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

Zhonglin Mou, Weihua Fan,1 and Xinnian Dong*


Alice Zhu
Lindsay Preston
Philip Lam

sciencewatch.com/.../2010/10febfbp/10febfbpKamo/
What is SAR?

• Caused by a signal transduction pathway

• Infected cells cause an immune response in uninfected regions of the plant [2]

• Causes targeted gene expression of pathogenesis related genes [2]

• When a SAR induced plant is infected again the normal pathogen interactions do not take place, similar to an immune response [2]

• This could be helpful to manipulate plants for disease resistance [2]

Salicylic Acid (SA)

Systemic Acquired Resistance (SAR) induction

Does anyone remember how???
Resistance (R) Proteins

Avirulence factors (avr)

Hypersensitive response
Creates ROS
Programmed cell death
SA in uninfected tissues

Expression of Pathogenesis related (PR) genes

SAR

The mechanism that relates SA and PR gene expression is the object of this study.
What are PR genes??

- Pathogenesis related genes
- Encode anti-microbial proteins
- Indicators of infection
- Other functions in development and abiotic stress responses

What other information did they have prior to this study?

**What they knew**

Mutants unable to make PR genes all had mutations in only one gene locus

![Image](image.png)

*npr1* (nonexpressor of PR genes)

What does it mean if all of these mutants are mutations of the same gene??

- NPR1 could be the only protein between SA and PR gene expression
- There could be functional redundancy in this pathway
- Mutations in other members of the pathway could be lethal
NPR1

- Has a nuclear localization sequence
- Localizes to the nucleus after SAR induction
- Has 10 cysteines
- Has no DNA binding domain
- Is a positive regulator of SAR
- Has 2 protein-protein interaction domains
- Probably not a TF
- Possible interaction with TFs
- Nuclear localization is needed for PR expression
- NPR1 is constitutively expressed
Previous studies by Chen et al, 1993; Noctor et al, 2002 and Vanacker et al, 2003 propose that increases in SA after infection could change the redox state of the cell.

**Hypothesis:** NPR1 conformation is affected by redox changes caused by the increase of SA after pathogen infection.

How could you test whether redox changes affect NPR1?

**Reducing Agents**

- (Dithiothreitol) DTT
  - Very strong reducing agent

- INA (2,6-dichloroisonicotinic acid)
  - Active analog of SA - because SA at high concentrations is toxic
  - Causes redox changes after application
To determine if cysteines in NPR1 effect protein conformation

- Shows that the conformation of NPR1 is not the same before and after SAR induction

- INA and DTT show similar results

- BUT.. it turns out the polyclonal antibody only hybridized to samples treated with reducing agents

To fix this they...

INA is representing SAR induction

DTT reduces (tests whether NPR1 is sensitive to redox)
Antibody Problems

1. Raised against the N terminus → hybridized to reduced NPR1 only
2. Raised against the C terminus → not specific enough
3. Used a commercial antibody for GFP → Ran further experiments with 35S::NPR1-GFP transformants of both npr1-1 mutants and WT.

4. **Why is it ok to use 35S in npr1-1 mutant?**
   1. *NPR1* is constitutively expressed in WT plants
   2. *npr1-1* mutants express non functional NPR1 protein (no background NPR1 effects)

Now they can redo the first blot using a functional antibody
<table>
<thead>
<tr>
<th></th>
<th>DTT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>INA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DTT</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Samples in DTT alone (strong reducing agent) only produce the small band. INA and DTT combined also only produce the 93 kDa band.

Suggests DTT is able to reduce NPR1 to a monomer.

INA treatment induces partial reduction of NPR1 to a monomer.

With no additives NPR1 is a large protein complex. INA (SAR induction) causes a 93 kDa band.
Did extraction cause a protein complex to form??

- Ran the same experiment
- Treated proteins with alkylating agents (NEM, IOD)
- Block the formation of nonspecific disulphide (S-S) bonds, leaving already established S-S intact.

(-) INA should only produce an oligomer

(+ ) INA should show both oligomer and monomer

Addition of NEM or IOD didn’t affect expected results

Complex is not caused by preparation process
What is this high molecular weight complex?

• SDS-PAGE with low levels of DTT to separate intermediates

This suggests that the oligomer is being reduced to trimer, dimer and monomer intermediates in the presence of low concentration of reducing agents.

What are these intermediates made of?

Hypothesis: NPR1 is a homooligomer

How can we test this again?
Immunoprecipitation

• Immunoprecipitated NPR1 using anti-GFP antibody
• Western blot using NPR1 antibody as the probe
Immunoprecipitation and Western Blot

• Anti-GFP precipitates NPR1-GFP and NPR1

• NOTE- this NPR1-GFP is not in a mutant background

• How does the antibody immunoprecipitate NPR1-GFP AND endogenous NPR1?

• The GFP antibody precipitates the oligomer, when treated with DTT causing monomerization revealing endogenous and GFP tagged components.

This supports the hypothesis that NPR1 is a homoligomer
Can NPR1 be reduced to a monomer in vivo?

Cyclohexamide- block protein synthesis

Why do they want to block protein synthesis?

• Blocking protein synthesis shows that these monomers are reduction products of the oligomer and not being synthesized after INA treatment

Is the monomer a reduction product or a protein synthesized after INA?

Answer: Reduction product

The presence of cyclohexamide does not inhibit the appearance of a monomer.

The oligomer is able to be reduced to a monomer with INA in vivo
All of these experiments suggest:

• NPR1 can be reduced from an oligomer to its monomeric form by reducing agents.

• NPR1 is a homoligomer.
So what if the monomer of NPR1 is a reduction product?

(reduced into monomer by reducing agents and cellular reduction potential)?

• Cellular redox changes are well-known indicators of plant stress and can be measured.
Glutathione: GSH and GSSG

- Glutathione: non-protein thiol-containing tripeptide antioxidant that exists in **reduced (GSH)** and **oxidized (GSSG)** states.

- **GSH** = major endogenous antioxidant produced by the cells, neutralizes free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms.

- GSH can participate not only in scavenging H2O2 through the AsA-GSH cycle but also in a direct reaction with other active oxygen species (May et al., 1998).

- In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the (oxidized) disulfide form (GSSG).

- GSH is regenerated from GSSG by the enzyme glutathione reductase and NADPH.
Ascorbate-Glutathione Cycle

2 H₂O → dehydro-ascorbate → H₂O₂

H₂O₂ → ascorbate → reduced glutathione (GSH)

GSH → oxidized glutathione (GSSG) → NADPH

NADP⁺
To test if there is a change in reduction potential

- 35S::NPR1-GFP plants treated with INA,
- treated tissues tested for GSH levels

- “the change in the GSH/GSSG ratio appeared to be influenced mainly by the concentration of GSH”

- Biphasic change in cellular reduction potential: SAR involves early burst of ROS followed by increase in reduction potential
Test: do similar (similar to INA-induced) redox changes occur in systemic tissues?
Test: do similar redox changes occur in systemic tissues?

- Half-leaves innoculated with *Pseudomonas* pathogen
- **uninfected halves** of infected leaves collected for glutathione measurements.

RESULT: increase in total glutathione and GSH/GSSG ratio in systemic tissues.

Results confirm that redox changes do occur following the induction of SAR.
Results confirm that redox changes do occur following the induction of SAR.

Q: What sort of experiment did they do to determine if these naturally-occuring redox changes in the cell alter the activity of NPR1?
To test whether observed increases in GSH/GSSG ratio could reduce NPR1 (in vitro):

Protein extracts from untreated 35S:NPR1-GFP plants incubated in defined [GSH/GSSG] before western blot

-NPR1-GFP monomer appears in significant amounts when GSH/GSSG ratio > 15:1.

-monomer oxidized to oligomer at GSH/GSSH ratio < 7.5:1

“...GSH/GSSG ratio required for in vitro NPR1 reduction (monomer) is similar to that reached in plants after SAR induction…”

(So far, data does not indicate that GSH/GSSG and NPR1 monomer/oligomer are in redox equilibrium. Only confirms that increased cellular reduction potential is correlated with increasing reduction (monomerization) of NPR1. Reducing agent of NPR1 has yet to be determined.)
Is NPR1 monomerization a regulatory mechanism by which SA turns on PR gene expression?

35S:NPR1-GFP plants treated with INA, then analyzed for reduced/monomeric NPR1

A time course experiment to follow kinetics of NPR1 reduction and expression of PR genes.

-Monomerization preceded expression of PR1

-“correlation between the 2 events supports the hypothesis that NPR1 protein monomerization is a regulatory step required for the activation of PR gene expression”
Contradiction:

- In the INA-treatments, NPR1 reduction/monomerization is observed before the expected increase in GSH/GSSG ratio (suspected to be correlated to if not causing NPR1 monomerization).

Explanation:

- The overall ratio of GSH/GSSG (from ground up tissues) might not reflect the redox environment of cellular compartment where NPR1 resides
- Either the redox environment in some cells is enough to reduce NPR1 but not in other cells.
- or GSH is not the direct reducing agent of NPR1
Update on redox-research

• “in plants, the high redox potential molecule ascorbate exists at comparable or higher concentrations and is used for scavenging hydrogen peroxide as an electron donor.”

• “Recently, examples that cannot be explained simply by the antioxidant activity of GSH have been increasing in number.”

Ascorbate-Glutathione Cycle

- 

- Reduced glutathione (GSH) reacts with NADP⁺ to produce NADPH.

- Oxidized glutathione (GSSG) is reduced by ascorbate to form glutathione.

- Dehydroascorbate is oxidized by hydrogen peroxide (H₂O₂) to form ascorbate.

- Ascorbate is oxidized by hydrogen peroxide (H₂O₂) to form dehydroascorbate.

http://www.uky.edu/~dhild/biochem/25/lect25.html
doi:10.1038/nchembio.85
What have they not done/confirmed yet?

- What sort of experiments have they overlooked so far?
To confirm that the observed NPR1 reduction is associated with naturally-occurring SAR (not artificially induced), collected uninfected half leaves and distal leaves -induced SAR with psm(avr) infection, collected uninfected half leaves and distal leaves. 

-NPR1 monomerization and PR1 gene expression was examined in the adjacent and distal tissues at 24 hours and 3 days after infection, respectively.

- A correlation was seen between presence of NPR1 monomer and PR1 expression (ambiguous).
SUMMARY
PPP provides **NADPH**: major donor of electrons for reductive reactions
6-AN Prevents Reductive Reactions

- $C_6H_7N_3O$, 6-Aminonicotinamide
- Inhibitor of NADP$^+$-dependent enzyme: 6-phosphogluconate dehydrogenase
- A competitive inhibitor of the G6PD of the oxPPP

- Production of a nonmetabolizable analogue of NADP

- Deplete cells of NADPH, and **prevent reductive reactions** since NADPH is major donor for reductive reaction
Is NPR1 reduction essential for PR gene activation?

- Q: What may happen to PR gene expression when 6-AN is added following INA induction?
- Ans: ↓PR gene expression.

NADPH depleted

No reductive reaction

NPR1 reduction inhibited

↓PR gene expression

Observed in result?
Indeed: 6-AN inhibits INA-induced changes in cellular Glutathione Pool, NPR1 Reduction and PR1 Expression

Effects of 6-AN:
• Diminish the increases in total glutathione and GSH/GSSG ratio induced by INA.

http://www.uky.edu/~dhild/biochem/25/lect25.html
Indeed: 6-AN inhibits INA-induced changes in cellular Glutathione Pool, NPR1 Reduction and PR1 Expression

Effects of 6-AN:
- Diminish the increases in total glutathione and GSH/GSSG ratio induced by INA.
- Partially inhibit the NPR1 monomerization
- Decrease PR1 gene expression

The reduction of NPR1 is possibly required for PR gene expression

Q: However, any possibility missing?
Ans: 6-An may prevent reduction of other unknown factors in PR gene-regulating pathway other than NPR1, which also need NADPH for reduction reactions.
**Causal Relationship:** NPR1 Monomerization is Sufficient for Activation of PR Gene Expression

**Amino Acid Sequence of NPR1 Protein**

1 MDTTIDGFAD SYEISSTSFV ATDNTDSSIV YLAAEQVLTG PDVSALQLLS
51 NSFESVFDSP DDFYSDAKLV LSDGREVSFH RCVLSARSSF FKSALAAAKK
101 EKDSNNTAAN KLELKEIAKD YEVGFSVVT VLAYVYSSRV RPPPKGVSEC
151 ADENCCHVAC RPAVDFMLEV LYLAFIFKIP ELITLYQRHL LDVVDKVVIE
201 DTLVILKLAN ICGKACMKLL DRCKIEIVKS NVDMVSLEKS LPEELVKEII
251 DRRKEGлей PKVKKHVSNV HKAEDDIE LVKLLLKEDH TNLDDACALH
301 FAVAYCNVKT ATDLLKLDSL DAKVHRNPRGY TVLHVAAMRK EPQLILSLE
351 KGASASEATL EGRTALKIAK QAATMAVCCNN IPEQCKHSK LGRCVEILEQ
401 EDKREQIPRD VPPSFAVAAD ELKMTLDLLE NRVALAQRLF PTEQAAMEI
451 AEMKGTCEFI VTSLEPDRLT GTRKTRSPGK IAEPRIEHE QSRLKALSKT
501 VELGKRFFPR CSAVLDQIMN CEDLTQLACG EDDTAEKRLQ KKQRMEIQE
551 TLKKAFOSEDN LEISGSSLTBD STSSTSKSTG GKRSNRLSH RRR

**10 conserved cysteines:** Introduce mutation into 35S::NPR1_GFP construct to substitute each cysteine *individually*

- Site-directed mutagenesis, achieved by using PCR-based Quick-Change Site-directed mutagenesis kit,
- confirmed by DNA sequencing.

(http://arabidopsis.org/servlets/TairObject?type=aa_sequence&id=1009107547)
PCR-based Quick-Change Site-directed mutagenesis


**One-Day Method**

**Mutant Strand Synthesis**
Perform thermal cycling to:
1) Denature DNA template
2) Anneal mutagenic primers containing desired mutation
3) Extend primers with PfuUltra DNA polymerase

**Dpn I Digestion of Template**
Digest parental methylated and hemimethylated DNA with Dpn I

**Transformation**
Transform mutated molecule into competent cells for nick repair

**Figure 1** Overview of the QuikChange® II XL site-directed mutagenesis method.
Introduce Mutagenesis in NPR1-GFP Protein

- Mutations that don’t deform the structure of NPR1 protein:
  1. C150, C155 → Tyrosine (Y)
  2. C223 → Serine (S)
  3. C82, C160, C212, C216, C306. C394, C511 → Alanine (A)

- 1 MDTTIDGFAD SYEISSTSFSV ATDNTDSSIV YLAAEQVLTG PDVSALQLLS
  51 NSFESVFDSDSP DDFYSDAKLV LSDGREVSFH RAVLSARSSF FKSA
  101 LAAKKK 151 ADENYCHVAARPAPVDFMEV LYLAFIFKIP ELITLYQRHL LDVV
  201 DKVKLLAN IAGKAMKLL DRSEKIVKS NVDMVSELEKS LPEELVKEII
  251 DRKELGLEV PKVKKHVSVN HKALSDDDIE LVKLLKEDH TNLDDACALH
  301 FAVAYAVKKT ATDLLKLDLA DVNRNPRGY TVLHVAAMRK EPQLILSLLE
  351 KGASAASEATL EGRTALMIAK QATMAVEC
  401 ENQU PEQCKHSLK GRLAVEILEQ
  451 EDKREQIPRD VPPSFAVAAD ELKMTLLDLE NRVALAQRFL PTEAA
  501 ASEATL EGRTA
  551 ASAVLDQIMN CEDLTQLACG EDDTAEKRLQ KKQRYMEIQE
  601 VELGKRF
  651 LAVLDQIMN CEDLTQLACG EDDTAEKRLQ KKQRYMEIQE
Is NPR1 Monomerization Sufficient for Activation of PR Gene Expression

- Agrobacteria 35S::NPR1-GFP or 35S::npr1Cys-GFP
- Transform
- Selected by herbicide Basta

- Average the total npr1Cys-GFP protein accumulated among multiple independent transformants
- Exam the conformation and functionality of the mutant npr1Cys-GFP protein in Transgenic Plants
Exam the conformation of the NPR1-GFP or npr1Cys-GFP protein and its effect on PR1 gene expression

- With DTT:
  1. C155Y and C160A yield less npr1Cys-GFP protein accumulation than control.
  2. The other 7 mutants (plus C150Y in npr1-2 mutant) have similar npr1Cys-GFP protein accumulation to control

- Without DTT:
  1. C82A and C216A have monomeric npr1Cys-GFP
  2. Constitutively elevated PR1 gene expression
Q: what does the presence of the monomer in C82A and C216 without DTT tell us?

- **monomeric form of NPR1** is a biologically active conformation sufficient for induction of PR gene expression.

- Formation of oligomers in C82A and C216 were **partially inhibited** by the C→A mutations.
How does NPR1 activate PR gene expression after monomerization?

• What is found:
  Only monomeric NPR1 present in the nuclei of INA-treated plants

→ Suggest:
  Monomerization of NPR1 might be sufficient for its nuclear accumulations

→ Suggest:
  Q: What would you predict that will happen to the mutants with constituent *monomeric npr1Cys-GFP*?
  Ans: monomers should be able to accumulate in the nucleus even in the ABSENCE of INA.
Is NPR1 Monomerization Sufficient for Activation of PR Gene Expression

Agrobacteria (35S::npr1Cys-GFP Or 35S::NPR1-GFP )

Transform

Selected by herbicide Basta

INA

Nuclear fractionation

Total Protein

Nuclear Protein

npr1-1 plant
Confirm the result by Nuclear Fractionation (isolate nuclei)

http://www.qiagen.com/Products/Protein/Proteomics/Qproteome/images/fc_NuklearProteinFractionationProcedure.gif
In NPR1-GFP transgenic plants:
Only Monomer Found in Nuclei after INA induction

- Detected by anti-GFP on immunoblot
- Found:
  - Treating with DTT in total protein: prove the existence of NPR1-GFP proteins in cells
  - However, only monomeric form of NPR1 appear in the nuclei of INA-treated plant
In leaf tissues from the mutants that constitute the monomeric NPR1(C82A and C216A), significantly enhanced nuclear fluorescence are observed compared to 35S::NPR1-GFP lines WITHOUT INA induction.

- accumulation of monomeric npr1Cys-GFP in nucleus
Confirm by nuclear fractionation experiment on the C82A and C216A mutatns

- About Equal amount of NPR1-GFP, npr1C82A-GFP and npr1C216A-GFP found in total protein in presence of DTT
- Only monomers of npr1C82A-GFP and npr1C216A-GFP found in nuclear fraction
In Conclusion

Reduction of disulphide bonds

Measured with GSH:GSSG

Redox change

Monomerization allows NPR1 to enter the nucleus (perhaps oligomer blocks NLS)

Demonstrated by nuclear fractionation

PR GENE Expression

- From infection
- INA
- DTT

NPR1 homooligomer

NPR1 monomer

Constitutive monomer mutants npr1-GFPCys82A and npr1-GFPCys216A
Family of transcriptional activators whose activity is influenced by redox state of the cell

Gene expression = SLOW

NPR1
- Oxidative stress
- Reduction of S-S bonds leads to nuclear localization

OxyR
- Oxidative stress
- Oxidation of S-S bonds leads to activation

Gene expression = FAST

yAP1
- Oxidative stress
- Oxidation of S-S bonds hides NES
TGA transcription factors

- NPR1 interacts with TGA family of basic leucine zipper transcription factors
- Found by yeast 2 hybrid

- Electromobility shift assays show that NPR1 increases TGA2 binding to PR-1 promoters
- Mutational analysis suggest TGA’s are functionally redundant
- Different TGA’s have different binding affinities with NPR1

Conclusion

• SA also plays an important role in maintaining the cellular redox state.
• If a stimulus can perturb the redox balance of the cell
  → induced downstream defense-related genes.
• SA (& analog)
  • inhibit catalase and ascorbate peroxidase activities or
  • serve as substrates for ascorbate peroxidase
Conclusion

- **Ascorbate peroxidases**
  1. detoxify peroxides (i.e. $H_2O_2$) by reducing peroxide
  2. Substrate = ascorbate
  3. products = dehydroascorbate and water

- **Catalase**
  - Catayze:
    $2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$

---

Inhibition $\rightarrow \uparrow \left(H_2O_2, \text{SA free radicals, other ROS}\right)$ = 2° messenger in defense signaling pathway

http://www.uky.edu/~dhild/biochem/25/lect25.html
**Conclusion**

- **Contradiction:**
  - Exogenous application or overproduction of H$_2$O$_2$ & prooxidants in plants lead to induction of PR1 expression, which can be **inhibited** by anti-oxidants, i.e. GSH
  - GSH itself can elicit expression of genes for ROS treatment; GSH and its precursor NAC could **induce** PR1 expression similarly to SA

- **Overcompensate to reach the redox environment for NPR1 activation = INDUCTION**
- **Oxidize the exogenous H$_2$O$_2$ & prooxidants to avoid the initial oxidative stress = INHIBITION**

![Graph showing GSH and GSSG levels over time after INA treatment](image)
Conclusion

• Cys82A and Cys216A are critical for the formation of oligomer,
• Other cysteines might are involved due to the observational partial inhibition.
• However, it is worth noting that upon SAR induction, the WT NPR1 protein is not all reduced to the monomer form.

**Plant Immunity Requires Conformational Charges of NPR1 via S-Nitrosylation and Thioredoxins**

Yasuomi Tada, *et al.*

*Science 321*, 952 (2008);
DOI: 10.1126/science.1156970

• S-nitrosylation of NPR1 by S-nitrosoglutathione (GSNO) at **cysteine-156** facilitates its oligomerization
• SA-induced NPR1 monomerization is catalyzed by thioredoxins (TRXs).
• Mutations in both NPR1 cysteine-156 and TRX compromised NPR1-mediated disease resistance.
• The regulation of NPR1 is through the opposing action of GSNO and TRX.