

# Mitogen-Activated Protein Kinases and Reactive Oxygen Species Signaling in Plants<sup>1</sup>

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In plants, reactive oxygen species (ROS) can be generated by various processes occurring in different cellular compartments. Under physiological steady-state conditions, ROS are scavenged by different antioxidative components, but the balance between production and scavenging of ROS may be perturbed by a number of adverse environmental factors, giving rise to rapid increases in intracellular ROS levels. Although high concentrations of ROS can cause irreversible damage and cell death, they can also influence signaling and gene expression, indicating that cells have evolved strategies to utilize ROS to control various biological programs (Apel and Hirt, 2004). Being small and able to diffuse over short distances, ROS are ideally suited to act as signaling molecules. Among different ROS, only hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) can cross plant membranes and can therefore directly function in cell-to-cell signaling. Plant cells possess still-unidentified specific ROS sensors that process and translate this information into respective biological output programs. In several systems, various pathways, particularly those involving mitogen-activated protein kinases (MAPKs), are modulated by ROS and will be the focus of this review.

MAPK cascades minimally consist of a MAPKKK-MAPKK-MAPK module that is linked in various ways to upstream receptors and downstream targets (Nakagami et al., 2005). Receptor-mediated activation of a MAPKKK can occur through physical interaction and/or phosphorylation by either the receptor itself, intermediate bridging factors, or interlinking kinases. Activation of MAPK modules generally occurs through sequential phosphorylation of its component kinases culminating in the generation of active MAPKs, which phosphorylate a variety of substrates including transcription factors, other protein kinases, and cytoskeleton-associated proteins. Specificity of MAPK cascades functioning within the same cell and employing iden-

tical components is achieved by docking domains found in diverse components of MAPK modules and by scaffold proteins.

### ROS AND STOMATA

The phytohormone abscisic acid (ABA) accumulates in response to dehydration and induces a range of stress adaptation responses including stomatal closure. Recent studies have implicated H<sub>2</sub>O<sub>2</sub> as an endogenous component of ABA signaling in *Arabidopsis thaliana* guard cells. ABA-stimulated ROS accumulation induces stomatal closure via activation of plasma membrane calcium channels (Pei et al., 2000), and changes in intracellular calcium levels were shown to mediate stomatal closure triggered by application of H<sub>2</sub>O<sub>2</sub> or the herbicide methyl viologen, which generates superoxide in chloroplasts (McAinsh et al., 1996). Complementary to the calcium-mediated stomatal closure triggered by H<sub>2</sub>O<sub>2</sub>, ozone exerts its function by inhibiting guard cell potassium channels that assist potassium uptake, which in turn drives stomatal opening (Torsethaugen et al., 1999). There is evidence for MAPKs to be involved in stomata regulation; *NtMPK4* is preferentially expressed in the epidermis and *NtMPK4*-silenced plants are hypersensitive to ozone due to an ABA-independent misregulation of stomatal closure (Gomi et al., 2005). Thus, *NtMPK4* might function at early stages of ROS signaling by controlling the entrance gate for ozone uptake from the environment. A MAPK that is activated by ABA was also identified in pea (*Pisum sativum*) guard cells (Burnett et al., 2000), providing further evidence for a role of MAPKs in stomatal control. MAPK modules do not only affect the aperture but also the density of stomata. Null mutations in the *Arabidopsis* MAPKKK *YODA* lead to excess stomata, whereas constitutive activation of *YODA* eliminated stomata (Bergmann et al., 2004). Microarray analyses revealed differential regulation of several genes in *yoda* mutants (Bergmann et al., 2004).

### ROS AND ROOTS

Roots and root hairs in particular assist water and nutrient uptake and help to anchor the plant in the soil. Because root hairs are not essential for plant

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growth under laboratory conditions, they have become an attractive model to study cell polarity and development in plants. Recently, *root hair defective 2* (*rhd2*), an Arabidopsis mutant forming root hair bulges, was found to lack the functional AtrbohC NADPH oxidase (Foreman et al., 2003). ROS were localized in the growing tips of root hairs of wild-type plants but not in the *rhd2* mutant. Possibly, the NADPH oxidase is controlled via small GTPases of the Rop family (Baxter-Burrell et al., 2002; Gu et al., 2003) in analogy to mammalian cells, where the small GTPase Rac regulates NADPH oxidase and ROS production. Two recent reports have implicated Arabidopsis OXI1 (oxidative stress inducible 1) belonging to the AGC family of protein kinases in root hair development. Mutant plants defective in OXI1 show strong reduction in number and length of root hairs. OXI1 kinase activity is induced by phosphatidic acid (Anthony et al., 2004) and by H<sub>2</sub>O<sub>2</sub> (Rentel et al., 2004), two factors known to be regulating root hair growth. *Oxi1* null mutants are impaired in the activation of the MAPKs MPK3 and MPK6 upon oxidative stress (Rentel et al., 2004), suggesting that OXI1 functions downstream of ROS but upstream of the MAPK module. An earlier study had already linked the stress-induced alfalfa (*Medicago sativa*) MAPK SIMK, the putative ortholog of Arabidopsis MPK6, to root hair growth (Samaj et al., 2002). In this work, inhibition of MAPK activity resulted in abrogation of root hair growth, whereas expression of constitutively active SIMK was correlated with increased root hair length. These findings suggest root hair development to be mediated by a ROS signaling pathway that depends on a number of protein kinases.

Not only the development of root hairs but also that of roots apparently relies on redox regulation. Kwak et al. (2003) found that AtrbohF and AtrbohD/F double mutants have shorter roots compared to wild-type plants, and in glutathione-deficient *root meristemless 1* mutants, roots fail to develop (Vernoux et al., 2000). In animals, the redox environment within the cell selectively regulates stress signaling through two MAPKKs, MEKK1 versus ASK1, and is assumed to participate in the induction of apoptosis by oxidative stress. This regulation is mediated by glutathionylation of Cys residues in the two MAPKKs (Cross and Templeton, 2004). It remains to be seen whether plant MAPKKs can also be directly redox regulated.

## ROS AND BIOTIC STRESS

Plants respond to pathogen attack by activating multistep defense responses. Among the many reactions, a hallmark to pathogen attack is the rapid production of ROS that is often followed by hypersensitive response (HR), a localized programmed cell death at the site of infection. A central role of MAPKs in the onset of pathogen defense is now firmly established (for review, see Nakagami et al., 2005). Infection of tobacco (*Nicotiana tabacum*) leaves by *Tobacco mosaic*

*virus* (TMV) activates both salicylic acid-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK; Zhang and Klessig, 1998) prior to the onset of HR-like cell death. Ectopic expression of SIPK is sufficient to yield active MAPK and induce HR (Zhang and Liu, 2001). Consistent with the finding that WIPK and SIPK are substrates of the MAPKK MEK2, overexpression of constitutively active MEK2 induces an oxidative burst and HR (Yang et al., 2001). Virus-induced gene silencing of MEK2, SIPK, or WIPK results in strong attenuation of *N* gene-mediated resistance against TMV (Jin et al., 2003). There appears to exist at least one more tobacco MAPK pathway in pathogen defense; virus-induced gene silencing of *MEK1* and its potential substrate MAPK *NTF6* attenuates *N*-mediated resistance of tobacco to TMV (Liu et al., 2003).

In Arabidopsis, MPK3, MPK4, and MPK6 are all activated by bacterial and fungal pathogen-associated molecular patterns and ROS (Kovtun et al., 2000; Nühse et al., 2000; Desikan et al., 2001). Arabidopsis *mpk4* mutants exhibit increased resistance to virulent pathogens, and MPK4 appears to be required for jasmonic acid-mediated gene expression (Petersen et al., 2000). In contrast, *MPK6*-silenced plants are compromised in resistance to avirulent *Peronospora parasitica* as well as to avirulent and virulent *Pseudomonas syringae* strains, while expression of most pathogenesis-related (PR) genes is similar to wild type (Menke et al., 2004). A combination of transient expression analysis with biochemical and genetic approaches revealed that the MEKK1-MKK4/MKK5-MPK3/MPK6 module acts downstream of the flagellin receptor *FLS2* and that transient overexpression of the MEKK1 kinase domain or constitutively active MKK4 or MKK5 renders leaves resistant to infection by bacterial and fungal pathogens (Asai et al., 2002).

## ROS AND ABIOTIC STRESSES

### Ozone

The production of ROS is a common response to virtually any biotic and abiotic stress. Among the abiotic stresses, ozone is a direct precursor of ROS. Ozone application triggers a programmed cell death that is highly reminiscent of biotic defense programs including HR and the synthesis of PR proteins (Overmyer et al., 2000). Ozone treatment of Arabidopsis leads to activation and nuclear translocation of MPK3 and MPK6 (Ahlfors et al., 2004), whereas RNAi lines silenced for either of these MAPKs are hypersensitive to ozone (Miles et al., 2005). Moreover, *MPK6*-RNAi plants display a stronger and prolonged activation of MPK3 compared to wild type. Reciprocally, *MPK3*-RNAi lines show a stronger and prolonged MPK6 activation (Miles et al., 2005). MPK3 and MPK6, however, are unlikely to have entirely redundant functions, as MPK3, but not MPK6, is additionally regulated by ozone treatment at the level of transcription and translation (Ahlfors et al., 2004).

Further evidence for the involvement of MAPKs in the ozone response comes from analyses of the ozone-sensitive *radical-induced cell death 1* (*rcd1*) mutant; ozone-induced cell death in *rcd1* was blocked by diphenylene iodonium (Overmyer et al., 2000), an inhibitor of NADPH oxidases, and *rcd1* mutants display enhanced resistance toward methyl viologen (Fujibe et al., 2004). Furthermore, ozone-exposed *rcd1* have increased salicylic acid levels and show earlier activation of MPK6 compared to wild-type plants (Overmyer et al., 2005).

Like MPK3 and MPK6 in Arabidopsis, the putative tobacco orthologs SIPK and WIPK also become activated by ozone and nitric oxide (Kumar and Klessig, 2000; Samuel et al., 2000). Paradoxically, overexpression as well as suppression of SIPK renders plants hypersensitive to ozone treatment (Samuel and Ellis, 2002). Overlapping but not completely interchangeable actions are known for SIPK and WIPK; WIPK activation usually is accompanied by SIPK activation, but some oxidative stresses preferentially activate SIPK and leave WIPK unaffected (Kumar and Klessig, 2000; Samuel et al., 2000). Plants overexpressing wild-type or constitutively active versions of SIPK, a SIPK-interacting MAPKK (Liu et al., 2000), exhibit enhanced ozone resistance (Miles et al., 2005), whereas *NtMPK4*-silenced plants are hypersensitive to ozone (see above).

### Cold, Salt, Drought, and Wounding

Many MAPKs are activated by osmotic stress, cold, salt, drought, and wounding (for review, see Nakagami et al., 2005). All of these conditions disturb the redox balance of plants. A role of the Arabidopsis MAPK module MEKK1-MKK2-MPK4/MPK6 was reported for cold and salt stress. MKK2 becomes activated by cold and salt stress, and *mkk2*-null mutants are hypersensitive to these stresses (Teige et al., 2004). *MEKK1* is transcriptionally induced by salt stress, drought, cold, and wounding (Mizoguchi et al., 1996) and can interact with MKK1, MKK2, and MPK4 (Ichimura et al., 1998). MEKK1 mediates pathogen-associated molecular pattern-induced activation of MPK3 and MPK6 through MKK4 and MKK5 (Asai et al., 2002) but activates MPK4 and MPK6 in an MKK2-dependent manner during abiotic stress (Teige et al., 2004). Because MKK1 is activated by wounding, cold, drought, and salt stress and can phosphorylate MPK4 (Matsuoka et al., 2002; Teige et al., 2004), MKK1 also seems to be involved in abiotic stress.

### Heavy Metals

ROS production is closely related to the response of plants to heavy metals. Although some heavy metals have physiological roles, higher concentrations are usually toxic and can cause severe cellular damage. Not only can heavy metals block functional groups or displace essential metal ions of enzymes, but they can also generate ROS through the Fenton reaction (for review, see Apel and Hirt, 2004). It is now clear that

heavy metals activate MAPKs in several plant systems (Jonak et al., 2004; Yeh et al., 2004). Depending on heavy metal identity, four alfalfa MAPKs become activated in a complex pattern (Jonak et al., 2004), indicating that besides their ROS-elevating effects, distinct heavy metals employ additional mechanisms to selectively activate particular MAPKs. Comparing these results with those from studies in parsley (*Petroselinum crispum*; Kroj et al., 2003) it appears that selectivity of heavy metals for a particular MAPK might vary between plant species.

## FUTURE PERSPECTIVES AND OPEN QUESTIONS

### Multiple ROS Pathways Lead to MAPK Activation

In Arabidopsis, there seem to exist multiple ways to activate MPK3 and MPK6 in response to ROS. The MAPKKK ANP1 mediates H<sub>2</sub>O<sub>2</sub>-induced activation of MPK3 and MPK6, and stable overexpression of ANP1 yields plants tolerant to heat shock, freezing, and salt stress (Kovtun et al., 2000). H<sub>2</sub>O<sub>2</sub> also increases expression of the Arabidopsis nucleotide diphosphate (NDP) kinase 2 (NDPK2), which, when overexpressed, reduces accumulation of H<sub>2</sub>O<sub>2</sub> and enhances tolerance to multiple stresses including cold, salt, and oxidative stress (Moon et al., 2003). The effect of NDPK2 might be mediated by MPK3 and MPK6, because NDPK2 can interact and activate these two MAPKs (Moon et al., 2003). Another upstream mediator of MPK3 and MPK6 is the OXI1 protein kinase (Rentel et al., 2004). *Oxi1* null mutants are hypersensitive to infection by virulent fungal pathogens and are compromised in ROS- and elicitor-induced MAPK activation. A further candidate to regulate MAPK pathways relies on the ability of ROS to oxidize labile thiol amino acid residues in Tyr phosphatases, as shown for Arabidopsis PTP1, which can suppress MPK6 activity and is inactivated by H<sub>2</sub>O<sub>2</sub> (Gupta and Luan, 2003).

### Are ROS the Missing Link between MAPKs and Stress Signaling?

Could ROS be responsible for mediating the activation of MAPKs by abiotic and biotic stresses? ROS are continuously produced in chloroplasts, peroxisomes, and mitochondria, and their cellular levels are strictly controlled. During pathogen attack, ROS production is beneficial for defense, and the cells increase ROS levels through the activation of ROS-generating oxidases and the simultaneous suppression of ROS scavenging enzymes. The activation of MAPKs by various pathogens might therefore be simply a consequence of higher ROS levels found in infected cells. However, if this was the case, then the ROS that mediate MAPK induction most probably originate from sources other than NADPH oxidases. Treatment of parsley cells with the fungal elicitor Pep13 triggers production of ROS and phytoalexins as well as the induction of three MAPKs and PR gene expression. Pep13-induced PR gene induction

requires MAPK activation. ROS and phytoalexin formation is blocked by diphenylene iodonium, but MAPK activation and PR gene induction remain unaffected (Kroj et al., 2003).

In contrast to the beneficial ROS effects, during abiotic stresses, ROS accumulation mainly seems to be detrimental and due to exhaustion of the cellular ROS scavenging potentials. In agreement with this concept, *OsMAPK5* RNAi rice (*Oryza sativa*) plants are less tolerant to drought, cold, and salt but display constitutive PR gene expression and increased resistance toward fungal and bacterial pathogens (Xiong and Yang, 2003).

Although diverse stresses result in cellular ROS accumulation, the final responses toward these stresses are fundamentally different. At first sight, it seems unlikely that ROS per se can encode the entire information for triggering different responses. However, the identity of a particular ROS as well as its subcellular location, concentration, diffusibility, and specific half life might specify distinct cellular responses. As indicated by the finding that H<sub>2</sub>O<sub>2</sub> can inhibit auxin-induced responses (Kovtun et al., 2000), further specificity can result from cross talk of ROS with other signaling and hormone pathways.

Another important issue relates to the fact that abiotic and biotic stresses have opposite effects on ROS levels and yet seem to employ the same MAPK signaling pathway. This raises the question of how specificity of a certain MAPK-mediated response can be achieved. Scaffolding proteins that support the formation of a particular MAPKKK-MAPKK-MAPK complex are known from yeast (*Saccharomyces cerevisiae*; O'Rourke and Herskowitz, 1998) and animals (Roy et al., 2002). MAPK modules with scaffolding function have also recently been identified in plants. The alfalfa MAPKKK OMTK1, which is activated by H<sub>2</sub>O<sub>2</sub> and initiates cell death, directly interacts with its downstream MAPK target MMK3 (Nakagami et al., 2004). The engagement of different scaffolding motifs might explain the discrepancy of finding more than 60 MAPKKKs but only 10 MAPKKs and 20 MAPKs in plant genomes.

### MAPKs: The Chicken or the Egg in ROS Signaling?

The stress-induced activation of MAPKs could be explained in most studies by the notion that ROS act upstream of MAPK pathways. However, an investigation of *Phytophthora infestans* infection of *Nicotiana benthamiana* showed that the MEK2 pathway might be part of an amplification cascade upstream of the NADPH oxidase genes, which are necessary for producing ROS in response to fungal infection (Yoshioka et al., 2003). Congruent with these studies, expression of constitutively active Arabidopsis MKK4 or MKK5, the orthologs of tobacco MEK2 (and activators of MPK3/6), resulted in generation of H<sub>2</sub>O<sub>2</sub> and cell death (Ren et al., 2002); protein kinase inhibitors blocked elicitor-induced cell death, oxidative burst, and expression of

defense genes in tobacco (Sasabe et al., 2000). Considering that two thioredoxins as well as an ascorbate oxidase-like protein were recently identified as potential substrates for MPK3 and MPK6 (Feilner et al., 2005), other scenarios for regulating ROS levels via MAPKs are conceivable. Manipulation of the ratio of ascorbate to dehydroascorbate or reduced to oxidized thioredoxin has an enormous impact. Overexpression of ascorbate peroxidase can suppress H<sub>2</sub>O<sub>2</sub>-induced cell death (Murgia et al., 2004), and silencing of thylakoidal ascorbate peroxidase enhances methyl viologen-induced photooxidative stress (Tarantino et al., 2005). Ascorbate-deficient mutants exhibit microlesions and constitutive PR gene expression, lack induction of H<sub>2</sub>O<sub>2</sub>-sensitive genes, and display higher tolerance to infection by *P. syringae* when compared to wild type (Pavet et al., 2005). Silencing of the tobacco thioredoxin *CITRX* resulted in enhanced pathogen-induced ROS accumulation, calcium-dependent protein kinase activation, and defense gene expression (Rivas et al., 2004). Moreover, there is a possible implication of the cytosolic thioredoxin *TRXh5* in response to pathogens and to oxidative stresses (Laloi et al., 2004). Overall, it appears that a number of redox-regulatory proteins are critical for establishing pathogen resistance. If these proteins are truly substrates for MAPKs, it is tempting to speculate that the phenotypes of plants with manipulated MAPK functions are due to the altered activities of certain redox-regulatory proteins. It is clear that many aspects on the connection between MAPK and ROS signaling remain elusive at the moment, and future investigations are necessary to unravel the factors and their particular mechanisms of action. We also have to learn more about the set of genes and metabolic processes that are affected by different MAPK pathways and ROS. The large set of available Arabidopsis T-DNA insertion mutants can undoubtedly be of tremendous help for a genetic analysis of these processes. In addition, genome-wide transcript and metabolite profiling techniques should make it possible to uncover the downstream targets of the ROS and MAPK pathways. On the basis of these data, we should be able to put the jigsaw puzzle together and better understand the regulatory functions of ROS and MAPK cascades in plant biology.

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