Disentangling the Complexity of Mitogen-Activated Protein Kinases and Reactive Oxygen Species Signaling

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For about 2 million years, molecular oxygen arising from photosynthetic processes has become pivotal to almost all organisms. Reactive oxygen species (ROS), the partially reduced or activated derivatives of oxygen (hydrogen peroxide [H_2O_2], HO·, $^1O_2$, O_2^-), are the highly reactive by-products of aerobic metabolism. They arise from various chemical reactions and can lead to oxidative damage of cells.

Plants possess a sophisticated ROS network, comprising antioxidative enzymes, antioxidants, and ROS-producing enzymes, which allow them to keep ROS levels under tight control. Moreover, as research of the past few years has shown, plants have developed efficient strategies for targeted production of ROS. For instance, ROS play a role in programmed cell death (PCD), development, and stress response. Mitogen-activated protein kinase (MAPK) cascades are key players in ROS signaling. Several studies have shown that MAPK signaling pathways are not only induced by ROS but can also regulate ROS production. MAPK cascades are signaling modules that minimally consist of a MAPK kinase kinase (MAPKKK/MEKK), a MAPK kinase (MAPKK/MKK), and MAPK. Upon a stimulus-triggered activation of a MAPKKK, the signal is transduced via phosphorylation-mediated activation of a corresponding downstream MAPKK, which in turn phosphorylates and thereby activates a specific MAPK. The Arabidopsis (*Arabidopsis thaliana*) genome contains more than 60 MAPKKKs, 20 MAPKs, and 10 MAPKs, which can, depending on the environmental stimulus or developmental stage, engage in different MAPK modules. With the characterization of mutants affected in pathogen response as well as the development and dynamics of stomata, the network of MAPK cascade activation and ROS is being disentangled. Here, we discuss the most recent insights into ROS production and perception involving MAPK-mediated signaling.

STOMATA

Regulation of Stomatal Spacing

Stomata are the major place of gas exchange. Because they represent the entry gate for any gaseous compound that can be potentially converted into or trigger the generation of ROS, their density and dynamics need to be tightly controlled. In Arabidopsis, stomatal distribution follows the one-cell spacing rule: adjacent stomata are separated from each other by at least one pavement cell (Nadeau and Sack, 2002). Stomatal patterning results largely from the position-dependent orientation of asymmetric divisions occurring next to a preexisting stoma or precursor (Geisler et al., 2000). The MAPKKK YODA has been associated with the regulation of stomata density. While loss of YODA leads to excess stomata formation and severe dwarfism, stomata formation is blocked by expression of a constitutively active YODA variant (Bergmann et al., 2004). Recent experimental evidence has led to the proposition of a complete MAPK cascade, consisting of YODA, MKK4/MKK5, and MPK3/MPK6, as a regulator of stomatal development (Wang et al., 2007). Transgenic lines silenced for MKK4/MKK5 or their targets MPK3/MPK6 have clustered stomata similar to those found in *yoda* mutants. This phenotype was found to be caused by the disruption of the coordinated cell fate specification of stomata versus pavement cells. Only plants that had been silenced for both MKKs or both MPKs displayed these abnormalities, indicating that MPK3 and MPK6, as well as MKK4 and MKK5, are functionally overlapping negative regulators of the stomatal development pathway. In compliance with this concept, overexpression of constitutively active variants of MKK4, MKK5, or the functionally interchangeable NtMEK2 from tobacco (*Nicotiana tabacum*; Ren et al., 2002) was found to suppress stomatal development in Arabidopsis wild-type plants and to rescue the stomatal cluster phenotype and dwarfism in *yoda* mutants (Wang et al., 2007).

In line with the perception of MPK6 as a negative regulator of stomatal development (Wang et al., 2007), *MPK6* native promoter-driven expression of a dominant-negative version of MPK6 (*MPK6*::*MPK6AEF*), which is unable to be phosphorylated by any of its activating MAPKKs, results in the formation of excessive stomata in transgenic plants (Bush and Krysan, 2007). However, stomatal development is only affected

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in plants lacking both MPK3 and MPK6 (Bush and Krysan, 2007; Wang et al., 2007). An explanation for the stomatal cluster formation in *MPK6*::*MPK6AEF* but not in single *mpk6* knockout plants (although both are wild type for *MPK3*) might be the ability of the dominant-negative MPK6 version to prevent other related MAPK isoforms from occupying the void created by the absence of wild-type MPK6 protein (Bush and Krysan, 2007).

The proposition of YODA as a specific upstream activator of MPK3 and MPK6 in regulating stomatal development is substantiated by the observation that MPK3 and MPK6 kinase activities are elevated in plants expressing a constitutively active YODA variant, whereas plants expressing gain- or loss-of function variants of the MAPKKKs ANP1 and MEKK1, known as stress-dependent activators of MPK3 and MPK6 (Kovtun et al., 2000; Asai et al., 2002), display no stomatal development defects (Wang et al., 2007).

Regulation of Stomatal Aperture

The well-coordinated opening and closure of stomata makes plants capable of withstanding harsh environmental conditions. For instance, stomatal closure limits water loss under drought stress. Likewise, closed stomata restrict the entry of harmful gases such as ozone. Stomatal opening and closure is primarily mediated by the phytohormone abscisic acid (ABA), and mutants affected in the synthesis or signaling of ABA display altered sensitivity to drought or ozone (Assmann, 2003; Verslues and Bray, 2006). ABA-triggered inhibition of stomatal opening is a distinct process from ABA-induced stomatal closure (Allen et al., 1999; Wang et al., 2001; Mishra et al., 2006). Both processes involve ROS as signaling intermediates and are regulated by the transport of osmotically active ions and metabolites across guard cell membranes (MacRobbie, 1998; Pei et al., 2000; Hetherington and Woodward, 2003; Yan et al., 2007).

There is increasing evidence for MAPK cascades to be involved in the regulation of stomatal aperture. Studies in pea (*Pisum sativum*) have revealed a role of MAPKs in ABA signaling in guard cells (Burnett et al., 2000). PD98059, which blocks activation of MAPKs, partially inhibited ABA-induced stomatal closure (Burnett et al., 2000) and also prevented ABA-induced H_2O_2 production in guard cells of *Vicia faba* (Pei et al., 2000). A physiological study showing that PD98059-triggered inhibition of MAPK activation prevents ABA-induced stomatal closure by reducing the ABA-triggered release of vacuolar ions implicates activation of MAPK(s) in the signaling chain linking ABA to tonoplast ion fluxes (MacRobbie and Kurup, 2007).

Substantial efforts have been undertaken to ascribe a role to individual MAPKKKs, MAPKKs, and MAPKs in stomatal regulation. MPK3, MPK4, and MPK6 are activated by ABA and H_2O_2 (for review, see Nakagami et al., 2005; Pitzschke and Hirt, 2006). In an elegant approach, the role of MPK3 in stomatal regulation has been investigated through guard cell-specific antisense inhibition of *MPK3* expression (Gudesblat et al., 2007). *MPK3*-silenced plants are impaired in ABA-induced inhibition of stomatal opening. However, silencing of *MPK3* affected neither ABA-induced H_2O_2 production nor stomatal closure. Yet, promotion of stomatal closure by exogenous application of H_2O_2 was abolished in these plants. This led to the proposition that MPK3 might be involved in the signaling of H_2O_2-induced stomatal closure by acting on ABA signaling of guard cells downstream of H_2O_2 (Gudesblat et al., 2007). However, provided that exogenously applied H_2O_2 is qualitatively comparable to H_2O_2 accumulating in response to ABA, MPK3 deficiency would be expected to also affect ABA-induced stomatal closure. A possible reason for the difference observed between ABA and H_2O_2 responsiveness with respect to stomatal closure might be that residual levels of MPK3 are sufficient for ABA-induced, but not for H_2O_2-induced, stomatal closure. Also, MPK3 originating from nonguard cells might, via signaling through additional components, contribute to the ABA-induced, but not to the H_2O_2-induced, stomatal closure. The inability of *mpk3* mutants to close stomata upon H_2O_2 exposure indicates that MPK3 cannot be fully compensated for by another MAPK or signaling component. This specificity is in contrast to the functional overlap of MPK3 and MPK6 in stomatal density regulation (see above) and in biotic stress signaling (Asai et al., 2002; for review, see Nakagami et al., 2005).

Very recent findings have implicated the MAPK module MKK1-MPK6 in ABA-dependent stomatal closure (Xing et al., 2008). Under normal conditions, neither MKK1-deficient nor MKK1-overexpressing plants have an apparent phenotype. However, MKK1-overexpressing plants display enhanced ABA sensitivity and drought tolerance, whereas the opposite holds true for *mkk1* mutants. These ABA-dependent phenotypes involve a misregulation of ROS balance: ABA-induced H_2O_2 production is elevated in *MKK*1-overexpressing plants but blocked in *mkk1* mutants. Similar abnormalities in ABA-induced H_2O_2 production have been observed in *MPK6*-overexpressing and *mpk6* mutants. These observations suggest a role of the MKK1-MPK6 module in ABA signaling. In addition, there is evidence for an alternative MKK1-MAPK module in ABA response.

mkk1 mutants were found to be compromised in ABA-induced activation of not only MPK6 but also MPK4. However, only *mkk1* and *mpk6*, but not *mpk4* mutants, are affected in ABA-induced H_2O_2 production (Xing et al., 2008). These observations suggest that ABA-activated MKK1 feeds into at least two distinct MAPK signaling pathways: one involving MPK6, the other involving MPK4. ABA-induced H_2O_2 production is apparently regulated specifically by the MKK1-MPK6 module. With respect to drought tolerance, which is achieved through the prevention of water loss via ABA-triggered H_2O_2 production and subsequent stomatal closure, *MKK1*- and *MPK6*-overexpressing

plants seem to benefit from enhanced H_2O_2 synthesis. A more efficient H_2O_2 production might render these plants more capable of closing their stomata. How can H_2O_2 be employed as a mediator of stomatal closure and yet be prevented from accumulating to toxic amounts? Evidence exists for an involvement of ROS-scavenging mechanisms. The expression of *CAT1*, encoding a major ROS-scavenging enzyme ($H_2O_2 \rightarrow H_2O + O_2$), is induced by ABA, and its induction can be blocked by PD98059, a specific inhibitor of MAPK activation. ABA-induced *CAT1* expression was found to be greatly diminished in *mkk1* and *mpk6* mutants and enhanced in *MKK1*- and *MPK6*-overexpressing plants (Xing et al., 2008). The activation of catalase, therefore, might function as negative feedback regulation of H_2O_2 signaling (Xing et al., 2008).

NITRIC OXIDE AND MAPKS IN STOMATAL REGULATION AND STRESS SIGNALING

A number of recent studies have provided evidence for a role of nitric oxide (NO) as a signaling molecule in various developmental and physiological processes (for review, see Neill et al., 2008). In Arabidopsis, at least three genes encode proteins involved in NO biosynthesis: NOA1 (for NO-ASSOCIATED1) and two nitrate reductases, NIA1 and NIA2. The biochemistry of these enzymes is poorly understood.

NO production is triggered by several abiotic stresses, including drought and osmotic stress. Recent data emphasize a role of NO in the ABA-induced stomatal closure. It had been shown earlier that exogenous application of NO or NO donors triggers stomatal closure in various plant species (Desikan et al., 2002; Neill et al., 2002). ABA-induced stomatal closure was later found to be dependent on NO production: removal of NO or the (pharmacological) prevention of NO generation, as well as the loss of nitrate reductase in the *nia1nia2* double mutant, inhibits ABA-induced stomatal closure (Desikan et al., 2002; Bright et al., 2006). The evidence for NO as a signaling molecule stems from its ability to induce MAPK and other kinase activities as well as alterations in Ca^{2+} and cGMP levels.

Based on the observations that exogenous H_2O_2 induces NO generation (Lum et al., 2002), that ABA-induced NO synthesis is dependent on prior H_2O_2 generation, and that removal of H_2O_2 or inhibition of its synthesis prevents NO generation and stomatal closure (Dong et al., 2005; Bright et al., 2006), a cascade consisting of ABA-H_2O_2-NO has been proposed (Neill et al., 2008). Moreover, since NO stimulates MAPK activities, as do ABA and H_2O_2 (Desikan et al., 2004; Zhang et al., 2007a), MAPK activation has been suggested as a convergence point for guard cell H_2O_2 and NO signaling during ABA-induced stomatal closure (Desikan et al., 2004).

In maize (*Zea mays*) mesophyll cells, the induction of antioxidant defenses provoked by drought or ABA was found to be dependent on H_2O_2 and NO generation and MAPK activation (Zhang et al., 2007a). Inhibition of NO synthesis or pretreatment with NO scavengers substantially reduced the ABA-induced NO production and activation of a MAPK (Zhang et al., 2007a). Bursts of NO can also be triggered by application of Ca^{2+}, and results from pharmacological and biochemical studies suggest that Ca^{2+} and calmodulin function both upstream and downstream of NO production in ABA- and H_2O_2-induced antioxidant defense in maize (Sang et al., 2008).

As has been substantiated in a recent study, there is also a strong interconnection between NO and MAPKs in the biotic stress response. In *Nicotiana benthamiana*, at least two distinct MAPK cascades participate in the regulation of the oxidative burst triggered by the oomycete elicitor INF1 (Asai et al., 2008). This oxidative burst involves the synthesis of NO by NOA1 and ROS production by the NADPH oxidase RBOHB. Gain-of-function and loss-of-function analyses have shown that the MEK2-SIPK/NTF4 (closest homologs in Arabidopsis: MKK4/5-MPK3/6) cascade specifically controls the NOA1-mediated NO burst, while the MEK1-NTF6 (Arabidopsis homologs: MKK6-MPK4) cascade regulates the NADPH oxidase-dependent ROS accumulation. Congruent with these observations, INF1- and SIPK-mediated NO bursts are compromised in *noa1* mutants. It remains to be seen whether such specificity of individual MAPK cascades for ROS bursts arising from NOA or NADPH oxidase activities also exist in Arabidopsis.

MPK3 AND MPK6 IN OZONE RESPONSE

Ozone is a potent ROS generator and a phytotoxic air pollutant, which triggers a rapid and transient activation of MAPKs in various plant species. Stomatal closure triggered by ozone is transient; in spite of continuing ozone exposure, guard cells regain their aperture within 40 min (Kollist et al., 2007). A ROS signaling cascade including (de)phosphorylation events is likely to be involved in ozone-induced stomatal closure. *abi2* mutants, which are defective in a protein phosphatase 2C, fail to close their stomata in response to ozone (Kollist et al., 2007). Similarly, tobacco plants silenced for *NtMPK4*, which is expressed in the epidermis, are compromised in stomatal closure and display ozone hypersensitivity (Gomi et al., 2005).

Clear evidence exists that Arabidopsis MPK3 and MPK6 are involved not only in the regulation of stomatal development and ABA-mediated stomatal closure (see above) but also in the response to ozone. Loss of MPK3 or MPK6 renders plants hypersensitive to ozone (Miles et al., 2005). Interestingly, constitutive activation of MPK6 by ectopic expression of *NtMEK2* in Arabidopsis also was found to confer ozone hypersensitivity, as did overexpression and silencing of the putative *MPK6* ortholog in tobacco, *SIPK* (Samuel and

Ellis, 2002). Apparently, MPK6/SIPK activity needs to be tightly controlled in order to withstand oxidative challenges. The need for a well-balanced ratio of MPK3 and MPK6 also seems plausible. That the perturbation of the MPK3-MPK6 equilibrium is detrimental to plants challenged with ozone has been substantiated in a recent study of the MAPK phosphatase 2 (MKP2) using an inducible RNA interference strategy (Lee and Ellis, 2007). Of the five MKPs present in Arabidopsis, only silencing of *MKP2* conferred an ozone-hypersensitive phenotype. MKP2 was found to inactivate MPK3 and MPK6, but not MPK12, another related MAPK tested, in vitro. Results from in vivo experiments are supportive of a specific regulation of MPK3 and MPK6 by MKP2 in the context of ozone response. In wild-type plants, ozone-triggered activation of MPK3 and MPK6 is transient (0.5–2 h), but it is sustained in *MKP2*-silenced plants. The studies by Lee and Ellis (2007) are thus in compliance with those by Miles et al. (2005) showing that constitutive activation of MPK6 confers ozone hypersensitivity. Because MKP2 inactivates both MPK6 and MPK3, it still needs to be investigated whether plants with enhanced MPK3 activity, but normal MPK6 activity, are also ozone hypersensitive.

Another level of complexity in the regulation of MPK3 and MPK6 became apparent from the observation that MKP2 activity is stimulated specifically by association with MPK3 or MPK6; this effect was found to be independent of MAPK activity (Lee and Ellis, 2007).

MKP2 is likely to be a regulator of MPK3 and MPK6 not only in the signaling of ozone but also of other stresses: *MKP2*-silenced plants are hypersensitive to treatment with harpin, a bacterial elicitor that triggers ROS production and induces MPK3 and MPK6 activation, resulting in a hypersensitive response-like cell death (Desikan et al., 2001). The pleiotropic importance of MKP2 is further substantiated by the finding that *MKP2*-silenced seedlings are severely hampered in their development (Lee and Ellis, 2007). Whether regulation of early development by MKP2 involves ROS signaling needs to be clarified.

SEARCH FOR MAPK TARGETS IN ROS SIGNALING AND STOMATAL DYNAMICS

The apparent key role that MAPK signaling plays in ROS-mediated stomatal dynamics poses questions about the downstream targets of the involved MAPKs. Candidate substrates would be proteins that function downstream of diverse ROS in mediating stomatal closure. The ozone-hypersensitive phenotype and lack of H_2O_2-induced stomatal closure observed in plants silenced for *MPK3* in guard cells (Gudesblat et al., 2007) suggest that a guard cell-specific substrate might be targeted by this MAPK. *SLAC1*, the ion channel preferentially expressed in guard cells (Vahisalu et al., 2008), might be a good candidate. Analysis of *slac1* mutants, previously referred to as *rcd3* (for *radical-induced cell death3*), revealed that SLAC1 is essential for stomatal closure in response to CO_2, ABA, ozone, light/dark transitions, humidity change, calcium ions, H_2O_2, and NO (Vahisalu et al., 2008). Ozone hypersensitivity in *slac1/rcd3* mutants, but not in other *rcd* mutants, is dependent on misregulation of stomata (Overmyer et al., 2000). The amino acid residue affected in the *slac1-1* mutant is a Ser and thus a potential phosphorylation site. However, since the adjacent amino acid is not a Pro (the MAPK substrate minimal consensus is Ser-Pro or Thr-Pro), SLAC1 is unlikely to be regulated by MPK3 or other MAPKs, at least at this particular residue. Whether SLAC1 can yet serve as a MAPK substrate (there are three sites matching the minimal MAPK target consensus) would have to be investigated. An indirect regulation of SLAC1 via a MAPK-controlled SLAC1-phosphorylating kinase also seems possible. The identification of MAPK-targeted proteins that play a role in stomatal regulation will help to dissect signaling pathways and thus increase our understanding of how changes in the redox status are translated into stomatal movement. Proteins encoded by genes identified from microarray analysis of guard cells (Leonhardt et al., 2004) represent a promising pool of potential MAPK substrates.

ASCORBIC ACID

Ascorbic acid (AA) is the most abundant antioxidant in plants. Ascorbate-deficient mutants exhibit microlesions and constitutive expression of pathogenesis-related (PR) genes. These mutants are compromised in the induction of H_2O_2-sensitive genes and display a higher tolerance to infection by the pathogen *Pseudomonas syringae* (Pavet et al., 2005). Similar phenotypes have been reported for the MAPK pathway mutants *mpk4* and *mekk1* (Petersen et al., 2000; Nakagami et al., 2006). An increasing body of evidence exists for an interconnection between AA and MAPK signaling, where feedback regulatory mechanisms are likely to be involved. For instance, microarray analysis has revealed a number of genes encoding enzymes involved in ascorbate biosynthesis and metabolism to be differentially expressed in some *mapk* pathway mutants (Pitzschke et al., 2009), suggesting that ascorbate levels might be controlled through MAPK modules. On the other hand, perturbations of the AA pool were found to affect MAPK activity (Pignocchi et al., 2006): the apoplastic redox state is mainly regulated by the cell wall-localized enzyme ascorbate oxidase (AO), which converts AA to dehydroascorbic acid (DHA). Overexpression of AO in tobacco resulted in highly elevated levels of apoplastic AA, while whole leaf AA content, the activity of antioxidative enzymes, and the ratio of reduced to oxidized glutathione remained largely unaffected (Pignocchi et al., 2003, 2006). These plants displayed enhanced protein kinase activity on myelin basic protein, an artificial substrate that is also phosphorylated by MAPKs. Moreover, *AO*-overexpressing

plants were found to be more susceptible to *P. syringae*, implying that the apoplastic redox state modulates defense responses by regulating signal transduction (Pignocchi et al., 2006). Whether the enhanced overall MAPK activity in *AO*-overexpressing plants is linked to the enhanced susceptibility remains elusive. It will be interesting to assess the activities of individual MAPKs in *AO*-overexpressing plants and to investigate whether MAPKs can be hyperactivated by stress.

The apoplastic AA pool can be modified not only by overexpression of AO but also by repression of dehydroascorbate reductase (DHAR), a key component of the ascorbate recycling system. Plants deficient for cytosolic DHAR have highly reduced levels of apoplastic ascorbate. The expression of cytosolic DHAR (cytDHAR), but not of the other two DHAR genes present in Arabidopsis, is induced by ozone, and *cytDHAR* mutants are hypersensitive to ozone, pointing to a key role of apoplastic ascorbate in ozone tolerance (Yoshida et al., 2006). In the study by Pignocchi et al. (2006), the effect of AO overexpression on ozone tolerance was not investigated. Likewise, MAPK activities have not been assessed in *cytDHAR* mutants (Yoshida et al., 2006). It is tempting to speculate that elevated MAPK activities, resulting from enhanced levels of apoplastic ascorbate, due to AO overexpression or cytDHAR deficiency, account for the altered ozone and pathogen tolerance in AO overexpressors and *cytDHAR* mutants, respectively. Interestingly, AO overexpression also affects stomatal dynamics: in contrast to wild-type plants, in which application of DHA or H_2O_2 rapidly induced stomatal closure, *AO* overexpressors did not respond to these treatments, which led to the suggestion that cell wall-localized AA plays a specific role in the perception of oxidative stress and that DHA acts as a regulator of stomatal dynamics (Fotopoulos et al., 2008). Together with the studies of Pignocchi et al. (2006) and Yoshida et al. (2006), it appears plausible that enhanced sensitivity to ozone and *Pseudomonas* might be, at least partly, due to the inability of apoplastic AA-accumulating plants to initiate ROS-induced closure of stomata, the entry gate for pathogens and gases. Judging from the key role that MAPK cascade components play in stomatal dynamics (see above) and the altered general MAPK activity found in AO-overexpressing plants (Pignocchi et al., 2006), it appears very likely that the hypersensitive phenotypes are related to perturbations in MAPK signaling. It will be the challenge of the future to dissect which of the players [MAPK(KK)s, AA, ROS] is acting upstream and/or downstream of the others.

A MAPK CASCADE ACTIVELY BLOCKS CARBON FIXATION

The well-controlled aperture and closure of stomata is essential for the maintenance of a proper balance of photosynthetic input (CO_2) and output (O_2). If CO_2 is not removed by fixation, excess excitation energy initiates a harmful process. As recently shown by Liu et al. (2007), activation of three tobacco MAPKs, SIPK/Ntf4/WIPK, by dexamethasone-induced expression of a constitutively active variant of their upstream MAPKK, *NtMEK2DD*, rapidly inhibits photosynthetic CO_2 fixation. At 2 h after induction of *MEK2DD* expression, leaves displayed strong accumulation of H_2O_2, mostly in the chloroplasts. More detailed studies revealed that MEK2DD-induced shutdown of carbon fixation triggers chloroplastic superoxide production, which is rapidly converted to H_2O_2 by superoxide dismutase. Prolonged activation of the MAPK cascade (10 h) leads to chloroplast damage in a light-dependent manner, resulting in a hypersensitive response-like cell death (Zhang and Klessig, 1998). When plants are challenged with tobacco mosaic virus, which activates SIPK and WIPK, ROS generation is greatly enhanced upon light. Examination of the kinetics of carbon fixation inhibition and the onset of cell death upon *MKK2DD* expression indicates that the MAPK cascade actively blocks carbon fixation, leading to excess excitation energy in illuminated plants and consequently to an accumulation of ROS. The sustained generation of ROS cannot be compensated for by the action of antioxidant enzymes, and their depletion leads to cell death (Liu et al., 2007).

NtMEK2 and its closest homologs in Arabidopsis, AtMKK4 and AtMKK5, have been implicated in biotic stress signaling (Ren et al., 2002). The shutdown of CO_2 fixation might contribute to the enhanced pathogen tolerance found in plants with hyperactive MAPKKs: by blocking the energy-consuming carbon fixation, forces can be subtracted and employed for a more efficient defense against the invader. As a consequence of MAPKK-mediated inhibition of CO_2 fixation, ROS accumulation can contribute to the establishment of the oxidative burst, which is an essential step of the defense response.

ROS IN BIOTIC STRESS RESPONSE

Similar to apoptosis in animal cells, a number of stress stimuli trigger the cell death pathway. PCD in plants as a response to pathogen attack is characterized by the generation of ROS, activation of specific proteases, and fragmentation of DNA, eventually leading to a hypersensitive response. ROS can trigger the deposition of lignin and callose, which serve to reinforce cell walls surrounding infection sites (Pontier et al., 1998). Thus, the hypersensitive response helps to restrict the attacked zone, thereby preventing pathogen spread. Ample evidence exists for MAPK components to be involved in plant pathogen response (for review, see Nakagami et al., 2006). The following sections will discuss the most recent highlights.

MKK3

MKK3 has been implicated in biotic stress signaling involving H_2O_2 (Doczi et al., 2007). In *mkk3* knockout plants, the growth of the pathogen *P. syringae* is increased, whereas it is decreased in *MKK3*-overexpressing plants. In line with this observation, expression of *PR1* is induced by MKK3, which itself is transcriptionally activated upon contact with *P. syringae*. By yeast two-hybrid analysis, coimmunoprecipitation, and protein kinase assays, MKK3 was shown to be an upstream activator of the MAPKs MPK1, MPK2, MPK7, and MPK14, all of which belong to the same subgroup of Arabidopsis MAPKs (Ichimura et al., 2002). In protoplast cotransfection studies, MPK6 and MPK7 are both activated by H_2O_2, but only MPK7 activation is further enhanced by MKK3. MPK6, but not MPK7, showed activation by the bacterial elicitor flagellin. MKK3 was proposed as an upstream regulator of MPK7 in the H_2O_2 signaling pathway that acts independently of the flagellin signaling pathway (Doczi et al., 2007). Depending on the environmental context, it appears that MKK3 can target either MPK6 or MPK7. Steroid-induced expression of constitutively active MKK3 or MKK4 in transgenic plants results in the accumulation of H_2O_2 and also of the stress- and senescence-associated plant hormone ethylene (Takahashi et al., 2007). However, the transcriptome profiles of these plants do not significantly overlap, suggesting that MKK3 and MKK4 have different roles in regulating gene expression. Based on the finding that MKK3 phosphorylates MPK6 in vitro and that *mkk3* and *mpk6* knockout mutants are hypersensitive to treatment with the wound-associated plant hormone jasmonic acid, MKK3 has been ascribed a role in the jasmonic acid-triggered activation of MPK6 (Takahashi et al., 2007). Similar to the situation in yeast and mammals (O'Rourke and Herskowitz, 1998; Yoshioka, 2004; Bhattacharyya et al., 2006), the preferential assembly of MKK3 with MPK6 or MPK7 might be assisted by scaffolding proteins that themselves underlie stimulus-dependent activation.

BIOTIC STRESS RESPONSE AND SALICYLIC ACID

A wide range of environmental stimuli, including bacterial and fungal elicitors as well as diverse abiotic stresses, can initiate MAPK cascades. They can be perceived by (mostly unknown) receptors that then transduce the signal to the MAPK cascade. However, secondary defense signals that are produced by the challenged plant also can be involved. Examples are the plant-derived peptide systemin, which is formed upon wounding, and the plant hormone salicylic acid (SA), which is synthesized in a stress-dependent manner and essential for many biotic stress responses. As previous studies have shown, stress, SA, ROS, and MAPK cascades are strongly interconnected.

MEKK1 has been implicated in mediating flagellin (flg22) signaling (Asai et al., 2002). *mekk1* homozygous knockout plants show a severe dwarfism (Nakagami et al., 2006). Their leaves accumulate high amounts of ROS, and this goes along with lesions reminiscent of PCD. A number of genes encoding redox-regulatory enzymes are misregulated in *mekk1* knockout plants. Apparently, *MEKK1* deficiency disturbs the redox balance. When developing true leaves, homozygous *mekk1* mutants display a lethal phenotype. Similarly, expression of redox-regulatory genes and the age-dependent lethal phenotype were found in homozygous *mpk4* mutants but not in *mpk3* or *mpk6* mutant plants. Moreover, MEKK1 is required for H_2O_2-induced activation of MPK4. Together with similar studies by Ichimura et al. (2006), these observations point to a role of MEKK1 and its downstream target MPK4 in the maintenance of ROS homeostasis. Previously, *mpk4* mutants had been shown to constitutively express SA-dependent stress genes, and this was found to be due to elevated levels of SA. The dwarfed phenotype of these plants could be largely rescued by the expression of the bacterial *NahG* gene (Petersen et al., 2000), which encodes an SA-degrading enzyme. The dwarfism of homozygous *mekk1* plants is likewise attenuated by *NahG* expression (Suarez-Rodriguez et al., 2007). Interestingly, expression of a kinase-impaired version of *MEKK1(K361M)* also largely rescued the dwarfism, *PR* gene expression, and callose-deposition phenotype of *mekk1* mutant plants (Suarez-Rodriguez et al., 2007). Ectopic expression of *MEKK1(K361M)* also rescued the *mekk1* phenotype with respect to flg22-induced activation of MPK4. Although the authors cannot exclude that a residual level of kinase activity may reside in the MEKK1(K361M) protein, it appears that the kinase activity of MEKK1 may be dispensable for many of its in planta functions (Suarez-Rodriguez et al., 2007). The rescue of the dwarfed and spontaneous-lesion phenotype of *mekk1* and *mpk4* by ectopic expression of *NahG* strongly suggests that the mutant phenotypes are predominantly due to elevated SA levels. This is different from the situation in the activation-tagged *bud1* mutant, which overexpresses the MAPKKK *MKK7*. *bud1* mutants have elevated SA levels and exhibit constitutive *PR* gene expression but no spontaneous lesions (Zhang et al., 2007b), strongly suggesting that the ROS generation can be uncoupled from SA signaling. Both *PR* gene expression and disease resistance of *bud1* plants were found to be due to constitutive *MKK7* expression, to be SA dependent, and to require the kinase activity of the MKK7 protein. Based on the observations that ectopic expression of *MKK7* in local tissues induces *PR* gene expression and pathogen resistance in systemic tissues and silencing of *MKK7*, Zhang et al. (2007b) propose that activation of MKK7 is sufficient for generating the mobile signal of systemic acquired resistance.

STRESS, ROS, AND MAPK GENE EXPRESSION

With an ever increasing set of microarray data becoming publicly available, a complex pattern of

MAPK regulation emerges. Not only are MAPK cascade components posttranslationally activated in the process of signal transduction from receptor to MAPK-targeted effector, finally leading to adaptation by appropriate alteration of the expression of certain genes, but several MAPK(KK)-encoding genes are subject to transcriptional regulation themselves. For instance, in a search for genes whose expression is rapidly induced upon wounding (5 min), *MKK9*, *MPK3*, as well as *AP2C1*, encoding a protein phosphatase that is a key regulator of MAPK signaling (Schweighofer et al., 2007), were found (Walley et al., 2007). Elevated transcript levels of diverse MAPK(KK) genes were also detected in microarray analyses of plants after short (several minutes) and prolonged (up to 48 h) exposure to various biotic and abiotic stresses (Kreps et al., 2002; Navarro et al., 2004; De Vos et al., 2005; Anand et al., 2007; Gust et al., 2007). These microarrays give cumulative evidence that transcriptional control of MAPK signaling components is involved in stress signaling. Some MAPK(KK) genes respond to very different types of stress (e.g. *MKK9* is induced both by wounding and by bacterial elicitors; Navarro et al., 2004; Walley et al., 2007). Whether the same or different MAPK gene promoter elements are employed in these stress responses remains elusive. Not unlikely, the common basis for MAPK(KK) gene induction, as well as for MAPK(KK) activation, by diverse types of stress might be ROS imbalances.

The fast kinetics of MPK3 activation upon a series of challenging conditions (Djamei et al., 2007; for review, see Nakagami et al., 2005) suggests that initially, already existing MPK3 protein is being used for signal transduction. The early transcriptional up-regulation of *MPK3* by stress (within 5 min; Walley et al., 2007) might be indicative of the need for a continuous supply of MPK3 enzyme, which would feed into the cascade and thereby contribute to amplifying the stress signal. Interestingly, in contrast to *MPK3*, its closest homolog *MPK6* underlies neither transcriptional nor translational control (Ulm et al., 2002). Why enhanced levels of some *MAPK(K)* transcripts persist long after MPK(K) activity has declined is a puzzling question.

An even more complex scenario of MAPK-regulated gene expression became apparent from the isolation of the Arabidopsis MAPKKK MEKK1 from a screen for proteins binding to the promoter of the *WRKY53* gene.

WRKY53 is a member of the plant-specific transcription factor family of WRKYs, which in Arabidopsis comprises 74 members, many of which are transcriptionally inducible by pathogen infection and other defense-related stimuli (Dong et al., 2003; Kalde et al., 2003). *WRKY53* expression is induced by H_2O_2 (Miao et al., 2004). Transient expression studies with *WRKY53* promoter deletion constructs suggest MEKK1 to be involved in the H_2O_2 response of *WRKY53* (Miao et al., 2007), an assumption that would be in accordance with the rapid induction of MEKK1 activity upon H_2O_2 (Nakagami et al., 2006).

MEKK1 not only interacts with the *WRKY53* promoter but also binds to and phosphorylates the *WRKY53* gene product (Miao et al., 2007). Previous studies have shown that WRKY53 induces the expression of stress- and defense-related genes as well as senescence-associated genes (Miao et al., 2004). Because binding to and phosphorylation of WRKY53 was found to enhance the DNA-binding capacity of WRKY53 (Miao et al., 2007), MEKK1 might be the direct regulator of WRKY53-induced stress-, defense-, and/or senescence-induced gene expression. The apparent shortcut in MAPK signaling by direct phosphorylation of a transcription factor is so far unique to MEKK1. *WRKY53* expression is also induced by the fungal cell wall-derived elicitor chitin (for review, see Montesano et al., 2003), known to activate MPK3 and MPK6 (Nühse et al., 2000; Gust et al., 2007), as well as by ectopic expression of the tobacco MAPKK NtMEK2 active mutant $NtMEK2^{DD}$ (Wan et al., 2004). Hence, *WRKY53* expression is apparently controlled not only by the MEKK1 shortcut but most likely also through a "classical" MAPK signaling pathway.

DEVELOPMENT

Numerous studies have given clear evidence for an involvement of MAPKs in signaling to PCD. PCD not only plays a role in stress response but also participates in developmental processes. One recent example is the regulation of the self-incompatibility response of poppy (*Papaver somniferum*; Li et al., 2007). Self-incompatibility, the rejection of incompatible ("self") pollen, is a mechanism that prevents inbreeding in higher plants. Upon contact of self pollen with the stigma, a Ca^{2+}-dependent signal is initiated that finally leads to PCD. P56, a poppy MAPK cross-reacting with Arabidopsis MPK3 but not MPK4 antibody, is involved in the initiation of self-incompatibility-induced PCD in incompatible pollen. The incompatibility response involving stimulation of caspase-like activity, followed by DNA fragmentation and finally PCD, is blocked by the MAPK inhibitor U0126 (Li et al., 2007). ROS production has been shown to trigger caspase activation as well as oxidative DNA damage, resulting in DNA fragmentation (for review, see Hoeberichts and Woltering, 2003). The role of P56, therefore, might involve the initiation of an oxidative burst whose consequences are DNA fragmentation and PCD. As Arabidopsis is a self-fertile plant, this particular role of poppy P56 in signaling the incompatibility response is not functionally paralleled by any of the 10 Arabidopsis MAPKs. Also in Arabidopsis, a MAPK has been implicated in flower development and/or fertility control. Homozygous *mpk6* knockout plants displayed reduced male fertility and abnormal anther development, and *MPK6* promoter-driven expression of a dominant-negative MPK6 variant led to abnormal sepal development (Bush and Krysan, 2007).

CONCLUSION

Over the last couple of years, considerable effort has been made to disentangle the networks regulating basal ROS levels and targeted ROS synthesis in plants. Several MAPK components were found to be involved in ROS signaling and homeostasis. MAPK pathways and ROS signaling play a key role in controlling normal development and dynamic processes such as flower development, stomatal patterning, and stomatal aperture. Particularly MPK3 and MPK6 appear to have pleiotropic functions in a number of ROS-controlled processes. Not only are they key regulators of the pathogen response, they also control stomatal development, stomatal dynamics, and ozone stress tolerance. Strikingly, in some, but not all, respects, these two highly homologous proteins functionally overlap.

Results from microarray analyses and biochemical studies have made us abandon the simplistic idea of linear signaling from MAPKKK to MAPKK to MAPK. A much more complex image arises: MAPK(KK) activities are regulated by a sophisticated network involving transcriptional, translational, and posttranslational control. Moreover, positive and negative feedback loops contribute to MAPK-mediated ROS signaling while keeping ROS levels at nontoxic concentrations. Very recent studies have discovered additional features of stress response or developmental regulation of and through MAPK(KK)s. An example is the complex mechanism by which MEKK1 directly regulates H_2O_2-induced *WRKY53* expression and WRKY53 activity. And surely, more surprises of MAPK-controlled ROS production and ROS-controlled MAPK activity will be disclosed in the future.

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