# Genetic dissection of systemic acquired resistance

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Significant progress has been made in the past year in understanding the mechanism of systemic acquired resistance. Mitogen-activated protein kinase cascades have been implicated as negative regulators of salicyclic acid accumulation and the induction of resistance. The salicylic acid signal is transduced through NPR1, a nuclear-localized protein that interacts with transcription factors that are involved in regulating salicylic-acid-mediated gene expression. Both promoter analyses and genetic studies have shown that gene expression in systemic acquired resistance requires not only the activation of a transcriptional activator(s) but also inhibition of a transcriptional repressor(s). Microarray experiments have been performed to search for those genes whose expression is transcriptionally regulated during systemic acquired resistance and to identify common promoter elements that control these genes.

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**Abbreviations**

| | |
|---|---|
| avr | avirulence |
| DEX | dexamethasone |
| HBD | hormone-binding domain |
| HR | hypersensitive response |
| ICS1 | ISOCHORISMATE SYNTHASE1 |
| INA | 2,6-dichloroisonicotinic acid |
| ISR | induced systemic resistance |
| JA | jasmonic acid |
| | bacterial salicylate hydroxylase gene |
| PR | pathogen-related |
| R | resistance |
| SA | salicylic acid |
| SAR | systemic acquired resistance |

## Introduction

Plants use different mechanisms to fend off pathogen infections. A significant number of genes in each plant genome encode leucine-rich-repeat-containing resistance (R) proteins that allow the organism to recognize the avirulence (avr) signals in pathogens [1,2,3••]. This 'gene-for-gene' interaction triggers a series of physiological changes at the site of infection, including programmed cell death (known as the hypersensitive response [HR]), production of reactive oxygen species, synthesis of antimicrobial phytoalexins, and accumulation of salicylic acid (SA) [4]. As a result of these events, the growth of the pathogen is restricted. Besides this gene-for-gene resistance, plants also employ inducible resistance mechanisms, such as systemic acquired resistance (SAR), to defend against a broad spectrum of pathogens [5]. SAR is induced after a local HR as a result of SA accumulation in both local and systemic tissues [6–8]. Removal of SA from transgenic plants expressing the bacterial salicylate hydroxylase gene (*nahG*) prevents the induction of SAR [9]. Exogenous application of SA or its analogs, such as 2,6-dichloroisonicotinic acid (INA) and benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), results in the induction of SAR [10–12]. A grafting experiment showed, however, that SA is not the systemic signal for SAR but rather a local signal that is required in systemic tissues [13]. Hence, the search is still on for a SAR systemic signal. The *Arabidopsis NPR1* (*NON-EXPRESSER OF PR GENES 1*; also known as *NIM1* [*NONINDUCIBLE IMMUNITY 1*]) gene has been shown to play a key role in SA signaling [14–17]. In *npr1* mutants, the SA-induced expression of pathogen-related (PR) genes and systemic resistance are completely blocked.

This review focuses on the signaling pathway that leads from a local HR to the accumulation of SA, followed by the activation of *NPR1* and the expression of PR genes. The emphasis is on genetic analyses, and unless otherwise specified, all of the mutants described are *Arabidopsis* mutants.

## Mutants affecting SA synthesis

The mechanism by which SA accumulates after a gene-for-gene interaction has yet to be revealed. Many mutants that form spontaneous HR-like lesions have elevated SA levels and enhanced disease resistance [18–21]. Cell death is, however, unlikely to be required for SA accumulation. The *dnd1* (*defense, no death 1*) mutant has increased SA concentrations and wild-type resistance to avirulent strains of *Pseudomonas syringae* but shows a reduced HR [22], which suggests that the HR can be uncoupled from SA synthesis and gene-for-gene resistance. In addition, the *dnd1* mutant shows enhanced resistance to virulent pathogens, probably because of its increased levels of SA. The *DND1* gene has been cloned and shown to encode a cyclic-nucleotide-gated ion channel [23•]. It remains to be determined how a loss-of-function mutation in this ion channel negatively affects the development of the HR and, at the same time, positively influences SA synthesis.

Another recently reported mutant, *mpk4*, also shows elevated SA concentrations in the absence of spontaneous lesions [24••]. This recessive mutation in the *MITOGEN-ACTIVATED PROTEIN KINASE4* (*MAPK4*) gene results in the constitutive accumulation of SA, SAR-related gene expression, and resistance. Thus, the wild-type MAPK4 is speculated to be a negative regulator of SAR.

The phenotype of *edr1* (*enhanced disease resistance 1*), a mutant that has a defective MAPKK kinase and that shows enhanced SA- and NPR1-dependent resistance to *P. syringae* and *Erysiphe cichoracearum*, is consistent with this hypothesis [25••]. There are also many reports suggesting that MAPK cascades positively regulate defense responses. Expression of a constitutively active MAPK kinase (NtMEK2) in tobacco results in the activation of two MAPKs, salicylic-acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK); expression of the *PAL* gene, which encodes phenylalanine ammonia lyase, the first enzyme in the phenylpropanoid pathway; and cell death [26••]. For a more in-depth discussion on studies of MAPK cascades in plant defense, see the excellent commentary by Bent [27••].

We must use caution before concluding that mutants with elevated SA levels, especially those forming spontaneous necrotic lesions, are affected in genes that are directly involved in defense responses. Lesion formation could be an indication of a stress that indirectly results in SA accumulation.

There are also several mutations that negatively affect SA accumulation. The *eds1* (*enhanced disease susceptibility1*) mutation abolishes resistance conferred by certain *R* genes, a phenotype that can be rescued by exogenous application of INA [28]. The discovery that *EDS1* encodes a protein that shares homology to eukaryotic lipases suggests that lipid metabolites are involved in regulating SA accumulation [29]. This hypothesis is consistent with the phenotype of *pad4* (*phytoalexin deficient4*), a mutant of another lipase homolog, which displays reduced SA accumulation after virulent pathogen infection [30,31]. Measurements of SA levels were used directly to identify the *sid1* (*salicylic acid induction-deficient1*) and *sid2* mutants [32]. After infection with the avirulent strain *P. syringae* pv. tomato DC3000/avrRpm1, SA levels in *sid1* and *sid2* were 10–20-fold lower than those in wild-type plants. Genetic complementation tests showed that *sid1* and *sid2* are allelic to the previously characterized *eds5* and *eds16*, respectively [33–35]. The *EDS16* (*SID2*) gene encodes ISOCHORISMATE SYNTHASE1 (ICS1) (MC Wildermuth, J Dewdney, FM Ausubel, G Wu, personal communication), indicating that SA might be synthesized not only by the previously proposed phenyl-propanoid pathway but also from chorismate by way of isochorismate, a pathway used by certain bacteria [36]. Indeed co-expression in tobacco of the *E. coli entC* gene (encoding an isochorismate synthase) and the *Pseudomonas fluorescens pmsB* gene (encoding an isochorismate pyruvate lyase) resulted in a 500–1000-fold increase in SA and SA glucoside levels and in enhanced resistance to tobacco mosaic virus and *Oidium lycopersicon* [37•].

## SA signaling through NPR1-dependent and NPR1-independent pathways

The SA-insensitive *npr1* mutants, the SA-deficient mutant *eds5* (*sid1*), and the SA-degrading transgenic *nahG* plants have been crossed with many mutants that have enhanced disease resistance to determine whether SA is required for the expression of their defense genes and for resistance. Interestingly, in many of the resulting double mutants, *eds5* and *nahG* seem to have a more dramatic effect on resistance responses than does *npr1* [19,20,38•]. For example, in *constitutive expresser of PR genes 6* (*cpr6*) ; *eds5* and *cpr6* ; *nahG* plants, *cpr6*-mediated resistance against both *P. syringae* and *Peronospora parasitica* is blocked, whereas in *cpr6* ; *npr1* plants only resistance to *P. syringae* is diminished [38•,39]. Hence, it is proposed that in mutants such as *cpr6*, an SA-dependent but NPR1-independent resistance response is activated. If such a response exists, application of SA to the *npr1* mutant should rescue the mutant phenotype and restore pathogen resistance. However, SAR induced by treatment with either SA or avirulent pathogen infection is blocked in *npr1*. To reconcile these observations, it is suggested that a second signal in addition to SA is required to activate this NPR1-independent pathway. So, what resistance response requires SA and an unknown signal but not NPR1? Likely not SAR, because SA alone has been shown to be sufficient to induce SAR. It is possible that mutants such as *cpr6* activate a response that is similar to gene-for-gene resistance. The fact that many mutants that have enhanced resistance form HR-like lesions is consistent with this speculation. Besides SA, which accumulates to high concentrations during a gene-for-gene response, many potential signal molecules, including reactive oxygen species, cell-wall fragments, and nitric oxide, are known to be produced at the site of pathogen infection. All of these molecules could potentially act as the second signal required for NPR1-independent resistance.

## Other mutants affecting SAR signaling

Mutations that can restore resistance in the *npr1* background are likely those that activate SA-dependent but NPR1-independent pathways. Mutants such as *cpr6*, *suppressor of SA insensitivity* (*ssi1*), and *acd6* all have elevated levels of SA and should not be considered as true suppressors of *npr1* [19,20,38•]. One mutant that may be a true *npr1* suppressor is *sni1* (*suppressor of npr1, inducible 1*) [40]. In the *sni1* mutant, SA levels are no greater than in wild-type plants, and in the *sni1* ; *npr1* double mutant, systemic induction of PR gene expression and resistance are restored. The phenotype of this recessive mutant suggests that wild-type *SNI1* is a negative regulator of SAR and that *NPR1* is required to alleviate *SNI1* repression. The *SNI1* gene encodes a novel protein sharing limited homology with the mammalian tumor suppressor RB (the retinoblastoma gene product). RB negatively regulates gene expression by sequestering transcriptional activators such as E2F and by recruiting histone deacetylase, which is involved in chromatin remodeling [41–43]. The mechanism by which *SNI1* negatively regulates SAR and the relationship between *NPR1* and *SNI1* have yet to be elucidated.

Another mutation that affects SAR signaling is *dth9* (*detachment 9*) [44••]. As in the *npr1* mutants, the induction of SAR

by local avirulent pathogen infection is blocked in *dth9* mutants. SA concentrations in *dth9* are slightly elevated compared to those in wild-type plants and application of SA does not restore disease resistance. The *dth9* mutant differs from the *npr1* mutant, however, in that its PR gene expression is unaffected. Furthermore, the *dth9* mutant has been shown to be insensitive to exogenous auxin treatment. On the basis of the phenotype of *dth9*, it is tempting to speculate that wild-type *DTH9* functions downstream of SA. It is more difficult to envision a function for DTH9 in a parallel SAR-inducing pathway because the presence of such a redundant pathway would have prevented the detection of both *dth9* and *npr1* mutant phenotypes. It will be interesting to characterize the *npr1* ; *dth9* and *sni1* ; *dth9* double mutants to determine their hierarchical relationships.

## Functional analysis of

Functional analysis of *NPR1* has shed light on the induction mechanism of SAR. Using an NPR1::green fluorescent protein (GFP) fusion and nuclear fractionation, NPR1 was shown to be localized to the nucleus [45••,46••]. Nuclear localization of NPR1 was demonstrated to be essential for SA-induced PR gene expression using a fusion between NPR1 and the hormone-binding domain (HBD) of the rat glucocorticoid receptor [46••]. In the absence of the steroid hormone dexamethasone (DEX), the NPR1::HBD protein is retained in the cytoplasm because of its association with the heat shock protein hsp90. Consequently, it is inactive in inducing PR gene expression even in the presence of INA. NPR1::HBD becomes biologically active when both INA and DEX are present. Interestingly, treatment with DEX alone, which is likely to cause the nuclear localization of NPR1::HBD, is insufficient for PR gene expression. This is consistent with the fact that NPR1 is detectable in the nucleus before SAR induction but PR gene expression is only observed after INA treatment [45••].

In the nucleus, NPR1 regulates PR gene expression through physical interactions with transcription factors. NPR1 itself contains no *bona fide* DNA-binding domains but rather protein–protein interaction domains. Using yeast two-hybrid screens, NPR1 was found to bind to the TGA transcription factor family [45••,47,48]. TGA transcription factors have been implicated in SA-induced gene expression in previous transcriptional studies [49–51]. A TGA-binding promoter element, *as-1*, has been shown to be required for SA-induced PR-1 expression, suggesting that the TGAs may be transcriptional activators [50]. Consistent with this hypothesis, NPR1 has been found to enhance TGA-factor binding to the *as-1* element in a gel-mobility shift assay using *in vitro* synthesized proteins [45••]. When nuclear extracts were used in this binding assay, however, a more complex pattern appeared. SA-dependent binding of a protein to *as-1* was detected when wild-type nuclear extracts were used in the gel mobility shift assay. Interestingly, when nuclear extracts were prepared from an *npr1* mutant, *as-1* protein-binding was detected in samples both with and without SA treatment. This result indicates that NPR1 is probably not required for TGA binding to the *as-1* promoter element, and that TGA binding alone is insufficient to cause the induction of PR genes.

## SAR-related gene expression

In addition to TGA transcription factors, other transcription factors may also play a role in SAR-related gene expression. In the *PR-1* gene, a negative promoter element has been identified; mutants in which this element is defective show heightened basal and induced gene expression [50]. This negative regulatory element contains a W-box, which is a binding site for the plant-specific WRKY transcription factors [52•]. Excitingly, multiple W-boxes (an average of 4.3) have been found in the upstream regions of many genes that are regulated in the same fashion as *PR-1* [53••]. This finding strongly suggests the involvement of WRKY transcription factors in SAR-related gene expression. It is still premature to assume that these W-boxes are all negative elements, as in *PR-1*, because there are approximately 75 WRKY transcription factors in *Arabidopsis*, some may be activators of SAR whereas others may be repressors [52•]. Evidence is accumulating, however, that argues for the presence of a negative regulatory mechanism in the control of SAR. The genetic identification of *SNI1*, a negative regulator of SAR, and the observation that TGA transcription factors can bind to the *as-1* element in the *npr1* mutant but fail to induce gene expression are indications that the induction of SAR involves both the activation of positive regulators and the inhibition of negative regulators.

The application of microarray technology has allowed genome-wide searches for downstream genes that are likely to be involved in conferring SAR [53••,54,55•]. After surveying 25–30% of all *Arabidopsis* genes, 31 genes were found that have expression patterns similar to that of *PR-1* [53••]. Besides those genes that are upregulated under various SAR-inducing conditions, genes whose expression is potentiated for induction during secondary pathogen challenge should not be ignored because they may also be involved in conferring resistance [56]. It is still possible that some resistance-determining genes are downregulated during SAR or not regulated transcriptionally at all. Establishing a causative relationship between the expression of a gene and SAR may be difficult because SAR is likely a result of the concerted expression of multiple downstream effector genes. Additional microarray analyses of mutants with diverse resistance profiles will allow us to identify the most likely candidate genes for functional analysis.

## SA-mediated resistance versus jasmonic-acid or ethylene-mediated resistance

In addition to SA, jasmonic acid (JA) and ethylene are also involved in various resistance responses [57]. Mutants that are insensitive to JA and ethylene have been found to be compromised in induced systemic resistance (ISR) elicited by root-colonizing bacteria [58]. Simultaneous induction of

ISR and SAR resulted in an additive level of resistance to *P. syringae* pv. tomato DC3000 [59•], indicating that JA/ethylene-mediated ISR functions in parallel with SA-mediated SAR. On the other hand, SA has been found to inhibit the JA-regulated wound response [60]. Antagonistic interaction between the SA- and JA-mediated responses is also displayed in the *mpk4* mutant in which SA-mediated SAR is constitutively activated whereas JA-mediated defensin PDF1.2 expression is blocked [24••]. Intriguingly, this blockage of PDF1.2 induction is not removed in the *nahG* background in which SA accumulation is inhibited and *mpk4*-mediated SAR is diminished. To date, we have found no consensus to describe the relationship between the SA and JA/ethylene pathways. It appears to vary among different resistance responses.

Even though SA has long been known to play a role in conferring gene-for-gene resistance, the effects of JA and ethylene in resistance against avirulent pathogens were first observed only recently. The SA- and ethylene-insensitive *npr1* ; *ein2* double mutant has been shown to be more severely compromised in gene-for-gene resistance to *P. syringae maculicola/avrRpt2* than either of the single mutants [37•]. Both JA-insensitive *coi1* (*coronatine-insensitive 1*) and *ein2* (*ethylene insensitive2*) mutants were found to be susceptible to strains of *Botrytis cinerea* and *Alternaria brassicicola*, which are avirulent on wild-type *Arabidopsis* [61,62]. A number of pathogens have also been tested on an ethylene-insensitive soybean mutant. Although this mutant responded normally to the avirulent *P. syringae/avrRpt2*, it was compromised in gene-for-gene resistance against certain avirulent races of *Phytophthora sojae* [63]. JA and ethylene may affect gene-for-gene resistance by regulating programmed cell death. In *Arabidopsis* protoplasts, cell death induced by the fungal toxin fumonisin B1 was found to require both the SA- and the JA/ethylene-signaling pathways [64•].

Besides the SA- and JA/ethylene-signaling pathways, novel defense pathways await discovery. For example, *RPP7*-mediated resistance against *P. parasitica* has been shown to be independent of SA and JA/ethylene signaling [65•]. SA- and JA/ethylene-independent resistance to *P. parasitica* can also be induced by treating plants with β-aminobutryic acid, a nonprotein amino acid [66•].

## Conclusions

It has become impossible to draw a linear pathway that describes the signaling events leading to a specific resistance response. More information must be collected to outline the plant defense network, which is also influenced by plant growth and development. The picture that has emerged from recent research shows redundancy and overlap between different disease and herbivore resistance responses. Signal molecules can be produced through different pathways, and resistance to the same pathogen can be achieved through different defense mechanisms. More standardized assays must be developed and utilized to better define each resistance response. A combination of genetic and genomic approaches will significantly broaden our view of plant disease resistance.

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