Nucleoredoxin guards against oxidative stress by protecting antioxidant enzymes

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Cellular accumulation of reactive oxygen species (ROS) is associated with a wide range of developmental and stress responses. Although cells have evolved to use ROS as signaling molecules, their chemically reactive nature also poses a threat. Antioxidant systems are required to detoxify ROS and prevent cellular damage, but little is known about how these systems manage to function in hostile, ROS-rich environments. Here we show that during oxidative stress in plant cells, the pathogen-inducible oxidoreductase Nucleoredoxin 1 (NRX1) targets enzymes of major hydrogen peroxide (H2O2)-scavenging pathways, including catalases. Mutant nrx1 plants displayed reduced catalase activity and were hypersensitive to oxidative stress. Remarkably, catalase was maintained in a reduced state by substrate-interaction with NRX1, a process necessary for its H2O2-scavenging activity. These data suggest that unexpectedly H2O2-scavenging enzymes experience oxidative distress in ROS-rich environments and require reductive protection from NRX1 for optimal activity.

Results

A Pathogen-Inducible NRX Regulates Plant Immune Responses. Pathogen infection and subsequent activation of plant immune responses is associated with an oxidative burst and expression of selected TRX gene family members. Infection of wild-type (WT) cells

Significance

Cellular accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H2O2) is associated with stress responses as well as aging. The reactive nature of ROS marks these molecules as a serious threat to cell integrity. Consequently, eukaryotic cells deploy numerous antioxidant enzymes that detoxify ROS to protect them from ROS-induced damage to proteins. Although the importance of antioxidant enzymes is well understood, how these proteins avoid becoming damaged in the hostile, ROS-rich environments in which they function remains unknown. We show that in plant cells the oxidoreductase Nucleoredoxin 1 (NRX1) protects antioxidant enzymes such as catalase from ROS-induced oxidation. Importantly, this protective effect of NRX1 boosted the H2O2 detoxification capacity of catalase, thereby protecting the plant cell from oxidative stress.


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plants with the bacterial leaf pathogen *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326 resulting in strong gene expression of a member of the NRX subfamily of TRX enzymes (Fig. 1A). *Psm* ES4326 infection also increased abundance of mixed disulfides between NRX1 and its substrates (Fig. 1B), indicating its oxidoreductase activity was pathogen-inducible. To investigate if NRX1 plays a functional role in plant immunity, we examined knockout lines (Fig. 1A and C). Mutant *nrx1* plants showed constitutive expression of pathogenesis-related (PR) genes but could be rescued by expression of Flag-tagged NRX1 from a constitutive 35S promoter (Fig. 1C and D). Elevated PR gene expression in *nrx1* was associated with enhanced disease resistance against *Psm* ES4326, which was comparable to WT plants immunized by the immune hormone salicylic acid (SA) or immunized by prior exposure to an avirulent pathogen (Fig. 1E and F). This autoimmune phenotype of *nrx1* mutants was dependent on SA, as mutation of the SA biosynthesis enzyme isochorismate synthase (ICS1) diminished immunity against *Psm* ES4326 (Fig. 1G).

**Two Active Sites Make Differential Contributions to NRX1 Disulfide Reductase Activity.** The NRX1 protein harbors three TRX-like domains, two of which contain an active site sequence consisting of WC(G/P)PC (Fig. 2A). Accordingly, NRX1 exhibits disulfide reductase activity (15), but the respective contributions of each active site have not yet been analyzed. In contrast to WT NRX1, mutation of either the first or third TRX-like domain in NRX1 (C55,58S) and NRX1(C375,378S) proteins, respectively, resulted in reduced disulfide reduction activity (Fig. 2B and Fig. S1). However, NRX1(C375,378S) was much more efficient than NRX1(C55,58S), indicating that the majority of disulfide reduction activity occurred at the first TRX-like domain of NRX1. Simultaneous mutation of both the first and third TRX-like domains in the NRX1(C55,58,378,375S) protein completely abolished its ability to reduce insulin, demonstrating that these two domains contain all necessary active sites for disulfide reduction (Fig. 2B and Fig. S1). Because NRX1 proteins can dimerize (15), we investigated if active sites from different NRX1 proteins cooperate during disulfide reduction. Interestingly, when equal amounts of NRX1(C55,58S) and NRX1(C375,378S) were combined in an attempt to reconstitute the full enzymatic capacity of native NRX1 protein, we observed only a low level of activity that was comparable to NRX1(C55,58S) monomer (Fig. 2B). This suggests that the two active sites do not function independent from one another. Moreover, a single cysteine in one active site was not sufficient to support the second active site, as the two single cysteine mutants, NRX1(C58S) and NRX1(C378S), both exhibited reduced activity (Fig. 2C and Fig. S1), indicating a requirement for both active-site resolving cysteines for full oxidoreductase activity. Accordingly, disulfide reduction activity of the mutant proteins NRX1(C55,58,378) and NRX1(C365,375,378S) (Fig. S1), in which only a single cysteine of one active site was left intact, was completely abolished (Fig. 2C). Taken together, these findings imply that NRX1 reduces disulfide bonds of insulin by a dicysteinic mechanism and that disulfide reduction may require cooperative or sequential interaction of a substrate with two different active sites of NRX1.

**Identification of Stress-Induced Substrates of NRX1.** Next we sought to identify NRX1 substrates using a previously designed capture strategy that exploits the mechanism by which disulfides are reduced by TRX family members (16, 17). Mutation of the second active-site cysteine of TRX enzymes prevents complete resolution of the disulfide reduction reaction, resulting in trapping of the substrate via an intermediate mixed disulfide bond. Therefore, we replaced the second cysteine with serine in each active site of NRX1. The resulting NRX1(C58,378S) mutant protein was unable to reduce insulin, confirming its inability to complete disulfide reduction reactions (Fig. 2D and Fig. S1). The NRX1(C58,378S) protein was then immobilized on a column containing NHS-activated resin and incubated with total protein extracted from *Psm* ES4326-inoculated plants. After trapping substrates via the formation of mixed disulfide bonds, the column was rigorously washed, substrates eluted with DTT, and finally identified by mass spectrometry (Fig. 2E). Two control columns were also included and treated identically. Nonspecific binding to the column was monitored by inclusion of a control column containing only NHS-activated resin and incubated with total protein extracted from *Psm* ES4326-inoculated plants. After trapping substrates.
quenched resin without any NRX1 protein. A second control column contained immobilized WT NRX1, allowing us to distinguish between targets that interacted as substrate or nonsubstrate. Compared with the WT NRX1 control column, 69 proteins were found to be enriched in the NRX1(C58,378S) column (P < 0.05, ratio > 1.5) (Fig. 2F, pink circle). Additionally, 23 proteins were enriched in comparison with the resin-only control column (P < 0.05, ratio > 1.5) (Fig. 2F, yellow circle). To further eliminate nonspecific background binding, a comparison was run between the WT NRX1 control column and the resin-only control column (P < 0.05, ratio > 1.5). This identified 19 proteins, 7 of which overlapped with those previously identified as potential NRX1(C58,378S) substrates (Fig. 2F, blue circle) and were therefore excluded from further analysis. Thus, a total of 74 proteins were uniquely identified as potential substrates on the NRX1(C58,378S) column (Fig. 2F, pink and yellow circles excluding overlap with blue circle; Dataset S1). A more stringent analysis still retained 45 potential NRX1 substrates with high confidence (Fig. S2A).

Gene ontology (GO) analysis of the identified targets revealed the most prominent biological processes were electron transport and energy pathways (Fig. 2G and Fig. S2B), consistent with the fact that these pathways often involve highly oxidative conditions. In agreement with our experimental approach, responses to stress as well as responses to abiotic or biotic stimuli were also found to be prominent biological processes (Fig. 2G and Fig. S2B). GO analyses for molecular function revealed that many potential NRX1 substrates exhibit structural molecule or enzymatic activity (Fig. 2H and Fig. S2C). As enzymes often contain active-site cysteines, these findings could suggest that NRX1 regulates their activity.

**NRX1 Interacts with Enzymes of the Antioxidant H_{2}O_{2}-Scavenging Pathway.** Upon further inspection of the entire list of identified potential NRX1 substrates, remarkably we identified many enzymes that respond to oxidative stress and participate in antioxidant pathways. For instance, we identified several enzymes involved in the glutathione (GSH) and thioredoxin (TRX) pathways, which are crucial for detoxifying reactive oxygen species (ROS) and maintaining cellular redox balance. These enzymes include glutathione peroxidase (GPx), glutathione reductase (GR), and thioredoxin reductase (TRXR). Their interactions with NRX1 suggest a regulatory role in modulating their activity, potentially through redox-switch mechanisms. Further studies will be needed to elucidate the functional significance of these interactions in the context of plant stress responses.

**Dataset S1.** A more stringent analysis still retained 45 potential NRX1 substrates with high confidence (Fig. S2A).

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**Fig. 2. Identification of substrates of NRX1 oxidoreductase activity. (A) Unique domain structures of AtNRX1 compared with AtTRXh5, a conventional immune-inducible TRX. (B–D) Oxidized insulin (130 μM) was incubated with 0.3 mM DTT either alone (control) or together with 6 μM native NRX1 or indicated NRX1 active-site mutants. Formation of reduced insulin was measured at 650 nm. Error bars indicate SD (n = 3). (E) Schematic of NRX1 substrate capture experiment, using immobilized mutant NRX1(C58,378S). (F) Venn diagram illustrating proteins identified in the substrate capture experiment. Each circle represents proteins found to be enriched (P < 0.05, ratio > 1.5) in one of the following comparisons between columns: NRX1(C58,378S) column compared with WT NRX1 control column (pink); NRX1(C58,378S) column compared with resin-only control column (yellow); WT NRX1 control column compared with resin-only control column (blue). (G and H) GO term analysis was performed on the 74 targets enriched in the immobilized NRX1(C58,378S) mutant column for biological process (G) and molecular function (H) using Classification Superviewer on bar.utoronto.ca. Normalized frequencies, SD, and P values were determined from absolute values as described in ref. 36. Error bars represent SD. P ≤ 0.05 are printed bold.**

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of the main cellular H$_2$O$_2$-scavenging pathway (Dataset S1). H$_2$O$_2$ is directly detoxified by both catalases and APX enzymes. Our quantitative mass spectrometry strategy identified as statistically significant several *Arabidopsis* catalases as well as the main cellular ascorbate peroxidase, APX1 (Fig. 3, Figs. S3, and Dataset S1). Albeit outside the statistically significant range ($P<0.05$), we also consistently found the ascorbate regenerating enzymes, monodehydroascorbate reductase (MDAR1) and dihydroascorbate reductase (DHAR3), that underpin APX1 activity (Fig. 3A and Dataset S1). The pattern emerging from these data implicates NRX1 in the regulation of antioxidant enzymes.

Because of their prominent role in H$_2$O$_2$ detoxification, we set out to validate the interaction of catalase enzymes with NRX1 in vivo. We performed a denaturing coimmunoprecipitation designed specifically to capture transient mixed disulfide interactions in vivo (see SI Materials and Methods) and found that catalase readily coimmunoprecipitated with Flag-tagged NRX1 in the WT but not in the *cat2 cat3* genetic background in which the two predominant catalase genes were knocked out (Fig. 3B) (18). These results confirm the mass spectrometry data and show that catalases are indeed substrates of NRX1 in vivo.

**NRX1 Protects Cells Against Oxidative Stress.** The identification of ROS-scavenging enzymes as targets of NRX1 prompted us to investigate if NRX1 plays a role in oxidative stress. We first assessed the cell death response of mutant *nrx1* plants treated with the ROS-generating chemical methyl viologen (MV). Compared with WT, mutant *nrx1* plants displayed faster and more pronounced MV-induced cell death than the WT (Fig. 3C). This is reminiscent of the ROS intolerant phenotype of mutant *cat2* plants (19), which, like *nrx1* plants also display autoimmune phenotypes (20). We considered the possibility that NRX1 and catalases may act within the same ROS detoxification pathway. To investigate this further, we studied the phenotypes of an *nrx1 cat2* double mutant. Mutation of NRX1 did not further aggravate the developmental phenotype of *cat2* mutants (Fig. 3D). We then assessed the cellular redox status of these mutants. Compared with WT, mutant *nrx1* plants accumulated significantly higher levels of total glutathione, but the ratio of oxidized versus reduced glutathione was normal (Fig. 3E). These data suggest that in absence of a stimulus, *nrx1* mutants experience low levels of oxidative stress that are managed by increased glutathione levels. Mutation of NRX1, however, was not able to aggravate further the redox status of *cat2* mutants, which as reported previously (19) contained very high levels of total and oxidized glutathione (Fig. 3E). These data suggest that NRX1 and CAT2 do not act additively to control the redox status of resting cells (Fig. 3E).

To assess if NRX1 and CAT2 function together or additively in stressed cells, we measured cell death in single and double mutants that had been treated with MV. As expected, both *nrx1* and *cat2* single mutants displayed increased levels of cell death in comparison with WT (Fig. 3F). However, the *nrx1 cat2* double mutant was as susceptible to oxidative stress as *cat2* single mutants, indicating that mutation of these genes also did not act additively during oxidative stress. Taken together, these data demonstrate that NRX1 is essential for protection against oxidative stress and suggest that it may act by modulating the activity of catalases.

**Catalase Activity Is Controlled by NRX1-Mediated Reduction.** Our findings suggest that catalases may be recipients of oxidative posttranslational modifications and may require NRX1 to remain in the reduced state. To examine this possibility, we performed a reductive switch assay in which specifically oxidized cysteines
undergo DTT-dependent labeling with biotin followed by pull-down with streptavidin. Indeed, catalase was subject to oxidative modification, and regardless of the treatment, the level of oxidized catalase was greatly enhanced in nrx1 mutants compared with WT, supporting the notion that NRX1 controls the oxidation state of catalase (Fig. 4A). To determine if NRX1 plays a role in modulating catalase activity, we examined catalase activity in protein extracts from untreated, MV-treated, and Psm ES4326-infected plants. Decomposition of H$_2$O$_2$ could be largely attributed to catalase activity, as cat2 cat3 double mutants displayed only residual activity (Fig. 4B and Fig. S4A). In striking contrast to WT, in all treatments nrx1 mutants displayed significantly decreased catalase activity (Fig. 4B and Fig. S4A), despite comparable catalase protein levels between genotypes (Fig. S4B). A direct link between NRX1 activity and the ability of catalase to decompose H$_2$O$_2$ was found by incubating plant extracts with recombinant WT NRX1 or mutant NRX1(C55,58,375,378S) protein. Addition of either recombinant protein to cat2 cat3 extracts did not increase H$_2$O$_2$ consumption, indicating NRX1 cannot detoxify H$_2$O$_2$ by itself (Fig. 4C and Fig. S4C). By contrast, addition of recombinant NRX1 but not mutant NRX1(C55,58,375,378S) to nrx1 extracts remarkably restored catalase activity to levels comparable to those found in WT extracts (Fig. 4C and Fig. S4C). Moreover, recombinant NRX1 significantly increased catalase activity in WT plant extracts, whereas mutant NRX1(C55,58,375,378S) protein was ineffective in this respect. In summary, these data demonstrate that NRX1 controls the oxidation status of catalase and thereby modulates its H$_2$O$_2$ dismutation activity.

**Discussion**

During periods of oxidative stress, ROS-scavenging by peroxidase enzymes is essential to maintaining cell integrity. However, how these enzymes withstand the toxic oxidizing conditions in which they function remains elusive. In this study, we demonstrate that in plants, NRX1, a member of the TRX superfamily of enzymes, maintains catalase enzymes in a reduced state, thereby protecting their H$_2$O$_2$-detoxifying activity and, importantly, ensuring efficient functioning of the antioxidant system during oxidative stress.

Using extracts from immune-induced plants, we identified 74 potential endogenous substrates of NRX1 (Fig. 2F). Although some of these are unlikely to be real substrates, strikingly we found that NRX1 engaged with enzymes of a major H$_2$O$_2$-scavenging pathway, including Arabidopsis catalases (Fig. 3A and Fig. S2). Indeed, we confirmed that catalase was a substrate of NRX1 in vivo, and epistasis experiments strongly suggested that NRX1 and the major leaf catalase CAT2 act together in the same H$_2$O$_2$-detoxification pathway (Fig. 3). The full extent to which catalase activity contributes to plant immune responses has only recently come to light. Catalases have previously been reported to bind SA, and a recent study showed that SA-induced suppression of catalase activity contributed to plant immune responses has only recently come to light (21). Here we show that additionally, during oxidative stress and pathogen infection, CAT2 activity is regulated by NRX1. Whether this is a conserved role of NRX1 in other eukaryotes remains to be seen, but in addition to this study, NRX1 has been implicated in immune responses in both a crop plant and mammals (22, 23).

Catalases are widely reported to localize to the peroxisomes where they remove toxic H$_2$O$_2$ generated in cellular metabolism. However, NRX1 lacks known peroxisomal targeting sequences (PTSs) and is localized to the cytoplasm and nucleus (15). Thus, it is unlikely that peroxisomal catalases are a substrate of NRX1. Nonetheless, using a coimmunoprecipitation trap, we found that NRX1 formed a mixed disulfide intermediate with catalase in vivo (Fig. 3D). In this respect, it should be noted that peroxisomal import of catalases is dependent on cytosolic interaction with the PTS import receptor, PEX5 (24, 25). PEX5 docks with the peroxisomal membrane where it releases its cargo into the peroxisome matrix. Under oxidizing conditions, however, PEX5 has been shown to undergo redox-dependent monoubiquitination at a cysteine residue in the N terminus, preventing it from being recycled back to the cytosol (26). Consequently, during periods of oxidative stress, PEX recycling is blocked, resulting in decreased peroxisomal import of PEX5 targets, including catalase (25). Accordingly, a recent report demonstrated that pathogen-mediated transient expression of CAT3 in tobacco leaves resulted in its localization to the peroxisome, cytoplasm, and plasma membrane (27). Our findings suggest that oxidation-induced retention of catalases in the cytoplasm may enable these critical detoxifying enzymes to become substrates of NRX1.

**Fig. 4. NRX1 regulates redox modification and activity of catalase. (A)** Protein extracts from untreated, MV- (5 μM), or Psm ES4326- (5 × 10$^5$ cells) treated plants were alkylated and incubated with or without DTT (2 mM). Free thiols were labeled with biotin and pulled down with streptavidin. Oxidized catalase (OX-CAT) was visualized by immunoblotting with an anticaltase antibody and shown relative to total catalase. (B) The ability of protein extracts from untreated, MV- (5 μM), or Psm ES4326- (5 × 10$^5$ cells) treated plants to decompose H$_2$O$_2$ (5 mM) was measured at A$_{240}$ and leaf catalase activity (nmol H$_2$O$_2$·mg protein$^{-1}$·min$^{-1}$) calculated using the extinction coefficient of 0.036 cm$^{-1}$μmol. Error bars represent SE (n = 3). **p < 0.05 and ***p < 0.005 for statistical differences with WT and cat2 cat3; **p < 0.05 for statistical differences with WT and nrx1 (Student’s t test). (C) As in B, but extracts were supplemented with 4 μM recombinant NRX1 or NRX1(C55,58,375,378S) and 0.33 mM DTT. pNRX1, recombinant NRX1 protein added to reaction mixture; mut., mutant recombinant NRX1 protein. Error bars represent SE (n = 3). *p < 0.05 and **p < 0.005 for statistical differences compared to mutant recombinant NRX1 protein (Student’s t test).
Catalases contain several surface-exposed cysteine residues that may be sensitive to oxidation (28–30). Therefore, it is surprising that these enzymes function efficiently in ROS-rich environments without incurring significant damage. Here we found that catalases are indeed subject to inhibitory oxidative modification and demonstrate that NRX1 may be dedicated to maintaining these enzymes in a reduced state to promote their H$_2$O$_2$-scavenging activity (Fig. 4). Studies on the green alga *Chlamydomonas reinhardtii* have demonstrated reversible, partial inactivation of a single cysteine residue that is conserved among *Arabidopsis* catalases (30). Oxidation of a single cysteine implies that rather than a disulfide, catalase may contain an S-nitrosothiol or sulfenic acid. Although the ability of NRX1 to reduce these modifications has not yet been demonstrated, they are targeted by conventional TRX family members (31–33). Regardless of these particulars, this study clearly demonstrates that NRX1-mediated reduction of catalase is an important mechanism for sustaining maximal catalase activity.

So does the advantageous effect of NRX1 on H$_2$O$_2$ scavenging extend beyond regulation of catalases? Extraordinarily our proteomic capture experiments recovered nearly all major enzymes of the cytosolic glutathione/ascorbate-dependent H$_2$O$_2$-scavenging pathway, including APX, MDAR, and DHAR (Fig. 3 and Dataset S1), suggesting that they have been identified virtually in other proteomic screens for substrates of conventional TRX enzymes (see ref. 34 and references herein). The importance of the cytosolic glutathione/ascorbate cycle in H$_2$O$_2$ scavenging is exemplified by reports that showed reduced levels of APX protein or ascorbate deficiency resulted in high levels of oxidative stress, spontaneous cell death, and hypersensitiveness to plant pathogens (11, 35). Given that cysteine oxidation appears prevalent in controlling activities of enzymes in this H$_2$O$_2$-scavenging pathway, it is highly likely that the interactions found here between NRX1 and these enzymes are of functional significance to cellular redox homeostasis. Thus, NRX1 may play an important novel role in directly regulating the cellular capacity for H$_2$O$_2$ detoxification, thereby protecting plant cells from oxidative stress triggered by environmental challenges.

**Materials and Methods**

Detailed procedures for plant materials and treatments, protein assays, and identification of NRX1 substrates by mass spectrometry are described in SI Materials and Methods.

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