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Current Opinion in Plant Biology

Seeing is believing: Understanding functions of NPR1 and its paralogs in plant immunity through cellular and structural analyses

Pei Zhou[1], Raul Zavaliev[2,3], Yezi Xiang[2,3] and Xinnian Dong[2,3]

Abstract

In the past 30 years, our knowledge of how nonexpressor of pathogenesis-related genes 1 (NPR1) serves as a master regulator of salicylic acid (SA)-mediated immune responses in plants has been informed largely by molecular genetic studies. Despite extensive efforts, the biochemical functions of this protein in promoting plant survival against a wide range of pathogens and abiotic stresses are not completely understood. Recent breakthroughs in cellular and structural analyses of NPR1 and its paralogs have provided a molecular framework for reinterpreting decades of genetic observations and have revealed new functions of these proteins. Besides NPR1's well-known nuclear activity in inducing stress-responsive genes, it has also been shown to control stress protein homeostasis in the cytoplasm. Structurally, NPR4's direct binding to SA has been visualized at the molecular level. Analysis of the cryo-EM and crystal structures of NPR1 reveals a bird-shaped homodimer containing a unique zinc finger. Furthermore, the $TGA3_2$-$NPR1_2$-$TGA3_2$ complex has been imaged, uncovering a dimeric NPR1 bridging two TGA3 transcription factor dimers as part of an enhanceosome complex to induce defense gene expression. These new findings will shape future research directions for deciphering NPR functions in plant immunity.

Addresses

[1] Department of Biochemistry, Duke University School of Medicine, Durham, NC 27708, USA

[2] Howard Hughes Medical Institute, Duke University, Durham, NC 27708, USA

[3] Department of Biology, PO Box 90338, Duke University, Durham, NC 27708, USA

Corresponding authors: Dong, Xinnian (xdong@duke.edu); Zhou, Pei (peizhou@biochem.duke.edu)

Current Opinion in Plant Biology 2023, **73**:102352

This review comes from a themed issue on **Biotic interactions**

Edited by **Li Yang** and **Eunyoung Chae**

For a complete overview see the Issue and the Editorial

Available online xxx

https://doi.org/10.1016/j.pbi.2023.102352

Keywords

Plant immunity, Systemic acquired resistance, Salicylic acid (SA), NPR1, NPR2, NPR3, NPR4, Structural analysis, Cryo-EM, Crystal structure, SA-binding domain (SBD), SA-induced NPR1 condensate (SINC).

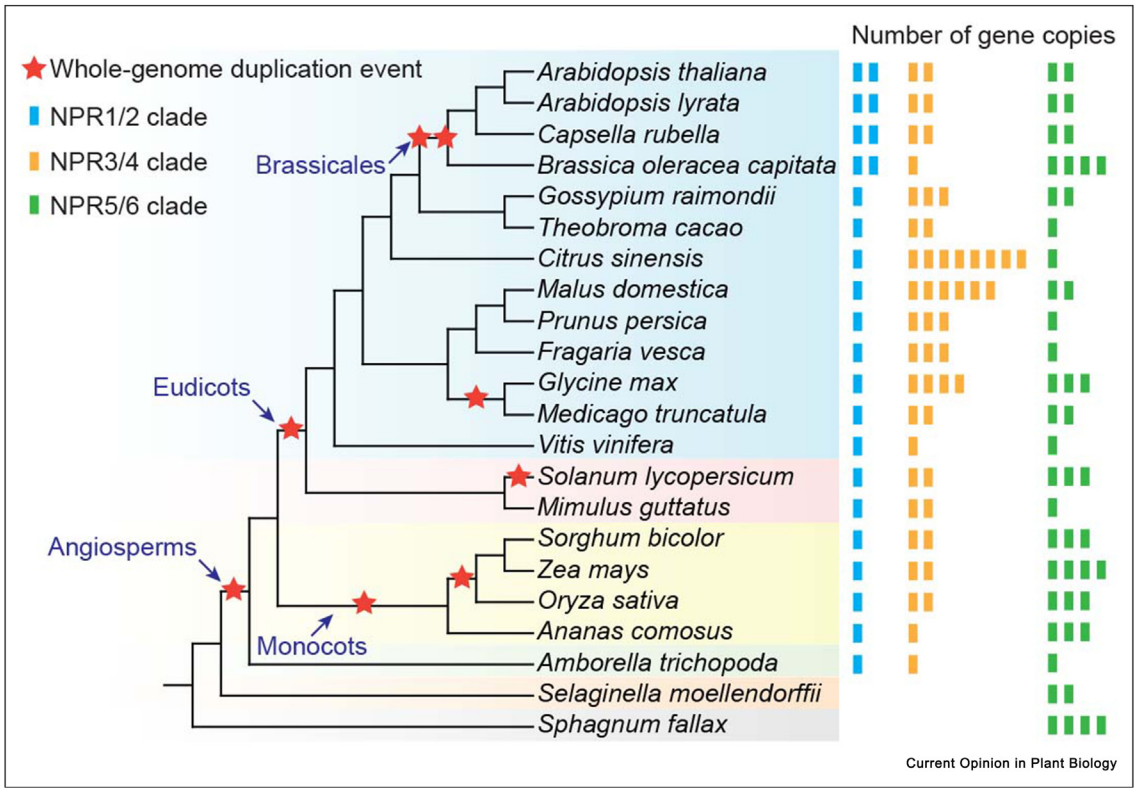
Introduction

Nonexpressor of pathogenesis-related genes 1 (NPR1)—a master immune regulator—was discovered through genetic screens for *Arabidopsis* mutants compromised in pathogen resistance mediated by salicylic acid (SA) [1–5]. *Arabidopsis npr1* mutants display decreased expression of pathogenesis-related (*PR*) genes and increased disease susceptibility. Conversely, overexpressing the *Arabidopsis NPR1* (*AtNPR1*) or its orthologs in both dicot and monocot plants could enhance disease resistance against a variety of pathogens [6,7]. These studies highlight a central role of NPR1 in plant immunity, allowing its potential use in engineering broad-spectrum disease resistance in crops.

AtNPR1 is the founding member of a unique family of proteins (clade I: NPR1 and NPR2; clade II: NPR3 and NPR4; and clade III: NPR5/BOP2 and NPR6/BOP1) in plants (Figure 1). In *Arabidopsis*, clades I and II are mainly involved in defense [1,4,5,8,9], whereas clade III mainly functions in leaf morphogenesis [10]. Orthology analysis revealed that these three clades had already diverged in the common ancestor of angiosperms and are retained in most angiosperm species. However, in early diverging land plants, such as mosses and ferns, only orthologs of clade III NPR genes could be found, suggesting their early emergence during land plant evolution (Figure 1). Though NPR-like sequences with defense activities have been found in a moss species [11], more data will be needed to determine whether they are orthologs of clade I or II NPRs. Unlike clades II and III, clade I gene ortholog occurs as a single copy in most of the angiosperm species sampled, indicating its functional conservation across lineages.

In response to SA-induced redox changes, the AtNPR1 protein is released from a quiescent oligomer to translocate into the nucleus [12–14], where it serves as a coactivator in complex with transcription factors

Figure 1



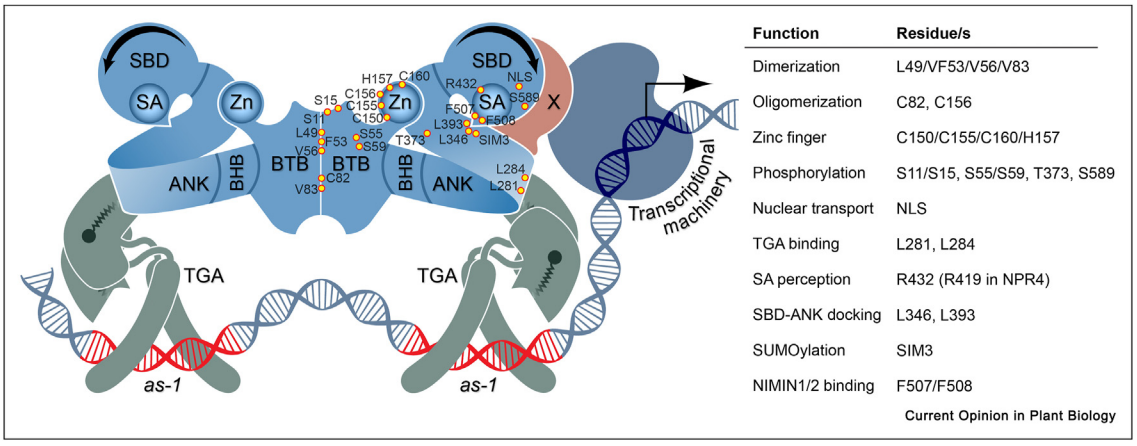
Origin of NPR gene family across land plants. A phylogenetic tree was constructed, and whole-genome duplication (WGD) events were annotated according to the 1 KP and other phylogenetic studies [46]. Full-length protein sequences from 62 representative algae and land plants (excluding Gymnosperms) with high-quality genome sequence data were downloaded from Phytozome v12. All-against-all BLASTP [47] were performed, and ortholog groups (OGs) were identified using OrthoMCL [48] (Supplemental Table 1). For simplicity, 22 of the 62 species were shown in the figure. From the analysis, AtNPR1, AtNPR2, and their 69 orthologs from 46 species were classified into one orthologous group (named here as the NPR1/2 clade); AtNPR3, AtNPR4, and their 121 orthologs from 47 species were classified into another orthologous group (named here as the NPR3/4 clade); AtNPR5, AtNPR6, and their 120 orthologs from 53 species were classified into the third orthologous group (named here as the NPR5/6 clade) (Supplemental Table 2). The number of rectangles represents the number of gene copies. Gene copies with the same color belong to the same orthologous group. The NPR genes not classified as orthologs are not shown.

(TFs), such as TGAs [15–18]. However, how NPR1 activates transcription remains a mystery. Opposite of NPR1, NPR3 and NPR4 are negative regulators of defense genes [8], whose activities are responsive to SA binding [19]. Two nonexclusive modes of actions have been proposed to explain their roles: one as transcription repressors based on the presence of the "EAR motif" known in other transcription repressors [20] and the other as adaptors for the Cullin 3 RING ubiquitin ligase (CRL3) complex to degrade substrates, such as NPR1 and JAZ1 [19,21]. Then, there were questions of whether NPR1, the *bona fide* SA receptor based on the genetic data, directly binds SA *in vivo*, despite its low binding efficiency *in vitro* when compared with NPR3 and NPR4 side by side [19,22], and how SA binding modulates the NPR1 transcriptional activity. Here, we describe advances made at cellular and structural levels in the past two years, which begin to address these fundamental questions.

Conservation of NPRs as SA receptors in diverse plant species

Although NPR proteins were discovered based on a loss of function in SA-mediated plant immunity response, how NPR proteins serve as SA receptors has been hotly debated [19,20,22–24]. The recent structural analysis of the C-terminal region of NPR4 showed that it contains an SA-binding core (SBC) consisting of a four-helix bundle, in which SA is embedded [22]. In particular, the R419 residue in the SBC of NPR4 binds SA by forming bidentate hydrogen bonds with the carboxylate group of the hormone, thus explaining its critical role in SA perception (Figure 2). As the SA-interacting residues are highly conserved within NPR proteins of immunity clades I and II, the structural analysis of the NPR4 SBC led to the classification of these NPRs as general SA receptors. It is worth noting that this SBC does not include the SA-binding residues, C521 and C529, identified in a previous report [24].

Figure 2



The enhanceosome model of the NPR1 dimer bridging two dimeric TGA transcription factors which recognize the *as-1* (activation sequence 1) *cis*-element in the promoters of defense genes. SA-induced SBD–ANK docking creates a new interface to facilitate post-translational modifications and/or recruitment of transcriptional regulators (X) for the activation of defense genes. The position of key residues in NPR1 (left) and the corresponding activation steps (right) are shown.

Despite the central role of NPR1 in plant immunity, details of its molecular features have remained elusive until recently. The combined use of single-particle cryo-EM and crystal structure analysis has revealed NPR1 as a bird-shaped homodimer [25] (Figure 2). It consists of the Broad-complex, Tramtrack, and Bric-à-brac (BTB) dimer in the middle, followed by the back-helix-bundle (BHB), and four ankyrin repeats (ANKs) forming the wings of the bird. A prominent feature of NPR proteins is the presence of a conserved cysteine cluster within the BTB domain. These cysteine residues together with a conserved histidine (C150, C155, C160, and H157 in AtNPR1) form a previously unrecognized zinc finger motif that bridges the BTB domain with the ANKs (Figure 2). Unlike the zinc coordinating cysteines, the surface exposed C156 residue of AtNPR1, involved in the redox-sensitive oligomer formation, is not conserved in species beyond the crucifer family, an observation supported by sequences recently reported for several additional plant species (note: in these studies, AtNPR1 C155 was mislabeled as C156) [26–28]. Thus, whether and how NPR1 orthologs in other plant species form redox-sensitive oligomers remain to be tested.

The cryo-EM structure of apo NPR1 shows the C-terminal region as disordered, whereas the protein refolded in the presence of SA shows the SA-binding domain (SBD) docked onto ANKs [25], with a conformation similar to that of the SA-bound SBD of NPR4 [22]. Importantly, crosslinking of this SBD-ANK docking conformation enhanced the NPR1-mediated *PR1* gene expression, providing the first evidence that SA plays a direct role in regulating the NPR1 conformation required for its transcriptional activity [25] (Figure 2). This initial conclusion needs to be further solidified by the identification of the possible chaperon/modification that would explain how SA gets into the enclosed SBD *in vivo* and how divergent residues between NPR1 and NPR4 affect their SA-binding efficiency and result in distinct outcomes in transcriptional regulation.

NPR1 as a transcription cofactor

NPR1 has been known to interact with both transcription activators (e.g., TGA) as well as repressors (e.g., certain WRKYs) to reprogram the transcriptome upon induction [15–18,29,30]. The crystal structure of the NPR1-interaction domain (NID) in TGA3 reveals a homodimer, each consisting of five long helices and three short helices with a palmitic acid embedded within [25]. Although the mechanistic connection of fatty acid and plant immunity requires further investigation, this observation provides strong support for the genetic relationship between lipid metabolism and plant immunity reported previously [31,32]. It has been suggested that TGA1 and TGA4 activity is regulated by a redox-mediated intramolecular disulfide bond between two cysteine residues (C260 and C266 in TGA1 and C256 and C263 in TGA4) [33] located in the NID, though this model has recently been challenged [34]. Based on the corresponding residues in the TGA3 structure, the cysteine residues in TGA1/4 are located on the same helix and separated by nearly two helical turns, which makes them unlikely to be engaged in disulfide bond formation. However, it remains possible that oxidized cysteines in TGA1/4 may perturb the folding and stability of their NID, thus affecting interaction with NPR1. Importantly, the cryo-EM structure of the NPR1-TGA3 complex shows that the NPR1 dimer activates transcription by bridging dimeric TGA3 molecules on each side (Figure 2). With its

dimeric architecture as well as the observation of the $(NPR1)_2$-$(TGA3)_2$ intermediate, it is conceivable that NPR1 could form a variety of TF complexes in a combinatorial manner, thus achieving a fine regulation of gene expression and crosstalk with other plant hormone signaling pathways [35,36]. Such a model is compatible with reported simultaneous interactions of NPR1 with multiple TFs revealed by pulldown studies, including both positive (e.g., TGA) [15–18] and negative (e.g., NIMINs) regulators [37] as well as histone modifiers (e.g., HAC1, histone acetyltransferase 1) [38], mediator components, and other key immune regulators, such as CDK8 [39] and EDS1 [40]. NPR1 has also been reported to interact with TFs involved in transcription mediated by other plant hormones, such as MYC2 required for jasmonic acid (JA)-responsive genes and EIN3 for ethylene signaling [35,36]. However, these components may not all be present in the same NPR1 complex. For example, the NPR1-WRKY interaction was observed in SA-induced NPR1 condensates (SINCs) in the cytoplasm rather than in the nucleus [30], as previously assumed [29]. Given its scaffolding role, NPR1 likely functions as a platform to nucleate multiple TFs in an enhanceosome complex. The core components of the NPR1 enhanceosome await to be revealed.

Examination of the $(TGA3)_2$-$(NPR1)_2$-$(TGA3)_2$ complex raises new questions: (1) Is there a component or modification that connects SBD to TGA activity? (2) How does the palmitate found in the TGA3 dimer regulate SA-mediated gene expression?

NPR1 and NPR4 in SA-mediated protein degradation

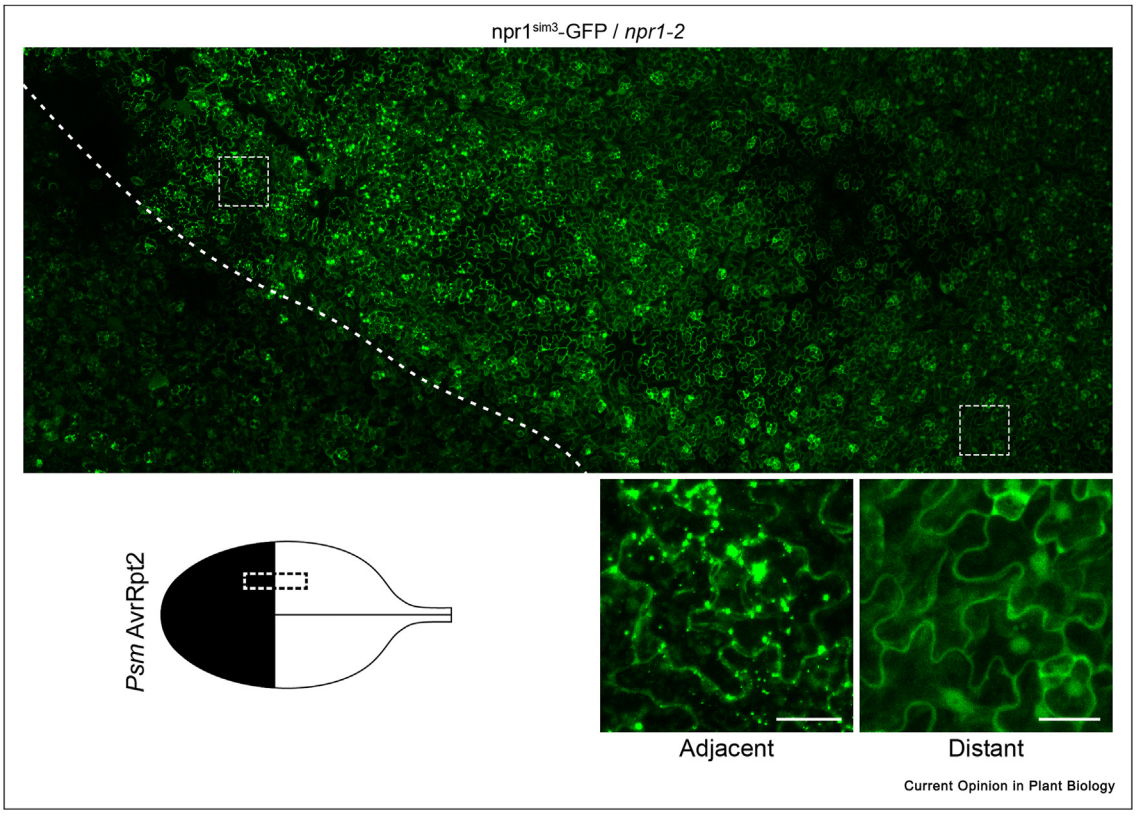
Another fascinating question about the NPR proteins is what makes the evolutionarily divergent NPR1/2-clade and NPR3/4-clade having opposing roles in defense gene transcription. The proposed EAR motif found in AtNPR4, but absent in AtNPR1, turned out to be present in NPR1 orthologs from other plant species, such as rice OsNH1 [22]. Moreover, while the presence of SA affects deuterium exchange in the SBD fragment of NPR4, it has no effect on fragments containing the EAR motif [22], suggesting that the EAR-motif in NPR4 is unlikely to be regulated by SA, and NPR4 may repress defense gene expression through a different mechanism.

The presence of the BTB domain in all NPRs suggests that they may be substrate adaptors for CRL3, like other BTB domain-containing proteins [41–44]. In contrast to the EAR motif, for which an SA-induced conformational change has not been observed, NPR3 and NPR4 interactions with NPR1 are enhanced and disrupted, respectively, by their binding to SA. These SA-mediated NPR3/4-NPR1 interactions have been demonstrated through multiple approaches, including yeast two-hybrid analysis, *in vitro* pull-down, AlphaScreen competition and titration assays, and IP-MS [9,22,30]. Moreover, NPR1 is by no means the only substrate for NPR3/4. During the effector-triggered immunity, both NPR1 and JAZ1 (a repressor of the defense hormone JA) are degraded in an SA-dependent manner to allow programmed cell death (PCD) to occur at the site of infection [21]. In contrast to the cell death zone, NPR1, a protein that promotes cell survival, accumulates in neighboring cells. The attempt to "see" NPR1 in these cells through microscopy has led to the surprising discovery that NPR1 not only forms SINCs in the nucleus but also in the cytoplasm [30] (Figure 3). Interestingly, these SA-induced cytoplasmic NPR1 condensates are not detected for other NPRs when expressed at similar levels [30]. Cell fractionation experiments showed that in the nucleus, where NPR3/4 are constitutively present, NPR1 is a major target of SA-induced ubiquitination, whereas, in the cytoplasm, NPR1 mediates SA-induced ubiquitination of other proteins. Consistent with its role in promoting cell survival in response to stress, IP-MS analysis showed that cytoplasmic SINCs contain many stress-response proteins, including nucleotide-binding leucine-rich repeat immune receptors and their downstream signaling components, such as EDS1, together with protein quality control and degradation machineries [30]. Therefore, NPR1 not only induces defense gene expression in the nucleus but also controls stress protein homeostasis through SINC formation in the cytoplasm.

In support of NPR1 serving as a CRL3 substrate adaptor, the plant growth hormone gibberellin (GA) receptor, GID1, was recently found to be another target, whose ubiquitination and degradation by NPR1 explain the reduced plant growth during the immune response [45]. This study, together with the report of SA-mediated degradation of the JA co-receptor JAZ1 by NPR3/4 during ETI [21] and the reports of NPR1 interactions with the MYC2 TF in JA signaling [35] as well as the EIN3 TF in ethylene signaling [36], provides additional examples for how plants could use a handful of hormones and their limited number of receptors and TFs to generate a myriad of regulatory activities through crosstalks, an important layer of regulation that has yet to be systemically studied (Figure 4).

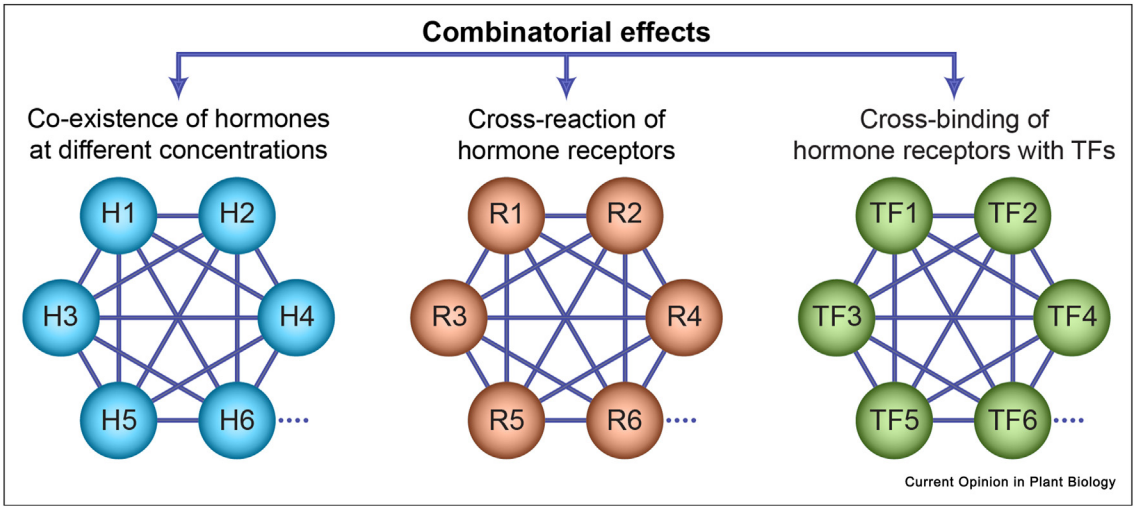
In summary, different facets of NPR proteins have been revealed through the recent cellular and structural studies. In both the nucleus and the cytoplasm, NPR1, which functions as a dimer, serves as a scaffolding platform to organize large numbers of proteins in the form of protein condensates for the regulation of gene transcription and protein homeostasis. Since these phase-separated membraneless entities have specific physical and biochemical properties, new approaches will be needed to further understand the functions of NPRs in these biomolecular condensates.

Figure 3



Subcellular localization npr1^{sim3}-GFP during ETI. The npr1^{sim3}-GFP/*npr1-2* transgenic plant was infected at the tip with *Psm* ES4326/AvrRpt2. At 24 hpi, tissue was sampled from the cell death-survival boundary (diagram). GFP signal was collected across the boundary between dead and surviving regions (dashed line). Enlargements from regions adjacent to and distant from the cell death zone (dashed rectangles) are shown in bottom panels. Scale bar = 100 μm (top panel); 20 μm (bottom panels). Modified from Ref. [30].

Figure 4



Proposed model for the hormone signaling network in plants. Based on the recent studies of NPR functions highlighted in this review, together with reports on other plant hormones, it is conceivable that a myriad of signaling diversity can be generated through combinatorial effects of interactions at the levels of hormone (H) production [21]; hormone receptor (R) cross-reactions [21,45]; and cross-binding of hormone receptors with TFs (TF) [35,36].

NPR1 has already been introduced into many crop species to enhance resistance against a variety of pathogens in both laboratory and field settings [6,7]. A better understanding of all its molecular functions will allow broader use of NPR1 to engineer crops resilient against both biotic and abiotic stresses without compromising yield.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: X.D. is a cofounder of Upstream Biotechnology and a scientific advisory board member of Inari Agriculture and Aferna Bio. The other authors declare no competing interests.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Jordan Powers and Dr. Xing Zhang for their helpful suggestions for the review. This work was supported by grants from the National Institutes of Health R01 GM145026 to P.Z., R35 GM118036 to X.D., and the Howard Hughes Medical Institute to X.D.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbi.2023.102352.

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