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. Environmental 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 MAPKs and encompass a recent review of this field.

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 H2O2 effect and initiates a phosphorylation cascade involving two stress responsive MAPKs, AtMPK3 and AtMPK6 (Kovtun et al. 2000). MAPKs are activated by MAPKKKs through phosphorylation of conserved serine and threonine residues in their activation loops. Plant MAPKs have the consensus S/TxxxxxS/T sequence, whereas animals and yeast enzymes have S/TxxxxS/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 MAPK, 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 MAPK 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),
shown in functional characteristics of the Arabidopsis MAPKKs are MKK9 and MKK10. A summary of the groupings and MKK5. Group D has four MAPKKs: MKK7, MKK8, MAPKK, MKK3. Group C contains two MAPKKs, MKK4 and MKK6. Group B has only one MKK1, MKK2 and MKK6. 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. Mizoguchi et al. (1999) 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), pathogenesis, 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 over-expressor 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. 2000; Nakagami et al. 2006). The absence or mutation of AtMKK6 is correlated with the down-regulation of AtMPK13 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-AtMPK6-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

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MAPK cascade signaling in Arabidopsis. Matsuoka et al.

Fig. 1 Classification of Arabidopsis MAPKKs and their functions. Phylogenetic 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences of inter DFG-SPE</th>
<th>Construction of active mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>M KK1</td>
<td>(208-235)</td>
<td>T218E/S224E, T218E/S224D</td>
</tr>
<tr>
<td>M KK2</td>
<td>(210-237)</td>
<td>T220D/T226E, T220E/S224E</td>
</tr>
<tr>
<td>M KK3</td>
<td>(225-252)</td>
<td>S235D/T241D</td>
</tr>
<tr>
<td>M KK4</td>
<td>(214-241)</td>
<td>T224D/S230E, T224D/S230D</td>
</tr>
<tr>
<td>M KK5</td>
<td>(205-232)</td>
<td>T215E/S221E, T215D/S221D</td>
</tr>
<tr>
<td>M KK6</td>
<td>(211-238)</td>
<td>S221D/T227E</td>
</tr>
<tr>
<td>M KK7</td>
<td>(183-210)</td>
<td>S193D/S199D</td>
</tr>
<tr>
<td>M KK8</td>
<td>(185-212)</td>
<td>S195D/S201E</td>
</tr>
<tr>
<td>M KK9</td>
<td>(185-212)</td>
<td>S201D</td>
</tr>
<tr>
<td>M KK10</td>
<td>(183-207)</td>
<td></td>
</tr>
</tbody>
</table>

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 MAPKKs indicate 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 bold faces. AtMKK9 has a unique sequence of this region, indicated red and bold 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 MAPKK-MAPKK signal pathways were indicated by black arrows. Possible ANP1/2/3-AtMKK6 pathways, which was predicted by the tobacco orthologous NPK1-NMEK1/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 MAPKs, only this MAPKK group has 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 AtMPK1 and 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 MAPK (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 orthologous signaling modules were identified in many plant species including tobacco (Yang et al. 2001), alfalfa (Kiegert et al. 2000), tomato (Pedley et al. 2004), potato (Katou et al. 2005) and parsley (Lee et al. 2004). Tobacco MAPKKKs 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 syringae* 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 MAPKK, was shown to be the upstream activator of this AtMKK4/5-AtMPK3/6 pathway (Wang et al. 2007).

FEATURES OF GROUP D MAPK (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 group 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 MAPKs.

CONCLUSION

As mentioned above, 60 MAPKKs, 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 MAPKs. MAPKs are phosphorylated and activated by upstream MAPKKs. These phosphorylation sites, S/TxxxxxS/T, are conserved in almost all MAPKs except AtMKK9 and AtMKK10 in Arabidopsis and constitutively active mutants of MAPKs 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 same 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 at different MAPK cascades.

Although the family of MAPKKs 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. Phylogenetic analysis based on the amino acid sequences of the protein kinase domain shows that plant MAPKKs fall into two major groups: 12 MEKK like and 48 Raf like MAPKK 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 MAPKKs 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 signal pathways are still limited. CTR1, an Arabidopsis MAPKK, interacts 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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