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Review

Genomic Analysis of MAP Kinase Cascades in *Arabidopsis* Defense Responses

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Abstract. The process of phosphorylation and dephosphorylation is a common mechanism of signal transduction in plants, connecting the perception of extracellular signals with the final responses to those signals. This paper will concentrate on the mitogen-activated protein (MAP) kinase pathway, one of the main phosphorylation pathways that plants use in biotic and abiotic stress resistance. It is a cascade consisting of several classes of kinases, each having a different role in signal integration and divergence. The cascade is regulated by various mechanisms, including not only transcriptional and translational regulations but also post-transcriptional regulations and protein-protein interactions. Recent detailed analysis of certain specific MAP kinase pathways has revealed the specificity of the kinases in the cascade, signal transduction patterns, identity of pathway targets, and the complexity of the cascade. Strategies in the study of phosphorylation pathways are discussed, and approaches integrating various genomics and proteomics technologies are suggested.

1. Introduction

Plant diseases have been known from the very beginnings of organized agriculture and have frequently been associated with hunger and suffering. One of the most famous examples in history is the Irish potato famine of the 1840s, caused by late blight of potato, whose agent, the fungus *Phytophthora infestans,* is the so-called "plant destroyer" (Holub, 2001). Since then, new ways to protect crops from disease and to increase their productivity have evolved, for example, through the use of pesticides and higher yielding plant varieties. Today, with the emergence of new genetic and biomolecular techniques, it becomes possible to understand more fully, and potentially to enhance, the plant's defense mechanism and thus produce crops that are more resistant to disease.

Arabidopsis thaliana was first used as a model plant for the study of plant-pathogen interactions about 20 years ago. Since then there has been exceptional progress in discovering the molecular and genetic basis for disease resistance in this plant (Buell, 1998). *Arabidopsis* was chosen as a model for several reasons. It

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exhibits all of the major defense responses found in other flowering plants, it has a relatively small genome that has been completely sequenced (The *Arabidopsis* genome initiative, 2000), it has a short generation time, and small size for easy screening tests. But perhaps the most valuable factor is that numerous mutants have been isolated, which made possible the identification of genes responsible for different phenotypes (Buell, 1998; Glazebrook et al., 1997; The *Arabidopsis* genome initiative, 2000).

Within the *Arabidopsis* genome there are approximately 1000 protein kinase genes and 200 phosphatase genes (Xing et al., 2002). The large pool of kinases and phosphatases indicates the importance of phosphorylation and dephosphorylation mechanisms in the growth and development of *Arabidopsis*. Indeed, phosphorylation is one of the main methods of post-translational modification that regulate protein stability, biological activity and cellular location. This process also has an effect on protein-protein interactions such as formation of protein complexes and protein docking. Phosphorylation affects serine, threonine, tyrosine and histidine residues in both eukaryotes and prokaryotes, and has been considered a universal regulator of cellular activities in all living systems (Huber et al., 1994). This paper gives an overview of mitogen-activated protein kinase (MAPK) pathways in plant defense responses in *Arabidopsis*.

2. MAP kinase cascade and defense responses in *Arabidopsis*

The basic composition of the MAPK pathway is a module with a minimum of three kinases, which is observed in all eukaryotes. Even though it seems that this cascade is universal for all eukaryotic organisms, some MAPK cascades have evolved uniquely in plants, such as those in cytokinesis and hormone signaling pathways (Caffrey et al., 1999). The three components are MAPKKK (MAPKK kinase), MAPKK (MAPK kinase) and MAPK (MAP kinase), which are linked in various ways to upstream receptors and downstream targets. There is also indication for the existence of MAPKKK kinases. The general accepted pathway has the following composition:

Stimulus → Receptor →→ MAPKKK → MAPKK → MAPK → Target → Response

The *Arabidopsis* genome contains 23 MAPKs, 10 MAPKKs and 60 MAPKKKs. These kinases are involved in a variety of functions, including growth, development and responses to environmental and endogenous stimuli as well as responses to plant hormones such as ethylene and auxin (Xing et al., 2002; Zhang and Klessig, 2001). Several cascades are induced by different stress stimuli such as pathogen infections, wounding, high and low temperatures, high salinity, UV radiation, ozone, reactive oxygen species, drought, and high or low osmolarity (Hirt, 2002; Jonak et al., 2002; Zhang and Klessig, 2001). The responses are diverse, and the responses to pathogen attack may include changes in redox chains, hypersensitive response (HR) cell death, generation of reactive oxygen species (ROS), systemic acquired resistance (SAR), activation of pathogenesis related (PR) genes and other protective genes (Xing et al., 2002; Zhang and Klessig, 2001).

All of the MAP kinases are classified based on two methods. The first is a phylogenetic analysis based on the *Arabidopsis* genome and EST sequencing project (MAPK project). The classification is comprised of six subfamilies, possibly representing at least six functional groups (Zhang and Klessig, 2001). This assumption has not yet been tested, and the classification remains to be further affirmed by molecular and biochemical studies. It is important to notice that the phylogenetic relationships may not represent functional relationships. The second method is based on functional analysis involving specific features and sequence signature motifs (Jonak et al., 2002). Table 1 summarizes the specialized signature motifs and the kinase classes they determine.

All MAPKs have a Thr-Glu-Tyr (TEY) activation motif, except MPK17/18/19, in which Asp (TDY) replaces the Glu residue, and a specific domain required for MAPKK docking (Zhang and Klessig, 2001). A specific feature of the TDY group of MAPKs, which the TEY group lacks, is their long carboxy terminal extension. Shared features of all MAPKKs include a putative MAPK docking domain at the N-terminus. MAPKKKs constitute the largest group of MAPK cascade kinases in *Arabidopsis* and are divided in two groups, one containing MEKK-like and ZR1-interacting kinases (ZIKs) which had been shown to function as true MAPKKKs, and the other composed of Raf-like protein kinases, to which no specific function has been assigned yet (Ichimura, 2002).

A specific nomenclature that unifies all *Arabidopsis* MAPKs has been proposed (Ichimura et al., 2002). For example, in this scheme, the gene for a MAPK is named MPK and the gene for a MAPKK is called MKK. Although the prefix At (for *Arabidopsis thaliana*) is not included in the official name of the gene, it is a useful species marker for comparative or homologue studies on MAPKs of different plant species. One drawback of this system is that it is not definitive yet because the structure and function of many members of the family are still not fully characterized. In addition, so far it has proven impossible to provide a nomenclature system that will unify the MAPKs of all plant species.

2.1. MAPKKKs and their function in the MAPK cascade

The sixty MAPKKKs identified in the *Arabidopsis* genome constitute the largest MAPK family. Based on their amino acid sequences, they can be divided into 3 subgroups (Table 1). Even though this group of kinases has the largest number of members, it is the least explored. For example, almost none of the MAPKKKs in *Arabidopsis* have been shown to function as MAPKK activators in the strict sense, which opens a possibility that not all of them are true MAPKKKs but are only similar in their amino acid sequence to those having this function (Jonak et al., 2002).

Mizoguchi et al. (1996) cloned and characterized a cDNA from *Arabidopsis* with high sequence homology to known mammalian MAPKKKs. This kinase was named AtMEKK1 (*Arabidopsis thaliana* ERK kinase kinase 1) and was found to share 46% sequence similarity to NDR1 from tobacco plants, 42% to Byr2 from *Schizosaccharomyces pombe* and 42% similarity to Bck1 from *Saccharomyces cerevisiae*, all of which are involved in pathogen induced responses. Its non-catalytic flanking region in the N-terminal domain was unique. The protein kinase ATP-binding region contains a glycine-rich stretch of residues in the vicinity of a

Table 1. A list of *Arabidopsis* MAPK signalling components based on Jonak, et al. (2002).

Kinase	No.	Class	Number	Named members	Signature motif
MAPK	23	A	3	MPK3/6/10	T(E/D)YFxTRWYRAPE(L/V)
		B	5	MPK 4/5/11/12/13	
		C	4	MPK 1/2/7/14	
		D	9	MPK 8/9/15/16/17/18/19/20	
		MHK	3	—	
MAPKK	10	A	3	MKK1/2/6	VGTxxYMSPER
		B	1	MKK3	
		C	2	MKK4/5	
		D	4	MKK7/8/9/10	
MAPKKK	60	MEKK-like	21	MEKK1, ANP-1, MAP3Kε1	G(T/S)Px(W/Y/F)MAPEv
		ZIK	11	ZIK1	GTPEFMAPE(L/V)Y
		Raf-like	48	EDR1, CTR1	GTxx(W/Y)MAPE
MAPKKKK	10	Ste20/PAK like	10	—	TFVGTPxWMAPEV

lysine residue, which has been shown to be involved in ATP binding. There is also a bipartite nuclear localization signal domain, which is a common motif found in most proteins and mediates the transport of nuclear proteins into the nucleus (Voet et al., 1998).

It is known that MAPKKKs can be regulated by other protein kinases or by binding to specific effectors. The receptor-mediated activation can occur through physical interaction and/or phosphorylation by the receptor itself (Jonak et al., 2002). In this particular cascade, that would be the putative flagellin receptor kinase (FLS2 LRR), and this receptor kinase could potentially activate the MAPKKK by phosphorylating the Ser/Thr residues found in this domain. However, no conclusive research has been done and most of the speculations remain unconfirmed.

A study on the interaction of this MAPKKK with other proteins revealed that AtMEKK1 has the ability to phosphorylate other kinases (Ichimura et al., 1998). When AtMEKK1 was expressed in *Arabidopsis* leaves, the defence responses of the plant were activated against both fungal (*B. cinerea*) and bacterial pathogens (*P. syringae*) (Asai et al., 2002). This was taken as a strong indication that this particular MAPKKK is in fact a part of a signal transduction cascade involved in pathogen resistance in *Arabidopsis*. It is known however that AtMEKK1 can interact and activate four different MAPKKs and thus transmit signals in four different cascades involved in touch, cold and water stress, in addition to the pathogen resistance cascade discussed here (Mizoguchi et al., 1996; Ichimura et al., 1998a). These data, in addition to the fact that the MAPKKK is the largest group of kinases in the MAPK cascade suggests that these members of the cascade function as divergent factors within the MAPK cascade module (Jonak et al., 2002).

2.2. MAPKKs and their function in the MAPK cascade

The ten MAPKKs are divided into four groups based on their structures (Table 1). A common characteristic for all MAPKKs is that they have a putative MAPK-docking domain at their N-terminus. Subgroups A, C and D encode relatively short proteins while subgroup B proteins are somewhat larger and have a specialized domain at their carboxy-terminus that mediates nuclear transport (Jonak et al., 2002). AtMKK1 (subgroup A) seems to mediate cold, drought and wounding signalling (Matsuoka et al., 2002). However, no functions are known for members of groups B and D, and only recently have members of the subgroup C, AtMKK4/5 been identified as being involved in pathogen resistance signalling (Asai et al., 2002).

Ichimura et al. (1998b) isolated and sequenced cDNAs and cloned them into *E. coli* to observe the expression of the genes. They isolated three MAPKKs with sequence homology to the previously known NPK2 genes from tobacco, known for their function in pathogen resistance. AtMKK3 had 85% similarity to this gene, while AtMKK4 and AtMKK5 were closely related to each other and shared 84% identity. Since AtMKK4/5 have been shown to be members of the flagellin-induced signal cascade (see below), and share very similar nucleotide and amino acid sequences, only AtMKK4 as a representative of the two kinases is explored in this section. AtMKK4 contains a putative MAPK-docking domain on the

N-terminus. This domain has the general structure: K/R-K/R-K/R-X $_{(1-6)}$-L-X-L/V/I, and its function is to assist in the binding of the MAPK to the MAPKK (Jonak et al., 2002). The precise function and mode of action of this domain is still not clear, except in *Medicago*, where this domain in SIMKK (stress-induced MAPKK) is required, but not sufficient, for MAPK activation. The Ser/Thr active site is crucial for the activity of the kinase. This site comprises an activation loop, which has the general structure S/TXXXXXS/T. MAPKK is activated by MAPKKK through phosphorylation at the Ser/Thr and Ser/Thr residues (Hirt, 1997; Xing et al., 2001). In transgenic studies, AtMKK4 and AtMKK5 were both shown to confer resistance to fungal and bacterial pathogens when activated by flagellin, even though their function could be redundant (Asai et al., 2002). It is also interesting that the same MAPKKs are involved in HR defense mechanism against pathogens (Ren et al., 2002). Thus, in addition to confirming the position of AtMKK4/5 in the cascade, this study also showed that this cascade could be activated by other elicitors.

A single MAPKK can interact with and activate more than one MAPK, and thus acts as another divergent factor in the module. This has been shown in studies of *Arabidopsis* as well as alfalfa, tobacco and tomato (Jonak et al., 2002; Zhang et al., 2000; Xing et al., 2001). Knowing that both MAPKKKs and MAPKKs have a divergent function, and considering the total number of different kinases in the plant, it is easy to grasp the complexity of the cascade.

2.3. MAPKs and their function in the MAPK cascade

The 23 MAP kinases are grouped into four sub-families (A-D) (Table 1). Those in groups A through C have a TEY phosphorylation motif in their active site, while group D kinases have a TDY motif and are characterized by a long carboxy-terminal extension. The fifth group (MHK) are not true MAPKs because, although they contain a TEY signature motif, they lack the MAPKK-docking domain present in all other MAPKs (Jonak et al., 2002). Most of the MAPKs characterized so far belong to groups A and B, which include AtMPK3/4/6 as the most studied members. Only a few MAPKs from group D have been characterized in rice but no known *Arabidopsis* homologues have been identified, and no group C kinases have been characterized at all.

The *Arabidopsis* MAPKs were the earliest members of the cascade to be cloned and characterized, including AtMPK6, which is involved in pathogen induced signalling (Mizoguchi et al., 1993). These *Arabidopsis* MAPKs have a high sequence similarity to MAPKs from yeast and other plant species. Furthermore, some members are highly similar to each other. For example AtMPK2 and AtMPK7 share 88.7% identity, and AtMPK3 and AtMPK6 share 94.4% identity. Similar to MAPKs in animals and yeast, the most important amino acid stretch involved in the action of the protein is the TEY sequence. Unlike MAPKKKs and MAPKKs, which are phosphorylated at a serine/threonine residue, AtMPK6 is phosphorylated on both the threonine and tyrosine residues present in its active site by the dual-specificity phosphorylation activity of AtMKK4 (Jonak et al., 2002; Zhang and Klessig, 2001; Ichimura et al., 2002).

Several studies have indicated that AtMPK6 has multiple activators. Kovtun et al. (2000) showed that AtMPK6 is activated by hydrogen peroxide. Ichimura et

al. (2000) showed that AtMPK6 is activated by cold, humidity, touch and wounding. The kinase was also found to be induced by three different elicitors from bacteria (flagellin) and fungi (xylanase and chitin) (Nuhse et al., 2000). Further study on the flagellin-induced pathway confirmed the role of AtMPK6 in the pathogen resistance cascade and also marked the position of AtMPK6 with respect to the other steps in the pathway, i.e., it is activated by AtMEKK1 and AtMKK4 (Asai et al., 2002).

Since a single MAPK is activated by several different elicitors through different MAPKKs and MAPKKKs, these kinases probably represent the converging point of the cascade. After their activation, they further phosphorylate different downstream targets so that different responses on the cellular level can be activated accordingly.

3. Targets of the pathway

The main target of cellular signal transduction is the nucleus, where numerous genes are activated. Disease resistance pathways are no exception, and it is known that MAPKs act by phosphorylating transcription factors (TFs), which subsequently activates transcription of other genes. In *Arabidopsis*, several families of transcription factors have been shown to regulate the expression of related-related genes, including TGA-bZIP, ERF, Myb, WRKY and Whirly (Table 2). In most cases consensus core motifs of the DNA binding sites for these proteins are known but the exact relationship between the transcription factors and the upstream components of the pathway are not completely established (Eulgem, 2005; Rushton and Somssich, 1998).

There seems no defined relationship between these transcription factors and their upstream components, but there are indications that some are regulated through phosphorylation. Among the structural features of ERF transcription factors is a putative MAP kinase-binding domain (Fujimoto et al., 2000). Although the only study showing that MAP kinase activates an ERF was in rice, this has been postulated as a possible common feature of all ERFs (Cheong et al., 2003). The situation is less clear for Myb TFs. The only indication that phosphorylation might be involved lies in the structural features of this protein, which suggest the involvement of phosphorylation in Myb activation (Jin and Martin, 1999). It is interesting that the PB element of the Whirly TF potentially overlaps with the W box recognised by WRKY TFs and with the TGA box, which is the binding site of the TGA TFs. The difference between them is that Whirly recognises single-stranded DNA while WRKY and TGA only bind to double-stranded DNA. But in any case, it is likely that there is interplay between these families, and possibly a similar mode of activation (Desveaux et al., 2005)

Probably the most studied family is the WRKY superfamily of transcription factors. Specifically, AtWRKY22 and AtWRKY29 have been found to mediate defence responses induced by fungal chitin and bacterial flagellin (Wan et al., 2004; Asai et al., 2002). Transgenic studies confirmed that the expression of these factors conferred resistance to the pathogen in both cases. A large number of pathogenesis-related (PR) genes as well as receptor-like protein kinases (RLK) are activated by certain WRKYs. Among the most studied are PR-1, a salicylic

Table 2. Defense-associated transcription factors.

Transcription factor	Size of family	Consensus core motif	Reference
ERF	56	GCCGCC (GCC box)	Fujimoto et al., 2000. Gutterson and Reuber, 2004
Myb	125	Type I: (T/C)AAC(T/G) Type II: G(G/T)T(A/T)G(G/T)T	Jin and Martin, 1999
TGA	10	TGACGTCA (TGA box)	Eulgem 2005. Rushton and Somssich, 1998
Whirly	3	GTCAAAA(A/T) (PB element)	Desveaux et al., 2005
WRKY	74	C/T- T-G-A-C-T/C	Ulker and Somissch, 2004, Eulgem et al., 2000

acid marker gene that controls the synthesis of salicylic acid in the infected tissue and induces systemic acquired resistance in the plant, and PR-2, which encodes a β-1,3-glucanase (an antifungal compound) (Rushton et al., 1996). Some RLKs, α-amylases, ethylene-induced DNA binding proteins, reverse transcriptases, protein kinases and different disease resistance gene products were also regulated by WRKYs (Du and Chen, 2000).

4. Proteomic approaches

Many kinases involved in plant signalling can be regulated at transcriptional, translational and post-translational levels, and the relative contribution of each to the overall response varies. Therefore a proteomic approach is valuable in understanding regulatory networks because it deals with identifying new proteins in relation to their function, and ultimately aims to unravel how their expression and modification is controlled.

Peck et al. used ^{32}P to pulse-label suspension-cultured cells of *Arabidopsis* in conjunction with 2-dimentional electrophoresis (2DE) and mass spectrometry (MS) to identify proteins that are rapidly phosphorylated in response to bacterial and fungal elicitors (Peck et al., 2001). One of these proteins, AtPhos43, was identified by nano-electrospray ionization (ESI) tandem MS and was found to be phosphorylated within minutes after treatment with flagellin. By measuring ^{32}P incorporation into AtPhos43 in defence response mutants, they found that phosphorylation of AtPhos43 after flagellin treatment was dependent on FLS2, a receptor-like kinase involved in flagellin reception. It has also been found that this protein was phosphorylated in response to both fungal and bacterial elicitors, and related proteins are phosphorylated in other monocot and dicot species (Peck, 2003; Peck et al., 2001). However, it is very interesting that in the examination of proteins that were phosphorylated in *Arabidopsis* upon treatment with flagellin only a few of the phosphoproteins were found to be regulated at the transcriptional level (Peck, 2003). This observation is consistent with other studies that have shown that the level of gene expression does not necessarily correlate with the protein levels in a cell (Gygi et al., 1999) or that the genes required for a response are not necessarily the same genes that are differentially regulated as a result of the response (Birrel et al., 2002; Giaever et al., 2002). For these reasons, it is believed that analysis of protein levels and protein modification profiles gives the best indication of the final players in a cellular response.

Proteomic approaches have been applied to monitor downstream components of specific MAPK pathways. tMEK2 is a known MAPK kinase in tomato and was previously shown to regulate the expression of β-1,3-glucanase and endochitinase genes in response to certain pathogen attacks (Xing et al., 2001). When 2DE was used to compare soluble proteins from wild-type and transgenic tomato plants carrying $tMEK2^{MUT}$, in which the tMEK2 is constitutively active (Xing et al., 2003), it was found that some of the proteins were phosphorylated in the $tMEK2^{MUT}$ transgenic tomato plant but not in wild-type plants. Eleven such proteins were identified by liquid chromatography (LC)-ESI MS and MALDI-TOF, including superoxide dismutase, glutathione peroxidase, GrpE, and calreticulin (C. Rampitsch and N. Bykova, unpublished).

5. Complexity of the pathogen activated MAPK cascade

Based on the number of MAPKKKs, MAPKKs and MAPKs, there can theoretically be numerous combinations ($23 \times 10 \times 60 = 13800$) of pathways. Recent comparative genomics analysis has indicated such complexity in MAPK pathways in two other species (rice and poplar) with completed genome sequences (Hamel et al., unpublished results). How can plants manage such a vast number of possibilities? It has been discovered that in mammalian systems different kinases are assembled into distinct modules by scaffold proteins. Scaffold proteins are important for preventing cross-talk between different cascades, and allow a given kinase to function in more than one module without affecting the specificity of the response (Morrison and Davis, 2003; Yoshioka, 2004). As recently shown for *Medicago* OMTK1, some MAPKKKs also seem able to act as scaffold proteins, assembling specific MAPK pathway components into particular modules (Nakagami et al., 2004). Scaffold proteins have not been identified in *Arabidopsis* MAP kinase pathways, but considering the fact that MAP kinases are conserved among eukaryotes it is plausible that *Arabidopsis* and other plants have the same mode of pathway assembly.

As shown in the analysis of the specific MAPK cascade components involved in pathogen induced signalling, one MAPK can be activated by multiple signals, and can itself phosphorylate more than one kinase. Taking the work by Asai et al. (2002) as an example, AtMEKK1 was shown to react with four different MAPKKs. One of them was AtMKK4, which also had the ability to phosphorylate more MAPKs. In turn, AtMPK6, the last kinase in the cascade, induced transcriptional factors that induced the activation of several genes. This shows only a small part of the entire MAPK signalling cascade. It can be concluded that there is no one distinct pathway that can be distinguished for one specific signal in plants. Instead, the MAP kinases act as a network of signalling components, and the final response of the plant depends on more conditions than just the type of pathogen that infects it.

Genetic knockout approaches are very effective in deciphering signalling pathways. However, as stress response cascades are obviously crucial for the well being of the plant, manipulating them might be detrimental for the health of the plant. This is probably one of the main reasons why not many *Arabidopsis* mutants are known for the genes encoding kinases in MAPK modules. Moreover, since the MAPKs are regulated post-translationally by phosphorylation, the loss of a functional gene product might not reveal the exact function of a MAPK cascade (Zhang and Klessig, 2001). A combination of genetic, biochemical, genomic and proteomic studies will reveal a much more complex picture of MAPK pathways in plant defense responses.

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