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Inducers of Plant Systemic Acquired Resistance Regulate NPR1 Function through Redox Changes

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Summary

NPR1 is an essential regulator of plant systemic acquired resistance (SAR), which confers immunity to a broad-spectrum of pathogens. SAR induction results in accumulation of the signal molecule salicylic acid (SA), which induces defense gene expression via activation of NPR1. We found that in an uninduced state, NPR1 is present as an oligomer formed through intermolecular disulfide bonds. Upon SAR induction, a biphasic change in cellular reduction potential occurs, resulting in reduction of NPR1 to a monomeric form. Monomeric NPR1 accumulates in the nucleus and activates gene expression. Inhibition of NPR1 reduction prevents defense gene expression, whereas mutation of Cys82 or Cys216 in NPR1 leads to constitutive monomerization, nuclear localization of the mutant proteins, and defense gene expression. These data provide a missing link between accumulation of SA and activation of NPR1 in the SAR signaling pathway.

Introduction

Plants, like animals, have evolved both innate and acquired immunity to counter attacks by microbial pathogens. Each plant genome encodes hundreds of so-called resistance (R) proteins that allow the plant to recognize specific pathogen-derived molecules known as avirulence (avr) factors. This R-avr recognition between plants and pathogens often triggers the hypersensitive response (HR), which involves programmed cell death, production of reactive oxygen species (ROS), synthesis of antimicrobial compounds at the site of infection, and ultimately leads to pathogen resistance (reviewed by Dangl and Jones, 2001). Thus, these R proteins determine the innate immunity of each plant species and individuals within a species. Concomitant with the appearance of an HR, a secondary resistance response known as systemic acquired resistance (SAR) is induced in the uninfected tissues (reviewed by Sticher et al., 1997). SAR provides long-lasting resistance throughout the plant to subsequent infections by a broad range of pathogens.

The establishment of SAR is associated with elevated levels of salicylic acid (SA) both at the site of infection and in systemic tissues. Exogenous application of SA or its biologically active analogs, such as 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), leads to the activation of SAR. In contrast, expression of a bacterial salicylate hydroxylase (*nah*G) gene, which inactivates SA by conversion to catechol, suppresses SAR. Therefore, SA is a necessary and sufficient signal molecule for SAR induction.

It is not completely understood how SA brings about SAR. SA has been shown to induce the concerted expression of genes known as pathogenesis-related (*PR*) genes, which encode small proteins that are either secreted from the cell or targeted to the vacuole (Sticher et al., 1997). An important aspect of understanding the induction mechanism of SAR is to identify factors that connect the SA signal with the expression of *PR* genes. Interestingly, mutants in only one genetic locus, *npr1* (nonexpressor of *PR* genes, also known as *nim1* and *sai1*), have been found to block SA signaling in *Arabidopsis thaliana* (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). This indicates that either NPR1 is the only protein component between SA increases and the onset of SAR or that other signal components are either functionally redundant or indispensable for plant growth and development.

The recessive *npr1* mutants fail to respond to various SAR-inducing agents, displaying little expression of *PR* genes, and exhibiting enhanced susceptibility to a wide range of pathogens. This suggests that the wild-type NPR1 protein is a positive regulator of SAR required for transducing the SA signal to activate downstream *PR* gene expression. This NPR1 signal transduction pathway is highly conserved in plant species as demonstrated by the ability of the *Arabidopsis NPR1* gene to function in heterologous plant species such as rice (Chern et al., 2001).

The *NPR1* gene encodes a protein containing a bipartite nuclear localization sequence (NLS) and two potential protein-protein interaction domains: one ankyrin-repeat domain and one BTB/POZ (*broad-complex*, *tramtrack*, and *bricà-brac*/poxvirus, zinc finger) domain (Aravind and Koonin, 1999; Cao et al., 1997; Kinkema et al., 2000). The mechanism by which NPR1 regulates transcription of SAR-related gene expression has been studied extensively. In our previous studies, the NPR1 cDNA was fused with the coding region of green fluorescent protein (GFP) to examine the subcellular localization of NPR1 in living plant cells (Kinkema et al., 2000). The biologically active NPR1-GFP fusion protein was seen accumulating in the nucleus in response to both chemical and biological inducers of SAR. Critically, nuclear localization of NPR1 was also demonstrated to be a prerequisite for its regulation of *PR* genes.

Even though NPR1 functions in the nucleus in regulating gene expression, it is unlikely to be a transcription factor, as it lacks any bona fide DNA binding domains. The presence of two protein-protein interaction domains in NPR1 suggests that it might regulate SAR-related gene expression through interaction with transcription factors. Indeed, NPR1 has been shown in multiple yeast

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two-hybrid screens to interact with the TGA subclass of basic leucine-zipper (bZIP) transcription factors (Després et al., 2000; Zhang et al., 1999; Zhou et al., 2000). These TGA factors can bind to the *as-1* element present in the *PR1* gene promoter, which is required for SA-responsiveness of the gene (Lebel et al., 1998).

Although the role of NPR1 in SA-mediated gene expression has been clearly demonstrated, the mechanism of NPR1 activation by SA is unknown. Expression of the *NPR1* gene is constitutive, influenced only moderately by SA (Cao et al., 1997). Therefore, SA must affect NPR1 function at the protein level. Using radioactively labeled SA, we found no SA binding activity using purified NPR1 protein (Xin Li and X.D., unpublished data), indicating that SA probably controls NPR1 through an indirect mechanism.

Previous studies have suggested that changes in SA concentration after pathogen infection could affect the redox state of the cell (Chen et al., 1993; Noctor et al., 2002; Vanacker et al., 2000). This, together with the observation that NPR1 and NPR1-like proteins from four plant species contains ten conserved cysteines (Mark Kinkema and X.D., unpublished data), led us to hypothesize that NPR1 protein conformation may be sensitive to cellular redox changes. To test this idea, we examined the NPR1 protein under different redox conditions and in the presence or absence of various SAR inducers. Here, we present the results from biochemical and genetic studies showing that in the absence of SAR induction, NPR1 exists as an oligomer formed through intermolecular disulfide bonds and is sequestered from transport into the nucleus. After SAR induction, following an initial oxidative burst, plant cells attain a more reducing environment due to the accumulation of antioxidants. Under these conditions, NPR1 is converted from an oligomeric to a monomeric state through reduction of intermolecular disulfide bonds. The monomeric NPR1 protein then moves into the nucleus to control SAR-related gene expression.

Results

NPR1 Is Reduced from an Oligomer to a Monomer after Treatment with SAR Inducers

To determine whether the cysteines in NPR1 affect the conformation of the protein before and after SAR induction, we performed protein extractions in the absence of reducing agents, such as dithiothreitol (DTT). As controls, DTT (50 mM) was later added to aliquots of the same extracts. Protein extracts from wild-type Columbia (Col-0) plants treated with the SAR inducer INA or with water were subjected to SDS-PAGE, followed by immunoblot analysis. In the absence of DTT, NPR1 protein (MW 66,000) was only observed in samples prepared from INA-treated plants but not from water-treated plants (Figure 1A). However, in the samples to which DTT was added after extraction, equal amounts of NPR1 protein were detected with or without INA treatment. INA treatment seemed to have the same effect on the NPR1 protein as DTT. Similar results were obtained from samples treated with two other SAR inducers, SA and BTH (data not shown). These results suggest that NPR1 may exist in a different state or conformation before and after SAR induction. Evidently, the polyclonal antibody raised against the N-terminal half of NPR1 could only recognize the INA-activated form of the protein.

To detect NPR1 in both INA-treated as well as untreated samples, we used another NPR1 antibody raised against a 16 amino acid peptide at the C terminus and found that it crossreacted with other homologs of NPR1 in *Arabidopsis* (data not shown). As an alternative approach, we tested the previously characterized transgenic line carrying the *35S::NPR1-GFP* transgene in the *npr1-1* mutant background (Kinkema et al., 2000). NPR1-GFP is regulated in a similar fashion to endogenous NPR1, allowing us to use this transgenic line to study the behavior of NPR1 using a commercially available antibody against GFP.

Protein samples extracted from *35S::NPR1-GFP (npr1-1)* plants treated with INA or water were subjected to SDS-PAGE and immunoblot analysis. As shown in Figure 1B, in the absence of DTT, the NPR1-GFP fusion protein was detected at the expected MW (93,000) only in the INA-treated sample but not in the untreated sample. This is consistent with the result obtained using the antibody against the endogenous NPR1. However, an additional protein band with a much higher MW was detected in both samples. The addition of DTT to both extracts eliminated this complex and reduced all the NPR1 protein to the monomeric form on the gel as shown by the downward shift of the high MW protein band to MW 93,000. This suggests that an NPR1-containing complex is formed through intermolecular disulfide bonds. Significantly, the disulfide bonds can be partially reduced as a result of INA induction.

The reduced monomeric form of NPR1 observed in the immunoblot analysis probably represents an in vivo conformation of the protein, because in a gel filtration experiment, under nondenaturing conditions, monomeric NPR1 was detected after SAR induction (data not shown). However, the high MW protein complex detected in the immunoblot analysis could be a product of nonspecific crosslinking caused by disulfide bond formation during sample preparation. To rule out this possibility, we homogenized plant tissue and denatured the protein in the presence of the alkylating agents, 2 mM iodoacetamide (IOD) or *N*-ethylmaleimide (NEM), which block free thiol groups from forming nonspecific disulfide bonds while keeping existing disulfide bonds intact (Murphy et al., 2002; Lee et al., 2002). The immunoblot of the resultant samples is shown in Figure 1C, revealing that treatment with IOD or NEM during sample preparation did not affect the formation of the high MW protein complex, confirming that the complex existed in intact plant cells prior to protein extraction. Identical results were obtained using 20-fold higher concentration of NEM. As an additional control, immunoblot analysis of samples from *35S::GFP* transgenic plants treated with INA or water showed that the GFP protein alone did not form a high MW complex (data not shown), indicating that the complex observed in the *35S::NPR1-GFP* transgenic line formed through the cysteines in NPR1.

To determine the nature of the high MW complex, we added a low concentration (0.5 mM) of DTT to the protein extraction buffer to artificially generate intermediates of the oligomer-to-monomer reaction. As shown in Figure

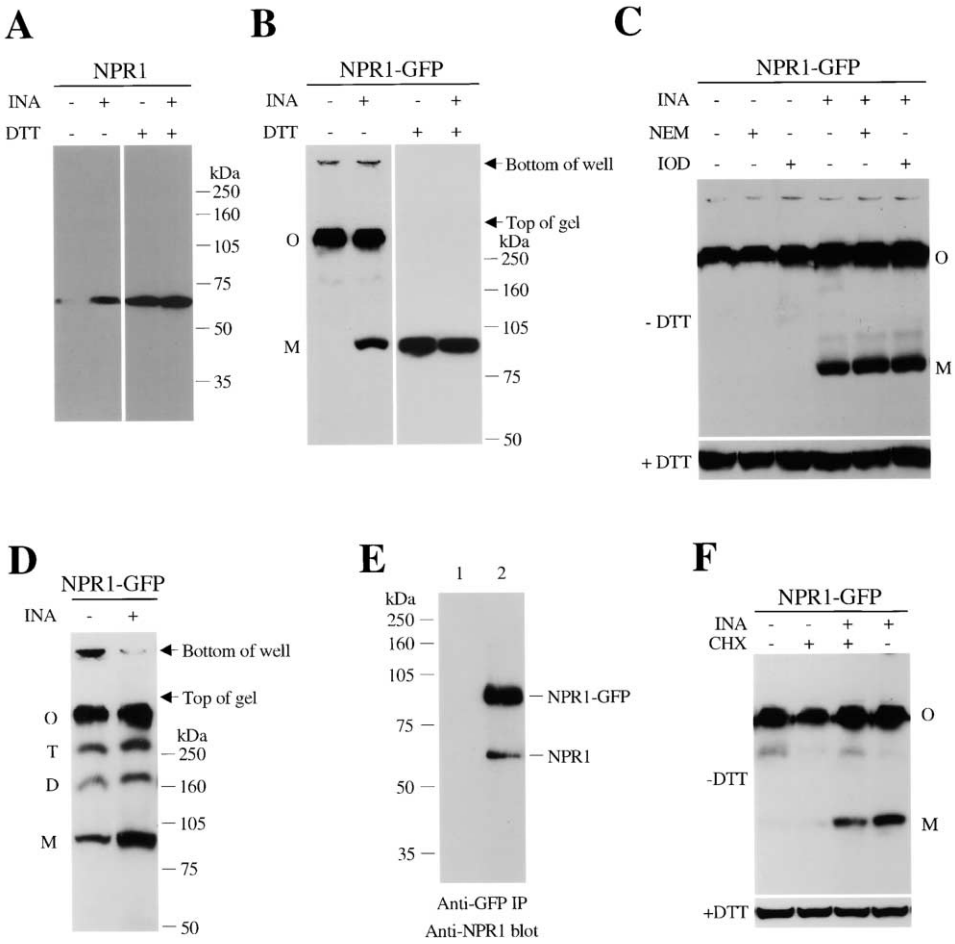

Figure 1. NPR1 Is Reduced from an Oligomer to a Monomer after INA Induction

(A) Total protein (100 μg) extracted from wild-type Columbia (Col-0) plants treated with (+) or without (−) 0.5 mM INA for 2 days was subjected to SDS-PAGE with (+) or without (−) DTT (50 mM) in the sample buffer and analyzed by immunoblot using a polyclonal anti-NPR1 antibody. Numbers represent MW.

(B) Total protein extracted from *35S::NPR1-GFP* transgenic plants (in *npr1-1*) was analyzed as in (A) using a monoclonal anti-GFP antibody. Both oligomeric (O) and monomeric (M) forms of NPR1-GFP were detected. The bottom of the wells and the top of the separating gel are shown for reference.

(C) Tissues from *35S::NPR1-GFP* transgenic plants (in *npr1-1*) treated with (+) or without (−) 0.5 mM INA were homogenized in the presence (+) or absence (−) of 2 mM iodoacetamide (IOD) or 2 mM *N*-ethylmaleimide (NEM). The protein was denatured by adding an equal volume of sample buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 25% glycerol, and 0.4% bromophenol blue), incubating at room temperature for 30 min, and heating at 60°C for 10 min. For the IOD or NEM treated samples, 2 mM IOD or 2 mM NEM was also included in the sample buffer. Immunoblot analysis was performed as in (B).

(D) Tissues from *35S::NPR1-GFP* transgenic plants (in *npr1-1*) treated with (+) or without (−) 0.5 mM INA were homogenized in the presence of a low concentration of DTT (0.5 mM). NPR1-GFP reduction intermediates, dimer (D) and trimer (T) as well as monomer (M) and oligomer (O), were detected using immunoblot. The bottom of the wells and the top of the separating gel are shown for reference.

(E) Total protein was extracted from wild-type (lane 1) and *35S::NPR1-GFP* transgenic (lane 2) plants. The extracts were immunoprecipitated using anti-GFP monoclonal antibody. The precipitated proteins were eluted and analyzed by immunoblot using the anti-NPR1 antibody.

(F) Total protein was extracted from *35S::NPR1-GFP* plants treated with (+) or without (−) 0.5 mM INA (24 hr) and 100 μM cycloheximide (CHX; 26 hr) and was subjected to SDS-PAGE with (+) or without (−) DTT (50 mM) in the sample buffer and analyzed by immunoblot analysis. A proteasome inhibitor MG132 (10 μM) was included in all treatments to prevent protein degradation.

1D, under these weakly reducing conditions, proteins with MWs of the NPR1-GFP dimer and trimer were detected. This result indicated that the high MW protein complex is either a homooligomer of NPR1 or a heterooligomer formed between NPR1 and a protein of similar MW. Immunoprecipitation performed using the anti-GFP monoclonal antibody showed that this antibody could not only precipitate NPR1-GFP but also the endogenous NPR1 (Figure 1E). This observation favors the hypothesis that the high MW complex is a homooligomer of NPR1. From the 8% SDS-PAGE gel in Figure 1D, it is difficult to determine precisely how many NPR1-GFP proteins are in the oligomer. It is possible that the high MW band on the gel consists of oligomers with varying numbers of NPR1. We cannot rule out the possibility that other proteins besides NPR1 are part of this complex.

To demonstrate that the NPR1 oligomer can be reduced to the monomer in vivo, we also performed an

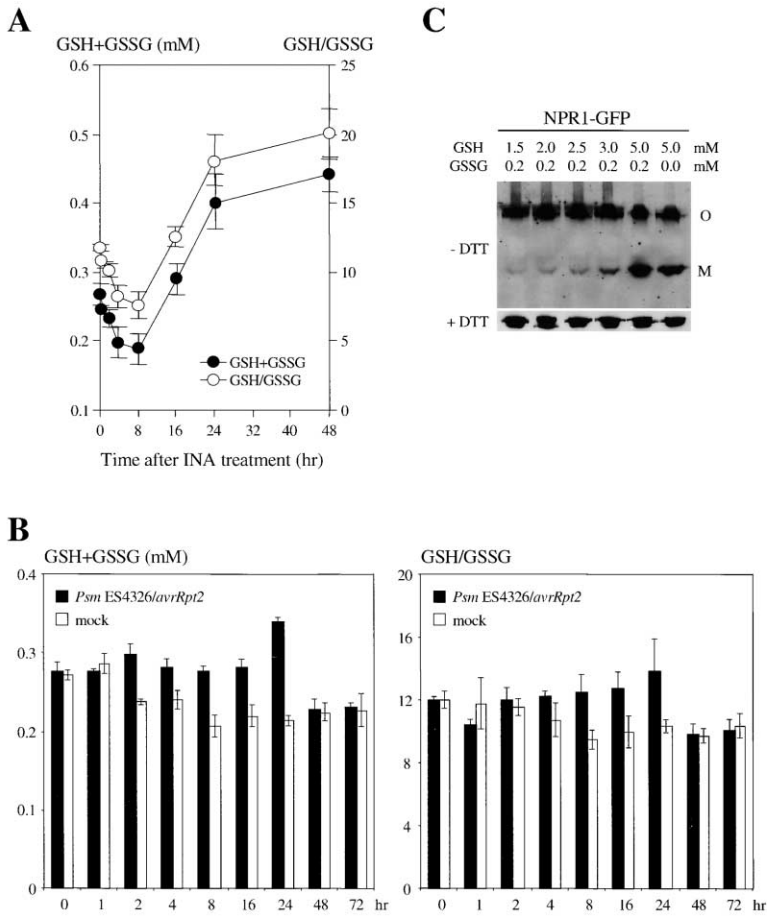

Figure 2. INA- and Pathogen-Induced Changes in Cellular Glutathione Pool and Corresponding Effects on NPR1 Conformation

(A) *35S::NPR1-GFP* transgenic plants (in *npr1-1*) were treated with 0.5 mM INA solution. Tissues were collected at the indicated time points after treatment. Total glutathione (GSH+GSSG) and GSSG were measured and the GSH/GSSG ratio was calculated (Rao and Ormrod, 1995). The error bars represent standard deviation of three samples.

(B) Half leaves were mock inoculated with $MgCl_2$ (10 mM) or infected with Psm ES4326/*avrRpt2* (OD_{600} = 0.02) and the uninoculated halves were collected at the indicated time points for glutathione measurements. The error bars represent standard deviation of three samples.

(C) Total protein (100 μg) extracted from *35S::NPR1-GFP* transgenic plants (in *npr1-1*) was incubated with the indicated amounts of GSH and GSSG at 0°C for 1 hr and then subjected to SDS-PAGE with (+) or without (−) DTT (50 mM) in the sample buffer. Immunoblot was performed as described in Figure 1.

INA induction experiment in the presence of 100 μM of cycloheximide to inhibit new protein synthesis (Osterlund et al., 2000; data not shown). Without protein synthesis, NPR1 monomer still appeared after INA treatment, indicating that the monomeric NPR1 came from reduction of the oligomer (Figure 1F).

SAR Induction Changes the Redox State of Plant Cells

The INA-induced switch of NPR1 protein from oligomer to monomer suggests that there might be a decrease in the reduction potential in plants following induction. To test this hypothesis, we measured the changes in the glutathione pool (GSH+GSSG) after INA treatment, because glutathione is a major thiol-disulfide redox buffer in plant cells (May et al., 1998; Schafer and Buettner, 2001). Measurements of total glutathione and GSSG levels have previously been made to estimate the redox environment in plants after pathogen attack (Vanacker et al., 2000). As shown in Figure 2A, INA treatment caused similar changes in kinetics in the total glutathione levels and the GSH/GSSG ratios, which first showed a dramatic decrease 8 hr after INA treatment, followed by a sharp increase reaching a plateau 24 hr after INA treatment. The change in the GSH/GSSG ratio appeared to be influenced mainly by the concentration of GSH.

To determine whether similar redox changes also occur in systemic tissues, we measured the total glutathione levels and the GSH/GSSG ratios in plants inoculated with the bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326 (Psm ES4326) carrying the avirulence gene, *avrRpt2*. Half leaves were infected with the pathogen and the uninfected halves were collected for the measurements. We found that in these uninfected tissues total glutathione and GSH/GSSG ratio were significantly increased, reaching peak levels of 0.34 mM and 13.9:1, respectively, 24 hr after infection (Figure 2B). These increases were not observed in mock-inoculated plants. We also measured glutathione concentrations in leaves distal to the infection site and did not observe consistent results, probably because the signals were too weak or too diluted.

To test whether the observed changes in the glutathione pool and GSH/GSSG ratio could result in reduction of the NPR1 oligomer in vitro, we incubated protein extracts from uninduced plants in defined concentrations of GSH/GSSG at 0°C for 1 hr before SDS-PAGE. As shown by immunoblot analysis (Figure 2C), the NPR1-GFP monomer began to appear in significant amounts when the GSH/GSSG ratio exceeded 15:1. To test whether this reaction is reversible, we also used protein extracts from INA-treated plants. At a GSH/GSSG ratio below 7.5:1, the monomeric NPR1 started to be oxidized to the oligomeric form (data not shown).

It is interesting to note that the GSH/GSSG ratio required for in vitro NPR1 reduction is similar to that

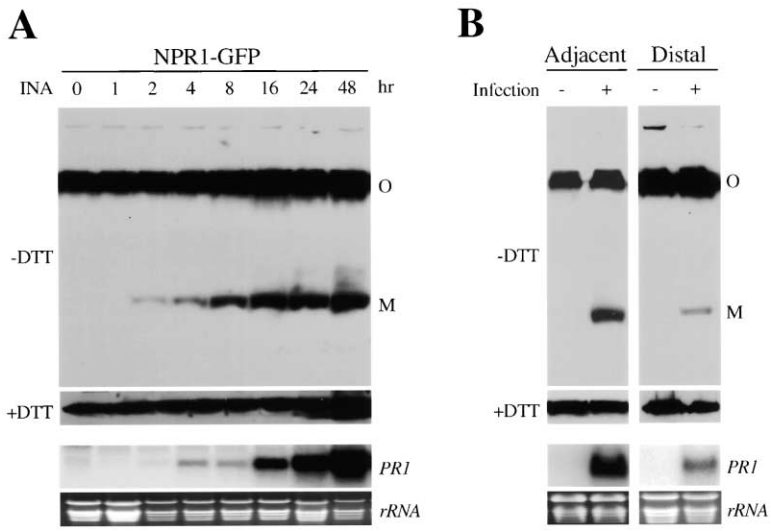

Figure 3. NPR1 Reduction Correlates with *PR1* Activation

(A) *35S::NPR1-GFP* transgenic plants (in *npr1-1*) were treated with 0.5 mM INA solution. Tissues were collected at the indicated time points after INA treatment. Total protein (100 μg) was extracted and analyzed using immunoblots as in Figure 1. Expression of the *PR1* gene was examined using RNA gel blot analysis. Loading of total RNA (20 μg) for each lane on the blot was represented by ethidium bromide staining of rRNA prior to blotting.

(B) Half leaves of 3-week-old *35S::NPR1-GFP* transgenic plant (in *npr1-1*) were inoculated with Psm ES4326/*avrRpt2* (OD_{600} = 0.02). The uninfected halves (adjacent) and distal leaves (distal) were collected 24 hr and three days after infection, respectively, and used for immunoblot and RNA gel blot analyses.

reached in plants after SAR induction. However, these data only show a correlation of the redox changes of these two redox couples, they do not indicate that GSH/GSSG and NPR1 monomer/oligomer are in redox equilibrium. These data confirm that SAR induction leads to a decrease in cellular reduction potential. Consequently, the cysteine residues in NPR1 are reduced, releasing the NPR1 monomer from the oligomeric complex. The reducing agent that is directly involved in this process has yet to be determined.

NPR1 Reduction Is Required for *PR* Gene Expression

SAR induction not only switches NPR1 protein from oligomer to monomer, but also activates downstream *PR* gene expression, suggesting that NPR1 monomerization might be a regulatory mechanism by which SA turns on NPR1-dependent gene expression. To test this hypothesis, a time course experiment was performed to follow the kinetics of NPR1 reduction and NPR1-dependent expression of *PR* genes. Following INA treatment, monomerization of the NPR1 protein preceded expression of *PR1*, as shown in Figure 3A. Significant amounts of NPR1 monomer were detected 8 hr after INA treatment while *PR1* gene expression became prominent 16 hr after INA treatment. The tight correlation between the two events supports the hypothesis that NPR1 protein monomerization is a regulatory step required for the activation of *PR* gene expression.

Even though NPR1 reduction and *PR1* gene activation (Figure 3) correlated with the cellular redox changes as measured using glutathione (Figure 2), NPR1 reduction seemed to appear earlier than the GSH/GSSG ratio increase. This might be explained by the nonsynchronous nature of induction. In the induced tissue, the redox environment in some cells might be reduced enough for NPR1 monomerization and *PR1* gene activation, while in other cells the reduction potential had yet to decrease. The overall GSH/GSSG ratio might not accurately reflect the redox environment of the cellular compartment (cytosol) where NPR1 resides. Glutathione concentrations of the endoplasmic reticulum, nuclei, mitochondria, and chloroplasts can all contribute to the overall GSH/GSSG ratio. An alternative explanation of this observation is that GSH is not the direct reducing agent of NPR1 in vivo. Besides glutathione, other redox-controlling molecules such as thioredoxin may be involved in regulating NPR1 reduction.

To confirm that NPR1 reduction is indeed a process associated with SAR, we performed biological induction of SAR using Psm ES4326/*avrRpt2*. Uninfected half leaves and distal leaves were collected and examined for NPR1 monomerization and *PR1* gene induction 24 hr and three days after inoculation, respectively. A correlation between the presence of NPR1 monomer and the expression of *PR1* was observed in the systemic tissues (Figure 3B).

To establish a causative relationship between the reduction of NPR1 and activation of *PR1* transcription, we sought a means to prevent NPR1 reduction after INA treatment. The pentose phosphate pathway (PPP) provides the cytosolic source of NADPH, which is a major donor of electrons for reductive reactions (Schafer and Buettner, 2001). An inhibitor of PPP, 6-aminonicotinamide (6-AN) has been used widely to deplete cells of NADPH and prevent reductive reactions (Garlick et al., 2002; Gupte et al., 2002; Hothersall et al., 1998). To demonstrate that NPR1 reduction is essential for *PR* gene activation, we treated plants with INA followed by 6-AN. As shown in Figure 4, 6-AN diminished the increases in total glutathione and GSH/GSSG ratio induced by INA, partially inhibited NPR1 monomerization, and decreased *PR1* gene expression. Therefore, we concluded that NPR1 reduction is likely a prerequisite for *PR* gene expression.

NPR1 Monomer Is Sufficient for *PR* Gene Expression

Although the PPP inhibitor, 6-AN, could prevent NPR1 reduction and decrease *PR* gene expression in response to INA induction, we could not rule out the possibility that 6-AN affected reduction of other unknown factors in the *PR* gene-regulating pathway. To provide further evidence to support our notion that NPR1 monomeriza-

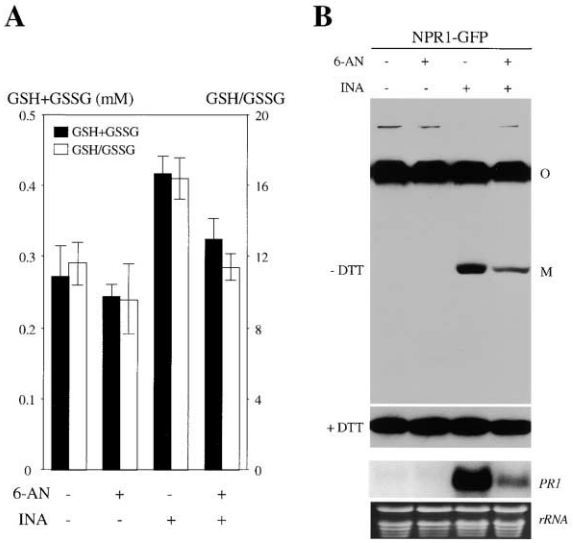

Figure 4. 6-AN Inhibits INA-Induced Changes in Cellular Glutathione Pool, NPR1 Reduction, and *PR1* Expression

Three-week-old *35S::NPR1-GFP* transgenic plants (in *npr1-1*) were transferred to culture wells with their roots submerged in a solution containing INA (0.5 mM) for 3 hr, then the roots were washed twice with water and submerged in water or a solution containing 6-AN (5 mM) for 24 hr. As controls, plants were treated with water or 6-AN (5 mM) for 24 hr. Plant tissues were then collected for the following experiments.

(A) Plant tissues (0.2 g) were used to measure the total glutathione (GSH+GSSG) pool and the GSH/GSSG ratios. Error bars represent standard deviation of three samples.

(B) Total protein (100 μg) was extracted and the effects of 6-AN on INA-induced NPR1-GFP monomerization and *PR1* gene expression were examined using immunoblot and RNA blot analysis, respectively.

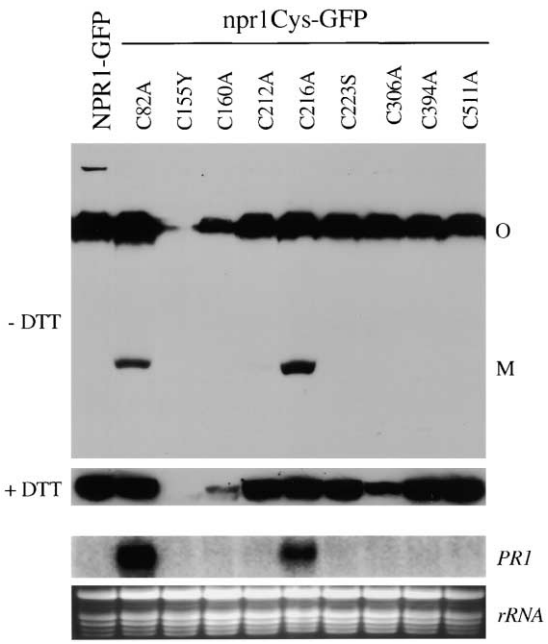

Figure 5. The *npr1C82A-GFP* and *npr1C216A-GFP* Mutants Show Constitutive Monomer Accumulation and Enhanced Expression of *PR1*

Total protein (100 μg) and total RNA (20 μg) extracted from the *35S::NPR1-GFP* and *35S::npr1Cys-GFP* (*C82A*, *C155Y*, *C160A*, *C212A*, *C216A*, *C223S*, *C306A*, *C394A*, and *C511A*) transgenic plants (in *npr1-1*) were analyzed using immunoblot and RNA gel blot, respectively, to examine the conformation of the NPR1-GFP or npr1Cys-GFP protein and its effect on *PR1* gene expression.

tion is a critical regulatory step for the activation of *PR* genes, mutations were introduced into the *35S::NPR1-GFP* construct to alter the sequences encoding each of the ten conserved cysteines. Besides three of the cysteines, which were changed to a tyrosine (C150Y, C155Y) or a serine (C223S), the rest of the cysteines were changed to an alanine (C82A, C160A, C212A, C216A, C306A, C394A, and C511A). The resulting constructs (*35S::npr1Cys-GFP*) were then transformed into the *npr1-1* background, and the conformation and the functionality of the mutant proteins were examined in the transgenic plants. For each mutant construct, multiple independent transformants were characterized and the average amount of total npr1Cys-GFP protein accumulated in plants was estimated using samples prepared in the presence of DTT. As shown in Figure 5, which includes one representative line from each construct, two mutants (*C155Y* and *C160A*) showed less protein accumulation than the wild-type while the other seven mutants (*C82A*, *C212A*, *C216A*, *C223S*, *C306A*, *C394A*, and *C511A*) showed almost the same levels of protein accumulation as the wild-type. In our previous experiments, no effect on protein accumulation was observed in the *npr1-2* mutant, which carries the *C150Y* mutation. To examine the conformations of the mutant proteins, we also prepared the protein samples in the absence of DTT. Without SAR induction, seven mutants (*C155Y*, *C160A*, *C212A*, *C223S*, *C306A*, *C394A*, and *C511A*) were present only in the oligomeric form, similar to the wild-type protein. However, in the *C82A* and *C216A* mutants, monomeric npr1Cys-GFP was detected constitutively. This indicated that formation of oligomers in these mutants was partially inhibited by the cysteine-to-alanine mutations. More importantly, the presence of monomeric npr1Cys-GFP in these two mutants coincided with constitutively elevated *PR1* gene expression (Figure 5). This result clearly demonstrated that the monomeric form of NPR1 is a biologically active conformation sufficient for induction of *PR* gene expression.

Monomerization Causes Nuclear Accumulation of NPR1

To investigate further the molecular mechanism underlying activation of NPR1 through monomerization, we performed nuclear fractionation experiment in the absence of DTT. We found that only monomeric form of NPR1 was present in the nuclei of INA-treated plants (Figure 6A). There was a correlation between NPR1-GFP monomerization and nuclear localization of the protein. This observation suggested that monomerization of NPR1 might be sufficient for its nuclear accumulation. If this is the case, monomerized npr1Cys-GFP in the *C82A* and *C216A* mutants should be able to accumulate in the nucleus in the absence of INA treatment. To test this hypothesis, we examined GFP fluorescence in plants transformed with the different *35S::npr1Cys-GFP* con-

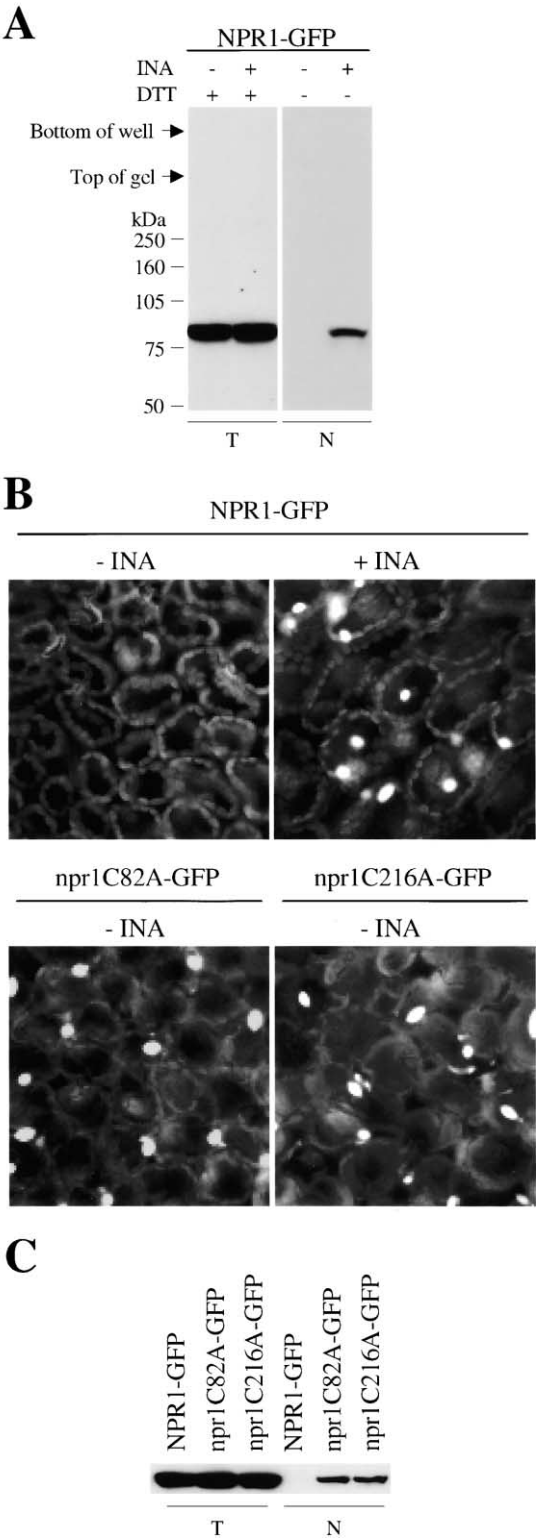

Figure 6. Monomerization of NPR1 Is Associated with Its Nuclear Accumulation

(A) Total protein (T) and the nuclear-fractionated protein (N) were extracted from *35S::NPR1-GFP* transgenic plants (in *npr1-1*) treated with (+) or without (−) 0.5 mM INA. The total protein (100 μg) and the nuclear-fractionated protein (12 μg) were subjected to SDS-PAGE with (+) and without (−) DTT (50 mM) in the sample buffer, respectively. NPR1-GFP was detected using immunoblot with the anti-GFP antibody.

(B) Leaf tissues from the transgenic lines carrying *35S::NPR1-GFP* treated with (+) or without (−) INA (0.5 mM) for 24 hr and untreated *35S::npr1C82A-GFP* or *35S::npr1C216A-GFP* were mounted in water and viewed with a fluorescence microscope.

(C) Total protein (T; 100 μg) and the nuclear-fractionated protein (N; 12 μg) from *35S::NPR1-GFP*, *35S::npr1C82A-GFP*, and *35S::npr1C216A-GFP* transgenic lines (in *npr1-1*) were extracted, subjected to SDS-PAGE with DTT (50 mM) in the sample buffer, and immunoblotted using the anti-GFP antibody.

structs. We found that without INA treatment, the *C82A* and *C216A* mutants indeed showed significantly enhanced nuclear fluorescence compared to wild-type and other mutants (Figure 6B). To confirm this result, we performed a nuclear fractionation experiment on the *C82A* and *C216A* mutants. The resulting nuclear protein extracts together with the total protein extracts were subjected to SDS-PAGE, followed by immunoblot analysis. As shown in Figure 6C, while equal amounts of NPR1-GFP, npr1C82A-GFP, and npr1C216A-GFP were detected in the total protein extracts, only npr1C82A-GFP and npr1C216A-GFP were found in the nuclear fraction. Therefore, we conclude that monomerization of NPR1 leads to its nuclear accumulation and subsequently *PR* gene induction.

Discussion

SA and NPR1 are two key components in the induction of SAR-related *PR* gene expression. In *npr1* mutants, induction of *PR* genes by SA is impaired (Cao et al., 1994), indicating that NPR1 functions downstream of the SA signal. Overexpression of NPR1 does not lead to constitutive *PR* gene expression in the absence of SAR induction (Cao et al., 1998), indicating that the NPR1 protein requires activation by SA to be functional. Our data show that this activation is accomplished through redox changes induced as a result of SA accumulation during SAR, which leads to conformational changes in NPR1 from the inactive oligomer to the active monomer.

SA and its functional analogs have been shown to have inhibitory effects on catalase and ascorbate peroxidase activities or serve as substrates for ascorbate peroxidase, which may lead to accumulation of H_2O_2, SA free radicals, and other ROS (Chen et al., 1993; Durner and Klessig, 1995; Kvaratskhelia et al., 1997). ROS can serve as second messengers in defense signaling pathways (Alvarez et al., 1998). Exogenous application or overproduction of H_2O_2 and several prooxidants in plants leads to induction of *PR1* expression (Chamnongpol et al., 1998; Klessig et al., 2000; Wu et al., 1997). This induction can be blocked by coapplication of antioxidants such as GSH (Klessig et al., 2000; May et al., 1998). Conversely, GSH by itself has been shown in some experiments to elicit expression of genes that are also responsive to ROS treatment (Wingate et al., 1988). Moreover, our unpublished data showed that GSH and its precursor N-acetyl cysteine (NAC) could induce *PR1* expression in a similar fashion to SA. These contradicting observations were reconciled by our finding that SAR induction involves an early burst of ROS and a transient increase in cell reduction potential, followed by a sharp decrease in reduction potential as a result

of accumulation of antioxidants such as GSH (Figure 2). This pattern is similar to that observed in barley during the HR after infection with the fungus *Blumeria graminis* (Vanacker et al., 2000). Fourteen hours after infection, H_2O_2 was detected in mesophyll cells underlying the epidermal cell attacked by the fungal spore. At the same time, dramatic changes in the leaf glutathione pool were observed. A transient decrease in GSH/GSSG ratio occurred 16 hr after infection, which was followed by a rapid increase in the total leaf glutathione pool at 18 hr after infection. Evidently, in response to the initial oxidative stress caused by pathogen infection, SAR inducer treatment, or ROS themselves, plant cells overcompensate with both enzymatic and nonenzymatic antioxidants that can scavenge ROS to protect against oxidative damage and at the same time, provide the redox environment for activation of transcriptional regulators such as NPR1. Therefore, any stimulus, a pathogen, chemical, oxidant, or antioxidant, that can perturb the redox balance of the cell, may serve as an inducer for the same set of defense-related genes.

SA not only triggers the redox changes described above, it also plays an important role in maintaining the cellular redox state, probably through induction of genes involved in the synthesis of antioxidants. In *nahG* plants, which express an enzyme that inactivates SA, the ozone (O_3)-induced increase in the glutathione reduction state (GSH/GSSG ratio) was diminished. As a result, there was a continuous decline in GSH/GSSG ratio after O_3-exposure and enhanced damage caused by O_3 (Rao and Davis, 1999).

The redox change induced as a result of SA accumulation during SAR brings about the conformational switch of NPR1 and thereby activation of *PR* genes (Figure 3). This is accomplished by reduction of the disulfide bonds formed between NPR1 protein molecules. The all-or-none pattern of this reduction suggests that an enzyme, such as thioredoxin or glutaredoxin, may be involved in catalyzing the reaction.

To form an oligomer, at least two cysteines in NPR1 are expected to be involved. Indeed, through site-directed mutagenesis, we found that Cys82 and Cys216 are critical for NPR1 oligomer formation. When these two cysteines were mutated to alanines, the monomeric form of the protein was detected without SAR induction or treatment with the reducing agent DTT (Figure 5). However, neither of these mutants could convert all of the oligomer to monomer, indicating that other cysteines may also be involved in the formation of additional disulfide bonds. Cys82 and Cys216 may be the residues forming the initial disulfide bond, which facilitates the formation of other disulfide bonds. It is worth noting that upon SAR induction, the wild-type NPR1 protein is not all reduced to the monomer form. Complete reduction of all the oligomers only occurred when DTT was added to the samples after preparation.

Monomerization of NPR1 is necessary and sufficient for its activation. Inhibition of NPR1 reduction by depleting the cellular NADPH pool using the PPP inhibitor, 6-AN, resulted in diminished *PR* gene expression (Figure 4), whereas prevention of oligomer formation through mutations in the disulfide bond-forming cysteines (*C82A* and *C216A*) led to constitutive *PR* gene expression (Figure 5). However, other SA-mediated activation steps are probably involved in complete induction of SAR. In the *npr1C82A-GFP* and *npr1C216A-GFP* mutants, *PR1* gene expression can be further upregulated in response to SAR induction (data not shown). When challenged with pathogens, such as Psm ES4326 and *Peronospora parasitica* Noco2, the *npr1C82A-GFP* and *npr1C216A-GFP* mutants showed only a moderate level of resistance compared to wild-type plants treated with INA (data not shown).

The biological significance of the conformational change in NPR1 may lie in the control of the subcellular localization of the protein. Only under SAR-induced conditions, when the oligomer is reduced to the monomer, was a significant amount of NPR1 detected in the nucleus using GFP fluorescence as well as nuclear fractionation (Kinkema et al., 2000; Figure 6). Furthermore, mutants with constitutive monomeric form of the protein (*npr1C82A-GFP, npr1C216A-GFP*) showed enhanced nuclear accumulation of the protein in the absence of any induction (Figure 6). These data suggest that the conformational change of NPR1 from oligomer to monomer is a prerequisite for nuclear transport of the protein. In the oligomeric form, the NLS of NPR1 is perhaps obscured, preventing the translocation of the protein to the nucleus. The oligomeric form of NPR1 is inaccessible to the antibody developed against the N-terminal half of the protein (Figure 1A), implying that it is markedly different from the monomeric form. Using the biologically active NPR1-GFP fusion allowed us to examine both forms of the protein and its subcellular localization.

This study adds NPR1 to a list of transcriptional regulators whose nuclear localization or activity is influenced by the redox state of the cell. Among these redox-regulated proteins, the bacterial transcriptional activator, OxyR, which regulates genes necessary for defense against oxidative stress, has been studied extensively (Delaunay et al., 2002; Kim et al., 2002; Zheng et al., 1998). The activity of OxyR is controlled by an intramolecular disulfide bond that can be oxidized as a result of H_2O_2 accumulation, leading to a conformational change and activation of the protein (Choi et al., 2001; Zheng et al., 1998). In yeast, activity of the transcription factor yAP1, which is responsible for expression of genes for defense against oxidative stress, is also controlled by redox signals (Kuge et al., 1997, 1998; Yan et al., 1998). Under nonstress conditions, yAP1 is constitutively shuttled in and out of the nucleus due to the presence of a nuclear export sequence (NES), leaving little yAP1 protein accumulating in the nucleus. In response to oxidative stress, an intramolecular disulfide bond forms in yAP1, which leads to a conformational change that conceals the NES from the nuclear export receptor. As a result of this conformational change, yAP1 accumulates in the nucleus and transcription of stress-responsive genes is activated.

Unlike oxyR- and yAP1-regulated ROS-defense gene expression that occurs minutes after exposure to oxidative stress, NPR1-mediated *PR* gene expression is much slower. Even though we cannot rule out a role for NPR1 in regulating genes involved in redox homeostasis, the biphasic GSH/GSSG ratio changes induced after INA treatment were identical in the *npr1* mutant and in wild-type plants (data not shown). This is consistent with our hypothesis that NPR1 activation occurs as a result of

these redox changes that serve as the secondary messenger connecting the SA signal with NPR1 activity. Identification of the redox mediator(s) involved in this process will advance our understanding of the induction mechanism of SAR.

Experimental Procedures

Antibody Development

The N terminus of NPR1 (nucleotides 1 to 1395) was cloned into pET-24c(+) (Novagen, Madison, WI) with NdeI and XhoI. The NPR1-His_6 fusion protein was purified from *Escherichia coli* by Ni^+ chelate chromatography, according to the supplier's instructions (Qiagen, Chatsworth, CA). The protein was further purified using an SDS-PAGE gel. Rabbit polyclonal anti-NPR1 antibody was made by immunizing rabbits with the NPR1-His_6 fusion protein (Pocono Rabbit Farm and Laboratory Inc., Canadensis, PA). The titer and specificity of the antibody were tested by immunoblot analysis.

Protein Analysis

Protein was extracted from 2- to 3-week-old plants by homogenizing in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA, 10% Glycerol, and protease inhibitors: 50 μg/mL TPCK, 50 μg/mL TLCK, and 0.6 mM PMSF). The extract was centrifuged at 14,000 *g* for 10 min. The protein concentration of the supernatant was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). To immunoprecipitate the NPR1-GFP protein, a green fluorescent protein (GFP) monoclonal antibody (Clontech, Palo Alto, CA) was added to the extracts (1:200) and incubated on ice for 1 hr. The antibody bound proteins were precipitated by adding protein A agarose beads (50 μL/mL) to the extracts, followed by incubation at 4°C with gentle rocking for 2 hr. The beads were then precipitated by brief centrifugation (6,000 rpm for 30 s) and washed 3 times with the extraction buffer. The proteins were eluted using 1 $\times$ SDS sample buffer.

To reduce disulfide bonds, 50 mM DTT was added to the protein extracts together with 2 $\times$ sample buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 25% glycerol, and 0.4% bromophenol blue). The protein samples were heated at 60°C for 10 min, loaded onto an SDS-PAGE gel, and transferred to nitrocellulose after electrophoresis. The blot was probed with anti-NPR1 antibody (1:6000) or anti-GFP antibody (1:1000). The antibody-bound proteins were detected using a horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz, Santa Cruz, CA) followed by chemiluminescence with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Nuclear fractionation was performed as described by Kinkema et al. (2000). The nuclei were resuspended in the extraction buffer and subjected to SDS-PAGE and immunoblot analysis in the absence of DTT as described above.

Glutathione Measurement

Plant leaf tissue (0.2 g) was homogenized in 2 ml of 2% metaphosphoric acid containing 2 mM EDTA and centrifuged at 4°C for 10 min at 14,000 *g*. The supernatant was used for glutathione measurement (Rao and Ormrod, 1995).

RNA Analysis

RNA extraction and RNA blot analysis were carried out as described by Cao et al. (1994).

Infection with Bacterial Pathogen

To induce SAR, 3-week-old *Arabidopsis* plants were inoculated with the avirulent bacterial pathogen *Pseudomonas syringea* pv. *maculicola* ES4326/*avrRpt2* at the concentration of $OD_{600} = 0.02$. Uninoculated tissues were collected for analysis.

Mutagenesis of *NPR1*

The *35S::NPR1-GFP* cassette, including the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase polyadenylation sequence, was excised from the pRTL2ΔN-NPR1-mGFPS65T construct (Kinkema et al., 2000) with SphI, blunt-ended using T_4 DNA polymerase and introduced into the SmaI site of the plant transformation vector pCB302 (Xiang et al., 1999). Site-directed mutagenesis of the conserved cysteines in NPR1 was performed in the pCB302-35S::NPR1-GFP construct using a PCR-based QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The presence of the expected mutations in the *35S::npr1Cys-GFP* constructs was verified by DNA sequencing.

Plant Transformation and Growth Conditions

The pCB302 plasmids carrying *35S::NPR1-GFP* and *35S::npr1Cys-GFP* were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90). The resulting *Agrobacteria* were used to transform *npr1-1* plants using a floral dipping method (Bent, 2000). Transformants were selected on soil by spraying with the herbicide Basta (glufosinate ammonium, dilution 1:2000).

Arabidopsis (ecotype Columbia) plants and *35S::NPR1-GFP* transgenic plants (in *npr1-1*) were grown in soil (Metro Mix 200; Grace-Sierra, Milpitas, CA). For induction, 3-week-old plants were transferred to culture wells with their roots submerged in a solution containing 2,6-dichloroisonicotinic acid (INA, 0.5 mM) or SA (0.5 mM) for 24 hr unless indicated otherwise. To inhibit protein synthesis, 100 μM cycloheximide and 10 μM MG132 (proteasome inhibitor) were added to the induction solution 2 hr before adding INA.

Microscopy

Arabidopsis leaf tissues were mounted in water and viewed with a Leica (Wetzlar, Germany) DMRB fluorescence microscope. GFP was visualized using an excitation wavelength of 488 nm and a bandpass 510 to 520 nm emission filter.

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