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

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Despite the recognition of H_2O_2 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 H_2O_2 signal is perceived and transduced in plant cells. We report here that H_2O_2 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 H_2O_2 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 H_2O_2 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 (H_2O_2), superoxide anion, and hydroxyl radicals (1–7). H_2O_2 is an active signaling molecule and its accumulation (oxidative stress) leads to a variety of cellular responses. Plant responses to H_2O_2 are dose dependent. High dosage of H_2O_2 results in a hypersensitive cell death (4, 8–10), whereas low dosage of H_2O_2 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 H_2O_2 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 H_2O_2 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 (10^6 per ml) was cotransfected with 30–50 μg of DNA of three plasmids containing a kinase, a reporter, and an

Abbreviations: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; NPK, *Nicotiana* protein kinase; ANP, *Arabidopsis* NPK1-like protein kinase; GST, glutathione-*S*-transferase; HSP, heat shock protein; ABA, abscisic acid; HA, hemagglutinin; MBP, myelin basic protein.

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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), *HSP18.2* (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 (10^4) was divided by the β-glucuronidase (GUS) activity (37) to normalize the data for variation in transformation efficiency and cell viability. The presented results are the means of triplicate samples ± SD.

In-Gel Kinase Activity Assay. Protoplast extracts containing 5 μg of protein were fractionated in an SDS/10% polyacrylamide gel containing 0.25 mg/ml myelin basic protein (MBP), a MAPK substrate. The protein denaturing, renaturing, and kinase activity assay in the gel were performed as described (37).

Kinase Constructs, Expression, and Immunocomplex Kinase Activity Assay. *Arabidopsis* cDNAs encoding MAPKKKs, ANP1–3 (31), and CTR1 (38), MAPKs, AtMPK2–7 (39), and serine-threonine protein kinases ASK1 and CK1–1 (37) were obtained by PCR from an *Arabidopsis* cDNA library and verified by DNA sequencing. The kinase-inactive ANP1 mutant (K98M) was generated by PCR using the following primers: TCTCGCCGTCAtgCAGGTTCTGATTGC and GCAATCAGAACCTGcaTGACGGCGAGAAG. The mutation (lowercase letters) was confirmed by DNA sequencing. All PCR products were sequenced and tagged with the double hemagglutinin (HA) epitope and inserted into a plant expression vector containing the *35SC4PPDK* promoter and the *NOS* terminator (37). Transfected protoplasts (10^5) were incubated in the presence of [^{35}S]methionine (200 μCi·ml^{-1}) for 4 h to allow expression and labeling of the ectopically expressed kinase proteins. The HA-tagged kinases were immunoprecipitated with an anti-hemagglutinin (HA) antibody (37), separated by SDS/PAGE (10%), and visualized by fluorography. The HA-tagged kinases were also immunoprecipitated from lysates of transfected protoplasts (10^5) with an anti-HA antibody and assayed for MAPK activity with MBP as a substrate (37). The ^{32}P-labeled MBP was separated by SDS/PAGE (15%) and visualized by autoradiography.

Plant Growth and Stress Tolerance Analysis. Wild-type *Nicotiana tabacum* SR1 and transgenic (37) F_2 seeds were germinated on ¼× Murashige and Skoog (MS) agar plates. The plantlets were grown at 23°C with a 10 h dark/14 h light (90 μmol·m^{-2}·s^{-1}) photoperiod before testing for tolerance to heat (48°C), freezing temperature (−10°C), and salt (300 mM NaCl in ¼× MS agar plates). After the stress treatments the plantlets were returned to the original growth conditions. At least 50 plantlets of each genotype were used for each treatment.

Results and Discussion

H_2O_2 Activates Oxidative Stress-Inducible Promoters and Two MAPK-Like Kinases in *Arabidopsis* Protoplasts. To initiate this study of oxidative stress signal transduction in plants, we first established that the *Arabidopsis* protoplast transient expression system is a suitable tool to study stress responses. Three *Arabidopsis* stress-responsive promoters, *GST6* (21), *HSP18.2* (33), and *RD29A* (34, 40), which are activated by oxidative stress, heat shock, and abscisic acid (ABA)/drought/cold, respectively, were fused to the *LUC* reporter and tested for their responses in transfected

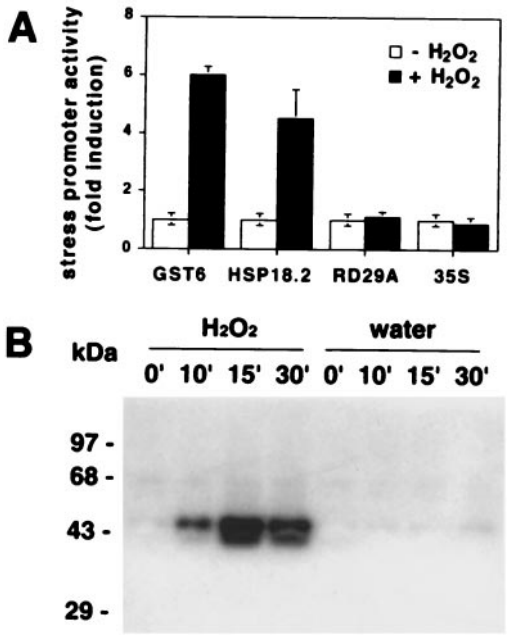

Fig. 1. Oxidative stress responses in *Arabidopsis* protoplasts. (*A*) H_2O_2 activated two oxidative stress-induced promoters. *Arabidopsis* protoplasts were transfected with *GST6-LUC* (GST6), *HSP18.2-LUC* (HSP18.2), *RD29A-LUC* (RD29A), or *CaMV35S-LUC* (35S) reporter constructs and incubated without (−) or with (+) 200 μM H_2O_2 for 3 h before the promoter activities were measured. Data are the results of triplicate samples and three independent experiments. (*B*) H_2O_2 induces two putative MAPKs. *Arabidopsis* protoplasts were treated with 200 μM H_2O_2 or water for 10, 15, and 30 min. The MAPK in-gel kinase activity assay was performed as described (37).

mesophyll protoplasts. Oxidative stress (H_2O_2) activated the *GST6* and the heat shock promoters in the transfected protoplasts (Fig. 1*A*). The results were similar to those previously reported for other systems (20, 21, 41). However, the ABA/drought/cold-inducible *RD29A* promoter was not affected by H_2O_2. The *RD29A* promoter was functional in the transfected protoplasts, since ABA could induce the promoter (see below). The H_2O_2 treatment also had no significant effects on *UBQ10* promoter activity, which served as an internal control, or on the *CaMV35S* promoter activity (Fig. 1*A*).

To determine whether H_2O_2 signaling is mediated through an evolutionarily conserved MAPK cascade, we performed a MAPK activity in-gel assay with extract from protoplasts challenged with H_2O_2. Within 10 min, treatment with H_2O_2, but not with water, activated two MBP (a common MAPK substrate) phosphorylation activities (Fig. 1*B*). The sizes and induction kinetics of the H_2O_2-activated kinases are similar to those reported for plant MAPKs (26–29).

ANP1 Activates Two Endogenous MAPKs. To determine the molecular identity and define the role of H_2O_2-activated MAPKs, we initiated a search for candidates that might participate in the oxidative stress-induced MAPK cascade. We chose to analyze MAPKKKs because they are the first conserved enzymes in the MAPK cascade and because a constitutively active form of these kinases can be generated by deleting the putative regulatory domains (31, 37, 42–44). The remaining kinase domain carries the specificity for its downstream target MAPKK, which, in turn, phosphorylates and activates specific MAPK(s) (43, 45). Three major classes of putative MAPKKK genes have been reported in *Arabidopsis*, *CTR1*, *AtMEKK1*, and *ANP*s (31, 38, 46). The CTR1 and AtMEKK1 classes of MAPKKKs are thought to mediate ethylene and touch/cold/drought signal transduction, respec-

tively (38, 46). Three closely related *Arabidopsis* MAPKKKs, ANP1, ANP2, and ANP3, are expressed in many tissues, but at a low level. They share high sequence similarity to tobacco NPK1, which is thought to be involved in cell cycle regulation (31, 47, 63, 64). We have recently found that constitutively active NPK1 can partially block embryo development (37). In animals, induction of an oxidative stress-activated MAPK also results in embryo arrest (48). In addition, several H_2O_2-induced MAPK cascades regulate cell cycle progression in nonstressed cells (25, 49), and oxidative stress can block cell cycle progression in yeast, mammals, and plants (11, 24, 25). Therefore, we reasoned that the NPK1/ANP class of MAPKKKs might mediate oxidative stress responses in plant cells.

To elucidate the function of ANPs, we first demonstrated that ectopically expressed ANPs could activate endogenous MAPKs in *Arabidopsis.* The coding regions of full-length (repressed), catalytic domain (constitutively active), or mutated (kinase-inactive) ANPs were expressed in *Arabidopsis* protoplasts as shown by [^{35}S]methionine labeling and immunoprecipitation. Because these ANPs are highly homologous in the kinase domain, we used an HA epitope tag and a specific anti-HA antibody to follow the expression of each individual ANP (Fig. 2*A*). An in-gel MAPK kinase assay indicated that constitutively active ANP1, ANP2, and ANP3 activated two endogenous MBP phosphorylation activities in transfected protoplasts (Fig. 2*B*). As expected, a mutation in the ATP-binding site abolished ANP1 action, whereas the presence of the regulatory domains diminished the ability of ANP1 to activate the putative MAPKs. The sizes of the ANP-activated kinases were the same as those activated by H_2O_2 (Fig. 1*B*).

ANP1 Initiates an H_2O_2-Activated MAPK Cascade. To identify downstream MAPKs of the ANP-mediated MAPK cascade, constitutively active ANP1 was cotransfected with one of six *Arabidopsis* MAPKs (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. 2*C*). In this assay, we relied on the endogenous MAPKKs to link between ectopically expressed ANP1 and AtMAPKs. Constitutively active ANP1 slightly changed the mobility of AtMPK3 and AtMPK6 detected by [^{35}S]methionine labeling, immunoprecipitation, and SDS/PAGE, suggesting that these MAPKs were phosphorylated (Fig. 2*C 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. 2*C 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_2O_2, but not auxin, activated AtMPK3 (Fig. 2*D Left*). H_2O_2 also activated AtMPK6 (data not shown). However, when the full-length ANP1 protein was ectopically expressed, only H_2O_2, not other stress stimuli,

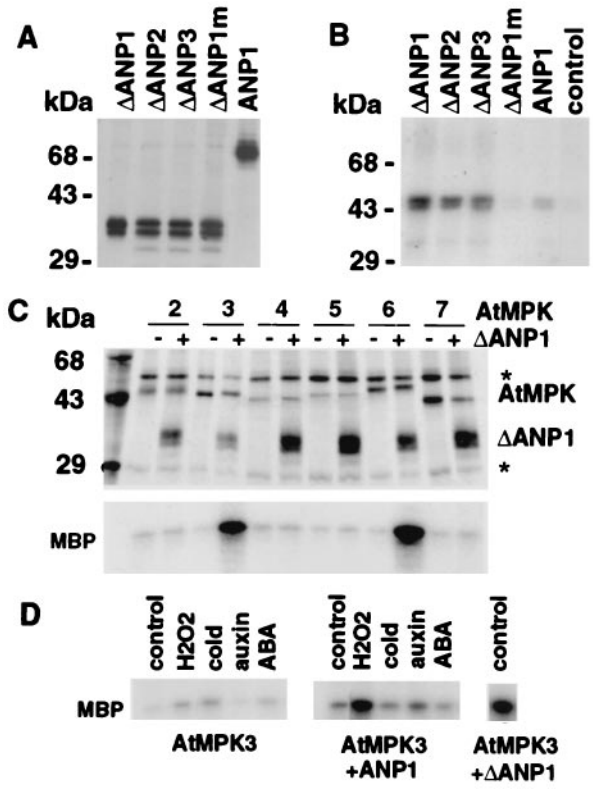

Fig. 2. ANP1 initiates an oxidative stress-inducible MAPK cascade. (*A*) Expression of the ANP kinases. *Arabidopsis* protoplasts were transfected with five different ANP constructs expressing various HA-tagged kinases: the catalytic domain of ANP1 (ΔANP1), the catalytic domain of ANP2 (ΔANP2), the catalytic domain of ANP3 (ΔANP3), the catalytic domain of ANP1 with the ATP-binding site mutation K98M (ΔANP1m), and a full-length ANP1 (ANP1). The HA-tagged kinases were labeled by [^{35}S]methionine, immunoprecipitated with an anti-HA antibody, separated by SDS/PAGE (10%) and visualized by fluorography. The ANPs were expressed as double bands which might be caused by the presence of two initiation sites. (*B*) ANPs activate two endogenous MAPKs. *Arabidopsis* protoplasts were transfected with the same five ANP constructs or with vector DNA (control). Activation of endogenous MAPKs in the transfected cells was detected by an in-gel kinase activity assay. (*C*) ANP1 activates AtMPK3 and AtMPK6 *in vivo*. *Arabidopsis* protoplasts were transfected with a construct expressing one of the *Arabidopsis* MAPKs (AtMPK2 to 7) alone, or cotransfected with another construct expressing ANP1 catalytic domain (ΔANP1). Protein levels of the ectopically expressed ANP1 and MAPKs were detected after immunoprecipitation with an anti-HA antibody (*Upper*). Asterisks indicate nonspecific bands. Activity of the MAPKs was assayed in immunocomplex with MBP as a substrate (*Lower*). The ^{32}P-labeled MBP was separated by SDS/PAGE (15%) and visualized by autoradiography. (*D*) Stress activation of AtMPK3 and ANP1. *Arabidopsis* protoplasts were transfected with AtMPK3 construct alone (*Left*) or cotransfected with full-length ANP1 (AtMPK3 + ANP1) (*Center*) or active ANP1 (AtMPK3 + ΔANP1) as a positive control (*Right*). The transfected protoplasts were incubated for 4 h to allow protein expression before treatment with 200 μM H_2O_2, 4°C (cold), 1 μM 1-naphthaleneacetic acid (auxin), or 100 μM ABA for 15 min. The AtMPK3 activity was assayed in immunocomplex. All experiments presented were repeated at least three times with similar results.

could further enhance the activation of AtMPK3 (Fig. 2*D Center*). Therefore, H_2O_2 can specifically induce the full-length ANP1 activity (Fig. 2*D Center*) to the level of the constitutively active ANP1 (Fig. 2*D Right*). The induction of AtMPK3 by stimuli unrelated to oxidative stress is probably mediated by an ANP-independent pathway (Fig. 2*D Left*). Thus, the data indicate that H_2O_2 can activate ANP1, which initiates a MAPK cascade leading to induction of at least two MAPKs, AtMPK3 and AtMPK6 in *Arabidopsis*.

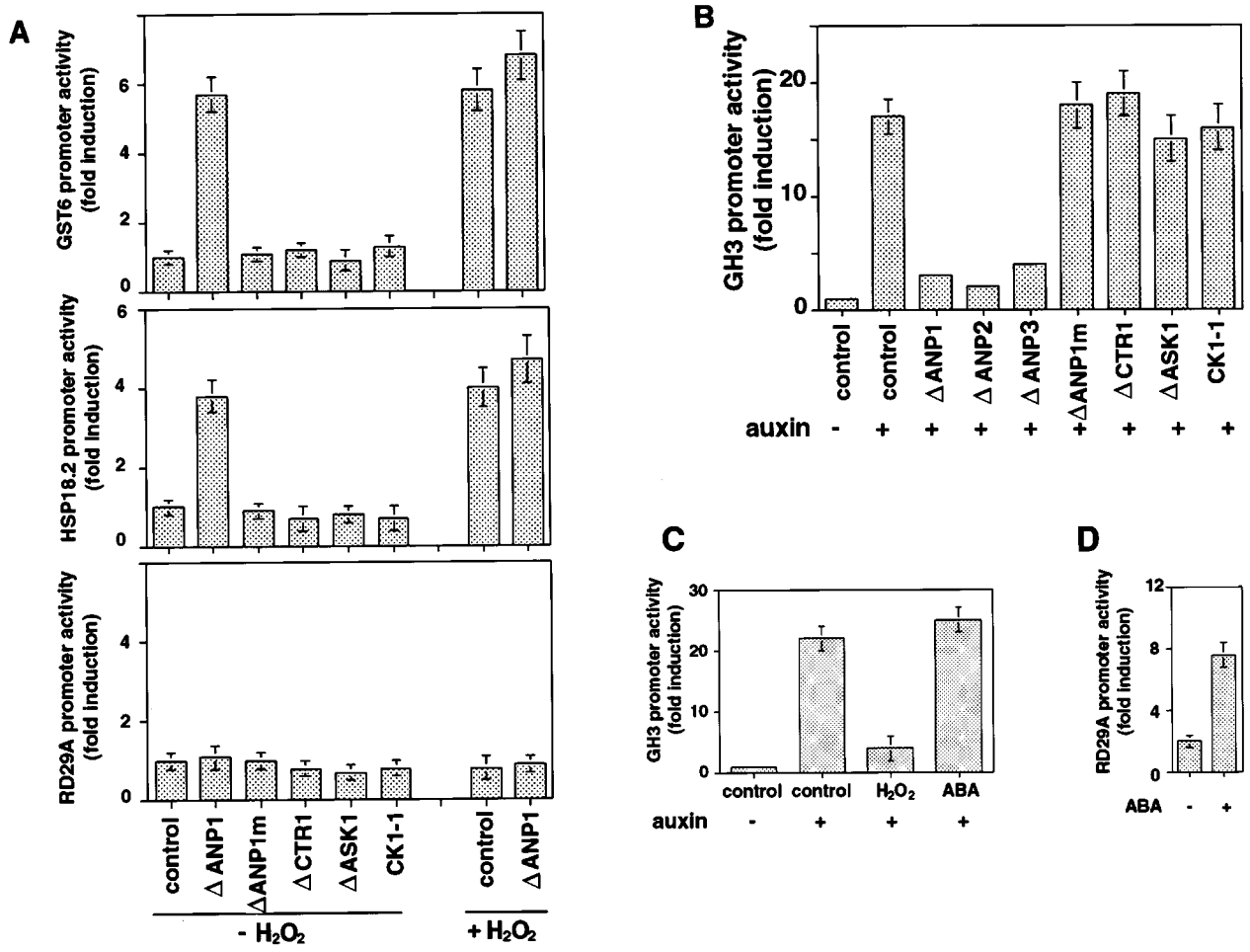

Fig. 3. The ANP pathway represents a molecular link between stress and auxin signaling. (*A*) ANP1 activates oxidative stress-inducible gene expression. *Arabidopsis* protoplasts were cotransfected with one of the reporter constructs: *GST6-LUC* (GST6), *HSP18.2-LUC* (HSP18.2), or *RD29A-LUC* (RD29A) and one of the ANP constructs as described in the legend of Fig. 2*A*. Other control kinase constructs were the catalytic domains of CTR1 (ΔCTR1), ASK1 (ΔASK1), and CK1-1 (CK1-1). Vector DNA was used as a control (control). The transfected protoplasts were incubated for 3 h to allow kinase expression before 200 μM H_2O_2 was added to induce the *GST6* and *HSP18.2* promoters. The cells were incubated for another 3 h before the promoter activities were measured. (*B*) ANPs repress the auxin response. *Arabidopsis* protoplasts were cotransfected with the *GH3-LUC* reporter construct and one of the kinase constructs as described in the legends of Figs. 2*A* and 3*A*. The transfected protoplasts were incubated for 3 h to allow kinase expression before 1 μM 1-naphthaleneacetic acid (auxin) was added to induce the *GH3* promoter. The cells were incubated for another 3 h before the *GH3* promoter activity was measured. (*C*) H_2O_2 suppresses the auxin-responsive *GH3* promoter. *Arabidopsis* protoplasts were transfected with the *GH3-LUC* reporter construct and incubated in the absence (− auxin) or presence of 1 μM 1-naphthaleneacetic acid (+ auxin) and 200 μM H_2O_2, or 100 μM ABA for 3 h before activity of the *GH3* promoter was measured. (*D*) ABA induces the *RD29A* promoter. *Arabidopsis* protoplasts were transfected with the *RD29A-LUC* reporter construct and incubated in the absence or presence of 100 μM ABA for 3 h before activity of the *RD29A* promoter was measured. All data presented on the figure are the results of triplicate samples and three independent experiments.

ANP1 Activates H_2O_2-Inducible Promoters. To provide further evidence for the specific involvement of ANPs in H_2O_2 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 H_2O_2 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. 3*A*). 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 H_2O_2, active ANP1, or both are comparable (Fig. 3*A*). These results indicate that ANP1 functions in H_2O_2 signal transduction.

Crosstalk Between H_2O_2 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. 3*B*) 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. 3*B*). Thus, ANPs may be functionally

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redundant orthologues of the tobacco NPK1 that can also suppress auxin signaling (37).

Because H_2O_2 can activate the ANP-mediated MAPK cascade, we reasoned that oxidative stress would be able to repress the *GH3* promoter activity. Indeed, H_2O_2 abolished the auxin response (Fig. 3*C*) without affecting the internal control *UBQ10-GUS* and the activity of *35S-LUC* (Fig. 1*A*). In contrast, the stress hormone ABA, which can activate the *RD29A* promoter in the system (Fig. 3*D*), did not appear to interfere with auxin signaling in leaf cells (Fig. 3*C*). As H_2O_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_2O_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. 3*A*), 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. 4*A*). However, transgenic plants recovered and regrew faster than did the wild-type plants after a freezing temperature treatment (Fig. 4*B*). Since ANP1 does not induce *RD29A* expression (Fig. 3*A*), 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. 4*C*). 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. 4*D*). 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. 4*A*). 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

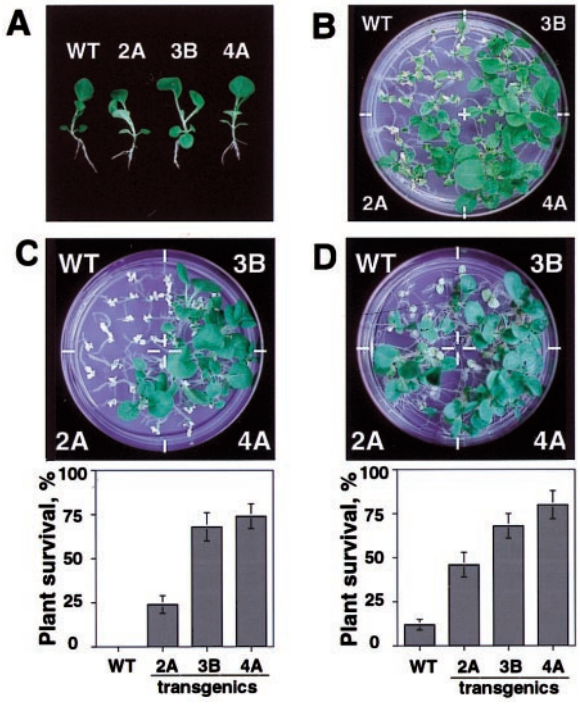

Fig. 4. Stress tolerance of transgenic tobacco plants expressing constitutively active NPK1. (*A*) Normal vegetative growth of NPK1 plants. Wild-type (WT) and transgenic (2A, 3B, 4A) plants were germinated and grown on a ¼× MS medium for 3 weeks. (*B*) Tolerance to freezing temperature. Plants were grown on plates for 10 days before freezing temperature treatment (−10°C, 3 h). The photograph was taken 11 days after the treatment. (*C*) Tolerance to heat shock. Plants were grown for 10 days before heat treatment (48°C, 45 min). The photograph was taken 18 days after the treatment. (*D*) Tolerance to salt stress. Plants were grown for 6 days and then transferred to plates containing 300 mM NaCl for 3 days. The photograph was taken 11 days after the plants were returned to ¼× MS medium plates. The graphic data are results of 50 plants of each genotype for *C* and *D*.

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). Additionally, the manipulation of this oxidative stress signaling regulator can protect plant cells from diverse environmental stresses, such as heat, freezing, and high salt (Fig. 4 *B–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 MAPKKKs mediate oxidative stress signal transduction in plants. For example, oxidative stress can activate ANP1. Constitutively active ANP-like 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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