components, black arrows direct activation, and grey arrows indirect connections. This scheme only shows major players up and downstream of the MAPK cascades.

The 6 members of the ERK family can be divided into 4 subfamilies of which ERK1 and 2 have been studied extensively. ERK1/2 have a role in cell proliferation, differentiation, cell cycle control and cell survival. They can be activated by the MKKs MEK1 and 2, which in turn most often get activated by MKKKs of the Raf family. Members of almost all other MKKK subfamilies are also reported to be able to activate MEK1/2. Upon activation,

cytoplasmic ERK1/2 both translocate to the nucleus (Lenormand *et al.*, 1993), and this translocation is important for their function (Brunet *et al.*, 1999). The mechanism of ERK translocation is not understood, but it is thought that MEK1 keeps inactive ERK1/2 in the cytoplasm (Fukuda *et al.*, 1997). Furthermore it is suggested that homodimerization of ERK1/2 could also play a role in their nuclear translocation (Khokhlatchev *et al.*, 1998).

ERK3 and 4 form the second ERK subfamily, but hardly anything is known about the function of these kinases. ERK3 is a constitutively nuclear kinase which can be activated by PKC kinases, and has an SEG motif instead of the common TXY motif in the activation loop (Cheng *et al.*, 1996). ERK4 has been shown to be activated by growth factors (Widmann *et al.*, 1999). The third and fourth subfamily consist of only ERK5 and ERK7, respectively. Both kinases have long distinct carboxyl-terminal extensions. ERK5 is able to induce c-Jun expression upon serum treatment, and is activated by oxidative and hyperosmolar stress. Cytoplasmic ERK5 is translocated to the nucleus upon activation and its C-terminus seems to inhibit its autophosphorylation activity (Zhou *et al.*, 1995). ERK7 can function as a negative regulator of cell growth, is a constitutively active nuclear kinase and requires its carboxyl-terminus for these functions (Abe *et al.*, 1999).

The SAPK/JNK group of MAPKs can be activated by stress, such as heat shock, hyperosmolar stress and ultraviolet irradiation. Furthermore, this group of kinases has a role in the cellular response to different extracellular ligands, like differentiation and growth factors. The SAPK/JNKs can be activated by the MKKs, MKK4 and MKK7, and activation of MKK4/7 occurs by MKKKs from almost all MKKK subtypes, except for members of the Raf subfamily. To date, all known substrates of the JNK pathway are transcription factors (Widmann *et al.*, 1999).

MAPKs of the p38 family are known to be activated by cytokines and certain cellular stresses, such as ultraviolet irradiation, osmotic shock, and heat shock. P38 MAPKs can be activated by MKK3 and MKK6, but also by MKK4. TAK1, ASK1 and SPRK can act as MKKKs in these pathways. Substrates for p38 MAPKs are MAPK-activated protein kinase 2 and 3 (MAPKAP2/3) and several transcription factors. Besides activation through cytokines, p38 also seems to be important for cytokine production (Widmann *et al.*, 1999).

The role of scaffold proteins in mammalian MAPK pathways is less clear, but potential mammalian MAPK-scaffold complexes have been described. The JNK-interacting protein 1 (JIP1) is known to interact with multiple components of the JNK signaling pathway and is thought to function in a similar way as STE5. Furthermore, MEKK1 might function as a scaffold protein in a PBS2-like way, since it is able to bind both MKK4 and JNK (Xu and Cobb, 1997; Whitmarsh and Davis, 1998). MP1 seems to function as a third class of scaffold proteins. It is able to bind MKK1 and ERK1 and facilitates the activation of ERK1 by MKK1 (Schaeffer *et al.*, 1998).

Despite an obvious lack of cross-talk between yeast MAPK pathways, single receptors can activate multiple MAPK cascades in mammalian cells, and one substrate can be regulated by different MAPK pathways. Some ligands also seem to activate one MAPK pathway while inhibiting another. Furthermore, other signaling pathways are present in cells and have the ability to interact (cross-talk) with each other and the MAPK cascades. This complex interplay between different signal transduction pathways gives mammalian cells the capacity to respond to extracellular stimuli in a sophisticated manner.

IV. MAPK cascades in plants: Structure and phylogenetic classification

IV.A. MAPKs

To date 28 full length MAPK cDNAs have been isolated from a variety of plant species (Table 1). A total of 7 cDNAs were published from *Arabidopsis*. Among the other MAPKs are 5 from tobacco, 5 from alfalfa, and 2 from maize and single genes from apricot, oat, pea, parsley, petunia, sweet potato, and wheat. Besides the cDNAs, additional sequences of MAPK homologues can be found in the databases. Besides the 7 *Arabidopsis* cDNAs in the database, 6 genomic and 7 EST sequences encoding additional MAPK homologues can be found in the database. Thus the *Arabidopsis* MAPK family consists of at least 20 members. Based on an additional preliminary search for MAPK genes, using several methods, it is postulated that the *Arabidopsis* genome may code for more than 30 different MAPK genes (Mizoguchi *et al.*, 1999).

The predicted amino acid sequences show the highest homology in the eleven kinase domains. The sequences outside the kinase domains are often more conserved in specific MAPKs from different species, than in MAPKs of different subfamilies of the same species. This makes it likely that similar MAPKs from different species also perform similar functions, and supportive evidence for this assumption exists for at least some MAPK groups as discussed in section V.

When compared with mammalian MAPKs, all plant MAPKs have highest homolgy to the ERK subfamily. Analysis of the plant MAPKs indicates that most of them cluster together in one group (PERKα). Only 3 plant MAPKs fall into a different group, denoted as PERKβ. According to sequence similarity, at least 5 different subfamilies can be established within PERKα, named PERKα1-5 (Fig. 3 and Table 1).

The different PERK groups have distinct lengths of their activation loops. The activation loops are 25, 22, and 21 amino acids long for the PERKβ, PERKα5 and PERKα1-4 groups, respectively.

Table 1. **Summary of the isolated plant MAPK sequences**

Name	Species	Accession No.	Amino acids no.	MW (kDa)	PI (pH units)	Subfamily	Reference
ATMPK1	*Arabidopsis thaliana*	D14713	370	42,7	6,8	PERKα5	Mizoguchi *et al.*, 1994
ATMPK2	*Arabidopsis thaliana*	D14714	376	43,1	6,1	PERKα5	Mizoguchi *et al.*, 1994
ATMPK3	*Arabidopsis thaliana*	D21839	370	42,7	5,5	PERKα2	Mizoguchi *et al.*, 1993
ATMPK4	*Arabidopsis thaliana*	D21840	376	42,9	5,7	PERKα3	Mizoguchi *et al.*, 1993
ATMPK5	*Arabidopsis thaliana*	D21841	376	43,1	5,4	PERKα3	Mizoguchi *et al.*, 1993
ATMPK6	*Arabidopsis thaliana*	D21842	395	45,1	5,2	PERKα1	Mizoguchi *et al.*, 1993
ATMPK7	*Arabidopsis thaliana*	D21843	368	42,4	6,8	PERKα5	Mizoguchi *et al.*, 1993
ATMPK8	*Arabidopsis thaliana*	unpublished	-	-	-	PERKβ	Mizoguchi *et al.*, 1997
ATMPK9	*Arabidopsis thaliana*	unpublished	-	-	-	PERKβ	Mizoguchi *et al.*, 1997
NtF3	*Nicotiana tabacum*	X69971	372	42,8	6,2	PERKα5	Wilson *et al.*, 1993
NtF4	*Nicotiana tabacum*	X83880	393	45,1	5,5	PERKα1	Wilson *et al.*, 1995
NtF6	*Nicotiana tabacum*	X83879	371	42,7	5,0	PERKα4	Wilson *et al.*, 1995
SIPK	*Nicotiana tabacum*	U94192	393	45,2	5,4	PERKα1	Zhang and Klessig, 1997
WIPK	*Nicotiana tabacum*	D61377	375	42,9	5,1	PERKα2	Seo *et al.*, 1995
MMK1	*Medicago sativa*	X66469	387	44,4	5,4	PERKα1	Jonak *et al.*, 1993; Duerr *et al.*, 1993
MMK2	*Medicago sativa*	X82268	371	42,3	6,1	PERKα3	Jonak *et al.*, 1995
MMK3	*Medicago sativa*	AJ224336	374	43,0	4,8	PERKα4	Bögre *et al.*, 1998
MMK4	*Medicago sativa*	X82270	371	43,0	5,6	PERKα2	Jonak *et al.*, 1996
MsTDY1	*Medicago sativa*	*AF129087*	608	68,9	9,8	PERKβ	Schoenbeck *et al.*, 1997
AsPK9	*Avena sativa*	S56638	369	42,9	5,4	?	Huttly and Phillips, 1995
IbMAPK	*Ipomoea batatas*	AF149424	365	41,7	5,5	PERKα2	Unpublished
ERMK	*Petroselinum crispum*	Y12785	371	42,8	5,7	PERKα2	Ligterink *et al.*, 1997
PhERK1	*Petunia x hybrida*	X83440	384	44,4	6,8	PERKα5	Decroocq-Ferrant *et al.*, 1995
PsD5	*Pisum sativum*	X70703	394	45,1	5,3	PERKα1	Stafstrom *et al.*, 1993
PaMAPK	*Prunus armeniaca*	AF134730	368	42,4	8,2	PERKα5	Unpublished
WCK1	*Triticum aestivum*	AF079318	369	42,8	5,3	?	Unpublished
ZmMPK4	*Zea mays*	AB016801	406	46,7	5,7	?	Unpublished
ZmMPK5	*Zea mays*	AB016802	399	44,9	5,2	PERKα1	Unpublished

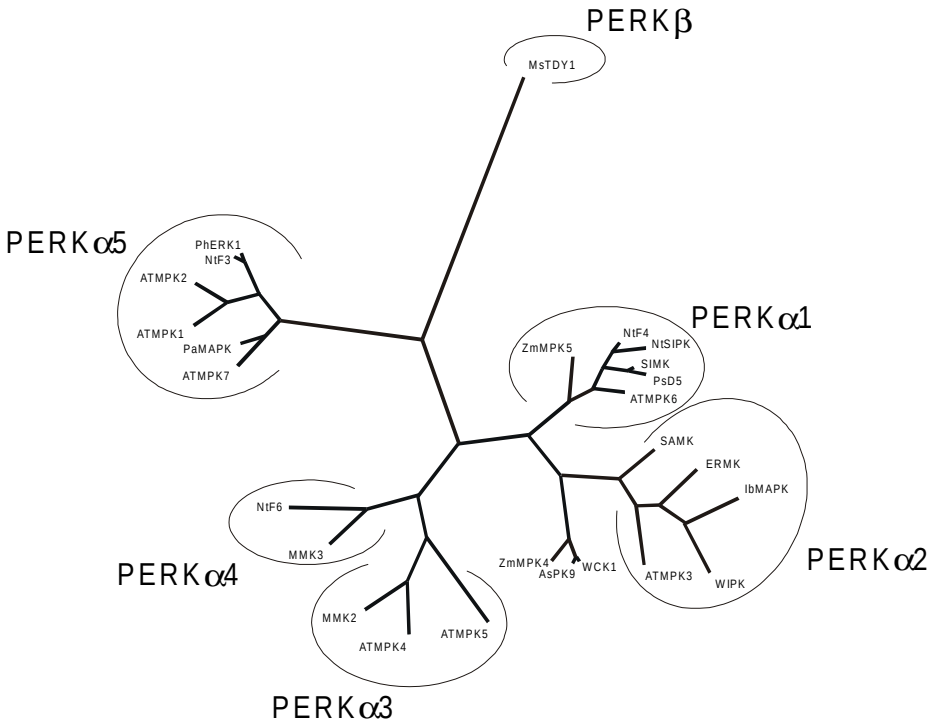

Fig. 3. **Phylogenetic tree based on the amino acid sequences of plant MAPKs (Table 1).** This tree was reconstructed using the neighbour-joining method implemented in version 4.0b1 of PAUP* written by David L. Swofford. The six different subfamilies are denoted as PERKα1-5 and PERKβ.

Almost all isolated plant MAPKs have the TEY motif as the dual phosphorylation site. The only exceptions are AtMPK8 and 9, and the recently discovered alfalfa MAPK TDY1, which have a TDY motif (Mizoguchi *et al.*, 1997; Schoenbeck *et al.*, 1997). So far, a TDY dual phosphorylation motif has only been found in some MAPKs from protozoa (Kültz, 1998). Interestingly, these 3 plant MAPKs also have a long C-terminal extension (Mizoguchi *et al.*, 1999, Schoenbeck *et al.*, 1997). Analysis of genomic sequences and ESTs coding for MAPKs has shown that additional MAPKs for this new group exist (Ligterink, 1999).

Table 2. **Summary of the isolated plant MKK sequences**

Name	Species	Accession nr.	Activation sites	Amino acids nr.	MW (kDa)	pI (pH units)	Subfamily	Reference
AtMEK1	*Arabidopsis thaliana*	AF000977	TSSLANS	354	39,2	7,5	PMKK1	Morris *et al.*, 1997
AtMMK2 / AtMAP2Kβ	*Arabidopsis thaliana*	AB015313 AJ006871	TAGLANT	363	39,9	6,0	PMKK1	Ichimura *et al.*, 1998b; Hamal *et al.*, 1999
AtMMK3	*Arabidopsis thaliana*	AB015314	SMAMCAT	520	57,5	5,3	PMKK3	Ichimura *et al.*, 1998a
AtMMK4	*Arabidopsis thaliana*	AB015315	TMDPCNS	348	40,1	9,7	PMKK2	Ichimura *et al.*, 1998a
AtMMK5/ AtMAP2Kα	*Arabidopsis thaliana*	AB015316 Y07694	TMDPCNS	348	38,3	8,8	PMKK2	Ichimura *et al.*, 1998a; Jouannic *et al.*, 1996
NPK2	*Nicotiana tabacum*	D31964	SIAMCAT	518	57,5	5,3	PMKK3	Shibata *et al.*, 1995
ZmMEK1	*Zea mays*	U83625	SIGQRDT	355	39,9	5,4	PMKK1	Hardin, Wolniak, 1998
LeMEK1	*Lycopersicon esculentum*	AJ000728	TSGLANT	357	39,7	5,5	PMKK1	Hackett *et al.*, 1998

IV.B. MKKs

Despite the rapidly accumulating information about plant MAPKs, little is known about their upstream activators. At the moment 8 MKK cDNA clones have been isolated from plants There are 5 MKKs from *Arabidopsis*, and one from tobacco, tomato, and maize (Table 2). In contrast to the MAPKs and MKKKs, no genomic sequences for additional MKKs can be found in the databases.

The first plant MKK was reported in 1995 and was named NPK2 (for *Nicotiana tabacum* protein kinase 2). Based on hybridization data, sequences with strong similarity to NPK2 are also expected in the genomes of tomato, potato, Atropa belladonna, sweet potato and *Arabidopsis* (Shibata *et al.*, 1995). In maize, at least 2 more MKKs highly related to ZmMEK1 are expected (Hardin and Wolniak, 1998), and rapeseed possesses at least three AtMAP2Kα homologous (Hamal *et al.*, 1999).

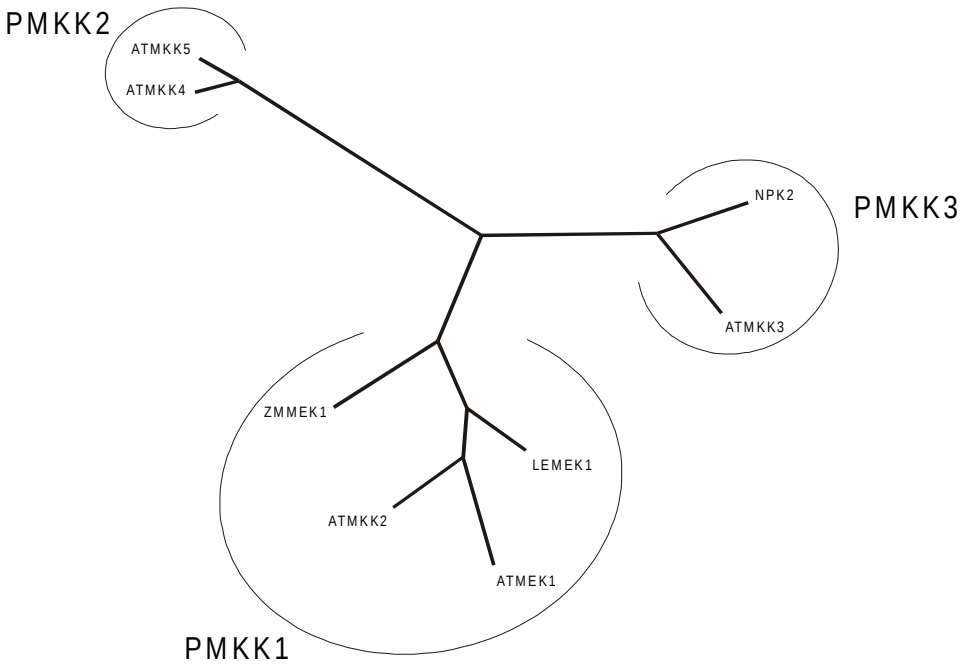

Fig. 4. **Phylogenetic tree based on the amino acid sequences of plant MKKs (Table 2).** This tree was reconstructed using the neighbour-joining method implemented in version 4.0b1 of PAUP* written by David L. Swofford. The two plant MKK subfamilies are denoted as PMEK1-3.

Similar to most isolated MAPK cDNAs, all isolated plant MKK cDNAs belong to one group when compared to MKK sequences from other species. Interestingly, the PMKK group can only be clustered together with a *Dictyostelium* MKK and not with animal MKKs.

The PMKK group in itself can be divided into three subfamilies (Table 2, Fig. 4). Since no functional data exist for plant MKKs it is not yet clear if members within one subfamily have similar functions, but the high homology in the kinase-unrelated regions within each subfamily suggests some functional similarities. Homology to yeast and animal MKKs could only be detected in the conserved kinase domains, but not in the kinase-unrelated regions.

Whereas most yeast and animal MKKs contain an $SXXX^{S}/_{T}$ motif as the phosphorylation sites in the activation loop, all plant MKKs have an $^{S}/_{T}XXXXX^{S}/_{T}$ motif. Such a motif has so far only been found in MKKs from *Candida albicans* and *Dictyostelium discoideum*. However, it should be noted that there is no experimental evidence that the serine/threonine residues in this motif become phosphorylated in plant MKKs.

Some characteristic features for the different PMKK groups can be found. The PMKK1 group and the *Dictyostelium* DdMEK1 share a conserved 15 amino acid motif in their amino-terminal kinase-unrelated domain and it is to be expected that this motif has some specific yet unknown function. Members of the PMKK2 group possess two proline-rich sequences in the amino- and carboxyl-terminal parts (Hamal *et al.*, 1999), which have been shown to be involved in efficient activation of MAPKs in animal systems (Dang *et al.*, 1998), and contain putative SH3-binding motifs (PXXP, Cohen *et al.*, 1995). PMKK3 members are different from other plant MKK sequences in that they have long non-catalytic carboxyl-terminal regions.

IV.C. MKKKs

17 plant cDNAs with homology to MKKKs have been cloned to date (Table 3). There is no direct evidence that these plant proteins also function as MKKKs in plants, but their homology to yeast and mammalian MKKKs, and the ability of AtARAKIN and NPK1 to complement yeast *ste11* and *bck1* mutants, respectively, strongly suggests that some plant MKKKs also function as activators of MKKs *in vivo* (Covic and Lew, 1996; Banno *et al.*, 1993a).

The isolated plant MKKKs seem to cluster mainly with the MEKK/STE11 and the Raf group, named PMEKK and PRaf respectively (Jouannic *et al.*, 1999b). The PMEKKs can be subdivided into 3 subfamilies and the PRaf group into 2 (Table 3, Fig. 5a).

In the databases, at least 19 additional genomic sequences for MKKK homologues can be found and several ESTs coding for putative MKKKs have also been identified. There are also cDNAs that share weak homology with both Raf and mixed lineage kinases (MLK) and they are candidates for an additional putative MKKK group in plants (Feng *et al.*, 1993; Tregear *et al.*, 1996; Ichimura *et al.*, 1997).

Table 3. **Summary of the isolated plant MKKK sequences**

Name	Species	Accession No.	Amino acids no.	MW (kDa)	pI (pH units)	Group / Subfamily	Reference
AtMEKK1	*Arabidopsis thaliana*	D50468	608	66,0	5,4	1 / PMEKK2	Mizoguchi *et al.*, 1996
AtMAP3Kα	*Arabidopsis thaliana*	AJ010090	582	63,6	9,7	1 / PMEKK3	Jouannic *et al.*, 1999
AtMAP3Kβ3	*Arabidopsis thaliana*	AJ010092	572	63,7	5,5	1 / PMEKK2	Jouannic *et al.*, 1999
AtMAP3Kγ	*Arabidopsis thaliana*	Y14316	-	-	-	1 / PMEKK3	Jouannic *et al.*, 1999
AtMAP3Kδ1	*Arabidopsis thaliana*	Y14199	-	-	-	2 / PRaf1	Jouannic *et al.*, 1999
ANP1L	*Arabidopsis thaliana*	AB000796	661	72,8	5,2	1 / PMEKK1	Nishihama *et al.*, 1997
ANP1S	*Arabidopsis thaliana*	AB000797	376	41,4	7,3	1 / PMEKK1	Nishihama *et al.*, 1997
ANP2	*Arabidopsis thaliana*	AB000798	642	70,8	5,3	1 / PMEKK1	Nishihama *et al.*, 1997
ANP3	*Arabidopsis thaliana*	AB000799	651	71,7	8,1	1 / PMEKK1	Nishihama *et al.*, 1997
AtARAKIN	*Arabidopsis thaliana*	L43125	-	-	-	1 / PMEKK2	Covic and Lew, 1996
CTR1	*Arabidopsis thaliana*	L08789	821	90,3	5,4	2 / PRaf2	Kieber *et al.*, 1993
BnMAP3Kα1	*Brassica napus*	AJ010091	591	64,5	9,3	1 / PMEKK3	Jouannic *et al.*, 1999
BnMAP3Kβ1	*Brassica napus*	AJ010093	575	62,6	5,9	1 / PMEKK2	Jouannic *et al.*, 1999
TCTR1	*Lycopersicon esculentum*	AF110518	829	91,9	6,4	2 / PRaf2	Unpublished
TCTR2	*Lycopersicon esculentum*	AJ005077	982	107,3	6,0	2 / PRaf1	Lin *et al.*, 1998
Put MEKK	*Lycopersicon esculentum*	Y13273	829	91,9	6,0	2 / PRaf2	Wang and Lin, 1997
NPK1	*Nicotiana tabacum*	D26601	690	76,2	7,9	1 / PMEKK1	Banno *et al.*, 1993

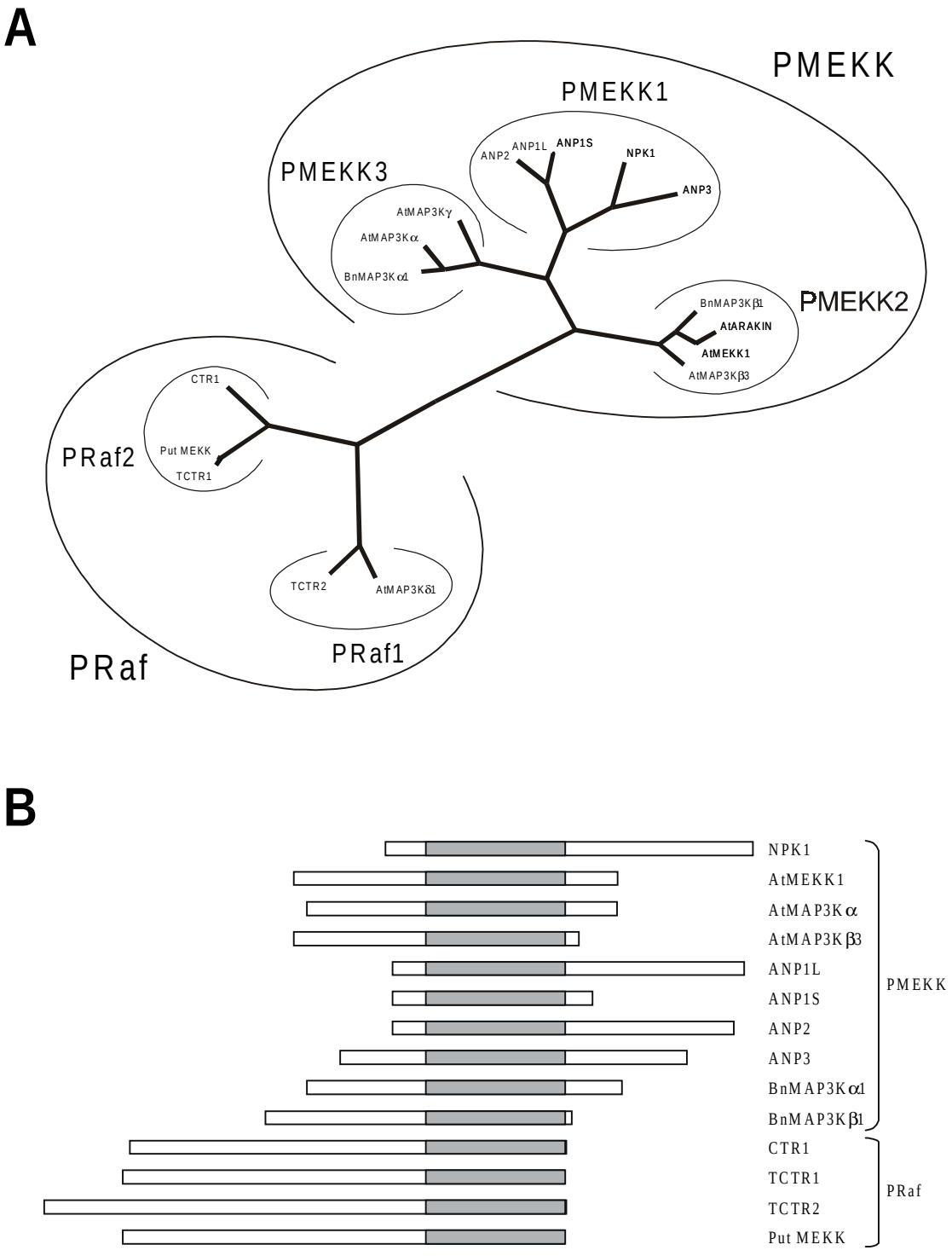

Fig. 5. **(A) Phylogenetic tree based on the amino acid sequences of plant MKKKs (Table 3).** This tree was reconstructed using the neighbour-joining method implemented in version 4.0b1 of PAUP* written by David L. Swofford. The two plant MKKK subgroups are denoted as PMEKK and PRaf. The six different subfamilies as PMEKK1-4 and PRaf1-2. **(B) Overview of the primary structures of plant MKKKs.** Catalytic domains are indicated as gray boxes.

Since MKKK sequences are very diverse, additional plant MKKK groups might still be discovered in the future. The considerable variability that can be observed in protein organization and in non-catalytic sequences of the plant MKKKs suggests, that like in other systems, plant MKKKs also have a wide variety of substrate specificities and regulatory mechanisms.

Whereas the PMEKK members do not have a general structure, both plant and animal proteins of the Raf family share the same structural organization with a carboxyl-terminal position of the catalytic domain and a long amino-terminal non-catalytic extension (Fig. 5b). Furthermore, weak homology can be observed in the non-catalytic domains of mammalian Raf and PRaf members, like serine- and cysteine-rich regions.

The largest group of plant MKKKs is formed by NPK1 and its homologues. Three *Arabidopsis* NPK1 homologues have been isolated (ANP1-3 for *Arabidopsis* NPK1-related protein kinase), but Southern blot analysis has shown that homologous genes are also present in maize and rice (Banno *et al.*, 1993a). *ANP1* transcripts exist in a long (ANP1L) and short (ANP1S) version, that are probably the result of differential splicing. The activity of ANP1S is higher than that of ANP1L and the differential splicing could be involved in the regulation of ANP1 activity (Nishihama *et al.*, 1997). A similar regulatory mechanism has been demonstrated for mammalian MEKK1, whose activity is altered by proteolytic cleavage (Cardone *et al.*, 1997).

IV.D. MAPK cascades

Various plant MAPKs, MKKs, and MKKKs have been identified, indicating that MAPK cascades as known for yeast and animals could also exist in plants. However, no MAPK cascade has been conclusively established in plants. A possible MAPK cascade has been identified for *Arabidopsis* by two-hybrid assays and yeast complementation experiments.

By yeast two-hybrid assay, specific interaction between AtMPK4 and the MKKs AtMKK2 and AtMEK1, and also specific interaction of AtMKK2 and AtMEK1 with AtMEKK1 has been shown (Mizoguchi *et al.*, 1998; Ichimura *et al.*, 1998b). AtMPK4 is not able to complement the yeast *mpk1* mutant, but complementation could be observed when AtMPK4 was expressed together with AtMEK1 (Mizoguchi *et al.*, 1998). Similarly, AtMKK2 was able to partially complement the yeast *pbs2* mutant when expressed together with AtMEKK1, and expression of AtMKK2 and AtMEK1 with an N-terminal truncated form of AtMEKK1 resulted in complementation in both cases. These results indicate that deletion of the N-terminus enhances the kinase activity of AtMEKK1, but also that AtMEKK1 is able to activate AtMKK2 and AtMEK1 in yeast (Mizoguchi *et al.*, 1998;

Ichimura *et al.*, 1998b). Therefore, AtMEKK1, AtMEK1, AtMKK2 and AtMPK4 could constitute a MAPK cascade, but there is no proof that these components also interact *in planta*. In contrast to the interaction data, expression data correlate AtMEKK1 with AtMPK3 (Mizoguchi *et al.*, 1996). This could either indicate that there is no functional meaning for their correlated expression, or that AtMEKK1 activates the ATMPK3 pathway via a different, yet unknown MKK.

Surprisingly, AtMEKK1 cannot only interact with both MKKs, but also with AtMPK4 (Ichimura *et al.*, 1998b). This could suggest a role for AtMEKK1 as a scaffold protein as has been postulated for the rat MEKK1 (Whitmarsh and Davis, 1998).

IV.E. Upstream of the MAPK cascade

In yeast and mammals, several kinases have been isolated that act upstream of MKKKs. STE20 was the first MKKK kinase isolated, and a large number of STE20-related kinases have been identified in various organisms. In general, there are two MKKK kinase subfamilies. The STE20/PAK subfamily possesses a C-terminal catalytic domain and an N-terminal domain with putative G-protein binding motifs. The second MKKK kinase subfamily is the GCK/SPS1 subfamily, which possesses an N-terminal catalytic domain, a long C-terminal kinase-unrelated region, and is mostly activated by stress (Fanger *et al.*, 1997). Two recently isolated putative MKKK kinases from rapeseed BnMAP4Kα1 and 2 (Leprince *et al.*, 1999), and the *Arabidopsis* SIK1 (for stress induced kinase 1) (accession nr. U96613) could be grouped into the GCK/SPS1 subfamily. However, several genomic sequences for plant MKKK kinases belonging to the STE20/PAK subfamily can also be found in the databases.

Other possible MKKK activators are PKC-like kinases. NPK1 can probably be activated by the yeast PKC1 (Banno *et al.*, 1993b), and this could indicate that a similar activation mechanism also occurs in plants. PKC-like activities have been reported to be involved in elicitor-induced defense responses in plants (Xing *et al.*, 1996; Subramaniam *et al.*, 1997), but to date, no plant PKC genes have been isolated.

Small G-proteins have an important role in the activation of mammalian MKKKs and several small G-proteins have been isolated from plants (reviewed in Bischoff *et al.*, 1999). To date, there is however no direct evidence if they play a role in activating plant MAPK cascades.

V. Functions of MAPK pathways in plants

V.A. The role of MAPK pathways in stress signaling

Due to their sessile habit, plants are exposed to a wide variety of environmental stresses, and they have developed a broad range of responses to resist these stresses. A function for MAPK pathways has been implicated in the signal transduction for a broad variety of stress responses.

V.A.1. Mechanical stress

Plants are exposed to different forms of mechanical stress like touch, wind and rain. These stresses are known to induce several physiological responses in plants, the majority of which result in differential growth, reduction of growth rates and thickening of the cell wall. The developmental changes in response to mechanostimulation are collectively named thigmomorphogenesis (Trewavas and Knight, 1994). Most of the responses serve to enhance resistance to subsequent mechanical stress.

In tobacco seedlings touch induces an immediate increase of cytoplasmic calcium (Knight *et al.*, 1991) and several calmodulin and calmodulin-related genes have been shown to be transcriptionally activated upon touch in *Arabidopsis* (Braam *et al.*, 1990). Changes in cytoplasmic calcium and protein phosphorylation are among the earliest responses of plants to mechanical stress, and several reports indicate that MAPKs are responsible for phosphorylating at least some of the proteins. Mechanical stimulation of *Arabidopsis* leaves results in transcript accumulation of both MAPK and MKKK genes (*AtMPK3* and *AtMEKK1*, respectively) (Mizoguchi *et al*., 1996). The transcriptional response is fast and transient with transcript levels starting to accumulate at 5 to 10 minutes after touching and reaching a maximum at around 20 minutes. These results suggest a role for a MAPK pathway in signaling mechanical stimuli. Evidence for such a role was provided by experiments with alfalfa leaves. Touching alfalfa leaves activated an alfalfa MAPK, named SAMK for stress-activated MAPK (previously called MMK4) (Bögre *et al.*, 1996). SAMK becomes activated within 1 minute after mechanical stimulation and activity disappears after 10 min. A constant SAMK activity could be seen in suspension cultured alfalfa cells. This is probably due to constant mechanical stimulation of the cells in suspension. Allowing the cells to rest for 1 hour abolished the SAMK activity, and after shaking the cells for only 2 seconds, SAMK activity was restored again (Bögre *et al.*, 1996). The fast activation of SAMK suggests this MAPK pathway to be one of the cell´s immediate responses to mechanical stimulation. Since SAMK and AtMPK3 are highly homologous it is likely that

AtMPK3 may be also activated at the post-translational level and have a similar function in mechanical signaling.

These results show that like in yeast and animal systems MAPKs are involved in mechanosignaling in plants, but their specific roles remain to be elucidated.

V.A.2. Wound signaling

Among the most severe environmental stresses plants have to cope with is wounding. Wounding can be the result of physical injury, herbivore, or pathogen attack, and induces a wide range of responses, mainly encompassing the activation of genes involved in healing and defense. Wound healing is associated with the onset of genes involved in cell cycle and cell differentiation, whereas defense is mediated by expression of genes encoding pathogen response (PR) proteins and proteinase inhibitors (PIN). Proteinase inhibitors are proposed to protect plants against pests and herbivores by decreasing the digestibility and nutritional quality of leaf proteins (Bowles, 1993).

Whereas some wound-induced genes are only expressed locally at the site of attack, others, such as *PR* and *PIN* genes, are also expressed systemically throughout the plant and protect the plant from attack at distant sites. The products of these genes are collectively named SWRPs for systemic wound response proteins (Bergey *et al.*, 1996). There are strong indications that the plant peptide systemin is a mediator of the systemic wound signal in tomato (Pearce *et al.*, 1991; reviewed in Ryan and Pearce, 1998). However, other signals, including jasmonates (Seo *et al.*, 1997), and changes in hydraulic pressure (Malone, 1992), or electrical potential (Wildon *et al.*, 1992), are also discussed as candidate systemic wound signals. The jasmonates jasmonic acid (JA) and its methyl ester (MeJA) have been implicated as important mediators of the wound signal, both at local and systemic sites (Seo *et al.*, 1997).

Compared to the systemic response to wounding, little is known about the primary sensing and signaling. Recently, protein kinases have been implicated to have a role in the primary wound signaling. The first suggestion for a role of protein kinases in wound signaling came from the observation that polygalacturonide, a local mediator of the wound response, induced phosphorylation of plasma membrane proteins of tomato (Farmer *et al.*, 1989). Since then, several reports have implicated MAPKs to have a role in signaling the wound response. In tobacco, a kinase activity of 46 kDa was detected after cutting of leaves (Usami *et al.*, 1995). This kinase activity appeared as early as 1 minute after wounding, reached its maximum at 2-5 minutes and decreased to basal levels within 30 minutes. The active form of the 46 kDa kinase was phosphorylated on both serine and/or threonine and tyrosine residue(s) and dephosphorylation inactivated the kinase. These phosphorylation

properties, together with the size of approximately 46 kDa and the ability to use myelin basic protein (MBP) as an artificial substrate, strongly suggested this kinase to be a MAPK. A similar wound-inducible kinase activity was detected in a broad range of plant species, including both monocots and dicots (Usami *et al.*, 1995).

It was shown that in tobacco leaves transcripts of a MAPK gene accumulate within 1 minute after wounding (Seo *et al.*, 1995). The corresponding MAPK was termed WIPK for wound-induced protein kinase. Accumulation of *WIPK* transcripts was not limited to the wounded leaves, but was also observed in adjacent and distant unwounded leaves at approximately the same level. These results showed that the *WIPK* gene is expressed systemically upon wounding, as it is known for many different wound-inducible genes. The fast kinetics of *WIPK* transcript accumulation suggested that WIPK could be one of the earliest responses to the wound-stress.

A recent study showed that *WIPK* transcript accumulation upon wounding is accompanied by an increase in WIPK activity (Seo *et al.*, 1999). Using a WIPK-specific antibody it was shown that WIPK is activated within 3 minutes after wounding and that activity returns to basal levels after 30 minutes. WIPK activation also occurred in adjacent unwounded leaves with a similar kinetics, although this activation was less pronounced. By using the same antibody it could be shown that WIPK protein levels do not change upon wounding (Seo *et al.*, 1999).

The alfalfa SAMK, which is very similar to the tobacco WIPK, also becomes activated upon wounding (Bögre *et al.*, 1997). Activation of SAMK can already be detected 1 minute after wounding of alfalfa leaves, and has a similar kinetics as WIPK activation. It was shown that activation of SAMK is a post-translational process, since α-amanitin and cycloheximide did not block the activation. However, these drugs did block SAMK inactivation, indicating that *de novo* transcription and translation of protein factors is required for inactivation of SAMK. An alfalfa PP2C-like phosphatase was isolated that was shown to be this or one of these factors (Meskiene *et al.*, 1998a), as discussed in chapter VI.

Similar to WIPK in tobacco, *SAMK* mRNA levels also increase upon wounding, although transcripts of the *SAMK* gene accumulate with a different kinetics, starting at 20 minutes after wounding. The accumulation of *SAMK* transcripts at a later time point than SAMK activation suggests that the *SAMK* gene may be a direct target of the SAMK pathway. At the moment the reason why transcript accumulation is not accompanied with an increase in SAMK protein levels is unclear, but could be explained by a more rapid turn-over rate of SAMK protein after activation. No evidence is yet available for such a model and further experiments are required to clarify this point.

Besides wound-induced transcriptional and post-translational activation of WIPK, another tobacco MAPK has been shown to be activated post-translationally by wounding. This 48 kDa MAPK, termed SIPK for salicylic acid-induced protein kinase, was shown to be

activated by wounding with a similar kinetics as WIPK (Zhang and Klessig 1998a). Unlike WIPK, there was no accumulation of *SIPK* transcripts upon wounding.

There is also evidence for a role of MAPKs in the wound response of tomato. Wounding of tomato leaves was shown to activate an MBP kinase of 48 kDa that was also found to be activated at lower levels in adjacent unwounded leaves (Stratmann and Ryan, 1997). This kinase was phosphorylated on tyrosine residues with a similar kinetics as the activity increased upon wounding, strongly suggesting it to be a MAPK. An activation of this kinase was also observed when the leaves were exposed to known secondary messengers of the wound signal, like systemin, polygalacturonic acid and chitosan (Stratmann and Ryan, 1997). Recently, it was shown that activation of the kinase and other defense responses by systemin could be suppressed by simultaneous addition of the N-terminal part of systemin, supporting the idea that the N-terminus of systemin is involved in receptor-binding, whereby the C-terminal part plays a role in the subsequent activation of the MAPK pathway (Meindl *et al.*, 1998). In alfalfa, it was shown that other proposed mediators of the wound signal, like MeJA and electrical signals, were not able to activate SAMK (Bögre *et al.*, 1997).

Activation data have given information about the involvement of MAPK pathways in the wound response and have helped to study some of the upstream components of this pathway. To elucidate the specific role of MAPKs and possible downstream targets, overexpression of sense-oriented WIPK cDNA was used in tobacco. In some transformants that were selected for high transgene expression, endogenous *WIPK* gene expression was silenced (Seo *et al.*, 1995). These transgenic plants showed a constitutive low level of MBP kinase activity, and wounding could not increase this activity. Wounding of leaves of the transgenic plants did not result in accumulation of the wound- and JA-inducible *PI-II* and basic *PR-1* genes. Moreover, no increase of JA and MeJA levels could be observed. In contrast, transgenic plants showed increased levels of salicylic acid and transcripts for acidic PR-1 and PR-2 proteins, responses which normally occur only after pathogen attack, but not wounding. A similar phenotype was found in transgenic plants over-expressing a small GTP-binding *rab*-related protein, and these authors showed that MeJA and SA accumulation antagonize each other (Sano *et al.*, 1994, 1996). Thus the different expression pattern in transgenic WIPK plants can be explained by the suppressed JA and MeJA synthesis and thereby a release of the otherwise repressed SA synthesis.

Extending these studies, Seo and colleagues (1999) also searched the WIPK-overproducing transformants for constitutive accumulation of *PI-II* gene transcripts. These transgenic plants consistently showed higher transgene *WIPK* transcript and WIPK activity levels. JA and MeJA amounts of the unwounded transgenic plants were three- to fourfold higher than in the wild-type plants. These results clearly demonstrated a role for WIPK in the production of jasmonates and their subsequent activation of wound-induced genes.

The mechanism by which WIPK induces the accumulation of JA and MeJA is not known, but it has been proposed that this could occur by a similar mechanism as the prostaglandin production in mammals (Bergey *et al.*, 1996). Prostaglandins are signaling molecules in the inflammatory stress response in mammals and the chemical structure of prostaglandins is similar to that of JA. Inflammatory stress in mammals is signaled by activation of MAPKs which phosphorylate and thereby activate cytoplasmic phospholipase A_2 ($cPLA_2$) (Lin *et al.*, 1993). $cPLA_2$ cleaves phospholipids to release arachidonic acid, which is then further converted to prostaglandins. By analogy to mammalian prostaglandin synthesis, one of the substrates of WIPK could be a $cPLA_2$-like enzyme. Although no plant PLAs have yet been isolated, increases in PLA activity have been observed after wounding and elicitor treatment (Lee *et al.*, 1997b; Chandra *et al.*, 1996).

A role for MAPKs acting upstream of the octadecanoid pathway also agrees with results of Stratmann and Ryan (1997). The wound-induced activation of the MAPK-like kinase in tomato was still possible in *def1*, a wound signaling-deficient tomato mutant. *def1* has a mutation in the octadecanoid pathway and is effectively blocked in the wound-induced synthesis of JA (Howe *et al.*, 1996). Since products of the octadecanoid pathway like JA and PDA were able to induce several wound-expressed genes, but not the kinase (Stratmann and Ryan, 1997), the MAPK is upstream of the octadecanoid pathway. The inability of JA to activate the wound-inducible SAMK in alfalfa supports these results (Bögre *et al.*, 1997). Overall, these data show that the MAPK cascade functions to activate the octadecanoid pathway.

Besides MAPKs, the *Arabidopsis* MKK AtMEK1 and its tomato homologue LeMEK1 have also been suggested to have a role in the wound response (Morris *et al.*, 1997; Hackett *et al.*, 1998). Transcript levels of *AtMEK1* increased remarkably after wounding of *Arabidopsis* leaves. Transcriptional activation was only observed in wounded leaves, starting to accumulate 6 hours after wounding, which is markedly slower than the transcriptional activation of the wound-induced MAPKs. Although no protein or activation data of AtMEK1 are available, the local and late transcriptional activation of *AtMEK1* makes it unlikely that AtMEK1 is the MKK upstream of the wound-induced kinases. It might be possible that AtMEK1 has no direct role in wound signaling, but functions in the re-entry of the cells into the cell cycle instead. Such a role would fit with AtMEK1's expression in meristematic regions (Morris *et al.*, 1997).

In general, it can be concluded that MAPK pathways play an important role in the wound response of plants and a preliminary model for its action can be made in which a MAPK pathway gets activated by wounding via secondary messengers of the wound response, like systemin. Subsequently the MAPK induces the production of JA and MeJA, which will activate a broad set of defense responses (Fig. 6).

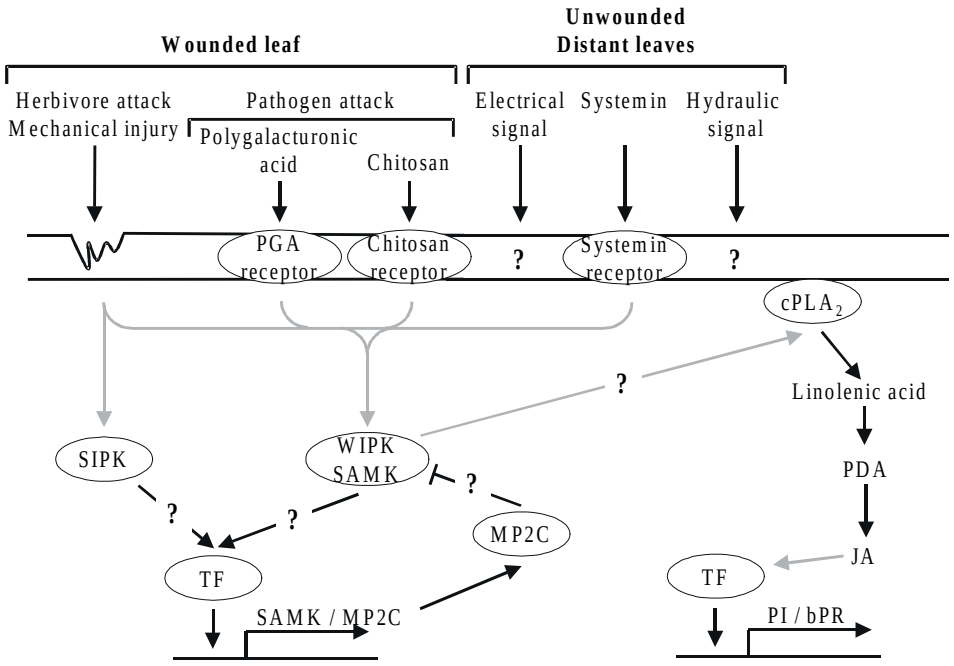

Fig. 6. **Schematic illustration of the wound response pathways in which MAPKs are involved.** Question marks indicate uncertain connections or unknown components. Black arrows indicate direct interaction / activation whereas grey arrows indicate indirect connections.

V.A.3. Abiotic stress signaling

Plants have to cope with extreme changes of the abiotic environmental conditions. These abiotic stresses include high and low temperature, drought and osmotic stress. The possible role of MAPK pathways in translating these stresses into cellular responses will be discussed below.

V.A.3.a. Drought stress

AtMEKK1 and *AtMPK3* are not only transcriptionally activated by mechanical stimulation, but also by dehydration (Mizoguchi *et al.*, 1996). The mRNA of the *AtPK19* gene, a ribosomal S6 kinase homologue, accumulated simultaneously under the same stress conditions (Mizoguchi *et al.*, 1996). Ribosomal S6 kinases have been shown to be phosphorylated and activated by MAPKs in several mammalian systems (Sturgill *et al.*, 1988; Gregory *et al.*, 1989; L'Allemain *et al.*, 1991; Sadoshima *et al.*, 1993) and these results could indicate the existence of a similar pathway in *Arabidopsis*, whereby AtPK19 could be phosphorylated and thereby activated by AtMPK3.

In accordance with a role of AtMPK3 during dehydration, the closely related alfalfa homologue *SAMK* is also transcriptionally upregulated upon drought stress (Jonak *et al.*,

1996). Besides transcriptional induction of the gene, SAMK is also activated at the post-translational level. However, in contrast to mRNA and SAMK activity levels, no changes in SAMK protein levels were observed upon dehydration (Jonak *et al.*, 1996).

V.A.3.b. Low temperature stress

Low temperature stress has been shown to have a strong effect on the phosphorylation status of several plant proteins. An important role of protein kinases in mediating low temperature responses is emphasized by the finding that kinase inhibitors are able to markedly inhibit cold-induced freezing tolerance (Monroy *et al.*, 1993). The finding that protein phosphatase 2A (PP2A) activity is down-regulated upon cold stress (Monroy *et al.*, 1998), underscores the importance of protein kinases in cold signaling.

AtMEKK1, AtMPK3, AtPK19, and SAMK react to cold stress in the same way as to dehydration and all show transcriptional upregulation (Mizoguchi *et al.*, 1996; Jonak *et al.*, 1996). Also the recently reported *Arabidopsis* MKKK, *AtMAP3Kβ3*, showed cold-induced transcript accumulation (Jouannic *et al.*, 1999a). In contrast, the transcript levels of the maize MKK, *ZmMEK1*, were substantially reduced after cold treatment of roots (Hardin and Wolniak, 1998).

V.A.3.c. High temperature stress

It was shown that heat stress is able to inactivate a 50 kDa protein kinase in tomato suspension cultured cells (Heider *et al.*, 1998). The ability of this kinase to phosphorylate MBP, together with its molecular mass, could be an indication that this kinase is a member of the MAPK family. Because transcriptional activation of heat shock genes by the human transcription factor Hsf1 is repressed by phosphorylation of Hsf1 by ERK1 (Chu *et al.*, 1996), the 50 kDa protein kinase could play a similar role in silencing heat-inducible genes in unstressed cells.

V.A.3.d. Osmotic stress

In the halotolerant green alga *Dunaliella tertiolecta*, protein kinases have an important role in conferring resistance to changes in external osmolarity. Hypo-osmotic stress induced a kinase of 40 kDa that could phosphorylate MBP and histone. In the same system, hyperosmotic stress induced, among other things, two kinases of 40 and 45 kDa, respectively (Yuasa and Muto, 1996). The 40 kDa kinase could use MBP, histone and casein as a substrate, whereas the 45 kDa could only phosphorylate MBP. Activity of the kinases could be shown to be dependent on their phosphorylation state. All three kinases were active in the absence of Ca^{2+}, proving that they are not CDPKs. The kinases could be MAPKs, although MAPKs are generally not able to use casein as a substrate. The use of histone as a substrate by plant MAPKs has been reported (Wilson *et al.*, 1995; Zhang and Klessig, 1997),

suggesting that hypo-osmotic stress activates a 40 kDa MAPK and hyperosmotic stress a 45 kDa MAPK. However, arguing against the possibility that the 40 and 45 kDa protein kinases are MAPKs was the inability to detect tyrosine phosphorylation of the proteins with an anti-phosphotyrosine antibody (Yuasa and Muto, 1996), leaving the possibility open that the kinases are not MAPKs.

D. tertiolecta cells change in volume if exposed to changes in external osmolarity, but its original volume is recovered within 30 minutes after treatment. Volume recovery was blocked by the kinase inhibitor K-252a, which also inhibited the hypo- and hyper-osmotic stress-induced protein kinase activities, suggesting that these kinases are involved in mediating osmotic tolerance of *D. tertiolecta* (Yuasa and Muto, 1996).

There are also indications for a role of MAPKs in the signaling of osmotic stress in higher plants. Hyperosmotic stress was able to activate MAPK-like kinases in tobacco (Usami *et al.*, 1995; Tena and Renaudin, 1998). Exposing tobacco suspension-cultured cells to hypo-osmotic stress was shown to activate 3 MBP-kinases with sizes of 50, 70 and 80 kDa (Takahashi *et al.*, 1997). The 50 kDa protein kinase has all the properties of a MAPK. It uses MBP as a substrate and gets tyrosine-phosphorylated upon activation. Protein kinase and phosphatase inhibitor studies suggested that activation and inactivation of the kinases is regulated by phosphorylation and dephosphorylation events, respectively (Takahashi *et al.*, 1997, 1998). It was shown that an increase in cytosolic Ca^{2+} is a prerequisite for the activation of the kinases and that the G-protein activator mastoporan is able to activate the same three kinases (Takahashi *et al.*, 1998). These results suggest a role for G-proteins, Ca^{2+} influx, and protein kinases in mediating hypo-osmotic stress in tobacco.

In yeast, hyperosmotic stress is sensed by so-called osmosensors. One hyperosmotic sensor in yeast is a two-component regulatory system, a well-known system of signal transduction pathways in prokaryotes. Two-component systems normally consist of a histidine kinase, that functions as a sensory kinase and a response-regulator protein. After autophosphorylation on a histidine residue, the sensor kinase transfers the phosphate group to a response-regulator protein, thereby activating the latter (Wurgler-Murphy and Saito, 1997; Chang and Stewart, 1998). In the yeast hyperosmotic signaling pathway, the two-component regulatory system consists of a phosphorelay between 3 proteins. The yeast osmosensor SLN1 is a fused two-component system that autophosphorylates on histidine in the N-terminal sensor domain and then transfers the phosphate group to an aspartate residue in the C-terminal located response-regulator domain. The phosphate is then further transferred to YPD1, which functions as a second histidine phosphorelay intermediate between SLN1 and the response regulator SSK1. SSK1 finally feeds into the HOG1 MAPK pathway by interaction with the MKKKs SSK2 and SSK22 (Posas *et al.*, 1996). A similar osmosensing system could also exist in plants, since the *Arabidopsis* SLN1 homologue

AtHK1 can function as an osmosensor in yeast and can complement SLN1-deficient yeast cells (Shinozaki and Yamaguchi-Shinozaki, 1997).

In yeast the hyperosmotic stress response is mediated through the HOG1 MAPK pathway. HOG1-deficient yeast cells are unable to survive under salt stress, demonstrating that the HOG1 pathway is essential for stress adaptation under hyperosmotic conditions. The pea PsD5 MAPK was able to complement a HOG1-deficient yeast mutant (Pöpping *et al.*, 1996). In accordance with these results, it was assumed that PsD5 may have a role in hyperosmotic stress signaling in pea. There is however no evidence for such a role and studies on the alfalfa MAPK MMK2 showed that care must be taken in using functional yeast complementation data to assign functions to plant MAPKs. The alfalfa MMK2 MAPK is specifically able to complement the yeast MPK1 kinase, which has a function in hypo-osmotic and heat stress signaling (Jonak *et al.*, 1995). Furthermore, MMK2, but not three other alfalfa MAPKs, was able to phosphorylate a 39 kDa protein that is part of or tightly associated with the cytoskeleton of carrot cells (Jonak *et al.*, 1995). Cytoskeletal-associated proteins have been shown to be the targets of several MAPKs (Ray and Sturgill, 1987), and several lines of evidence suggest a role for cytoskeletal components in the activation of the MPK1 pathway (Kamada *et al.*, 1995). These results could suggest a similar role of MMK2 in the hypo-osmotic and heat stress response in alfalfa, but no evidence could be found for this assumption (Jonak *et al.*, 1996).

Interestingly, some stress-activated MAPKs like AtMPK3 and SAMK seem to be involved in mediating multiple abiotic stresses. Both genes become transcriptionally activated upon touch, drought, and cold stress, and *AtMPK3* also shows transcriptional upregulation upon salt stress (Mizoguchi *et al.*, 1996). Although these stresses look to be very different at the first sight, drought, cold, and high salt stress all result in dehydration. Therefore, the AtMEKK1/AtMPK3 and SAMK pathways could function in signaling general dehydration.

V.A.4. Pathogen response

Plants are threatened by a wide variety of pathogens and have developed many ways to protect themselves against them. Plants are protected by chemical and physical barriers and also have defenses that are induced upon pathogen attack, like re-inforcement of cell walls, the production of phytoalexins, and transcriptional activation of defense genes (Hammond-Kosack and Jones, 1996; Yang *et al.*, 1997; Somssich and Hahlbrock, 1998). Induction of defense response is mostly a result of interaction between pathogen-produced signals (elicitors) and plant receptors. Elicitors can be involved in the induction of race-specific incompatible (gene-for-gene interactions), species-specific compatible, or non-host

incompatible responses (Ebel and Mithöfer, 1998). An important feature of plant defense responses is the hypersensitive response (HR) that can be recognized by a localized host cell death at the infection sites (Dangl *et al.*, 1996; He, 1996). HR is often associated with the onset of a broad-spectrum, long-lasting systemic resistance to subsequent infections, called systemic acquired resistance (SAR) (Ryals *et al.*, 1996).

Extensive research has been done to elucidate the components of the signaling pathways that lead to these defense responses, and G-proteins, ion fluxes, calcium homeostasis, and the formation of reactive oxygen species are thought to play imporant roles (Blumwald *et al.*, 1998; Yang *et al.*, 1997).

A number of reports have demonstrated an essential role of phosphorylation and dephosphorylation events in the activation of defense genes and the hypersensitive response. Elicitor treatment of plant cells resulted in changes in phosphoprotein profiles (Dietrich *et al.*, 1990; Felix *et al.*, 1991; Grab *et al.*, 1989), and inhibition of certain defense responses were observed by applying inhibitors of both, protein kinases (Felix *et al.*, 1991; Groskopf *et al.*, 1990; Schwacke and Hager, 1992; Viard *et al.*, 1994), and protein phosphatases (Dunigan and Madlener, 1995; Conrath *et al.*, 1997). In some cases phosphatase inhibitors were also able to activate defense responses on their own (Chandra and Low, 1995; Felix *et al.*, 1994; Levine *et al.*, 1994; MacKintosh *et al.*, 1994). Furthermore, several groups have reported the elicitor-induced phosphorylation and thereby activation of transcription factors (Dröge-Laser *et al.*, 1997; Stange *et al.*, 1997; Subramaniam *et al.*, 1997).

The first indication for a possible function of MAPKs in the pathogen response of plants was provided by Suzuki and Shinshi (1995). Treatment of tobacco suspension cultured cells with a fungal elicitor, derived from the cell walls of *Phytophthora infestans* (PiE), resulted in a fast and transient activation of a 47 kDa MAPK-like kinase, and staurosporine was able to block both, elicitor-induced MAPK activity (Suzuki and Shinshi, 1995), and defense gene accumulation (Suzuki *et al.*, 1995).

Direct proof for the involvement of MAPKs in pathogen response came from studies of parsley suspension cultured cells treated with the elicitor Pep13. Most responses of parsley cells to the fungal pathogen *Phytophthora sojae* are mimicked by this elicitor, that is a 13 amino acid long oligopeptide fragment derived from a 42 kDa extracellular glycoprotein from *P. sojae*. Treatment of parsley suspension cultured cells with Pep13 results in binding of Pep13 to a specific plasma membrane receptor and subsequent activation of plant defense responses, such as phytoalexin synthesis and activation of defense genes (Nürnberger *et al.*, 1994). Ion fluxes and the oxidative burst were shown to be involved in the signal transduction pathway leading from the Pep13 receptor to the defense responses (Jabs *et al.*, 1997). A MAPK was found to become activated at both the post-translational as well as at the transcriptional level after elicitor treatment of parsley cells, and it could be shown that this activation was the result of the binding of Pep13 to its specific

receptor (Ligterink *et al.*, 1997). Moreover, inhibitor studies could place the MAPK downstream of elicitor-induced ion channel activation, and upstream or parallel of the oxidative burst. Similar to many mammalian and yeast MAPKs (Chen *et al.*, 1992; Sanghera *et al.*, 1992; Ferrigno *et al.*, 1998), elicitor-induced activation of the parsley MAPK resulted in translocation to the nucleus. MAPKs phosphorylate transcription factors in the nucleus and thereby regulate gene transcription (Treisman, 1996). The parsley MAPK may play a similar role and could be the link between cytosolic signal transduction and nuclear activation of defense genes.

The tobacco SIPK was isolated because of its transient activation upon treatment of tobacco suspension cultured cells with salicylic acid (SA) (Zhang and Klessig, 1997). Salicylic acid is known to play an important role in signaling pathogens, and is able to activate several plant defense responses (Dürner *et al.*, 1997). SIPK is also activated by treatment of the cells with different elicitors, like parasiticein, cryptogein, and a cell wall-derived carbohydrate elicitor from the pathogenic fungus *Phytophthora parasitica*, but in these cases its activation is more prolonged (Zhang *et al.*, 1998). Besides activating SIPK, parasiticein and cryptogein are also able to activate WIPK and a 40 kDa MBP kinase (Zhang and Klessig, 1998b; Zhang *et al.*, 1998). WIPK, but not the 40 kDa kinase, was also activated by the fungal cell wall elicitor, but activation occurred to a much lesser extent (Zhang *et al.*, 1998). Cryptogein had already been shown to induce protein phosphorylation in tobacco cells (Viard *et al.*, 1994), and recently, Lebrun-Gracia *et al.* (1998) showed activation of two MAPKs with molecular masses of 50 and 46 kDa, that are likely to correspond to SIPK and WIPK, respectively.

Besides activation by fungal elicitor and SA, SIPK also gets activated upon treatment of tobacco plants with tobacco mosaic virus (TMV) in an *N* gene-dependent manner (Zhang and Klessig, 1998b). Besides SIPK, also WIPK is activated by this treatment with a similar kinetics. Similar to the wounding response of WIPK (Seo *et al.*, 1995), TMV treatment resulted in elevated *WIPK* transcript levels. In contrast to the wound system, WIPK protein levels increased upon TMV treatment, and transcript accumulation preceded the increase of WIPK protein amounts, which in turn preceded the activation of WIPK (Zhang and Klessig, 1998b). These results suggest that activation of WIPK by TMV not only requires post-translational phosphorylation, but also *de novo* transcription and translation. WIPK activation is independent of SA, because TMV-induced WIPK activation was not altered in a transgenic tobacco line expressing the *NahG* gene, in which SA-mediated defense responses are blocked (Gaffney *et al.*, 1993; Zhang and Klessig, 1998b).

Experiments with a tobacco cell line expressing the tomato Cf-9 resistance gene elicited with the corresponding avr-protein Avr9 from the fungal pathogen *Cladosporium fulvum*, suggests that MAPKs also play an important role in signaling gene-for-gene interaction-dependent defense responses (Romeis *et al.*, 1999). In this system, both SIPK

and WIPK become activated upon Avr9 treatment in a Cf-9 dependent manner. *WIPK* is also transcriptionally upregulated, but unlike the TMV-induced WIPK activation, no accumulation of WIPK protein is observed (Romeis *et al.*, 1999).

Not only fungal elicitors or viruses are able to activate MAPKs, but also harpins from the bacteria *Erwinia amylovora* and *Pseudomonas syringae* pv *syringae* were able to activate a MAPK-like kinase in tobacco leaves (Ádám *et al.*, 1997). Recent data suggest that this kinase is identical to SIPK (Hoyos *et al.*, 1997).

Besides pathogen-induced MAPK activation in tobacco and parsley, a MAPK-like activity was also detected in soybean cells treated with a β-glucan elicitor isolated from the pathogenic fungus *Phytophthora sojae* (Ebel and Mithöfer, 1998).

Elicitors and pathogens that are able to activate WIPK, such as cryptogein and parasicticein (Zhang *et al.*, 1998), the harpin-producing bacterial pathogen *Pseudomonas syringae* pv. *Syringae*, and TMV (Zhang and Klessig, 1998b), all induce HR-like host hypersensitive cell death (hcd). This could indicate a role for MAPKs in hcd, similar to mammalian cells, where the SAPK/ JNK and p38 subfamilies of MAPKs play an important role in stress-induced apoptosis (Ichijo *et al.*, 1997; Schwenger *et al.*, 1997; Verheij *et al.*, 1996). Since kinase inhibitors are able to block hcd, protein kinases are definitely involved in the signaling pathway leading to hcd (Levine *et al.*, 1994; He *et al.*, 1994). Suzuki and co-workers (1999) gave additional correlative data for a role of MAPKs in hcd. Treatment of tobacco suspension cultured cells with the fungal elicitor xylanase from *Trichoderma viride* (TvX) was able to activate both hcd and a 47 kDa MAPK (Suzuki *et al.*, 1999). The kinase was activated to the same level as after treatment with the *P. infestans* elicitor PiE (Suzuki and Shinshi, 1995), but with a more prolonged kinetics. Interestingly, only TvX is able to induce hypersensitive cell death (Yano *et al.*, 1998). Similar to the role of JNK in apoptosis of mammalian cells (Chen *et al.*, 1996a,1996b), sustained activation of the 47 kDa MAPK may trigger hypersensitive cell death in plants. In accordance with such a role, it was shown that the phosphatase inhibitor calyculin A can both induce a prolonged activation of the 47 kDa MAPK and a hypersensitive cell death. Moreover, staurosporine can selectively block PiE-induced activation of the kinase and the induction of defense gene accumulation by both PiE and TvX elicitor without affecting TvX-induced MAPK activation and hcd (Suzuki and Shinshi, 1995; Suzuki *et al.*, 1995, 1999).

In general, MAPK activation has been shown for a large number of plant-pathogen interactions, during both race-specific and non-host resistance responses. However, still little is known about the other components of the elicitor-induced MAPK pathways. Several groups have tried to address this question by the use of different inhibitors and activators of known components of the signaling pathways in plant-pathogen interactions. From these results it can be concluded that MAPK cascades in most cases function downstream of a Ca^{2+} influx. It is known that Ca^{2+} plays an important role in many signaling pathways (Bush,

1995) and an important role of Ca^{2+} in the pathogen response of plants has been proposed by different groups (Dietrich *et al.*, 1990; Viard *et al.*, 1994; Nürnberger *et al.*, 1994; Zimmerman *et al.*, 1997). Although influx of extracellular Ca^{2+} appears to be necessary, it is not sufficient for elicitor-induced MAPK activation in several systems (Suzuki and Shinshi, 1995; Ádám *et al.*, 1997; Lebrun-Gracia *et al.*, 1998; Romeis *et al.*, 1999). Two reports showed that MAPKs act upstream or independent of the oxidative burst (Ligterink *et al.*, 1997; Lebrun-Gracia *et al.*, 1998), and recently, Romeis and co-workers (1999) demonstrated that in their system activation of both WIPK and SIPK occurs independently of the oxidative burst, since both processes could be blocked separately. Treating cells with a PLA inhibitor resulted in a strongly reduced Avr9-induced MAPK activation (Romeis *et al.*, 1999), suggesting that phospholipases may also be involved in MAPK activation. These results are summarized in Figure 7, but it should be noted that the signaling pathways activated by the different elicitors are not necessarily composed of the same components.

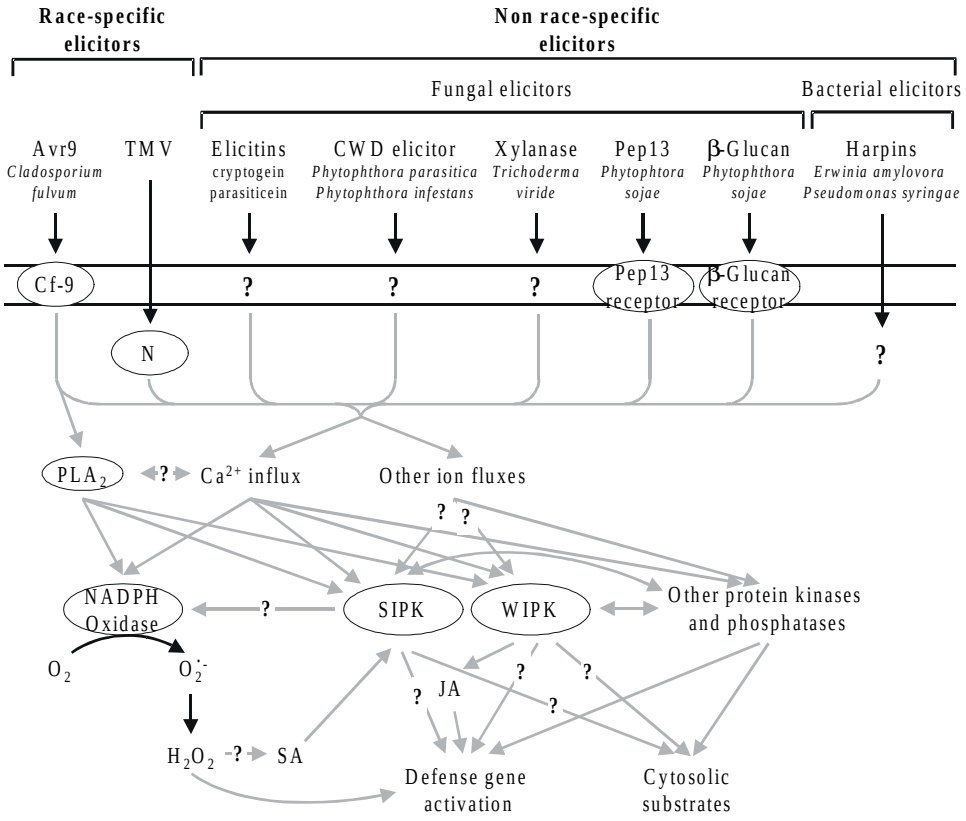

Fig. 7. **Schematic illustration of the major components involved in pathogen-induced MAPK activation.** Question marks show uncertain connections or unknown components. Black arrows indicate direct interaction / activation whereas grey arrows indicate indirect connections. See text for details.

V.B. The role of MAPK pathways in signaling of plant hormones

The focus of investigations concerning MAPK pathways in plants has been on stress responses. It is known that MAPK pathways also play an important role in the stress signaling in yeast and mammals, but mammalian MAPK pathways certainly play an equally important role in the signaling of hormones. In plants, five "classical" hormones are known. The plant hormones cytokinin, gibberellin, and auxin are known to have a global effect in promoting, and ethylene and abscisic acid in inhibiting growth and cell division (Kende and Zeevaart, 1997). Little is known about the role of MAPK pathways in signaling plant hormones, but there is culminating information that suggests at least some roles for MAPKs in the signaling pathways of all of these hormones (Fig. 8).

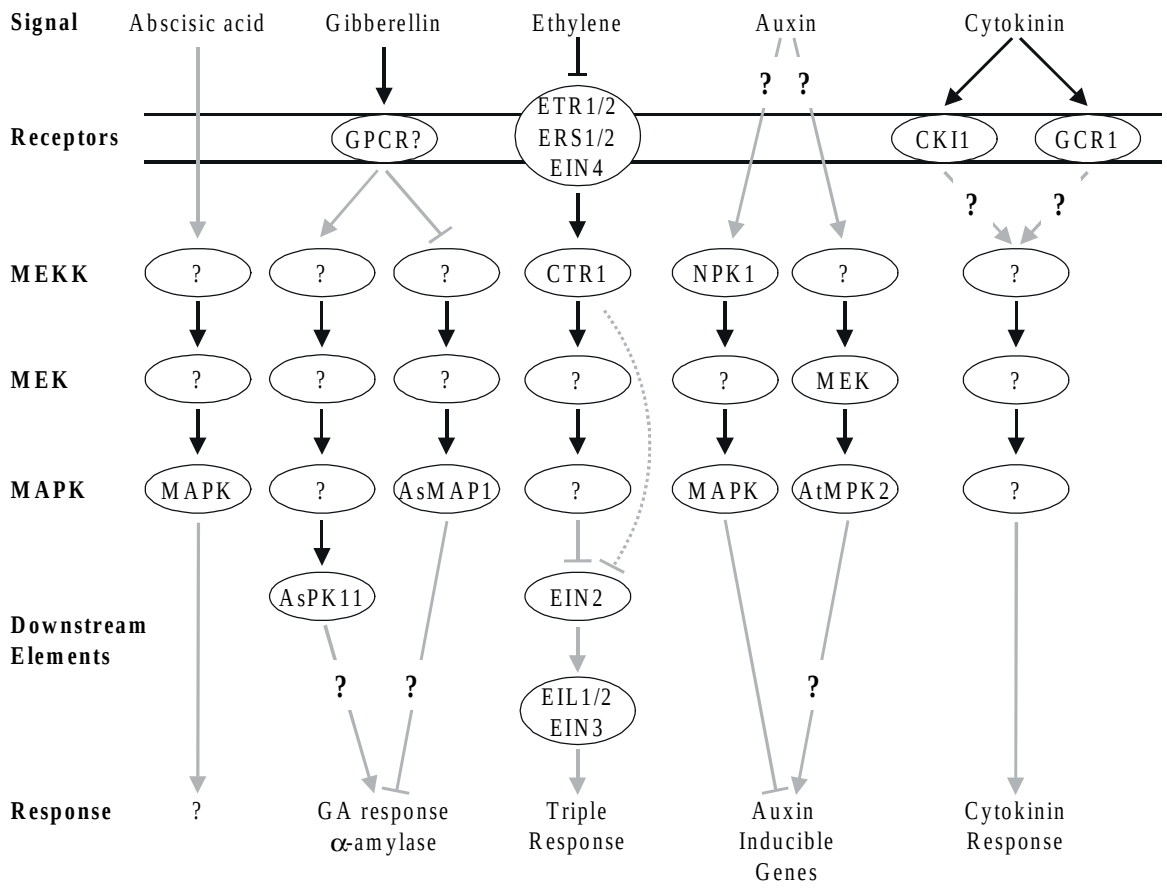

Fig. 8. **Schematic illustration of the (possible) role of MAPK cascades in plant hormone signaling.** Question marks show uncertain connections or unknown components. Black arrows indicate direct interaction / activation, whereas grey arrows indicate indirect connections. See text for details.

Besides the five "classical" plant hormones, other plant hormones have been discovered (Creelman and Mullet, 1997), including brassinosteroids and compounds that play an important role in the defense response in plants, like SA, JA and systemin (Dürner *et al.*, 1997; Wasternack and Parthier, 1997; Ryan and Pearce, 1998), and the possible role of MAPKs in the signaling pathway of these hormones have already been discussed in section V.A.

V.B.1. Abscisic acid

Abscisic acid (ABA) plays a role in seed maturation and germination, stomatal regulation, and many abiotic stress responses. Evidence exists for a role of ABA in response to dehydration, salt, osmotic, and low temperature stress (Leung and Giraudat, 1998). The important role for ABA in stress adaptation is emphasized by its ability to induce certain stress-induced genes in the absence of stress (Shinozaki and Yamaguchi-Shinozaki, 1996).

Making use of protein kinase and protein phosphatase inhibitors, a role for phosphorylation in ABA-mediated signaling has been shown in several systems (Chapman *et al.*, 1975; Hey *et al.*, 1997). Several ABA-responsive kinases (Anderberg and Walker-Simmons, 1992; Gómez-Cadenas *et al.*, 1999; Hwang and Goodman, 1995), and phosphatases (Leung *et al.*, 1997) have also been cloned. Important information about ABA signaling mechanisms has come from the analysis of ABA-insensitive *Arabidopsis* mutants (reviewed in Bonetta and McCourt, 1998). Among these are the well studied *abi1* and *abi2* mutants, which are affected in ABA-mediated effects on germination, stomatal closure and root growth, implying ABI1 and 2 in the early steps of ABA signaling. ABI1 and 2 turned out to be protein phosphatases of type 2C (PP2Cs) (Leung *et al.*, 1997). Components of MAPKs pathways have been shown to be possible substrates for PP2Cs in plants and other organisms (Meskiene *et al.*, 1998a; Hanada *et al.*, 1998), but there are no indications of such a role for ABI1 or 2. It is sure that ABA signaling involves a complex interplay between various kinases and phosphatases and this complexity is illustrated by the fact that both kinase and phosphatase inhibitors block ABA-mediated gene expression of the ABA-responsive dehydrin gene in pea (Hey *et al.*, 1997).

Although no ABA-responsive MAPKs have been cloned so far, there are indications for a role of MAPKs in ABA signaling. Aleurone protoplasts are a model system for the analysis of ABA signaling. In this system, ABA inhibits the production of hydrolitic enzymes and apoptosis (Grill and Himmelbach, 1998; Wang *et al.*, 1996). Analysis of MAPK activity in barley aleurone protoplasts identified a MAPK-like protein that cross-reacted with an ERK1 antibody and was activated rapidly and transiently after ABA treatment (Knetsch *et al.*, 1996). ABA treatment in the presence of the protein tyrosine

phosphatase inhibitor phenylarsine oxide (PAO) resulted in a complete block of both MAPK activity (Knetsch *et al.*, 1996), and ABA-induced gene expression (Heimovaara-Dijkstra *et al.*, 1996). These results suggest a model in which a tyrosine phosphatase activates a MAPK pathway that in turn activates transcription of several ABA-response genes. Although MAPK pathways are usually activated by protein kinases, there are examples for phosphatase regulation of MAPK pathways in *Xenopus*, mammals, and yeast (Tang *et al.*, 1995; Sun and Tonks, 1994; Nguyen and Shiozaki, 1999).

Another system often used to study ABA signaling is guard cells. Guard cells respond to ABA with osmotic-controlled shrinking, resulting in stomatal closure (Grill and Himmelbach, 1998). In guard cells of fava bean, a calcium-independent protein kinase of 48 kDa was activated upon ABA-treatment, and named ABR kinase for ABA-responsive protein kinase (Mori and Muto, 1997). This kinase used preferentially MBP as a substrate and was poorly phosphorylating histone or casein. Considering its size and its substrate specificity, this kinase could be a MAPK. However, the kinase could not be immunoprecipitated by an anti-phosphotyrosine antibody (Mori and Muto, 1997). Li and Assman (1996) detected a kinase of similar molecular mass, which was named AAPK for ABA-activated protein kinase that could efficiently use histone as a substrate. These results indicate a clear role for serine/threonine protein kinases in ABA signaling in guard cells, but whether these kinases are MAPKs remains to be resolved.

The fact that many abiotic stresses can induce MAPKs (see section V.A.3) and that ABA was able to activate a MAPK-like kinase, might suggest that MAPK activation by all abiotic stresses is mediated by ABA. However, it was clearly shown that the alfalfa SAMK is not activated by ABA (Jonak *et al.*, 1996), making this mechanism unlikely to be generally valid. Compared to ABA accumulation, MAPKs are activated much earlier by abiotic stresses. Therefore, MAPKs might be involved in activating ABA synthesis. On the other hand, it is equally likely that the stress-activated MAPKs act on ABA-independent pathways. Future investigations are required to differentiate between these possibilities and may be helped by a genetic analysis of MAPK mutants.

V.B.2. Gibberellin

Gibberellins (GA) have an important role in the regulation of plant growth. They affect germination, leaf expansion, shoot elongation, flowering, fruit development and mobilization of seed reserves (reviewed in Hooley, 1994). Some components of the signaling pathways that GA uses to perform its function have been identified and many more have been suggested to play a role. Among these are heterotrimeric G-proteins, calmodulins, CDPKs, and transcription factors (Bethke and Jones, 1998).

Huttly and Phillips (1995) isolated several kinase genes from oat aleurone cells. Among these, two turned out to be responsive to GA. The first one turned out to code for a MAPK named AsMAP1. Aleurone cells incubated in the absence of GA had much higher *AsMAP1* transcript levels as had cells incubated in the presence of GA (Huttly and Phillips, 1995). Aleurone cells are known to synthesize and secrete hydrolitic enzymes into the endosperm and start the breakdown of stored aleurone reserves in response to gibberellin. AsMAP1 could have a role as negative regulator of GA-induced events, preventing premature degradation of the grain resources. The finding that treatment of aleurone cells with the phosphatase inhibitor okadaic acid leads to insensitivity to GA (Kuo *et al.*, 1996), supports such a model, since okadaic acid has been shown to be able to block MAPK pathway inactivation (Haystead *et al.*, 1994; Chajry *et al.*, 1996).

The other gene, *AsPK11*, has some homology to mammalian ribosomal S6 kinases and its transcripts are strongly induced by GA. As discussed earlier, ribosomal S6 kinases are known MAPK substrates in mammals and a similar pathway could exist in plants. Since G-proteins are known to activate yeast and mammalian MAPK cascades (Herskowitz, 1995; Gutkind, 1998), the involvement of heterotrimeric G-proteins in gibberellin-induced gene expression could be a further indication for a role of MAPKs in this process (Jones *et al.*, 1998), but more work is required to clarify the role of MAPK pathways in GA signaling.

V.B.3. Ethylene

The plant hormone ethylene plays a role in many signaling pathways in plants. Besides a role in plant growth and developmental aspects, like fruit ripening, senescence of leaves and flowers, and sex determination, it also has a function in the induction of certain defense responses, such as after flooding, wounding and pathogen attack (Johnson and Ecker, 1998).

Raz and Fluhr (1993) were the first to show a role of protein phosphorylation in ethylene signaling. Ethylene treatment of tobacco leaves induced rapid protein phosphorylation and the kinase inhibitor K252a could block both protein phosphorylation and ethylene-induced defense responses.

Besides these reports, most of our knowledge about ethylene signaling has come from analysis of mutants with an altered ethylene response (Solano and Ecker, 1998; Fluhr, 1998).

Dark-grown dicot seedlings show a shortening and radial swelling of the hypocotyl, inhibition of root elongation, and exaggerated apical curvature when exposed to ethylene. These responses are collectively called the “triple response” and can easily be used to screen for mutants with an altered ethylene response. The mutants that are insensitive to ethylene

are termed *etr* for ethylene-resistant (ETR1 and 2) or *ein* for ethylene-insensitive (EIN1-7). Another class of mutants show a constitutive triple response in the absence of ethylene. This class can be divided into 2 subgroups. One group of mutants overproduces ethylene, whereas the other group does not produce more ethylene than normal and therefore must be affected in the perception or transduction of ethylene. Several mutants of the second group fall into a single complementation group termed *ctr*1 for constitutive triple response (Kieber *et al.*, 1993).

Of the genes responsible for the ethylene insensitive mutants, *ETR1* and *2*, and *EIN4* turned out to code for proteins with homology to two-component histidine receptor kinases and are thought to be ethylene receptors. Based on their homolgy, several additional members of the ethylene receptor family have been isolated, among which are ERS1 and 2 for ethylene response sensor. Two-component receptors consist of a sensor protein with a histidine kinase domain and a response regulator protein (Wurgler-Murphy and Saito, 1997; Chang and Stewart, 1998). In ETR1, 2 and EIN4 these two components are present in the same protein. ERS1 and 2 lack the response regulator. Of the ethylene receptor family, ETR1 was the first member to be cloned and most information is available for this receptor. Besides the fact that *etr1* mutants bind significantly less ethylene than wild type plants, it was shown that ETR1 has histidine kinase activity *in vitro* (Gamble *et al.*, 1998) and is able to bind ethylene with its N-terminus when expressed in yeast (Schaller and Bleecker, 1995).

The *CTR1* gene codes for a protein with strong homology to the Raf family of protein kinases. *ctr1* mutants are recessive loss-of-function or reduction-of-function mutations (Kieber *et al.*, 1993), and CTR1 must therefore act as a negative regulator of ethylene signaling. By epistasis analysis it was shown that CTR1 is downstream of the isolated ethylene receptors (Hua and Meyerowitz, 1998). Furthermore, it was shown that CTR1 can interact with ETR1 and ERS1 *in vitro* (Clark *et al.*, 1998). From these results it is speculated that the ethylene receptors bind to and subsequently activate CTR1 by yet unknown mechanisms. However, also additional factors could be involved in CTR1 activation. A 14-3-3 protein is a candidate to be one of these factors, since certain isoforms were found to interact with both CTR1 and ETR1 in a two-hybrid assay (Solano and Ecker, 1998). CTR1 homologues are expected to be present in a range of other plant species (Kieber *et al.*, 1993), and it is clear that at least in tomato a similar ethylene response pathway exists. The tomato ERS1 homologue is encoded by the *Never-ripe* gene (Wilkinson *et al.*, 1995), and two CTR1 homologues from tomato have been isolated (TCTR1 and 2) (Johnson and Ecker, 1998; Lin *et al.*, 1998). TCTR1, which is 95% identical to CTR1, is transcriptionally upregulated upon ethylene treatment (Johnson and Ecker, 1998).

Besides the ethylene receptor family, all other ethylene insensitive mutants act downstream of CTR1. EIN2, 5, 6 and 7 are thought to have a role in mediating the ethylene signal from the putative MAPK cascade to the nucleus. EIN3 is a protein with sequence

features similar to transcription factors and possesses a functional nuclear localization signal (Chao *et al.*, 1997). Furthermore, EIN3 showed transactivation activity in yeast. Besides EIN3, two other proteins with similar characteristics have been cloned that were named EIL1 and 2 (for EIN3-like). It has been proposed that these proteins represent a novel class of transcriptional regulators involved in the regulation of ethylene response genes (Chao *et al.*, 1997).

At the moment the only pathway known to contain both a two-component phosphorelay system and a complete MAPK cascade is the hyper-osmosensing pathway of yeast (Posas *et al.*, 1996). Interestingly, SLN1, the receptor kinase of this pathway, is inactivated upon sensing high osmolarity, similar to the proposed inactivation of the ethylene receptors upon ethylene binding (Hua and Meyerowitz, 1998). It will be interesting to find out if the similarity of these two pathways can be extended and whether a complete MAPK cascade exists for ethylene signaling. To date, no MKKs or MAPKs have been identified that could be downstream targets of CTR1. However, ethylene treatment of tobacco leaves resulted in fast activation of a 50 kDa MBP kinase (Sessa *et al.*, 1996). This kinase could be a candidate for a MAPK downstream of CTR1. It was, however, suggested that this kinase activity could come from the ethylene-responsive PK12 kinase. PK12 is a dual-specificity kinase of the LAMMER family and was shown to be activated by ethylene with a similar kinetics as the 50 kDa MBP kinase (Sessa *et al.*, 1996). It should be remembered that there are also cases in mammals where MKKKs activate pathways not involving MAPKs (Lee *et al.*, 1997a), and this could also be the case for the CTR1 pathway.

V.B.4. Auxin

Many important aspects of plant growth and development are regulated by auxin. Among these are apical dominance, tropic responses, lateral root formation, root hair formation and vascular tissue differentiation (reviewed in Abel and Theologis, 1996; Hobbie *et al.*, 1994). Little is known about the signaling pathway(s) that mediate the multiple auxin effects.

Cumulating evidence suggests a role for protein kinases and/or phosphatases in auxin signaling. In 1978, Murray and Key reported for the first time an effect of auxin on protein phosphorylation. Treatment of soybean with auxin led to an enhanced phosphorylation of nuclear proteins. Other indications for a role of kinases or phosphatases in auxin signaling came from experiments with pea epicotyl segments and bean plants, which also showed auxin-induced changes in protein phosphorylation (Reddy *et al.*, 1987; Poovaiah *et al.*, 1988).

The first indication that a MAPK could be one of the kinases involved in auxin signaling came from experiments, where it was shown that treatment of auxin-starved tobacco BY-2 cells with the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in a rapid and transient activation of an MBP protein kinase of 46 kDa (Mizoguchi *et al.*, 1994). Moreover, the activity of a protein kinase, which was able to phosphorylate recombinant AtMPK2 protein, also increased rapidly after auxin treatment. These results suggest that MKKs and MAPKs could have a role in auxin mediated cell cycle re-entry. However, in a recent report, Tena and Renaudin (1998) could not reproduce the protein kinase activation upon auxin treatment. Only treatment of cells with very high and toxic 2,4-D concentrations resulted in an activation of a 47 kDa MBP kinase, but the same activation was also observed after treatment with the inactive auxin analogue 2,3-D. It was also shown that the activated MBP kinase is most probably a MAPK, since it could be detected by MAPK-specific antibodies. Activation of this MAPK was shown to be correlated and probably triggered by a cytosolic acidification (Tena and Renaudin, 1998). The activation of a MAPK pathway by such a mechanism is likely to be of physiological relevance, because elicitor treatment of cells was shown to induce a cytosolic acidification (Mathieu *et al.*, 1996).

MAPKs may also be involved in negatively regulating auxin responses, because expression of the tobacco MKKK, NPK1, in maize cells results in inhibition of auxin-induced gene expression (Kovtun *et al.*, 1998). Blocking of the auxin activation of the GH3 and ER7 auxin-responsive promoters by NPK1 was shown to be dependent on NPK1 kinase activity. Furthermore, there was a correlation between the kinase activity of the different NPK1 mutants and a MAPK activity in the maize cells. Deactivation of this MAPK by a MAPK-specific phosphatase (mouse MPK1) completely eliminated the NPK1 effect (Kovtun *et al.*, 1998). These results indicate that NPK1 is able to activate an endogenous MAPK cascade in maize, which in turn is responsible for inhibiting auxin-induced gene expression. Interestingly, *NPK1* was also reported to be transcriptionally activated upon auxin treatment of tobacco roots (Nakashima, 1998), and therefore it could be postulated that NPK1 may serve a role in a negative feedback mechanism for auxin-induced gene expression.

V.B.5. Cytokinin

Cytokinins are suggested to have a role in a wide variety of developmental processes like photomorphogenesis, chloroplast biogenesis and maintenance, and senescence. In combination with auxin, it is also regulating several aspects of growth and development (Hobbie *et al.*, 1994). Little is known about the mechanisms of cytokinin action and the

signaling pathways that lead to these responses. However, the isolation of mutants for cytokinin requirement has given some information about its signal transduction pathway (reviewed in Kakimoto, 1998).

At the moment there is no indication for a role of a MAPK pathway in cytokinin signaling. However, a possible upstream component of such a pathway has been isolated. Two *Arabidopsis* mutants were isolated that exhibit a constitutive cytokinin response in the absence of cytokinin (Kakimoto, 1996). One of the genes responsible for such a phenotype turned out to be a homologue of the ethylene receptor ETR1, consisting of a histidine kinase domain, a receiver domain and a putative input domain. It turned out that overexpression of this gene, named *CKI1* for cytokinin insensitive 1, was responsible for the constitutive cytokinin response phenotype (Kakimoto, 1996), suggesting that CKI1 could function as a cytokinin receptor.

Several cytokinin-induced genes encode proteins with similarity to response-regulators of two-component systems and may function together with CKI1. The same proteins also have regions that could function as transcriptional activators (Kakimoto, 1998). Thus, cytokinin signaling could function similar to the *E. coli* EnvZ-OmpR system, where EnvZ is the sensory kinase that activates the response-regulator OmpR which functions directly as a transcription factor (Chang and Stewart, 1998). However it is also feasible that CKI1 could be the start of a signaling pathway, leading into a MAPK cascade, as is proposed for the ethylene pathway (Fluhr, 1998), and is known for the yeast osmosensing pathway (Posas *et al.*, 1996).

Besides the CKI1 signaling pathway, an additional cytokinin pathway has been proposed recently. A putative *Arabidopsis* seven transmembrane receptor was isolated (GCR1) and antisense GCR1 plants show a reduced cytokinin response (Plakidou-Dymock *et al.*, 1998). This makes GCR1 another candidate for a cytokinin receptor. At the moment nothing is known about possible downstream components of the GCR1 pathway, but yeast and animal signaling pathways are known where seven transmembrane receptors feed into MAPK cascades via heterotrimeric G-proteins (Herskowitz, 1995; Gutkind, 1998).

V.C. The role of MAPK pathways in plant development

V.C.1. The cell cycle

Mammalian and yeast MAPKs also function in regulating the cell division cycle. In fact the name “mitogen-activated protein kinase” refers to their ability to function in transducing signals that induce proliferation of cell cycle-arrested mammalian cells, but MAPKs are also involved in other cell cycle events. In *Xenopus*, a MAPK gets activated

upon entry of meiosis (Gotoh *et al.*, 1991) and is required for the spindle assembly checkpoint (Takenaka *et al.*, 1997). ERK1 and ERK2 play an important role in cell cycle re-entry (Pagès *et al.*, 1993; Brunet *et al.*, 1999), and FUS3 and KSS1 from *Saccharomyces cerevisae* and SPK1 from *Schizosaccharomyces pombe* are yeast MAPKs involved in cell cycle regulation (Gustin *et al.*, 1998).

The first evidence for a role of protein phosphorylation in the regulation of the plant cell cycle was the finding that kinase inhibitors can block the entry into mitosis and the disassembly of the preprophase band (Katsuta and Shibaoka, 1992). Furthermore, protein kinase inhibitor treatment prolongs metaphase (Wolniak and Larsen, 1995).

Several studies have tried to look for plant MAPKs with a function in the cell cycle or in cell cycle re-entry. The cDNA for the pea MAPK PsD5 was isolated from a growing bud library, and therefore it was hoped to have some role in cell cycle control (Stafstrom *et al.*, 1993). It could be shown that *PsD5* transcript accumulation was correlated with cell proliferation, but not with a particular phase of the cell cycle (Devitt and Stafstrom, 1995).

A tobacco homologue of PsD5 could also have a function in controlling the cell cycle. Tobacco suspension cultured cells can be arrested in G_1 by phosphate starvation. After re-addition of phosphate the cells re-enter the cell cycle, and a MAPK is activated fast and transiently during this process (Wilson *et al.*, 1998). Activation occurred prior to the entry of the cells into S-phase, and could therefore have a function in the reactivation of the cell cycle. There is, however, no direct proof for such a role for this MAPK. Additional data that suggest a role for PsD5 homologues in cell cycle control came from the finding that the alfalfa PsD5 homologue the stress-induced MAPK (SIMK, previously named MsK7) is expressed in a cell cycle-dependent manner (Jonak *et al.*, 1993). *SIMK* transcripts could be detected at low levels in G_1, but transcript levels increased during S and G_2 phase. A clone identical to SIMK was isolated from the infectable zone of *Rhizobium*-inoculated alfalfa roots, but there are no indications for a specific role of SIMK in the mitogenic induction in symbiotic root nodules (Duerr *et al.*, 1993). Besides these MAPKs, two isolated MKKK kinases, BnMAP4Kα1 and 2, also show a cell cycle-dependent expression with highest expression in G_2 (Leprince *et al.*, 1999).

Receptor-like kinases (RLKs) from plants are structurally similar to mammalian receptor protein kinases (RPKs), and are thought to have an important role in plant development (Becraft, 1998; Lease *et al.*, 1998). The best characterized RLK is CLAVATA1 (CLV1), which has been proposed to maintain the proper balance between proliferation and differentiation of meristematic cells (Clark *et al.*, 1997). It was found that upon activation, CLV1 forms a protein complex together with a PP2C-like phosphatase (KAPP), a Rho GTPase-related protein, and other unknown factors (Trotochaud *et al.*, 1999). The fact that mammalian RPKs often feed into MAPK pathways and the apparent involvement of small G-proteins in CLV1 signaling, might suggest a role for MAPKs in the CLAVATA pathway.

The best indication for an involvement of plant MAPKs in the cell cycle have been derived from studies on the alfalfa MMK3 MAPK and its tobacco homologue *Nicotina tabacum* FUS3-like kinase 6 (NtF6) (Bögre *et al.*, 1999, Calderini *et al.*, 1998). It was shown that the activation levels for these MAPKs are fluctuating in a cell cycle-dependent manner. The kinases were exclusively active in ana- and telophase. Furthermore, destabilizing microtubules blocked their activation in anaphase. During mitosis they are localized at the phragmoplast in late anaphase and stay localized to the cell plate during telophase. These results strongly suggest that MMK3 and NtF6 have a function in cytokinesis, but their targets are so far unknown. The kinases could function in phragmoplast construction or in regulating the vesicle transport to the cell plate. Both the localization (Shapiro *et al.*, 1998; Zecevic *et al.*, 1998) and the activation (Tamemote *et al.*, 1992) of MMK3 and NtF6 during mitosis in plants are similar to that of ERK1/2 in mammals and these results suggest a similar role for these MAPKs in mitosis. Recent data on the mRNA and protein localization of NtF6 homologues in pepper and onion support a role for NtF6 in mitosis. Both protein and mRNA levels were significantly higher in proliferating cells compared to quiescent cells (Préstamo *et al.*, 1999).

The tobacco MKKK, NPK1, was shown to have the highest expression levels in suspension cultured cells during the logarithmic growth phase (Banno *et al.*, 1993a), and in meristematic and developing tissues (Nakashima *et al.*, 1998). Furthermore, it could be shown that *NPK1* transcripts accumulate after induction of cell proliferation (Nakashima *et al.*, 1998). In addition, the *Arabidopsis* NPK1 homologues (*ANP1-3*) also showed higher transcript levels in proliferating tissues (Nishihama *et al.*, 1997). During the cell cycle, NPK1 protein and mRNA is present from S to M phase, with slightly higher levels in M phase (Machida *et al.*, 1998).

Two kinesin-like proteins were isolated that can interact with NPK1 and are also able to activate NPK1 in a yeast complementation assay and were therefore named NACK1 and 2, for NPK1 activating kinesin-like proteins (Machida *et al.*, 1998). Interestingly ERK1 and 2 are also complexed with a microtubule motor protein, CENP-E (Zecevic *et al.*, 1998). Similar to CENP-E, NACK1 accumulates only in the M phase and is localized to the center of the phragmoplast together with NPK1. NACK1 is not only a possible activator of NPK1, but may also be its substrate, implying some kind of feedback mechanism. An MKK belonging to the NACK1-NPK1 pathway has also been identified (Machida *et al.*, 1998), but the identity of the MAPK that functions in this pathway is not yet known. Since MMK3 and NtF6 have a similar localization as NACK1 in mitosis, these kinases could be good candidates to belong to the same pathway.

V.C.2. Pollen development

Changes in protein phosphorylation during pollen development have been reported by several groups (Clarke *et al.*, 1998; Hiscock *et al.*, 1995; Op den Camp and Kuhlemeier 1998). A role for protein kinases in the onset of embryogenesis after starvation of immature tobacco pollen has been proposed (Garrido *et al.*, 1993). Although these kinases have not been identified, MAPKs could be candidates. MAPKs have been shown to play an important role in the reactivation of the cell cycle in quiescent cells in other systems and plant homologues of upstream components of mammalian MAPK pathways, like small G-proteins and a receptor-like kinase have been shown to be involved in pollen development (Lin and Yang, 1997; Lee *et al.*, 1996).

Direct evidence for a function of MAPKs in pollen development were provided by the observation that transcripts of the tobacco MAPK *NtF4* could solely be found from the mid-bicellular to the mature stage of pollen development, and during germination of pollen a strong increase in NtF4 kinase activity could be observed (Wilson *et al.*, 1997). The activation of the kinase could be correlated with the rehydration of the pollen and was not dependent on germination of the pollen (Wilson *et al.*, 1997). NtF4 activation occurs before the pollen tube emerges and is therefore likely to play a role in early events of pollen activation. Since one of the earliest events that follows pollen hydration is a rapid rearrangement of the cytoskeleton, it could be possible that NtF4 has a function in this process. Such a role for MAPKs has been shown in mammalian systems (Reszka *et al.*, 1995; Morishima-Kawashima and Kosik, 1996). It should be mentioned that it is not completely clear if NtF4 activation has a specific function in pollen germination or that its activation is only a stress response to the swelling of pollen upon hydration. However, activation of NtF4 as a response to hypo-osmotic stress would not necessarily rule out a function in pollen germination.

V.D. The role of MAPK pathways in sugar signaling

In plants, sugars not only have metabolic functions, but are also important signals. It is known that sugars are able to regulate the expression of genes encoding sink-specific enzymes, such as extracellular invertase, and specific pathogen- and stress-response genes, such as proteinase inhibitor genes and the gene for phenylalanine ammonia-lyase (*PAL*). On the other hand, genes involved in photosynthesis are repressed by sugars (Koch, 1996).

Treatment of *Chenopodium rubrum* suspension cultured cells with glucose results in the accumulation of several transcripts, encoding enzymes, like PAL and extracellular invertase (CIN1). Furthermore, high steady state mRNA levels of the ribulose biphosphate

carboxylase (*RbcS*) gene were strongly reduced upon glucose treatment (Ehness *et al.*, 1997). Besides transcriptional effects, glucose treatment of the cell culture also resulted in the activation of a 44 and 46 kDa MBP kinase (Ehness *et al.*, 1997). The two kinases could be MAPKs, and since the same kinase activity and transcriptional changes seem also to be triggered by different elicitors, it is tempting to speculate that they could be the homologues of WIPK and SIPK, the two identified elicitor-induced MAPKs in tobacco (Zhang and Klessig, 1998b; Zhang *et al.*, 1998).

VI. MAPK pathway inactivation

As important as the activation of MAPK pathways is their inactivation. Inactivation of MAPKs is mediated by threonine and/or tyrosine dephosphorylation of the TXY motif. Several yeast and mammalian phosphatases have been cloned and characterized that are able to inactivate MAPK pathways. They can be divided into at least 3 groups: (i) The dual-specificity phosphatases that can dephosphorylate the MAPKs on both tyrosine and threonine residues (DsPTPases) (Sun *et al.*, 1993; Doi *et al.*, 1994). (ii) The tyrosine phosphatases that can solely dephosphorylate tyrosine residues (PTPases) (Wurgler-Murphy *et al.*, 1997; Van Vactor *et al.*, 1998). (iii) The serine/threonine phosphatases (PPases) like PP2A and PP2C type phosphatases (Alessi *et al.*, 1995; Millward *et al.*, 1999; Shiozaki and Russell, 1995; Takekawa *et al.*, 1998).

A broad range of plant protein phosphatases has been isolated, but little is known about their interaction with MAPK pathways. Most information about the function of phosphatases in the deactivation of plant MAPK pathways has come from inhibitor studies. Both serine/threonine and tyrosine phosphatases were shown to inactivate plant MAPKs *in vitro* (Usami *et al.*, 1995; Ádám *et al.*, 1997; Zhang and Klessig, 1997, 1998b). Furthermore it could be shown that activation of MAPKs can be sustained (Suzuki and Shinshi, 1995), or even induced solely by the use of phosphatase inhibitors (Suzuki and Shinshi, 1999), indicating a role for phosphatases in downregulating basal MAPK activity. Several groups also showed a requirement for *de novo* protein sythesis for MAPK inactivation (Suzuki and Shinshi, 1995; Bögre *et al.*, 1997; Ádám *et al.*, 1997).

Several plant protein phosphatases have been isolated that could be involved in inactivation of MAPK pathways. Among them are members for all three MAPK phosphatase classes. The first report of a possible MAPK phosphatase in plants came from Haring and co-workers (1995). They isolated a dual-specificity phosphatase from *Chlamydomonas eugametos* referred to as VH-PTP13, that had homology to members of the vaccinia virus H1 (VH)-like PTPs. VH-like PTPs can dephosphorylate and thereby inactivate MAPKs efficiently. A role for VH-PTP13 as a MAPK phosphatase was suggested by its ability to

inactivate the alfalfa MAPKs SIMK and MMK2 *in vitro* (Harding *et al.*, 1995). Futhermore, VH-PTP13 has relative high homology to the human CL-100 MAPK phosphatase and a similar expression pattern, being restricted to the beginning of the G_1-phase (Keyse and Emslie, 1992).

A role in inactivating plant MAPK pathways has also been proposed for the *Arabidopsis* dual-specificity protein phosphatase AtDsPTP1 (Gupta *et al.*, 1998), which also seems to be a member of the VH-like PTPase subfamily. AtDsPTP1 was shown to be an active phosphotyrosine and phosphoserine/threonine phosphatase and was able to dephosphorylate and inactivate the *Arabidopsis* AtMPK4 *in vitro*. AtDsPTP1 was demonstrated to be constitutively expressed in various tissues and under various conditions leaving the possibility open that AtDsPTP1 may be exclusively regulated at the post-translational level as the tyrosine phosphatases PTP2 and 3 from yeast (Wurgler-Murphy *et al.*, 1997). So far, such a mechanism was however not seen for other VH-PTPs.

The *Arabidopsis* protein tyrosine phosphatase AtPTP1 has high homology to cytoplasmic mammalian PTPases (Xu *et al.*, 1998), and is able to dephosphorylate and thereby deactivate a MAPK *in vitro* (Luan, 1998). Expression of AtPTP1 was strongly induced by high-salt conditions and its transcript levels declined under low-temperature conditions. Recently also pea and soybean homologues of AtPTP1 have been isolated (Fordham-Skelton *et al.*, 1999). There are, however, no indications for these PTPs to be MAPK phosphatases and it should be remembered that protein tyrosine phosphorylation events are implicated in many signaling cascades other than MAPK pathways, and there are indications that protein tyrosine phosphorylation is a relatively frequent event in plants (Barizza *et al.*, 1999; Trojanek *et al.*, 1996).

Tyrosine phosphorylation in correlation with MAPK activation has been shown for MsERK1 (Duerr *et al.*, 1993), NtF3, 4 and 6 (Wilson *et al.*, 1995; Wilson *et al.*, 1997), SIPK (Zhang and Klessig, 1997, 1998a) and MAPK-like activities in ABA signaling (Knetsch *et al.*, 1996), elicitor response (Suzuki and Shinshi, 1995, 1999; Ádám *et al.*, 1997), and wounding (Usami *et al.*, 1995; Stratmann and Ryan, 1997). Surprisingly, Romeis (1999) reported a transient activation of WIPK and SIPK upon Avr9 treatment of Cf9 tobacco cells, whereas tyrosine phosphorylation of these MAPKs was sustained. This would suggest an important role for threonine dephosphorylation in the MAPK inactivation in this system.

The only putative serine/threonine MAPK phosphatase isolated to date is the alfalfa protein phosphatase 2C, MP2C (Meskiene *et al.*, 1998a). MP2C was isolated by a functional yeast screen, where it could intervene with the pheromone-induced MAPK pathway, and it was able to inactivate the wound-induced SAMK pathway *in vitro*. The *MP2C* gene was transcriptionally activated upon wounding of alfalfa leaves and the kinetics of this transcriptional activation correlated very well with the inactivation of SAMK.

It was shown that the inactivation mechanism is induced after activation of SAMK, preventing reactivation of the SAMK pathway for a certain time (Bögre *et al.*, 1997). This refractory period extends over a time period of approximately the same length as the presence of *MP2C* transcripts (Meskiene *et al.*, 1998a). These results suggest that MP2C is one of the factors that is able to reset the SAMK pathway to make it accessible for subsequent sensing of changes in the environment. Furthermore, it is possible that the expression of the *MP2C* gene is regulated by the SAMK pathway itself and that it is part of a negative feedback loop (Meskiene *et al.*, 1998b), as has been shown for other systems (Sun *et al.*, 1993; Doi *et al.*, 1994; Brondello *et al.*, 1997).

VII. Concluding remarks and perspectives

Over the last few years many components of plant MAPK cascades have been isolated and information on their function is rapidly accumulating. It is clear that some MAPKs can be activated by a whole range of stimuli, and that some environmental conditions can activate more than one MAPK. This leaves the question open, how one MAPK can give rise to different responses. From our knowledge of MAPK pathways in mammals and yeast, activation of one MAPK can lead to different cellular responses and different levels and kinetics of activation may determine the outcome of the signal transduction process. Such a mechanism has been shown for both the human ERK1 MAPK cascade and the yeast SMK1 pathway. ERK1 can activate both cell proliferation and differentiation in the same cell type (Traverse *et al.*, 1992; Cowley *et al.*, 1994), depending on the amplitude and kinetics of the ERK1 activation (Marshall, 1995). SMK1 has an important role in spore morphogenesis, and distinct steps of spore morphogenesis were shown to be directly correlated with the magnitude of SMK1 activity. Increasing SMK1 activity levels allowed more and later spore morphogenesis events (Wagner *et al.*, 1999). Differences in plant MAPK activation kinetics by different stimuli have been observed and further research will have to clarify the importance of these observations.

Furthermore, specificity can be achieved by the physical separation of signaling pathways. This can happen through specific cellular localization of components of each pathway, or by differential expression of certain signal pathway components and substrates in specific cells, but probably the most important way to generate specificity occurs with the help of scaffold proteins that tether specific signaling components into a complex. Therefore, the isolation of plant scaffold proteins will be important to understand the exact regulation of the plant MAPK pathways.

Despite the increasing amount of data on the functions of certain components of MAPK cascades, little is yet known on the interaction between different components, and on

their upstream activators and downstream targets. To date, not a single MAPK substrate has been identified. Several plant transcription factors have been isolated (reviewed in Liu *et al.*, 1999), and the effect of phosphorylation on the function of some of these is also known, but none of these could be unequivocally assigned to be a MAPK substrate. Little is known about the possible upstream activators of plant MAPK cascades. There are, however, some indications that the upstream activators will be at least as diverse as in yeast and animals. Finally, despite the isolation of multiple MAPKs, MKKs and MKKKs, a placement of these components into distinct signaling pathways remains to be established. The use of both biochemical and genetic approaches should reveal the role and function of different MAPK pathways, finally unravelling the complexity of signal transduction networks that link appropriate responses to distinct extracellular signals.

Chapter 2

MAP kinases in plant signal transduction: How many, what for?

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Contents

Summary

Mitogen-activated protein kinase (MAPK) pathways are protein kinase cascades that have a function in the transduction of extracellular signals to intracellular targets in all eukaryotes. Distinct MAPK pathways are regulated by different signals and have a role in a wide variety of physiological processes. In plants there is evidence for a role of MAPKs in the signaling of pathogens, abiotic stresses, plant hormones, and cell cycle cues. A large number of distinct MAPKs in plants have been identified that are all most similar to the animal ERK MAPKs. By sequence alignment all available full length plant MAPKs can be grouped into 5 subfamilies. Functional data exist for members of 4 subfamilies and show that different subfamilies encode MAPKs for specific functions. Analysis of partial MAPK sequences from full length, truncated cDNAs and expressed sequence tags (ESTs) revealed the presence of two new subfamilies in the plant MAPK superfamily. Signature sequences valid for the superfamily of plant MAPKs and each subfamily were derived and should help in future classification of novel MAPKs. The future challenge is to unambiguously assign functions to each MAPK and decipher the other partners of their signaling pathways.

1. Introduction

Mitogen-activated protein kinases (MAPK) are serine/threonine protein kinases that can be found in all eukaryotes. The MAPK family belongs to the eukaryotic protein kinase superfamily. Within this superfamily they group together with the cyclin-dependent kinases (cdk), glycogen synthase kinases 3 (gsk3), and cell division cycle-dependent (cdc)-like kinases (Hanks and Hunter 1995).

MAPKs have a function in transducing a broad set of extracellular signals to cellular targets and perform their function as part of a protein kinase cascade. Besides MAPKs this cascade is formed by MAPK kinases (MAPKKs) and the MAPKK kinases (MAPKKKs). Signaling into the MAPK cascade can occur via tyrosine kinase receptors, G-protein-linked receptors and protein kinase C-dependent pathways. Downstream of the MAPK cascade the signal is mediated to cellular responses. MAPKs may be translocated to the nucleus to phosphorylate and thereby activate specific transcription factors or stay in the cytoplasm and phosphorylate certain enzymes like protein kinases and phosphatases or cytoskeletal components.

MAPKs are activated through phosphorylation by the dual-specificity MAPKKs also named MAPK/ERK kinases (MEKs) on both the tyrosine and threonine residues in the conserved TXY motif (Marshall CJ 1994). Phosphorylation on both residues is required for full activation of the MAPKs. Inactivation of MAPKs occurs through dephosphorylation by serine/threonine phosphatases, tyrosine phosphatases or dual specificity phosphatases (Canagarajah et al 1997).

MAPKKs are activated through phosphorylation by MAPKKKs also named MEK kinases (MEKKs) on serine and threonine/serine residues in the conserved $SXXX^{S}/_{T}$ motif. MAPK cascades have been identified in yeast and animals. Recently the first possible MAPK cascade in plants has been identified (Mizoguchi et al 1998).

Specificity of MAPK pathways is obtained by the activation through distinct MAPKKs and by the substrate specificity of the MAPKs. Much of the specificity of MAPK pathways is also obtained by physical separation of MAPK modules. Scaffold proteins have been identified that hold specific components together. Furthermore, particular MAPK pathways can mediate different extracellular signals to a broad range of responses by differential expression of certain signal pathway components and substrates in specific cells.

2. The structure of MAPKs

Sequence comparison was used to define conserved sequence motifs, establishing 11 subdomains that are present in all serine/threonine protein kinases (Hanks et al 1988).

Crystallographic analysis of ERK2 in the inactive as well as in the dual phosphorylated active conformation determined the folding of these conserved subdomains and has defined the 3-D architecture (Figure 1) (Zhang et al 1994, Canagarajah et al 1997). ERK2 has a two lobed structure, where the active site is found at the domain interface (Goldsmith and Cobb 1994). The N-terminal domain is mainly composed of β-strands whereas the C-terminal domain consist mainly of α-helices. This overall topology of ERK2 is similar to that of cAMP-dependent protein kinase and CDK2 (Taylor and Radzio-Andzelm 1994).

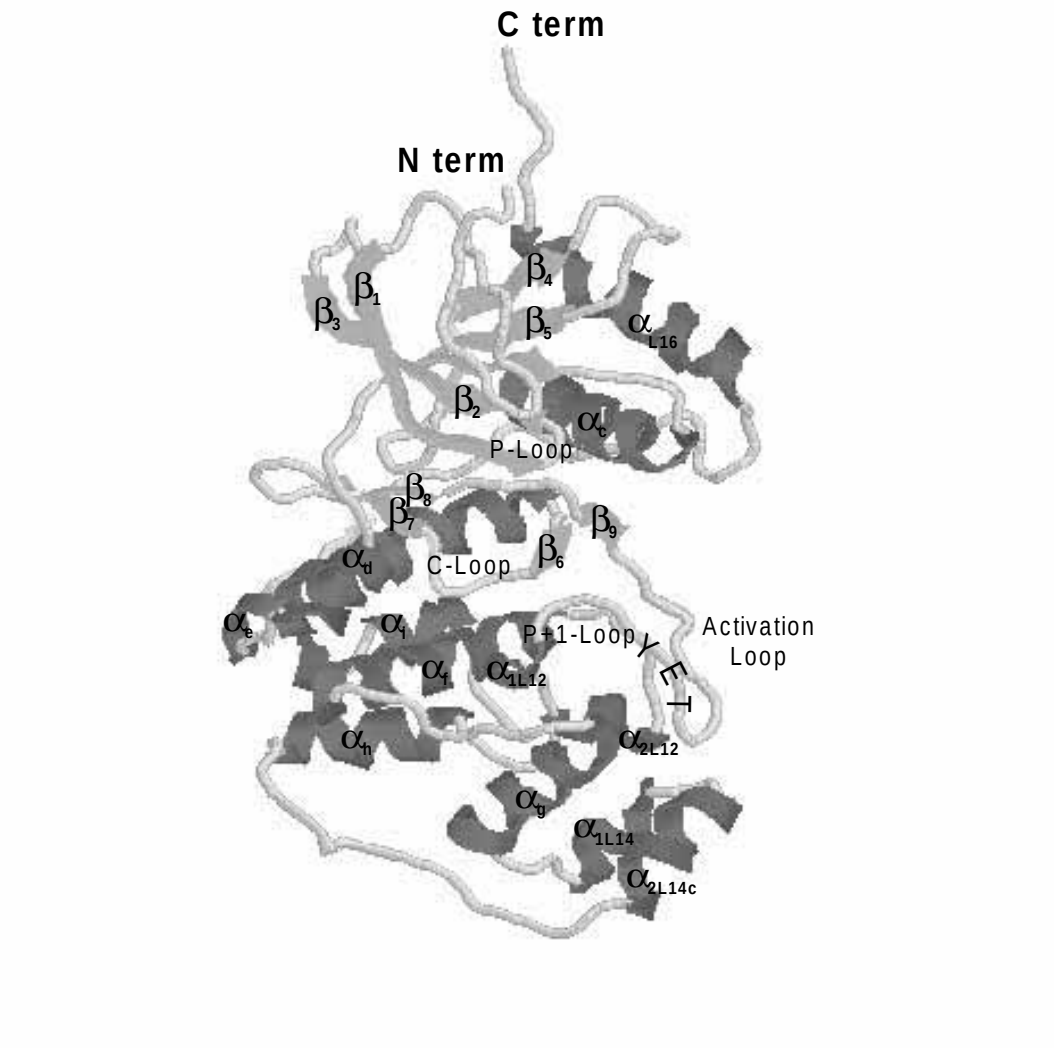


Fig. 1. **Ribbon model of the three-dimensional structure of ERK2 in the inactive, unphosphorylated state.** The image was drawn using Rasmol from Roger Sayle and the coordinates from rat ERK2 (PDB ID = 1ERK). Labels for the secondary structural elements are based on Zhang et al (1994). β strands are drawn in light grey and α helices in dark grey.

The loops between β-strands 6 and 7 and between β-strands 8 and 9 are important for forming the ATP binding pocket. The loop between β-strands 6 and 7 is called the catalytic loop (C-loop in Figure 1) and contains the aspartate that functions as the catalytic base, the lysine that binds the γ-phosphate of ATP and the asparagine that may bind a metal ion. The loop between β-strands 8 and 9 contains the aspartate that chelates the primary Mg^{2+} ion that bridges the α- and γ-phosphates of ATP. This loop may also have a function in stabilising contacts between the 2 lobes of the MAPK (Taylor and Radzio-Andzelm 1994).

Another important secondary structure element in the active site of MAPKs is the phosphate anchor loop (P-loop in Figure 1). The P-loop consists of the conserved sequence motif GXGXXG and helps to anchor the non-transferable phosphates of ATP.

MAPKs are only able to phosphorylate substrates that contain proline in the P+1 site. The P+1 site is the residue that immediately follows the serine or threonine residue of MAPK substrates. This substrate specificity is regulated by the P+1 loop. In ERK2, specific binding of substrate proline by this loop is possible after activation of the kinase.

One of the most important and least stable secondary structure element of MAPKs is the Activation Loop containing the TXY motif (Fig. 1). The Activation Loop forms the mouth of the active site. The name of this loop is based on its importance for the activation of MAPKs. Both phosphorylation sites, T at position 183 and Y at position 185 in ERK2, are located in the Activation Loop (Zhang et al 1994). In inactive ERK2, Y185 is not accessible and binding of the MEK to the MAPK already induces conformational changes so that both T183 and Y185 become accessible for phosphorylation. Comparison of the inactive and the activated ERK2 structures revealed strong conformational changes of the Activation Loop and of the L16 helix. These conformational changes align the catalytic residues and remodel the P+1 loop resulting in an active kinase (Canagarajah et al 1997).

3. MAPKs in yeast and animals

Only six MAPKs are found in the fully sequenced yeast genome. Five of these MAPKs have been assigned to specific pathways: pheromone signaling, filamentous growth, adaptation to high osmolarity, cell wall remodelling, and sporulation (Madhani and Fink 1998).

Presently, there are 37 human MAPK protein sequences available in the database, but many of these sequences only differ in a single amino acid or are splicing variants, reducing the number to a total of 12. The ERK kinases are known to transmit a large array of signals stimulating proliferation or differentiation. Members of the c-Jun amino-terminal kinases/stress-activated protein kinases (JNKs/SAPKs) and of the p38 kinases are involved in mediating various stresses.

4. Plant MAPKs

23 full length MAPK cDNAs have been isolated from a variety of plant species (Table 1). Among these are 9 Arabidopsis, 5 tobacco, and 4 alfalfa genes and single genes from pea, parsley and petunia. There are two single representatives from monocot species, oat and wheat. The predicted amino acid sequences of these plant MAPKs show a highly conserved overall structure with sequence similarities between 50 and 98% (Fig. 2). Highest similarity is observed in the 11 kinase subdomains (denoted by roman numerals in Fig. 2). As a reference, the animal ERK2 and p38 MAPKs are included in the alignment. Positions of β strands (β_{1-9}), α helices (α_{c-i}), and the connecting loops (L0-L16) as defined for ERK2 (Zhang *et al.* 1994) are also shown.

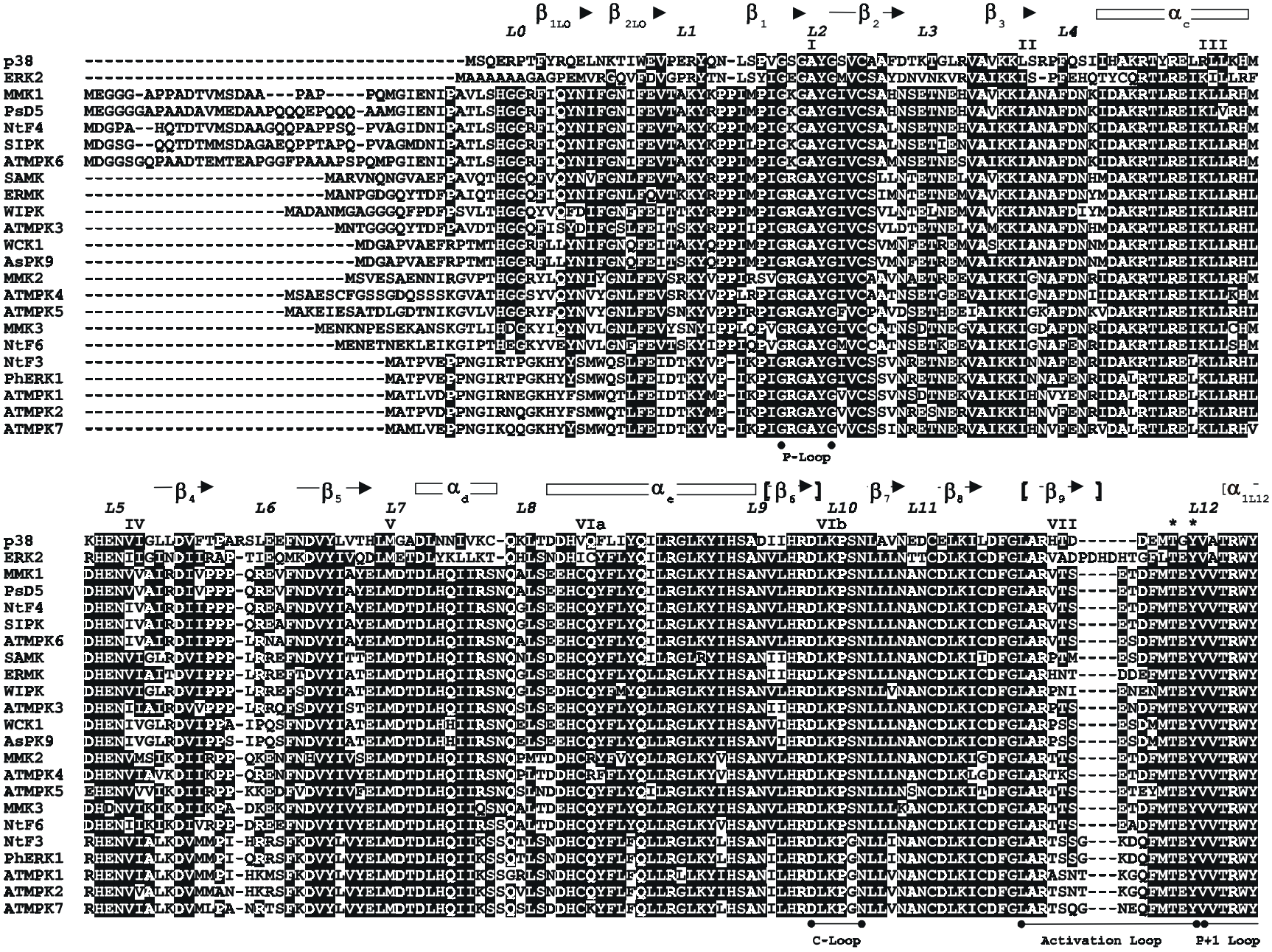
The entire MAPK family including all yeast, animal and plant sequences can be divided into 3 major subgroups: The stress-activated protein kinases (SAPKs), the extracellular signal-regulated kinases (ERKs) and the MAPK3 subgroup (Kültz 1998). Animal and fungal MAPK members can be found for all 3 subgroups. However, all plant MAPKs fall within the ERK subfamily and were therefore denoted as plant extracellular regulated protein kinases (PERK). Based on this phylogenetic analysis it was suggested that the ancestor of plant MAPKs split off from the animal/fungal lineage before the SAPK and MAPK3 subgroups evolved.

The presence of unique amino acid residues at the same positions in all members within a single subgroup can help to characterise new members. The PERK subfamily only has one unique residue if compared to the entire MAPK family (P39 in ATMPK3, Kültz 1998). This is an indication that the PERKs signal a broader range of responses than any of the individual subfamilies in animals, which are defined by a higher number of unique residues.

A striking sequence difference among members of ERK and SAPK subgroups was found in the Activation Loop. ERK2 has a 6 amino acid insertion in this loop when compared to p38, a SAPK member. PERKs contain an insertion of only 2 to 3 amino acids at this position (Fig. 2). A characteristic motif in the Activation Loop is defined by the amino acid in between the T and Y MAPK phosphorylation sites. ERK2 has an E at this position (TEY) while p38 has a G (TGY). All plant MAPKs have a TEY motif. The function of the insertion in the Activation Loop and the TEY versus TGY motif was studied by site directed mutagenesis (Jiang *et al.* 1997). It was found that the dual phosphorylation motif and the insertion loop influence substrate specificity but are not crucial for the specificity of activation by upstream signals, while the length of the insertion loop plays a role in controlling autophosphorylation.

Table 1. **Summary of the isolated plant MAPK sequences**

Name	Species	Accession no.	Amino acids no.	MW (kDa)	pI (pH units)	Group / subfamily	Reference
ATMPK1	*Arabidopsis thaliana*	D14713	370	42.7	6.8	3 / PERK5	Mizoguchi *et al.* (1994)
ATMPK2	*Arabidopsis thaliana*	D14714	376	43.1	6.1	3 / PERK5	Mizoguchi *et al.* (1994)
ATMPK3	*Arabidopsis thaliana*	D21839	370	42.7	5.5	1 / PERK2	Mizoguchi *et al.* (1993)
ATMPK4	*Arabidopsis thaliana*	D21840	376	42.9	5.7	2 / PERK3	Mizoguchi *et al.* (1993)
ATMPK5	*Arabidopsis thaliana*	D21841	376	43.1	5.4	2 / PERK3	Mizoguchi *et al.* (1993)
ATMPK6	*Arabidopsis thaliana*	D21842	395	45.1	5.2	1 / PERK1	Mizoguchi *et al.* (1993)
ATMPK7	*Arabidopsis thaliana*	D21843	368	42.4	6.8	3 / PERK5	Mizoguchi *et al.* (1993)
ATMPK8	*Arabidopsis thaliana*	unpublished	-	-	-	4	Mizoguchi *et al.* (1997)
ATMPK9	*Arabidopsis thaliana*	unpublished	-	-	-	4	Mizoguchi *et al.* (1997)
NtF3	*Nicotiana tabacum*	X69971	372	42.8	6.2	3 / PERK5	Wilson *et al.* (1993)
NtF4	*Nicotiana tabacum*	X83880	393	45.1	5.5	1 / PERK1	Wilson *et al.* (1995)
NtF6	*Nicotiana tabacum*	X83879	371	42.7	5.0	2 / PERK4	Wilson *et al.* (1995)
SIPK	*Nicotiana tabacum*	U94192	393	45.2	5.4	1 / PERK1	Zhang and Klessig DF (1997)
WIPK	*Nicotiana tabacum*	D61377	375	42.9	5.1	1 / PERK2	Seo *et al.* (1995)
MMK1	*Medicago sativa*	X66469	387	44.4	5.4	1 / PERK1	Duerr *et al.* (1993) Jonak *et al.* (1993)
MMK2	*Medicago sativa*	X82268	371	42.3	6.1	2 / PERK3	Jonak *et al.* (1995)
MMK3	*Medicago sativa*	unpublished	374	43.0	4.8	2 / PERK4	Bögre *et al.* (1999)
MMK4	*Medicago sativa*	X82270	371	43.0	5.6	1 / PERK2	Jonak *et al.* (1996)
AsPK9	*Avena sativa*	S56638	369	42.9	5.4	1 / ?	Huttly and Phillips (1995)
ERMK	*Petroselinum crispum*	Y12785	371	42.8	5.7	1 / PERK2	Ligterink *et al.* (1997)
PhERK1	*Petunia x hybrida*	X83440	384	44.4	6.8	3 / PERK5	Decroocq-Ferrant *et al.* (1995)
PsD5	*Pisum sativum*	X70703	394	45.1	5.3	1 / PERK1	Stafstrom *et al.* (1993)
WCK1	*Triticum aestivum*	AF079318	369	42.8	5.3	1 / ?	Takezawa (1998)


β1LO β2LO β1 β2 β3 αc
L0 L1 L2 L3 L4
I II III
p38
ERK2
MMK1
PsD5
NtF4
SIPK
ATMPK6
SAMK
ERMK
WIPK
ATMPK3
WCK1
AsPK9
MMK2
ATMPK4
ATMPK5
MMK3
NtF6
NtF3
PhERK1
ATMPK1
ATMPK2
ATMPK7
P-Loop
β4 β5 αd αe β6 β7 β8 β9 α1L12
L5 L6 L7 L8 L9 L10 L11 L12
IV V VIa VIb VII
C-Loop
Activation Loop
P+1 Loop

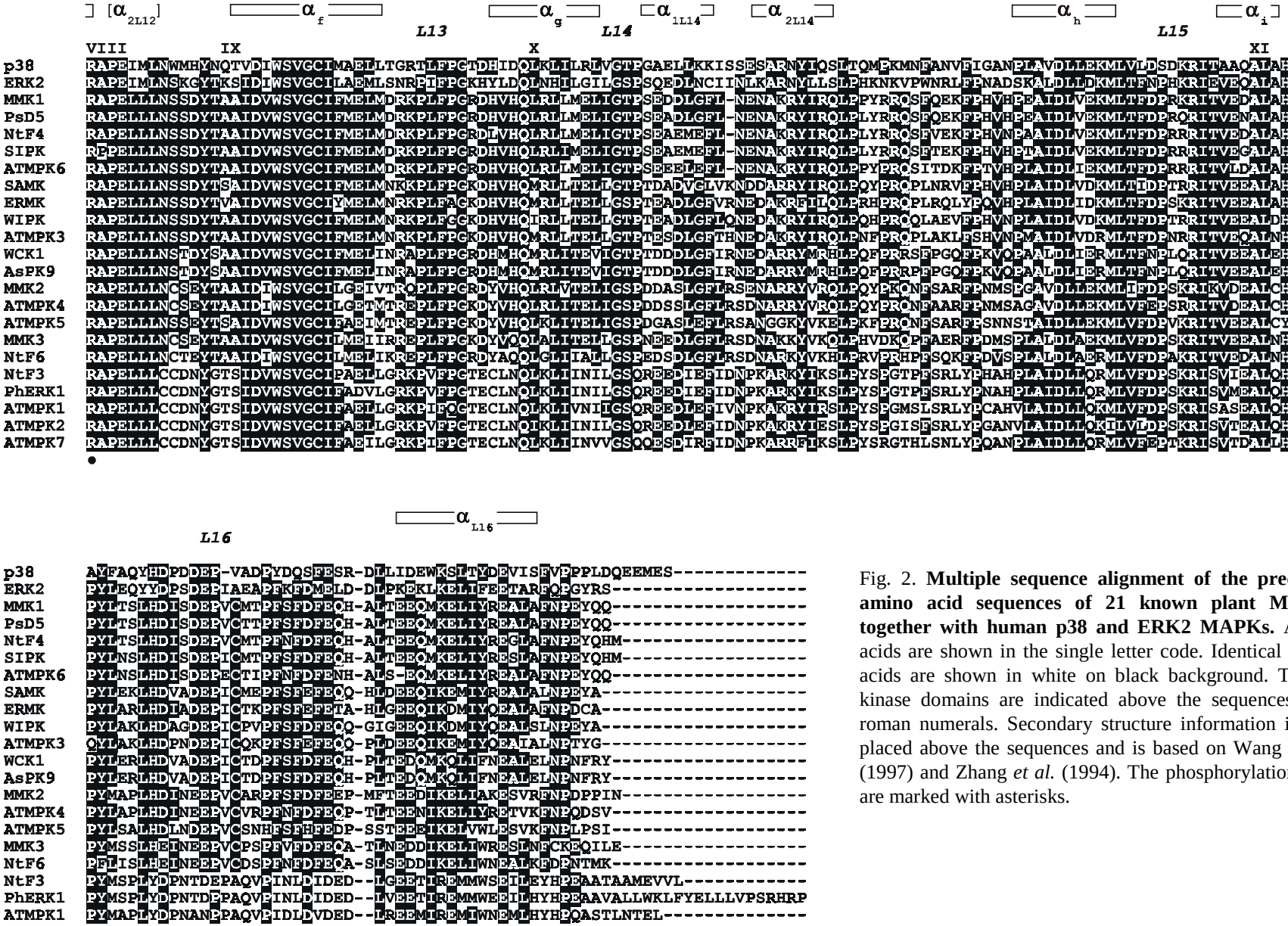


Fig. 2. **Multiple sequence alignment of the predicted amino acid sequences of 21 known plant MAPKs together with human p38 and ERK2 MAPKs.** Amino acids are shown in the single letter code. Identical amino acids are shown in white on black background. The 11 kinase domains are indicated above the sequences with roman numerals. Secondary structure information is also placed above the sequences and is based on Wang *et al* . (1997) and Zhang *et al.* (1994). The phosphorylation sites are marked with asterisks.

Besides the variable N- and C- terminal sequences found in plant MAPKs, short stretches of variable sequences are interspersed between the conserved eleven subdomains. These variable sequences occur mainly in the loops that join the secondary structural elements, and are typically found on the surface of the molecule in the ERK2 and p38 kinases. These variable parts might be important to determine the binding of a particular MAPK to upstream regulators or specific substrates. In chimeras, constructed between p38 and ERK2, an N-terminal region of 40 residues, composed of the α_c, L5, β_4, L6, β_5 structural units, was found to be involved in specifying the response of the kinase to different external signals, whereas the carboxy-terminal half of the molecule specified substrate recognition (Brunet and Pouysségur 1996).

5. Plant MAPKs can be classified into 5 groups

Acording to sequence similarity of predicted proteins, at least 3 different subgroups could be established within the plant MAPKs (Group 1-3) which could be further subdivided into at least 5 subfamilies, named PERK1-5 (Fig. 3 and Table 1). Only the 2 known MAPKs of monocots (AsPK9 and WCK1) could not be assigned unequivocally to any of these 5 subfamilies.

In addition to the phylogenetic analysis, a search was performed for unique residues characteristic for members within a group or subfamily. Due to the high sequence variability in group 1 MAPKs, no unique residues could be identified. This variability might reflect functional heterogeneity, suggesting that members of this group transmit a wide range of stimuli.

Members in the PERK1 subfamily have 13 unique residues: G3, D11, M14, A17, S41, I55, K71, V214, D260, M278, S285, R335 and A373 in ATMPK6. When compared to the other plant MAPKs, all members within the PERK1 subfamily contain a prolonged N-terminus (Fig. 2), resulting in higher calculated molecular masses when compared to other plant MAPKs (Table 1). Analysis of chimeras between ERK2 and p38 MAPKs indicated that the N-terminal region is involved in binding to upstream regulators (Brunet and Pouysségur 1996). The N-terminal extensions of the PERK1 subfamily might have a similar role. The deletion of one amino acid between subdomains X and XI was also recognised in all PERK1 subfamily members (Fig. 2).

Only 4 unique residues were found in members of the PERK2 subfamily (Q20, T58, L108, and D302 in ATMPK3). If the 2 monocot MAPKs, AsPK9 and WCK1 are also included into the PERK2 subfamily, there are 5 additional unique residues (M77, T119, N234, T259, and D270 in ATMPK3).

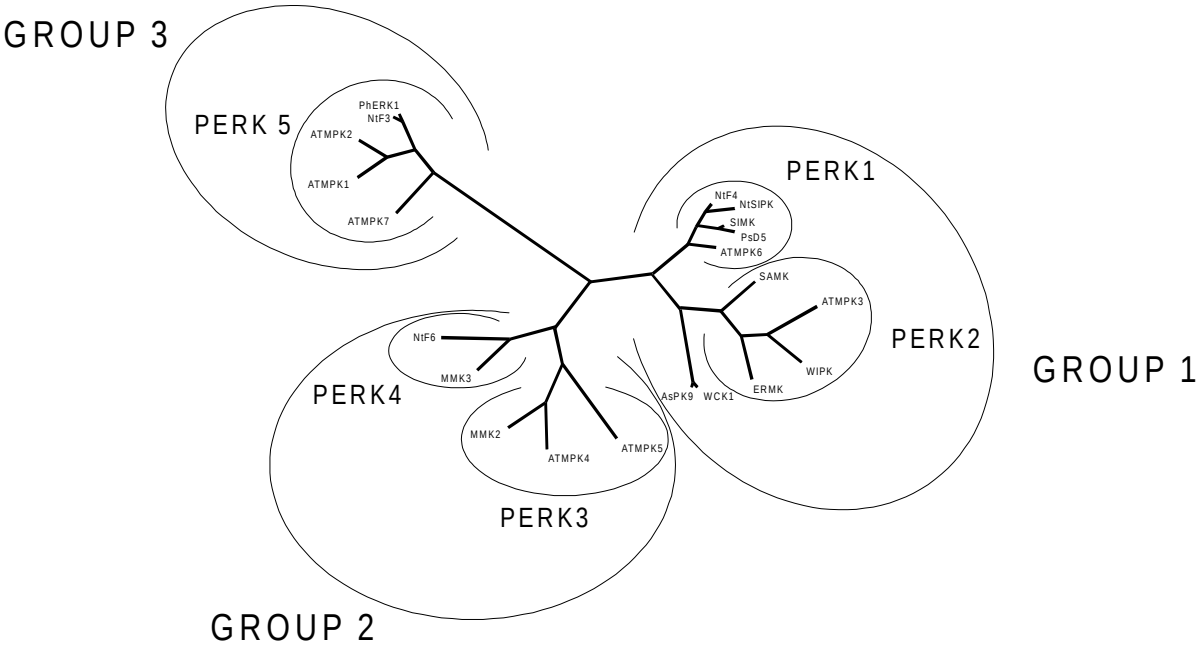


Fig. 3. **Phylogenetic tree based on the amino acid sequences of all described 21 plant MAPKs (Table 1).** This tree was reconstructed using the neighbor-joining method implemented in version 4.0.0d64 of PAUP* written by David L. Swofford. The three plant MAPK subgroups are denoted as GROUP 1-3, the 5 different subfamilies as PERK 1-5.

The PERK3 and PERK4 subfamilies are too small to define subfamily-specific unique residues. Therefore unique residues are given only for the entire group 2. These are G18, G75, E220, Y249, S273, and V280 in ATMPK4.

A total number of 55 unique residues was identified in the PERK5 subfamily (V5, N9, G10, G15, K16, H17, Y18, S20, M21, W22, G23, D29, K36, S49, L80, R87, M98, K107, L111, K125, F140, G162, Q188, C207, D209, N210, G211, T212, S213, L228, G229, T237, E238, C239, L240, N241, N248, Q253, P264, K265, S272, Y275, S276, G278, A288, Q297, S309, A331, I335, L337, D340, R349, Y359, H360, and A363 in ATMPK1). Another characteristic feature of the PERK5 subfamily is the shorter N-terminus and extended C-terminus when compared to other PERKs. PERK5 members also contain an aditional amino acid in the Activation Loop. The high sequence conservation within the PERK5 subfamily might indicate that these MAPKs are involved in some common signaling pathway and transmit related signals.

6. PERK groups: Towards a functional classification system of plant MAPKs

Information on possible functions is available for members of the PERK1, 2, 3, and 4 subfamilies. The kinase activity of NtSIPK of the PERK1 subfamily is transiently activated by salicylic acid, wounding and by pathogens (Zhang and Klessig 1997; 1998a; 1998b). Though NTF4 is highly similar to SIPK, it is most abundant in pollen and responds to rehydration during pollen germination (Wilson *et al.* 1997). The alfalfa member of the PERK1 subfamily, *MMK1* is transcriptionally upregulated in the G2-phase of the cell cycle, possibly indicating a function in cell cycle regulation (Jonak *et al.* 1993).

A characteristic feature for the members in the PERK2 subfamily is that a transcriptional upregulation of the gene acompanies the activation of the respective protein kinase. Some of these signals activate both PERK1 and PERK2 members, but there are also signals which appear to be subfamily-specific. WIPK, ATMPK3 and MMK4 all appear to be involved in mediating signaling of various stresses. The transcription and protein kinase activity of the MMK4 alfalfa MAP kinase was induced by cold, drought, wounding and mechanical stress (Bögre *et al.* 1996; 1997; Jonak *et al.* 1996). The same stresses, including salt stress, induce the transcription of the ATMPK3 Arabidopsis MAP kinase gene (Mizoguchi *et al.* 1996). WIPK (Zhang and Klessig 1998b), and ERMK (Ligterink *et al.* 1997) are activated by elicitors from different pathogens. With a reverse genetic approach it was shown that downregulation of WIPK mRNA levels in leaves interferes with wound-activation of MAPK and resulted in altered responses, abrogating the transcription of certain wound-induced genes and the production of jasmonic acid (Seo *et al.* 1995).

MMK2, a member of the PERK3 subfamily is able to complement the yeast MPK1 mutation, involved in cell wall biosynthesis and actin organisation (Jonak *et al.* 1995). Consistent with a role in regulating actin organisation was the finding, that a 39 kD protein in cytoskeleton preparations is a substrate for MMK2 (Jonak *et al.* 1995).

The two known members of the PERK4 subfamily appear to function in mitosis. Both MMK3 and NTF6 are transiently activated as cells pass through ana- and telophase and were found to localise to the cell division plane in telophase cells (Bögre *et al.* 1999; Calderini *et al.* 1998). So far, no function for members of the PERK5 subfamily have been defined.

It should be noted that MAPKs almost certainly play a role in many other processes. Based on biochemical data, but without identification of the respective MAPK, a number of reports imply an involvement of MAPKs in the signal transduction of wounding (Stratman and Ryan 1997; Usami *et al.* 1995), pathogen defense (Adam *et al.* 1997; Suzuki and Shinshi 1995; Zhang et al 1998), and several plant hormones. ABA is able to induce a MAPK-like activity in barley aleurone protoplasts (Knetsch *et al.* 1996). Besides inducing specific genes, ABA is known to inhibit giberellic acid (GA)-induced effects in aleurone cells. Whereas

ABA stimulates MAPK activation in aleurone cells, GA may do the reverse, as indicated by the negative effect of GA on transcript accumulation of a MAPK gene in oat aleurone cells (Huttly and Phillips 1995).

Whereas auxin starvation arrests cell division in a tobacco cell suspension culture, readdition starts the cell cycle. During this process, a MAPKK and a protein kinase that has the properties of a MAPK are activated (Mizoguchi *et al.* 1994). A MAPK pathway may also be a negative regulator of auxin because expression of NPK1, a tobacco MAPKKK, in maize cells results in inhibiton of auxin-induced gene expression (Kovtun *et al.* 1998).

Genetic analysis of the ethylene pathway in Arabidopsis indicates a possible involvement of a MAP kinase module. A number of mutants have been isolated that show a constitutive triple response (CTR) in the absence of ethylene. The *CTR1* gene was isolated and found to encode a protein with similarity to Raf1, a MAPKKK (Kieber *et al.* 1993).

7. How many out there? - Plant ESTs with similarity to MAPKs

To estimate how many additional MAPKs might exist beside the 23 published full length cDNAs, expressed sequence tag (EST) databases were searched with sequences from all 5 PERK subfamilies. In total, 45 ESTs were found that showed sequence similarity to MAPKs. Most of these ESTs are from Arabidopsis (24) and rice (9). All plant ESTs and partial cDNAs presently found in the databases are listed in Table 2 indicating length and position of the partial sequences relative to MMK1.

Out of the 24 Arabidopsis ESTs eleven showed identity with the 7 published full length clones. The other ESTs do not necessarily encode 13 other MAPKs in Arabidopsis, because these ESTs only encode N-terminal, central, or C-terminal MAPK regions that cannot be aligned with each other. The closest full length MAPK homolog of each EST and three additional partial MAPK cDNA clones are also indicated in Table 2. Phylogenetic analysis of these sequences and the 21 full length MAPKs revealed that the majority of the ESTs can be grouped into one of the five previously described PERK subfamilies (Fig. 4). At least one EST was found in each of the 5 subfamilies. Surprisingly, no Arabidopsis EST was found that could be grouped into the PERK4 subfamily. A reason for this could be that cDNAs for PERK4 members are not present or have a low abundance in the EST cDNA libraries. In contrast, many Arabidopsis ESTs were grouped in the PERK2 subfamily. In addition to the known full length sequences, the EST analysis identified novel Arabidopsis MAPKs in the PERK2, 3 and 5 subfamilies.

Table 2. **Summary of all isolated plant MAPK EST and partial plant MAPK cDNAs**

Name	Accession No.	Species	Nucleotides (bp)	ORF (aa)	Closest homology	Position of orf (relative to MMK1)
AtEST1	AA404767	*Arabidopsis thaliana*	476	73	ATMPK7	316-
AtEST2	AA605427	*Arabidopsis thaliana*	515	96	ATMPK3 (ident.)	296-
AtEST3	AA721921	*Arabidopsis thaliana*	492	104	ATMPK6 (ident.)	1-96
AtEST4	AA713093	*Arabidopsis thaliana*	408	116	SlORF2	35-152
AtEST5	H36168	*Arabidopsis thaliana*	466	150	ATMPK3 (ident.)	183-330
AtEST6	H37019	*Arabidopsis thaliana*	525	145	SlORF1	355-
AtEST7	N97150	*Arabidopsis thaliana*	535	105	SlORF1	352-
AtEST8	R90476	*Arabidopsis thaliana*	363	83	FsMAPK (*Nectria aematococca*)	314-
AtEST9	N96333	*Arabidopsis thaliana*	510	133	SlORF2	35-168
AtEST10	N65559	*Arabidopsis thaliana*	500	73	ATMPK3 (ident.)	20-90
AtEST11	T04165	*Arabidopsis thaliana*	429	119	SlORF1	377-
AtEST12	Z24542	*Arabidopsis thaliana*	323	104	PMK1 (*Magnaporthe grisea*)	196-296
AtEST13	R30629	*Arabidopsis thaliana*	466	102	ATMPK2 (ident.)	23-125
AtEST14	T21003	*Arabidopsis thaliana*	356	86	SlORF1	334-
AtEST15	R90032	*Arabidopsis thaliana*	400	64	ATMPK3	20-82
AtEST16	T14181	*Arabidopsis thaliana*	359	115	ATMPK5 (ident.)	246-360
AtEST17	Z24497	*Arabidopsis thaliana*	306	59	SlORF1	336-
AtEST18	T21678	*Arabidopsis thaliana*	300	79	ATMPK3 (ident.)	132-208
AtEST19	T22107	*Arabidopsis thaliana*	371	134	ATMPK5 (ident.)	246-378
AtEST20	AI100610	*Arabidopsis thaliana*	375	54	ATMPK3	331-386
AtEST21	T22988	*Arabidopsis thaliana*	501	126	SpSPM1 (*S.pombe*)	212-335
AtEST22	T41567	*Arabidopsis thaliana*	479	144	ATMPK1 (ident.)	23-167
AtEST23	T45144	*Arabidopsis thaliana*	482	164	ATMPK3 (ident.)	168-330

AtEST24	T46244	*Arabidopsis thaliana*	487	60	ATMPK7 (ident.)	23-83
OsEST1	AA750536	*Oryza sativa*	243	80	SlORF	379-
OsEST2	AA753878	*Oryza sativa*	541	109	WCK1	281-387
OsEST3	AA753910	*Oryza sativa*	498	95	WCK1	295-387
OsEST4	C22363	*Oryza sativa*	462	153	ASPK9	22-176
OsEST5	C22364	*Oryza sativa*	463	84	WCK1	304-387
OsEST6	C22686	*Oryza sativa*	463	40	ATMPK1	350-
OsEST7	C23546	*Oryza sativa*	464	143	ASPK9	20-144
OsEST8	C23554	*Oryza sativa*	458	148	NtF3	23-133
OsEST9	D41944	*Oryza sativa*	390	129	ATMPK7	23-78
ZmEST1	AA979888	*Zea mays*	904	215	PsD5	179-
ZmEST2	T18821	*Zea mays*	272	90	NtF3	58-148
GhEST1	AI054813	*Gossypium hirsutum*	596	140	ATMPK6	-3-116
GhEST2	AI055089	*Gossypium hirsutum*	760	200	ERMK	18-207
GhEST3	AI055345	*Gossypium hirsutum*	621	154	FsMAPK (*Nectria aematococca*)	174-322
GhEST4	AI055221	*Gossypium hirsutum*	773	168	SlORF1	286-
BrEST1	AT000688	*Brassica rapa* ssp. pekinensis	178	57	NtF6	207-264
BrEST2	AT000946	*Brassica rapa* ssp. pekinensis	196	64	ATMPK4	222-285
BnEST1	H07751	*Brassica napus*	236	78	SlORF1	328-
MdEST1	AT000385	*Malus domestica*	265	72	MMK2	20-68
PaEST1	Z92691	*Picea abies*	557	130	PhERK1	23-150
LeEST1	AA824827	*Lycopersicon esculentum*	603	69	NtF6	319-387
Other MAP kinase partial sequences						
OsMAP1	D21288	*Oryza sativa*	280	90	NtF3	247-335
SlORF1	U96717	*Selaginella lepidophylla*	746	230	NtF6	354-
SlORF2	U96716	*Selaginella lepidophylla*	709	196	PhERK1	38-231

Sixteen sequences (10 from Arabidopsis, 2 from cotton, 1 from rapeseed and rice, and 2 partial cDNA sequences from *S. lepidophylla)* could not be grouped to any of the 5 established PERK subfamilies. Instead, these sequences formed two new groups named EST1 and EST2 (Fig. 4). We are not certain whether the EST1 and EST2 groups really denote different subfamilies, because all the sequences in the EST1 group are derived from N-terminal or central MAPK parts, while all the members of the EST2 group originate from C-terminal MAPK sequences. No full length MAPKs have been published which belong to these EST groups. Some members of the EST1 group (ATEST12, ATEST21, GHEST3, and SLORF2) contain the dual phosphorylation motif TDY in place of TEY that is present in all other PERKs. A TDY dual phosphorylation motif has so far only been found in MAPKs from protozoa. We also noted that members of the EST1 group (ATEST4, ATEST9, and SLORF2) do not contain the characteristic unique residue for other plant MAPKs (P39 in ATMPK3, Kültz 1998). Another feature for many members of the EST2 group is a long C-terminal extension, predicting these MAPKs to have a total length of between 434 and 560 amino acids similar to members of the MAPK3 subfamily in animals which are also substantially larger than other MAPKs (Kültz 1998).

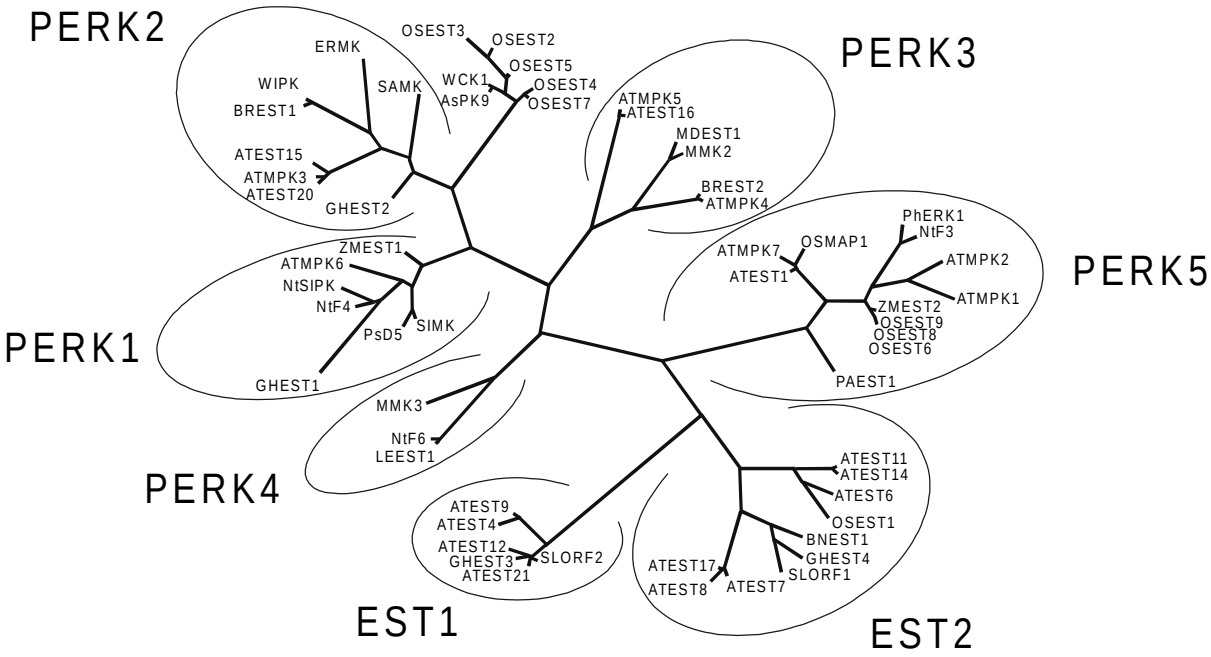


Fig. 4. **Phylogenetic tree of all isolated plant MAPK ESTs, partial plant MAPK cDNAs and the 21 full length plant MAPKs (Table 2).** Subfamilies are marked as in Figure 3. Two possible new MAPK groups, consisting only of EST and partial MAPK cDNAs are denoted as EST1 and EST2. The phylogenetic analysis was done with a heuristic search method as implemented in version 4.0.0d64 of PAUP* written by David L. Swofford.

A single signature sequence was mentioned to be characteristic for all MAPKs and sufficient to distinguish MAPKs from other members of the protein kinase superfamily. This signature sequence is the 16 amino acid long sequence of the activaiton loop [L/I/V/M][T/S]XX[L/I/V/M]XT[R/K][W/Y]YRXPX[L/I/V/M][L/I/V/M], where the 2nd and 4th residues represent the phosphorylation sites (Kültz 1998). This signature does not hold for the putative MAPKs of the EST1 group. The sequence of members of this group is very conserved in this region and consists of the sequence WTDYVATRWYRXPELC. Both the first and the last residues do not fit with the general MAPK signature sequence.

Considering the complete sequence information on plant MAPKs, the PERK signature sequences proposed by Kültz (1998) have to be modified. The first proposed signature is ELMDTDLHXII[K/R]SXQ located at the L7 loop. This signature is not valid for MMK3 that has a Q at position 12, and ATMPK1 that has a G instead of a Q at position 15. Modification of this sequence to ELMDTDLHXIIXS would give a signature sequence which would fit for every plant MAPK in the database, but is also present in some fungal MAPKs. The second proposed signature motif for plant MAPKs is HCX[F/Y]F[L/I/V/M][F/Y]Q[L/I/V/M]LRGL[K/R]Y[L/I/V/M]HSAN located at the α_e helix. This signature is not valid for ATMPK1, which has a K instead of a G at position 12 of the signature. Modification of the signature sequence to HCX[F/Y]F[L/I/V/M][F/Y]Q[L/I/V/M]LRXL[K/R]Y[L/I/V/M]HSAN gives a signature sequence that is valid for all plant MAPKs in the database and is not present in any other protein. However, this signature sequence would probably fail to recognize MAPKs belonging to the EST1 and/or EST2 group. The only member of these groups that includes amino acid sequences in this region (SLORF2) has a different sequence. This again would suggest that the newly identified group(s) are not part of the general PERK subgroup of MAPKs.

Taken together, the grouping of plant MAPKs into subfamilies appears to provide a functional classification system. So far, a functional assignment was based on the transcriptional upregulation of the respective MAPK gene or the activation of the kinase by defined stimuli. An understanding of the biological function of the MAPKs will require the identification of the targets and upstream kinases of the MAPKs but, last not least, their context with other signaling pathways, appropriately termed neuronal networks, that make biological systems so wonderful.

Chapter 3

Wounding induces the rapid and transient activation of a specific MAP kinase pathway

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ABSTRACT

Mechanical injury in plants induces responses that are involved not only in healing but also in defense against a potential pathogen. To understand the intracellular signalling mechanism of wounding, we have investigated the involvement of protein kinases. Using specific antibodies, we showed that wounding of alfalfa leaves specifically induces the transient activation of the p44^{MMK4} kinase, which belongs to the family of mitogen-activated protein kinases. Whereas ativation of the MMK4 pathway is a post-translational process and was not blocked by α-amanitin and cycloheximide, inactivation depends on de novo transcription and translation of protein factor(s). After wound-induced activation, the MMK4 pathway was subject to a refractory period of 25 min, during which time restimulation was not possible, indicating that the inactivation mechanism is only transiently active. After activation of the p44^{MMK4} kinase by wounding, transcript levels of the *MMK4* gene increased, suggesting that the *MMK4* gene may be a direct target of the MMK4 pathway. In contrast, transcripts of the wound-inducible *MsWIP* gene, encoding a putative proteinase inhibitor, were detected only several hours after wounding. Abscisic acid, methyl jasmonic acid and electrical activity are known to mediate wound signaling in plants. However, none of these factors was able to activate the p44^{MMK4} kinase in the absence of wounding, suggesting that the MMK4 pathway acts independently of these signals.

INTRODUCTION

Mechanical injury (wounding) of plant organs results in the rapid activation of genes that play a role in two types of responses (reviewed in Bowles, 1993). A local response occurs in cells in the vicinity of the wound site and involves induction of genes involved in healing. Via a communication system(s) that may involve electrical signals and/or chemical factors, cells near the wound site trigger a systemic response in distal parts of the plant. Here, a set of genes that is responsible for the defense reaction against herbivorous pathogens becomes activated. The induction of the majority of defense proteins involves transcriptional activation and either can be restricted to the wound site or can occur systemically throughout the plant. The regulation of the expression of several of these genes is positively mediated by oligosaccharides, jasmonic acid, abscisic acid (ABA), and a peptide called systemin but is negatively affected by auxin. Although all of these compounds are involved in the wound response, the sequence of events and the causal relationship between these factors and the transcriptional activation of wound-induced genes are as yet unclear.

Protein phosphorylation is one of the major mechanisms for controlling cellular functions in response to external signals in animals and yeast. A specific class of serine/threonine protein kinases, designated mitogen-activated protein (MAP) kinases, is involved in many of these processes. In animals, these kinases are activated in response to mitogenic stimuli (Ray and Sturgill, 1987; Hoshi *et al.*, 1988; Rossomando *et al.*, 1992), meiosis (Gotoh *et al.*, 1991; Posada *et al.*, 1991), differentiation (Gotoh *et al.*, 1990; Boulton *et al.*, 1993), or various stresses (Ely *et al.*, 1990; Stratton *et al.*, 1991; Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994). MAP kinase activation requires phosphorylation on tyrosine and threonine residues (Anderson *et al.*, 1990; Posada *et al.*, 1991) and is mediated by a single dual-specific activator protein kinase, MAP kinase kinase (Alessandrini *et al.*, 1992; Crews *et al.*, 1992; Matsuda *et al.*, 1992). Activation of the MAP kinase activator occurs by phosphorylation on serine residues by other protein kinases, Raf-1, mos and MAP kinase kinase kinases (Hattori *et al.*, 1992; Kyriakis *et al.*, 1992; Lange-Carter *et al.*, 1993). This set of three functionally interlinked protein kinases has been identified in yeast, animals, and plants and appears to be conserved in modular form in all eukaryotes (reviewed in Hirt, 1997).

MAP kinases have also been implicated in signal transduction in plants. Touch stimuli induce the activation of a MAP kinase (Bögre *et al.*, 1996) as well as the accumulation of transcripts encoding different protein kinases, including a MAP kinase and a MAP kinase kinase kinase (Mizoguchi *et al.*, 1996). Cold and drought were also shown to lead to accumulation of steady state transcripts of a MAP kinase gene (Jonak *et al.*, 1996; Mizoguchi *et al.*, 1996) as well as to activation of the MAP kinase enzyme (Jonak *et al.*,

1996). Several hormones have been reported to positively or negatively affect the enzymatic activity of MAP kinases. ABA induces the activation of a MAP kinase in barley aleurone cells (Knetsch *et al.*, 1996) but not in alfalfa leaves (Jonak *et al.*, 1996). Giberellic acid might affect the aleurone MAP kinase pathway in a negative way, because transcript levels of a MAP kinase gene in oat aleurone cells decreased when treated with gibberellic acid (Huttly and Philips, 1995). Because refeeding auxin to auxin-starved cells leads to MAP kinase activation (Mizoguchi *et al.*, 1994), it has been suggested that a MAP kinase might be involved in auxin signal transduction. Finally, it is possible that ethylene signaling might be transmitted by a MAP kinase cascade. The evidence rests on the isolation of the constitutive triple response mutant *ctr1*. *CTR1* encodes a Raf homolog and potentially acts as a MAP kinase kinase activator (Kieber *et al.*, 1993).

Recently, mouthing evidence has shown that MAP kinases might also be involved in signaling pathogens. After treating tobacco cells with a fungal elicitor, Suzuki and Shinshi (1995) activated a 47-kD MAP kinase-like protein. Cutting leaves induced the activation of a 46-kD protein kinase in tobacco and a variety of other plant species (Seo *et al.*, 1995; Usami *et al.*, 1995). Although circumstantial evidence highly suggests a role for a MAP kinase in pathogen signal transduction, so far no direct evidence shows the direct activation of a MAP kinase protein in response to a pathogen.

In this article, we report the involvement of a particular MAP kinase in mediating wound signal transduction in alfalfa. By using specific antibodies, we showed that wounding activates the $p44^{MMK4}$ MAP kinase (where *MMK4* stands for *Medicago* MAP kinase gene 4). After wounding, the activity of the $p44^{MMK4}$ kinase rose within 1 min but decreased to basal levels within 30 min. Inhibitor studies indicated that two distinct mechanisms control the $p44^{MMK4}$ kinase: an activating and an inactivating mechanism. Restimulation of the MMK4 pathway is not possible over a refractory period of 30 min. Finally, we tested several upstream candidates that are known to mediate wound signaling in plants and might be responsible for activation of the MMK4 pathway. However, ABA, methyl jasmonic acid (MeJA), and electrical activity were unable to stimulate $p44^{MMK4}$ kinase activation in the absence of wounding.

RESULTS

Wounding transiently activates a MAP kinase pathway

In an attempt to investigate whether protein phosphorylation events are part of the wound signal transduction pathway, we investigated protein kinase activation in extracts of wounded leaves. For this purpose, alfalfa leaf protein extracts were analyzed at different

times after wounding by in-gel protein kinase assays with myelin basic protein (MBP) as artificial substrate. Comparing to unwounded leaves (Fig. 1A, at 0 min) with wounded ones, we found that wounding induced rapid activation of an MBP kinase (Fig. 1A, at 1 min). Within 60 min after wounding, MBP kinase had decreased to noninduced levels (Fig. 1A, at 60 min). In comparison with marker proteins, the relative mobility of the protein with MBP kinase activity after being electrophoresed on an SDS-polyacrylamide gel was determined to be ~45 kD, reminiscent of the molecular mass of many MAP kinases.

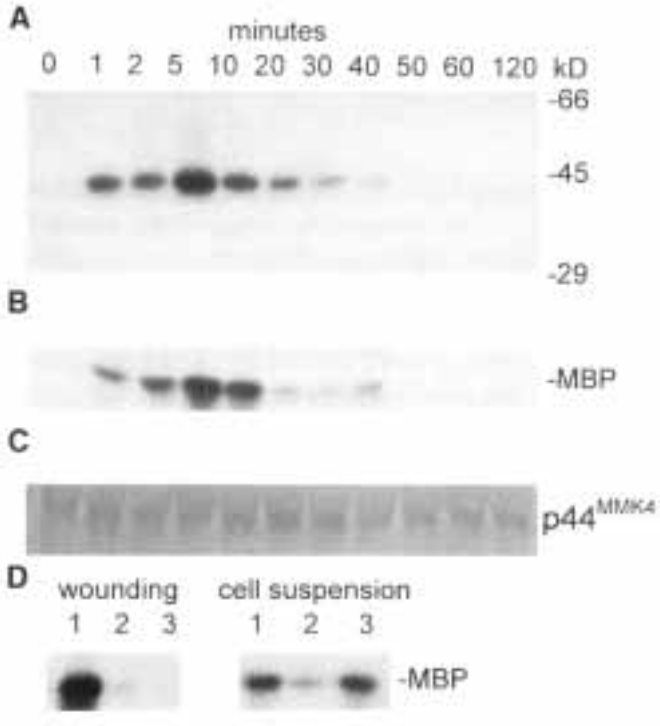

Fig. 1. **The MMK4 MAP kinase is induced by wounding.** Alfalfa leaves were mechanically wounded by cutting the lamina with a razor blade. Leaf extracts were prepared at 0, 1, 2, 5, 10, 20, 30, 40, 50, 60, and 120 min after wounding. **(A)** An MBP kinase is activated by wounding. Each lane contains 20 µg of total protein from leaf extracts. The protein was separated on an SDS-polyacrylamide gel containing 0.5 mg/mL MBP. After protein renaturation, kinase reactions were performed in the gel and analyzed by autoradiography. Numbers at right indicate the molecular masses of the marker proteins. **(B)** The $p44^{MMK4}$ kinase is activated by wounding. Leaf extracts containing 100 µg of total protein were immunoprecipitated with 5 µg of the protein A-purified M7 antibody. This antibody was raised against a synthetic peptide encoding the C-terminal 10 amino acids of the alfalfa MMK4 kinase. Kinase reactions of the immunoprecipitated protein were performed with 0.5 mg/mL MBP, 0.1 mM ATP and 2 µCi of γ-^{32}P-ATP. The phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE. **(C)** $p44^{MMK4}$ protein amounts remain constant after wounding. Leave extracts containing 20 µg of total protein were separated by SDS-PAGE, blotted, and probed with the M7 antibody. **(D)** $p44^{MMK4}$ kinase, but not MMK2 or MMK3 kinase, is specifically activated by wounding. Leaf extracts containing 100 µg of total protein at 5 minutes after wounding (left) or from nonwounded suspension-cultured cells (right) were immunoprecipitated with M7 (lanes1), M11 (lanes 2), and M14 (lanes 3) antibodies. These antibodies were raised against peptides encoding the C-terminal 10 amino acids of MMK4, MMK2, and MMK3, respectively. The kinase activity of the immunoprecipitated proteins was determined as described in **(B)**.

To determine whether the MBP kinase activity correlated with the activation of a MAP kinase, we immunoprecipitated the same leaf extracts with the polyclonal antibody M7. The M7 antibody was raised against a synthetic peptide encoding the C-terminal 10 amino acids of the alfalfa MMK4 kinase (Jonak *et al.*, 1996). The MBP kinase activity of the immunoprecipitated $p44^{MMK4}$ kinase was analyzed by SDS-PAGE and subsequent autoradiography (Fig. 1B). The $p44^{MMK4}$ kinase was activated at 1 min, and kinase activity decreased to nondetectable levels at 30 min after wounding. Protein gel blotting of these leaf extracts with the M7 antibody detected a single band of 44-kD in all extracts (Fig. 1C and 2). In contrast to the changes in immunoprecipitated MBP kinase activities, the 44-kD MMK4 protein amounts stayed constant over the experimental period of 120 min and did not parallel the changes in $p44^{MMK4}$ kinase activity. The similar kinetics of the changes in activity detected for the in-gel MBP kinase and the immunoprecipitated $p44^{MMK4}$ kinase indicate that wounding transiently activates a MAP kinase. These results agree with a recent report on the rapid activation of a 46 kD MBP kinase in leaves of a variety of plants after cutting (Seo *et al.*, 1995; Usami *et al.*, 1995) and identify a MAP kinase pathway to be a general mechanism in wound-induced signal transduction in plants.

Wound-specific activation of the MMK4 pathway

To date, several MAP kinase genes have been identified from alfalfa (Jonak *et al.*, 1993, 1995, 1996). Because the predicted molecular mass of the alfalfa MAP kinases do not differ by >5 kD, we investigated the possibility that only a particular alfalfa MAP kinase is activated in the wounding process. For this purpose, cell extracts of wounded leaves were immunoprecipitated with three different antibodies, M7, M11, and M14, that were raised against synthetic peptides encoding the C-terminal 10 amino acids of the alfalfa MMK4, MMK2 and MMK3 MAP kinases, respectively. Immune kinase assays of protein extracts of leaves at 5 min after wounding (Fig. 1D, left) showed that only the M7 antibody that was raised against the MMK4 C terminus had immunoprecipitated active kinase complexes (Fig. 1D, lanes 1). To prove that immunoprecipitation with the different antibodies did not inactivate the kinases, protein extracts of suspension-cultured alfalfa cells expressing the MMK2, MMK3, and MMK4 kinases (L. Bögre, I. Meskiene, and H. Hirt, unpublished results) were also immunoprecipitated with the M7, M11, and M14 antibodies. Immune complex kinase assays (Fig. 1D, right) showed that all kinases had retained activity after immunoprecipitation. These data indicate that wounding activates a particular MAP kinase pathway.

Specificity of the M7 antibody

The specificity of the M7 antibody was tested by immunoblotting different alfalfa MAP kinases. For this purpose, the *MMK2*, *MMK3*, and *MMK4* gene products were expressed as glutathione S-transferase (GST) fusion proteins in bacteria. After affinity purification, we seperated equal amounts of proteins by SDS-PAGE and immunoblotted these with the M7 antibody. Preincubation of the M7 antibody with the M7 peptide completely blocked the reaction (Fig. 2, GST-MMK4 plus the peptide). Protein gel blots of crude protein extracts from nonwounded leaves showed a single band with a molecular mass of 44 kD that disappeared when the M7 antibody was preincubated with the M7 peptide (Fig. 2, leaf without and with the peptide, respectively). M7 antibody immunoblots of crude protein extracts of leaves at 10 min after wounding revealed the same amount of the 44-kD band, and the antibody reaction fully competed with the M7 peptide (Fig. 2, wounded leaf, without and with the peptide, respectively).

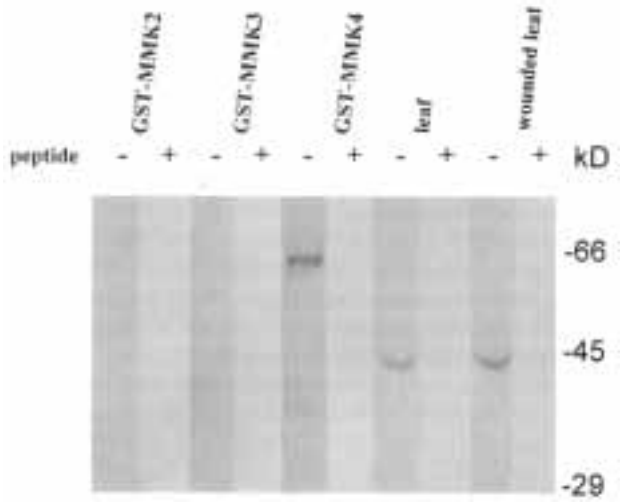

Fig. 2. **Specificity of the M7 antibody.** Glutathione S-transferase (GST) fusion constructs of the *MMK2*, *MMK3*, and *MMK4* gene products were expressed in bacteria and affinity purified. The GST-MMK2, GST-MMK3, and GST-MMK4 proteins as well as crude protein extracts of alfalfa leaves and wounded leaves were seperated by SDS-PAGE, blotted, and probed with the M7 antibody, which was preincubated without (-) or with (+) an excess of the peptide that was used to raise the M7 antibody. Numbers at right indicate the molecular masses of the marker proteins in kilodaltons.

These results show that the M7 peptide specifically recognizes the product of the *MMK4* gene. To test whether the M7 antibody was able to immunoprecipitate active MAP

kinases, we immunoprecipitated the affinity purified GST-MMK2, GST-MMK3, GST-MMK4 kinases with the M7 antibody. Subsequently, the activity of the immunoprecipitated proteins was determined by in vitro kinase reactions using MBP as a substrate. In contrats to immunoprecipitated GST-MMK4 kinase, no kinase activity was detected in the other alfalfa MAP kinases (data not shown). These results show that the M7 antibody specifically immunoprecipitates GST-MMK4 but not GST-MMK2 and GST-MMK3 kinases.

Rewounding does not stimulate the MMK4 pathway during the refractory period

The transient nature of the activation of the p44^{MMK4} kinase was investigated further by rewounding leaves at different times after the initial mechanical injury. Because the p44^{MMK4} kinase activity is maximal at 5 min after wounding, the rewounded leaves were collected at 5 min after the rewounding treatment and analyzed for p44^{MMK4} kinase activity by immune kinase assays. As shown in Fig. 3, rewounding leaves over the first 25 min after the first wounding treatment did not induce p44^{MMK4} kinase activation above the levels induced by the initial stimulation. At 25 min after the first wounding, the p44^{MMK4} kinase activity had decreased to nondetectable levels and could not be restimulated by a second wounding treatment. At 35 min and later, however, rewounding induced increasing levels of p44^{MMK4} activation. These results indicate that after initial stimulation, the MMK4 pathway is refractory to restimulation for ~25 min, suggesting that wounding also induces a mechanism to inactivate the MMK4 pathway (see below) that is active for 25 min.

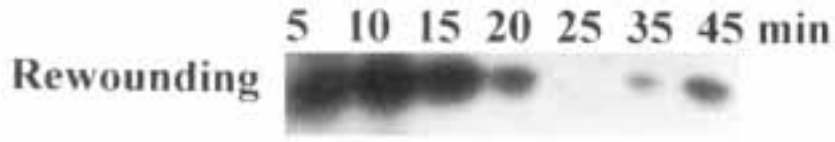

Fig. 3. **Activation of the MMK4 pathway is transiently refractory to rewounding.** Alfalfa leaves were mechanically wounded by cutting the lamina with a razor blade. At 5, 10, 15, 25, 35, and 45 min after wounding, leaves were rewounded. At 5 min after the rewounding treatment, leaf extracts were prepared. Leaf extracts containing 100 µg of total protein were immunoprecipitated with 5 µg protein A-purified M7 antibody. p44^{MMK4} kinase activity was assayed by in vitro kinase reactions with MBP and γ-^{32}P-ATP, as given in Figure 1.

Wounding specifically induces *MMK4* transcript accumulation

Wounding has been shown to induce the transcription of a variety of genes. To study the transcriptional events after wounding, we extracted RNA from alfalfa leaves at different times. After blotting to nylon filters, the RNA was hybridized with radiolabeled fragments of *MsWIP*, an alfalfa homolog to the maize *WIP1* gene that encodes a Bowman-Birk-type proteinase inhibitor and becomes transcriptionally induced within 30 min after wounding in maize (Rohrmeier and Lehle, 1993). No *MsWIP* transcript was detected in nonwounded alfalfa leaves (Fig. 4). One hour after wounding, *MsWIP* transcripts became detectable and strongly increased for at least 12 hr (Fig. 4). To test whether MAP kinase genes are transcriptionally induced by wounding, we subsequently blotted the same blot with radiolabelled probes of the four alfalfa MAP kinase genes. Nonwounded leaves contained low levels of *MMK4* mRNA but not of the other MAP kinases (Fig. 4). In contrast to the *MMK1*, *MMK2*, and *MMK3* mRNAs, *MMK4* transcript amounts increased strongly at 20 min after wounding and decreased to the basal levels of the nonwounded leaves by 4 hr. *MMK4* transcript accumulated after $p44^{MMK4}$ kinase activation (Fig. 1B), suggesting that transcriptional induction of *MMK4* gene expression is not necessary for the activation of the MMK4 pathway but might be a downstream target of the pathway.

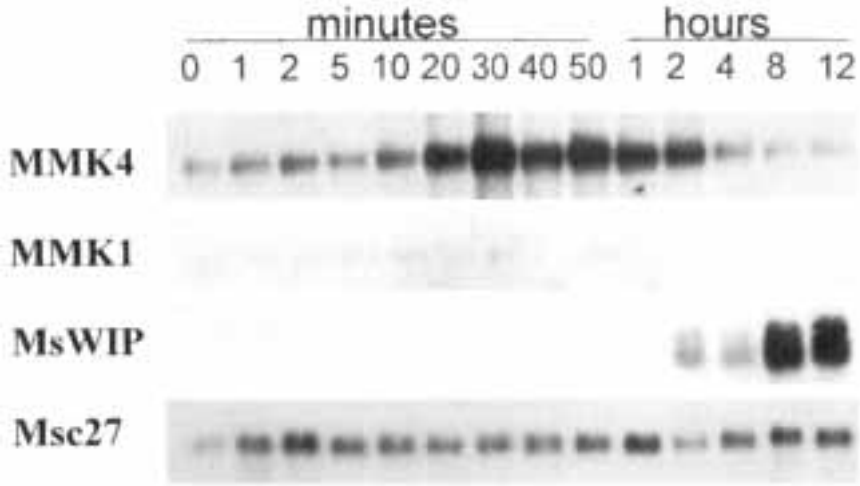

Fig. 4. **Transcriptional induction of the *MMK4* and the *MsWIP* gene by wounding.** RNA was extracted from leaves at 0, 1, 2, 5, 10, 20, 30, 40, and 50 min and at 1, 2, 4, 8, and 12 hr after wounding. Two micrograms of poly(A)$^+$ RNA was separated per lane on a denaturing formaldehyde gel. After blotting to nylon membranes, the blot was sequentially hybridized with fragments containing the 3´ nontranslated regions of the *MMK4* and the *MMK1* genes, respectively, and the coding region of the *MsWIP* gene. As a control, the blot was hybridized with a radiolabeled fragment of the constitutively expressed *Msc27* gene.

Interestingly, at the time when *MMK4* transcripts accumulated, p44^{MMK4} kinase activity and protein did not show an increase (Fig. 1B and 1C, respectively). At first glance, these results are puzzling but could be explained by the different turnover rates in various cellular compartments or by post-transcriptional regulation events that require further study.

Activation of the MMK4 pathway is independent of de novo transcription and translation

Because plant MAP kinases appear to be induced transcriptionally by wounding (Seo *et al.*, 1995), we also investigated whether wound-induced MMK4 activation depends on de novo transcription or translation of the *MMK4* gene. Under conditions in which transcription of the wound-induced genes was completely inhibited after preincubation of detached leaves with α-amanitin, the timing of activation and activity levels of p44^{MMK4} kinase was similar for leaves containing no inhibitor (Fig. 5), indicating that wound-induced activation of the MMK4 pathway is not dependent on de novo transription of genes. To investigate whether translation is necessary for the activation of the MMK4 pathway, we incubated detached leaves with cycloheximide before wounding. In contrast to the work reported by Usami *et al.* (1995), neither α-amanitin nor cycloheximide induced activation of the p44^{MMK4} kinase in the absence of wounding. Under conditions that inhibited incorporation of ^{35}S-methionine into proteins by >95%, the p44^{MMK4} kinase remained fully activatable by wounding (Fig. 5). Although a dose-dependent inhibition of transcription and translation was obtained when different concentrations of either α-amanitin and/or cycloheximide were used (data not shown), the wound-induced activation of the p44^{MMK4} kinase was not affected. These results support the idea that wounding activates the MMK4 pathway by a post-translational mechanism.

Inactivation of the MMK4 pathway depends on de novo synthesis of protein factor(s)

Although α-amanitin treatment of detached leaves before wounding did not affect the activation of the p44^{MMK4} kinase by wounding, inactivation of the wound-induced p44^{MMK4} kinase was completely inhibited (Fig. 5), indicating that the inactivation depends on the transcription of specific gene(s). Gene transcription alone is not sufficient to inactivate wound-induced p44^{MMK4} kinase, however, because pretreating detached leaves with cycloheximide led to sustained activation of p44^{MMK4} kinase. These results show that the inactivation of the wound-induced MMK4 pathway requires the transcription and the translation of protein factor(s). The inactivation mechanism appears to be active for a

limited period of time, as indicated by the inability to restimulate an activated p44^{MMK4} pathway over a refractory period of 25 min.

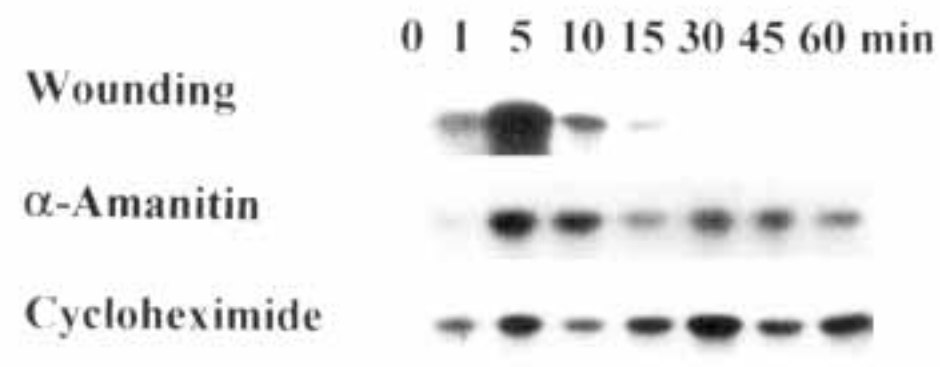

Fig. 5. **Inactivation of the wound-induced MMK4 kinase requires de novo synthesis of protein(s).** Before laminar wounding, we detached alfalfa leaves at the petioles and preincubated for 2 hr in medium containing 100 µM translation inhibitor cycloheximide and 100 µM transcription inhibitor α-amanitin. Leaf extracts were prepared at 0, 1, 5, 10, 15, 30, 45, and 60 min after wounding. Extracts containing 100 µg of total protein were immunoprecipitated with the M7 antibody. Kinase reactions of immunoprecipitated MMK4 proteins were performed with γ-^{32}P-ATP and MBP as a substrate and analyzed by autoradiography after separation by SDS-PAGE.

ABA and MeJA do not activate the MMK4 pathway

ABA and MeJA are known to be involved in mediating the wound response and can induce several wound-inducible genes in the absence of a wound signal (Bowles, 1993). To test the idea that ABA or MeJA could activate the p44^{MMK4} kinase pathway, we treated intact leaves or leaf pieces with ABA and Me-JA and determined the p44^{MMK4} kinase activity by immune complex kinase assays. No activation was observed over a range of concentrations or time periods (data not shown). To exclude the possibility that the lack of p44^{MMK4} kinase activation was a result of the fact that ABA and MeJA were not taken up by the leaf tissues, we analyzed ABA- and MeJA-treated leaves for the induction of an ABA-inducible alfalfa gene, *MsABA1*, encoding a homolog of the ABA-inducible maize pMAH9 gene (Gomez *et al.*, 1988). ABA as well as MeJA induced activation of the alfalfa *MsABA1* gene within 10 min (data not shown; Jonak *et al.*, 1996), indicating that the failure to activate the p44^{MMK4} kinase was not caused by uptake problems of the plant hormones.

DISCUSSION

Wounding triggers the rapid activation of a specific MAP kinase in alfalfa, suggesting that a MAP kinase pathway is involved in the intracellular signal transduction of the wound stimulus. This wound-signaling mechanism appears to be highly conserved in plants, because protein kinases with all the properties of MAP kinases were shown to be activated by wounding in a variety of plants (Seo *et al.*, 1995; Usami *et al.*, 1995).

Wounding has been shown to stimulate transcription of specific genes that are thought to play a role in healing and defense response to pathogens. We show that the *MMK4* and the *MsWIP1* genes qualify as wound-inducible genes, because transcripts of these genes specifically accumulate upon wounding. The accumulation of *MMK4* transcripts after wounding agrees with the report on the wound-induced transcription of the tobacco *WIPK* gene encoding a MAP kinase (Seo *et al.*, 1995). Although these authors found no basal transcript levels of the *WIPK* gene in unwounded leaves, *WIPK* mRNA accumulation was observed almost immediately. Accumulation began at 1 min after wounding with maximal accumulation between 30 and 60 min after wounding. Because wounding activated an MBP kinase with the same kinetics, these results might be taken as evidence that the WIPK protein is produced de novo before activation of the MAP kinase pathway occurs. In alfalfa, the wound-induced activation of the MMK4 kinase clearly precedes the accumulation of transcripts of the *MMK4* gene. Whereas the highest $p44^{MMK4}$ kinase activation was observed at 5 min after wounding of alfalfa levaes, *MMK4* transcript levels did not increase before 20 min after wounding. At this time, $p44^{MMK4}$ kinase activity had almost decreased to basal levels. Furthermore, we showed that $p44^{MMK4}$ protein is already present in unwounded leaves and that the activity but not the steady state protein levels of $p44^{MMK4}$ changed after wounding.

To date, MAP kinase pathways in all eukaryotes are activated by a post-translational mechanism culminating in the phosphorylation of a threonine and tyrosine residue in kinase domain VIII of the MAP kinases. The inhibition of transcription or protein synthesis did not prevent $p44^{MMK4}$ kinase activation by wounding. These results are consistent with the idea that wounding activates the $p44^{MMK4}$ MAP kinase by a post-translational mechanism and that de novo transcription and translation are not required.

The inactivation of MAP kinase pathways is less well understood but involves serine/threonine protein phosphatases, such as PP2A as well as dual-speificity phosphatases that dephosphorylate tyrosine and threonine residues. Wounding induces a transient activation of the $p44^{MMK4}$ kinase, indicating that two specific mechansims must operate: one that activates the kinase and one that inactivates it. The action of the two distinct mechanisms was shown by experiments in which leaves were preincubated with either α-amanitin or cycloheximide before wounding. Although $p44^{MMK4}$ kinase was fully

activatable by wounding, sustained activation of the kinase was obtained under these conditions. These data show that wound-induced activation of the MMK4 pathway does not depend on transcription and translation but that inactivation of the p44^{MMK4} kinase is completely dependent on de novo synthesis of a protein factor(s).

A second wounding did not lead to further activation of the p44^{MMK4} kinase over a time period of ~25 min. This indicates that once stimulated, the inactivation mechanism remains fully operational over the refractory period, probably by immediately inactivating any newly stimulated p44^{MMK4} kinase. Obviously, the inactivation mechanism has to be switched off as well, otherwise the plant would be unable to respond to a second wound stimulus. This predicts that the inactivating protein factor might only be produced over a short time after activation of the MMK4 pathway and/or that the de novo-synthesized protein(s) must be highly unstable. Both prediction appear to be correct, because a recently isolated protein phosphatase meets all these criteria (Meskiene *et al.*, 1998a).

The fast activation of the MMK4 kinase after wounding suggests that the stimulation of the MMK4 pathway must be one of the cells' immediate responses to the wound stimulus. In the search for possible candidates that may act upstream of this pathway, it became clear that only those factors that have faster or similar kinetics could qualify. ABA and MeJA are considered to play a role in wound signaling and rapidly accumulate after wounding. Although treating leaves with these substances induced gene transcription of an ABA- and MeJA-inducible gene, neither substance was able to activate the p44^{MMK4} kinase in the absence of wounding. These data suggest that the activation of the p44^{MMK4} kinase is upstream of ABA and/or MeJA or that the MMK4 and the ABA and/or MeJA pathways act independently of each other. A genetic analysis on the *WIPK* gene from tobacco (Seo *et al.*, 1995) convincingly demonstrated that the wound-induced MAP kinase acts upstream of the MeJA pathway.

The activation of the MAP kinase pathway by electrical signals, which are one of the earliest responses of plants to tissue damage, is another possible factor that was considered. Electrical signals can propagate systemically and have been shown to induce proteinase inhibitor genes at distant sites (Wildon *et al.*, 1992). Alfalfa leaves were electrically stimulated over a range of conditions, but no activation of the p44^{MMK4} kinase was observed (data not shown). Although these data might indicate that the wound induction of the MMK4 pathway is not mediated by changes in electrical potentials, more rigorous tests are necessary to prove this point unequivocally.

Taken together, we have presented evidence that a specific MAP kinase pathway is rapidly and transiently activated in alfalfa leaves by wounding. Evidence from other groups suggests that this mechanism is a highly conserved and general mechanism by which plants sense and respond to wounding (Seo *et al.*, 1995; Usami *et al.*, 1995). Surprisingly, the same MAP kinase pathway is activated by other seemingly unrelated signals. Drought and cold

were found to activate the p44^{MMK4} kinase in alfalfa (Jonak *et al.*, 1996). Touch and / or mechanical manipulation also activate the MMK4 pathway (Bögre *et al.*, 1996). The common denominator of these inducers is that they are different forms of stress, suggesting that the p44^{MMK4} kinase may be part of a general stress-induced signaling pathway. Therefore, we propose to rename the p44^{MMK4} kinase as SAM, for stres-activated MAP kinase.

Stress-induced MAP kinase pathways are also present in metazoans. The SAPK (stress-activated protein kinase) or JNK (Jun N-terminal kinase) and the p38 kinase are MAP kinases that become activated by a variety of different stresses, including heat stress, osmotic stress, pathogen components, and proinflammatory signals (Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994; Kyriakis *et al.*, 1994). One obvious question is how a given MAP kinase pathway can be activated by different signals. In metazoans, it appears that different receptors can feed into the same MAP kinase pathway. A major convergence point appears to occur at the level of the Raf, mos and MAP kinase kinase kinases. Another question is how a given MAP kinase pathway can give rise to different responses. Convincing evidence from work with mammalian cells indicates that the activation level and the total time of activation of a MAP kinase are critical factors influencing the outcome of the signal transduction process. In PC12 cells, the epidermal growth factor produces a short transient activation of the ERK MAP kinase, resulting in cell proliferation. In contrast, the nerve growth factor induces a prolonged activation of the ERK kinase, leading to differentiation (Traverse *et al.*, 1992). These and other results, including the expression of mutant components of the ERK pathway (Cowley *et al.*, 1994), suggest that the time and activation levels of ERK are crucial factors determining the direction of development in these cells. Cold, drought, touch, and wounding also induce different activation levels and kinetics of the p44^{MMK4} kinase, making it plausible that similar mechanisms may also operate in plants. Definitive answers to these speculations require further investigations and the identification and study of the factors that regulate the SAM kinase pathway. The multiple involvement of the SAM kinase pathway in mediating different signals predicts that understanding signal transduction is not a linear matter but probably requires the study of interactions (cross-talk) between different pathways that previously have been considered to act independently of one another. In this respect, we may compare signal transduction processes with neuronal networks that also have linear and interlinked components. The final goal in both systems is the same: to respond to changes in the environment in an optimal way. To define what is optimal requires the integration of different signals and the potential to respond at multiple levels. Seen from this point of view, studying signal transduction should help us to understand plant physiology.

METHODS

Plant culture conditions and treatments

Alfalfa (*Medicago sativa* ssp. *varia*, cv Rambler, line A2) plants were grown in soil or under sterile conditions on hormone-free MS (Murashige and Skoog, 1962) medium. Suspension cultured alfalfa cells were cultivated in MS (Murashige and Skoog, 1962) medium containing 1mg/L 2,4 dichlorophenoxyacetic acid and 0.1mg/L kinetin.

Alfalfa leaves were mechanically wounded by cutting the lamina with a razor blade. Leaf extracts were prepared at 0, 1, 2, 5, 10, 20, 30, 40, 50, 60, and 120 min after wounding in extraction buffer (25 mM Tris.HCl, pH 7.5, 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol, 0.1 % NP40, 15 mM p-nitrophenylphosphate, 60 mM ß-glycerophosphate, 0.1 mM $NaVO_3$, 1 mM NaF, 1 mM phenylmethylsulfonylfluoride, 10 µg/ml of each leupeptine, aprotinine, soybean trypsin inhibitor and 5 µg/ml of antipain, chimostatin, and pepstatin). The cleared supernatant after the centifugation at 100.000 g for 1 hour was used.

For activation studies of $p44^{MMK4}$ kinase in the absence of wounding, leaves were detached at the petioles and preincubated for 2 hours in MS medium (Murashige and Skoog, 1962) containing 1, 10 , and 100 µM ABA (Sigma, USA) or Me-JA. Electrical stimulation was performed as described (Wildon *et al.*, 1992).

Inhibitor studies

Before laminar wounding, alfalfa leaves were detached at the petioles and preincubated for 2 hours in MS medium (Murashige and Skoog, 1962) containing 1, 10 or 100 µM of the translation inhibitor cycloheximide, or 1, 10 or 100 µM of the transcription inhibitor α-amanitin. To study the effect of cycloheximide on protein synthesis, leaf pieces were pulse-labeled for one hr with 100 µCi [^{35}S]-methionine. The degree of inhibition of protein synthesis was quantified by acinitillation counting of TCA-precipitated total protein from untreated and cycloheximide-treated samples. For a qualitative analysis, autoradiography was performed on protein extracts that were separated on SDS-PAGE. A linear relationship betwen the amount of cycloheximde and the amount of incorporated label was obtained, whereby 100 µM cycloheximide for more than 97 % inhibited incorporation of label into proteins and was therefore used for further studies.

For the analysis of the effect of α-amanitin on transcription, RNA gel blot analysis was performed on untreated and α-amanitin-treated samples comtaining 20 µg ot total RNA. After blotting the RNA to nylon membranes, the filters were hybridized with a radiolabeled EcoRI-XhoI fragment of the constitutively expressed *Msc27* gene (Pay *et al.*, 1992). Phosphoimager analysis was used to quantify the degree transcription inhibition by α-amanitin. A more or less linear relationship between the concentration of α-amanitin and the degree of transcriptional inhibition was observed. A 100 µM concentration of α-amanitin showed more than 90 % inhibition and was used for further experiments.

In-gel protein kinase assays

For in-gel protein kinase reactions, leaf extracts containing 20 µg of total protein per lane were separated by SDS-polyacrylamide gel electrophoresis. Myelin basic protein (0.5 mg/ml) was used as a substrate that was polymerized in the polyacrylamide gel. After protein renaturation, the kinase reactions were carried out in the gel as described (Usami *et al.*, 1995).

Antibody production

The following peptides (VRFNPDPPIN, LNFCKEQILE, and EALALNPEYA), corresponding to the C termini of the MMK2, MMK3, and MMK4 kinases (Jonak *et al.*, 1995; 1996), were produced synthetically and conjugated to a purified derivative protein of tuberculin. Polyclonal antiserum was raised in rabbits and finally purified by protein A column chromatography.

Immune kinase assays

Leaf extracts containing 100 µg of total protein were immunoprecipitated with 5 µg protein A-purified M11, M14, and M7 antibody, that was produced against synthetic peptide encoding the carboxyl terminal 10 amino acids of either the alfalfa MMK2, MMK3, and MMK4 MAP kinases, respectively (Jonak *et al.*, 1995; 1996). The immunoprecipitated protein was washed three times with wash buffer I (20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 1 % Triton X-100), once with the same buffer but containing 1 M NaCl, and once with kinase buffer (20 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT). Kinase reactions of the immunoprecipitated protein were performed in 15 µl of kinase buffer containing 0.5 mg/ml MBP, 0.1 mM ATP and 2 µCi [γ-^{32}P]ATP. The protein kinase reaction was carried out at room temperature for 30 minutes. The reaction was stopped by addition of SDS sample buffer. The phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE.

Immunoblotting

Immunoblotting was performed as described (Bögre *et al.*, 1995). Briefly, leaf extracts containing 20 µg of total protein were separated by SDS-PAGE, immunoblotted to polyvinylidene difluoride membranes (Millipore) and probed with the M7 antibody at a 1:1000 dilution. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody and the reaction was visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as substrate.

RNA gel blot analysis

RNA was extracted from leaves as described (Meskiene *et al.*, 1995). 2 µg of poly(A)$^+$ RNA was separated per lane on a denaturing formaldehyde gel. After blotting to nylon membranes, the blot was sequentially hybridized with radiolabeled XhoI-SphI and EcoRI fragments, containing the 3´ nontranslated regions of the *MMK4* and the *MMK1* genes, respectively, and the coding region of the *MsWIP* gene. As a control, the blot was hybridized with a radiolabeled EcoRI-XhoI fragment of the constitutively expressed *Msc27* gene (Pay *et al.*, 1992).

ACKNOWLEDGMENTS

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Chapter 4

Receptor-mediated activation of a MAP kinase in pathogen defense of plants

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ABSTRACT

Parsley cells recognize the fungal plant pathogen *Phytophthora sojae* through a plasma membrane receptor. A pathogen-derived oligopeptide elicitor binds to this receptor and thereby stimulates a multicomponent defense response through sequential activation of ion channels and an oxidative burst. An elicitor-responsive mitogen-activated protein (MAP) kinase was identified that acts downstream of the ion channels but independently or upstream of the oxidative burst. Upon receptor-mediated activation, the MAP kinase is translocated to the nucleus where it might interact with transcription factors that induce expression of defense genes.

Plants react to pathogen attack with a variety of defense responses, including transcriptional activation of defense genes, accumulation of phytoalexins and pathogen-related (PR) proteins, and impregnation of the cell wall with phenolic substances and specific proteins (Kombrink and Somssich, 1995). Infection of parsley leaves with spores from the soybean pathogen *Phytophthora sojae* leads to small necrotic lesions resulting from hypersensitive cell death, incorporation of phenolic compunds into, and apposition of callose onto, cell walls at the infection site, as well as local and systemic activation of defense-related genes and secretion of fouranocoumarin phytoalexins into the infection droplet (Scheel *et al.*, 1996; Schmelzer *et al.*, 1989). Cultured parsley cells show most of these defense reactions when treated with elicitor preparations from the fungus and have been used as a model system to study the plant-pathogen interactions (Dangl *et al.*, 1987; Somssich *et al*, 1989; Nürnberger *et al.*, 1994). An extracellular 42-kD fungal glycoprotein was identified in these preparations as the principal elicitor of the multicomponent defense response in parsley cells (Parker *et al.*, 1991) An oligopeptide fragment of 13 amino acids in length (Pep13) within this glycoprotein is necessary and sufficient to induce the same reactions as the intact glycoprotein (Nürnberger *et al.*, 1994; Sacks *et al.*, 1995). Pep13 specifically interacts with a plasma membrane target site in the plant and initiates a signal transduction cascade leading to the transient activation of plant defense genes and the accumulation of phytoalexins (Nürnberger *et al.*, 1994).

Elicitor signal transduction in parsley cells involves Ca^{2+}-dependent transient changes in protein phosphorylation, suggesting the participation of protein kinases in defense gene activation (Dietrich *et al.*, 1990). To detect specific protein kinases that catalyze such reactions, we treated cultured parsley cells with Pep25, a larger fragment of the elicitor that includes the Pep13 sequence and induced an identical response but was more stable in the culture medium than Pep13 (Nürnberger *et al.*, 1994). A protein kinase that phosphorylated by myelin basic protein (MBP) was activated within 5 min after elicitor treatment (Fig. 1A). From its relative mobility on SDS-polyacrylamide gels, the apparent molecular mass of this enzyme was estimated to be ~45 kD, similar to that of known plant mitogen-activated protein (MAP) kinases (Mizoguchi *et al.*, 1994; Mizoguchi *et al.*, 1996; Jonak *et al.*, 1996; Seo *et al.*, 1995; Suzuki and Shinshi, 1995; Bögre *et al.*, 1996).

To determine whether the elicitor-activated protein kinase might belong to the class of MAP kinases, we incubated the same cell extracts used for activity assays with three different antisera, M7, M11, and M14, that were raised against synthetic peptides representing the COOH-terminal 10 amino acids of the alfalfa MMK4, MMK2, and MMK3 MAP kinases, respectively (Jonak *et al.*, 1996). Elicitor treatment exclusively activated a protein kinase that was immunoprecipitated by the M7 antiserum (Fig. 1B). The similarity of the activation kinetics in the kinase and immunoprecipitation assays indicate that elicitor treatment activates a specific MAP kinase pathway in parsley cells.

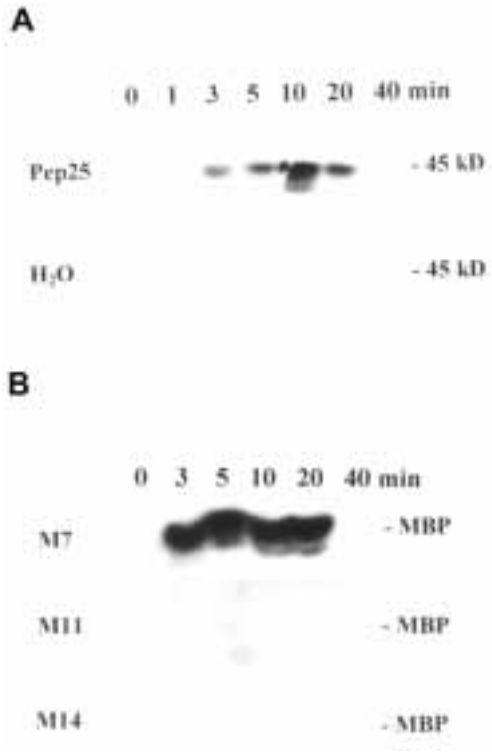

Fig. 1. **A specific MAP kinase is activated by elicitor.** Suspension cultured parsley cells were treated with the synthetic peptide elicitor, Pep25 (175 nM), or water alone. Cell extracts were prepared at 0, 1, 3, 5, 10, 20, and 40 min after initiation of elicitor treatment in extraction buffer [25 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol (DTT), 0,1% NP-40, 15 mM *p*-nitrophenylphosphate, 60 mM ß-glycerophosphate, 0,1 mM $NaVO_3$, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml each of leupeptine and aprotinin]. After centrifugation at 100,000*g* for 1 hour, the cleared supernatant was used. **(A)** In-gel protein kinase assay. Each lane contained 20 µg of total protein from cell extracts, which was separated by SDS-polyacrylamide gel electrophoresis (PAGE). MBP (0.5 mg/ml) was used as a substrate and was polymerized in the polyacrylamide gel. After protein renaturation, the kinase reactions were carried out in the gel with [γ-^{32}P]adenosine 5'-triphospate (ATP) as described (Jonak *et al.*, 1996). **(B)** Immunoprecipitation of an elicitor-responsive MAP kinase. Cell extracts containing 100 µg of total protein were immunoprecipitated with 5 µg protein A-purified M7, M11, and M14 antibodies that were produced against synthetic peptides encoding the COOH-terminal 10 amino acids of the alfalfa MMK4, MMK2, and MMK3 MAP kinases, respectively (Jonak *et al.*, 1996). The immunoprecipitated proteins were washed three times with wash buffer I (20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100), once with the same buffer but containing 1 M NaCl, and once with kinase buffer (20 mM HEPES (pH 7.5), 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT). Kinase reactions of the immunoprecipitated proteins were performed in 15 µl of kinase buffer containing MBP (0.5 mg/ml), 0.1 mM ATP and 2 µCi of [γ-^{32}P]ATP at room temperature for 30 min. The reactions were stopped by the addition of SDS sample buffer. The phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE.

Because the M7 antiserum specifically recognized the elicitor-responsive MAP kinase from parsley, a radiolabeled fragment of the alfalfa MMK4 gene was used to screen a cDNA library prepared from RNA isolated from cultured parsley cells. A 1.6-kb cDNA fragment was isolated that contained an open reading frame of 1.113 nucleotides potentially encoding a protein of 371 amino acids and a molecular mass of 43 kD. The deduced amino acid sequence is most similar to those of the MAP kinases from *Arabidopsis* (MPK3, 83 %)

(Mizoguchi *et al.*, 1996), alfalfa (MMK4, 81 %) (Jonak *et al.*, 1996), and tobacco (WIPK, 83 %) (Seo *et al.*, 1995). The overall structure of the parsley, tobacco, *Arabidopsis*, and alfalfa kinases is highly conserved (Fig. 2).

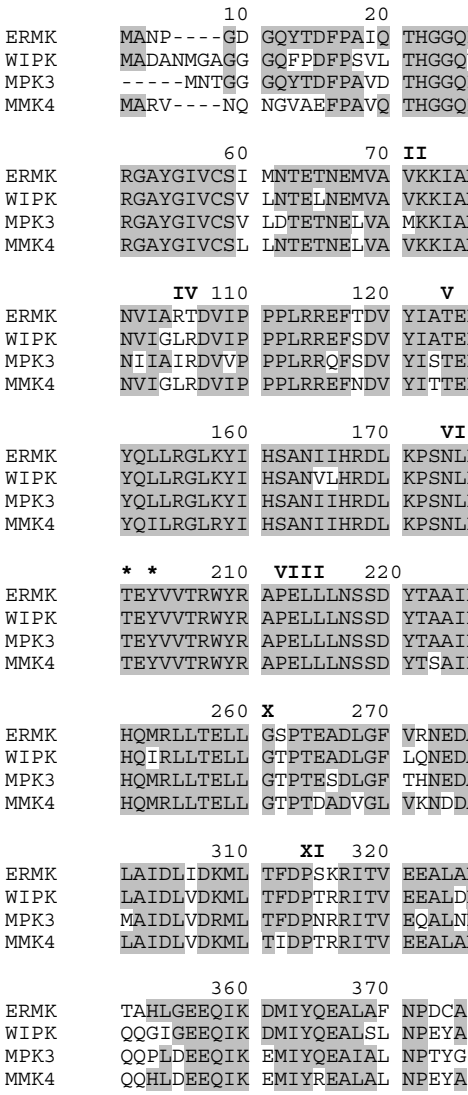

Fig. 2. **Primary structure of an elicitor-responsive MAP (ERM) kinase from parsley.** The nucleotide and predicted amino acid sequence of the ERM kinase has been deposited with GenBank, DNA Data Base Japan, and European Molecular Biology Laboratory databases (accession number Y12875). The primary sequence of ERM kinase was deduced from the sequence of a cDNA clone isolated by standard methods (Sambrook *et al.*, 1989) from a parsley cDNA library constructed in the λ-ZAP vector (Stratagene), by use of a 1.1 kb random primed ^{32}P-labeled DNA probe (megaprime labelling kit, Amersham), representing the near full-length open reading frame of MMK4 (Jonak *et al.*, 1996). The positive clone thus isolated is aligned with its closest homologs, MPK3 from *Arabidopsis thaliana* (Mizoguchi *et al.*, 1996), MMK4 from *Medicago sativa* (Jonak *et al.*, 1996), and WIPK from *Nicotiana tabacum* (Seo *et al.*, 1995). Shaded areas represent identical sequences. Roman numerals indicate kinase subdomains (Cobb and Goldsmith, 1995). Conserved phosphorylation sites are marked with an asterix.

DNA gel blot analysis of parsley cells with the radiolabeled kinase cDNA fragment under high-stringency hybridization conditions revealed the parsley kinase to be present as a single-copy gene (data not shown). RNA gel blot analysis of cultured parsley cells with radiolabeled fragments, containing either the coding region or the 3'-untranslated region of the kinase cDNA, showed a severalfold increase of kinase transcript levels within 30 min after elicitor treatment (data not shown). When the parsley cDNA was expressed as a fusion protein with glutathione-S-transferase (GST) in *Escherichia coli*, the recombinant protein catalyzed its autophosphorylation and phosphorylated MBP. In immunoblots, the GST-MAP kinase fusion protein was exclusively recognized by the M7 antiserum that recognized the elicitor-responsive protein kinase from cultured parsley cells. In contrast, the M11 and M14 antisera did not decorate the parsley kinase fusion protein. These results suggest that the cDNA isolated from parsley cells indeed encodes the elicitor-activated kinase detected in the activity and immunocomplex assays, which is therefore denoted ERM kinase for elicitor-responsive MAP kinase.

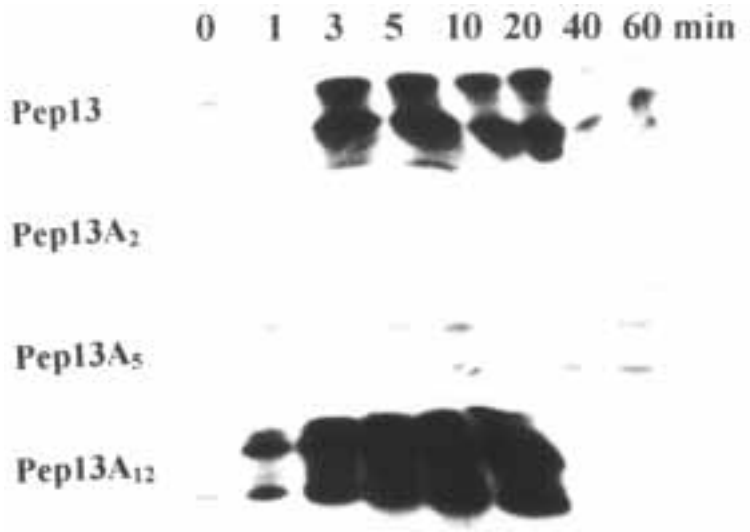

Fig. 3. **ERM kinase is exclusively activated by active peptide elicitor.** Suspension-cultured parsley cells were treated with the synthetic peptide elicitor Pep13 (50 nM), and with inactive ($Pep13A_2$, 50 nM, and $Pep13A_5$, 50 nM) and active ($Pep13A_{12}$, 50 nM) derivatives. Cell extracts were prepared at 0, 1, 3, 5, 10, 20, 40, and 60 min after elicitor treatment. Cell extracts containing 100 µg of total protein were immunoprecipitated with M7 antibody. The kinase activity of the immunocomplexes was determined by in vitro kinase assays with MBP as substrate as described in Fig. 1B.

To investigate whether Pep13 activates this kinase through the same receptor that is used for the induction of the other defense responses, we determined ERM kinase activation upon treatment of parsley cells with four different but structurally related elicitor oligopeptides. Pep13 and Pep25, both corresponding to the wild-type sequence of the 42-kD *P. sojae* glycoprotein elicitor, activated the elicitor-responsive MAP kinase in an identical manner (Fig. 1 and 3). Two Pep13 derivatives in which the second ($Pep13A_2$) or the fifth ($Pep13A_5$) amino acid had been replaced by alanine did not activate ERM kinase, whereas a derivative with an alanine substitution in position 12 ($Pep13A_{12}$) was as active as Pep13 and Pep25 (Fig. 3). These results correlate well with binding and elicitor studies (Nürnberger *et al.*, 1994) with the same Pep13 derivatives, which showed that $Pep13A_{12}$ competes with binding of Pep13 to its receptor and elicits a normal pattern of defense reactions. In contrast, $Pep13A_2$ and $Pep13A_5$ were inactive in both assays, indicating that the Pep13 receptor that initiates the multicomponent defense response is also engaged in ERM kinase activation.

Binding of Pep13 to its receptor induces phytoalexin synthesis, defense gene activation, in vivo phosphorylation of proteins, and the producton of an oxidative burst, which all depend on the integrity of specific ion channels mediate rapid ion fluxes across the cell membrane in response to elicitor (Nürnberger *et al.*, 1994; Dietrich *et al.*, 1990; Jabs *et al.*, 1997; Zimmermann *et al.*, 1997). To investigate whether ERM kinase activation also depended on the activity of these ion channels, we incubated parsley cells with the ion channel blocker, anthracene-9-carboxylate (A9C), which inhibits the elicitor-stimulated ion fluxes, thereby blocking all subsequent defense responses (Jabs *et al.*, 1997). Under these conditions, Pep13 activation of the ERM kinase was completely inhibited, indicating that ion channel activation was also necessary for this reaction (Fig. 4). Amphotericin B, which mimics elicitor-induced ion fluxes and thereby induces the full set of defense responses (Jabs *et al.*, 1997), also activates the ERM kinase in the absence of elicitor (Fig. 4). Activation of ERM kinase (Fig. 4), ion fluxes, and the oxidative burst (Jabs *et al.*, 1997) by amphotericin B all occur after a delay of about 30 min. Thus, ERM kinase activation depends on the state of specific ion channels, and activation of these channels is necessary and sufficient for ERM kinase activation as it is for the induction of the other elicitor responses in this system.

The elicitor-stimulated production of reactive oxygen species is thought to be catalyzed by an NADH [nicotinamide adenine dinuceotide (reduced)] or NADPH [nicotinamide adenine dinuceotide phosphate (reduced)] oxidase that is inhibited by diphenylene iodonium (DPI) (Mehdy *et al.*, 1996). In elicitor-treated parsley cells DPI blocked the oxidative burst, defense gene activation and phytoalexin accumulation without affecting ion fluxes (Jabs *et al.*, 1997). Together with the results from gain-of-function experiments with KO_2 which stimulated phytoalexin production in the absence of elicitor, this placed the oxidative burst downstream of the ion channels within the elicitor signal transduction cascade (Jabs *et al.*, 1997). Pep13 activation of the ERM kinase was not

inhibited by DPI, indicating that this kinase acts either upstream or independently of the oxidative burst (Fig. 4).

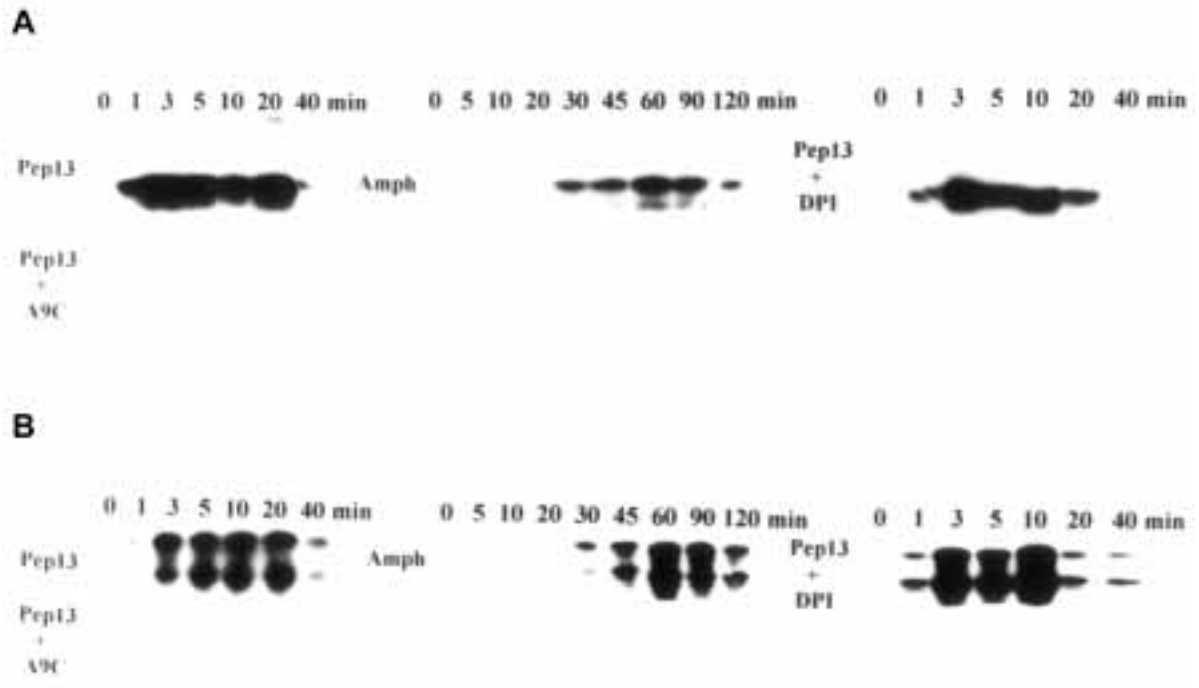

Fig. 4. **ERM kinase activation depends on elicitor-stimulated ion-channel activity but not on an oxidative burst.** Suspension-cultured parsley cells were pre-incubated with 100 µM of the ion channel blocker, anthracene-9-carboxylate (A9C), with 50 µM of the polyene antibiotic, amphotericin B (Amph), or with 50 µM of diphenylene iodonium (DPI), an inhibitor of the oxidative burst, followed by addition of Pep13 (50 nM) to A9C- and DPI-treated cells 30 min later. After the indicated periods of treatment the cells were harvested, and total protein was extracted and analyzed by **(A)** in-gel MBP kinase assays and **(B)** M7 antibody-precipitated immunocomplex kinase assays as described in Figure 1.

Certain MAP kinases are translocated into the nucleus upon activation, where they may catalyze phosphorylation of transcription factors and thereby regulate gene transcription (Chen *et al.*, 1992; Lenormand *et al.*, 1993; Sanghera *et al.*, 1992). The subcellular location of ERM kinase was determined with M7 antiserum in immunofluorescence microscopy before and after treating parsley cells with Pep25 elicitor. Within 3 to 10 min after Pep25 treatment, ERM kinase was translocated into the nucleus (Fig. 5C) Because no nuclear localization signal is present in the ERM kinase, translocation of the activated kinase into the nuclear compartment may be initiated by its interaction with another protein, perhaps a transcription factor. In parsley, several elicitor-responsive genes have been identified and have led to the identification of cis elements and transcription factors that may be involved

in mediating pathogen-induced transcription (Kombrink and Somssich, 1995; Rushton *et al.*, 1996). Although it has not yet been shown that phosphorylation of these transcription factors is responsible for elicitor-induced transcription of PR genes, the elicitor-induced relocation of ERM kinase into the nucleus might link cytosolic signal transduction to nuclear activation of plant defense genes.

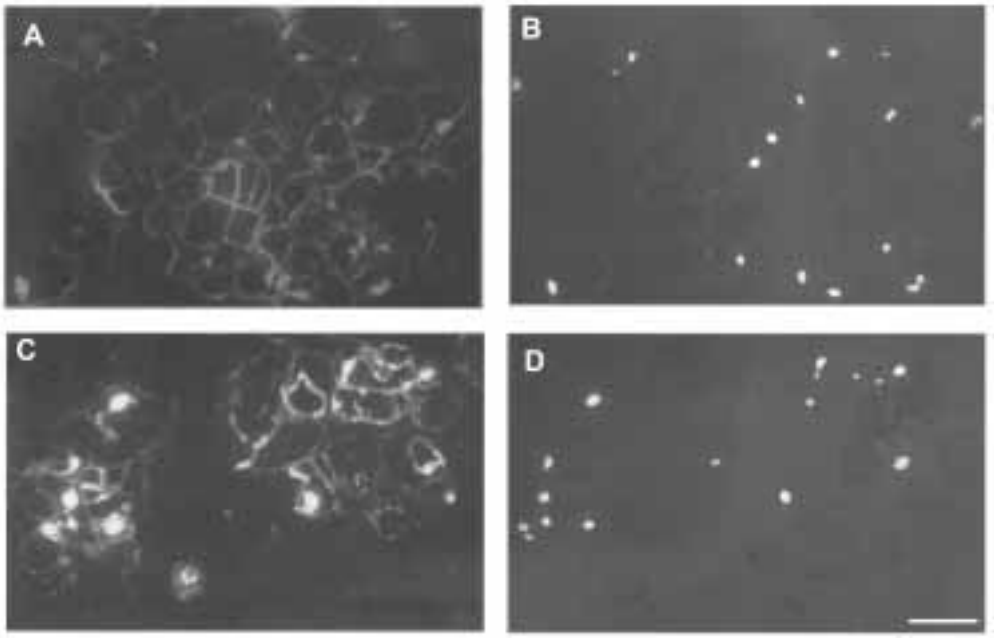

Fig. 5. **ERM kinase is translocated to the nucleus upon elicitor activation.** Cultured parsley cells were treated with Pep25 (175 nM) and harvested before **(A and B)** or 5 min after initiation of treatment **(C and D)**. Sections (2µm) across cell clusters were fixed with 4% formaldehyde, embedded in polyethylene glycol (Lammeren *et al.*, 1985), and either stained with the M7 antiserum **(A and C)**, specifically recognizing ERM kinase, or with 4',6'-diamidino-2-phenylindole (DAPI) **(B and D)** to visualize nuclei. Biotinylated secondary antibody, streptavidin-horseradish peroxidase, and fluorescein tyramid reagent were used to visualize the primary antibody bound to ERM kinase according to the manufacterer's instructions (Tyramid Signal Amplification Systems, TSA-Direct-Green, Du Pont, NEN, Boston, Massachusetts). After treatment with Pep25 most nuclei were decorated by the M7 antibody **(B)**, whereas no or little staining was detectable in untreated cells **(A)**, in cells treated with water instead of Pep25 or when the M7 antibody was replaced by preimmune serum (data not shown). Bar (D), 50µm.

MAP kinases were first found in yeast and animals, where they participate in signaling cascades linking plasma membrane receptors that perceive extracellular signals to a variety of cellular response mechanisms (Herskowitz, 1995; Marshall, 1994). The MAP kinases known in plants are activated by environmental stresses and plant hormones (Hirt, 1997; Mizoguchi, 1997). Our results demonstrate posttranslational and transcriptional activation of a plant MAP kinase within a signal transduction pathway that mediates the

response to a pathogen. Activation of ERM kinase follows input from receptor-regulated ion channels of the plasma membrane and precedes or parallels the formation of O_2^- radicals, which in turn activate defense gene and phytoalexin synthesis (Jabs *et al.*, 1997).

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Chapter 5

Yeast elicitor activates WIPK and SIPK homologs in alfalfa cells: Medium alkalinisation and oxidative burst are mediated by protein kinase pathways distinct from MAP kinase cascades

Ligterink, W., Jonak, C., Barker, P.J., Huskisson, N.S., Pühler, A., Niehaus, K., and Hirt, H.

Summary

Plant cells respond to a variety of elicitors by inducing defense responses such as expression of defense genes and production of phytoalexins. These reactions are dependent on the activity of protein kinase pathways. Recently, two mitogen-activated protein (MAP) kinases SIPK (salicylic acid-induced protein kinase) and WIPK (wounding-induced protein kinase) have been identified to be rapidly activated by various elicitors in tobacco cells. In an attempt to extend these analyses to other plant species, analysis of yeast elicitor-treated alfalfa cells revealed that protein kinases of relative molecular masses of 44-kD and 46-kD are rapidly and transiently activated. The elicitor-activated 44-kD and 46-kD protein kinases were identified as MAP kinases, encoded by the *SAM* (stress-activated MAP) and *SIM* (stress-inducible MAP) kinase genes. SIMK and SAMK are most closely related to tobacco SIPK and WIPK, respectively, suggesting that these MAP kinases perform homologous functions in different plant species. Yeast elicitor-induced medium alkalinisation, oxidative burst, and MAP kinase activation was blocked in alfalfa cells by the protein kinase inhibitor K252a, demonstrating that protein kinase pathways are responsible for mediating these elicitor responses. However, SIM and SAM kinase pathways are not involved in elicitor-induced medium alkalinisation or oxidative burst, because staurosporine, another protein kinase inhibitor, did not affect elicitor-induced activation of SAM and SIM kinase pathways but totally inhibited medium alkalinisation and production of reactive oxygen species. These data show that multiple protein kinase pathways exist in plants that are responsible for elicitor-mediated defense response signaling. Whereas elicitor-induced medium alkalinisation and oxidative burst depend on protein kinase pathways, these protein kinases lie on separate pathways from elicitor-activated SAM and SIM kinase cascades.

Introduction

As sessile organisms, plants must cope with a number of adverse environmental conditions, including abiotic stresses, such as cold, drought, and UV irradiation, but also biotic challenges by pathogens. To withstand pathogen attack, plants have developed the ability to sense elicitors, signals that are derived from pathogens or degradation products of plant cell walls. Sensing of elicitors is followed by intracellular signal transduction resulting in defense reactions such as ion fluxes, the generation of reactive oxygen species (oxidative burst), expression of genes encoding pathogen-related proteins, the synthesis of phytoalexins, and programmed cell death. Elicitor signaling was shown to involve protein phosphorylation (Dietrich *et al.*, 1990; Felix *et al.*, 1991, 1994; Viard *et al.*, 1994), suggesting that protein kinase cascades are involved in the intracellular information transfer. The importance of protein kinases was demonstrated by the ability of protein kinase inhibitors to block a variety of defense responses including the elicitor-induced medium alkalinisation, oxidative burst, and defense gene transcription (Groskopf *et al.*, 1990; Felix *et al.*, 1991; Viard *et al.*, 1994; Suzuki *et al.*, 1995). A specific class of serine/threonine protein kinases, denoted as MAP kinases, was shown to be activated by elicitors. In tobacco cells, MAP kinases are activated by elicitors derived from fungi (Suzuki and Shinshi, 1995; Zhang *et al.*, 1998) and from the bacterial pathogen *Erwinia amylovora* (Adam *et al.*, 1997). An elicitor derived from the fungus *Phytophtora sojae* was shown to activate a MAP kinase in parsley cells (Ligterink *et al.*, 1997). In intact tobacco, and in dependence of a functional product of the disease resistance *N* gene, TMV infection leads to the activation of the MAP kinases SIPK and WIPK (Zhang and Klessig, 1998b). Most recently, we showed that the *Cladosporium fulvum* avirulence gene product Avr9 can induce activation of SIPK and WIPK in tobacco cells that express a functional copy of the tomato disease resistance *Cf9* gene (Romeis *et al.*, 1999). These data indicate that MAP kinase cascades are involved in defense signaling and may constitute a major mechanism for activation of defense responses by various elicitors.

MAP kinases have also been implicated in mediating other signals. Touch stimuli induce the activation of the SAM kinase in alfalfa (Bögre *et al.*, 1996) as well as the accumulation of transcripts encoding different protein kinases, including a MAP kinase and a MAP kinase kinase in Arabidopsis (Mizoguchi *et al.*, 1996). Cold and drought were shown to lead to accumulation of steady state transcripts of MAP kinase genes (Jonak *et al.*, 1996; Mizoguchi *et al.*, 1996), as well as to activation of the MAP kinase enzyme (Jonak *et al.*, 1996). Whereas abscisic acid induces the activation of a MAP kinase in barley aleurone cells (Knetsch *et al.*, 1996), transcript levels of a MAP kinase gene in oat aleurone cells decrease upon treatment with giberellic acid (Huttly and Philips, 1995). Although refeeding auxin to auxin-starved cells was originally reported to activate MAP kinase (Mizoguchi *et*

al., 1994), cytosolic acidification but not auxin at physiological concentrations was later shown to be responsible for MAP kinase activation (Tena and Renaudin, 1998). Moreover, expression of a truncated MAP kinase kinase kinase negatively affects auxin-induced gene transcription (Kovtun *et al.*, 1998). Some aspects of ethylene signaling might be mediated by a MAP kinase cascade, because the constitutive triple response mutant CTR1 encodes a Raf homolog that functions as a MAP kinase kinase kinase in mammals (Kieber *et al.*, 1993). MAP kinases are also involved in wound signaling. Mechanical wounding of leaves induces the activation of MAPKs in alfalfa (Bögre *et al.*, 1997), tobacco (Seo *et al.*, 1995; Usami *et al.*, 1995), and tomato (Stratmann and Ryan, 1997). The MAPKs were identified as the *SAM* kinase gene in alfalfa (Bögre *et al.*, 1997), and the *WIPK* (Seo *et al.*, 1999) and *SIPK* genes (Zhang and Klessig, 1998a) in tobacco.

In this study, we investigated the possibility, that homologues of tobacco SIPK and WIPK are also involved in elicitor signaling in other plant species. Treating alfalfa cells with yeast elicitor induced typical defense responses, such as the rapid alkalinization of the medium and the production of reactive oxygen species (oxidative burst). Analysis of cell extracts by in-gel kinase assays indicated that two protein kinases with relative molecular masses of 44-kD and 46-kD are rapidly and transiently activated after elicitor treatment. Using specific antibodies, the elicitor-activated 44-kD and 46-kD protein kinases were identified as MAP kinases encoded by the *SAM* and *SIM* kinase genes, previously denoted as *MMK4* and *MMK1* genes, respectively (Jonak *et al.*, 1993; 1996). Although elicitor-induced medium alkalinisation and oxidative burst are dependent on protein kinase pathways, these defense responses are not mediated by the SAM or SIM kinase pathways, indicating the functioning of several independent protein kinase pathways in elicitor signaling.

Results

Fungal elicitor induces medium alkalinisation, oxidative burst, and protein kinase activation in alfalfa cells

Cells respond to highly different elicitors by the induction of a set of defense-like responses including a very rapid medium alkalinisation. When alfalfa cells were treated with different concentrations of a fungal cell wall preparation (50-500 μg/ml), a dose-dependent change in the extracellular pH could be observed (Fig. 1). At all elicitor concentrations, a change in pH was already detectable at 3 min, showing maximal alkalinisation at approximately 20 min (Fig. 1). A near maximal alkalinisation was obtained at a concentration of 200 μg/ml of fungal elicitor. Therefore, all subsequent studies were performed at this elicitor concentration.

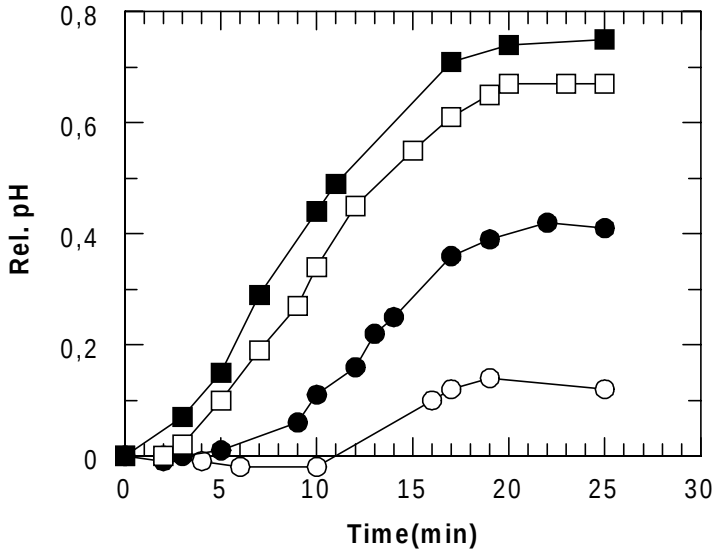

Fig. 1. **Yeast elicitor induces medium alkalinisation in suspension cultured cells.** Suspension cultured alfalfa cells were treated with 50, 100, 250, and 500 µg/mL of yeast elicitor. Changes in culture medium pH are given in relative pH units representing the pH change in comparison to the initial pH. Treatment with 50, 100, 250, and 500 µg of elicitor /mL medium is represented by empty circles, filled circles, empty squares, and filled squares, respectively.

Another typical response of cells to elicitors is the production of reactive oxygen species. When alfalfa cells were treated with different concentrations of fungal elicitor, a rapid and transient synthesis of reactive oxygen species was measured (Fig. 2). Although an increase in reactive oxygen species was detected as early as 5 min after elicitor treatment, maximal accumulation was observed at 15 to 20 min. In contrast to the relatively slow recovery of the elicitor-induced medium alkalinisation, reactive oxygen species levels decreased to nearly non-induced levels within 40 min.

MAP kinases have been reported to be activated in response to elicitors in parsley (Ligterink *et al.*, 1997) and tobacco cells (Adam *et al.*, 1997; Suzuki and Shinshi, 1995; Zhang *et al.*, 1998). As a first test for the activation of a MAP kinase by elicitor, aliquots of alfalfa cells were collected at different times after elicitor treatment. The cell extracts of these aliquots were analysed by in-gel kinase assays using myelin basic protein (MBP) as substrate. Comparing non-treated cells (Fig. 3, at 0 min) with elicitor-treated ones, a 44-kD and a 46-kD protein kinase were identified to become strongly activated at 5 min and showed complete inactivation at 40 min. These data show that fungal elicitor rapidly induces medium alkalinisation, production of reactive oxygen species, and activation of protein kinase pathways in alfalfa cells. Elicitor-induced medium alkalinisation, oxidative burst, and

protein kinase activation was observed in at least three independent experiments and gave similar results.

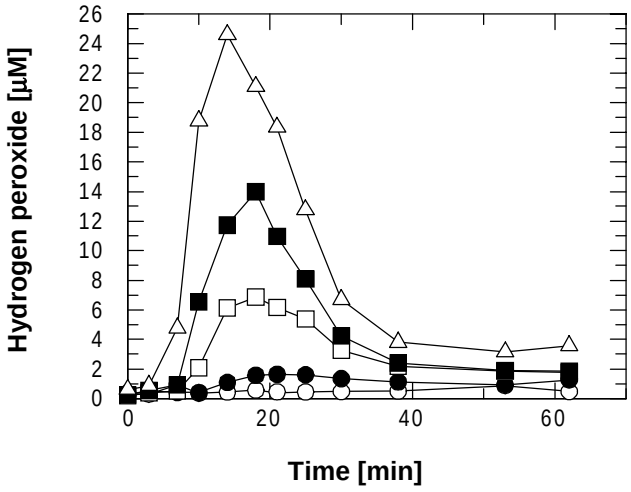

Fig. 2. **Yeast elicitor induces oxidative burst in suspension cultured cells.** Suspension cultured alfalfa cells were treated with 0, 50, 100, 250, and 500 µg/mL of fungal elicitor. Induction of oxidative burst was determined by measuring the concentration of hydrogen peroxide. Treatment with 0, 50, 100, 250, and 500 µg/mL elicitor is represented by empty circles, filled circles, empty squares, filled squares, and triangles, respectively.

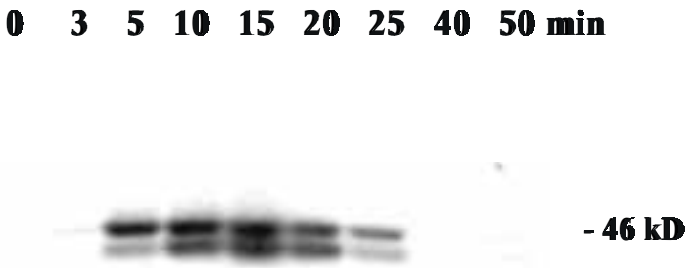

Fig. 3. **Yeast elicitor induces activation of 44-kD and 46-kD protein kinases in suspension cultured cells.** Suspension cultured alfalfa cells were treated with 200µg/mL of yeast elicitor. At the indicated times, cell extracts were prepared. Extracts of 100µg/mL total protein were separated by SDS-PAGE containing 1 mg/mL myelin basic protein. After protein renaturation, kinase reactions were preformed in the gel and analysed by autoradiography. The relative distance migrated by a 46-kD marker protein is indicated

The M23 and M24 antibodies specifically recognize the 46-kD SIM and 44-kD SAM kinases

A variety of bacterial and fungal elicitors, as well as infection with tobacco mosaic virus, showed activation of MAPKs in tobacco (Adam *et al.*, 1997; Lebrun-Garcia *et al.*, 1998; Romeis *et al.*, 1999; Suzuki and Shinshi, 1995; Zhang and Klessig, 1998b; Zhang *et al.*, 1998). The SIPK and WIPK MAP kinases were identified as being activated by several of these elicitors (Romeis *et al.*, 1999; Zhang and Klessig, 1998b; Zhang *et al.*, 1998). In parsley, a fungal elicitor was shown to activate ERM kinase, the closest relative of the tobacco WIPK MAP kinase (Ligterink *et al.*, 1997), suggesting that these two classes of MAP kinases might be general mediators of elicitor-induced responses in plants. To determine whether the 44-kD and 46-kD kinase activities in alfalfa cells correlate with the activation of the SIPK- and WIPK-related SIM and SAM kinases, cell extracts were analyzed by immunokinase assays using the SIM and SAM kinase-specific antibodies M23 and M24, respectively.

M23 antibody was raised against a synthetic peptide encoding the C-terminal 7 amino acids of the alfalfa SIM kinase (Jonak *et al.*, 1993). M24 antibody was raised against a synthetic peptide encoding the C-terminal 6 amino acids of the alfalfa SAM kinase (Jonak *et al.*, 1996). The specificity of the M23 and M24 antibodies was tested by immunoblotting different alfalfa MAP kinases. For this purpose, the *SIM* and *SAM* kinase genes, as well as the MMK2 (Jonak *et al.*, 1995) and MMK3 (Bögre *et al.*, 1999) genes, were expressed as glutathione-S-transferase fusion proteins in bacteria. After affinity purification, equal amounts of proteins were separated by SDS-PAGE (Fig. 4A) and immunoblotted with either M23 or M24 antibody. As shown in Fig. 4B, only GST-SIMK was recognized by M23, and only GST-SAMK was recognized by M24 antibody. Preincubation of the M23 and M24 antibodies with the M23 and M24 peptides, respectively, blocked the reaction (Fig. 4C, GST-SIMK + peptide and GST-SAMK + peptide, respectively).

The specificity of the M23 and M24 antibodies to immunoprecipitate active SIM and SAM protein kinases was also tested. Affinity-purified autoactivated GST-SIMK, GST-MMK2, GST-MMK3, and GST-SAMK were immunoprecipitated with both antibodies. Subsequently, the activity of the immunoprecipitated proteins was determined by in vitro kinase reactions using myelin basic protein as substrate. Whereas M23 was only able to immunoprecipitate active GST-SIM kinase, M24 antibody exclusively immunoprecipitated active GST-SAM kinase (data not shown). These results demonstrate that the M23 and M24 antibodies specifically recognize the SIM and SAM kinases, respectively. Since the antibodies are also able to immunoprecipitate the active kinases, the antibodies can be used for the quantification of denatured SIMK and SAMK protein on immunoblots as well as for the determination of in vivo protein kinase activities of SIM and SAM kinases, respectively.

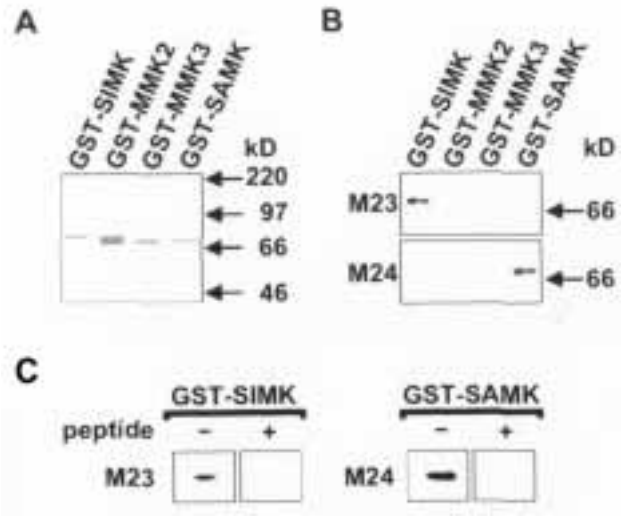

Fig. 4. **Specificity of SIMK and SAMK antibodies. (A)** Glutathione S-transferase (GST) fusion constructs of the *SIM* and *SAM* kinase genes were expressed in bacteria. Affinity purified GST-SIMK, GST-MMK2, GST-MMK3, and GST-SAMK were separated by SDS-PAGE and stained with Coomassie Blue. **(B)** M23 and M24 antibodies were raised against synthetic peptides encoding the C-terminal 7 and 6 amino acids of alfalfa SIMK and SAMK, respectively. The specificity of the antibodies was determined by immunoblotting GST-SIMK, GST-MMK2, GST-MMK3, and GST-SAMK with M23 (top panel) and M24 (lower panel) antibody. **(C)** Recognition of GST-SIMK by M23 antibody and GST-SAMK by M24 antibody can be specifically blocked by peptide competition. Preincubation of M23 antibody with an excess of M23 peptide blocked the immune reaction to GST-SIMK. Preincubation of M24 antibody with an excess of M24 peptide blocked the immune reaction to GST-SAMK.

The elicitor-activated 44-kD and 46-kD protein kinases are MAP kinases encoded by the *SAM* and *SIM* kinase genes

Elicitor-treated alfalfa cell extracts that were analyzed by in-gel kinase assays in Figure 3 were analyzed for the activity of SIM and SAM kinases. For this purpose, the MAP kinases were immunoprecipitated with the SIM and SAM kinase-specific antibodies, M23 and M24, respectively. The activity of the immunoprecipitated protein kinases was determined using MBP as substrate. Phosphorylated MBP was analysed by autoradiography after SDS-PAGE. As shown in Figure 5A, SIM kinase was activated at 5 min after elicitor treatment. SIM kinase activity decreased to non-induced levels at 40 min. SAM kinase was also transiently activated by fungal elicitor, showing a similar kinetics of activation and inactivation.

Western blotting of these extracts with M23 and M24 antibodies detected a single band of 46-kD and 44-kD, respectively (Fig. 5B). In contrast to the changing kinase activities, protein amounts of $p46^{SIMK}$ and $p44^{SAMK}$ did not change considerably over the experimental period of 50 min. These data show that fungal elicitor transiently activates the

SIM and SAM kinase pathways and that the activation of both kinases occurs by a post-translational mechanism.

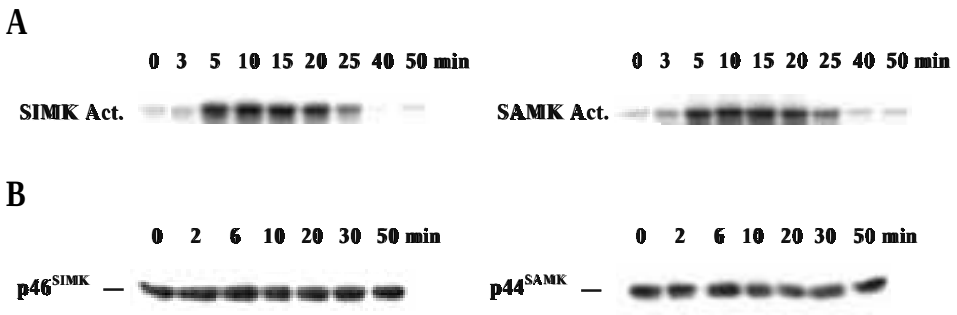

Fig. 5. **Yeast elicitor activates 44-kD SAM and 46-kD SIM kinases. (A)** Elicitor-induced activation of SIM and SAM kinases. Immunokinase assay of SIM and SAM kinase, respectively. Extracts from elicitor-treated alfalfa cells, containing 100 µg/mL of total protein, were immunoprecipitated with 5 µg of protein A-purified M23 and M24 antibody, respectively. Kinase reactions with the immunoprecipitated SIM and SAM kinases were performed with 1 mg/mL MBP as substrate, 0.1 mM ATP, and 2 µCi [^{32}P]-γ-ATP. Phosphorylation of MBP was analysed by autoradiography after SDS-PAGE. **(B)** SIM and SAM kinase protein amounts do not change after elicitor treatment. Elicitor-treated cell extracts were separated by SDS-PAGE, blotted to membranes and decorated with M23 and M24 antibody.

K252a inhibits elicitor-induced activation of SIM and SAM kinase pathways as well as medium alkalinisation and oxidative burst

To further investigate the role of the SIM and SAM kinase pathways with respect to the elicitor-induced defense responses, we pretreated alfalfa cells for 30 min with the protein kinase inhibitor K252a. Treating alfalfa cells with increasing concentrations of K252a resulted in a dose-dependent inhibition of elicitor-induced medium alkalinisation and the oxidative burst, showing almost complete inhibition of these defense responses at a 1 µM concentration of K252a (Fig. 6A and 6B, respectively). Because both, fungal elicitor-induced medium alkalinisation and oxidative burst was blocked at this concentration of K252a, the elicitor-induced activation of the MAPK pathways was investigated in cells that were pretreated with 1 µM K252a for 30 min. At this dose, the elicitor-induced activation of the SIM and SAM kinases was considerably inhibited (Fig. 6C). These results demonstrate that protein kinases are involved in elicitor-induced medium alkalinisation and oxidative burst.

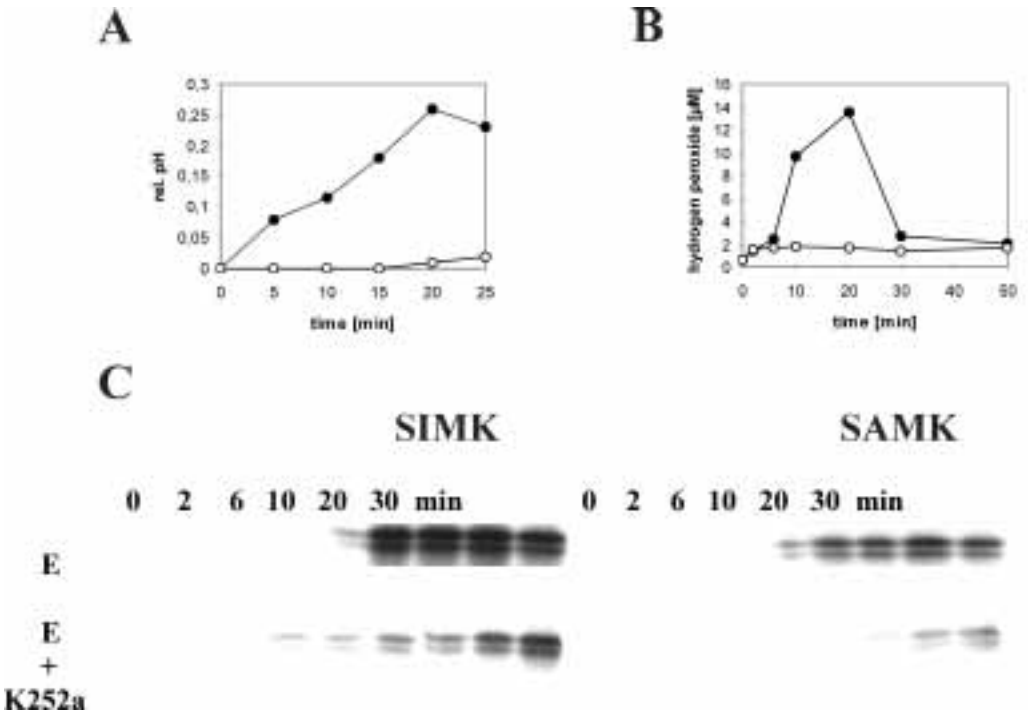

Fig. 6. **Elicitor-induced medium alkalinisation, oxidative burst, SIM, and SAM kinase activation are inhibited by K252a. (A)** Elicitor-induced medium alkalinisation is blocked by preincubating cells for 30 min with 1µM K252a. Medium alkalinisation induced by elicitor in the absence (filled circles) and in the presence of K252a (open circles). **(B)** Elicitor-induced oxidative burst is blocked by 1µM K252a. Production of hydrogen peroxide induced by elicitor in the absence (filled circles) and in the presence of K252a (open circles). **(C)** Elicitor-induced SIM and SAM kinase activation is blocked by 1µM K252a. Activation of SIMK and SAMK by elicitor (E) and by elicitor in the presence of K252a (E+K252a).

Staurosporine inhibits elicitor-induced alkalinisation and oxidative burst but not SIM and SAM kinase pathways

Because inhibition of the SIM and SAM kinase pathways was correlated with inhibition of the elicitor-induced medium alkalinisation and oxidative burst, we investigated whether there exists a causal relationship between these events. For this purpose, we also tested the protein kinase inhibitor staurosporine. Pretreating alfalfa cells for 30 min with increasing concentrations of staurosporine resulted in a dose-dependent inhibition of elicitor-induced medium alkalinisation and oxidative burst. At a concentration of 1 µM staurosporine, both elicitor-induced medium alkalinisation and oxidative burst were strongly inhibited (Fig. 7A and 7B, respectively). However, the elicitor-induced activation of the SIM and SAM kinases was not inhibited under these conditions (Fig. 7C), demonstrating that the SIM and SAM kinases are not involved in mediating elicitor-induced alkalinisation and oxidative burst. Instead, other protein kinase pathways exist, that are responsible for

inducing these defense reactions. The data also reveal that neither medium alkalinisation nor oxidative burst are required for SIM and SAM kinase activation.

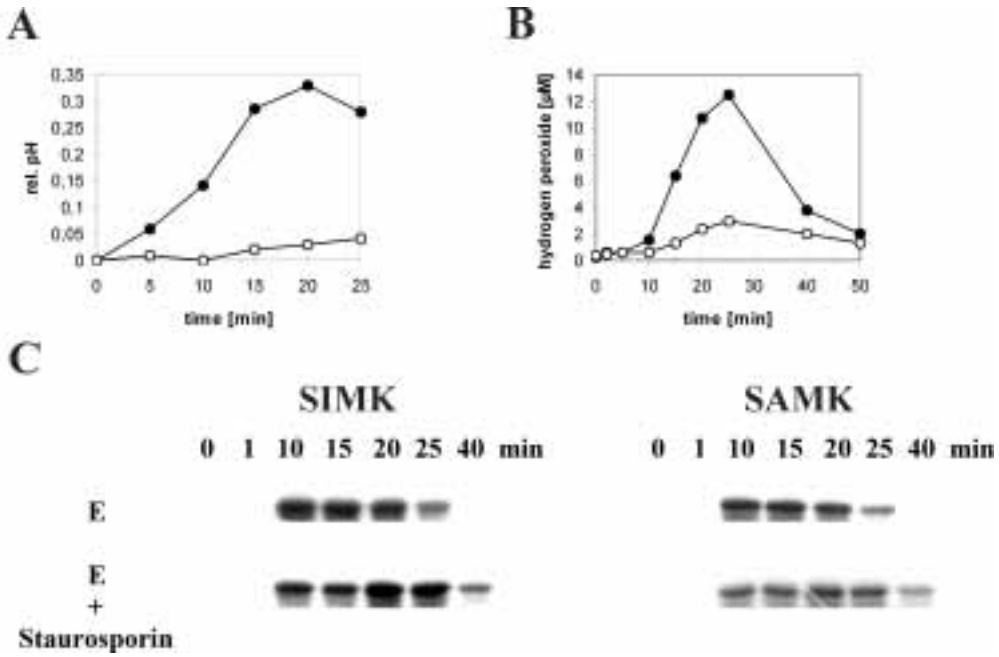

Fig. 7. **Elicitor-induced medium alkalinisation and oxidative burst are mediated by protein kinase pathways distinct from SIM and SAM kinase cascades. (A)** Elicitor-induced medium alkalinisation is blocked by preincubating the cells for 30 min with 1µM staurosporine. Medium alkalinisation induced by elicitor in the absence (filled circles) and in the presence of staurosporine (open circles). **(B)** Elicitor-induced oxidative burst is blocked by 1µM staurosporine. Production of hydrogen peroxide induced by elicitor in the absence (filled circles) and in the presence of staurosporine (open circles). **(C)** Elicitor-induced SIM and SAM kinase activation is not blocked by 1µM staurosporine. Activation of SIMK and SAMK by elicitor (E) and by elicitor in the presence of staurosporine (E+staurosporine).

Discussion

Protein phosphorylation has been shown to be involved in mediating elicitor-induced defense responses in a number of systems. Although the responsible protein kinases and phosphatases are still largely unidentified, recent evidence in tobacco indicates that the two specific MAP kinases SIPK and WIPK are involved in elicitor signaling (Romeis *et al.*, 1999; Zhang and Klessig, 1998b; Zhang *et al.*, 1998). To test whether this class of MAP kinases is a general mediator of elicitor signal transduction, alfalfa cells were treated with an elicitor that was derived from yeast cell walls and was able to induce typical defense-related responses, including medium alkalinisation and production of reactive oxygen species. Our data reveal that the non host-specific elicitor from yeast cell walls activates the SAM and

SIM kinases in alfalfa. Whereas the SAM kinase is most closely related to the elicitor-inducible parsley ERMK and tobacco WIPK (Ligterink *et al.*, 1997; Romeis *et al.*, 1999; Zhang and Klessig, 1998b), the SIM kinase is most similar to the tobacco SIPK that is activable by different elicitors (Romeis *et al.*, 1999; Zhang and Klessig, 1998b; Zhang *et al.*, 1998). These results support the concept that these two classes of MAP kinases are general mediators of defense signaling in plants, and suggest that these MAP kinases integrate signals from different elicitor-induced pathways. How can this integration of pathways be imagined to occur at the molecular level? In animals, MAP kinase pathways are also activated in response to many extracellular signals (for review, see Robinson and Cobb, 1997). MAPK activation is mediated by an activator protein kinase, MAPKK (MAP kinase kinase), that is itself activated by a MAPKKK (MAP kinase kinase kinases), and this module of three functionally interlinked protein kinases can integrate a variety of upstream signals through interaction with other protein kinases or GTP-binding proteins, which physically couple the MAPK module to specific membrane-located receptors. The available evidence makes it likely that plant MAPK modules may function in similar ways to integrate diverse elicitor signaling pathways.

Activation of MAP kinases belongs to the most rapid cellular responses to elicitors. Therefore, elicitor-induced MAPK signaling pathways might be suitable candidates for mediating subsequent defense responses. In accordance with such a role, inhibition of the MAP kinase pathways with the protein kinase inhibitor K252a also blocked yeast elicitor-induced medium alkalinisation and oxidative burst. On the other hand, because cytosolic acidification was shown to be sufficient for MAPK activation in tobacco cells (Tena and Renaudin, 1998), these results could not rule out the possibility that the inhibition of cytosolic acidification, as revealed by the alkalinisation of the medium, was not the effect but the cause of the observed failure of elicitor-induced MAPK activation. Experiments using the protein kinase inhibitor staurosporine unambiguously showed that elicitor-induced MAP kinase activation occurs independently of cytosolic acidification. When cells were treated with the protein kinase inhibitor staurosporine, elicitor-induced medium alkalinisation and oxidative burst were completely blocked, but SIM and SAM kinase activation were unaffected. In agreement with our findings in *Cf9*-expressing tobacco cells where Avr9-induced activation of WIPK and SIPK was found to act on other targets than those responsible for the production of the oxidative burst (Romeis *et al.*, 1999), these data demonstrate that the MAP kinase cascades are not involved in activating medium alkalinisation and production of reactive oxygen species in response to yeast elicitor. Taken together, it appears that not the MAP kinases, but yet other, unidentified protein kinase pathways are responsible for mediating medium alkalinisation and oxidative burst in response to specific and non-specific elicitors.

If elicitor-induced medium alkalinisation and oxidative burst are signaled independently of the MAP kinases, what function could the SIPK/SIMK and WIPK/SAMK pathways have in defense? Several reports show that SAM kinase is activated by a number of stresses, such as cold, drought, touch, and wounding (Jonak *et al.*, 1996; Bögre *et al.*, 1996; 1997). The SAM kinase homolog in tobacco, WIPK, has been shown to play a role in wound-induced production of jasmonic acid and derivatives (Seo *et al.*, 1995; 1999). Because a number of wound- and pathogen-induced genes are induced by jasmonates, WIPK/SAMK might link various stresses to jasmonate-induced gene expression. In analogy to mammalian cells, where stress-activated MAP kinases can induce prostaglandin synthesis via phospholipase A2, Seo *et al.* (1995) suggested that WIPK might activate lipases of the jasmonate pathway. In parsley cells, the WIPK/SAM kinase homolog ERMK (elicitor-responsive MAP kinase) is rapidly activated by a fungal elicitor (Ligterink *et al.*, 1997). Importantly, activation of ERMK is correlated with its nuclear translocation, suggesting that this class of MAP kinases might be directly involved in activating transcription of genes involved in jasmonate biosynthesis.

Compared to the possible functions of the SAMK/WIPK/ERMK subfamily of MAPKs, much less is known on the role of SIMK/SIPK and its homologs in other plant species. Besides an obvious function in pathogen defense signaling, tobacco SIPK was also linked to signaling of abiotic stresses, such as wounding (Zhang and Klessig, 1988a). So far, however, no targets for the class of SIP/SIM kinases have been identified to suggest a specific function for these kinases.

In summary, it appears that specific MAP kinase pathways are involved in mediating responses to various elicitors and stresses. Our results support the notion that elicitor-induced oxidative burst and medium alkalinisation are mediated by protein kinase pathways independent of the SIM and SAM kinase cascades. In analogy to animals and yeast, which also use specific MAP kinase pathways for stress signaling, it can be expected that the SIMK/SIPK and SAMK/WIPK/ERMK subfamilies play equally important roles in mediating stress responses in plants. The molecular identification of these MAP kinases as mediators of a variety of elicitors and abiotic stresses enables us now to investigate a number of important questions. One of the primary tasks will be to define their biological functions with respect to pathogen and/or stress resistance. To understand the molecular basis of their functions will require the identification of the molecular targets that are regulated by these kinases. Another challenge is how these MAP kinases can be activated by so many different extracellular signals. This will necessitate the isolation and study of the upstream components of these pathways. Both approaches should pave the way for an understanding of how plants sense, transduce, and orchestrate the sequence of events that make up the full complexity of physiological responses to pathogen attack.

Experimental Procedures

Plant cell culture conditions

Suspension cultured alfalfa cells were produced from callus of *Medicago sativa* "Du Puits" root cuttings. Cells were cultivated in MS (Murashige and Skoog, 1962) medium containing 1mg/L 2,4 dichlorophenoxyacetic acid and 0.1mg/L kinetin.

Preparation of yeast elicitors

The elicitor was derived from a yeast cell wall preparation. One kg of baker's yeast was stirred for 30 min in 1.5 L of citrate buffer (20 mM citrate, pH 7.5). After autoclaving (30 min, 121°C, 1.2 bar) the preparation was centrifuged for 10 min at 8000xg. One vol EtOH (96%v/v) was added to the filtered supernatant and stirred o/n at 4°C. After centrifugation at 8000xg for 10 min, the pellet was dissolved in water and centrifuged again. The final supernatant was collected and dialyzed (Spectra-Pore MWCO 1000, Serva) o/n at 4°C and freeze-dried for storage. 1 mg of the elicitor corresponded to a glucose equivalent of 185 µg.

Measurement of pH in the medium of plant cell suspension cultures

Alfalfa cell suspension cultures (approximately 0.2 g of fresh cell mass in 3 ml suspension) were incubated in open vials on an orbital shaker. The pH of the medium was continuously registered with a glass pH electrode.

Determination of reactive oxygen species

The production of reactive oxygen species was monitored by chemiluminescence from the ferricyanide-catalyzed oxidation of luminol. One week after subcultivation, alfalfa cells were harvested on a filter paper funnel. The cells were immediately transferred to sterile pre-incubation medium containing 3% w/v sucrose and 4% v/v MS-mediun (Sigma). The transfer was carried out on a balance in order to adjust the suspension to 0.2 g of cells per mL pre-incubation medium. Prior to elicitation, the cell cultures were incubated for 2 to 4 hours on a shaker (120 rpm) in the dark at 24°C. Generally, 8 mL of the preincubated cell suspension was treated with elicitor. For inhibitor treatments, cells were preincubated for 30 min in the presence of inhibitor. 200 µl aliquots were removed at the given times and mixed with 700 µL potassium phosphate buffer (50 mM, pH 7.9) and 100 µL luminol (1.21 mM in the same buffer). After the addition of 100 µL potassium ferrocyanide (14 mM $K_4Fe(CN)_6$) chemiluminescence was immediately recorded in a

luminometer (model 1250, Pharmacia-LKB). The maximal peak height, reached immediately after addition of the potassium ferrocyanide, was used as a measure of activated oxygen species.

In-gel protein kinase assays

Alfalfa cell extracts were prepared at different times after elicitor treatment in extraction buffer (25 mM Tris.HCl, pH 7.5, 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol, 0.1 % NP40, 15 mM p-nitrophenylphosphate, 60 mM ß-glycerophosphate, 0.1 mM $NaVO_3$, 1 mM NaF, 1 mM phenylmethylsulfonylfluoride, 10 µg/ml of each, leupeptine and aprotinine). The cleared supernatant after centrifugation at 20 000 g for 45 min was used.

For in-gel protein kinase reactions, cell extracts containing 20 µg of total protein per lane were separated by SDS-polyacrylamide gel electrophoresis. Myelin basic protein (0.5 mg/ml) was used as a substrate that was polymerized in the polyacrylamide gel. After protein renaturation, the kinase reactions were carried out in the gel as described (Usami *et al.*, 1995).

Antibody production

The following synthetic peptides, FNPEYQQ and LNPEYA, corresponding to the carboxyl terminal 7 or 6 amino acids of either the alfalfa SIM and SAM kinases, respectively (Jonak *et al.*, 1993; 1996), were conjugated to a purified derivative protein of tuberculin. Polyclonal antiserum was raised in rabbits and finally purified by protein A column chromatography.

Immune kinase assays

Cell extracts containing 100 µg of total protein were immunoprecipitated with 5 µg protein A-purified M23, and M24 antibody. The immunoprecipitated protein was washed three times with wash buffer I (20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 1 % Triton X-100), once with the same buffer but containing 1 M NaCl, and once with kinase buffer (20 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT). Kinase reactions of the immunoprecipitated protein were performed in 15 µl of kinase buffer containing 1 mg/ml MBP, 0.100 mM ATP and 2 µCi [^{32}P]-γ-ATP. The protein kinase reaction was carried out at room temperature for 30 minutes. The reaction was stopped by addition of SDS sample buffer. The phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE.

Immunoblotting

Protein blotting was performed as described (Bögre *et al.*, 1995). Briefly, cell extracts containing 20 µg of total protein were separated by SDS-PAGE, immunoblotted to polyvinylidene difluoride membranes (Millipore) and probed with the M23 or M24 antibody at a 1:10.000 dilution. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody and the reaction was visualized by fluorography using CDP-Star (Amersham Life Sciences) as a substrate.

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Chapter 6

Concluding remarks

In the last few years many putative components of plant MAPK pathways have been isolated. In accordance with the diverse roles of MAPKs in yeast and animals, plant MAPKs seem also to be involved in signaling a whole range of stimuli. In this thesis, evidence is given for the involvement of some plant MAPKs in the stress response of plants.

The results in chapter 3 show the activation of SAMK (Stress activated MAPK) upon wounding of alfalfa leaves. Activation of SAMK occurs both at the transcriptional as well as on the post-translational level. Interestingly, *SAMK* transcript levels increase at a later time point than SAMK activation, suggesting that the *SAMK* gene may be a direct target of the SAMK pathway. At the moment the reason why the transcript accumulation is not accompanied with an increase in SAMK protein levels is unclear, but could be explained by a more rapid turn-over rate of SAMK protein after activation. No evidence is yet available for such a model and further experiments are required to clarify this point.

It was shown that activation of SAMK is a post-translational process, since α-amanitin and cycloheximide did not block the activation. However, these drugs did block SAMK inactivation, indicating that *de novo* transcription and translation of protein factors is required for inactivation of SAMK. An alfalfa PP2C-like phosphatase (MP2C) was isolated that was shown to be this or one of these factors (Meskiene *et al.*, 1998a). MP2C is able to inactivate the wounding-induced SAMK pathway *in vitro*. Furthermore, the *MP2C* gene was transcriptionally activated upon wounding of alfalfa leaves and the kinetics of this transcriptional activation correlated very well with the inactivation of SAMK. It was demonstrated that an inactivation mechanism is induced after activation of SAMK that prevents a reactivation of the SAMK pathway for a certain time (Chapter 3). This refractory period is of approximately the same length as the presence of *MP2C* transcripts (Meskiene *et al.*, 1998a). Taken together, these results suggest a role for MP2C as one of the factors that is able to reset the SAMK pathway to make it accessible for subsequent sensing of changes in the environment. We speculate that the expression of the *MP2C* gene is regulated by the SAMK pathway itself and that it is part of a negative feedback loop (Meskiene *et al.*, 1998b), as also has been shown for other systems (Sun *et al.*, 1993; Doi *et al.*, 1994; Brondello *et al.*, 1997).

The role of SAMK activation upon wounding and its activation mechanism is not known. It was shown that proposed mediators of the wound signal, like MeJA and electrical signals, were not able to activate SAMK (Bögre *et al.*, 1997). There is, however, good evidence that the tobacco SAMK homologue, WIPK, regulates the wound-induced accumulation of jasmonic acid (Seo *et al.*, 1995; Seo *et al.*, 1999) and SAMK could have a similar role in the wound response of alfalfa. In tomato, wounding activates a MAPK-like kinase, and this kinase was also activated by known secundary messengers of the wound signal, like systemin, polygalacturonic acid and chitosan (Stratman and Ryan, 1997).

Therefore, these compounds are likely to have a role in the systemic activation of wound-induced MAPKs.

Besides wound-induced transcriptional and post-translational activation of WIPK, another tobacco MAPK (SIPK for salicylic acid-induced protein kinase) has been shown to be activated post-translationally by wounding (Zhang and Klessig 1998a). Recently, the alfalfa SIPK homologue, SIMK (stress induced MAPK) was also shown to be involved in the wound response (unpublished results). In accordance with data on the tobacco SIPK, SIMK activation only occured at the post-translational level and not at the transcriptional level. In summary, the results presented in this thesis together with results from other groups suggest that MAPK pathways are a highly conserved and general mechanism by which plants sense and respond to wounding.

Besides a role of MAPKs in the wound-response, the results presented in this thesis also show the involvement of MAPKs in the defense of plants against pathogens. The parsley ERMK (elicitor responsive MAPK) was the first MAPK that was demonstrated to be involved in the elicitor response of plants (Chapter 4). Treatment of parsley suspension cultured cells with the Pep13 elicitor from the fungal pathogen *Phytophthora sojae* results in an activation of specific ion channels, an oxidative burst and ERMK (Jabs *et al.*, 1997; Chapter 4). ERMK gets activated at both the post-translational as well as at the transcriptional level after elicitor treatment and it could be shown that this activation was the result of the binding of Pep13 to its specific receptor (Chapter 4). Moreover, inhibitor studies could place ERMK downstream of elicitor-induced ion fluxes and upstream or parallel of the oxidative burst.

Similar to many mammalian and yeast MAPKs (Chen *et al.*, 1992; Sanghera *et al.*, 1992; Ferrigno *et al.*, 1998), elicitor-induced activation of the parsley MAPK resulted in translocation to the nucleus. MAPKs phosphorylate transcription factors in the nucleus and thereby regulate gene transcription (Treisman, 1996). Several parsley transcription factors have been identified that are likely to have a role in elicitor-mediated transcriptional activation (Kombrink and Somssich, 1995; Rushton *et al.*, 1996). Although it has not yet been shown that phosphorylation of these transcription factors is responsible for elicitor-induced transcription of PR genes, these transcription factors could be MAPK substrates and be the link between elicitor-induced MAPK activation and activation of PR genes.

The involvement of MAPKs in the pathogen response was not only demonstrated for parsley, but also for tobacco and alfalfa. In chapter 5 the activation of both SIMK and SAMK upon treatment of alfalfa suspension cultured cells with a yeast cell wall elicitor is reported. Also the tobacco SIPK and WIPK, which are most closely related to SIMK and SAMK, respectively, have been identified to be rapidly activated by various elicitors (Zhang and Klessig, 1998b; Zhang *et al.*, 1998; Romeis *et al.*, 1999), suggesting that these MAP kinases perform homologous functions in different plant species. In general, MAPK

activation has been shown for a large number of plant-pathogen interactions, during both race-specific and non-host resistance responses and there seem to be at least two classes of MAPKs that are general mediators of defense signaling in plants. Despite accumulating evidence on the involvement of MAPKs in pathogen response of plants, little is still known about the other components of the elicitor-induced MAPK pathways. By the use of different inhibitors, it could be established that MAPK cascades in most cases function downstream of a Ca^{2+} influx. It is known that Ca^{2+} plays an important role in many signaling pathways (Bush, 1995) and an important role of Ca^{2+} in the pathogen response of plants has been proposed by different groups (Dietrich *et al.*, 1990; Viard *et al.*, 1994; Nürnberger *et al.*, 1994; Zimmerman *et al.*, 1997). Although influx of extracellular Ca^{2+} appears to be necessary, it is not sufficient for elicitor-induced MAPK activation in several systems (Suzuki and Shinshi, 1995; Ádám *et al.*, 1997; Lebrun-Gracia *et al.*, 1998; Romeis *et al.*, 1999). The data presented in chapter 4 and 5 of this thesis together with a report from Lebrun-Garcia and co-workers (1998) showed that MAPKs act upstream or independent of the oxidative burst and recently, Romeis and co-workers (1999) demonstrated that activation of both WIPK and SIPK, as a result of Avr9 treatment of tobacco suspension cultured cells expressing Cf9, occurs independently of the oxidative burst, since both processes could be blocked separately. Not only the oxidative burst seems to be independent of the elicitor response, but in alfalfa also the medium alkalinisation after elicitor treatment is not necessary for MAPK activation (Chapter 5).

The involvement of SIMK and SAMK in the signaling pathway of wounding and elicitor response, but also in signaling several abiotic stresses (Jonak *et al.*, 1996; Bögre *et al.*, 1996; unpublished results), indicates that the signaling pathways of these stresses cannot be considered as a linear chain of events, since several signaling pathways converge to activate the same kinases. In metazoans, different receptors can also feed into the same MAPK pathway and the major convergence point appears to occur at the level of MKKKs in these systems. Therefore, it will be interesting to identify the MKKKs that function in the different pathways involved in SIMK and SAMK activation.

The question remains open, how one MAPK can give different responses. From our knowledge of MAPK pathways in mammals and yeast, it is known that activation of one MAPK can lead to different cellular responses. It has been shown that different levels and kinetics of activation may determine the outcome of the signal transduction process. Such a mechanism has been shown for several human MAPK cascades and the yeast SMK1 pathway. SMK1 has an important role in spore morphogenesis, and distinct steps of spore morphogenesis are shown to be directly correlated with the magnitude of SMK1 activity. Increasing SMK1 activity levels allow more and later spore morphogenesis events (Wagner *et al.*, 1999). JNKs and the p38 kinases are 2 different mammalian MAPK groups that become activated by different environmental stresses, including heat stress, osmotic stress,

pathogen components and proinflammatory signals, and the kinetics of activation of both kinases have been shown to be important for the determination of cell fate (Xia *et al.*, 1995; Chen *et al.*, 1996a, 1996b; Goillot *et al.*, 1997; Nishina *et al.*, 1997). For example, whereas the T-cell activation signal induces a rapid and transient activation of JNK and the proliferation of T-cells, UV radiation induces a delayed and persistent activation of JNK and apoptotic cell death (Chen *et al.*, 1996a, 1996b). Differences in the kinetics of MAPK activation upon different stresses have been observed for plant MAPKs. In general MAPK activation upon mechanical stress is very transient (Chapter 3; Bögre *et al.*, 1996; Seo *et al.*, 1995; Zhang and Klessig, 1998a) compared to a more prolonged activation of MAPKs upon elicitor treatment (Chapter 4,5; Romeis *et al.*, 1999; Zhang and Klessig, 1998b; Zhang *et al.*, 1998). Furthermore, the prolonged activation of a MAPK in tobacco has been proposed to be involved in the onset of programmed cell death (Suzuki *et al.*, 1999), which would be in accordance with the importance of the duration of activation of JNK and p38 MAPKs in determination of cell fate in mammalian cells. Treatment of tobacco suspension cultured cells with the fungal elicitor xylanase from *Trichoderma viride* (TvX) was able to activate both an HR-like host hypersensitive cell death (hcd) and a 47 kDa MAPK (Suzuki *et al.*, 1999). The kinase was activated to the same level as after treatment with the *P. infestans* elicitor PiE (Suzuki and Shinshi, 1995), but with a more prolonged kinetics. Interestingly, only TvX is able to induce hypersensitive cell death (Yano *et al.*, 1998). Furthermore, it was shown that the phosphatase inhibitor calyculin A can both induce a prolonged activation of the 47 kDa MAPK and a hypersensitive cell death, and staurosporine can selectively block PiE-induced activation of the kinase and the induction of defense gene accumulation by both PiE and TvX elicitor without affecting TvX-induced MAPK activation and hcd (Suzuki and Shinshi, 1995; Suzuki *et al.*, 1995, 1999).

Another way in which specificity of MAPK cascades can be achieved is by the physical separation of signaling pathways. This can happen through specific cellular localization of components of each pathway, but also by differential expression of certain signal pathway components and substrates in specific cells. In addition, and probably the most important way to separate specific pathways is by scaffold proteins that tether specific signaling components into a complex. It is tempting to speculate that also in plants distinct responses are accomplished by creating analogous stimulus- and pathway-specific multikinase complexes, and the isolation of plant scaffold proteins will be an important step to understand the exact regulation of the plant MAPK pathways. The ability of the *Arabidopsis* MKKK, AtMEKK1 to bind both the MKK, AtMEK1, and the MAPK, AtMPK4 (Ichimura *et al.*, 1998b), could indicate a role for AtMEKK1 as scaffold protein as has been postulated for the rat MEKK1 (Whitmarsh and Davis, 1998).

One of the primary tasks for the next few years will be to define the biological functions of the stress activated MAPKs with respect to pathogen and/or stress resistance. To

understand the molecular basis of their functions will require the identification of the molecular targets that are regulated by these kinases. To solve the question how these MAP kinases can be activated by so many different extracellular signals, it will be necessary to isolate and study upstream and regulating components of these pathways. Both approaches should pave the way for an understanding of how plants sense, transduce, and orchestrate the sequence of events that make up the full complexity of physiological responses to changes in their environment.

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Samenvatting

Planten staan bloot aan een groot aantal verschillende extracellulaire stimuli en gebruiken een breed spectrum aan signaalwegen om de juiste reacties daarop te geven. Mitogeen geactiveerde protein kinasen (MAPKs) spelen een belangrijke rol in de signaaltransductie van gisten en dieren en een toenemende hoeveelheid informatie lijkt te wijzen op een gelijke rol voor MAPKs. MAPKs functioneren als deel van protein kinase cascades, bestaande uit een MAPK, een MAPK kinase (MKK), en een MAPK kinase kinase (MKKK). MKKKs kunnen MKKs activeren door ze te fosforyleren op geconserveerde threonine of serine residuen, en vervolgens kunnen MKKs de MAPKs activeren door fosforylatie van sterk geconserveerde tyrosine en threonine residuen.

De introductie geeft een kort overzicht van de MAPK wegen die gebruikt worden door gisten en dieren en een uitgebreider overzicht van de huidige kennis over de functie van MAPKs in planten. Analyse van alle geïsoleerde plant MAPK sequenties laat zien dat deze kunnen worden verdeeld in tenminste 5 subfamilies (hoofdstuk 2). Voor sommige van deze groepen is het bewezen dat MAPKs met een homologe sequentie ook een ongeveer gelijke functie hebben. Analyse van "expressed sequence tags" (ESTs) en gedeeltelijke cDNAs, die voor MAPKs coderen, heeft het bestaan van een nieuwe MAPK subfamilie getoond.

Eén van de meest ingrijpende stress factoren waaraan planten kunnen worden blootgesteld is verwonding. Verwonding kan het gevolg zijn van fysische verwonding, maar ook van blootstelling aan herbivoren of pathogenen en activeert een breed spectrum aan reacties, voornamelijk gebaseerd op de activatie van genen die een rol hebben in reperatie en verdedigings processen. In hoofdstuk 3 wordt de rol van een MAPK in de reactie van alfalfa op verwonding beschreven. Er kon worden aangetoond dat verwonding de stress geactiveerde MAPK (SAMK) van alfalfa zowel post-translationeel als transcriptioneel activeert. The inactivatie, maar niet de activatie van SAMK is afhankelijk van "*de novo*" transcriptie en translatie van eiwit factor(en).

Een andere belangrijke stress factor waaraan planten worden blootgesteld is de aanval door pathogenen. De verschillende verdedigings reacties van planten op pathogenen worden normaal gesproken ook geactiveerd door specifieke van de pathogenen afkomstige factoren (elicitors). Behandeling van peterselie cellen met een 13 aminozuren lange oligopeptide fragment van een 42 kDa extracellulair glycoprotein van de pathogene schimmel *Phytophthora sojae*, resulteert in de activatie van een breed spectrum aan verdedigings reacties. De signaalwegen die leiden tot deze antwoorden bestaan o.a. uit de activatie van verschillende ion kanalen en de productie van reactieve zuurstof moleculen. Ook een MAPK, die kan worden geactiveerd door de elicitor behandeling, kon worden geïdentificeerd. Deze MAPK werkt "downstream" van de activatie van verschillende ion kanalen en "upstream" of onafhankelijk van de "oxidative burst". Na activatie door de elicitor wordt de MAPK verplaatst naar de celkern en zou daar transcriptie factoren kunnen activeren, die verantwoordelijk zijn voor de activatie van verdedigings genen.

Bij een poging om vergelijkbare resultaten te verkrijgen voor andere plantensoorten, werden de reacties van alfalfa cellen op een gist elicitor bekeken. Behandeling van alfalfa cellen met de gist elicitor resulteert in de snelle en tijdelijke activatie van twee protein kinasen met een relatieve moleculaire massa van 44-kD en 46-kD. Deze kinasen werden respectievelijk geïdentificeerd als SAMK en SIMK (stress-induceerbare MAPK). De door gist elicitor geactiveerde medium alkalinisatie, "oxidative burst", en MAPK activatie kon worden gestopt door de protein kinase remmer K252a. Dit laat zien dat protein kinase wegen verantwoordelijk zijn voor de doorgave van deze elicitor reacties. SAMK and SIMK zijn echter niet betrokken bij de elicitor-geactiveerde medium alkalinisatie of "oxidative burst", omdat staurosporine, een andere protein kinase remmer, geen effect heeft op de activatie van de SAMK en SIMK signaaltranductiewegen door de elicitor, maar wel de medium alkalinisatie en de "oxidative burst" volledig stopt. Deze resultaten laten zien dat de, door de elicitor geactiveerde, medium alkalinisatie en "oxidative burst" afhankelijk zijn van protein kinasen, maar dat deze protein kinasen in andere signaaltranductiewegen werken dan de elicitor-geactiveerde SAMK and SIMK.

Samenvattend kunnen we concluderen dat de resulaten die in dit proefschrift worden beschreven een bewijs leveren voor de betrokkenheid van verschillende MAPKs in de reacties van planten op meerdere stressen, maar dat verder onderzoek nodig is om de exacte rol van deze MAPKs in hun respectievelijke transductiewegen te definieren.

Curriculum vitae

Jan Willem Ligterink (Wilco) werd op 17 juli 1969 geboren te Winterswijk. In 1987 behaalde hij het VWO diploma aan de rijksscholengemeenschap “Hamaland” te Winterswijk en datzelfde jaar begon hij aan de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. Het doctoraal examen werd behaald in november 1992 en omvatte afstudeervakken bij de vakgroep Microbiologie (Dipl. Ing. J. Nölling en Prof. Dr. W.M. de Vos) en Erfelijkheidsleer (Drs. P. van den Broek en Prof. Dr. C. Heyting), alsmede een stage bij het Europees Moleculair Biologisch Laboratorium (EMBL) in Heidelberg, Duitsland in de groep van Dr. H. Stunnenberg. Na afsluiting van zijn studie heeft hij tot juli 1995 onderzoek gedaan op vrijwillige basis bij achtereenvolgens de vakgroep Erfelijkheidsleer in de groep van Dr. T. Goossen en bij de vakgroep Biochemie in de groep van Dr. W.J.H. van Berkel.

Van augustus 1995 tot september 1999 is hij in het kader van een promotie-onderzoek verbonden geweest aan het Insituut voor Microbiologie en Genetica van de Universiteit van Wenen, waar hij onder supervisie van Dr. H. Hirt het onderzoek heeft uitgevoerd zoals beschreven in dit proefschrift. Vanaf november 1999 zal hij als post-doc werkzaam zijn bij het laboratorium voor fytopathologie van de Landbouwuniversiteit Wageningen.

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