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

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Introduction: Plant Pathogen Interactions

- Plants have R (resistance) proteins which enable them to detect pathogen-derived molecules known as avr (avirulence) factors.
- R-avr interaction can trigger an HR (hypersensitive response) for pathogen resistance, which can take the form of:
  - Programmed cell death
  - Production of reactive oxygen species (ROS)
  - Synthesis of antimicrobial compounds
- An HR can lead to a secondary response known as a systemic acquired resistance (SAR) in the uninfected tissue of plants, which is associated with elevated levels of salicylic acid (SA).
Introduction: Role of Salicylic Acid

- Applications of exogenous SA can induce SAR, and expression of nahG, which inactivates SA, suppresses SAR
  - SA is necessary and sufficient
- SA has been shown to induce a suite of a pathogenesis related genes
  - But it is important to understand the factors which connect the SA signal with expression of *PR* genes
- Thus far, the only link that can be found between SA and PR genes through mutational analysis is the NPR1 gene, AKA NIM1 and SAI1
Introduction: NPR1

➢ Traits of npr1:
  ○ Does not respond to various SAR-inducing agents
  ○ Displays little expression of PR genes
  ○ Exhibits enhanced susceptibility to a wide range of pathogens

➢ Evidence suggests NPR1 is a positive regulator of SAR and required to complete the signalling pathway between SA and PR gene expression
  ○ The NPR1 signal pathway is highly conserved between species
Introduction: NPR1 continued

- *NPR1* gene encodes a protein containing a bipartite nuclear localization sequence and two potential protein-protein interaction domains
  - Studies of NPR1 cDNA fused to GFP showed the protein accumulating in the nucleus in response to both chemical and biological inducers of SAR
  - Nuclear localization was also shown to be a prerequisite for regulation of *PR* genes
  - Likely not a transcription factor, as it lacks DNA binding domains
    - Probably interacts with a transcription factor through its protein-protein binding domains (likely TGA subclass of transcription factors)
Introduction: NPR1 and Salicylic Acid

- Expression of *NPR1* is constitutive and unaffected by SA; therefore, SA must affect the NPR1 protein itself
  - However, radioactively labelling SA showed that it did not bind directly to NPR1
- Previous studies that showed SA may affect the cellular redox potential combined with the presence of ten conserved cysteines on the NPR1 protein led the authors to hypothesize that the protein conformation may be sensitive to cellular redox changes
  - To test this idea, authors examined NPR1 under different redox conditions and in the presence or absence of various SAR inducers
Introduction: What the Authors Showed

Results of biochemical and genetic studies:

➢ In the absence of SAR inducers, NPR1 exists as an oligomer formed through intermolecular disulfide bonds and is sequestered from transport into nucleus
➢ After SAR induction, following an initial oxidative burst, plant cells attain a more reducing environment due to the accumulation of antioxidants
➢ Under these redox conditions, NPR1 is converted from an oligomeric state to a monomeric state through reduction of intermolecular disulfide bonds
➢ The monomeric NPR1 protein moves into the nucleus to control SAR-related gene expression
NPR1 is reduced from an oligomer to a monomer after treatment with SAR inducers

- Performed protein extractions in the absence of reducing agents such as DTT (which was then later added).
- Protein extracts from WT Col (Col-0) treated with the SAR inducer INA or with water were subjected to SDS-PAGE and immunoblot analysis.
  - In absence of DTT, NPR1 was only found in samples prepared from INA-treated plants.
  - Equal amounts of NPR1 were detected in samples to which DTT were added, regardless of INA/water treatment.
  - Similar results were found using other SAR inducers, SA and BTH.
  - These results suggest that NPR1 may exist in a different state or conformation before and after SAR induction.
  - Results achieved using a polyclonal antibody raised against the N-terminal half of NPR1.
NPR1 is reduced from an oligomer to a monomer after treatment with SAR inducers

➢ To detect NPR1 in both INA-treated and untreated samples, another NPR1 antibody raised against a 16 amino acid peptide at the C terminus was used, but it crossreacted with NPR1 homologs
➢ To get around this, they used a previously created transgenic line with the gene 35S::SNPR1-GFP in the npr1-1 mutant background
  ○ The NPR1-GFP gene is regulated similar to NPR1
  ○ This allowed them to use an antibody for GFP instead of NPR1
NPR1 is reduced from an oligomer to a monomer after treatment with SAR inducers

➢ Once again, in the absence of DTT, NPR1-GFP was found only in the INA-treated sample
  ○ However, a second band of higher molecular weight appeared in both samples
➢ Adding DTT to both extracts eliminated the second band, and the lower molecular weight band appeared in both samples
  ○ This suggests that an NPR1-containing complex is formed through intermolecular disulfide bonds, and these bonds can be partially reduced as a result of INA induction
NPR1 is reduced from an oligomer to a monomer after treatment with SAR inducers

- The authors confirmed that the lower molecular weight, monomeric form of NPR1 observed in the immunoblot analysis probably represents the in vivo conformation of the protein by performing a gel filtration experiment under nondenaturing conditions
  - This resulted in monomeric NPR1 after SAR induction
NPR1 is reduced from an oligomer to a monomer after treatment with SAR inducers

➢ The authors then confirmed that the higher molecular weight, oligomeric form of NPR1 observed in the immunoblot analysis was not due to nonspecific crosslinking caused by disulfide bond formation preparation by denaturing the protein in the presence of alkylating agents which block the formation of nonspecific disulfide bonds while keeping existing disulfide bonds
  ○ This resulted in the oligomeric NPR1 being detected
NPR1 is reduced from an oligomer to a monomer after treatment with SAR inducers

➢ The authors also confirmed that the high molecular weight protein was not due to interactions with the GFP protein by performing the same INA treatment and immunoblot analysis on samples from 35S::GFP plants
   ○ No high molecular weight complexes were found
NPR1 is reduced from an oligomer to a monomer after treatment with SAR inducers

➢ The authors then added very small amounts of DTT to samples from both control and INA treated plants and ran SDS-PAGE with immunoblot analysis
  ○ This resulted in four different NPR1 molecules being detected, which the authors surmised were the monomeric, dimeric, trimeric, and oligomeric forms of NPR1
  ○ Using immunoprecipitation with the anti-GFP monoclonal antibody, the authors were also able to conclude that it is most likely a homooligomer
  ○ However, they could not rule out the possibility that other proteins besides NPR1 are part of this complex
NPR1 is reduced from an oligomer to a monomer after treatment with SAR inducers

- The authors also concluded that it was extremely likely that the NPR1 oligomer was a homooligomer and not a heterooligomer
  - This is due to a immunoprecipitation/immunoblot analysis, which not only isolated the NPR1-GFP protein, but also endogenous NPR1
    - This was done by first performing an immunoprecipitation for GFP-tagged protein, and then using their original NPR1 antibody to perform an immunoblot analysis on the protein they had isolated
NPR1 is reduced from an oligomer to a monomer after treatment with SAR inducers

- The authors also confirmed that the NPR1 monomer was a result of oligomer reduction, and not new protein synthesis, by doing an INA induction experiment in the presence of cycloheximide (which inhibits new protein synthesis)
  - This resulted in monomeric NPR1 being detected
SAR induction changes the redox state of plant cells

- Authors hypothesized that the switch of NPR1 from an oligomer to monomer was the result of a decrease in reduction potential following INA-induction
- To test this hypothesis, the authors measured the changes in the glutathione pool after INA treatment
  - The “pool” consists of glutathione as GSH and GSSG
  - Glutathione is a major thiol-disulfide redox buffer in plant cells
  - This method had been used previously to estimate the redox state of plants after pathogen attack
SAR induction changes the redox state of plant cells

➢ Authors saw a dramatic decrease in total glutathione levels (GSH+GSSG) as well as GSH:GSSG ratios 8 hours after INA treatment, followed by a sharp increases in glutathione levels and GSH:GSSG ratios from 8-24 hours after treatment.
SAR induction changes the redox state of plant cells

➢ The authors then tested the glutathione levels and GSH:GSSG ratios of systemic tissues by inoculating half leaves with P. syringae pv maculicola ES4326 carrying the avirulence gene avrRpt2
  ○ The uninfected halves had significantly increased levels of glutathione and GSH:GSSG ratios 24 hours after infection
  ○ However, they were not able to replicate these results with distal leaves
SAR induction changes the redox state of plant cells

- The authors also tested whether the observed changes in the GSH:GSSG ratios could result in the reduction of NPR1 by incubating protein extracts in different defined concentrations of GSH:GSSG for one hour before performing SDS-PAGE
  - This resulted in the presence of the NPR1 monomer at concentrations exceeding a ratio of 15:1
  - The results also showed monomeric NPR1 being oxidized to oligomeric NPR1 at ratios less than 7.5:1
SAR induction changes the redox state of plant cells

- The authors noted that the GSH:GSSG ratio required for NPR1 to be monomerized is similar to the ratio reached in plants after SAR induction
  - However this is only corollary based on the data here
- The data here can confirm:
  - SAR induction leads to a decrease in the cellular redox potential
  - A decrease in the cellular redox potential reduces the cysteine residues in NPR1
  - Reducing the cysteine residues releases the NPR1 monomer from the oligomeric complex
- The authors are unsure of what exactly is the reducing agent
Is NPR1 reduction required for PR gene expression?

What we know:

- SA and INA induce NPR1 protein monomerization
- SA and INA activate PR gene expression
- npr1 mutants: impaired SA induction of PR genes
  - Indicates that NPR1 functions downstream of SA signal
Questions:

What is the relationship between NPR1 and PR1 gene expression?

- Is NPR1 reduction required for PR gene expression?
  - Is this process of NPR1 reduction associated with SAR?
  - Is the relationship between NPR1 and PR1 gene expression causative?
Is NPR1 monomerization required for PR gene expression?

Time Course experiment:

- 35S::NPR1-GFP transgenic plants
  - Treatment with 0.5mM INA

- Immunoblot analysis (*NPR1*)
  - Western blotting

- RNA gel blot analysis (*PR-genes*)
  - Northern blotting
Is NPR1 monomerization required for PR gene expression?

Results

➢ 8hr: NPR1 monomer
➢ 16hr: PR gene expression

Monomerization of NPR1 preceded PR gene expression.
Is NPR1 monomerization required for PR gene expression?

Results

Aside:

➢ NPR1 reduction appeared earlier than any changes in the GSH/GSSG ratio.
   ○ Why?
Is NPR1 monomerization required for PR gene expression?

Is NPR1 reduction a biological process that is associated with SAR?

- Induce SAR using *P. syringae*
- Collect half leaves (adjacent) and distal leaves
Is NPR1 monomerization required for PR gene expression?

Is NPR1 reduction a biological process that is associated with SAR?

➢ Examine:

  ○ NPR1 monomerization
    ■ Immunoblot (Western Blotting)

  ○ PR-gene induction
    ■ RNA gel blot (Northern Blotting)
Is NPR1 monomerization required for PR gene expression?

Is NPR1 reduction a biological process that is associated with SAR?

Results:

- Correlation between the presence of NPR1 monomers and PR1 gene expression in systemic tissues following infection.
Is NPR1 monomerization required for PR gene expression?

Is NPR1 reduction a biological process that is associated with SAR?

- Monomerization of NPR1 preceded PR gene expression
- Correlation between the presence of NPR1 monomers and PR1 gene expression in systemic tissues following infection.

But is it **required**? What can you do to test this?
Is NPR1 monomerization required for PR gene expression?

- Prevent NPR1 reduction after inducing defence response
  - Induce with INA
  - Inhibit PPP with 6-AN
    - Produces non-metabolizable analogue of NADP

- Will PR1 still be expressed if NPR1 reduction is diminished?
Is NPR1 monomerization required for PR gene expression?

Results:

➢ 6-AN application lowered total glutathione and INA induced GSH/GSSG ratio
  ○ NPR1 monomerization partially inhibited
  ○ PR1 gene expression decreased
Is NPR1 monomerization required for PR gene expression?

**Results:**

- 6-AN application lowered total glutathione and INA induced GSH/GSSG ratio
  - NPR1 monomerization partially inhibited
  - PR1 gene expression decreased

Therefore, NPR1 reduction is likely **required** for *PR1* expression.
Is NPR1 monomer is sufficient for PR gene expression?

➢ What if 6-AN affects other steps in the PR-gene regulating pathway?
  ○ How can we be sure?
Is NPR1 monomer is *sufficient* for PR gene expression?

➢ What if 6-AN affects other steps in the PR-gene regulating pathway?
  ○ How can we be sure?
  ○ Mutate the sequences encoding for conserved cysteines in 35S::NPR1-GFP
    ■ Recall: 10 cysteines
    ■ Resultant constructs: 35S::npr1Cys-GFP
Is NPR1 monomer is **sufficient** for PR gene expression?
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<th>Point mutation</th>
<th>NPR1 protein Conformation</th>
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<td>Cysteine → Tyrosine</td>
<td>Oligomeric</td>
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Is NPR1 monomer sufficient for PR gene expression?
Is NPR1 monomer is *sufficient* for PR gene expression?

- In the absence of a reducing agent...
  - Cysteine mutants that constitutively expressed NPR1 monomers also showed elevated PR1 gene expression

- Therefore...

NPR1 monomer is *sufficient* for the induction of PR gene expression
How does NPR1 monomerization lead to activation and PR gene expression?

**Recall**: Prerequisite relationship between NPR1 monomerization and PR gene expression

➢ *How does NPR1 monomerization lead NPR1 activation, and therefore PR gene expression?*
Nuclear Protein Fractionation Procedure

1. Lyse
2. Supernatant containing cytosolic proteins
3. Resuspend nuclei
4. Supernatant containing nucleic-acid binding proteins
5. Pellet containing "insoluble" nuclear proteins
How does NPR1 monomerization lead to activation and PR gene expression?

- **Nuclear fractionation (− DTT, +INA)**
  - Only monomeric form of NPR1 protein found in INA-treated plants

- **Is monomerization of NPR1 sufficient for nuclear localization?**
How does NPR1 monomerization lead to activation and PR gene expression?

➢ Is monomerization of NPR1 sufficient for nuclear localization?

**Recall:** Two mutants, C82A and C216A have constitutively elevated levels of NPR1 monomer and PR1 gene expression

➢ Where would you expect to see the proteins for these two mutants in the absence of INA?
How does NPR1 monomerization lead to activation and PR gene expression?

➢ Is monomerization of NPR1 sufficient for nuclear localization?

**Recall:** Two mutants, C82A and C216A have constitutively elevated levels of NPR1 monomer and PR1 gene expression

➢ Monomerized npr1Cys-GFP in C82A and C216A mutants should be able to accumulate in the nucleus in the absence of INA
How does NPR1 monomerization lead to activation and PR gene expression?

➢ Monomerized npr1Cys-GFP in C82A and C216A mutants should be able to accumulate in the nucleus in the absence of INA

   ○ Transform plants with 35S::npr1Cys-GFP constructs for C82A and C216A mutants
   ○ Examine GFP fluorescence of leaf tissues
How does NPR1 monomerization lead to activation and PR gene induction?

➢ Monomerized npr1Cys-GFP in C82A and C216A mutants should be able to accumulate in the nucleus in the absence of INA

Results:

➢ In absence of INA:
  ○ C82A and C216A showed significantly more fluorescence compared to wild type

➢ But how can we confirm that these proteins are within the nucleus?
How does NPR1 monomerization lead to activation and PR gene induction?

➢ Monomerized npr1Cys-GFP in C82A and C216A mutants should be able to accumulate in the nucleus in the absence of INA

Nuclear Fractionation!!! *(again)*

➢ Fractionate the nuclei of C84A and C216A mutants
  ○ SDS page (nuclear protein and total protein)
  ○ Immunoblot analysis
How does NPR1 monomerization lead to activation and PR gene induction?

- Monomerized npr1Cys-GFP in C82A and C216A mutants should be able to accumulate in the nucleus in the absence of INA.

**Total protein extract:**
- WT, C82A, and C216A protein found in similar amounts.

**Nuclear protein extract:**
- No WT found.
- C82A and C216A found.
How does NPR1 monomerization lead to activation and PR gene induction?

➢ Monomerized npr1Cys-GFP in C82A and C216A mutants should be able to accumulate in the nucleus in the absence of INA

Therefore…

➢ Monomerization of NPR1 protein allows for nuclear localization of the protein, and induces PR gene expression
How does SA activate NPR1?

Is NPR1’s protein conformation sensitive to cellular redox changes?

How does this tie into SAR?
How does SA activate NPR1?

Is NPR1’s protein conformation sensitive to cellular redox changes?

How does this tie into SAR?
How does SA activate NPR1?

Is NPR1’s protein conformation sensitive to cellular redox changes?

How does this tie into SAR?

**Recall:** C82A and C216A

- At least two cysteines are involved in oligomer formation
  - Other cysteines may be involved
  - Might be the residues forming initial disulphide bond
How does SA activate NPR1?

Is NPR1’s protein conformation sensitive to cellular redox changes?

How does this tie into SAR?

➢ Interesting note:
  ○ Monomerization of NPR1 is necessary and sufficient for activation of the protein
    ■ Depleting NADPH pool = **diminished** PR gene expression
    ■ Prevention of oligomer formation = **constitutive** PR gene expression
  
  ○ DATA NOT SHOWN
  ○ In *npr1C82A-GFP* and *npr1C216A-GFP* mutants
    ■ PR1 gene expression further upregulated with SAR induction
    ■ But only moderate resistance to pathogens

Other SA mediated activation steps are involved in **complete** SAR induction.
How does SA activate NPR1?

Is NPR1’s protein conformation sensitive to cellular redox changes?

How does this tie into SAR?

➢ Oligomeric NPR1 likely has an obscured NLS
  ○ Inaccessible to the antibody developed against N-terminal half of protein
  ○ Large structural difference from monomeric form
NPR1 as a transcriptional regulator

➢ A transcriptional regulator whose nuclear localization/activity regulated by cellular redox state

➢ Other example:
  ○ yAP1
    ■ Accumulation in nucleus in response to oxidative stress
    ■ Faster than NPR1

➢ Even in npr1 mutants, however, GSH/GSSG ratios experienced same biphasic change
  ○ Indicates very likely possibility that these redox changes are a secondary messenger that connects the SA signal with NPR1 activity
ROS as secondary messengers

Initial oxidative stress from pathogen attack

- Plant responds by overcompensating with antioxidants (GSH)
  - Protects against oxidative stress
  - Provides redox environment for transcriptional regulators (NPR1)

SAR induction:

Early burst of ROS

- Transient increase in cell reduction potential
- Sharp decrease in cell reduction potential

Due to accumulation of antioxidants like GSH
Further Questions

➢ If NPR1 does not have a DNA binding domain (therefore it is not a transcription factor), what is its role in activating PR gene expression?

○ What does NPR1 protein associate with inside the nucleus?
  ■ FIND OUT NEXT TUTORIAL!

○ Looking at the structure of NPR1 protein, how is that utilized in the activation of PR genes?
  ■ Roles of the BTB/POZ and ankyrin-repeat domains?

➢ If GSH is not the direct reducing agent, what may be? Thioredoxin like suggested?