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Interaction of SOS2 with Nucleoside Diphosphate Kinase 2 and Catalases Reveals a Point of Connection between Salt Stress and H_2O_2 Signaling in *Arabidopsis thaliana*[▽]

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SOS2, a class 3 sucrose-nonfermenting 1-related kinase, has emerged as an important mediator of salt stress response and stress signaling through its interactions with proteins involved in membrane transport and in regulation of stress responses. We have identified additional SOS2-interacting proteins that suggest a connection between SOS2 and reactive oxygen signaling. SOS2 was found to interact with the H_2O_2 signaling protein nucleoside diphosphate kinase 2 (NDPK2) and to inhibit its autophosphorylation activity. A *sos2-2 ndpk2* double mutant was more salt sensitive than a *sos2-2* single mutant, suggesting that NDPK2 and H_2O_2 are involved in salt resistance. However, the double mutant did not hyperaccumulate H_2O_2 in response to salt stress, suggesting that it is altered signaling rather than H_2O_2 toxicity alone that is responsible for the increased salt sensitivity of the *sos2-2 ndpk2* double mutant. SOS2 was also found to interact with catalase 2 (CAT2) and CAT3, further connecting SOS2 to H_2O_2 metabolism and signaling. The interaction of SOS2 with both NDPK2 and CATs reveals a point of cross talk between salt stress response and other signaling factors including H_2O_2.

Among the mechanisms known to be important in abiotic stress responses in plants are the *s*alt *o*verly *s*ensitive (SOS) ion homeostasis and signaling pathway (66, 67) and reactive oxygen species (ROS) accumulation and signaling (16, 41). The SOS pathway is currently one of the most extensively studied mechanisms in controlling salt stress response in plants. The *SOS1*, *SOS2*, and *SOS3* loci were first identified through forward genetic screens for salt-hypersensitive growth (67). SOS1 is a plasma membrane Na^+/H^+ antiporter that is essential for Na^+ efflux from roots (48, 55). SOS2 belongs to subgroup 3 of the sucrose-nonfermenting-related kinases (SnRK3s) (34), of which there are 25 encoded by the *Arabidopsis thaliana* genome (27). SOS3 is a myristoylated calcium-binding protein that likely responds to salt-induced Ca^{2+} oscillations in the cytosol (33). A total of nine SOS3-like Ca^{2+} binding proteins (SCaBPs) are encoded by the *Arabidopsis* genome (21).

The SOS2 kinase has emerged as an especially important regulatory component through its interactions with other signaling proteins. First, as part of the SOS signaling pathway, the regulatory region of SOS2 was shown to interact with SOS3 (25). This interaction activates SOS2 protein kinase activity in a Ca^{2+}-dependent manner and recruits the SOS2-SOS3 complex to the plasma membrane, where it phosphorylates SOS1 and activates Na^+ efflux (48, 50). Specific interactions between other SnRK3s (also referred to as calcineurin B-like protein-interacting protein kinases [CIPK]) and SCaBPs (also referred to as calcineurin B-like proteins [CBL]) have also been detected and are involved in signal transduction controlling abcisic acid (ABA) sensitivity, cold response, sugar response, and cellular pH (6, 11, 21, 32, 46).

Previous work has also shown that SOS2 interacts with the ABA-insensitive 2 (ABI2) protein phosphatase 2C (PP2C) through a specific protein phosphatase interaction domain (44). ABI2 and the highly homologous ABI1 PP2C are negative regulators of a broad range of ABA responses (15). PP2C genes also comprise a large gene family which, in *Arabidopsis*, has 76 members and 9 in the subgroup that includes the ABI1 and ABI2 genes (52). It was also found that three other SnRK3s interact with either ABI1 (SnRK3.6) or ABI2 (SnRK3.1, SnRK3.13, and SnRK3.15 [24, 44]) but not both. The many possible interactions of SOS2-like SnRK3s, PP2Cs, and SCaBPs, combined with different patterns of expression of these proteins, suggest that this may be a step where downstream signaling specificity is generated from common upstream signals (3, 35). Finding additional proteins that interact with SOS2 or other SnRK3s remains a promising approach in better understanding stress signaling.

Work on the SOS pathway has indicated the possibility of cross talk between SOS-mediated salt signaling and ROS signaling based on the interaction of the C-terminal cytoplasmic tail of SOS1 with radicle-induced cell death 1 (30). Also, the *enh1* mutant, which was isolated as an enhancer of the salt sensitivity of *sos3-1*, suggests a link between the SOS pathway and superoxide metabolism (65). However, an overall understanding of the molecular factors that sense changes in ROS,

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particularly H_2O_2, and integrate changes in H_2O_2 with other stress-related signaling mechanisms remains elusive. Among the proteins that are known to influence ROS accumulation are both ROS-metabolizing enzymes and other proteins whose connection to ROS is less clear. One of these latter factors is nucleoside diphosphate kinase 2 (NDPK2). In addition to its basic enzymatic role in phosphotransfer and regeneration of nucleoside triphosphates, NDPK2 has been shown to be involved in several signaling pathways including phytochrome and auxin signaling (8, 9, 54) and H_2O_2 signaling. *NDPK2* expression was induced by H_2O_2, and knockout of *NDPK2* led to greater ROS accumulation and stress sensitivity, while *NDPK2* overexpression decreased H_2O_2 and stress sensitivity (42). The effect of NDPK2 on H_2O_2 and stress sensitivity may be mediated at least in part by the interaction of NDPK2 with two H_2O_2-responsive mitogen-activated protein kinases (AtMPK3 and AtMPK6) and stimulation of their kinase activity (42).

Metabolism of H_2O_2 itself is controlled by a complex set of enzymes. Catalases (CATs), along with ascorbate and ascorbate peroxidases and glutathione peroxidases, are key components of H_2O_2 catabolism (41). CATs have been thought to be particularly important in detoxifying H_2O_2 formed during photosynthesis and photorespiration (61), but whether or not they also may have other functions in H_2O_2 regulation or signaling is not known. *CAT* gene regulation and the control of CAT activity are complex. The active form of CAT is a tetramer, and *Arabidopsis* contains three *CAT* genes, which are differentially expressed and can form up to six different isozymes (18, 64). CATs are themselves damaged by light and reactive oxygen and must be continually regenerated (13). Of the three *Arabidopsis CAT* genes, *CAT1* is expressed at only a low level in vegetative tissues but is more highly expressed in seeds. *CAT2* is the most highly expressed in vegetative tissues and is under circadian control, with the highest expression during the light period, consistent with a primary role in detoxifying H_2O_2 derived from photosynthesis or photorespiration (64). Interestingly, *CAT3* is also circadianly regulated but in the opposite manner as *CAT2*: *CAT3* expression is highest in the dark period (64), suggestive of a different molecular function than the detoxification of photosynthesis-derived H_2O_2, presumably performed by *CAT2*.

We have continued to investigate the protein-protein interactions of SOS2 and have found that it interacted with NDPK2, CAT2, and CAT3, but not CAT1. Interestingly, interaction of SOS2 with NDPK2 occurred at the 21-amino-acid FISL motif, the same motif required for SOS2 interaction with SOS3. Interaction with SOS2 inhibited NDPK2 autophosphorylation, indicating that NDPK2 activity was modulated by its interaction with SOS2. A *sos2-2 ndpk2* double mutant was more sensitive to salt stress than *sos2-2* and *ndpk2* single mutants. SOS2 interaction with CAT2 and CAT3 was detected both in vivo by purification of tandem affinity purification (TAP)-tagged SOS2 protein complexes and in yeast two-hybrid assays, further indicating a connection between SOS2 and H_2O_2. These results suggest that SOS2 is part of a signaling node that connects salt stress response with H_2O_2-dependent signaling.

MATERIALS AND METHODS

Construction of plasmids and yeast two-hybrid screening. pAS-SOS2, pAS-SOS2 K40N, pAS-SOS1, pAS-PKS3 (SnRK3.1), pAS-PKS11 (SnRK3.13), pAS-PKS18 (SnRK3.6), pASPKS24 (SnRK3.15), pAS-SOS3, and deletion constructs of *SOS2* in pAS were constructed previously (22, 25, 44). Deletion constructs of pACT-NDPK2 were made by PCR amplification using the original pACT-NDPK2 isolated from the SOS2 library screening (44) as a template and insertion into SmaI and EcoRI sites of pACT2. The following primer sets were used: for pACT-NDPK2-1/79, forward, 5′-AATGGTGGGAGCGACTGTAG-3′; reverse, 5′-GCGAATTCAAGAAGCTACAAGGTGAGGAAGGAAG-3′; for pACT-NDPK2-80/231, forward, 5′-AATGGAGGACGTTGAGGAGAC-3′; reverse, 5′-GCGAATTCACTCCCTTAGCCATGTAG-3′; for pACT-NDPK2-80/140, forward, 5′-AATGGAGGACGTTGAGGAGAC-3′; reverse, 5′-GCGAATTCAAAAGAATGATTTAGCACTAAG-3′; and for pACT-NDPK2-141/231, forward, 5′-TCCTAACCTGATTGAGTAC-3′; reverse, 5′-GCGAATTCACTCCCTTAGCCATGTAG-3′.

The pACT-CAT2 construct was obtained by PCR amplification followed by digestion of an EcoRI-*CAT2*-XhoI fragment generated using sequence-specific primers (forward, 5′-CCGGAATTCGAATGGATCCTTACAAGTATCG-3′; reverse, 5′-CCGCTCGAGTTAGATGCTTGGTCTCACG-3′) and the U19716 cDNA clone obtained from the Arabidopsis Biological Resource Center (ABRC) as a template. The fragment was then ligated in a digested pACT2 plasmid (Clontech). The *CAT3* coding sequence was amplified using *Arabidopsis* cDNA prepared from total RNA. The amplified fragment was then ligated in a TOPO plasmid (Invitrogen), and the resulting CAT3-TOPO vector was used as a template for PCR to generate a *CAT3*-SacI fragment using the forward primer 5′-ATGGATCCTTACAAGTATCGTCC-3′ and reverse primer 5′-CCCGAGCTCCTAGATGCTTGGCCTCACGTTC-3′. The fragment was then digested with SacI and inserted into a pACT2 vector digested with NcoI, filled in, and subsequently digested with SacI. For *CAT1*, the full-length coding region was PCR amplified from clone U24477 (obtained from ABRC) using the following oligonucleotides: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATCCATACAGGGTTCGTCC-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGAAGTTTGGCCTCACGTTAAG-3′. The amplified fragment was cloned into the entry vector pDONR207 and moved to the destination vector pDEST22 using the Gateway cloning system (Invitrogen).

Yeast two-hybrid experiments using bait constructs in either pAS2 or pDEST32 and prey constructs in either the pACT2 or pDEST22 vector were performed using *Saccharomyces cerevisiae* strain Y190 as previously described (20, 36).

RNA extraction and RNA gel blot analysis were performed using previously described techniques (24, 34). Twenty micrograms of total RNA was loaded in each lane, and blots were probed for *Actin* expression as a loading control.

Expression of AtNDPK2 and SOS2 in *Escherichia coli* and in vitro interaction experiments. *E. coli* BL21 codon$^+$ was transformed with pGEX-2TK-NDPK2, pGEX-2TK-SOS2, pGEX-2TK-SOS3, or pGEX-2TK-GGT1 and grown in 2× yeast extract tryptone agar media with ampicillin and chloramphenicol. Cells harvested from 1-liter cultures were resuspended in 50 ml of 1× phosphate-buffered saline containing 1.0 mM phenylmethylsulfonyl fluoride, 1.0 μM leupeptin, and 0.3 μM aprotinin, sonicated, and centrifuged (3,000 rpm for 10 min at 4°C). Glutathione *S*-transferase (GST) fusion protein was recovered by adding the supernatant to glutathione-Sepharose 4B resin. Radiolabeled SOS2 proteins were produced from pET14b-SOS2 using the TNT coupled reticulocyte lysate system (Promega) for in vitro transcription and translation, with [^{35}S]methionine as the sole source of methionine. In vitro pull-down assays were performed as previously described (22).

In vitro phosphorylation assay. Autophosphorylation assays were performed with GST-NDPK2 and GST-SOS2 as previously described (20) with the following modifications. The reaction buffer contained 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM dithiothreitol. After addition of appropriate amounts of each recombinant protein in buffer on ice, 2 μl cold 200 μM ATP and 0.5 μl [γ-^{32}P]ATP (5 μCi) were added and the reaction volume was adjusted to 20 μl with distilled H_2O. The reaction mixture was incubated at 30°C for 30 min, and the reaction was stopped by addition of 1 μl of 0.5 M EDTA. After addition of an equal volume of sodium dodecyl sulfate (SDS)-containing 2× sample buffer, samples were run on 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels.

Analysis of NTAPi-SOS2 protein complexes. SOS2 was cloned into the NTAPi vector (51) and used to transform *sos2-2* as described previously (2). Expression of NTAP-SOS2 complemented the salt sensitivity of the *sos2-2* mutant, indicating that the NTAP-SOS2 recombinant protein retained wild-type SOS2 functionality. SOS2-containing protein complexes were purified from either un-

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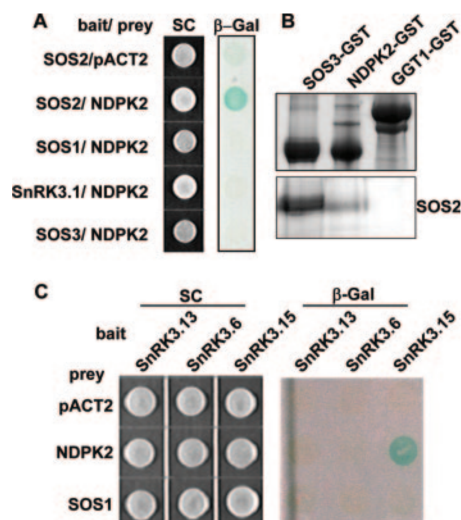

FIG. 1. SOS2 interacts with NDPK2. (A) Interaction of NDPK2 prey with SOS2 but not with SOS1, SOS3, or SnRK3.1 (PKS3) bait constructs in yeast two-hybrid assays. The pAS-SOS2/pACT2 (empty vector), and pAS-SOS1/pACT-NDPK2 combinations are shown as negative controls. Yeast grown on synthetic complete plates (SC) and results of a β-galactosidase filter assay (β-Gal) are shown. (B) SOS2 and NDPK2 interact in vitro. Radiolabeled SOS2 was incubated with GST-SOS3 (positive control), GST-NDPK2, or GST-GGT1 (glutamate glyoxylate transferase 1, a negative control). (Top) Coomassie blue-stained SDS-PAGE gel. (Bottom) Autoradiogram of the same gel. (C) Yeast two-hybrid assay of the interaction between NDPK2 and other SnRK3s. NDPK2 interacted with SnRK3.15 (PKS24) but not SnRK3.13 (PKS11) or SnRK3.6 (PKS18).

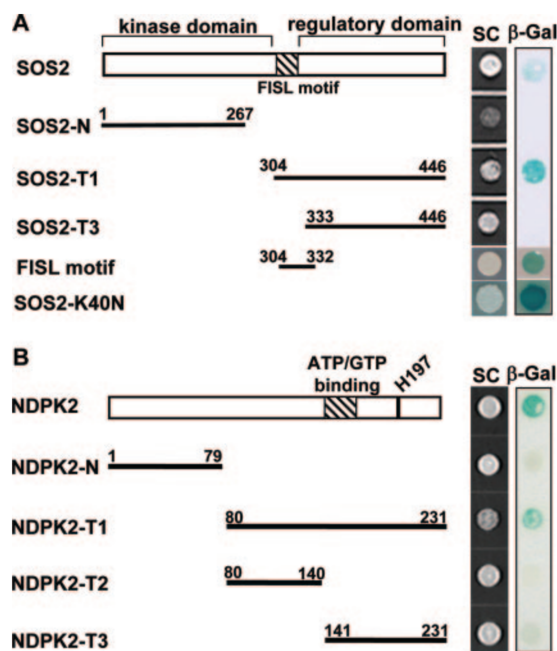

FIG. 2. Mapping of the SOS2 and NDPK2 motifs required for interaction. (A) Mapping of the SOS2 motif required for interaction with NDPK2. The indicated regions of SOS2 were cloned into pAS2 bait plasmid and cotransformed with pACT-NDPK2 prey plasmid. SOS2-K40N is full-length, catalytically inactive SOS2. SC and β-Gal are as defined for Fig. 1. (B) Mapping of the NDPK2 domain required for SOS2 interaction. The pAS-SOS2 bait plasmid was contransformed with pACT2 prey plasmids containing the indicated portions of NDPK2.

stressed plants or plants treated with 150 mM NaCl for 24 h using the protocols described in reference 51. The purified proteins were separated by SDS-PAGE, and protein bands detected by Coomassie blue staining were excised, trypsin digested, and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (2).

Phenotypic and H_2O_2 analysis. The *ndpk2* transferred DNA line was a generous gift from G. Choi (9) and has an insertion in an intron between codons 128 and 129 of *NDPK2*. The *sos2-2 ndpk2* double mutant was obtained by crossing homozygous *sos2-2* and *ndpk2* single mutants and subsequent PCR screening.

For seedling analysis, seedlings of each genotype were routinely grown by surface sterilizing seed and plating onto half-strength Murashige and Skoog medium (MS) with 6 mM MES (morpholineethanesulfonic acid; pH 5.7). Seeds were stratified for 4 days at 4°C and then transferred to a growth chamber maintained at 23°C and continuously lighted (70 μmol m^{-2} s^{-1}). Salt treatments were performed by adding salt to the media that the seeds were germinated on or by transferring 7-day-old seedlings from control media to salt-containing media. Seedling H_2O_2 content was assayed using an Amplex Red H_2O_2 assay kit (Invitrogen) as previously described (56).

RESULTS

SOS2 interacts with NDPK2. Using a yeast two-hybrid approach, we screened a λ-ACT Arabidopsis seedling cDNA library for proteins that interacted with the bait protein SOS2 (44). One of the interacting clones was found to encode NDPK2. Subsequent analysis demonstrated that, while NDPK2 interacted strongly with SOS2, it did not interact with SOS1, SOS3, or the SOS2-like kinase SnRK3.1 (Fig. 1A). The SOS2-NDPK2 interaction was confirmed in vitro by demonstrating that GST fusion proteins of NDPK2 or the positive control SOS3 both pulled down SOS2 (Fig. 1B) while the unrelated negative control glutamate glyoxylate transferase 1 did not. Further tests found that NDPK2 interacted with SnRK3.15 but not SnRK3.13 or SnRK3.5 (Fig. 1C). These results demonstrated that, while NDPK2 interacts with more than one SnRK3, the interaction was specific to certain members of the SnRK3 family. This is similar to the pattern seen for the interaction of SOS3-like SCaBPs and PP2Cs (ABI1 and ABI2) with SOS2 and other SnRK3s (22, 44).

We next used deletion constructs of SOS2 to determine the SOS2 domain required for interaction with NDPK2 (Fig. 2A). The N-terminal kinase domain (SOS2-N) containing the SOS2 catalytic site (25, 27) did not interact with NDPK2. In contrast, the C-terminal regulatory domain (SOS2-T1) containing the FISL motif (also referred to as a NAF domain) was sufficient for interaction. Removal of the FISL motif (SOS2-T3) abolished the interaction with NDPK2. The FISL motif by itself showed an interaction, demonstrating that the FISL motif is both required and sufficient for interaction with NDPK2. Thus, NDPK2 interacts with the same domain of SOS2 that is required and sufficient for interaction with SOS3 (22). A full-length, catalytically inactive SOS2, SOS2-K40N (25), also interacted, demonstrating that the phosphorylation activity of SOS2 was not required for interaction with NDPK2.

A similar strategy was used to identify the NDPK2 domain required for interaction with SOS2 (Fig. 2B). The N-terminal region of NDPK2 (NDPK2-N) was not sufficient for interaction with full-length SOS2. However, the C-terminal portion of NDPK2 (NDPK2-T1) was sufficient for interaction. Two additional constructs (NDPK2-T2 and NDPK-T3) containing por-

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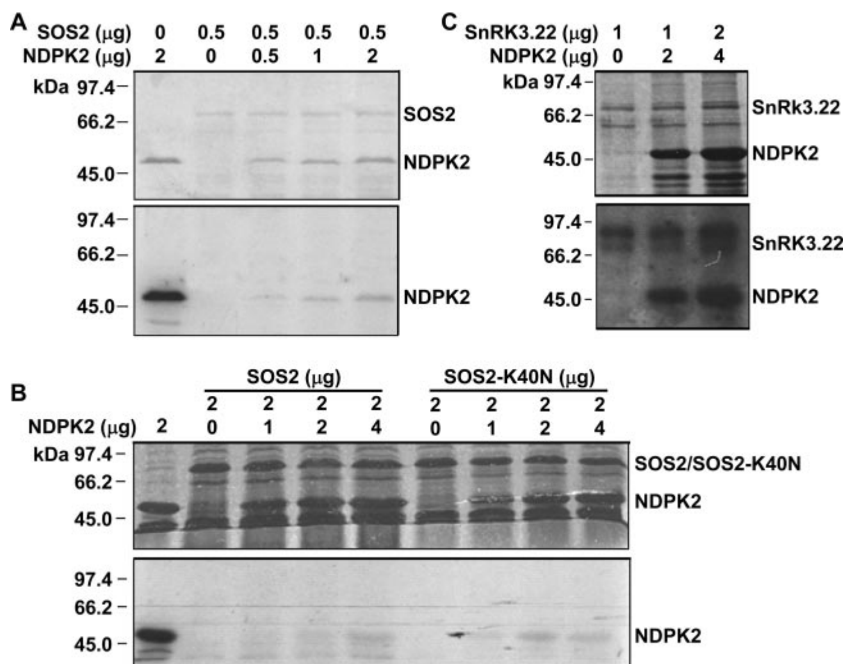

FIG. 3. SOS2 inhibits NDPK2 autophosphorylation. (A) Inhibition of NDPK2 autophosphorylation by SOS2. (Top) Coomassie blue-stained gel. (Bottom) Autoradiogram of the same gel after in vitro phosphorylation assay. The amounts of SOS2 and NDPK2 loaded in each line are indicated across the top of the gel. (B) Inhibition of NDPK2 autophosphorylation by catalytically inactive SOS2. Top and bottom panels show the Coomassie-stained gel and autoradiogram, respectively, from in vitro phosphorylation assay mixtures containing the indicated amounts of wild-type SOS2 or the catalytically inactive SOS2-K40N mutated protein. (C) SnRK3.22 (PKS5) does not inhibit NDPK2 autophosphorylation. Top and bottom panels show the Coomassie-stained gel and autoradiogram, respectively, from in vitro phosphorylation assay mixtures containing the indicated amounts of SOS2 and SnRK3.22.

tions of the C-terminal region were not sufficient for interaction. Thus, the ATP/GTP binding motif, as well as the catalytically active His-197, was within the portion of NDPK2 required for interaction with SOS2. Interaction of NDPK2 with phytochrome A also occurred through the C-terminal portion of NDPK2, although in that case it was NDPK2 amino acids 214 to 221 that were especially important (54).

SOS2 inhibits NDPK2 autophosphorylation. To determine the effect of SOS2 interaction on NDPK2 function, we analyzed the effect of SOS2 on NDPK2 autophosphorylation. NDPK2 alone had high autophosphorylation activity (Fig. 3A, lane 1). When 0.5 μg of NDPK2 was coincubated with 0.5 μg of SOS2, phosphorylation of NDPK2 was almost completely eliminated (Fig. 3A, lane 3). It should be noted that the molar ratio of SOS2 to NDPK2 in this case was approximately 1 to 2. Decreasing the SOS2/NDPK2 molar ratio to approximately 1 to 4 (0.5 μg SOS2 and 2 μg NDPK2) recovered only a small amount of NDPK2 phosphorylation. This is consistent with previous reports that wild-type NDPK2 exists almost exclusively in a hexameric form (28), and our results imply that SOS2 inhibited autophosphorylation of the NDPK2 hexamer or another multimeric form of NDPK2.

To test whether SOS2 kinase activity was required for inhibition of NDPK2 phosphorylation, NDPK2 was incubated with either SOS2 or the catalytically inactive SOS2-K40N. SOS2-K40N suppressed NDPK2 kinase activity to a similar extent as SOS2 (Fig. 3B). This also confirmed that the low level of NDPK2 phosphorylation observed in the presence of SOS2 was autophosphorylation instead of phosphorylation by SOS2. Together with recent data suggesting that *Arabidopsis* NDPK2 is autophosphorylated only on the active-site His (53), this suggests that SOS2 regulates NDPK2 kinase activity by inhibition of active-site phosphorylation rather than by regulatory phosphorylation at another site.

To determine whether this inhibitory effect on NDPK2 autophosphorylation was specific to SOS2, we assayed NDPK2 phosphorylation in the presence of the SOS2-related kinase SnRK3.22. SnRK3.22 has functions in pH homeostasis but not salt tolerance (Y. Guo and J.-K. Zhu, unpublished data). SnRK3.22 had no effect on the autophosphorylation of AtNDPK2 (Fig. 3C). This demonstrated that a specific interaction was required to inhibit NDPK2 autophosphorylation. We also note that SnRK3.22 itself had a much higher autophosphorylation activity (Fig. 3C) than SOS2 (Fig. 3B).

Salt sensitivity and H_2O_2 content of *ndpk2* and *sos2-2 ndpk2* mutants. We next examined whether knockout of *NDPK2* could affect salt tolerance and how this effect would interact with the known salt sensitivity phenotype of the *sos2-2* mutant (34). When *ndpk2*, *sos2-2*, and *sos2-2ndpk2* mutants were plated on media containing 50 mM NaCl, growth of the *sos2-2 ndpk2* double mutant was more inhibited than that of either the *sos2-2* or *ndpk2* mutant (Fig. 4A).

To further quantify the salt sensitivity of these mutants, we also tested the salt sensitivity of wild-type and mutant seedlings that were germinated on control media for 4 days and then transferred to salt-containing media for an additional 6 days. Quantification of seedling fresh weight at the end of the salt treatment again showed an increased salt sensitivity of the

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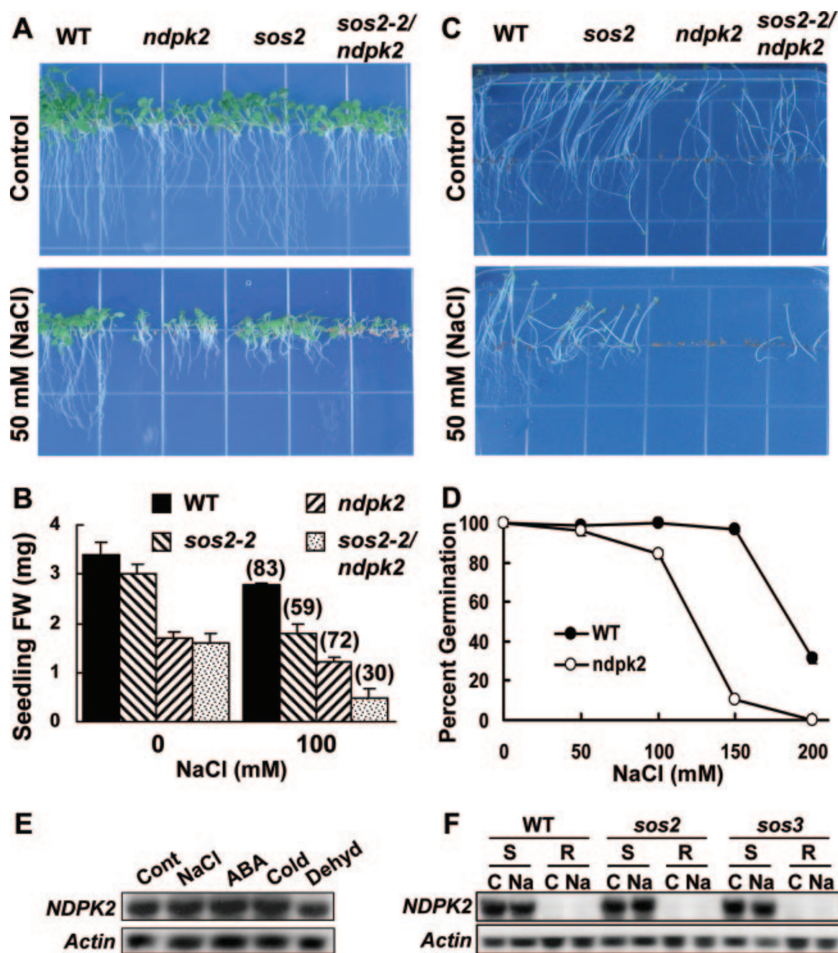

FIG. 4. Salt stress sensitivity of *sos2-2*, *ndpk2*, and *sos2-2ndpk2* seedlings and *NDPK2* expression. (A) Wild-type (WT), *ndpk2*, *sos2*, and *sos2-2 ndpk2* seedlings after 7 days of growth on control media (half-strength MS with 0.5% sucrose) or media containing 50 mM NaCl. (B) Fresh weights (FW) of wild-type, *ndpk2*, *sos2-2*, and *sos2-2 ndpk2* seedlings. Seedlings were grown for 4 days on control media and then transferred to either fresh control plates (half-strength MS without sucrose) or plates containing 100 mM NaCl for 6 days before measurement of seedling fresh weight. Each measurement involved five to seven seedlings, with the total fresh weight divided to obtain a per-seedling fresh weight. Data shown are means ± standard errors ($n = 3$ or 4). Numbers in parentheses above each bar of the 100 mM NaCl data are the fresh weights of seedlings of the different genotypes following NaCl treatment expressed as percentages of their unstressed fresh weights shown on the left side of the graph. (C) Salt response of etiolated seedlings. Conditions were as in panel A except that the plates were wrapped in foil to maintain darkness. (D) Sensitivity of wild-type (Columbia) and *ndpk2* seed germination to NaCl. Germination was scored at 4 days after the end of stratification. (E) RNA blot analysis of the effect of stress or ABA on *NDPK2* expression. RNA was extracted from seedlings grown on MS media (3% sucrose) and treated with 300 mM NaCl for 5 h, 100 μM ABA for 3 h, 0°C for 24 h, and dehydration for 24 h. (F) RNA blot analysis of the expression of *NDPK2* in root and shoot tissue of the wild type and *sos2* and *sos3* mutants under either control (C) or salt stress (Na) conditions.

sos2-2 ndpk2 double mutant (Fig. 4B). The *ndpk2* mutant grew less than the wild type in the absence of salt stress; however, the effect of transfer to 100 mM NaCl on seedling fresh weight was only slightly more in the *ndpk2* mutant than in the wild type (fresh weight of salt-treated wild-type seedlings was 83% that of the control, while for the *ndpk2* mutant it was 72%; Fig. 4B). For the *sos2-2 ndpk2* double mutant, however, fresh weight of seedlings on 100 mM NaCl was only 30% that of *sos2-2 ndpk2* seedlings on control media (Fig. 4B) compared to 59% for the *sos2-2* single mutant. This analysis showed that the *ndpk2* mutant by itself did not have substantially increased sensitivity to salt but rather was constitutively growth impaired. The *sos2-2 ndpk2* double mutant, on the other hand, was severely salt sensitive, indicating that NDPK2 has an effect on salt tolerance that is more apparent in the absence of SOS2. It should also be noted that we have previously reported increased ABA response of root growth and proline accumulation in the *ndpk2* mutant compared to the wild type (58), which may be caused either by direct action of *NDPK2* or indirectly by the increased H_2O_2 content of the *ndpk2* mutant.

The involvement of NDPK2 in phytochrome signaling has been well characterized (8, 9, 54); thus, we tested whether the response of *ndpk2* to salt was altered in etiolated seedlings. Etiolated wild-type and the *sos2-2* seedlings germinated and grew on 50 mM NaCl; however, the root growth of the *sos2-2* mutant was inhibited compared to that of the wild type (Fig. 4C). *sos2-2 ndpk2* seedlings developed similarly to *sos2-2* seedlings under these conditions. *ndpk2* seedlings, however, failed to develop even on this relatively low level of salt (Fig. 4C). Additional experiments found that, although *ndpk2* seedling

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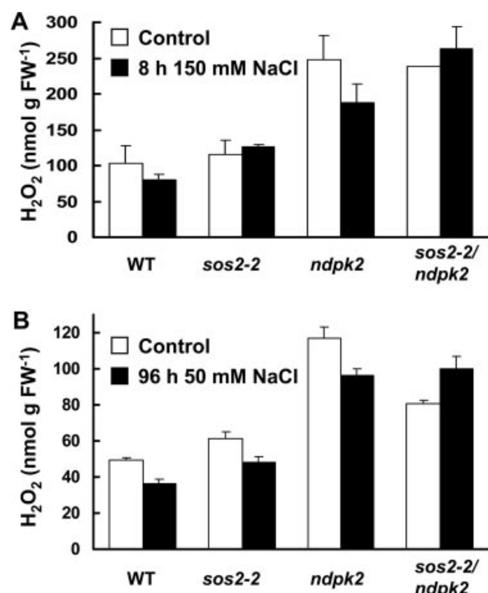

FIG. 5. H_2O_2 content of the wild type (WT), *sos2-2* mutant, *ndpk2* mutant and *sos2-2 ndpk2* double mutant. (A) Seedlings were grown on control media (half-strength MS, 0.5% sucrose) and transferred to either fresh control media or media containing 150 mM NaCl for 8 h before H_2O_2 assay. (B) Seedlings were grown on control media without sucrose and transferred to fresh media or media containing 50 mM NaCl for 96 h before H_2O_2 quantification. In panels A and B, H_2O_2 contents of seedlings were quantified by Amplex Red assay and data are means ± standard errors (n = 3 or 4).

germination was inhibited more than that of the wild type at higher salt concentrations, it was not affected at 50 mM NaCl, where nearly all seeds germinated (Fig. 4D). Thus, these experiments suggested that NDPK2, perhaps because of its interaction with phytochrome, has a strong effect on the salt response of etiolated seedlings.

To determine if altered *NDPK2* gene expression could have a role in salt resistance, the gene expression pattern of *NDPK2* was examined. We found that NDPK2 expression was not induced by salt, ABA, cold, or dehydration treatment (Fig. 4E) and that expression was not altered in the *sos2* or *sos3* mutant either under control conditions or after salt stress treatment (Fig. 4F). Consistently with its previously described role in phytochrome A signaling (9, 54), *NDPK2* is expressed in all shoot tissues including leaf, stem, flower, and silique tissue, but not in roots (Fig. 4F; data not shown).

ndpk2 has previously been shown to have altered sensitivity to H_2O_2 along with elevated H_2O_2 levels, and *NDPK2* gene expression was induced by exogenous H_2O_2 (42). H_2O_2 is also believed to be involved in a number of stress and ABA signaling events (30, 40, 41). Therefore, we quantified the H_2O_2 content of entire seedlings under control conditions or after transfer to salt-containing media to determine if altered H_2O_2 levels could be a factor in the salt sensitivity of the *sos2-2 ndpk2* double mutant. H_2O_2 was quantified after a short-term salt shock (150 mM NaCl; Fig. 5A) and after longer-term exposure to 50 mM NaCl, a condition where the *sos2-2 ndpk2* double mutant had inhibited growth but did still survive (Fig. 5B; growth conditions for seedlings used in this assay were the same as those used in the experiment shown in Fig. 4A). Under these experimental conditions, the H_2O_2 content of the salt-stressed *sos2-2* mutant was slightly greater than that of the salt-stressed wild type for both salt treatments (Fig. 5A and B). The *ndpk2* mutant, in contrast, had constitutively elevated levels of H_2O_2 even under control conditions, as has been observed previously (42, 58), but its H_2O_2 content was slightly decreased by salt stress (Fig. 5A and B). Interestingly, the *sos2-2 ndpk2* double mutant had H_2O_2 levels generally similar to that of *ndpk2* (Fig. 5A and B), but salt stress tended to increase its H_2O_2 content slightly, in contrast to seedlings of other genotypes (Fig. 5A and B). Since no large differences in H_2O_2 content between *ndpk2* and *sos2-2 ndpk2* seedlings were found, it can be concluded that increased build-up of toxic H_2O_2 could not explain the greater salt sensitivity of *sos2-2 ndpk2* seedlings than of *ndpk2* seedlings. It should be noted that the 150 mM NaCl experiments (Fig. 5A) were conducted using media containing 0.5% sucrose while the 50 mM NaCl experiments (Fig. 5B) were conducted on media without sucrose (same conditions as used for Fig. 4B). The presence of sucrose in the media led to a higher basal level of H_2O_2 in seedlings of all genotypes; however, the differences between seedlings with the different genotypes were similar and consistently observed in both conditions.

Both *ndpk2* and *sos2* mutants have previously been reported to have increased tissue damage when exposed to exogenous H_2O_2 or other ROS (43, 65). We tested whether *sos2-2 ndpk2* seedlings had greater sensitivity to H_2O_2 than *ndpk2* seedlings and found that again the phenotype of *sos2-2 ndpk2* seedlings was similar to that of *ndpk2* seedlings grown at a range of H_2O_2 concentrations (data not shown). This suggested that it was not greater susceptibility to H_2O_2-induced damage that was responsible for the greater salt sensitivity of *sos2-2 ndpk2* seedlings but leaves open the possibility that altered H_2O_2 signaling is a factor.

SOS2 interacts with CAT2 and CAT3. We also detected two additional SOS2-interacting proteins that support a connection between H_2O_2 and the SOS pathway. The yeast two-hybrid interaction screen (25) also identified CAT3 as a SOS2-interacting protein (data not shown). In addition, subsequent experiments to detect SOS2-interacting proteins by purifying protein complexes containing NTAPi-SOS2 (2) identified both CAT2 and CAT3 as SOS2-interacting proteins. LC-MS/MS analysis detected a number of peptides from both CAT2 and CAT3 in protein complexes isolated from salt-stressed plants (Fig. 6A and B). Interestingly, when SOS2-containing protein complexes were isolated from unstressed plants, we were not able to detect any CAT3 peptides and found only a relatively low level of a single CAT2 peptide. This suggests that salt stress may be required to promote the interaction of SOS2 with CAT2 and CAT3.

Direct interaction of SOS2 with both CAT2 and CAT3 was confirmed by yeast two-hybrid assays (Fig. 6A and B), with CAT3 having an especially strong interaction with SOS2. Both CAT2 and CAT3 interacted with the catalytically inactive SOS2-K40N mutant, indicating that SOS2 kinase activity was not required for the interaction (Fig. 6A and B). We also tested whether SOS2 could interact with CAT1 in the yeast two-hybrid system and found that it did not (Fig. 6C). In addition we found that neither CAT2 nor CAT3 interacted with NDPK2 in the yeast two-hybrid system (Fig. 6D). These

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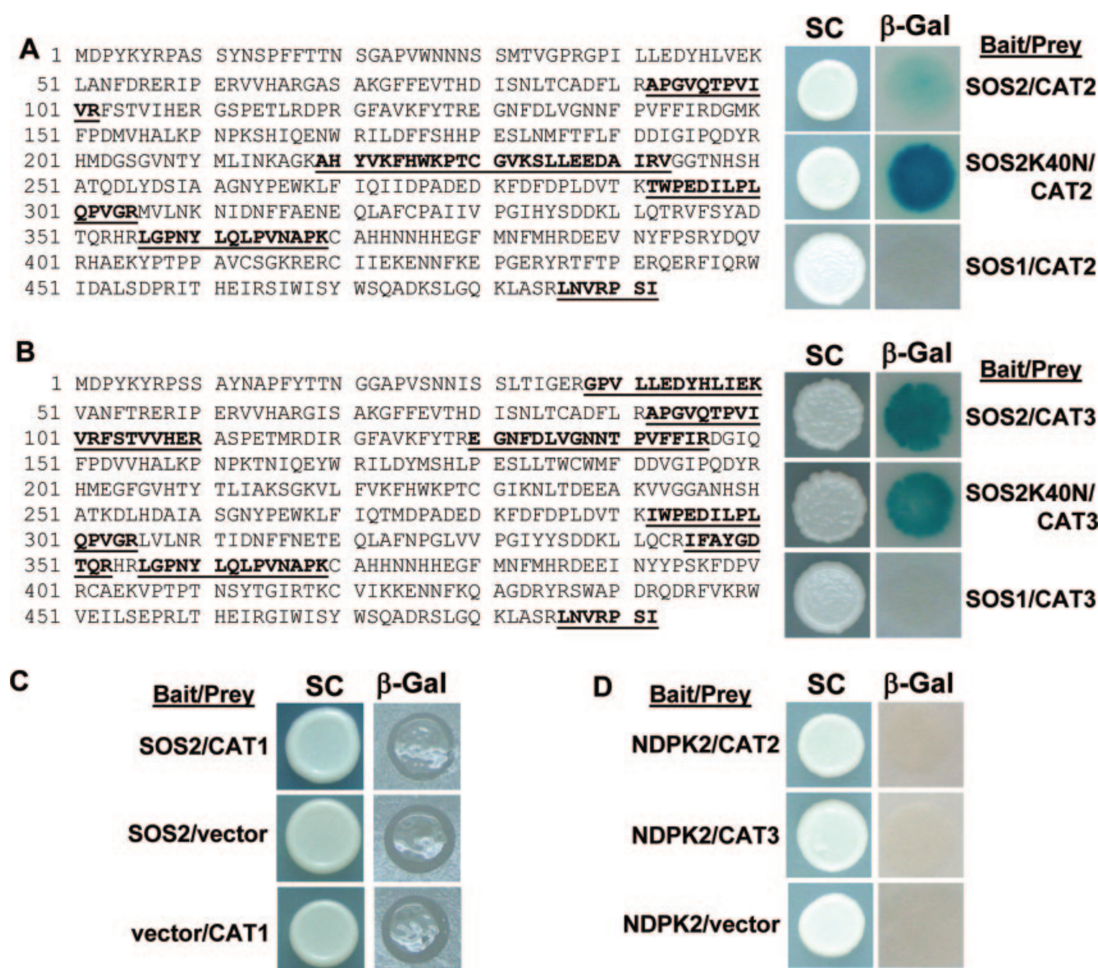

FIG. 6. Interaction of SOS2 with CAT2 (A) and CAT3 (B) detected in vivo by purification of NTAPi-SOS2 protein complexes and in yeast two-hybrid assays. (A and B) Protein sequences of CAT2 and CAT3, respectively, are shown at the left, with the peptides identified by LC-MS/MS analysis of NTAPi-SOS2 complexes underlined. Interaction of SOS2 and the catalytically inactive mutant SOS2-K40N in the pAS2 bait plasmid with CAT2 or CAT3 in the pACT2 prey plasmid is shown at the right. Lack of interaction of CAT2 or CAT3 with SOS1 bait is shown as a negative control. SC and β-Gal are as defined for Fig. 1. (C) SOS2 does not interact with CAT1. Interaction was tested using $pDEST_{32}SOS2$ as the bait vector and $pDEST_{22}CAT1$ as the prey vector. Empty bait and prey vectors were used as controls. (D) NDPK2 does not interact with CAT2 or CAT3. Interaction was tested using pAS2 and pACT2 bait and prey vectors. An empty prey vector was used as a control.

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results support the conclusion that SOS2 interacts specifically with CAT2 and CAT3 and provides an additional link between SOS2 and H_2O_2.

DISCUSSION

An emerging paradigm in signal transduction is that of a series of "nodes" where several signals interact rather than a collection of linear pathways each with a defined function. It is also becoming increasingly apparent that enzymes previously thought to have general housekeeping or detoxification functions can, in addition, be key signal transduction components. Examples of this latter point are the now well-defined role of hexosekinase 1 in sugar sensing and signaling as well as sugar metabolism (7, 43) and the recently described role of *Arabidopsis* glutathione peroxidase 3 (AtGPX3) in ABA signaling (40). In terms of which proteins might form parts of nodes for stress-related signaling, SOS2 has emerged as a good candidate.

In addition to the well-established role of SOS2 in regulating ion transport (5, 48–50, 68), the interaction of SOS2 with ABI2 suggests a connection to other aspects of stress signaling (44). Other SnRK3 kinases may also be involved in signaling mechanisms controlling responses to the environment or hormone response, particularly ABA signal transduction (24). Here we show that NDPK2 interacts with SOS2 and SnRK3.15. This interaction, together with the salt sensitivity phenotype of the *sos2-2 ndpk2* double mutant, suggests that NDPK2 has a role in salt stress signaling through its interaction with SOS2. NDPK2 is known to affect H_2O_2 accumulation and sensitivity, and the interaction of SOS2 with CAT2 and CAT3 also suggests a connection between SOS2 and H_2O_2. Taken together with previous results, these data suggest a role for SOS2 beyond direct regulation of ion transport. The interactions of SOS2 characterized to date (excluding membrane transporters) are summarized in Fig. 7. Of special interest is the observation that SOS3 and NDPK2, as well as other SOS3-related SCaBPs (21),

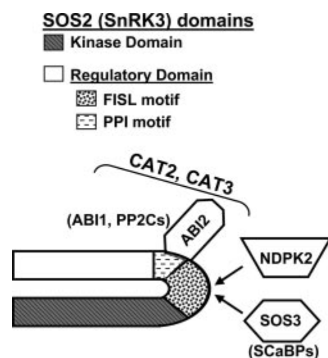

FIG. 7. Summary of SOS2 interactions potentially important in stress signaling (excluding SOS2 interactions with membrane transport proteins). SnRK3s consist of an N-terminal kinase domain and a C-terminal regulatory domain. Part of the SOS2 regulatory domain has also been shown to include a FISL motif, required for interaction with SOS3, and a PPI motif, required for interaction with ABI2 and possibly other PP2Cs. Data presented here demonstrate that NDPK2 also interacts with the FISL motif, suggesting that competition between SOS3 and NDPK2 may occur in tissue where they are both expressed. CAT2 and CAT3 interact with SOS2 at an unknown location. Protein names in parentheses are those that have been shown to interact with SnRK3s other than SOS2.

can interact with the same domain present in SOS2 and related SnRK3 kinases. This suggests a complex regulatory interaction between these proteins and salt stress, H_2O_2, ABA, and light signaling.

Potential roles of SOS2-NDPK2 interaction. In plants, NDPK2 has been implicated in diverse physiological processes including growth; phytochrome A signaling; and UV-B, heat stress, and oxidative stress adaptation (8, 9, 14, 42, 45, 69). We have also shown recently that the *ndpk2* mutant has enhanced ABA response in root growth and proline accumulation (58), although this may be an indirect effect of the elevated H_2O_2 content of the *ndpk2* mutant. NDPKs have been shown to be an important signaling component in mammalian systems where NDPK (nm23) can act as a tumor suppressor, possibly through a direct role in transcriptional activation (1, 36, 47). These multiple functions are likely to be due to specific protein phosphorylation activity of NDPK (12, 17, 37, 59) rather than its role in NDP phosphotransfer, and mammalian NDPKs have been shown to interact with proteins including key signal transducers such as AMPK and CK2 (10).

Autophosphorylation is essential for NDPK2 activity, and recent evidence has suggested that only the active-site histidine is autophosphorylated in NDPK2 (53). Therefore, SOS2 inhibition of NDPK2 autophosphorylation is likely to block NDPK2-dependent phosphorylation of downstream targets such as AtMPK3 and AtMPK6. In contrast, interaction with phytochrome A stimulated NDPK2 autophosphorylation activity (54); thus, interaction with SOS2 and phytochrome A may have opposite effects on NDPK2 activity, and this may explain why etiolated *ndpk2* seedlings were particularly salt sensitive. Although we did not observe changes in NDPK2 gene expression in response to salt stress, dehydration, or ABA or in the *sos2-2* or *sos3* mutants, it remains possible that NDPK2 protein levels could be increased by salt stress (31). Thus, the protein-protein interactions of NDPK2 with both activators and repressors, as well as its abundance, can determine its activity in response to salt stress or other stimuli.

It is of particular interest to note that both SOS3 and NDPK2 bind to the FISL motif of SOS2 (22, 25). Reverse transcription-PCR analysis has shown that both SOS2 and SOS3 are expressed at a low level in shoot tissue (J.-K. Zhu, unpublished data) in addition to their expression in roots. Therefore, in shoots it is possible that SOS3 and NDPK2 compete for binding to SOS2. However, in roots, where NDPK2 is not expressed, SOS3 is the only protein known to bind to the SOS2 FISL domain. Thus, competition between SOS3 and NDPK2 for SOS2 binding may be significant in root versus shoot responses to salt stress. It is also possible that modification of SOS2 under salt stress (2; H. Fujii and J.-K. Zhu, unpublished observations) may promote its interaction with SOS3 and/or ion transporters over NDPK2, thus releasing the repression of NDPK2. However, it must be noted that overexpression of a kinase-activated mutant form of SOS2 lacking the FISL domain could largely complement the *sos2-2* mutant salt sensitivity phenotype (23). The most likely explanation is that, when such a constitutively active SOS2 is overexpressed, it no longer needs SOS3 for either activation or targeting to the membrane for SOS1 phosphorylation. Since the FISL domain is required for interaction with NDPK2, this would suggest that the SOS2/NDPK2 interaction is not critical for obtaining a nearly wild-type level of salt tolerance under the conditions used for those tests. Further study of the activation state of NDPK2 under various conditions with and without SOS2 present will be needed to verify these hypotheses. One must also consider that NDPK2 interacts with at least one other SnRK3; thus, the effect of any specific interacting protein, such as SOS2, on NDPK2 phosphorylation in vivo in response to specific stimuli is likely to be localized and transient.

The interactions reported here also potentially place NDPK2 in the same protein complex as CAT2 and/or CAT3. This is interesting in light of previous observations that *Arabidopsis* NDPK1, which is closely related to NDPK2, can interact with all three *Arabidopsis* CATs (19) and that *Neurospora crassa* NDPK1 can also interact with a CAT (60, 63). At the gene expression level, upregulation of *CAT3*, as well as other ROS-metabolizing enzymes, has been observed in *Arabidopsis* plants overexpressing *NDPK2* (62). Thus, other lines of evidence, in addition to our finding of a common interaction partner, suggest a functional relationship between CATs and NDPKs.

NDPK2 interaction with SOS2 was detected through yeast two-hybrid screening and confirmed by in vitro pull-down assays. It should be noted that NDPK2 was not detected in NTAP-SOS2 protein complexes from either control or salt-stressed plants. This is perhaps not surprising, as it is known that TAP tagging methods do not identify all possible protein-protein interactions (51). This may be due to low expression levels of some proteins, membrane localization or low solubility that prevents extraction and solubilization in the low-detergent conditions used in isolating TAP complexes, or a conditional interaction that is dependent on some factor that is disrupted during the TAP purification. As an example, we also did not find SOS3, whose interaction with SOS2 is well characterized, in NTAP-SOS2 protein complexes. This may be due

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to the low expression of SOS3 or the fact that SOS3 is myristoylated and may not be separated from the membrane and solubilized by the mild conditions used for extracting the NTAP-SOS2 protein complexes. It is also consistent with the idea that SOS2 may compete with other proteins, such as other SnRK3s, for interaction with NDPK2. Thus, the TAP tagging and yeast two-hybrid approaches employed in our laboratory are complementary approaches that have allowed us to conduct a more complete search for SOS2-interacting proteins.

Interaction with SOS2 suggests a cytoplasmic role for CAT2 and CAT3. Both purification of TAP-tagged SOS2-containing protein complexes and yeast two-hybrid assays indicated an interaction of SOS2 with CAT2 and CAT3. The interaction of CAT2 and CAT3 with cytoplasmically localized SOS2 suggests that CAT2 and CAT3 function in the cytoplasm in addition to their roles in H_2O_2 detoxification in the mitochondria and peroxisome. While the targeting of CAT1 to the peroxisome has been confirmed (29), the targeting of other CATs, the portion of CAT protein that remains in the cytoplasm, and whether stress or other factors can change CAT localization are unclear.

While it is not known whether CAT2 or CAT3 is present in the same protein complexes as NDPK2 in vivo, their common interaction with SOS2 raises an interesting possibility that CAT activity may play a role in NDPK2 function. Song et al. (57) have shown that H_2O_2 inactivated human NDPK A (Nm23) by the reversible formation of disulfide cross-linking within the protein. They proposed that oxidative modification of NDPK may be a regulatory mechanism to control its activity (57). Modulation of NDPK2 activity could occur by CAT activity creating an H_2O_2-depleted zone inside or around a protein complex containing NDPK2. This scenario is possible because the catalytic rate of CAT is near the diffusion-limited maximum (4). If the loss of SOS2 prevents NDPK2 from assembling into a CAT-containing protein complex, this could lead to its more rapid deactivation. Such a scenario would also be consistent with the salt hypersensitivity of the *sos2-2 ndpk2* double mutant.

Alternatively, it has also been observed that CATs can be divided into two classes based on whether they possess significant peroxidase activity in addition to CAT activity (26) and that some CAT monomers can have peroxidase activity (4). If one or more of the *Arabidopsis* CATs possessed peroxidase activity toward NDPK2, this could alter their activities and hence stress and ABA responses. However, we are not aware of any attempt to test *Arabidopsis* CATs for peroxidase activity. Adding to this complexity is the fact that our results cannot distinguish whether it is CAT3 and CAT2 homotetramers, heterotetramers of CAT2 and CAT3, or CAT2 and CAT3 monomers that interact with SOS2 in vivo.

When taken together with previous results showing the interaction of SOS2 and other SnRK3s with ABI1 and ABI2 (24, 44), our results also raise the possibility that CAT2 and CAT3 could be in the same protein complex as ABI1 and ABI2. ABI1 and ABI2 have also been shown to be sensitive to inactivation by H_2O_2 in vitro (38–40), and Miao et al. (40) have recently provided evidence that H_2O_2-dependent inactivation of ABI1 and ABI2 is important for ABA signaling in vivo and may be mediated through ABI2 interaction with AtGPX3. Thus, if CAT2 or CAT3 were in the same protein complex as ABI1 or ABI2, both could also affect ABI1 or ABI2 protein phosphatase activity by the same mechanisms described above for NDPK2. Our finding that both NDPK2 and CATs interact with SOS2 identifies an additional set of proteins (SOS2, NDPK2, and CATs) that are involved in the junction between H_2O_2 and abiotic stress response. These results now make possible a range of targeted experiments to further define the molecular mechanisms by which these proteins connect H_2O_2 to salt stress responses.

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