Cytoplasmic H₂O₂ prevents translocation of NPR1 to the nucleus and inhibits the induction of PR genes in Arabidopsis

Smadar Peleg-Grossman, Naomi Melamed-Book, Gil Cohen and Alex Levine*

Department of Plant and Environmental Sciences; The Hebrew University of Jerusalem; Givat-Ram-Campus; Jerusalem, Israel

Key words: salicylic acid, hydrogen peroxide, pathogenesis, NPR1, catalase, PR1

Plants activate a number of defense reactions in response to pathogen attack. One of the major pathways involves biosynthesis of Salicylic Acid (SA), which acts as a signaling molecule that regulates local defense reaction at the infection site and in induction of systemic acquired resistance (SAR). SA is sensed and transduced by NPR1 protein, which is a redox sensitive protein that acts as a central transcription activator of many pathogenesis related and defense related genes. In its uninduced state NPR1 exists as an oligomer in the cytoplasm. Following pathogen attack and SAR induction, cells undergo a biphasic change in cellular redox, resulting in reduction of NPR1 to a monomeric form, which moves to the nucleus. Recently, it was shown that pathogen attack or SA treatment cause S-nitrosylation of NPR1, promoting NPR1 oligomerization and restricting it in the cytoplasm. We used *A. thaliana* mutants in cytosolic *ASCORBATE PEROXIDASE*, *apx1* and plants expressing antisense *CATALASE* gene, as well as the CATALASE inhibitor 3-amino-1,2,4-triazole, to examine the effect of H₂O₂ on the pathogen-triggered translocation of the NPR1 to the nucleus. Our results show that the pathogen-triggered or SA-induced nuclear translocation is prevented by accumulation of H₂O₂ in the cytosol. Moreover, we show that increased accumulation of cytoplasmic ROS in *apx1* mutants reduced the NPR1-dependent gene expression. We suggest that H₂O₂ has a signaling role in pathogenesis, acting as a negative regulator of NPR1 translocation to the nucleus, limiting the NPR1-dependent gene expression.

Introduction

Classical genetic studies have established that plant-pathogen interactions are characterized by a gene-for-gene hypothesis, which posits that resistance (*R*) genes in the host interact with avirulence (*Avr*) genes in the pathogen.^{1,2} Plants activate a variety of defense responses when challenged by pathogens, such as production of reactive oxygen species (ROS), biosynthesis of antimicrobial compounds and activation of hypersensitive cell death, resulting in the limitation of pathogens' dispersion.³⁻⁵ Following the HR, plants develop a secondary, systemic defense program that provides protection against a wide range of pathogens, called Systemic Acquired Resistance (SAR).⁶

The SAR process is associated with elevated levels of Salicylic Acid (SA), which mediates the induction of pathogenesis related (PR) genes, both locally and systemically.⁷ Disease resistance in plants is regulated by multiple signal transduction pathways in which SA plays a central role.⁸ SA is sensed and transduced by a Redox-sensitive protein that contains Ankyrin rich repeats and a BTB-POZ domain, called Nonexpressor of PR Genes1 (NPR1). The NPR1 protein acts as a transcriptional coactivator regulating SA-dependent gene expression.⁹ In a quiescent state the NPR1

protein exists in the cytosol in oligomeric form.¹⁰ However, during pathogen attack the NPR1 protein is reduced to its monomeric state and translocated to the nucleus, where it binds a bZIP transcription factor of the TGA/OBF family.¹¹⁻¹³ The oligomeric state of the NPR1 protein is sustained by disulfide S-S bonds that involve cys82 and cys216.^{10,14} Mutations of these cysteines caused accumulation of monomers, resulting in constitutive localization of the NPR1 in the nucleus and *PR* gene expression, even in the absence of pathogens.

The formation and dissipation of disulfide bonds is regulated by redox changes, caused by ROS production on one hand, and biosynthesis of antioxidants on the other hand.¹⁵ Here, we used pharmacological and genetic manipulations to test the possibility that the cytoplasmic ROS function in maintaining the NPR1 complex in its oligomeric state. We show that accumulation of ROS in the cytoplasm excludes the NPR1 from the nucleus, inhibiting the *PR* gene expression.

Results and Discussion

Suppression of catalase activity inhibits NPR1 translocation to the nucleus. To analyze the subcellular localization of NPR1

*Correspondence to: Alex Levine; Email: alexl@cc.huji.ac.il

Submitted: 08/02/10; Accepted: 08/02/10

Previously published online: www.landesbioscience.com/journals/psb/article/13209

DOI: 10.4161/psb.5.11.13209

Figure 1. The effect of suppression of catalase activity on the localization of NPR1-GFP protein in the nucleus. (A–C) Roots of eight day-old NPR1-GFP (A and B) or NPR1-GFP plants that were crossed with antisense *Catalase* (C) were inoculated with *P. putida* (B and C) or left intact (A). Nuclear localization of NPR1 was analyzed 24 hours after inoculation, using DAPI stain. Brightfield light image (left part), UV filter (central part), narrow-pass GFP filter (right part). Bar = 25 μ m. (D) Seedlings of wild-type, mutants of *ASCORBATE PEROXIDASE1* (*apx1*) and antisense *CATALASE* (CAT AS) transgenics were infected with *P. putida*. ROS levels were analyzed 24 hpi as described in Methods. (E) Percentage of NPR1 localization in the nucleus 24 h.p.i. with *P. putida*. One hundred root hairs from 6 seedlings of NPR1-GFP and NPR1-GFP crossed with antisense *CATALASE* were scored in each treatment. Error bars indicate standard deviation of the mean.

during pathogen attack we tracked its localization in *A. thaliana* seedlings by the expression of GFP-labeled NPR1 protein. The NPR1-GFP plants were infected with *P. putida* bacteria and the roots analyzed by epifluorescence microscopy. Our previous data showed that the accumulation of ROS returned to normal level, as seen in uninfected seedlings, 24 hours post-inoculation (h.p.i.) of this soil bacteria into either *A. thaliana* or *M. truncatula* plants, after peaking at 5 h.p.i.¹⁶ Analysis of the NPR1-GFP protein localization 24 hours post infection showed that the protein was present in the nucleus, as has been reported in plants inoculated with pathogenic bacteria¹⁰ (Fig. 1B).

To study the role of ROS in the localization of NPR1, we crossed the transgenic *NPR1-GFP* seedlings with plants transformed with the antisense *Catalase* gene, which have reduced catalase activity by almost 90%.^{17,18} The hybrid plants were infected with *P. putida* in the roots region, and ROS production was analyzed 24 h.p.i., when the levels of ROS in wild-type plants returned to normal, as recorded prior to the bacterial inoculation (Fig. 1D). Plants that expressed the antisense *Catalase* gene failed to translocate the NPR1 protein to the nucleus (Fig. 1C). These results strongly suggest that the accumulation of ROS in the cytoplasm prevented breaking of the sulfhydryl bonds that keep the NPR1

monomers together (compare Fig. 1B with C). Quantitative analysis of the NPR1 localization in 100 cells (from six seedlings) of each genotype showed that the NPR1 was present in the nucleus in about 80% of root hairs of the plants inoculated with *P. putida*, three folds higher than uninfected. The expression of antisense *CATALASE* almost completely inhibited the translocation of NPR1 to the nucleus (Fig. 1E).

To substantiate the catalase-dependent inhibition of NPR1 translocation to the nucleus after challenging by pathogens, we replaced the *P. putida* with salicylic acid (SA) treatment. SA was shown to cause intracellular redox changes that were similar to infection with avirulent pathogens.^{10,19,20} Seedlings expressing the GFP-labeled NPR1 and hybrids expressing the GFP-labeled NPR1 with antisense *CATALASE* (NPR1-GFP AS-CAT) were treated with 0.5 mM SA for 24 hours. Plants were stained with DAPI and analyzed by confocal microscopy. The SA treatment caused NPR1 translocation to the nucleus with similar efficiency as the bacterial infection (Fig. 2). Moreover, SA treatment allowed us to assay the nuclear translocation also systemically, which occurred in the leaves, as well as in the roots (Fig. 2A and B). However, in plants that expressed the antisense *CATALASE* gene, the NPR1-GFP protein was detected in the cytoplasm. Quantification of the results showed that the effect

Figure 2. The effect of absence of catalase on the NPR1-nuclear localization. Eight-day-old NPR1-GFP (A and B), or NPR1-GFP crossed with antisense *CATALASE* (C and D) plants were exposed to Salicylic acid for 24 hours and analyzed for NPR1 nuclear localization in leaves (A and C) and roots (B and D) by confocal microscopy using DAPI stain. Brightfield light (BF), DAPI (nuclei, UV filter), GFP (narrow-pass GFP filter), RED (Chlorophyll autofluorescence, RED filter). Right column shows merged pictures. Note the greenish color in the NPR1-GFP plants and the lack of green color in plants crossed with antisense *CATALASE*. The experiment was repeated three times with very similar results. Quantification of the results is shown in Supplemental Figure S1. Bar = 20 μm.

of SA was suppressed by catalase inhibition (Sup. Fig. S1). These data are in line with the reported nuclear translocation of NPR1 in *A. thaliana* after bacterial challenge or after SA treatment.¹⁰

We also attempted to reduce catalase activity by a biochemical/pharmacological approach, using the herbicide *CATALASE* inhibitor, 3-amino-1,2,4-triazole (3ATA).²¹ Eight-day-old NPR1-GFP seedlings were either treated with 3ATA or left intact and were infected with *P. putida* or treated with SA after two hours. The 3ATA treatment prevented the translocation of NPR1 to the nucleus (Fig. 3, right two parts), while bacterial inoculation or SA treatment, induced nuclear NPR1 translocation (Fig. 3, left two parts).

Cytoplasmic ROS inhibit NPR1-dependent gene expression. To further analyze the effect of cytoplasmic ROS in the pathogenesis response, we examined the effect of cytoplasmic ROS on the *PR* gene expression. To study the effect of increased H₂O₂ accumulation in the cytoplasm we analyzed mutants in the major cytosolic antioxidant enzyme, the Ascorbate Peroxidase (APX).²² Comparison of ROS accumulation in wild-type and *apx1* mutants 24 hours after inoculation of *P. putida* showed that the level of ROS in the *apx1* mutants was more than twice that was seen in the wild-type plants (Fig. 1D).

The expression of *PR1* gene in wild-type and *apx1* mutants was analyzed in plants infected 24 h.p.i. with *P. putida* at 24 hours

after inoculation. As expected, inoculation of *P. putida* caused a major increase in the *PR1* gene expression in the wild-type plants, in agreement with results of infections by a variety of pathogenic microorganisms.^{23,24} In contrast, expression of *PR1* in the *apx1* mutants remained low (Fig. 4A).

Next, we analyzed the effect of high levels of increased ROS production on the SA-induced expression of NPR1-dependent genes, *PR1* and *WAK1* (Wall Associated Kinase1).²⁵ Eight-day-old wild-type and *apx1* mutants seedlings were either treated with SA or left intact and the expression of *PR1* and *WAK1* genes was tested 24 h.p.i. Very high expression of both genes was seen 24 hours after SA treatment in the wild-type plants, but in the *apx1* mutants the expression of these genes was strongly reduced (Fig. 4B). The results from both genes that were obtained by quantitative Real-time PCR results were also observed by semi-quantitative RT-PCR (Fig. 4B and inset).

In addition, we tested *WAK1* expression in plants expressing the antisense *CATALASE* gene after treatment with SA. Eight-day-old wild-type and antisense *CATALASE* transgenics were either treated with SA or left intact. Very high expression of *WAK1* was seen 24 hours after SA treatment in the wild-type plants. However, in the antisense *CATALASE* plants the *WAK1* expression was the same as in untreated plants (Fig. 4C), which is consistent with the results of *apx1* mutants (Fig. 4B). Thus,

Figure 3. The effect of inhibition of catalase on the NPR1-nuclear localization. Eight-days-old NPR1-GFP seedlings were transferred to medium containing 20 μ M 3ATA and after 2 hours either infected with *P. putida* or exposed to Salicylic acid. After another 24 hours the roots were analyzed for NPR1 nuclear localization, using DAPI staining as described in Materials and Methods. The assay was repeated three times, each with four repeats, with very similar results. Bar = 40 μ m.

we show that inhibition of both enzymes that function specifically in the detoxification of H_2O_2 induced NPR1-dependent gene expression.

Materials and Methods

Biological material and plant treatment. *A. thaliana* seeds were grown on agar plates containing 1/2 MS medium, placed in 4°C for 48 hours and then transferred to 25°C. Seedlings were treated with one of the described treatments 8 days after germination by transferring them to medium containing 1/2 MS supplemented with 3-ATA or SA. Bacteria were grown in LB at 28°C and inoculated on the roots at a concentration of 10^7 cells.

GFP-labeled NPR1 *A. thaliana* seeds were a gift from Xinnian Dong (Duke University, North Carolina, USA), antisense *CATALASE* plants were from Wim Van Camp (University of Gent, Belgium) and *apx1* mutant seeds were a gift from Ron Mittler (University of Nevada, USA).

ROS production in plants. ROS were detected by 10 μ M 2', 7'-dichlorodihydrofluorescein diacetate and quantified using the ImagePro Plus analysis package (Media Cybernetics, USA) as described by ref. 27. Roots were photographed with Nikon Coolpix 4500 camera attached to Olympus IX70 microscope. The fluorescent light pass settings used narrow-band cube (Omega Optical Inc., Brattleboro, VT USA). The pixels of mean density were collected from representative images for statistical analysis ($N = 12$). Confocal microscopy: roots were viewed using a MRC-1024 confocal microscope (Bio-Rad) equipped with 40x oil immersion objective (N.A. 1.3). Time series sections were collected once every 30 sec.

RT-PCR assay. Total RNA was extracted from roots before and after treatment. Roots were frozen in liquid nitrogen; RNA was extracted with Tri Reagent (Molecular Research Center, Inc.) and transcribed into cDNA using oligo dT as a primer with SuperScript II reverse transcriptase (Invitrogen). cDNA was amplified by semi-quantitative PCR using Taq polymerase and the following primers: *PR1*(At2g14610): forward AGG CAA CTG CAG ACT CAT ACA C and reverse CAC ATC CGA GTC TCA CTG ACT T; *WAK1*(At1g21250): forward GCA CAT GAC TTC TTT TCA CGA and reverse GCG GTA ACC AGA TTG ACA CTT; *Actin2*, forward taa ccc aaa ggc caa cag ag; reverse ctt ggt gca agt gct gtg at. *Actin2* was used to normalize RNA amounts. For RealTime PCR RNA was extracted and transcribed to cDNA using a Rotor Gene 2000 thermocycler (Corbett Research), and the following primers: *Actin-2* gene was used for normalization. L-5'-CTG CTT GGT GCA AGT GCT GTG ATT-3'; R-5'-AGA AGT CTT GTT CCA GCC CTC GTT-3'; *PR1*: forward AAG GAG CAT CAT ATG CAG GA; reverse ATT TAA ATA GAT TCT CGT AAT CTC AGC; *WAK1*: forward ACT GCA TGC TTG TCA TTA TGC; reverse AAG CGT AGG TGC AAG GAC TAA. For quantification, calibration curves were run simultaneously with experimental samples, and Ct calculations were performed by the Rotor-Gene 5.0 software (Corbett Research).

Concluding Remarks

The above results show that cytoplasmic H_2O_2 prevented the NPR1 translocation to the nucleus, resulting in suppression of NPR1-dependent *PR* gene induction. Recently, it was suggested that the

Figure 4. The effect of ROS accumulation on NPR1-dependent gene expression. (A) Wild-type and *apx1* mutants were infected with *P. putida* (*P.p*) and RNA was isolated after 24 hours. Gene expression was analyzed by semi-quantitative RT-PCR with *PR1* primers. Bands were quantified using ImagePro Plus software package. Error bars indicate standard deviation of the mean from repeats (N = 5). (B) Eight days old wild-type (WT) and *ASCORBATE PEROXIDASE1* (*apx1*) mutants were treated with 0.5 mM SA for 24 hours. Gene expression was analyzed by Real-Time PCR or semi-quantitative PCR (inset) using primers for *PR1* or *Wall Associated Kinase1* (*WAK1*). Error bars indicate standard deviation of the mean from 6 repeats (N = 6). RNA samples for all assays were normalized according to the expression of the β -actin gene. All experiments were repeated 3 times with very similar results. (C) Eight days old wild-type (WT) and antisense *CATALASE1* (*CAT*) plants were treated with 0.5 mM SA or left intact. RNA from roots was isolated from the roots 24 hours later and analyzed by quantitative Real-Time PCR using *WAK1* primers. Error bars indicate standard deviation of the mean of 6 repeats (N = 6).

oxidizing force that restricts the NPR1 protein in the cytoplasm as an oligomer, preventing its nuclear translocation is the nitric oxide (NO) donor *S*-nitrosoglutathione (GSNO).²⁶ However, that conclusion was based on in vitro studies, using a cell-free system, in which the pro-oxidant compounds were added to a total protein extract. Therefore, the in vitro approach failed to detect the secondary and indirect effects. For example, Tada and co-workers showed that H₂O₂ had no effect on the state of the NPR1 protein when added to the total protein extract in vitro.²⁶ However, our

results show that the increased H₂O₂ concentration caused by suppression of *CATALASE* gene expression or by inhibition of catalase activity by 3ATA prevented translocation of NPR1 to the nucleus (Figs. 1–3), and inhibited the NPR1-dependent gene expression (Fig. 4). Moreover, the expression of *PR1* and *WAK1* gene after either SA treatment or inoculation of *P. putida* was suppressed in *apx1* mutants (Fig. 4). Therefore, we conclude that H₂O₂ inhibits the breakdown of the disulfide bonds in the NPR1 oligomer. It is possible that in vivo H₂O₂ interacts with the GSNO.

Acknowledgements

The work was supported by grants from Israel Science Foundation (ISF 437/07) and from Goldinger Trust. We thank the Canadian Friends of the Hebrew University for graduate students support.

Note

Supplemental materials can be found at:
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