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Perception of the plant immune signal salicylic acid

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Salicylic acid (SA) plays a central role in plant innate immunity. The diverse functions of this simple phenolic compound suggest that plants may have multiple SA receptors. Several SA-binding proteins have been identified using biochemical approaches. However, genetic evidence supporting that they are the bona fide SA receptors has not been forthcoming. Mutant screens revealed that NPR1 is a master regulator of SA-mediated responses. Although NPR1 cannot bind SA in a conventional ligand-binding assay, its homologs NPR3 and NPR4 bind SA and function as SA receptors. During pathogen challenge, the SA gradient generated at the infection site is sensed by NPR3 and NPR4, which serve as the adaptors for the Cullin 3-based E3 ubiquitin ligase to regulate NPR1 degradation. Consequently, NPR1 is degraded at the infection site to remove its inhibition on effector-triggered cell death and defense, whereas NPR1 accumulates in neighboring cells to promote cell survival and SA-mediated resistance.

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Introduction

Salicylic acid (SA) is one of the major plant hormones that regulates various stress responses and development, such as resistance to pathogens, flowering, thermogenesis, senescence, and abiotic stress responses [1,2]. Among them, the most well studied role of SA is in plant immune response to pathogens. The plant immune system consists of different layers of active defense responses, including MAMP-triggered immunity (MTI), effector-triggered immunity (ETI) and systemic acquired resistance (SAR). Many studies have demonstrated that SA plays a central role in these responses [3,4]. In 1979, White found that treatment of tobacco with SA, or its derivative aspirin (acetyl-salicylic acid), dramatically enhanced its resistance to tobacco mosaic virus (TMV) [5]. Later studies found that blocking SA accumulation by expressing a bacterial enzyme, salicylate hydroxylase (NahG), compromised both ETI and SAR in tobacco as well as in *Arabidopsis* [6,7]. A central question related to SA is how it activates disease resistance. Studies in the past 20 years have greatly improved our understanding of the SA signaling pathway. This review focuses on the mechanisms by which the SA signal is perceived in plants.

Biochemical search for SA-binding proteins

As an immune signal, SA must be able to bind to cellular targets or receptors in order to activate downstream signaling events. This idea led to great efforts in the past 20 years to identify the SA receptor. Klessig and his colleagues found potential SA receptors by isolating SA-binding proteins (SABPs) using biochemical approaches. The first identified SABP was the tobacco catalase with a dissociation constant (K_d) of 14 μM [8–10]. It was proposed that SA could bind and inhibit catalase, resulting in an elevated level of reactive oxygen species, such as H_2O_2, which could further activate downstream defense mechanisms. However, Ruffer *et al.* presented evidence against the specific binding of SA to plant catalase [11]. They found that the catalases from fungi and animals could also bind SA. Further studies showed that SA could bind to iron-containing enzymes such as aconitase, lipoxidase and peroxidase as well [11]. These enzymes are therefore general cellular targets of SA, but unlikely specific SA receptors in plants.

Compared to catalase, SABP2 in tobacco has a much higher SA-binding affinity (K_d = 90 nM) [12]. Through structural and biochemical studies, SABP2 was found to have methyl salicylate (MeSA) esterase activity with SA as a potent product inhibitor [13,14]. *Arabidopsis* carries at least 18 potential SABP2 homologs. Among them, AtMES9 showed the highest SA binding activity (about 50% of tobacco SABP2) [15]. Although SABP2 is required for SAR, it does not function as a receptor for SA but rather converts the biologically inactive MeSA to the active SA in the systemic tissues during SAR [13].

SABP3 was identified as a carbonic anhydrase (CA) localized in chloroplasts. It has moderate SA-binding activity with apparent K_d of 3.7 μM [16]. Although SA is synthesized in the chloroplasts, its receptors are unlikely in this organelle because SA needs to be exported by its transporter, EDS5, to the cytoplasm in order to regulate immune responses [17•].

As an alternative to the traditional biochemical approach using tobacco plants, Klessig's group recently isolated

additional SABPs in *Arabidopsis* using a combined photo-affinity labeling and surface plasmon resonance-based technology [18•]. These SABPs were the E2 subunit of α-ketoglutarate dehydrogenase and the glutathione S-transferases GSTF2, GSTF8, GSTF10 and GSTF11. It was noted that these proteins had little or no SA-binding activities in the traditional ligand binding assays using radioactive SA, indicating that they are SABPs with low affinity and/or transient interactions. The significance of these proteins in SA responses remains to be tested.

More recently, Popescu and her colleagues used protein microarrays to identify SABPs in *Arabidopsis* [19•]. In this study, they used a functional SA analog, 4-azido SA (AzSA) to probe the *Arabidopsis* protein microarray and identified 65 proteins interacting with AzSA. They further characterized the thimet metalloendopeptidase (TOP) and found that SA could bind and inhibit this enzyme. However, in the traditional SA-binding assay, 10 mM non-radioactive SA could only compete away 50% of the binding activity of 300 nM radioactive SA, raising concerns about the binding specificity of TOP.

Genetic screens identified NPR1 as a master regulator of SA-mediated responses

In contrast to the biochemical approaches, several genetic screens for mutants defective in SA responses independently identified the same gene, *NPR1* (*Nonexpresser of PR genes 1*, *a.k.a. NIM1*, *SAI1*), as a key regulator of the SA signaling pathway [20–25]. NPR1, which contains two conserved protein–protein interaction domains: BTB (Bric-a-brac, Tramtrack, Broad-complex) domain and ankryin repeat domain, was found in yeast two-hybrid screens to interact with TGA transcription factors, and proposed to function as a transcription co-activator of SAR gene expression [26,27]. This hypothesis was supported first by the observation that NPR1 was translocated to the nucleus upon SA induction [28]. In the absence of SA, NPR1 forms an oligomer in the cytosol to prevent untimely defense activation. Upon pathogen infection or SA treatment, NPR1 is reduced to monomers as a result of SA-induced redox changes in the cell and is translocated into the nucleus to regulate defense gene expression [29]. The role of NPR1 as a transcription cofactor was also consistent with a genome-wide gene expression study in which the *npr1* mutant was found to be almost completely defective in SA-mediated transcriptional reprogramming of approximately 10% of the *Arabidopsis* transcriptome and compromised in SA-induced disease resistance [30]. These genetic data strongly suggest that NPR1 is an SA receptor.

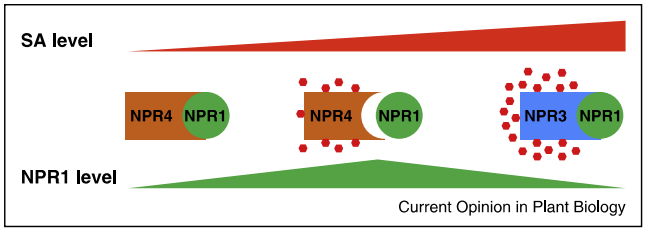
Is NPR1 an SA receptor?

NPR1 would be a perfect candidate for being an SA receptor if only it could bind SA. Unfortunately, in the Fu *et al.* study, no considerable SA-binding activity for *Arabidopsis* NPR1 was detected using a traditional ligand-binding assay, in which significant binding activities were observed for other NPR proteins [31••]. However, Wu *et al.* reported that NPR1 could bind SA in an equilibrium dialysis assay [32••]. It was suggested that binding of SA requires copper as a cofactor through two key cysteine residues (Cys521 and Cys529). This raised the possibility that the failure to detect the SA-binding activity in the Fu *et al.* binding assay was due to the lack of copper in the binding buffer. However, addition of copper to the binding buffer failed to improve the SA-binding activity of NPR1 (unpublished data). If the two cysteine residues were important for SA-binding, one would expect them to be conserved in the NPR1 homologs of other plant species. However, this is not the case, raising the question how other plants perceive SA through NPR1. In a separate study by the same research group [33], oxidation of the same cysteine residues was found to be required for reporter gene induction in a transient expression assay, leading to another question of how these cysteine residues bind copper, as in the large majority of cases metal binding activity requires cysteines to be in the reduced state [34]. According to Wu *et al.*, NPR1 is a high affinity SA-binding protein with the K_d value about 140 nM, much higher than the K_d values of SABP and SABP3. If this is the case, then the traditional ligand binding assay conducted by Fu *et al.* should have been sensitive enough to detect SA-binding activity of the NPR1 protein. Therefore, the jury is still out on whether *Arabidopsis* NPR1 is an SA receptor despite strong genetic data supporting this hypothesis.

The NPR1 homologs NPR3 and NPR4 are SA receptors

The failure to detect SA-binding activity of NPR1 leads to the hypothesis that other components controlling NPR1 may be the SA receptors. Spoel *el al.* found that proteasome-mediated NPR1 degradation plays dual roles in plant immunity [35]. In the absence of SA or pathogen infection, NPR1 is degraded to prevent spurious activation of defense responses. Upon induction, NPR1 degradation is also required to achieve maximum activation of defense gene expression likely by continuously refreshing the transcription initiation complex. Spoel *et al.* further demonstrated that NPR1 degradation required the Cullin 3-based E3 ligase, which utilizes BTB-domain containing proteins as the substrate adaptors [36]. The NPR1 homologs NPR3 and NPR4 are good candidate adaptors because both of them contain BTB domains and more importantly, the *npr3 npr4* double mutant was shown to have enhanced disease resistance rather than compromised resistance observed in the *npr1* mutant [37]. Indeed, Fu *et al.* found that NPR3 and NPR4 could interact with both NPR1 and Cullin 3 and were required for the NPR1 degradation [31••]. Interestingly, the interaction between NPR3/4 and NPR1 was regulated by SA. While SA disrupted the interaction between NPR4

Figure 1



NPR3 and NPR4 bind salicylic acid to control NPR1 level. NPR1 is a key positive regulator of SA-mediated responses. NPR3 and NPR4 are the adaptor proteins mediating NPR1 degradation. NPR4 is a high affinity SA receptor and NPR3 is a low affinity SA receptor. SA blocks NPR4–NPR1 interaction and facilitates NPR3–NPR1 interaction. When SA level is very low, NPR1 level is low because NPR4 mediates its degradation. When SA level is very high, NPR1 level is also low because NPR3 mediates its degradation. At the medium SA level, NPR1 level is the highest because SA is enough to disrupt NPR4–NPR1 interaction, but not enough to facilitate NPR3–NPR1 interaction.

and NPR1, it facilitated the interaction between NPR3 and NPR1. These results suggest that NPR3 and NPR4 may bind SA to regulate their interactions with NPR1 at different SA levels. Consistent with this hypothesis, Fu *et al.* demonstrated that NPR3 and NPR4 bound SA with different affinities. NPR4 had higher binding affinity with a K_d around 50 nM and NPR3 had lower binding affinity with a K_d around 1000 nM. It has been shown that pathogen infection creates an SA gradient around the infection site [38] and NPR1 suppresses programmed cell death during ETI [39]. Based on these facts, Fu *et al.* proposed that NPR3 and NPR4 are the SA receptors that sense the SA gradient during pathogen infection to determine cell death and survival. In infected tissues, the SA level is high enough to facilitate NPR3 and NPR1 interaction to degrade NPR1, allowing cell death and ETI to occur. In the surrounding tissues, the lower SA level is sufficient to disrupt NPR4–NPR1 interaction but not high enough to mediate NPR3–NPR1 interaction, allowing the accumulation of NPR1 protein, which promotes cell survival and SA-mediated resistance (Fig. 1).

Are there other SA receptors?

Although NPR1 plays a major role in SA-mediated transcriptional reprogramming, a large body of evidence indicates that there are SA-dependent but NPR1-independent pathways to regulate defense gene expression [40]. For example, in a genetic screen for the suppressors of *npr1*, SNI1 and SNC1 were identified as negative regulators of defense responses [41,42]. In the *sni1 npr1* and *snc1 npr1* double mutants, expression of the SA-mediated defense gene was restored and constitutively activated, respectively. Recently, SNI1 was discovered to be a subunit of the SMC5/6 complex involved in DNA damage responses [43•]. Further studies demonstrated that SA could activate the DNA damage responses to potentiate defense gene expression in an NPR1-independent manner. Hence, it is likely there are other SA receptors than NPR3 and NPR4 to mediate NPR1-independent pathways.

In addition to regulating defense gene expression, SA has other physiological effects in plants. It has been shown that SA treatment can rapidly induce the generation of superoxide anion, followed by a transient increase in cytosolic free calcium concentration in tobacco suspension culture [44]. Recently, SA was shown to interfere with clathrin-mediated endocytic protein trafficking [45•]. Compared to transcriptional regulation, these responses are very rapid (10 s to 10 min), indicating the presence of other SA receptors in these early SA responses.

SA and its synthetic derivatives such aspirin have broad medicinal effects in humans. Besides cyclooxygenase and IκB kinase, which have been shown to be cellular targets in humans, a recent study found that salicylate could directly bind and activate the AMP-activated protein kinase (AMPK), a cellular energy sensor conserved across all the eukaryotes [46••]. The activated AMPK will then promote the ATP-generating pathways and decrease the ATP-consuming pathways. In *Arabidopsis*, there are 38 AMPK homologs called Snf1-related kinase (SnRK) [47]. Two of the SnRKs have been shown to function as central integrators of transcriptional networks in stress and energy signaling [48]. Since the SA-activated defense response is also an energy-demanding process, it is worthwhile to test whether SnRKs can function as SA receptors. A good candidate is PKS5, which has been shown to interact and phosphorylate NPR1 [49].

How do plants with high basal level of SA perceive SA signal?

Compare to *Arabidopsis*, some plants have much higher basal level of SA. For example, rice has two orders of magnitude higher levels of SA than *Arabidopsis*. The studies in rice suggest that SA is not an effective signal to induce defense gene expression. Rather, SA plays an important role in protecting rice from oxidative damage during pathogen infections [50]. Although the main function of SA is different between rice and *Arabidopsis*, rice has all the homologs of *Arabidopsis* NPR1, NPR3 and NPR4 [51]. Similar to *Arabidopsis* NPR1, rice NPR1 (called OsNPR1 or NH1) is also a positive regulator of defense response [51,52]. The NPR3 homolog in rice (NH3) shares the highest homology with *Arabidopsis* NPR3 and NPR4. Interestingly, while *Arabidopsis* NPR3 and NPR4 negatively regulate immune responses [37], NH3 plays a positive role in rice [53]. In addition to NPRs, rice WRKY45 plays a crucial role in SA signaling [54]. It will be interesting to test whether rice perceives SA signal through NPRs homologs or through other components such as WRKY45.

Conclusion and future prospective

SA plays a central role in plant immunity, in which the master regulator NPR1 has the intriguing functions of controlling both cell death and cell survival. The identification of NPR3 and NPR4 as SA receptors is a major step forward in our understanding of the SA signaling pathway [55–57]. This discovery explains how SA functions through binding with NPR3 and NPR4 to control NPR1 level to determine cell death and survival during pathogen infection. There are many interesting questions still remaining. For example, why NPR1 cannot bind SA while its homologs NPR3 and NPR4 can? Why NPR3 and NPR4 have such different SA-binding affinities? What are the SA receptors in the NPR1-independent pathways? What are the SA receptors mediating the early SA responses? Further genetic, biochemical and structural studies are needed to address these questions.

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