ELSEVIER

# NPR1, all things considered

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Recent work has shown that the *Arabidopsis* NPR1 protein not only plays an essential role in salicylic acid (SA)-mediated systemic acquired resistance and rhizobacterium-triggered induced systemic resistance, but also is involved in crosstalk inhibition of jasmonic acid (JA)-mediated defense responses. Molecular characterization has revealed that activation of NPR1 and certain TGA transcription factors occurs under the reducing conditions that follow an initial oxidative burst after the induction of defense responses. In addition to NPR1 and TGA, the single-stranded DNA-binding transcription factor AtWhy1 and the WRKY70 transcription factor were recently found to be involved in SA-mediated defense and SA–JA crosstalk, respectively.

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**Abbreviations**

| | |
|---|---|
| ***AtWhy1*** | Arabidopsis thaliana *Whirly1* |
| **BTH** | benzothiadiazole *S*-methyl ester |
| **INA** | 2,6-dichloroisonicotinic acid |
| **ISR** | induced systemic resistance |
| **JA** | jasmonic acid |
| ***npr1*** | *nonexpressor of PR genes1* |
| ***PR*** | *PATHOGENESIS-RELATED* |
| **SA** | salicylic acid |
| **SAR** | systemic acquired resistance |

## Introduction

Systemic acquired resistance (SAR) is a plant immune response that is often induced after a local infection. Unlike the immune response in animals, which is specific to the inducing pathogen, SAR protects the plant against bacterial, fungal and viral infections [1,2]. The onset of SAR requires the accumulation of salicylic acid (SA) and the coordinated expression of *PATHOGENESIS-RELATED* (*PR*) genes, which encode small secreted or vacuole-targeted proteins that have antimicrobial activities [3–7]. In fact, exogenous application of SA, or of one of its functional analogs (2,6-dichloroisonicotinic acid [INA] or benzothiadiazole *S*-methyl ester [BTH]), can activate *PR* gene expression and resistance in plants without pathogen inoculation [1,8,9].

It has been ten years since the *Arabidopsis nonexpressor of PR genes1* (*npr1*) mutant was isolated in a genetic screen for plants that failed to express *PR* genes after SAR induction [10]. Additional *npr1* alleles (also known as *nim1*) were found in multiple screens for components of the SAR signaling pathway [10–13]. These *npr1* alleles are insensitive to all inducers of SAR, including SA, INA, BTH and avirulent pathogens. They are compromised not only in SAR but also in basal resistance, showing enhanced disease symptoms when infected with virulent pathogens. Originally, the *npr1* mutant was thought to be deficient only in SA-mediated defense. It soon became clear, however, that *NPR1* plays a role in other defense-signaling pathways. In the *npr1* mutant, the triggering of induced systemic resistance (ISR) by non-pathogenic rhizobacteria is blocked. Interestingly, this resistance response is independent of SA but requires regulators of ethylene and jasmonic acid (JA) signaling, ETR1 and JAR1, respectively [14].

*NPR1* was cloned in 1997 [15,16] and a significant amount of work has gone into understanding its molecular function. The observation that *NPR1* is constitutively expressed, and levels of its transcripts are increased only two-fold following SA treatment, suggested that it is regulated at the protein level [17]. Indeed, treating plants with SA induces the nuclear localization of NPR1, which is essential for the induction of *PR* genes [18]. Although lacking a canonical DNA-binding domain, NPR1 is known to regulate *PR* gene expression through interaction with TGA transcription factors [19–22]. Analysis of the *PR1* promoter indicates that it is regulated by both positive and negative *cis*-elements [23]. One negative regulator of SAR is *SNI1*, which was identified in a genetic screen for suppressors of *npr1* [24]. SA-induced *PR* gene expression is restored in the *npr1 sni1* double mutant. The interactions between NPR1, SNI1, TGAs and other transcription factors remain to be determined. Another gap in our understanding of the SAR signaling pathway is the mechanism by which SA activates the NPR1 protein. Over the past year, several significant findings have been made that begin to answer these questions. In this review, the focus will be on three areas: the mechanism by which SA activates NPR1, the mechanism by which NPR1 induces *PR* gene expression, and the role of NPR1 in the overall defense network.

## Activation of NPR1 by SA

Two major approaches have been used to elucidate the mechanism by which SA activates SAR. Genetic screens have yielded multiple alleles of *npr1* but no mutations at other loci [10–13]. This implies either that SA regulates NPR1 directly or that other regulatory components in the SA signaling pathway are either essential for plant viability or functionally redundant. A biochemical approach used radioactively labeled SA to look for SA-binding proteins. This led to the identification of several SA-binding proteins: a catalase, an ascorbate peroxidase, a carbonic anhydrase and a lipase [25–27,28••]. The lipase, SABP2, has a much higher affinity for SA ($D_d$ = 90 nM) than do the other SA-binding proteins ($D_d$ = 3.7–14 μM), and its enzymatic activity is increased by the presence of SA. Upon silencing of *SABP2*, SAR is abolished [28••], suggesting that SABP2 is probably the long-sought-after SA receptor required for this defense response. The lower-affinity SA-binding proteins are antioxidants and SA inhibits their activities, contributing to an accumulation of reactive oxygen species. The link between this oxidative burst and the activation of NPR1 was uncovered recently.

In an attempt to purify NPR1-containing protein complexes biochemically, Mou and co-workers [29••] found that, under non-denaturing and non-reducing conditions, NPR1 protein could only be detected in SAR-induced samples. This suggested that, in the uninduced state, constitutively synthesized NPR1 protein was present in a complex that was too large to enter the size-filtration column. This large complex was shown to be an oligomer of NPR1, formed through intermolecular disulfide bonds. Following SAR induction with INA there is a rapid oxidative burst. The cellular redox state then recovers and rebounds to a reduced environment. The NPR1 monomer appeared under these reducing conditions, and the appearance of this monomer was followed by activation of *PR* gene expression. Blocking the establishment of the reducing state with an inhibitor of the pentose phosphate pathway, the major source of cellular reducing power, decreased the formation of the NPR1 monomer and *PR* gene expression. On the other hand, mutations in the cysteine residues that cause monomer accumulation resulted in constitutive nuclear localization of the mutant proteins and constitutive *PR* gene expression. These results demonstrated that the NPR1 monomer is the biologically active form, and that the oligomer-to-monomer switch controls NPR1 nuclear transport.

The involvement of cellular redox in controlling SAR was demonstrated independently in a study of TGA transcription factors. Després and colleagues [30••] found that although TGA1 and TGA4 do not interact with NPR1 in yeast two-hybrid assays, both interact with NPR1 *in planta* following SA treatment. By comparison with other TGA factors, which interact with NPR1 constitutively both in yeast and *in planta*, TGA1 and TGA4 contain two unique cysteine residues (Cys-260 and Cys-266). Using a clever differential labeling method, Després' group found that, in the uninduced state, these two cysteines are oxidized, forming an intramolecular disulfide bond. Upon SA induction, the disulfide bond is broken, allowing the proteins to interact with NPR1 to activate gene expression. If TGA1 and TGA4 are involved in SAR, which is yet to be proven experimentally, the work of this group is in complete agreement with that of Mou and colleagues [29••], suggesting that SA-mediated gene expression occurs under reducing conditions.

One question still remains to be answered is how the initial oxidative burst triggered by SA accumulation leads to the subsequent establishment of a reducing state. Ozone-induced reducing redox state is diminished in transgenic *nahG* plants, which do not accumulate SA [31]. Therefore, it is possible that during SAR, SA not only triggers the initial oxidative burst but is also required for the establishment of reducing conditions, perhaps by activation of genes that encode antioxidants. SA is known to induce two waves of gene expression. Before NPR1-dependent *PR* gene expression, which occurs 12–16 h after induction, there is a wave of gene expression that peaks about 2–3 h after SA treatment [32]. The early inducible genes that are expressed in this wave encode detoxifying enzymes, such as glutathione-*S*-transferase and glucosyltransferase, that help to protect plant cells against oxidative stress [33•]. The major source of reducing power in plants and animals is the pentose phosphate pathway. The gene that encodes the rate-limiting enzyme in this pathway glucose-6-phosphate 1-dehydrogenase (*G6PDH*; **At5g40760**) is induced by pathogen infection (N Weaver, X Dong, unpublished). Furthermore, inhibition of G6PDH enzymatic activity using 6-amino nicotinamide blocked the establishment of the reducing state, NPR1 monomer formation and *PR* gene expression following SAR induction [29••]. It will be interesting to determine whether the induction of the *G6PDH* gene is SA-dependent but NPR1-independent. It will also be worth examining the phenotype of knockout mutants of this gene and other genes in the pathway. Another question that should be answered is whether specific redox mediators are involved in controlling NPR1, TGA1 and TGA4 oxidation/reduction exchange. Identification of such mediators may require both biochemical and genetic approaches.

## NPR1 activation of *PR* gene expression

The interaction of NPR1 with TGA transcription factors, discovered in multiple yeast two-hybrid screens, suggests that TGA factors may be responsible for NPR1-mediated *PR* gene expression [19–22]. Chromatin immunoprecipitation experiments, conducted by Johnson and co-workers [34•], showed that TGA factors are recruited to the *PR1* promoter *in vivo* in an SA- and NPR1-dependent

manner. Direct genetic evidence that supports a role for TGA factors in *PR* gene expression has been difficult to obtain, however, because there are ten *TGA* genes in *Arabidopsis*, some of which have high sequence similarity and functional redundancy. For example, TGA2, TGA5 and TGA6 all interact with NPR1 in the yeast two-hybrid analysis and belong to a subgroup that have high sequence similarity. To add to the problem, the *TGA2* and *TGA5* genes are adjacent on Chromosome V, making it virtually impossible to generate the *tga2 tga5* double mutant through genetic recombination. This problem was solved when a deletion that spans both genes was identified by Zhang *et al.* [35•]. They found that SA-induced *PR* gene expression was significantly blocked only in the *tga2 tga5 tga6* triple knockout mutant. This triple mutant also showed reduced tolerance of high levels of exogenous SA, a phenotype that is also observed in the *npr1* mutant. Interestingly, unlike *npr1*, the triple mutant does not show significantly enhanced susceptibility to virulent pathogens, indicating that other NPR1-interacting TGA factors (including the redox-sensitive TGA1 and TGA4) may be responsible for the expression of genes that are involved in basal resistance.

Besides TGAs, WRKY transcription factors have been suggested to play a role in controlling *PR* gene expression. The *cis*-element that is recognized by WRKY factors, the W-box, is over-represented in the *PR1* regulon [36], and mutations in a W-box led to depression of the *PR1* promoter [23]. It is thus hypothesized that WRKY factors are negative regulators of *PR* genes. The *WRKY* family consists of many more genes than the *TGA* family [37]; therefore, it will be difficult to identify a specific WRKY factor that is involved in *PR* gene expression. One WRKY factor that has been shown to affect *PR* gene expression is WRKY70 [38•]. Overexpression of *WRKY70* leads to constitutive *PR* gene expression, indicating that this transcription factor is a positive regulator of *PR* genes.

Restoration of inducible SAR in the *npr1 sni1* double mutant indicates that an NPR1-independent but SA-dependent transcription factor might be involved in controlling *PR* gene expression [24]. A recent study by Desveaux and colleagues [39••] suggests that the transcription factor Whirly1 is a likely candidate. A knockout mutation in the Arabidopsis thaliana *Whirly1* (*AtWhy1*) gene is lethal, which explains why this transcription factor was not identified in previous mutant screens. Fortunately, two lines that carry point mutations in this gene, *atwhy1.1* and *atwhy1.2*, are viable. Studies have shown that these mutants are compromised in SA-induced *PR* gene expression and resistance to *Peronospora parasitica*. The unusual feature of the Whirly1 transcription factor is its single-stranded DNA-binding activity. Its binding elements GTCAAAA/T are enriched in some of the *PR* gene promoters. The SA-induced AtWhy1 DNA-binding activity is NPR1-independent. It will be interesting to examine the phenotype of the *npr1 sni1 atwhy1* triple mutant. If AtWhy1 is indeed an NPR1-independent, SA-dependent transcription factor that regulates *PR* genes, the triple mutant is expected to have a *PR* gene expression pattern that resembles that of *atwhy1*.

## The role of NPR1 in the overall defense network

Stepping back from examining the specific role of NPR1 in regulating *PR* gene expression, several recent studies have provided new insights into the position of NPR1 in the overall defense network. NPR1 is thought to be a signal component whose activity is found downstream of *R*-gene-mediated defense. The constitutive resistance observed in *snc1* and *ssi4*, which are *R*-gene mutants that were isolated as suppressors of *npr1*, is clearly NPR1-independent [40•,41]. However, silencing of the *NPR1* gene in tomato enabled *Pseudomonas syringae* pv. *tomato* (*Pst*DC3000), carrying the avirulence gene *avrPto*, to develop disease symptoms in the *RG–PtoR* background, which normally shows *Pto*-mediated resistance to this bacterium [42•]. This suggests that NPR1, together with several other downstream proteins (such as TGA1a and TGA2.2) that were also tested in the study, plays a role in *Pto*-mediated resistance. It is possible, however, that the enhanced disease symptoms observed in the *NPR1*-silenced plants were due to a loss of basal resistance to *Pst*DC3000 carrying *avrPto*.

With regards to induced defense pathways, the SAR and ISR pathways are independent but have an overlapping requirement for NPR1 [43]. Recently, the role of NPR1 in ISR was reaffirmed using the *Pseudomonas fluorescens* CHA0 strain as the inducer and *Peronospora parasitica* Noco as the challenging pathogen [44•]. ISR is initiated in roots, whereas SAR is initiated in leaves, suggesting that these two responses may not be in competition for NPR1. SAR- and JA-mediated resistance are not independent, however, and may compete for NPR1 in leaves. When SA and JA are applied together to leaves, the presence of SA inhibits JA synthesis and signaling. This inhibition is alleviated in the *npr1* mutant, indicating that *NPR1* is part of the crosstalk control between signaling pathways [45••]. Interestingly, nuclear localization of NPR1 is not essential for this inhibition to occur. Mutation of the nuclear localization sequence of *NPR1* produces a protein that is defective in *PR*-gene induction [18] but is still able to inhibit the JA signaling pathway (SH Spoel, X Dong, unpublished). In the cytoplasm, SA-activated NPR1 may bind a positive regulator of the JA signaling pathway and prevent it from being transported to the nucleus. It is equally possible that NPR1 is required for the activity of a negative regulator of JA signaling.

The biological significance of this crosstalk control was elegantly demonstrated in studies of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) infection. SA-mediated

defense responses are more effective against *Pst*DC3000 infection than JA-mediated responses. In wildtype *Arabidopsis*, infection with *Pst*DC3000 leads to SA accumulation and signaling. In *nahG* or *npr1-1* plants, however, these responses are blocked and JA synthesis and signaling are significantly upregulated [45••]. This indicates that *Pst*DC3000 infection has the capability to induce the JA signaling pathway but, in the presence of SA and NPR1, this pathway is repressed. In tomato, *Pst*DC3000 uses the type-III secretion system and the phytotoxin coronatine to activate the JA signaling pathway and to repress *PR* gene expression to promote disease [46•]. These studies support the idea that crosstalk between the SA and JA pathways plays an important role in fine-tuning the plant defense response to *Pseudomonas* infection.

Microarray analysis is another method that is used to examine the role of NPR1 in the overall defense network. Using hierarchical clustering of microarray data, Glazebrook *et al.* [47••] found that the expression of SA-mediated genes and of a much larger group of genes, whose expression requires JA and ethylene signaling, was affected in the *npr1-1* mutant (which is likely to be a null). Interestingly, in the *npr1-3* mutant, which lacks the carboxy-terminal 194 amino acids that contain the nuclear-localization signal (NLS), only SA-mediated gene expression was compromised. This is consistent with the observation that *npr1-3* is cytoplasmically localized and is still partially active in crosstalk (SH Spoel, X Dong, unpublished).

In a study of SA- and JA-mediated gene expression, Li and co-workers [38•] demonstrated that the WRKY70 transcription factor plays a role in crosstalk control. Overexpression of *WRKY70* resulted in constitutive, SA- and NPR1-independent expression of *PR* genes. Conversely, JA-induced expression of *PDF1.2* was inhibited in *WRKY70*-overexpressing plants. Interestingly, the repression activity of WRKY70 was NPR1-dependent. Consistent with these data, *WRKY70* antisense lines showed reduced *PR* gene expression but constitutively activated expression of the JA-inducible genes *AtCOR1* and *AtVSP*. Future work should address the question of whether WRKY70 and NPR1 work together or independently in SA–JA crosstalk.

## Conclusions

NPR1 is a key regulatory component that is positioned at the crossroads of multiple defense pathways. Future challenges include the determination of how a single protein plays multiple roles in these defense responses. NPR1 may interact with different partners in different tissues, with the redox-regulated oligomer-to-monomer exchange controlling its subcellular localization in the cytoplasm or the nucleus. It is crucial that the NPR1 protein complexes are isolated and characterized soon as this will validate the genetic data and identify new NPR1-interacting proteins.

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Using different mutant strains of *Pseudomonas fluorescens* CHA0, the authors demonstrated that the production of 2,4-diacetylphloroglucinol in this rhizobacterium is important for triggering ISR against *Peronospora parasitica* in plants. This resistance response requires the functions of NPR1, JAR1 and EIR1.

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In the work described in this paper, a large collection of defense-related mutants were put into three groups using a microarray-based 'global expression phenotyping' method. The first group affects only SA-mediated genes, the second group affects a much larger set of genes that require JA and ethylene signaling in addition to SA, and the third group affects only the JA- and ethylene-mediated genes. This work provides a novel way of modeling the defense network and allows the placement of previously uncharacterized mutants in the network.