Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants

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Despite the recognition of H2O2 as a central signaling molecule in stress and wounding responses, pathogen defense, and regulation of cell cycle and cell death, little is known about how the H2O2 signal is perceived and transduced in plant cells. We report here that H2O2 is a potent activator of mitogen-activated protein kinases (MAPKs) in Arabidopsis leaf cells. Using epitope tagging and a protoplast transient expression assay, we show that H2O2 can activate a specific Arabidopsis mitogen-activated protein kinase kinase kinase, ANP1, which initiates a phosphorylation cascade involving two stress MAPKs, AtMPK3 and AtMPK6. Constitutively active ANP1 mimics the H2O2 effect and initiates the MAPK cascade that induces specific stress-responsive genes, but it blocks the action of auxin, a plant mitogen and growth hormone. The latter observation provides a molecular link between oxidative stress and auxin signal transduction. Finally, we show that transgenic tobacco plants that express a constitutively active tobacco ANP1 orthologue, NPK1, display enhanced tolerance to multiple environmental stress conditions without activating previously described drought, cold, and abscisic acid signaling pathways. Thus, manipulation of key regulators of an oxidative stress signaling pathway, such as ANP1/NPK1, provides a strategy for engineering multiple stress tolerance that may greatly benefit agriculture.

Destined to reside in the habitats of germination, plants are frequently exposed to unfavorable environmental conditions. Extreme temperature, drought, salinity, pollution, and pathogens greatly affect plant growth, development, and productivity. To survive, plants have developed a complex signaling network that senses and protects them from an ever-changing environment. A common plant response to different abiotic and biotic stresses, such as heat, chilling, excessive light, drought, wounding, ozone exposure, UV-B irradiation, osmotic shock, and pathogens is the accelerated generation or/and accumulation of reactive oxygen species, including hydrogen peroxide (H2O2), superoxide anion, and hydroxyl radicals (1–7). H2O2 is an active signaling molecule and its accumulation (oxidative stress) leads to a variety of cellular responses. Plant responses to H2O2 are dose dependent. High dosage of H2O2 results in a hypersensitive cell death (4, 8–10), whereas low dosage of H2O2 blocks cell cycle progression (11) and functions as a developmental signal for the onset of secondary wall differentiation (12). Additionally, preexposure to abiotic or biotic stresses, which induce H2O2 production or/and accumulation, can trigger a protective function and “immunize” plants against different stress conditions, thus enhancing tolerance to multiple stresses and pathogens (10, 13–16).

One of the mechanisms contributing to oxidative signal-induced stress and pathogen tolerance is the activation of detoxification and protection/defense gene expression. For example, Arabidopsis plants respond to oxidative stress with an increase in production of antioxidant enzymes, including glutathione-S-transferases (GSTs), peroxidases, superoxide dismutases, and catalases, as well as the activation of protective genes encoding heat shock proteins (HSPs) and pathogenesis-related proteins (1, 4, 17–20). Several oxidative stress-responsive elements have been identified in plant gene promoters (19, 21–23), and some transcription factors that bind to the cis-elements have been reported (21). However, the redox-sensing mechanisms and signaling pathways that regulate activity of these transcription factors are still obscure.

In many eukaryotes, the transduction of oxidative signals is controlled by protein phosphorylation involving mitogen-activated protein kinases (MAPKs) (24, 25). MAPK and immediate upstream activators, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK), constitute a functionally interlinked MAPK cascade (24, 25). Although many plant MAPK, MAPKK, and MAPKKK homologues have been identified on the basis of sequence conservation and functional complementation in yeast, their precise physiological functions in plants are mostly unknown (26–29). Elevation of MAPK activity has been detected in plant cells after exposure to various stresses and mitogenic stimuli (26–29). However, it remains to be determined whether these MAPK activation events are mediated through MAPK cascades consisting of specific MAPKs, MAPKKs, and MAPKKKs in plant cells.

Genetic and biochemical analysis of plant signaling cascades is not straightforward because the key regulators are typically functionally redundant, expressed at low levels, or have indispensable roles for cell viability (30). Here, we used an alternative approach, protoplast transient expression assays, to unravel the function of a redundant class of MAPKKKs in oxidative stress signaling. We show that the ANP class of MAPKKKs from Arabidopsis (31) can be induced specifically by H2O2 and can activate a specific class of stress-induced MAPKs (ANP, Arabidopsis NPK1-like protein kinase, in which NPK is a Nicotiana protein kinase). The activated MAPK cascade plays a dual role in regulation of gene expression: it activates stress-response genes that protect plants from diverse environmental stresses, and it represses auxin-inducible promoters. Thus, the ANP-mediated MAPK cascade represents a molecular link between oxidative stress and the plant growth hormone auxin.

Materials and Methods

Arabidopsis Protoplast Transient Expression Assays. Arabidopsis protoplasts were isolated and transfected by a modified polyethylene glycol method as described (32). Typically, 0.2 ml of protoplast suspension (106 per ml) was cotransfected with 30–50 µg of DNA of three plasmids containing a kinase, a reporter, and an
internal control. The transfected protoplasts were incubated at 23°C for 4 h before collection unless specified otherwise. All transient expression experiments were repeated at least three times with similar results.

**Reporter Constructs and Activity of the Stress- and Auxin-Responsive Promoters.** *Arabidopsis GST6* (21), *HSPIB.2* (33), and *RD29A* (34), as well as soybean *GH3* (35), promoters were fused to the firefly luciferase gene to create *GST6-LUC, HSP18.2-LUC, RD29-LUC, and GH3-LUC* reporter constructs. The *UBI10-GUS* construct (36) was used as an internal control in each transfection. The luciferase activity of the lysate from the transfected protoplasts (10⁵) was divided by the transfection. The luciferase activity of the lysate from the transfected protoplasts (10⁴) was divided by the transfection. The luciferase activity of the lysate from the transfected protoplasts (10⁴) was divided by the transfection. The luciferase activity of the lysate from the transfected protoplasts (10⁴) was divided by the transfection.

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ANP1 Initiates an H$_2$O$_2$-Activated MAPK Cascade. To identify downstream MAPKs of the ANP-mediated MAPK cascade, constitutively active ANP1 was cotransfected with one of six Arabidopsis MAPKKs (AtMPKs), representing three different classes (26–29). Active ANP1 initiated a MAPK cascade that could be assayed by measuring the activity of an individual epitope-tagged AtMPK after immunoprecipitation (Fig. 2C). In this assay, we relied on the endogenous MAPKKs to link between ectopically expressed ANP1 and AtMPKs. Constitutively active ANP1 slightly changed the mobility of AtMPK3 and AtMPK6 detected by [35S]methionine labeling, immunoprecipitation, and SDS/PAGE, suggesting that these MAPKs were phosphorylated (Fig. 2C Upper). Notably, active ANP1 dramatically increased the activity of only these two MAPKs in protoplasts by an in vitro MAPK activity assay using MBP as a substrate (Fig. 2C Lower). Active ANP2 and ANP3, but not CTR1, a different class of MAPKKK (38), also induced AtMPK3 and AtMPK6 activity (data not shown), indicating that CTR1 and ANPs activate different MAPK cascades. The constitutively active CTR1 used in this study can activate MAPK activity in plant cells (44) and inhibit the activity of an ethylene-inducible enhancer in the transfected Arabidopsis protoplasts (data not shown).

AtMPK3 and AtMPK6 are most similar to the tobacco and alfalfa MAPKs implicated in stress and pathogen signal transduction (26–29). The ability of ANPs to activate stress-related MAPKs suggests that the ANP-mediated MAPK cascade is involved in stress signaling. To define the stress signals that can regulate the ANP1-mediated MAPK cascade, HA epitope-tagged AtMPK3 was transfected into Arabidopsis protoplasts, and the protoplasts were then challenged with different stresses. Phosphorylation activity of AtMPK3 was measured after immunoprecipitation with an anti-HA antibody by using MBP as a substrate. Several stress signals, including H$_2$O$_2$, but not auxin, activated AtMPK3 (Fig. 2D Left). H$_2$O$_2$ also activated AtMPK6 (data not shown). However, when the full-length ANP1 protein was ectopically expressed, only H$_2$O$_2$, not other stress stimuli, could further enhance the activation of AtMPK3 (Fig. 2D Center). Therefore, H$_2$O$_2$ can specifically induce the full-length ANP1 activity (Fig. 2D Center) to the level of the constitutively active ANP1 (Fig. 2D Right). The induction of AtMPK3 by stimuli unrelated to oxidative stress is probably mediated by an ANP-independent pathway (Fig. 2D Left). Thus, the data indicate that H$_2$O$_2$ can activate ANP1, which initiates a MAPK cascade leading to induction of at least two MAPKs, AtMPK3 and AtMPK6 in Arabidopsis.
ANP1 Activates H₂O₂-Inducible Promoters. To provide further evidence for the specific involvement of ANPs in H₂O₂ signaling and to investigate their downstream targets, we tested the effect of constitutively active ANP1 on the activity of the GST6, HSP18.2, and RD29A promoters. Active ANP1 could substitute for H₂O₂ to induce the GST6 and HSP18.2 promoters, but it did not change the expression of the ABA-, cold-, or drought-responsive RD29A promoter (Fig. 3A). Activation of the GST6 and HSP18.2 promoters required ANP kinase activity, since a single amino acid mutation in the ATP-binding site abolished the ANP1 effect on the promoters. However, the activation was not due to nonspecific protein phosphorylation, because three other Arabidopsis protein kinases, including constitutively active CTR1 (38), did not affect the promoter activities. The tested protein kinases were expressed equally well and displayed kinase activity similar to ANP-like MAPKKks in transfected cells when casein was used as a nonspecific substrate (37). The levels of promoter activity induced by H₂O₂, active ANP1, or both are comparable to that previously reported in other systems (35, 50). In Arabidopsis protoplasts, auxin, 1 μM 1-naphthaleneacetic acid (data not shown), dramatically increased GH3 promoter activity. The magnitude of GH3 promoter activation in Arabidopsis protoplasts was comparable to that previously reported in other systems (35, 50). Constitutively active ANP1, ANP2, and ANP3, but not other tested protein kinases, effectively suppressed the GH3 promoter induction by auxin (Fig. 3B). Thus, ANPs may be functionally involved in H₂O₂ signal transduction.

Crosstalk Between H₂O₂ and Auxin Signaling. We have recently reported that a tobacco ANP homologue, NPK1 (42), initiates a MAPK cascade that represses activities of several promoters responsive to auxin, a plant mitogen and growth hormone (37). To test whether ANPs are functional homologues of NPK1 in Arabidopsis, we assayed the effect of the kinases on activity of a well-characterized auxin-responsive promoter, GH3 (35, 50). In Arabidopsis protoplasts, auxin, 1 μM 1-naphthaleneacetic acid (Fig. 3B) or 1 μM indole-3-acetic acid (data not shown), dramatically increased GH3 promoter activity. The magnitude of GH3 promoter activation in Arabidopsis protoplasts was comparable to that previously reported in other systems (35, 50). Constitutively active ANP1, ANP2, and ANP3, but not other tested protein kinases, effectively suppressed the GH3 promoter induction by auxin (Fig. 3B). Thus, ANPs may be functionally involved in H₂O₂ signal transduction.
Because H$_2$O$_2$ can activate the ANP-mediated MAPK cascade, we reasoned that oxidative stress would be able to repress the GH3 promoter activity. Indeed, H$_2$O$_2$ abolished the auxin response (Fig. 3C) without affecting the internal control UBQ10-GUS and the activity of 35S-LUC (Fig. 1A). In contrast, the stress hormone ABA, which can activate the RD29A promoter in the system (Fig. 3D), did not appear to interfere with auxin signaling in leaf cells (Fig. 3C). As H$_2$O$_2$ can arrest the cell cycle (11), whereas auxin promotes it (51), there may be shared mechanisms in oxidative stress and auxin signaling. Our finding that the H$_2$O$_2$-induced MAPK cascade can repress auxin responses provides a molecular link between oxidative stress and auxin signal transduction. The ANP-mediated MAPK cascade may help stressed plants to shift energy from auxin-dependent activities to stress protection and survival.

Constitutively Active NPK1 Enhances Tolerance to Multiple Stresses in Transgenic Tobacco. Oxidative stress-activated GSTs and HSPs encode conjugation enzymes and molecular chaperones, respectively. They play essential roles in detoxification and stabilization of damaged proteins, thereby assisting cell recovery from stresses (20, 22, 52). Constitutive expression of individual GSTs or HSPs in transgenic tobacco and Arabidopsis has been shown to enhance plant resistance to low temperature, salt, or heat (53, 54). Since constitutively active ANP1 induces expression of GST6 and HSP18.2 promoters (Fig. 3A), it is possible that transgenic plants ectopically expressing the active ANP-like protein might be more tolerant to multiple stresses. Several transgenic tobacco lines (2A, 3B, 4A), expressing different levels of the constitutively active tobacco ANP orthologue, NPK1, (37) were examined. Phenotypically, the transgenic plants did not differ from wild-type plants under normal growth conditions (Fig. 4A). However, transgenic plants recovered and regrew faster than did the wild-type plants after a freezing temperature treatment (Fig. 4B). Since ANP1 does not induce RD29A expression (Fig. 3A), the basis of the observed freezing tolerance is different from the previously reported one that relied on overexpression of transcription factors that activate the RD29A promoter (55, 56). Thus, plants can employ distinct mechanisms to protect themselves from low temperature. We have also tested sensitivity of the NPK1 transgenic plants to heat shock. Exposure to 48°C heat shock killed all the wild-type plants, but 24% of 2A, 68% of 3B, and 74% of 4A plants survived (Fig. 4C). In addition, only 12% of the wild-type, but 46%, 68%, and 80% of 2A, 3B, and 4A plants, respectively, survived a 3-day exposure to high salt (300 mM NaCl) (Fig. 4D). The stress tolerance of the NPK1 transgenic plants was proportional to the level of NPK1 transgene expression (37). Thus, the NPK1 transgenic plants seem to have a combined advantage of overproducing GSTs and HSPs (53, 54) and are more tolerant to salt, cold, and heat than are the wild-type plants. Further analysis of these transgenic plants will be required to reveal other downstream targets of the NPK1/ANP signaling pathway and their tolerance to other abiotic and biotic stresses.

Although NPK1 represses transcription of several auxin early response genes, it does not appear to affect development of vegetative tissues in the transgenic plants (Fig. 4A). It is possible that the transgene expression levels are not sufficient to cause abnormal phenotypes in vegetative tissues. However, the NPK1 transgenic plants produced some seeds defective in embryo development (37), a stage when auxin plays an essential role (57). It is likely that ectopic NPK1 expression could have different accumulation levels and distinct effects in different cell types at different developmental stages. The absence of obvious growth defects in postembryonic development of the transgenic plants suggests that the achieved level of NPK1 expression is not deleterious, but rather beneficial in vegetative tissues. This is an advantage over the ectopic expression of stress-inducible transcription factors that appear to interfere with normal plant growth and development (55, 56). In addition, the manipulation of this oxidative stress signaling regulator can protect plant cells from diverse environmental stresses, such as heat, freezing, and high salt (Fig. 4B–D). This approach may even be applicable for plant protection against other environmental stresses, such as UV-B, ozone, photooxidation, herbicides, pathogens, drought, and chilling that also involve oxidative stress damage (1, 7, 15).

Molecular genetic approaches have previously been used to enhance plant tolerance to stresses through alteration of osmolytes, osmoprotectants, membrane fatty acids, channels, transcription factors, and enzymes that scavenge active oxygen species by transferring or mutating individual stress target genes (55, 56, 60–62). Manipulation of key regulators that constitute the signaling core of multiple stress responses and control expression of several protective genes might provide an alternative or even more effective strategy. Since a common consequence of many abiotic and biotic stresses is the generation or accumulation of oxidative signals, manipulation of key regulators of an oxidative stress signaling pathway, such as ANP/NPK1, in vegetative tissues may provide a novel strategy for cross-protection from multiple stresses in agriculturally important plants.

Future analyses of ANP transgenic plants and knockout mutants in Arabidopsis will likely yield more insights into the function of this oxidative stress-activated MAPK cascade in...
plant development and stress tolerance. The completion of the *Arabidopsis* genome sequence (58) and the availability of microarray gene expression profiles (59) will facilitate functional analysis of genes encoding MAPK cascade components in diverse plant signaling transduction pathways by using the cellular system we established in this study.

**Summary.** Our studies uncovered molecular connections from a specific signal to MAPKKKs and MAPKs, and to downstream gene expression programs in plants. We have presented several lines of evidence indicating that the ANP/NPK1 type of MAPKKs mediate oxidative stress signal transduction in plants. For example, oxidative stress can activate ANP1. Constitutively active NPK1-type MAPKKKs mimic oxidative stress signal by inducing stress MAPKs and protective gene expression, as well as by repressing an auxin-responsive promoter. Further analysis of the ANP cascade might reveal additional MAPKs and target genes. These cellular studies can support and complement analyses in ANP transgenic plants and mutants in the future. Since ANP/NPK1 proteins are found at high levels in meristems (31, 42, 47), these MAPKKKs might mediate a natural tolerance of meristems to diverse stresses, and play a dual role in both cell cycle regulation (63, 64) and oxidative stress signal transduction in plants.

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