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Spotlight

ZAR1 resistosome and helper NLRs: Bringing in calcium and inducing cell death

To protect themselves from infectious diseases, plants have deployed a two-tiered surveillance system to recognize invading pathogens (Fu and Dong, 2013). In the first layer of the immune system, plants evolved extracellular pattern recognition receptors to detect conserved pathogen-associated molecular patterns (PAMPs) and activate PAMP-triggered immunity (PTI) (Boller and Felix, 2009). The second layer of the immune system, which is termed effector-triggered immunity (ETI), relies on an array of nucleotide-binding leucine-rich repeat receptor (NLR) proteins to detect pathogen effectors, either directly or the activities of these effectors indirectly (Figure 1A) (Sun et al., 2020). NLR activation often triggers a rapid localized cell death called hypersensitive response (HR).

In general, NLRs are divided into two functional groups: sensor NLRs that are involved in the recognition of pathogen effectors and helper NLRs that are required by sensor NLRs for ETI activation (Jubic et al., 2019; Sun et al., 2020). Two major types of sensor NLRs are defined as TIR (Toll/interleukin-1 receptor)-NLR (TNL) and CC (coiled-coil)-NLR (CNL) based on their N-terminal TIR or CC domain. HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) is a sensor NLR containing a canonical CC domain that acts as a sensor for a number of pathogen effectors, such as HopZ1a, HopF1, HopX1, HopO1, AvrAC, etc. (Laflamme et al., 2020). In many flowering plants, N REQUIRED GENE1 (NRG1) and ACTIVATED DISEASE RESISTANCE 1 (ADR1), two sequence-related protein groups, function as helper NLRs that are required for the full function of several TNL and CNL receptors (Sun et al., 2020). Some NLRs, such as ZAR1, function as both sensors and executors, and largely do not require helper NLRs to activate ETI (Adachi et al., 2019). Despite the importance of ZAR1 resistosome, NRG1, and ADR1 in plant immunity, the precise biochemical functions of these proteins have remained elusive until the recent studies published by Bi et al. (2021) and Wan et al. (2021).

The funnel-shaped structure formed by the ZAR1 resistosome makes sense that it functions as a channel pore (Figure 1B). Bi et al. (2021) tested this hypothesis by expressing wild-type ZAR1 along with AvrAC, RKS1, and PBL2 in *Xenopus oocytes* and performing two-electrode voltage clamp recordings. They observed strong current traces upon application of voltage, demonstrating that the activated ZAR1 resistosome indeed possesses a channel activity (Bi et al., 2021). Furthermore, they found that the channel activity is dependent on Glu11, a conserved acidic residue in all ZAR1 proteins from different plant species (Wang et al., 2019; Bi et al., 2021). To further determine the channel activity of the ZAR1 resistosome, the authors implemented planar lipid bilayer-based electrophysiology studies. Pre-assembled ZAR1 resistosomes were reconstituted into planar lipid bilayers, and single-channel measurements were conducted. They found that the ZAR1 channel was permeable to Na^+, K^+, Cs^+, Mg^{2+}, and Ca^{2+} with distinctive current amplitudes (Bi et al., 2021).

The next question is whether ZAR1 activation in the plant cell can lead to increased Ca^{2+} in the cytosol. To answer this question, the authors tested the Ca^{2+} influx in the protoplast of a *zar1* aequorin line expressing ZAR1 along with RKS1, PBL2, and AvrAC (Bi et al., 2021). Aequorin is a calcium-sensitive photoprotein, which is used to measure calcium concentration. They found that the ZAR1 activation by AvrAC is sufficient to induce Ca^{2+} influx in the protoplast, and that Glu11 is required for the ZAR1-mediated Ca^{2+} influx (Bi et al., 2021). Through observation of Ca^{2+} influx in the *zar1* aequorin line complemented with ZAR1 after infiltration with *Pseudomonas syringae* pv. *tomato* DC3000 carrying *HopZ1a*, they also confirmed that activation of ZAR1 can trigger Ca^{2+} influx in leaves during infection (Bi et al., 2021).

To further reveal how ZAR1 oligomerizes in the plant cell, the authors implemented state-of-the-art technology, single-molecule imaging, to visualize ZAR1 resistosome subcellular localization. Through this technology, they provided direct evidence that the AvrAC-induced ZAR1 complex indeed forms pentamers in living protoplasts (Bi et al., 2021). Furthermore, they observed that the ZAR1-mEGFP signal in the presence of AvrAC had a similar distribution as the plasma membrane (PM) marker PIP2;1-mCherry, demonstrating that the ZAR1 resistosome can be localized in the PM (Bi et al., 2021). Cell staining results showed that propidium iodide, which stains and enters into the dead cell after activation of ZAR1, indicating that the PM-localized ZAR1 resistosome causes a loss of PM integrity once activated (Bi et al., 2021).

HR-associated cell death is often accompanied by oxidative burst. Thus, the authors examined reactive oxygen species (ROS) production and PM integrity and tracked the process by live-cell imaging. They found that protoplasts expressing ZAR1, RKS1, PBL2, and AvrAC showed a continuous increase of ROS production before cell death (Bi et al., 2021), indicating that activation of ZAR1 induces ROS production followed by a loss of PM integrity (Figure 1B).

To summarize, Bi et al. demonstrated that the activation of ZAR1 in the plant cell led to Glu11-dependent Ca^{2+} influx, production of reactive oxygen species, perturbation of PM integrity, and eventually cell death. The milestone discovery that ZAR1 resistosome

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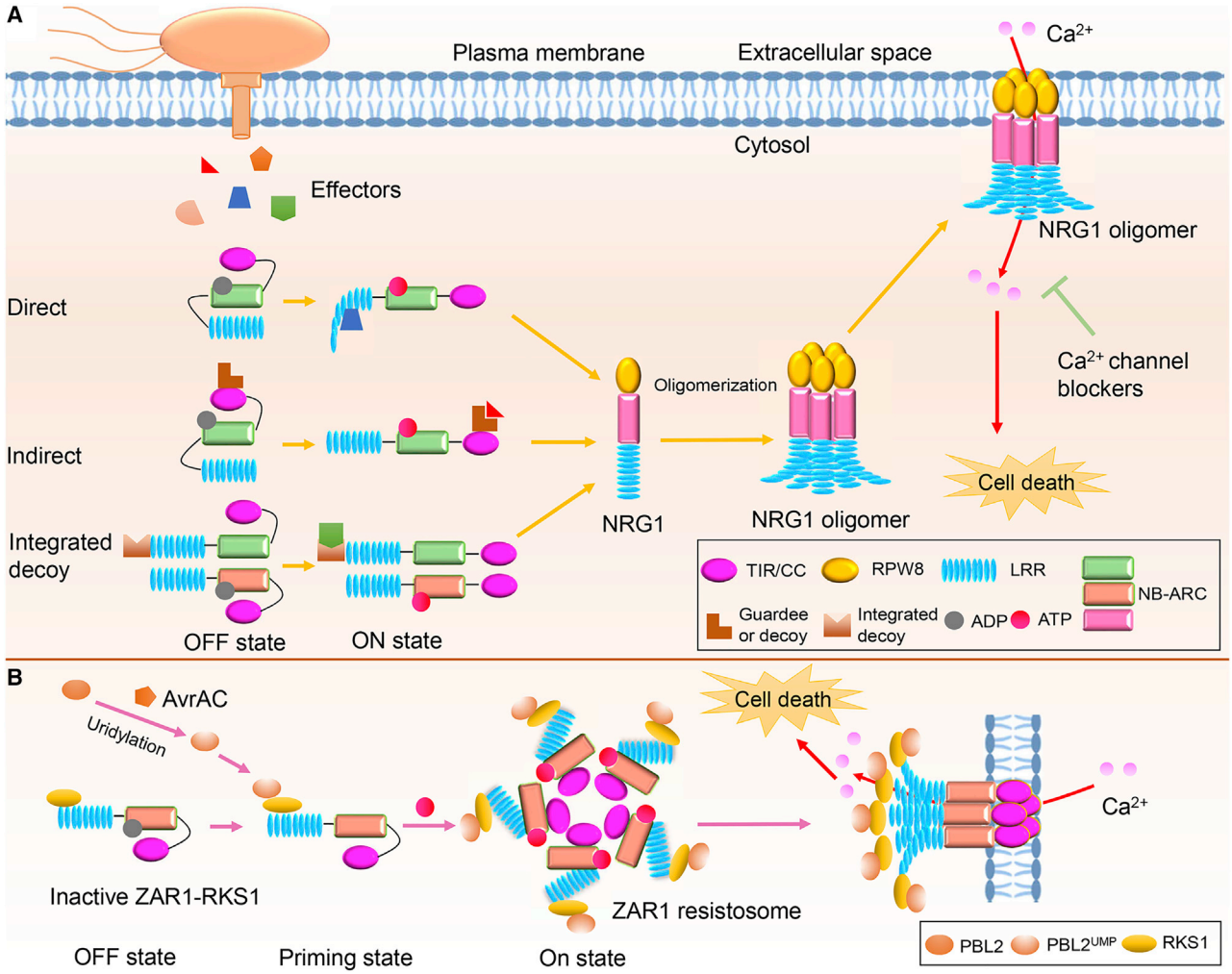

Figure 1. A simplified model for pathogen effector recognition by sensor NLRs and the functions of NRG1 and ZAR1 resistosome as calcium channels in cell death activation.

(A) NRG1 activation followed by effectors recognition. Pathogen recognition by sensor NLRs can be explained by three models, including "direct recognition," "indirect recognition," and "integrated decoy." The helper NLR NRG1 functions downstream of TIR- or CC- NLRs. NRG1 oligomerizes upon pathogen effector recognition and translocates to the plasma membrane to form a pore-like calcium channel, which allows the influx of calcium ions. As a consequence, cell death occurs. Ca^{2+} channel blockers inhibit the cell death induced by NRG1 oligomers.

(B) ZAR resistosome activation. In the resting state, RKS1 interacts with the sensor NLR protein ZAR1 that is bounded to ADP. Upon pathogen infection, the type III effector from the plant bacterial pathogen *Xanthomonas campestris* pv. *campestris* AvrAC uridylates PBL2. Uridylated PBL2 is recruited by ZAR1-RKS1 heterodimer. $PBL2^{UMP}$ activates RKS1, which facilitates ADP release from ZAR1 by inducing conformational changes in ZAR1, enabling ZAR1 binding with ATP or dATP. Subsequently, a wheel-like structure termed a "resistosome," consisting of five heterotrimeric ZAR1-RKS1-$PBL2^{UMP}$ protomers, forms. ZAR1 resistosome translocates to the plasma membrane and forms a calcium channel allowing influx of Ca^{2+}, leading to production of ROS and eventually cell death.

functions as a calcium-permeable channel provided new insights into the functions of NLRs in plant immunity and changed our view of ETI-triggered cell death.

More recently, Jacob et al. (2021) discovered that two helper NLRs have similar biochemical activities as the ZAR1 resistosome. They obtained X-ray crystal structures of two mutant NRG1.1 CC-R domains (residues 1–124), K94E/K96E/R99E/K100E/R103E/K106E/K110E (7K(R)/E) and K94E/K96E (2K/E). The structures of these two mutants superimposed well with the N-terminal four-helical bundle of the resting-state CC domain of ZAR1 and the cation channel-forming domain of mammalian mixed-linage kinase domain-like. Because the wild-type NRG1.1 is inactive without NLR activation, the authors introduced the auto-active NRG1.1 D485V (DV) allele and demonstrated that NRG1.1 DV is sufficient to trigger cell death *in planta* (Jacob et al., 2021). NRG1.1 DV oligomerized and was enriched in PM (Figure 1A). These interesting observations prompted them to investigate whether NRG1.1 forms pores and functions as a channel. The authors found that NRG1.1 DV induced cell death in HeLa cells. Using scanning electron microscopy, they observed significantly increased numbers of PM pores in dying HeLa cells.

Using Ca^{2+} reporter GCaMP3 transgenic *Nicotiana benthamiana* plants, Jacob et al. (2021) showed that both NRG1.1 DV and

ADR1 triggered Ca^{2+} influx *in planta*. Therefore, the authors drew the conclusion that the active NRG1.1 forms Ca^{2+} permeable channels (Figure 1A). These breakthrough discoveries uncovered the mysterious biological functions of the helper NLRs NRG1.1 and ADR1.

The fact that ZAR1 functions as a calcium channel does not mean that all CNLs function as calcium channels. Some CNLs in *Arabidopsis* require NDR1 for their functions, and NLR-REQUIRED FOR CELL DEATH (NRC) is required for cell death of some CNLs in solanaceous plants. It remains to be determined whether these CNLs, NDR1 or NRC, function as calcium channels. If they do not form calcium channels, what are the biochemical functions of these proteins?

Future studies may focus on how calcium influx induces cell death. Elevated cytoplasmic Ca^{2+} levels can certainly cause Ca^{2+} influx into mitochondria and these higher levels of Ca^{2+} could disrupt mitochondria metabolism, leading to cell death (Ermak and Davies, 2002). Ca^{2+} could also bind to calmodulin to modulate the activity of many cytosolic and nuclear proteins as well as gene transcription to promote cell death. In addition, we still do not know how effector recognition triggers the activation of helper NLR. TIR domains in TNL receptors have been shown to function as NAD^+ cleavage enzymes, whose activity is indispensable for the TNL protein-dependent cell death response (Horsefield et al., 2019; Wan et al., 2019). It may be worth of exploring the possible connection between TIR domain's NAD^+ cleavage activity and helper NLR's calcium channel formation.

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REFERENCES

Adachi, H., Derevnina, L., and Kamoun, S. (2019). NLR singletons, pairs, and networks: evolution, assembly, and regulation of the intracellular immunoreceptor circuitry of plants. Curr. Opin. Plant Biol. **50**:121–131. https://doi.org/10.1016/j.pbi.2019.04.007.

Bi, G., Su, M., Li, N., Liang, Y., Dang, S., Xu, J., Hu, M., Wang, J., Zou, M., Deng, Y., et al. (2021). The ZAR1 resistosome is a calcium-permeable channel triggering plant immune signaling. Cell https://doi.org/10.1016/j.cell.2021.05.003.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu. Rev. Plant Biol. **60**:379–406. https://doi.org/10.1146/annurev.arplant.57.032905.105346.

Ermak, G., and Davies, K.J. (2002). Calcium and oxidative stress: from cell signaling to cell death. Mol. Immunol. **38**:713–721. https://doi.org/10.1016/s0161-5890(01)00108-0.

Fu, Z.Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. Annu. Rev. Plant Biol. **64**:839–863. https://doi.org/10.1146/annurev-arplant-042811-105606.

Horsefield, S., Burdett, H., Zhang, X., Manik, M.K., Shi, Y., Chen, J., Qi, T., Gilley, J., Lai, J.S., Rank, M.X., et al. (2019). NAD(+) cleavage activity by animal and plant TIR domains in cell death pathways. Science **365**:793–799. https://doi.org/10.1126/science.aax1911.

Jacob, P., Kim, N.H., Wu, F., El-Kasmi, F., Chi, Y., Walton, W.G., Furzer, O.J., Lietzan, A.D., Sunil, S., Kempthorn, K., et al. (2021). Plant "helper" immune receptors are Ca^{2+}-permeable nonselective cation channels. Science, eabg7917. https://doi.org/10.1126/science.abg7917.

Jubic, L.M., Saile, S., Furzer, O.J., El Kasmi, F., and Dangl, J.L. (2019). Help wanted: helper NLRs and plant immune responses. Curr. Opin. Plant Biol. **50**:82–94. https://doi.org/10.1016/j.pbi.2019.03.013.

Laflamme, B., Dillon, M.M., Martel, A., Almeida, R.N.D., Desveaux, D., and Guttman, D.S. (2020). The pan-genome effector-triggered immunity landscape of a host-pathogen interaction. Science **367**:763–768. https://doi.org/10.1126/science.aax4079.

Sun, Y., Zhu, Y.X., Balint-Kurti, P.J., and Wang, G.F. (2020). Fine-tuning immunity: players and regulators for plant NLRs. Trends Plant Sci. **25**:695–713. https://doi.org/10.1016/j.tplants.2020.02.008.

Wan, L., Essuman, K., Anderson, R.G., Sasaki, Y., Monteiro, F., Chung, E.H., Osborne Nishimura, E., DiAntonio, A., Milbrandt, J., Dangl, J.L., et al. (2019). TIR domains of plant immune receptors are NAD(+)-cleaving enzymes that promote cell death. Science **365**:799–803. https://doi.org/10.1126/science.aax1771.

Wang, J., Hu, M., Wang, J., Qi, J., Han, Z., Wang, G., Qi, Y., Wang, H.-W., Zhou, J.-M., and Chai, J. (2019). Reconstitution and structure of a plant NLR resistosome conferring immunity. Science **364**:eaav5870. https://doi.org/10.1126/science.aav5870.