# Cell Signaling and Response via Mitogen-Activated Protein Kinase (MAPK) Cascade in Arabidopsis

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## ABSTRACT

Plant cells respond specifically to environmental conditions such as temperature, light, pH and parasite attacks. The involvement of signal transduction is inevitable in relation to activated chains of intracellular signaling responses. The intracellular signal processing depends on specific interactions between proteins. Protein phosphorylation is amongst events that play significant roles in the protein-protein communication to make up signal relay. The mitogen-activated protein kinase (MAPK) is one of many kinds of plant protein kinases that have been reported in the past decade. MAPK cascade plays essential roles in the signal transduction process. In plants, the cascade has been implicated in relaying signal of various plant hormones and stress stimuli. It consists of MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). Activated MAPKKK phosphorylates MAPKK, thus activated MAPKK in turn activates MAPK through phosphorylation. The extracellular signaling is relayed through this MAPK cascade resulting in switching gene expression on/off or activation/inactivation of cell response matters, and by doing so physiological and morphological changes to adapt external situation are fulfilled. In *Arabidopsis thaliana*, genome analysis reveals 20 different genes encoding MAPKs, 10 MAPKK genes and 60 MAPKKK genes, suggesting complicated signal networks of this cascade. Recently, each set of plant MAPK cascade begins to be investigated and several evidences are shown. We have done research projects especially on *Arabidopsis* MAPKK (AtMEK1) and showed AtMEKK1-AtMEK1-AtMPK4 cascade that becomes active and works on a wounding stimulus. This review will discuss the mechanism and roles of plant protein phosphorylation via three-component MAPK cascade, focus especially on *Arabidopsis* MAPKKs and encompass a recent review of this field.

## CONTENTS

## INTRODUCTION

Mitogen-activated protein kinase (MAPK) cascade is one of signal modules that connect the perception of various extra- and intracellular stimuli to physiological cellular responses. The cascade is conserved throughout eukaryotes and functions to control of developmental and physiological processes, such as the growth and differentiation of cells and adaptation to stresses. In plants, MAPK pathways have been implicated in responses to various biotic and abiotic stresses, plant hormones, cell division and developmental processes (see reviews in Takahashi *et al.* 2004; Nakagami *et al.* 2005). MAPK cascades are composed of three protein kinases: MAPKs, MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs), which are linked in various ways to upstream receptors and downstream targets. Although the activation mechanisms of MAPKKKs are still not clear in many cases, physical interaction and/or phosphorylation by receptor itself or interlinking proteins are thought to be a regulator of MAPKKK activities. MAPKKKs contain the regulatory regions in their N-terminus or C-terminus adjacent to kinase domain. By deleting the regulatory regions,

the constitutively active MAPKKKs are generated and used as a powerful tool of identifying their downstream targets. Transient overexpression of the ANP1 kinase domain, constitutively active ANP1, mimics the $H_2O_2$ effect and initiates a phosphorylation cascade involving two stress responsive MAPKs, AtMPK3 and AtMPK6 (Kovtun *et al.* 2000). MAPKKs are activated by MAPKKKs through phosphorylation of conserved serine and threonine residues in their activation loops. Plant MAPKKs have the consensus S/TxxxxxS/T sequence, whereas animals and yeast enzymes have S/TxxxS/T. MAPKK proteins also can be generated a constitutively active form by substituting both serine or threonine residues in the consensus sequence to acidic amino acid residues. Expression of constitutively active mutant of tobacco MAPKK, NtMEK2, induced HR-like cell death in tobacco, which is preceded by the activation of endogenous SIPK and WIPK (Yang *et al.* 2001). It was the first manipulation of generating a constitutively active MAPKK in plants, and then many kinds of works have been done in various plant species including Arabidopsis (Asai *et al.* 2002; Ren *et al.* 2002; Teige *et al.* 2004; Hua *et al.* 2006), tobacco (Jin *et al.* 2003; Kim *et al.* 2003; Gomi *et al.* 2005),

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tomato (Ekengren *et al.* 2003; Pedley *et al.* 2004) and parsley (Lee *et al.* 2004) in order to identified downstream targets and/or clarify the function of the MAPKKs. Active MAPKKs dual-phosphorylate the threonine and tyrosine residues of the conserved TxY sequence of their downstream MAPKs. Animal MAPKs have TEY, TGY and TPY motifs in their activation loops, whereas plants have TEY and TDY motifs. Interestingly, human malaria parasite, *Plasmodium falciparum* has a TDY-type activation loop (Lin *et al.* 1996). Plant TEY-type MAPKs also have shown that these conserved threonine and tyrosine residues are phosphorylated and then activated by their upstream MAPKKs, however, TDY-type MAPKs have no CD domain, which is thought to be a interacting region with MAPKKs, phosphatases and protein substrates (Tanoue *et al.* 2000). Moreover, TDY-type MAPKs have an extended C-terminal region relative to TEY type. It has been reported that rice BWMK1 and alfalfa TDY genes are induced by both blast fungus and wounding. Rice BWMK1 was induced by the blast fungus *Magnaporthe grisea* and upon mechanical wounding in leafs (He *et al.* 1999). Alfalfa TDY1 expression was observed in leaf mesophyll surrounding areas of mechanical wounding and fungal pathogen *Phoma medicaginis* invasion (Schoenbeck *et al.* 1999). No upstream activator MAPKKs have been identified and the functions of this type MAPKs in plants or the downstream targets still have not been clarified. Recently rice BWMK1 phosphorylated OsERBP1 transcription factor and mediated pathogenesis-related gene – *PR1*, *PR2*, *PR3*, *PR4* and *PR5* – expression (Cheong *et al.* 2003).

On the basis of the fully sequenced Arabidopsis genome, 20 MAPKs, 10 MAPKKs and 60 MAPKKKs were identified (MAPK group 2002). Recently, flagellin signaling, which is through the Arabidopsis FLS2-MEKK1-MKK4/5-MPK3/6-WRKY22/20 pathway, has been shown by combining transient expression analysis with biochemical and genetic approaches (Asai *et al.* 2002). We have identified the AtMEKK1-AtMEK1-AtMPK4 cascade as signal transducers of wounding (Matsuoka *et al.* 2002; Toto *et al.* 2006). It was also reported that AtMEKK1 activates the AtMKK2-AtMPK4/6 signal modules by cold and salt stresses (Teige *et al.* 2004). In tobacco plant, NPK1-MEK1-NTF6 pathway has a dual-function in pathogen defense and cytokinesis (Calderini *et al.* 2000; Soyano *et al.* 2003). To date, functional data are available for relatively few of the corresponding proteins. Furthermore, crosstalk systems among them remain largely unknown.

The limited number of MAPKKs compared with a relatively large number of MAPKKKs suggests that MAPKKs are converging points among plant MAPK cascade. In this review, we focus on the functions of MAPKKs in Arabidopsis and discuss the complex network of plant MAPK signaling modules.

## CLASSIFICATION OF PLANT MAPKKS

By the phylogenic analysis inferred from the amino acid sequence alignments of catalytic domain, plant MAPKKs have been classified into four groups (MAPK group 2002). In the Arabidopsis genome, there are 10 MAPKKs divided into four groups: Group A consists of three MAPKKs: MKK1, MKK2 and MKK6. Group B has only one MAPKK, MKK3. Group C contains two MAPKKs, MKK4 and MKK5. Group D has four MAPKKs: MKK7, MKK8, MKK9 and MKK10. A summary of the groupings and functional characteristics of the Arabidopsis MAPKKs are shown in **Fig. 1**. In general, the protein kinases classified into the same clade are thought to have similar functions and substrate specificities (Hanks and Hunter 1995). We review the roles of plant MAPKKs in each group, especially focussing on Arabidopsis MAPKKs.

## DUAL-FUNCTION OF GROUP A MAPKKS (AtMKK1, AtMKK2 AND AtMKK6)

AtMKK1 (renamed from MEK1) was cloned by cDNA library screening in 1997. The transcript of the gene was enhanced by wounding and not detected in etiolated seedlings (Morris *et al.* 1997). It indicated that expression of the gene is regulated by developmental processes and by stress signals. Mizoguchi *et al.* identified the AtMEKK1-AtMKK1-AtMPK4 cascade based on pair-wise yeast two-hybrid analysis and functional complementation tests of yeast mutants. This was the first demonstration of a possible MAPK cascade in plants (Mizoguchi *et al.* 1998). Biochemical properties of this cascade were confirmed by using bacterially expressed recombinant proteins (Huang *et al.* 2000; Matsuoka *et al.* 2002). Analysis of AtMKK1 using a specific antibody revealed that wounding (Matsuoka *et al.* 2002), pathogenic elicitors and reactive oxygen species (Teige *et al.* 2004) activated AtMKK1. AtMKK1 was also phosphorylated by immunoprecipitated AtMEKK1 from wounding treated Arabidopsis seedlings (Toto *et al.* 2006). AtMKK1 mutant Arabidopsis showed no obvious growth defect but was more sensitive for pathogen attack (Mészáros *et al.* 2006). AtMKK2 was identified as an AtMEKK1 interacting protein by yeast two-hybrid screening and also interacts with downstream AtMPK4 (Ichimura *et al.* 1998a). In Arabidopsis protoplasts, AtMKK2 was specifically activated by cold (0°C) and salt (280 mM NaCl) stress and by the stress-induced AtMEKK1. Yeast two-hybrid and biochemical analysis revealed AtMKK2 directly targets AtMPK4 and AtMPK6. Gain of function and loss of function mutants of AtMKK2 indicated the formation of AtMEKK1-AtMKK2-AtMPK4/6 cascade mediated cold and salt stress tolerance in Arabidopsis (Teige *et al.* 2004). AtMKK2 null and overexpressor plants had no obvious phenotype (Teige *et al.* 2004) like the AtMKK1 null mutant (Mészáros *et al.* 2006). Because the mutants of AtMPK4 (Petersen *et al.* 2000) or AtMEKK1 (Ichimura *et al.* 2006; Nakagami *et al.* 2006), which are downstream or upstream of both AtMKK1 and AtMKK2, showed a dwarf phenotype, AtMKK1 and AtMKK2 may have a functional redundancy in developmental processes but they have distinct roles in stress signal transduction. AtMKK6 was identified by the Arabidopsis genome project. Arabidopsis plants with a mutation in the AtMKK6 (renamed from ANQ1) gene, display a dwarf phenotype, with unusually large cells that contain multiple nuclei and cell-wall stubs in various organs (Soyano *et al.* 2003). Functional complementation of the cell lysis phenotype of mutant yeast strain, AtMKK6 activates AtMPK13 (Mekikant *et al.* 2004). The transcripts of AtMKK6 and AtMPK13 were co-expressed in roots, flower buds and stems (Mekikant *et al.* 2004). The tobacco ortholog of AtMKK6, NQK1/NtMEK1 was known to form the NPK1-NQK1/NtMEK1-NRK1 cascade and have a dual function in pathogenesis and cytokinesis. A reverse-genetic approach using multiple-knockout mutants of the Arabidopsis ANP1/2/3, tobacco NPK1 ortholog, genes revealed their function as a positive regulator of cytokinesis and a possible negative regulator of oxidative stress responses (Kovtun *et al.* 2000; Krysan *et al.* 2002). In addition, ANPs and NPK1 function in oxidative-stress-response signaling and as negative regulator of the auxin-response pathway (Kovtun *et al.* 1998, 2000). These indicate the formation of the ANP1/2/3-AtMKK6-AtMPK13 signaling cascade. In summary, group A MAPKKs have a dual function in stress and developmental signal pathways.

## UNUSUAL STRUCTURAL FEATURE OF GROUP B MAPKK (AtMKK3)

Molecular cloning of the AtMKK3 gene was performed by cDNA library screening using the NPK2-like PCR product as a probe. The AtMKK3 gene was expressed in all organs and the GST-fusion protein of AtMKK3 possessed kinase activities (Ichimura *et al.* 1998b). Although tobacco NPK2
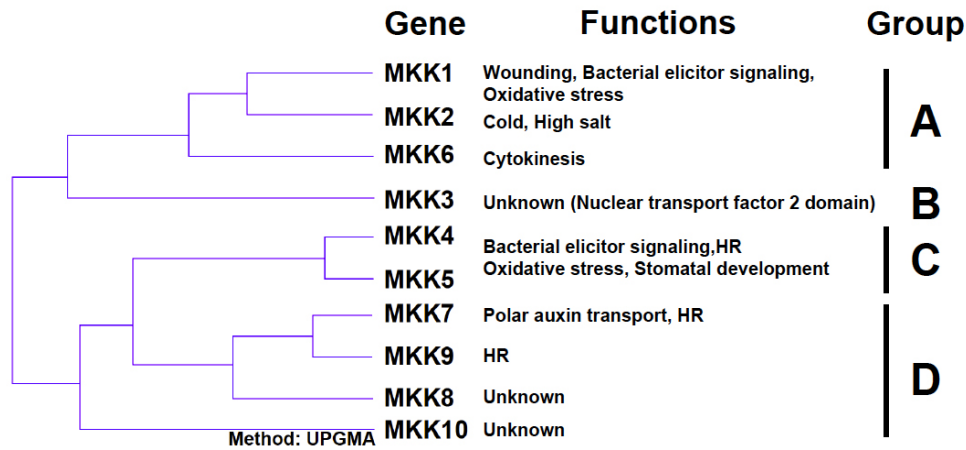
**Fig. 1 Classification of Arabidopsis MAPKKs and their functions.** Phylogenic tree of Arabidopsis MAPKK was created using GENTYX-MAC software in UPGMA method inferred from their amino acid sequence alignments of kinase domain. Each MAPKK was classified into four groups. The reported function of each MAPKK was shown together.

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Gene                 Sequences of inter DFG-SPE       Construction of active mutants
MKK1  (208-235)      DFGVSKILTSTSSLANSFVGTYPYMSPE     T218E/S224E, T218E/S224D
MKK2  (210-237)      DFGVSTVMTNTAGLANTFVGTYNYMSPE     T220D/T226E, T220E/S224E
MKK3  (225-252)      DFGISAGLENSMAMCATFVGTVTYMSPE     S235D/T241D
MKK4  (214-241)      DFGVSRILAQTMDPCNSSVGTIAYMSPE     T224D/S230E, T224D/S230D
MKK5  (205-232)      DFGVSRILAQTMDPCNSSVGTIAYMSPE     T215E/S221E, T215D/S221D
MKK6  (211-238)      DFGVSASLASSMGQRDTFVGTYNYMSPE     S221D/T227E
MKK7  (183-210)      DFGVSKIITRSLDYCNSYVGTCAYMSPE     S193D/S199D
MKK8  (185-212)      DFGVSKIVVRSLNKCNSFVGTFAYMSPE     S195D/S201E
MKK9  (185-212)      DFGVSKILVRELDSCNSYVGTCAYMSPE          S201D
MKK10 (183-207)      DFGASRIVAGG--DYGS-NGTCAYMSPE           -
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**Fig. 2 Amino acid sequence alignments of Arabidopsis MAPKKs in their activation loops and constitutively active mutants of Arabidopsis MAPKKs.** Amino acid sequence alignments of Arabidopsis MAPKK inter DFG-SPE region were shown. The amino acid number of this region was shown in light side of each MAPKK. The previously reported constitutively active mutants of each MAPKK were shown. The consensus serine or threonine residues were indicated in bald faces. AtMKK9 has a unique sequence of this region, indicated red and bald faces. There is no constitutively active AtMKK10 mutant because of lacking the consensus S/TxxxxxS/T motif.
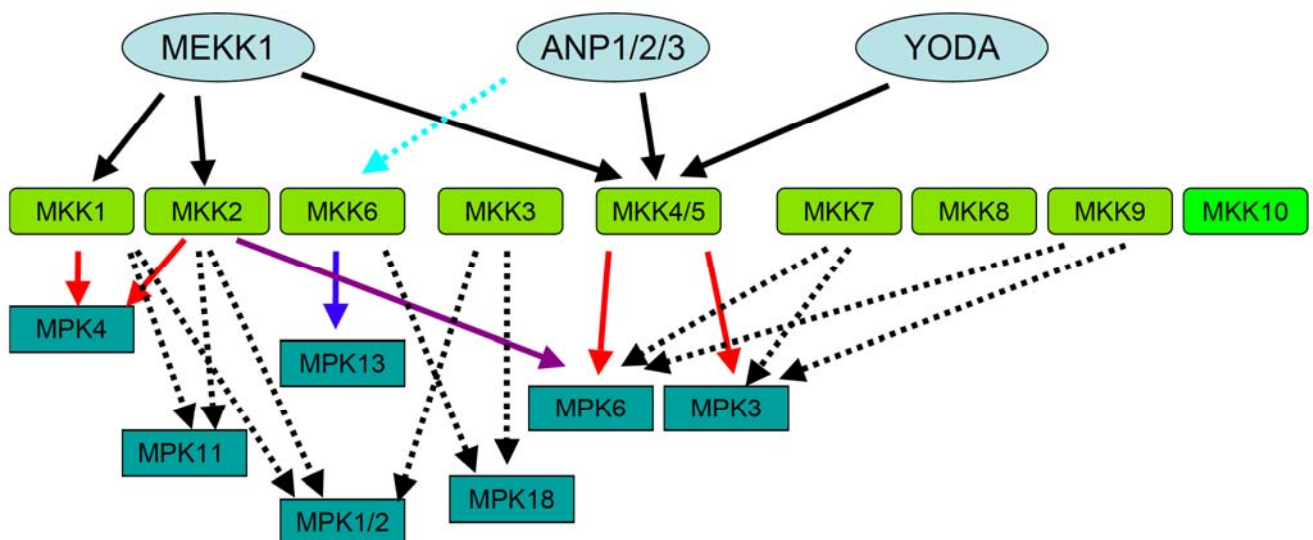


**Fig. 3 Arabidopsis MAPK signaling networks.** The confirmed MAPKKK-MAPKK signal pathways were indicated by black arrows. Possible ANP1/2/3-AtMKK6 pathways, which was predicted by the tobacco orthologus NPK1-NtMEK1/NQK1 pathway, was indicated dotted blue arrow. The dotted arrows in MAPKK-MAPK signal pathway were determined by transient co-expression of a pair of active MAPKK and wild type MAPK in tobacco leaves. Red arrows indicated the MAPKK-MAPK pathways that confirmed by the plural methods. The AtMKK6-AtMPK13 signal module, indicated by blue arrow, was confirmed by the functional complementation of yeast mutant and kinase assays using bacterially expressed proteins. AtMKK2-AtMPK6 pathway, indicated by purple arrow, showed a different result in each researcher. Respective cascade shown here was referred in the text.

was the first identified MAPKK in plants (Shibata *et al.* 1995), the functions of this group MAPKK have not been clarified yet. In plant MAPKKs, only this MAPKK group haa a C-terminal extended domain of nuclear transport factor 2 (NTF2). NTF2 is a small protein that mediates the nuclear import of Ran-GDP and binds to both Ran-GDP and FxFG-repeat-containing nucleoporins (Suyama *et al.* 2000). Recently, transiently expressed active mutant of AtMKK3 in tobacco leaves phosphorylated the co-transformed AtMPK1and AtMPK2 and activated RD29A/GUS and RD29B/GUS reporter genes (Hua *et al.* 2006). This may lead to the functional identification of AtMKK3.

## THE ROLES OF GROUP C MAPKK (AtMKK4 AND AtMKK5) IN STRESS SIGNALING

AtMKK4 and AtMKK5 genes were screened from a Arabidopsis cDNA library by using a partial fragment of the Arabidopsis EST clone 127H15T7 as probe. The levels of AtMKK4 and AtMKK5 transcripts were relatively higher in stems and leaves than in flowers and roots (Ichimura *et al.* 1998b). Expression of constitutively active AtMKK4 and AtMKK5 in tobacco by using a steroid-inducible promoter results in generation of hydrogen peroxide and cell death (Ren *et al.* 2002). Flagellin signaling, which is through the Arabidopsis FLS2-MEKK1-MKK4/5-MPK3/6-WRKY22/20 pathway, has been identified by combining transient expression analysis with biochemical and genetic approaches (Asai *et al.* 2002). The orthologus signaling modules were identified in many plant species including tobacco (Yang *et al.* 2001), alfalfa (Kiegerl *et al.* 2000), tomato (Pedley *et al.* 2004), potato (Katou *et al.* 2005) and parsley (Lee *et al.* 2004). Tobacco MAPKKKα was identified as an upstream activator of NtMEK2, a tobacco ortholog of AtMKK4/5, and functions as a key regulator of cell death associated with plant immunity and the bacterial pathogen *Pseudomonas syrigae* susceptibility (Pozo *et al.* 2004). Loss of function of AtMKK4/5 or AtMPK3/6 disrupts the coordinated cell fate specification of stomata versus pavement cells, resulting in the formation of clustered stomata (Wang *et al.* 2007). Moreover, YODA, an Arabidopsis MAPKKK, was shown to be the upstream activator of this AtMKK4/5-AtMPK3/6 pathway (Wang *et al.* 2007).

## FEATURES OF GROUP D MAPKK (AtMKK7, 8, 9 AND 10)

The Arabidopsis group D MAPKK was also identified by the genome sequence project. The consensus sequence S/TxxxxxS/T is not found in AtMKK9 and AtMKK10 because of amino acid substitution of former S/T residue to glutamic acid in AtMKK9 and the deletion of three amino acid residues in AtMKK10, respectively (**Fig. 2**). Constitutively active mutant of AtMKK7 and AtMKK9 phosphorylated the co-transformed AtMPK6 and can cause hypersensitive response in transiently expressed tobacco leaves (Hua *et al.* 2006). Enhanced expression of AtMKK7 resulted in reduced apical dominance and showed bushy and dwarf phenotypes (Dai *et al.* 2006). This MAPKK groups may have dual-functions in stress signaling and determine the morphological shape although the findings are still few and further analysis are necessary to clarify the roles of this group D MAPKKs.

## CONCLUSION

As mentioned above, 60 MAPKKKs, 10 MAPKKs and 20 MAPKs exist in the Arabidopsis genome. The numbers of MAPKKs are fewer than other two components of the MAPK cascade. MAPKKs locate in the middle of the MAPK cascade. It is reasonable that the function of each MAPK cascade is classified according to the MAPKKs. MAPKKs are phosphorylated and activated by upstream MAPKKKs. These phosphorylation sites, S/TxxxxxS/T, are conserved in almost all MAPKKs except AtMKK9 and

AtMKK10 in Arabidopsis and constitutively active mutants of MAPKKs can be produced by substitutions of the serine or threonine residues to acidic residues, which mimics the phosphorylated form of MAPKK (**Fig. 2**). AtMKK9 has a unique ExxxxxS sequence in the activation loop. Therefore, constitutively active mutant is constructed by S201D mutation. As a result, nine active forms of ten Arabidopsis MAPKKs can be constructed. Constitutively active mutants of the enzymes are extremely powerful tools for the analysis of downstream events and identification of MAPKKs functions. In addition, loss of function mutant, yeast two hybrid analysis, and biochemical approaches revealed the features of several MAPK cascades. **Fig. 3** shows the present known Arabidopsis MAPK cascades. AtMKK4/5- AtMPK3/6 signal pathway has several upstream activator, thus the plural signals integrate the single MKK-MPK module. How is the signaling specificity of different MAPK cascades sharing the same MKK-MPK modules maintained? In yeast, Ste11, a MAPKKK, has dual functions in mating and high osmolarity signaling pathways (Park *et al.* 2003). Scaffold proteins Ste5 and Pbs2 maintain the specificity of each MAPK cascade. It is likely to resemble plant MAPK cascades. Tissue- or cell-specific expression of input signaling modules may play a critical role in maintaining the signaling specificity of different MAPK cascades.

Although the family of MAPKKKs forms the largest and most heterogeneous group of MAPK pathway components, it has only been shown in a few cases that these kinases function as the activator of a MAPKK. Phylogenic analysis based on the amino acid sequences of the protein kinase domain shows that plant MAPKKKs fall into two major groups: 12 MEKK like and 48 Raf like MAPKKK groups. The functions of a few MAPKKs, like CTR1 (Kieber *et al.* 1993) or EDR1 (Frye *et al.* 2001), are identified by the analysis of their loss of function mutant in the Raf-like group but evidence that they act as MAPKKKs is still lacking. An unknown signaling mechanism seems to play a role in plant cells.

As described above, MAPK cascade is one of important pathways in stress or developmental signaling in plants. However, it is known that there are many other signaling components such as phytohormones, sugars, CDPKs and PGIPs in plant cellular signaling. What is the link between these components and MAPK signaling networks? Although, there are many researches that suggested a possible involvement of these components that participated in the common signal networks, the evidences that clarified the direct interaction of MAPK cascade components with other signaling pathways are still limited. CTR1, an Arabidopsis MAPKKK, interacted with ETR1 and ERS ethylene receptors (Clark *et al.* 1998). Arabidopsis MPK6 phosphorylated the ACC synthases ACS2 and ACS6, thus involved ethylene biosynthesis (Liu and Zhang 2004). To know the roles of MAPK signaling networks in plants, it is necessary to analyze not only MAPK cascade components but also the interaction with other signaling pathways.

Research on MAPK cascade has progressed by using the two-hybrid system, molecular genetics approaches, and biochemical analysis. Further information on the functions of each MAPK cascade component will be provided by continuing these analyses. However, it is predicted that each MAPK cascade would be interconnected in a complex crosstalk. Novel methods are desired to analyze the profile of cellular reactions or sites and tissue-specific investigations in living plants.

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