

REVIEW ARTICLE

Arabidopsis MAPKs: a complex signalling network involved in multiple biological processesJean COLCOMBET and Heribert HIRT¹

Unité de Recherche en Génétique Végétale, INRA–CNRS–UEVE, 2 rue Gaston Crémieux, 91057 Evry Cédex, France

Many changes in environmental conditions and hormones are mediated by MAPK (mitogen-activated protein kinase) cascades in all eukaryotes, including plants. Studies of MAPK pathways in genetic model organisms are especially informative in revealing the molecular mechanisms by means of which MAPK cascades are controlled and modulate cellular processes. The present review highlights recent insights into MAPK-based signalling in

Arabidopsis thaliana (thale cress), revealing the complexity and future challenges to understanding signal-transduction networks on a global scale.

Key words: *Arabidopsis thaliana* (thale cress), biotic and abiotic stresses, mitogen-activated protein kinase (MAPK), phosphorylation cascade, signalling.

INTRODUCTION

As plants are sessile organisms unable to escape changing environmental conditions, they developed mechanisms to sense and adapt their physiology, growth and development. These adaptations involve a rapid and dynamic regulation of enzymatic activities and the modification of gene-expression programmes.

Phosphorylation/dephosphorylation of proteins is among the most common post-translational modification in all organisms. Protein phosphorylation is carried out by protein kinases that are typically organized in signalling cascades. It is estimated that about 30% of all proteins are phosphorylated in a eukaryotic cell. Not surprisingly, about 5% of the genomes of green plants (Viridiplantae) code for protein kinases [1,2]. Roughly 10% of all plant kinases are involved in MAPK (mitogen-activated protein kinase) pathways. In the present review we discuss the function of plant MAPKs, with a particular focus on *Arabidopsis thaliana* (thale cress) studies, and Table 1 gives an overview of our current knowledge. Genetic studies coupled with biochemical approaches have allowed breakthroughs in this area and have shown that MAPKs are not only involved in biotic and abiotic signalling, but also in processes such as hormonal and developmental signalling. Astonishingly, the same kinases are often involved in distinct physiological processes. Moreover, MAPK modulators such as phosphatases were found to be involved in the control of signal strength and the duration of signalling processes.

BACKGROUND

MAPK cascades are conserved signalling modules found in all eukaryotic cells, including plants, fungi and animals. A MAPK cascade minimally consists of three kinases: a MAP3K (MAP2K

kinase), a MAP2K (MAPK kinase) and a MAPK, which phosphorylate, and therefore activate, each other in a specific way. MAP3Ks are serine/threonine kinases phosphorylating two amino acids in the S/T-X₃₋₅-S/T motif of the MAP2K activation loop. MAP2Ks are dual-specificity kinases that activate a MAPK through double phosphorylation of the T-X-Y motif in the activation loop. MAPKs are serine/threonine kinases able to phosphorylate a wide range of substrates, including other kinases and/or transcription factors. A fourth level of kinases, named MAP4Ks (MAP3K kinases), may act as adaptators linking upstream signalling steps to the core MAPK cascades. Interactions between kinases within a MAPK cascade occur through docking sites present in the kinases and/or with the help of external scaffolding proteins. MKPs (MAPK phosphatases) are involved in the time-dependent control or in the shut-down of the pathway after signalling.

Complete sequencing of the *Arabidopsis* genome has revealed a large number of genes coding for MAPK-related kinases: 20, 10 and 80 kinases share significant similarity with the MAPK, MAP2K and MAP3K families found in animals and fungi respectively [3]. A similar repertoire of genes was observed in the sequenced genomes of other plants, such as rice (*Oryza sativa*), poplar (*Populus* sp.) or grapevine (*Vitis vinifera*). In general, MAPKs can be found in a large number of plant species, indicating that MAPK cascades are also very conserved signalling mechanisms among higher plants. MAPKs and MAP2Ks constitute homogeneous kinase families, mechanistically behaving like their homologues in animals and yeast and divided into four subfamilies (A–D) based on sequence similarities [3]. The 80 MAP3Ks are a much more heterogeneous group of protein kinases [4] that can be further divided into three main subgroups: the MEKK (MAPK/ERK kinase kinase)-like MAP3Ks, for which

Abbreviations used: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ACS6, ACC synthase 6; ANP1, *Arabidopsis* NPK1 (*Nicotiana* protein kinase 1)-related kinase 1; AOS, active oxygen species; BAK1, BRI1-associated kinase 1; bZIP, basic leucine zipper; CERK1, chitin elicitor receptor kinase 1; CTR1, constitutive triple response 1; EDS1, enhanced disease susceptibility 1; EF-Tu, elongation factor thermo-unstable; EIN2, ethylene-insensitive 2; ET, ethylene; Flg22, a 22-amino-acid-long peptide derived from flagellin; ETR1, ET response 1; FLS2, FLAGELLIN SENSING 2; HR, hypersensitive response; JA, jasmonic acid; MAPK/MPK, mitogen-activated protein kinase; MAP2K, MAPK kinase; MAP3K, MAP2K kinase; MeJA, methyl jasmonate; MEKK, MAPK/ERK kinase kinase; MKP, MAPK phosphatase; MKS1, MPK4 substrate 1; NLP, Nep1-like protein; OX11, oxidative signal-induced 1; PAD4, phytoalexin-deficient 4; PAMP, pathogen-associated molecular pattern; PCD, programmed cell death; *PR1*, pathogenesis-related 1 gene; RbohD, respiratory-burst oxidase protein D; RLK, receptor-like kinase; RNAi, RNA interference; SA, salicylic acid; VIP1, VirE1-interacting protein 1.

¹ To whom correspondence should be addressed (email heribert.hirt@evry.inra.fr).

Table 1 An integrative list of our knowledge on *Arabidopsis* MAPK, MAP2K and MAP3K

Abbreviation: ND, not determined.

Name	Activated by:	Genetic data	Functional cascade	Target(s)
MAPK				
MPK1	JA [44]; ABA [44]	ND	MKK3–MPK1/2/7/14 [40]	ND
MPK2	JA [44]; ABA [44]	ND	MKK3–MPK1/2/7/14 [40]	ND
MPK3	H ₂ O ₂ [27]; O ₃ [67]; PAMPs [17]; osmotic shock [66]; ABA [63]; ET [54]	Wild-type-like plant, except: ABA-dependent; H ₂ O ₂ -induced stomatal closure impaired [63]; hypersensitive to O ₃ -induced oxidative burst [68]; redundant with MPK6; <i>mpk3mpk6</i> dwarfed with <i>yoda</i> -like stomatal patterning [20]	MEKK1–MKK4/5–MPK3/6 [17]; YODA–MKK4/5–MPK3/6 [20]; MKK9–MPK3/6 [54]	VIP1 [23] + 48 candidates [24]
MPK4	O ₃ [67]; PAMPs [16]; osmotic shock [16]; cold [27]; salt [27]	Dwarf [30,59]; SA overproducer [30,59]; resistant to pathogens [30,59]; MeJA-dependent gene expression impaired [30,59]; resistant to osmolarity [16]	MKK2–MPK4 [27]; MKK1–MPK4 [27]; MEKK1–MKK2–MPK4/6 [27]	MKS1 [30,78]
MPK6	H ₂ O ₂ [27]; O ₃ [67]; PAMPs [17]; osmotic shock [66]; JA [58]; ET [54]	Wild-type-like plant except: embryo development impaired [72]; stomatal patterning slightly affected [72]; smaller flowers [72]; hypersensitive to O ₃ -induced oxidative burst [68]; redundant with MPK3: <i>mpk3mpk6</i> dwarf with <i>yoda</i> -like stomatal patterning [20]	MEKK1–MKK4/5–MPK3/6 [17]; YODA–MKK4/5–MPK3/6 [20]; MEKK1–MKK2–MPK4/6 [20]; MKK3–MPK6 [58]; MKK9–MPK3/6 [54]	ACS6 [21,22]; EIN3 [54] + 39 candidates [24]
MPK7	ND	ND	MKK3–MPK1/2/7/14 [40]	ND
MPK14	ND	ND	MKK3–MPK1/2/7/14 [40]	ND
MAP2K				
MKK1	H ₂ O ₂ [27]; flg22 [27,28]; laminarin [27]	Wild-type-like plant [28]; reduced flg22-dependent activation of MPK3 and MPK6 [28]; no flg22-dependent activation of MPK4 [28]	MEKK1–MKK1–MPK4 [28]; MKK1–MPK4 [27]	
MKK2	Salt [27]; cold [27]	Wild-type-like plant [27]; unable to acclimate to freezing temperatures [27]; hypersensitive to salt [27]; reduced cold-induced activation of MPK4 and MPK6 [27]	MEKK1–MKK2–MPK4/MPK6 [27]; MKK2–MPK4 [27]	
MKK3	Pathogen [40]; JA [58]	Wild-type-like plant [40,58]; hypersensitive to <i>Pseudomonas</i> [40]; hypersensitive to JA [58]; JA-dependent activation of MPK6 impaired [58]	MKK3–MPK1/2/7/14 [40]; MKK3–MPK6 [58]	
MKK4	PAMPs [17]; H ₂ O ₂ [39]	<i>mkk4mkk5</i> RNAi; plant: dwarf with <i>yoda</i> -like stomatal patterning [20]	MEKK1–MKK4/5–MPK3/6 [17]; YODA–MKK4/5–MPK3/6 [20]	
MKK5	PAMPs [17]; H ₂ O ₂ [38]	<i>mkk4mkk5</i> RNAi; plant: dwarf with <i>yoda</i> -like stomatal patterning [20]	MEKK1–MKK4/5–MPK3/6 [17]; YODA–MKK4/5–MPK3/6 [20]	
MKK7				
MKK9	ET [54]	ACC inhibition of hypocotyl elongation impaired [54]; reduced ACC-dependent induction of ERF1 [54]; increased NaCl-sensitivity [54]; no ACC-dependent activation of MPK3 and MPK6 [54]	MKK9–MPK3/6 [54]	
MAP3K				
ANP1	H ₂ O ₂ [38]	Truncated ANP1 mimics H ₂ O ₂ -induced gene expression	ANP1–?–MPK3/6	
ANP2	Cell division [38]	<i>anp2anp3</i> show cytokinesis defects	ND	
ANP3	Cell division [38]	<i>anp2anp3</i> show cytokinesis defects	ND	
YODA	ND	Dwarf [70]; embryo development impaired [70]; leaf stomatal-patterning impaired [20,71];	YODA–MKK4/5–MPK3/MPK6 [20]	
MAP3Kε2/ M3K6	ND	Wild-type-like [77]; redundant with M3K7: <i>m3k6/m3k7</i> pollen fails to mature [77]	ND	
MAP3Kε1/ M3K7	ND	Wild-type-like [77]; redundant with M3K6: <i>m3k6/m3k7</i> pollen fails to mature [77]	ND	
MEKK1	H ₂ O ₂ [43]; flg22 [18,19]	<i>mekk1</i> dwarf over-producer of SA [18,19,43]; MPK4 activation impaired [18,19,43]; reduced root hair elongation [43]; overaccumulation of H ₂ O ₂ [18,43]; spontaneous cell death [18]; dwarfism reverted by higher temperature [18,19] and in SA-depleted background [18]	MEKK1–MKK1/2–MPK4 [18,19,43]; MEKK1–MKK4/5–MPK3/MPK6 [17]	

we have functional evidence that they act as MAP3Ks *in planta*, and the Raf-like and ZIK-like subgroups, for which the only functional evidence comes from non-plant systems. In addition to their kinase activity, plant MAP3Ks often contain long N- or C-terminal regions that might function in regulation or scaffolding to recruit MAP2Ks and MAPKs, or in the integration of the input signals. Apart from identifying a MAP3K as a scaffold, no specific scaffolding protein was yet reported in plants. The *Arabidopsis* genome contains a large number of various phosphatases putatively involved in kinase dephosphorylation (for

a review, see [5]). Among these, five genes of the dual-specificity MKP family exist which should be modulators of the MAPKs.

MAPK CASCADES ARE INVOLVED IN PATHOGEN SIGNALLING

Plant–pathogen interactions involve very complex mechanisms ensuring survival in the competition arena. During evolution, higher plants developed an innate immune system to detect and rapidly respond to pathogen aggression [6]. After pathogen infection and recognition of PAMPs (pathogen-associated molecular

patterns) in the plant cell environment, multiple levels of defence are activated. PAMPs are small molecules usually derived from abundant pathogen structures such as structural proteins or cell-wall components that are shared by a large range of pathogen varieties. Within minutes of pathogen-derived PAMP recognition, the plant modifies enzymatic activities and gene-expression patterns to synthesize a large set of anti-microbial reagents, including AOS (active oxygen species) and phytoalexins. In order to suppress the innate immune response of plants, pathogens inject a number of virulence factors into the host cell. In specific cases, however, plants learnt to recognize virulence factors, resulting in the so-called 'gene-for-gene resistance reactions'. Recognition of virulence factors occurs by plant NBS-LRR (nucleotide binding site-leucine-rich repeat) receptors to switch on defence signalling cascades. Whereas PAMP recognition is not specific to a given pathogen variety, gene-for-gene interactions are based on the recognition of a given pathovar. Among others factors, resistance against different pathogen types generally involves a regulation of the balance between SA (salicylic acid)- and ET/JA (ethylene/jasmonic acid)-dependent defence mechanisms and thereby long-term responses to pathogens [7]. SA is linked to resistance to biotrophic pathogens and is important to trigger the HR (hypersensitive response), a PCD (programmed cell death) to locally counteract pathogen attack and progression. ET and JA play a role in the control of PCD spreading [8] and regulate resistance against necrotrophic pathogens. All three hormones regulate distinct sets of pathogen-related genes and are involved in inducing SAR (systemic acquired resistance), a long-range process of priming pathogen resistance in unaffected tissues.

In recent years, the number of publications revealing a function of MAPK cascades in pathogen signalling increased greatly. A set of three MAPKs – MPK3, MPK4 and MPK6 – was found to be activated on interaction with pathogens, explaining why they are by far the most well known plant MAPKs. Other MAPKs might also be involved in plant–pathogen signalling, but they have been only rarely observed so far, possibly solely because of a lack of appropriate tools such as specific antibodies to detect low protein amounts or kinase activities. Efforts to identify single and multiple knock-out lines for these kinases should also help to by-pass these limitations.

Given the heterogeneity in the protocols, the variety of biological systems and the favourite assays and topics of research of each laboratory, an integrative view of theoretical pathogen signalling pathways is still difficult. Nevertheless, MAPKs are unambiguously implicated in the signalling processes linked to innate immunity, whereas no function has so far been revealed for gene-for-gene defence mechanisms.

Flagellin, a model PAMP, helps in dissecting the MAPK cascades involved in innate immune responses

Flg22 is a 22-amino-acid-long peptide derived from flagellin, the main structural protein of the eubacterial flagellum [9]. Flg22 acts as a PAMP on various plant species, inducing a wide range of pathogen-related responses. In *Arabidopsis*, the signal perception occurs at the plasma membrane through a receptor-like kinase complex composed of the proteins FLS2 (FLAGELLIN SENSING 2) and BAK1 (BRI1-ASSOCIATED KINASE 1) [10–12]. FLS2 alone carries the specificity toward flg22. Once bound to flg22, the FLS2–BAK1 complex initiates a signalling cascade, triggering a set of pathogen-related responses, including an early oxidative burst [13], the activation of various kinases, the consecutive modification of the phosphorylation state of many cellular proteins [14] and broad gene modulations [15].

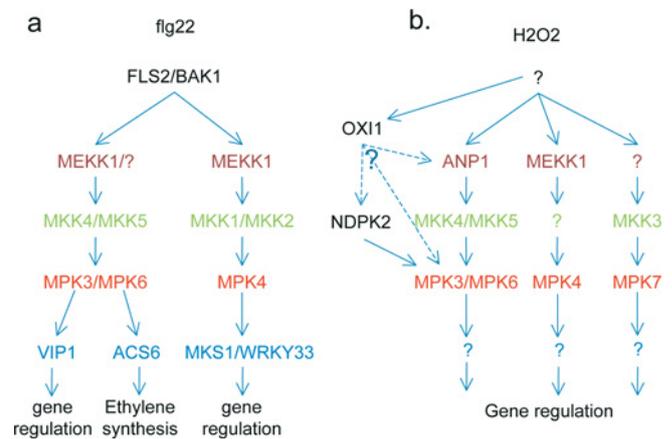


Figure 1 Model of (a) flg22 and (b) H₂O₂-induced MAPK signalling

(a) Upon stimulation of the FLS2 receptor by flg22 peptide, the MEKK1–MKK1/2–MPK4 and the MEKK1?–MKK4/5–MPK3/6 modules are activated to phosphorylate the substrates MKS1/WRKY33, VIP1 and ACS6 respectively. (b) H₂O₂ activates the three MAPK modules (ANP1–MKK4/5–MPK3/6, MEKK1–MPK4 and MKK3–MPK7) either directly or via the protein kinases NDPK2 (nucleoside diphosphate kinase 2) and OX11.

In *Arabidopsis*, MPK3, MPK4 and MPK6 are activated by flg22 and other PAMPs [16]. This activation occurs within 5 min of treatment, even in the presence of the translation inhibitor cycloheximide, indicating a direct link between receptors and the initiation of the MAPK signalling pathways [17]. Flg22 perception by *Arabidopsis* became one of the main models to study the early signalling events upon pathogen recognition and is now broadly used in the scientific community.

Antagonistic control of flg22-induced defence responses by two MAPK cascades

Transient expression in protoplasts, together with biochemical and genetic approaches, identified the first MAPK cascade acting downstream of the FLS2–BAK1 receptor complex [17] (Figure 1a). This cascade consists of the MAP3K MEKK1 (named also MAP3K08), two MAP2Ks, namely MKK4 and MKK5, and two MAPKs, namely MPK3 and MPK6, and allows the early flg22-induced expression of *WRKY29* (*WRKY DNA-BINDING PROTEIN 29*) and *FRK1* (*FLG22-INDUCED RECEPTOR KINASE 1*). Expression of constitutively active forms of MEKK1 or MKK4 activate downstream events, mimicking flg22 treatment in terms of gene regulation. Interestingly, in the *mekk1* background, flg22 is still able to activate MPK3 and MPK6, suggesting redundancy at the level of the MAP3K step in the MEKK1–MKK4/MKK5–MPK3/MPK6 signalling pathway [18,19]. It is presently unclear whether this kinase is YODA, which was recently shown as the MAP3K upstream of a developmental signalling pathway otherwise consisting of MKK4/MKK5 and MPK3/MPK6 [20].

Flg22-regulated MPK3/MPK6 targets start to be unveiled. For example, upon flg22 treatment, MPK6 specifically phosphorylates – and therefore stabilizes – ACS6 [ACC (1-amino-cyclopropane-1-carboxylic acid) SYNTHASE 6], an enzyme involved in biosynthesis of ET [21,22]. Interestingly, ET production is triggered by flg22. More recently, VIP1 (VirE1-INTERACTING PROTEIN 1), a bZIP (basic leucine zipper) transcription factor, was shown to be specifically phosphorylated by MPK3 upon flg22 treatment, inducing its re-localization from the cytoplasm to the nucleus to activate defence-related genes such as *PRI*

(*PATHOGENESIS-RELATED 1*) [23]. The question which of the two signalling pathways regulates flg22-induced early genes *WRKY29* and *FRK1* remains to be addressed. However, MPK3 and MPK6 probably also target other proteins. Indeed, using a protein-chip approach, several putative targets were phosphorylated *in vitro* by these MAPKs, but this has not yet been confirmed *in vivo* [24].

The second MAPK cascade recruited by flg22 consists of the MAP3K MEKK1 and MPK4 (Figure 1a). MPK4 activity increases within a few minutes upon flg22 treatment [16], and this activation is abolished in *mekk1* mutants [18,19]. *mpk4* and *mekk1* mutants have related phenotypes. Both mutants exhibit dwarfism, a phenotype which is likely linked to the overproduction of SA and H₂O₂ [18,19,25]. This dwarf phenotype is partially reversed by overexpression of the bacterial SA hydrolase *nahG* or by mutations in genes involved in the SA signalling pathways or biosynthesis [19,25]. Moreover, *mekk1* and *mpk4* mutants show spontaneous cell death on leaves and constitutive expression of pathogenesis-related genes such as *PR1* and *PDF1.2* (*PLANT DEFENSIN 1.2*). Consequently they are both more resistant to infection from pathogens such as *Pseudomonas syringae* or fungi [19,25]. Interestingly, yeast two-hybrid experiments showed that the MAP3K MEKK1 is able to interact with MPK4 through a short motif in the MEKK1 N-terminal tail [26]. Taken together, these results show that MEKK1 and MPK4 are important elements of a flg22-induced signalling cascade involved in negative regulation of pathogen- or PAMP-induced cell death. Despite extensive investigations, no MAP2K mutants have yet been identified showing a *mpk4*- or *mekk1*-like phenotype, suggesting functional redundancy at this level. However, MKK1 and MKK2 were both identified to be specific upstream regulators of MPK4 [27], and Meszaros et al. [28] showed that flg22 fails to activate MPK4 in *mkk1* mutants. Using a hyperactive form of MKK2, Brader et al. [29] showed enhanced MPK4 activation and altered pathogen responses, suggesting that MKK1 and MKK2 could perform redundant MAP2K functions on the flg22-induced MEKK1–MPK4 cascade.

The currently known targets of MPK4 are MKS1 (MPK4 substrate 1), as well as WRK25 and WRK33, two WRKY type transcription factors [30]. Even if not tested upon flg22 treatment, these substrates could be targets of the flg22-activated MPK4 cascade. This is supported by the fact that MKS1-overexpressing plants show a weak *mpk4* phenotype in terms of *PR1* gene expression, SA accumulation, dwarfism and resistance to pathogens [30].

Are MPK3/6 and MPK4 cascades common signalling modules for various PAMPs?

So far, different studies have revealed that two MAPK signalling pathways are activated by the PAMP flg22. MKK4/MKK5–MPK3/MPK6 activates pathogen responses, whereas MEKK1–MPK4 acts negatively on the same responses. Some results suggest that these two distinct cascades are more interconnected than expected. For example, in *mkk1* mutants [28], flg22-dependent activation of MPK3–MPK6 is impaired, which could indicate that MKK1 acts redundantly in the MPK3/6 branch of the signalling cascade. An alternative explanation could be that in the *mkk1* mutant background, players in the MPK3/6 cascade are not expressed/regulated in the same way. Moreover, it was recently shown that the protein phosphatase AP2C1 is able to regulate both MPK6 and MPK4, confirming that neither of the MAPK cascades is regulated autonomously [31].

EF-Tu (elongation factor thermo-unstable), a peptide from the *Escherichia coli* elongation factor, was recently identified to trigger various pathogen-related events from activation of MAPKs

to gene regulation [32]. The authors identified EFR (EF-Tu RECEPTOR) as the EF-Tu sensor, belonging to the superfamily of RLKs (RECEPTOR-LIKE KINASES). Strikingly, despite being sensed by independent receptors, flg22 and EF-Tu regulate a very similar set of genes, indicating that they use the same signalling pathways [33]. This hypothesis is supported by the fact that these two elicitors do not have additive effects, indicating the saturation of the signalling pathways. Very recently, using a reverse genetic approach on candidate RLKs, the receptor CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1), sensing the fungal elicitor chitin, was identified [34]. Chitin also activates MPK3 and MPK6, and this activation is totally impaired in the *cerk1* mutant. This result indicates that flg22-activated MAPK pathways could be also recruited by fungus-derived PAMPs and, more importantly, that such a signalling network could be downstream of many PAMP-sensing receptors. Further characterization of other kinases upon chitin treatment will also help us to understand to what extent the signalling networks overlap. As a small step, Miya et al. [34] have shown that chitin in wild-type plants regulates a set of pathogen-related genes whose overlap with the ones regulated by flg22 and EF-Tu could help to test this hypothesis.

In contrast with flg22, EF-Tu and chitin, harpin from *Ps. syringae* and NLPs (Nep1-like proteins) from various oomycetes trigger HR-related PCD in *Arabidopsis* and other species. Desikan et al. [35,36] showed that harpin activates both MPK4 and MPK6 in *Arabidopsis* cell cultures, and NLPs induce activation of MAPKs as well [37]. Even if these kinases are not always identified at the molecular level, these results suggest that the same MAPKs might be targeted by harpin, NLPs, flg22 and EF-Tu. Moreover, about 50 % of the flg22-regulated genes are also regulated on treatment with NLP [37]. A question that needs to be addressed is whether the relative strength of the signal spreading through either the MPK6 or the MPK4 branches might determine different responses, such as the occurrence of PCD. Another possibility is that HR might not be determined by the MPK4 and MPK6 pathways alone, but also by the activity of other, yet unknown, cascades or components. In any case, it is clear that the flg22 system has helped us to map some signalling pathways, but our knowledge is clearly not sufficient to understand the whole range of responses observed after a plant–microbial interaction.

Are AOS acting as early second messengers of PAMP responses?

AOS production is one of the earliest known signalling step in response to environmental stimuli, such as pathogen interactions – or PAMP treatment – and abiotic stresses, but also in response to various hormones and in developmental processes. The plant AOS content controls the expression of many genes and the activity of cellular enzymes. In addition, upon pathogen attack, an oxidative burst occurs, which is supposed to have an antimicrobial effect, be involved in cell-wall strengthening to counteract pathogen progression and to promote HR-related cell death. Exogenous H₂O₂ was shown to activate various *Arabidopsis* MAPK cascades, including MPK3/MPK6 (Figure 1b). Using a protoplast system, Kovtun et al. [38] showed H₂O₂-dependent activation of ANP1 [*Arabidopsis* NPK1 (*Nicotiana* protein kinase 1)-related kinase 1; also termed MAP3K01] and the downstream activation of MPK3 and MPK6. The MAP2Ks involved in this signalling pathway could, once again, be MKK4/MKK5. Indeed, Ren et al. [39] showed that the expression of hyperactive forms of MKK4 and MKK5 in *Arabidopsis* activates MPK3/6 and thereby triggers H₂O₂ production and cell death. MKK4 and MPK6 seem to be part of a feed-forward loop, since Doczi et al. [40] recently showed that MKK4 is involved in H₂O₂-dependent activation of MPK6. Several other investigations completed the picture

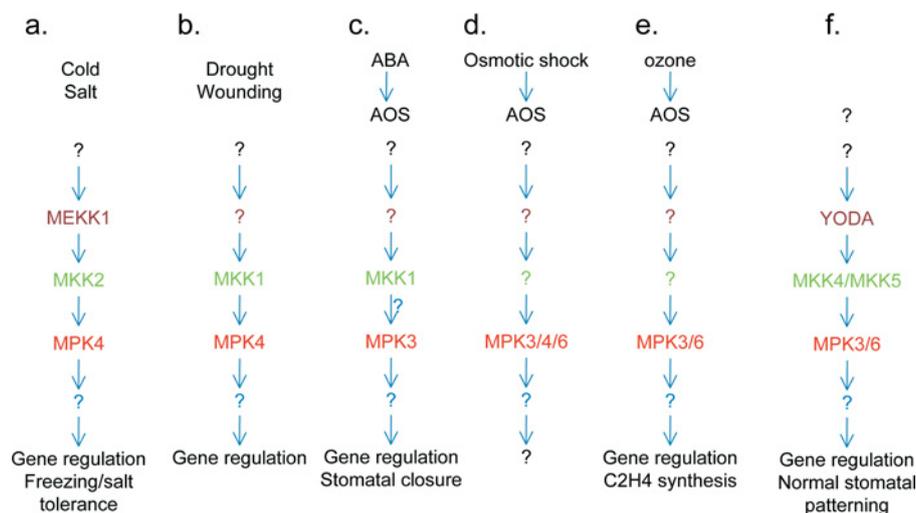


Figure 3 Abiotic-stress-induced MAPK signalling cascades

(a) Cold/salt stress induces the MEKK1–MKK2–MPK4 cascade. (b) Drought and wounding induces MKK1–MPK4. (c) ABA-induced H_2O_2 production activates MKK1–MPK4. (d) Osmotic shock activates MPK3/4/6 via AOS production. (e) O_3 -induced AOS activates MPK3/6. (f) Stomatal patterning is controlled by YODA–MKK4/5–MPK3/6.

MAP3K upstream of MKK9 is still a matter of debate: CTR1 belongs to the Raf-kinase family, which acts in other systems as an MAP2K activator, but genetic data indicate that CTR1 is a negative regulator of ET signalling. Direct physical interaction between CTR1 and MKK9 could help to clarify this.

Many abiotic and biotic factors activate MPK6 and might thereby regulate stress-induced ET production [22] (Figure 2a). This hypothesis is supported by the identification of ACS6 as a substrate of MPK6 [21,22]. The upstream activators of this ET-producing signalling pathway are probably MKK4 or MKK5, since expression of hyperactive MKK4 or MKK5 triggers MPK3 and MPK6 activation and sustained ET production. Interestingly, inducible expression of hyperactive MKK5 also triggers ET-dependent PCD, probably corresponding to ET overaccumulation above a threshold [57].

The question how these two ET-related cascades, despite sharing kinases, do not interfere, remains to be investigated. One explanation could be that the proteins involved in each of the cascades are not located in the same compartment. This idea is supported by the fact that activated MKK9 relocates from the cytosol to the nucleus, whereas active MKK4 is located in the cytosol [54]. Each of these MAP2Ks could possibly activate a subpopulation of MPK6, which is located in both compartments and could therefore phosphorylate distinct targets, namely ACS6 in the cytosol and EIN3 in the nucleus.

MKK3-dependent MPK6 activation was recently identified as part of a negative regulator of JA signalling [58] (Figure 2b). MPK1 and MPK2 are also activated by MeJA (methyl jasmonate) [44]. Together with MPK7 and MPK14, these two MAPKs belong to the C subgroup of *Arabidopsis* MAPKs. Since MPK7, but not MPK6, was shown *in planta* to physically interact with MKK3 [40], MeJA activation of MPK6 could be a secondary consequence of MeJA-dependent MKK3–MPK1/2/7/14 activation.

MPK4 is involved in the modulation of the balance between SA and JA/ET-related defences [59]. An *mpk4* mutant accumulates SA, constitutively expresses SA-related PR genes and is impaired in ET- and MeJA-dependent gene regulation. Interestingly, *eds1* (*enhanced disease susceptibility 1*) and *pad4* (*phytoalexin deficient 4*) mutants and transgenic plants with decreased SA contents partially revert the *mpk4* phenotype: double mutants are less dwarfed than *mpk4*, contain much less SA and show

partial restoration of MeJA-dependent *PDF1.2* expression. These results indicate that MPK4 acts on the hormonal balance through the EDS1/PAD4 module [59]. Given the mutant phenotype similarities and the fact that MEKK1 physically interacts with MPK4 in yeast two-hybrid assays [26], MEKK1 could act upstream of MPK4 to modulate the defence-related hormonal balance. Interestingly, expression of a hyperactive form of MKK7 also triggers a higher SA content, constitutive *PR1* expression and pathogen resistance [60]. This suggests that an unknown MAPK cascade could antagonistically modulate this balance. Given the antagonistic roles of the MEKK1–MPK4 and MKK4/MKK5–MPK3/MPK6 cascades during the immune response and MPK6 regulation of ACS6 activity and ET synthesis, the MPK3/MPK6 branch is of course a very good candidate for such a role.

MAPKS INVOLVED IN ABIOTIC STRESSES

In several species, including *Arabidopsis*, MAPK cascades have also been shown to be involved in signalling pathways activated by abiotic stresses such as cold, salt, touch/wind, wounding, heat, UV or osmotic shock. Once again, the main activated kinases are MPK3, MPK4 and MPK6, or their orthologues in other species.

MAPK cascades in salt and cold stresses

The most complete MAPK cascade functioning in abiotic stresses consists of the MAP3K MEKK1 activating MKK2 and MPK4/MPK6 [27] (Figure 3a). In the *mkk2* background, cold and salt activation of MPK4 and MPK6 are impaired and *mkk2* mutant plants are hypersensitive to cold and salt stresses. Using the protoplast expression system, the authors showed that whereas MKK2 activates MPK4 in response to cold and salt, activation of MPK4 by H_2O_2 , *flg22* and β -glucan is triggered by MKK1 (Figure 3b). This result is apparently in contradiction with genetic data suggesting that MEK1 (MKK1) also functions in salt tolerance [61].

Water stress, ABA (abscisic acid) and osmotic shock

ABA is a phytohormone involved in various physiological processes, including the adaptation to water stress and the control of

seed dormancy. The fact that a MAP2K inhibitor, PD98059, was able to decrease ABA-induced stomatal closure indicates that a MAPK cascade could function in ABA signalling [62] (Figure 3c). More direct evidence came from guard-cell-specific *MPK3* silencing in *Arabidopsis* [63]. In these RNAi (RNA interference) lines, ABA partially failed to maintain stomatal closure upon opening conditions, indicating a function of MPK3 in ABA-dependent inhibition of the stomatal opening machinery, but not of ABA-induced closure. Additionally, H₂O₂, known to be an important second messenger of ABA in the closure of stomata, gave very similar results, indicating MAPK activation downstream of ABA-induced AOS production. Stomata of knock-out plants of the *MAP2K* gene *MEK1* (also named *MKK1*) were also found to be unable to respond to ABA and to be impaired in ABA-induced expression of the leaf catalase gene *CAT1* [61]. To our knowledge, MKK1 has not been shown to regulate MPK3 activity in response to ABA. More indirect genetic data of a function for a MAPK cascade in ABA signalling comes from the study of two mutants impaired in putative MKPs: *ibr5* (*indole-3-butyric acid response 5*) showed partial resistance to ABA in a root elongation assay but was unfortunately not tested for its stomatal response to ABA [64]. By contrast, *phs1-3* (*propryamide-hypersensitive 1-3*) exhibits ABA hypersensitivity in seed germination and stomatal opening [64a]. ABI1 (ABA-INSENSITIVE 1), a member of the PP2C (protein phosphatase 2C) family known to function in ABA signalling, was also shown to interact with MPK6 *in vitro*, in yeast two-hybrid assays and *in planta* [65]. Additionally, expression of dominant-negative forms of MPK6 rendered plants hypersensitive to ABA [65]. Direct proof of MAPK activation by ABA was obtained recently by Ortiz-Masia et al. [44] for MPK1 and MPK2, who used an *Arabidopsis* protoplast expression system and revealed these two MAPKs as putative candidates mediating ABA signalling in plant cells.

An important component of water stress is the osmotic shock created by washing or concentration of the extracellular medium. In suspension-cultured *Arabidopsis* cells, hyperosmotic and hypo-osmotic shock were shown to activate MPK3, MPK4 and MPK6 [16,66] (Figure 3d). Interestingly, even if MPK4 is not activated by hyperosmotic shock in plantlets, *mpk4* mutants exhibit resistance to osmotic stress in germination and root-growth assays, indicating a negative function in osmotic tolerance [16]. Interestingly, in *Arabidopsis* and other species, osmotic shock is known to trigger a strong AOS production, which could be the input signal activating the MAPK network.

O₃ (ozone)-induced MAPK cascade

The major pollutant O₃ was shown to activate a MAPK signalling pathway through AOS production and to modify ET, SA and JA contents, leading to a PCD similar to HR. These similarities between responses to pathogens, H₂O₂ and O₃ allowed researchers to use O₃ to manipulate the H₂O₂ content of plants. In *Arabidopsis*, Ahlfors et al. [67] observed MPK3 and MPK6 activation within 30 min of exposure to O₃ (Figure 3e). MAPK activation was normal in ET, SA and JA signalling mutants, indicating (i) the existence of a hormone-independent pathway for MPK3 and MPK6 activation and (ii) that O₃-induced hormone changes could be a long-term process under the control of the MAPK cascade. Plants with diminished MPK6 or lacking MPK3 exhibit O₃ hypersensitivity, as shown by O₃-induced leaf damage [68]. Interestingly, in each of these mutant backgrounds, the other MAPK remained activated longer [68]. Recently, MKP2 was identified as an important regulator for controlling both O₃-induced MPK3, and MPK6 and MKP2 RNAi plants were shown to exhibit hypersensitivity to O₃ [69].

STRESS-UNRELATED FUNCTIONS FOR MAPK CASCADES

A number of studies have also revealed the role of MAPK pathways in other processes, including developmental and auxin signalling.

YODA-MKK4/MKK5-MPK3/MPK6 regulates stomatal patterning in epidermis

The main finding concerning MAPK function in development came from studies on the MAP3K YODA (also named MAP3K04) and its downstream MAPK components MKK4/5 and MPK3/6 (Figure 3f). *yoda* was initially identified in a genetic screen for plants impaired with respect to normal cell division during early embryo development [70]. Another study revealed that *yoda* mutants also exhibit typical clustering of stomata in the leaf epidermis corresponding to a transgression of the 'one-cell spacing' rule [71]. Using a reverse-genetic approach, MKK4-MKK5 and MPK3-MPK6 were then shown to be redundant downstream steps of this MAPK signalling cascade: both *mkk4mkk5* and *mpk3mpk6* double mutants fail to develop further than the cotyledon stage and show a typical *yoda*-like stomatal patterning at the epidermis [20]. Using a dominant-negative form of MPK6 to by-pass functional redundancy, Bush confirmed MPK6 function in stomatal patterning and also published work suggesting a convincing role for it during embryo and inflorescence development [72]. The embryo-development phenotype in *mkk4mkk5* and *mpk3mpk6* double mutants still remains to be studied. Despite the upstream signal of YODA-MKK4/5-MPK3/6 remains still being unknown, it defines unambiguously a function for MAPKs in the intercellular communication during development.

MAPK cascades and auxin signalling

Interesting results on the function of MAPKs in auxin signalling came from the phenotypic study of the semi-dominant *bushy and dwarf1* (*bud1*) *Arabidopsis* mutant, which turned out to be an overexpresser of MKK7 [73]. Auxin was known to trigger MAPK-like activities in tobacco (*Nicotiana tabacum*) [74] and *Arabidopsis* [75]. Using tobacco and maize (*Zea mays*) protoplasts, however, Kovtun et al. previously showed a negative function for a MAP3K NPK1-dependent MAPK cascade in auxin signalling [76]. Genetic evidence for a role of the *Arabidopsis* NPK1 homologues ANP1, ANP2 and ANP3 as positive regulators in cytokinesis was provided in a reverse-genetic approach. Whereas single-mutant plants displayed no obvious abnormal phenotypes, double-mutant combinations displayed defects in cell division and growth [76a]. Whether ANPs have a role in auxin signalling is currently unclear and remains to be determined. A role for MKK7 in auxin transport was recently unravelled by analysing the phenotypes of the *bud1* mutants which exhibit several auxin-related phenotypes, such as less ramifications in root architecture, a simpler venation of leaves, an enhanced curvature of the hypocotyls in response to gravity and a modified polar auxin transport. MKK7 could thus act as a negative regulator of auxin transport [73]. Later the same authors reported a MKK7 function in defence regulation [60]. Studies of loss-of-function *mkk7* alleles should help to clarify the auxin-related MKK7 function *in planta*. Support for the notion that auxin signalling could involve a MAPK cascade also comes from studies of the MKP mutant *ibr5*, which was isolated on the basis of its auxin resistance and which shows, in common with some other auxin-related mutants, aberrant vascular patterning, longer roots and

shorter hypocotyls [64]. The IBR5-inactivated MAPKs remain to be identified.

In *Arabidopsis mek1* and *mpk4* mutants, Nakagami et al. [43] showed an impaired auxin-induced activation of the auxin-regulated genes *IAA3* (indole-3-acetic acid 3), *IAA5* and *IAA14*, a phenotype that was suggested to arise as a consequence of H₂O₂ down-regulation of auxin signalling. Despite extensive forward-genetic approaches, no mutants of MAPK components were isolated with an auxin phenotype, probably attributable to redundancy in the extensive MAPK gene family.

MAP3K ϵ 1 and MAP3K ϵ 2 function during pollen maturation

A recent study [77] has shown that MAP3K ϵ 1 and MAP3K ϵ 2 (also known as MAP3K07 and MAP3K06) function redundantly during male gametophyte maturation. Whereas single mutants do not show any obvious phenotype, homozygous double mutants never recover, because of a lack of viable pollen grains. Additionally, the authors showed that double-mutant pollen grains often fail to go beyond the second mitosis, showing, as they do, additional plasma-membrane structures and pollen-wall modifications. Interestingly, MAP3K ϵ 1 and MAP3K ϵ 2 were previously reported to be expressed acutely during the cell cycle [77a]. Chaiwongsar et al. [77] did not exclude the possibility that MAP3K ϵ 1 and MAP3K ϵ 2 could also act redundantly in mitosis during the sporophytic phase. This question remains difficult to tackle because of the lack of viable double-mutant plants.

CONCLUSIONS

Many kinases with unknown functions

On the 120 kinases putatively involved in MAPK cascades, only a handful have been characterized so far. By far the most studied MAPKs are MPK3, MPK4 and MPK6, which were identified in many distinct processes ranging from stress responses to developmental processes. Some activity data are now available for some of the other 17 MAPKs, but, to our knowledge, not a single loss-of-function mutant has been reported yet, even though insertion lines for almost all genes are available in various insertion mutant databases. An open question remains as to why it is so easy to monitor the activity of MPK3, MPK4 and MPK6 from plant extracts by in gel kinase assays, whereas the other MAPK activities are so rarely observed. Could this be for technical reasons? Are those MAPKs activated in such discrete conditions that they escaped detection? The situation is better for the ten MAP2Ks: the smaller number of genes, together with the possibility to generate constitutively active forms, enabled one to attribute functions to the majority of the MAP2Ks. Only the functions of MKK8 and the atypical MKK10 remain unknown. Nevertheless, gain-of-function approaches, such as strong overexpression of hyperactive kinases, indicate roles in pathogen responses, but they could also hide more subtle functions *in planta*. With the notable exception of some well studied kinases such as CTR1, MEKK1 and YODA, very little is known about the function or mechanism of action of the plant MAP3Ks on their downstream MAP2Ks. Moreover, no information is yet available to explain how the MAP3Ks are connected to their respective sensors and receptors.

A few kinases do all?

Plants use the same signalling pathways in very different signal-transduction processes. For example, MPK6 is involved in O₃, PAMP, H₂O₂, ET, ABA and JA signalling pathways, but also in important developmental processes such as epidermal patterning

and anther and embryo development. At this time it is difficult to conclude whether the involvement in many signalling pathways is a common feature of the 20 MAPKs or is specific for the three well-known MAPKs, namely MPK3, MPK4 and MPK6.

Nevertheless, one of the explanations for MPK6's various roles could be that all input signals in fact recruit a common second messenger, which activates a conserved signalling pathway. In this scenario, AOS production could be a good candidate, since an oxidative burst is known to be generated in response to many stresses, to several phytohormones and also during developmental processes such as root-hair growth. To our knowledge, H₂O₂ has not yet been shown to be involved in positioning-dependent differentiation during epidermal patterning, but such processes should be discrete and therefore difficult to detect. Even though the notion cannot be excluded, it is difficult to imagine that the set of genes and responses regulated by MPK6 during a developmental process or during a stress response are the same. Specificity could be under control of parallel signalling pathways that remain to be identified.

A second explanation why MPK6 is a common mediator of several distinct cascades could be that the other members and targets of the pathway are only expressed in particular cell types, in particular subcellular compartments, at particular developmental stages or under particular environmental conditions. This notion is supported, for example, by the fact that functional interaction of MPK6 was demonstrated for a wide set of MAP2Ks such as MKK2 [27], MKK3 [58], MKK4, MKK5 [17] and MKK9 [54]. The fact that MPK4 activity increases in response to an osmotic shock in *Arabidopsis* suspension cells but not in plantlets supports the idea of cell-specificity for MAPK cascades [16]. ET-related functions of MPK6 could provide an example of subcellular-dependent function: MKK4–MPK6–ACS6 is used to control ET production, whereas MKK9–MPK6–EIN3 is involved in ET signalling. Yoo et al. [54] showed that active MKK4 is mainly a cytoplasmic protein, whereas active MKK9 re-localizes in the nucleus. Both MAP2Ks could target subpopulations of MPK6, either in the nucleus or in the cytoplasm, which thereby phosphorylate distinct targets and initiate different functional responses. Integration of expression data of MAPK-related kinases will help to simplify the system and highlight kinases susceptible to meet and build conditional cascades. In this scenario, scaffolding proteins have particularly important functions, as they help kinases to build functional cascades.

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