

Plant Immune Mechanisms: From Reductionistic to Holistic Points of View

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ABSTRACT

After three decades of the amazing progress made on molecular studies of plant-microbe interactions (MPMI), we have begun to ask ourselves “what are the major questions still remaining?” as if the puzzle has only a few pieces missing. Such an exercise has ultimately led to the realization that we still have many more questions than answers. Therefore, it would be an impossible task for us to project a coherent “big picture” of the MPMI field in a single review. Instead, we provide our opinions on where we would like to go in our research as an invitation to the community to join us in this exploration of new MPMI frontiers.

Key words: extracellular immunity, pattern-triggered immunity (PTI), effector-triggered immunity (ETI), heterogeneity in immune responses, translational regulation, circadian clock

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EXTRACELLULAR IMMUNITY AT THE BATTLEFRONT OF PLANT DEFENSE

Plants encounter a great number of microbes with different lifestyles throughout their lifetime. The extracellular microbes, from beneficial to pathogenic, are the first challenge faced by the plant immune system. Plants can launch immunity to restrict the colonization and entry of these microbes (Figure 1; Melotto et al., 2006; Boller and He, 2009; Hawes et al., 2012). At the same time, some of these beneficial microbes and successful pathogens have evolved sophisticated strategies to either rapidly evade or actively disrupt these surveillance mechanisms (Melotto et al., 2006, 2017; Liang et al., 2013), thereby achieving successful colonization and entry into plants.

At this battlefield, stomatal immunity against leaf-associated microbes and rhizospheric immunity against root-associated microbes are essential layers of the plant's extracellular defense system. Even after entry into plants, many plant pathogens proliferate in the apoplastic space outside of plant cells. Therefore, immune responses that occur in the apoplast are also considered to constitute extracellular immunity. Significant progress has been made in understanding the immune mechanisms in aerial tissues (Melotto et al., 2006; Boller and Felix, 2009; Zeng and He, 2010; Zeng et al., 2011; Dubiella et al., 2013; Gao et al., 2013; Kadota

et al., 2014; Li et al., 2014; Liang et al., 2018; Sun and Zhang, 2020; Zhou and Zhang, 2020). However, in roots, with constant exposure to soil microbes, how plants differentiate beneficial microbes from pathogenic ones and establish rhizospheric immunity remain mostly unknown.

STOMATAL IMMUNITY

Stomata are natural pores surrounded by specialized guard cells that control gas exchange and water loss in plants. They are typically found in the leaf epidermis and play critical roles in plant growth and development. However, they also provide natural openings for entry of some filamentous and bacterial pathogens (Melotto et al., 2006; Zeng and He, 2010; Zeng et al., 2011). Upon contact, conserved molecular signatures derived from microbes, named microbe-associated molecular patterns (MAMPs) (Ausubel, 2005; Boller and Felix, 2009), are recognized by plant surface pattern-recognition receptors (PRRs). The perception of MAMPs induces stomatal closure, which is important for restricting the entry of microbes lacking a penetration apparatus (Melotto et al., 2006; Thor et al., 2020; Ye et al., 2020). MAMP perception also triggers complicated crosstalk among multiple

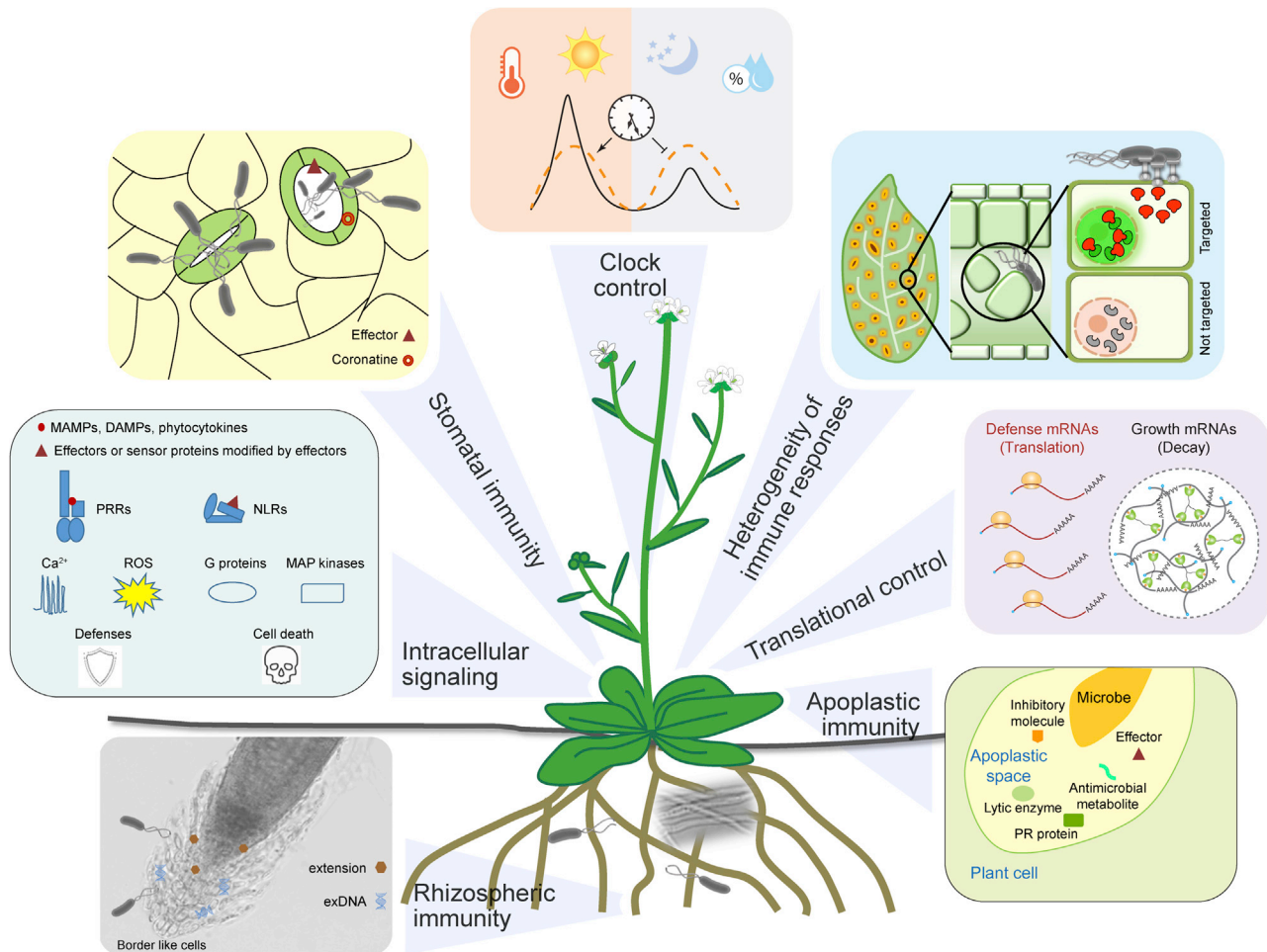


Figure 1. Plant Immune Responses.

The front line of plant defense occurs in the stomata, rhizosphere, and apoplast. The recognition of microbe-associated molecular patterns (MAMPs) can trigger stomatal closure to restrict the entry of microbes into plants. To counteract this, microbes produce effectors and coronatine to promote stomatal opening. In roots, extensins, border and border-like cells, and extracellular DNAs (exDNAs) contribute to rhizospheric immunity that functions in attracting beneficial microbes while repelling pathogens. In the apoplastic space, plants produce lytic enzymes to release immune-inducing MAMPs and antimicrobial pathogenesis-related (PR) proteins and metabolites to restrict the proliferation of invading pathogens. In the same space, microbes can use effectors and inhibitory molecules to counteract plants' apoplastic immunity. The recognition of MAMPs and pathogen effectors activate pattern-recognition receptors (PRRs) on the cell surface and nucleotide-binding leucine-rich repeat immune receptors (NLRs) inside the cell to trigger pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively. These immune receptors signal through Ca^{2+} , reactive oxygen species (ROS), G proteins, and MAP kinase cascades to confer resistance, in which ETI is often associated with cell death. The induction of both PTI and ETI involves reprogramming of the plant proteome through the decay of housekeeping mRNAs (perhaps inside stress granules; circle) and the activation of translation of defense proteins. Heterogeneity exists in plant responses to infection due to differential pathogen distribution and host immune sensitivity. Plant immune responses are also regulated by the circadian clock, which not only helps plants anticipate infection but also gates immune responses when infection occurs to minimize effects on plant physiology and fitness.

plant hormone signaling pathways. It has been well documented that the plant hormone abscisic acid (ABA) induces stomatal closure (Sirichandra et al., 2009; Joshi-Saha et al., 2011), whereas a derivative of jasmonate (JA), (+)-7-iso-jasmonoyl-L-leucine (JA-Ile), induces stomatal opening (Okada et al., 2009). In addition, salicylic acid (SA)-deficient mutants exhibit compromised stomatal defense mechanisms, indicating a positive role of SA in stomatal defense (Melotto et al., 2006; Zeng and He, 2010; Zeng et al., 2011).

In addition to MAMP perception, stomatal development mediated by the ERECTA (ER)-YODA (YDA) signaling pathway also

indirectly contributes to plant immunity (Shpak et al., 2005; Nadeau, 2009; Meng et al., 2015). Moreover, this signaling pathway can act in parallel with the canonical PRR signaling pathway to confer broad-spectrum immunity with the ER serving to recognize yet-to-be-identified MAMPs (Godiard et al., 2003; Llorente et al., 2005; Adie et al., 2007; Shpak, 2013; Häffner et al., 2014; Sopena-Torres et al., 2018). These findings indicate dual roles of ER-YDA signaling in stomatal development and immunity, and suggest that the key regulators of stomatal development might be co-opted for stomatal immunity during evolution to resolve the dilemma of stomata allowing gas exchange as well as pathogen entry.

To counteract the plant immune response, successful foliar pathogens and some beneficial microbes have evolved strategies to interfere with stomatal immunity. Several pathovars of *Pseudomonas syringae* produce the phytotoxin coronatine (COR) to induce an interaction between CORONATINE INSENSITIVE1 (COI1) and JASMONATE ZIM-DOMAIN proteins (JAZs). This induced interaction triggers the degradation of JAZs and the activation of NO APICAL MERISTEM [NAM], ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR [ATAF], and CUP-SHAPED COTYLEDON [CUC] (NAC) transcription factors (TFs), thereby inhibiting SA accumulation and promoting stomatal opening (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007, 2009; Katsir et al., 2008; Melotto et al., 2008, 2017; Sheard et al., 2010; Zheng et al., 2012; Gimenez-Ibanez et al., 2017). COR also targets the *Arabidopsis* RPM1-INTERACTING4 (RIN4) and plasma membrane H⁺-ATPases, AUTOINHIBITED H⁺-ATPASE ISOFORM 1 (AHA1) and AHA2, to induce stomatal opening (Zhang et al., 2008a; Liu et al., 2009).

Bacterial pathogens secrete effectors to prevent stomatal closure triggered by the perception of MAMPs. The *P. syringae* effectors HopZ1, AvrB, and HopX1 all target COI1-JAZ complexes to promote stomatal opening (Jiang et al., 2013; Gimenez-Ibanez et al., 2014; Lee et al., 2015; Ma et al., 2015; Zhou et al., 2015b). HopF2 has been reported to target the MITOGEN-ACTIVATED PROTEIN KINASE KINASE5 (MAPKK5), BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1), and RIN4 to suppress MAMP-induced stomatal closure (Wang et al., 2010; Wilton et al., 2010; Hurley et al., 2014; Zhou et al., 2014). The *Xanthomonas oryzae* effector XopR targets *Arabidopsis* RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) and interferes with stomatal defense (Wang et al., 2016). These examples of pathogen effector-mediated evasion of stomatal immunity reinforce its important function in plant defense.

Developmentally related to stomata, hydathodes found in leaves of many plants direct the colonization of phytopathogens into the xylem, including *Xanthomonas campestris* (Cerutti et al., 2017). Although hydathode pores do not respond to flagellin treatment, it remains unclear whether hydathodes contribute to sensing and restricting microbial entry.

RHIZOSPHERIC IMMUNITY

Although less understood than leaf defense, it is conceivable that the plant rhizosphere is equipped with sophisticated immune mechanisms to differentiate commensal and beneficial microbes from pathogens to allow normal interactions with microbes that are important for plant health.

Extensins are cell-wall glycoproteins that are predominantly expressed in plant roots where they play essential roles in plant defense (Esquerré-Tugayé et al., 1979; Merkouropoulos and Shirsat, 2003). The composition and structure of extensins in the roots of infected plants undergo significant alterations upon the perception of pathogenic microbes (Xie et al., 2011; Plancot et al., 2013; Wu et al., 2017b), resulting in the strengthening of the plant cell wall to restrict infection. In addition to playing a direct role in modulating the structure and rigidity of the cell wall, extensins and extensin-like molecules are secreted into

the rhizosphere and extracellular rhizospheric mucilage (Koroney et al., 2016).

In addition to molecules such as extensins, border cells are detached cell populations released from the root tip into the rhizosphere (Hamamoto et al., 2006; Hawes et al., 2012). Pathogenic infection can cause an increase in the release of border cells from the root tip (Zhao et al., 2000; Cannesan et al., 2011; Hawes et al., 2012). Accumulating evidence indicates a potential role of border cells in attracting and repelling certain microbes (Gunawardena et al., 2005; Curlango-Rivera and Hawes, 2011; Driouich et al., 2013). Border and border-like cells secrete mucilage containing antimicrobial molecules, polysaccharides, and extracellular DNAs (exDNAs). Both treatment of roots with DNase I and mutation of the pathogen's exDNase-encoding genes reduce root tip immunity, suggesting a positive role of exDNAs in root immunity (Curlango-Rivera et al., 2013; Hawes et al., 2016; Tran et al., 2016). What are the functional mechanisms of exDNAs in this process? Do attraction and repulsion of microbes confer specificity? How is specificity achieved? These are major questions that remain to be addressed.

Extensins and extensin-like molecules, together with root border and border-like cells, have been proposed to form the “root extracellular trap” that functions as a defense network (Brinkmann et al., 2004; Driouich et al., 2013). Additional components that contribute to the formation of the root extracellular trap await further investigation. This protective network has potential roles in eliminating pathogenic microbes and facilitating the attachment of beneficial microbes (Brinkmann et al., 2004; Driouich et al., 2013). Secreted extensins and extensin-like molecules can interact with pectins and arabinogalactan proteins to reinforce the mucilage network, thereby weakening the attachment of pathogenic microbes to plant roots (Oosterveld et al., 2002; Immerzeel et al., 2006; Cannesan et al., 2012). During symbiotic interactions, the accumulation of extensins in infected nodules is suggestive of their putative roles in facilitating the attachment of beneficial microbes (Sujkowska-Rybkowska and Borucki, 2014). It remains unknown whether secreted extensins and extensin-like molecules can serve as signals to modulate microbial behavior. The underlying mechanism of the root extracellular trap in mediating interactions with soil microbes awaits further investigation.

APOPLASTIC IMMUNITY

The apoplast is an enclosed active battlefield between plants and microbes and one of the first environments encountered after the pathogen invades the plant body. The plant cell wall is a formidable physical barrier that restricts pathogen colonization. Pathogen attack usually triggers modifications in the plant cell wall and alterations in cell-wall integrity (Malinovskiy et al., 2014; Bacete et al., 2018; De Lorenzo et al., 2019). The deposition of callose at infection sites is a typical defense response that strengthens the plant cell wall (Stone and Clarke, 1992). Pathogens secrete cell-wall-degrading enzymes to modify or hydrolyze cell-wall components. The impairment of cell-wall integrity and degradation of cell wall causes damage or produces pattern signals that are sensed by plants to activate immune signaling, indicating an active role of the plant cell wall

in immunity rather than a passive role as a barrier only (Malinovskiy et al., 2014; Bacete et al., 2018; De Lorenzo et al., 2019).

In addition to the cell wall, plants also secrete pathogenesis-related (PR) proteins, lytic enzymes, and antimicrobial secondary metabolites into the apoplastic space upon pathogen challenge to restrict the colonization of both foliar and rhizospheric microbes (van Loon et al., 2006; Shabab et al., 2008; van Esse et al., 2008). Some MAMPs buried within supramolecular microbial surface structures or larger proteins need to be released by the host lytic enzymes for perception to occur (Liu et al., 2014; Buscaill et al., 2019). Therefore, these enzymes not only serve to destruct microbial structures but also release immunogenic patterns to trigger plant defenses (Doehlemann and Hemetsberger, 2013; Liu et al., 2014; Jashni et al., 2015; Buscaill et al., 2019).

Roots can also sense MAMPs in the apoplastic space and mount an immune response (Millet et al., 2010; Beck et al., 2014; Wyrsh et al., 2015; Stringlis et al., 2018). However, the perception of the immunogenic peptides flg22 and, nlp20, as well as the medium-chain 3-hydroxy fatty acid, is spatially restricted in *Arabidopsis* roots, with the differentiated zone exhibiting very low to no response (Zhou et al., 2020). It has also been observed that there is a higher expression of PRR genes in the inner cellular layers than in the outer cellular layers of *Arabidopsis* roots (Beck et al., 2014). The lack of flagellin responsiveness correlates with the lack of *FLAGELLIN SENSING2* (*FLS2*) expression in the differentiated zone. Laser-induced cell ablation of small clusters of cells was shown to be sufficient in inducing *FLS2* expression and conferring MAMP responsiveness in neighboring cells (Zhou et al., 2020). These observations suggest a spatial differentiation of immune responses in root cells that provides a strategy to reduce their responsiveness to beneficial and free-living microbes, while enabling the initiation of a specific and strong immune response to pathogenic microbes through damage associated with the infection. These results highlight the interplay between stresses and suggest a “damage-gated” mechanism for the differential response to beneficial and pathogenic microbes.

Microbes employ diverse strategies to evade or disrupt host recognition in the apoplast. Some of them evade host recognition via the divergence of MAMPs. For instance, the nitrogen-fixing symbiont *Sinorhizobium meliloti* possesses variations in the core sequences of flagellin, evading recognition by the flagellin receptor *FLS2* in *Arabidopsis* (Felix et al., 1999). Another common strategy to suppress apoplastic immunity is through the disruption of the recognition of MAMPs by their cognate receptors. Foliar pathogens can secrete effector proteins into the apoplast to achieve this goal. For example, some *P. syringae* and *Pseudomonas aeruginosa* strains secrete the alkaline protease AprA that can degrade flagellin monomers, thereby inhibiting flagellin perception (Bardoel et al., 2011; Pel et al., 2014). *P. syringae* can also secrete inhibitory molecules to suppress the activity of β -galactosidase 1 that functions in the release of immunogenic peptides from glycosylated flagellin (Buscaill et al., 2019). Moreover, *P. syringae* strains possess glycan polymorphisms on flagellin that can evade hydrolysis by

plants (Buscaill et al., 2019). The fungal pathogen *Cladosporium fulvum* secretes the chitin-binding effector proteins Avr4 and Ecp6 to prevent chitin hydrolysis or its recognition by lysin motif domain-containing receptors (van den Burg et al., 2006; de Jonge et al., 2010). Although less documented for their specific functions, AprA homologs and Ecp6-like proteins are present in a wide range of rhizospheric beneficial bacterial (Pel et al., 2014) and fungal (Bolton et al., 2008) species. The soil-borne pathogen *Verticillium dahliae* secretes the polysaccharide deacetylase, VdPDA1, to deacetylate chitin oligomers and disrupt chitin perception by plants, providing evidence for the prevention of MAMP recognition by root-infecting fungal pathogens (Gao et al., 2019). In addition, the plant growth-promoting rhizobacterium *Piriformospora indica* produces the β -glucan-binding lectin, FGB1, to disrupt host perception of chitin (Wawra et al., 2016).

In addition to proteins, microbes can also produce other molecules to suppress the MAMP-activated immune response. *Pseudomonas* spp. uses organic acids to lower the environmental pH and inhibit the flagellin-activated immune response (Yu et al., 2019a), while *Bradyrhizobium japonicum* produces nodulation (Nod) factors to suppress the MAMP-induced immune response in soybean and *Arabidopsis* (Liang et al., 2013). Furthermore, the *S. meliloti* lipopolysaccharide has been shown to suppress MAMP-induced reactive oxygen species (ROS) burst in *Medicago truncatula* (Scheidle et al., 2005; Tellstrom et al., 2007).

The above evidence demonstrates that the apoplast is a place where the battle between the pathogen and the host intensifies. Despite this progress, the mechanisms that prevent overstimulation of immune responses against beneficial and free-living microbes remain a challenging area for future research.

SENSING IMMUNOGENIC SIGNALS BY IMMUNE RECEPTORS ON THE CELL SURFACE

PRRs are cell surface immune receptors that include a large number of receptor kinases (RKs) and receptor-like proteins (RLPs) (Tang et al., 2017; Zipfel and Oldroyd, 2017). In addition to MAMPs, PRRs also sense host-derived and damage-associated molecular patterns (DAMPs) such as structures that are released from plant cell walls, extracellular ATP, and NAD (Choi et al., 2014; Wang et al., 2017a; Bacete et al., 2018). These immunogenic patterns are characteristic of a pathological state caused by pathogen invasion (Cook et al., 2015). In addition to MAMPs and DAMPs, plants also produce immune-modulating peptides called phytocytokines whose receptors either directly stimulate immune responses or interact with other PRRs to modulate immune responses (Gust et al., 2017; Segonzac and Monaghan, 2019). The perception of immunogenic patterns by PRRs activates pattern-triggered immunity (PTI), which plays a key role in fending off potential pathogens (Figure 1).

Detailed analyses of several *Arabidopsis* PRRs, such as *FLS2*, *EF-TU RECEPTOR* (*EFR*), and *LysM-CONTAINING RECEPTOR-LIKE KINASE5* (*LYK5*), have uncovered molecular

mechanisms underlying the activation of early signaling events downstream of PRRs. Associating with PRRs are receptor-like cytoplasmic kinases (RLCKs), including BOTRYTIS-INDUCED KINASE1 (BIK1), PBS1-like kinases (PBLs), and BR-SIGNALING KINASE1 (BSK1), that directly connect PRRs to downstream components (Lu et al., 2010; Zhang et al., 2010; Shi et al., 2013; Rao et al., 2018; Majhi et al., 2019). The phosphorylation state and/or stability of BIK1 is regulated by MAP4 kinase, PP2C, CPK28, as well as PLANT U-BOX PROTEIN25 (PUB25) and PUB26, thereby allowing the fine-tuning of responsiveness and degree of immune output (Monaghan et al., 2014; Couto et al., 2016; Wang et al., 2018a; Zhang et al., 2018). A recent study has shown that BIK1 is also monoubiquitinated by the E3 ubiquitin ligases, RING-H2 FINGER A3A (RHA3A) and RHA3B, and that this modification is necessary for BIK1 to dissociate from PRRs and activate downstream signaling (Ma et al., 2020). Do different PRRs employ the same RLCKs for function? A systematic analysis of RLCK higher-order mutants showed that while some RLCKs are redundantly required for multiple PRRs, others are specifically required for particular PRRs (Rao et al., 2018). Unlike most other RLCKs that positively regulate PRR-dependent immunity, PBL13 negatively regulates FLS2 function (Lin et al., 2015). Moreover, BIK1 was also found to negatively regulate signaling mediated by RLP23, a receptor for the epitope nlp20 derived from fungal, oomycete, and bacterial NLP proteins, through an unknown mechanism (Wan et al., 2019b).

Upon activation by PRRs, RLCKs phosphorylate downstream components, initiating an array of early signaling events, including the rapid influx of calcium ions, production of ROS, activation of heterotrimeric G proteins, and activation of MAP kinase cascades. BIK1 and its paralog in rice can phosphorylate several cyclic nucleotide-gated channels (CNGCs) to positively regulate calcium influx, although these findings only partially explain MAMP-triggered calcium burst (Tian et al., 2019; Wang et al., 2019b). The increased calcium influx is profoundly important in immune activation, as a number of key immune regulatory proteins are directly controlled by calcium. These include calcium-dependent kinases (CDKs), RBOHs, TFs such as CALMODULIN BINDING PROTEIN 60-LIKE G and CAMODULIN-BINDING TRANSCRIPTION ACTIVATOR3 (Wang et al., 2009; Huang et al., 2020), CALCIUM-DEPENDENT PROTEIN KINASES (CPKs; Romeis et al., 2001), and calcium-dependent metacaspases (Hander et al., 2019; Shen et al., 2019). BIK1 also directly phosphorylates the N terminus of RBOHD to promote the rapid production of ROS, which serve as a secondary signal for immune activation (Kadota et al., 2014; Li et al., 2014). By contrast, PBL13 phosphorylates the C terminus of RBOHD to promote its degradation and dampen its activity (Lee et al., 2020). BIK1 additionally phosphorylates components of the heterotrimeric G-protein complex, including REGULATOR OF G-PROTEIN SIGNALING1 and EXTRA LARGE G PROTEIN2 (XLG2), to activate heterotrimeric G proteins (Liang et al., 2016, 2018). Furthermore, BIK1, multiple PBLs, and BSK1 phosphorylate specific residues within the C or N terminus of MAP kinase kinases to activate MAP kinase cascades, thereby inducing defense (Yamada et al., 2016; Bi et al., 2018; Yan et al., 2018). Despite these advances, we still know little about how these early signaling events are linked to downstream

defense. For instance, what are the target proteins regulated by ROS and G proteins? How do these proteins regulate defense outputs?

SENSING MICROBIAL EFFECTOR PROTEINS INSIDE HOST CELLS

In addition to PRRs, plants also possess intracellular immune receptors that are nucleotide-binding leucine-rich repeat receptors (NLRs; Jones et al., 2016). NLRs recognize pathogen effectors delivered into the host cell, which initially evolved as virulence factors. This layer of immunity is defined as ETI (effector-triggered immunity) (Figure 1). The recognition occurs either directly via a physical interaction between an NLR and an effector or indirectly via an interaction between an NLR and an effector-target protein in the host cell. NLR proteins can be broadly divided into three major classes based on the N-terminal domain: coiled-coil, TOLL-INTERLUKIN1 RECEPTOR, or RPW8, and are thus called CNL, TNL, and RNL (Shao et al., 2016). Recent advances show that these NLRs play different roles in the perception of effectors and immune signaling. Some TNLs and CNLs act as singletons in effector perception and signaling while others act as physically interacting pairs, with one acting as an effector sensor and the other serving as a helper for signaling (Adachi et al., 2019b). RNLs are a small class of highly conserved NLRs divided into the *Nicotiana benthamiana* N REQUIREMENT GENE1 (NRG1) and ACTIVATED DISEASE RESISTANCE1 (ADR1) clades (Pearl et al., 2005; Bonardi et al., 2011; Collier et al., 2011; Wang et al., 2011) and act downstream of all studied TNLs and some CNLs as “executors” of the hypersensitive response (HR) or SA production (Wang et al., 2011; Qi et al., 2018; Gantner et al., 2019; Lapin et al., 2019). Solanaceous plants carry an additional class of slow-evolving CNLs called NLR-REQUIRED FOR CELL DEATH (NRCs) that function as a network required for the function of different sensor NLRs (Wu et al., 2017a).

Recent breakthroughs are starting to unveil mechanisms by which TNLs and CNLs activate immune signaling upon effector perception. Two reports show that the TIR domain of TNLs possesses NADase activity upon dimerization, and an unidentified metabolite(s) generated in the reaction is proposed to act as a signal that functions through ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) before activating NRGs and ADRs through unknown mechanisms (Horsefield et al., 2019; Wan et al., 2019a). Another study on ZAR1, a singleton NLR with a canonical coiled-coil domain, showed that, upon the perception of effector activity, it oligomerizes to form a pentameric complex (Wang et al., 2019a, 2019c). In this complex, which was named the resistosome, the N-terminal α 1 helix of ZAR1 forms a funnel-shaped structure, and the resulting pore on the plasma membrane has been found to be required for cell death and antibacterial immunity. The fact that the structural requirements for the *in vitro* assembly of the resistosome are also needed for effector-induced oligomerization observed in protoplasts supports the formation of the resistosome during immune activation. How does this pore-forming activity trigger cell death and the immune response? Does it simply cause cell lysis or create an ion channel? Is it also localized to the membranes of organelles? These are urgent questions that need to be addressed. The

mechanism underlying ZAR1 resistosome-mediated immune signaling appears to be shared by a group of CNLs, including *N. benthamiana* NRC proteins and some canonical CNLs, which carry an MADA-motif within the N terminus, as this motif possesses signaling activity similar to that of the ZAR1 α 1 helix (Adachi et al., 2019a). The coiled-coil domain of RNLs, referred to as CC_R in recent literature, appears to have a fold comparable with that of ZAR1's CC and may also form a pore on membranes, a possibility that remains to be tested. This raises an interesting possibility that TNLs and CNLs may use analogous mechanisms for immune execution.

CROSSTALK AMONG DIFFERENT RECEPTORS AND IMMUNE ACTIVATION

It has become increasingly clear that the integration of different immunogenic signals is critical for proper plant immune output. Two very recent studies have shown that activation of NLRs in the absence of PRR activation results in a mild immune response (Ngou et al., 2020; Yuan et al., 2020). When both PRRs and NLRs are stimulated, the plant mounts a strong defense characterized by much greater ROS production, defense gene transcription, the HR, and fully induced disease resistance. It appears that activation of the two classes of immune receptors can mutually potentiate each other in defense outputs. A study has shown that activation of PRRs and NLRs gives rise to overlapping phosphoproteomes, with some key signaling components phosphorylated at similar sites, suggesting very early crosstalk of PRR and NLR signaling pathways (Kadota et al., 2019). It is interesting to note that BIK1-dependent phosphorylation is required but not sufficient for activating CNGCs, RBOHD, heterotrimeric G proteins, and MAP kinase kinase kinases (Kadota et al., 2014; Li et al., 2014; Liang et al., 2016; Bi et al., 2018; Tian et al., 2019). It is possible that multiple inputs at the post-translational level are required for the activation of these key signaling components. Indeed, the activation of RBOHD also requires phosphorylation by CPKs and likely EF-hand-dependent calcium binding at the N terminus of this protein (Dubiella et al., 2013). Do PTI and ETI employ common signaling pathways? Although RBOHD and MAP kinases are known to be required for both PTI and ETI (Su et al., 2018; Kadota et al., 2019), it is unclear whether additional PTI components are also required for ETI. In addition, it is unknown whether different signal inputs are integrated via direct receptor–receptor interactions or at the post-translational modification level of signaling components.

Under field conditions, plants are frequently subjected to simultaneous infection with different pathogen classes. Crosstalk among immune receptors/co-receptors also plays an important role in cross-protection during complex infections. A recent study has shown that CERK1, a co-receptor for chitin, associates with BAK1, which is a co-receptor for multiple proteinaceous MAMPs, including flagellin (Gong et al., 2019). The infection of *Arabidopsis* with bacterial pathogens leads to the BAK1-dependent phosphorylation of CERK1 in the juxtamembrane domain, which potentiates chitin perception and antifungal immunity. Another receptor-like kinase, NUCLEAR SHUTTLE PROTEIN-INTERACTING KINASE1 (NIK1), is a positive player in antiviral immunity. NIK1 interacts with FLS2, and the activation of FLS2

can lead to NIK1 phosphorylation to potentiate antiviral immunity (Li et al., 2019a).

The observation of “damage-gated” MAMP perception in the differentiated zone of *Arabidopsis* roots represents another example of how different signals are integrated to activate defenses only in response to pathogen infection (Zhou et al., 2020). This unique control of immunity may be particularly important for root microbiota, as constant stimulation of the immune response is not desirable for the interaction with commensal or beneficial microbes. It is currently unknown, however, how cell damage leads to PRR gene expression in the neighboring cells, as the application of known DAMPs failed to induce PRR expression in differentiated zones.

DEFENSE MEDIATED BY NONCANONICAL RESISTANCE GENES

In contrast to PRRs and NLRs, other resistance proteins belong to diverse families and often confer broad-spectrum disease resistance. Durable resistance to soybean cyst nematode conferred by *Rhg1* is linked to copy-number variation in a gene cluster encoding an α -soluble *N*-ethylmaleimide-sensitive factor attachment protein, a wound-inducible domain protein 12, and an amino acid transporter (Cook et al., 2012). *Rhg1* exhibits a strong epistatic interaction with *Rhg4*, a serine hydroxymethyltransferase, to confer full nematode resistance in the Peking background (Meksem et al., 2001; Liu et al., 2012). Disease resistance can also be conferred by mutations in susceptibility genes (van Schie and Takken, 2014). For example, loss of function of rice *Bsr-k*, encoding a tetratricopeptide repeat protein, induces overaccumulation of defense-related OsPAL mRNAs, resulting in resistance to *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* (Zhou et al., 2018). Naturally occurring TF alleles, such as the rice *Broad-Spectrum Resistance-Dingu 1* (*Bsr-d1*), can confer broad-spectrum resistance (Li et al., 2017). The rice *Ideal Plant Architecture 1* (*IPA1*) gene encodes an SQUAMOSA promoter binding protein-like TF (Jiao et al., 2010; Miura et al., 2010). The *ipa1-1D* allele is resistant to targeting by microRNA 156 (miR156) and miR5269, resulting in higher *IPA1* transcript and protein accumulation (Jiao et al., 2010; Wang et al., 2015b). The presence of *ipa1-1D* is able to increase both yield and disease resistance to the fungal pathogen *M. oryzae* (Wang et al., 2018b). However, phosphorylation can change this TF's DNA-binding specificity; unphosphorylated IPA1 binds the *DEP1* promoter to enhance growth and yield, whereas phosphorylated IPA1 binds the *WRKY45* promoter to enhance defense gene expression (Wang et al., 2018b). This elegant example illustrates how a mechanistic understanding of broad-spectrum resistance can be used to enhance crop production. In wheat, *WHEAT KINASE START1* (*WKS1*) encodes a kinase fused to an START lipid-binding domain and renders partial resistance to stripe rust caused by *Puccinia striiformis* f. sp. *tritici* (Fu et al., 2009). *WKS1* localizes to chloroplasts where it phosphorylates a thylakoid-associated ascorbate peroxidase, reduces the ability of cells to detoxify ROS, and contributes to the death of infected cells (Gou et al., 2015). Another noncanonical resistance gene, *Yr19/Lr34/Sr57/Pm38*, encodes an ABA ABC transporter and controls partial resistance against powdery mildew, leaf rust,

stripe rust, and stem rust (Krattinger et al., 2019). *Yr46/Lr67/Sr55/Pm39* encodes a nonfunctional hexose transporter that confers partial resistance to powdery mildew and all three wheat rusts (Moore et al., 2015). *Fhb7* encodes a glutathione S-transferase that confers broad resistance to *Fusarium* species by detoxifying trichothecenes, such as the deoxynivalenol (DON) toxin in *Fusarium graminearum* (Wang et al., 2020). It seems that each of these noncanonical resistance genes mediates a unique defense mechanism. Additional studies are required to elucidate which genes directly perceive the presence of the pathogen and how they activate disease resistance.

An emerging class of broad-spectrum disease-resistance genes has been shown to encode tandem kinase proteins (TKPs), which represent a novel protein family that is widely present in the plant kingdom (Klymiuk et al., 2018). TKPs evolved by either duplication or fusion of two kinase domains, with the most common functional domains belonging to WALL-ASSOCIATED KINASES (WAKs) and cysteine-rich kinases. WHEAT TANDEM KINASE1 (WTK1) possesses kinase-pseudokinase domains and is likely derived from a fusion of WAK and RLCK subfamily VIII domains. WTK1 confers broad-spectrum resistance against more than 3000 genetically diverse *P. striiformis* f. sp. *tritici* isolates (Klymiuk et al., 2018). WTK3 also possesses kinase-pseudokinase domains and confers resistance to powdery mildew caused by *Blumeria graminis* f. sp. *tritici* (Lu et al., 2020). WTK2 and barley RPG1 possess tandem kinase domains and confer resistance to wheat and barley stem rusts, respectively (Brueggeman et al., 2002; Chen et al., 2020). Many outstanding questions remain about this novel class of resistance proteins. How are effector(s) or pathogen components recognized? Do pseudokinase domains serve as decoys for pathogen effectors? What downstream signaling events are initiated by diverse TKPs? Does TKP function extend beyond immunity?

IMMUNE RESPONSES IN VASCULAR TISSUE

Not only can diverse pathogens of all classes infect plants, but their distribution and disease phenotypes are also variable within different tissues. Various plant pathogens are capable of colonizing specific host tissues, and some can invade distinct vascular cell types. Phloem-limited pathogens comprise vector-borne viruses and bacteria whose distribution is influenced by climate change and interconnected with production practices (Juroszek et al., 2020). Bacterial and fungal pathogens, such as *Xylella*, *Ralstonia*, *Clavibacter*, *Fusarium*, and *Verticillium*, employ diverse strategies, including vector transmission, root invasion, and seed/wound entry, to gain entry into and colonize xylem vessels (Michielse and Rep, 2009; Bae et al., 2015).

Fundamental questions remain about mechanisms regulating plant immune responses to pathogens that colonize vascular tissues. Multiple NLR immune receptors that recognize effectors from xylem-colonizing pathogens have been identified. These include RPS4/RRS1-R and ZAR1 that recognize the bacterial pathogens *Ralstonia solanacearum* and *Xanthomonas campestris*, respectively (Narusaka et al., 2009; Wang et al., 2015a). Using a split-GFP system to visualize effector delivery during

early infection, the recognized *R. solanacearum* effector PopP2 accumulates in the nuclei of host cells surrounding the site of lateral root emergence, correlating with the sites of invasion before xylem entry (Henry et al., 2017). The soil-borne fungus *Fusarium oxysporum* f. sp. *lycopersici* (Fol) is recognized in tomato by two surface-localized immune receptors (the receptor-like protein I and the receptor-like kinase I-3) as well as one intracellular NLR (I-2) (Takken and Rep, 2010). Tomato lines with surface-localized immune receptors I and I-3 were more effective at restricting fungal spread than the NLR I-2, but all three only inhibited colonization after *Fusarium* reached xylem tissues (van der Does et al., 2019). These intriguing results expose multiple outstanding questions. For example, which plant cell types are responsible for immune perception? Are specific cell types targeted for effector delivery?

The phloem environment contains carbohydrates, proteins, amino acids, and sugars, representing a specialized niche for pathogens. Some of the most devastating plant pathogens and insects reside and feed, respectively, in phloem sieve elements, including the bacterium *Candidatus Liberibacter asiaticus* that has severely affected the citrus industry worldwide, causing billions of dollars in economic losses (Wang et al., 2017b). To fight infection, the phloem transports mobile defense signals required for systemic acquired resistance (SAR) and systemic wound responses. Recent studies on glutamate, which acts as a DAMP, have provided striking images of the speed and systemic spread of calcium after glutamate perception (Toyota et al., 2018). Waves of Ca^{2+} , electrical signals, and ROS form a network to promote rapid systemic signaling in plants (Choi et al., 2016). Phloem-specific forisomes and P proteins are also activated by Ca^{2+} , which causes them to rapidly expand in size, and have been hypothesized to occlude sieve pores (Knoblauch et al., 2014). However, a detailed investigation of sieve element occlusion-related (SEOR) P proteins did not reveal alterations in phloem translocation, and future studies will be required to understand their physiological roles (Knoblauch et al., 2014). Callose deposition at sieve plates and plasmodesmata is a common defense in response to pathogens and insect feeding, which blocks viral spread but not the movement of Ca. *Liberibacter* (Hao et al., 2008; Achor et al., 2020). The distribution of the bacterial pathogens, phytoplasmas, and Ca. *Liberibacter* in vascular tissues cannot be completely explained by source-sink relationships either. Future research is required to understand how both the pathogen and the host alter tissue tropism during an infection.

It remains unclear whether phloem sieve elements or companion cells are capable of direct pathogen recognition through PRRs or NLRs. There is evidence that phloem-feeding insects can trigger plant immunity while possessing effectors that suppress defense responses. Hemipteran insects use their stylets to occasionally pierce host cells to sample plant material, feed from the phloem, and secrete watery saliva (Tjallingii, 2006; Will et al., 2009). Watery saliva not only contains MAMPs from bacterial symbionts and pathogens that can be perceived by the host but also contains insect effectors that manipulate host physiology and suppress defense (Chaudhary et al., 2014; Elzinga et al., 2014). For plant defense, a cluster of three lectin RKs was found to confer resistance to both brown and white

back planthopper insect pests (Liu et al., 2015). Future research identifying additional RLKs or RLPs involved in the active perception of piercing-sucking insects and investigating their roles in controlling plant disease would provide significant advances in our understanding of immune responses.

HETEROGENEITY OF IMMUNE RESPONSES WITHIN TISSUES AND CELLS

Variations in cellular responses and tissue types determine the outcome of plant–pathogen interactions. Heterogeneity in pathogen distribution and, thus, uneven targeting of host cells during infection must influence plant responses (Figure 1). For example, microscopical analyses with fluorescently tagged *P. syringae* reveal preferential colonization of particular sites (substomatal cavity, epidermal cell junctions) (Boureau et al., 2002; Rufián et al., 2018). The fungal pathogen *Ustilago maydis* induces tumor formation in aerial maize organs by deploying distinct effectors for colonization and virulence in specific organ types (Hemetsberger et al., 2012; Schilling et al., 2014).

One of the most striking demonstrations of the spatial and temporal dynamic of a plant defense response occurs during typical ETI, where effector-triggered cell death is sharply restricted to the site of infection. The cell death and survival decisions are modulated through the spatial and temporal expression of SA and JA at the infection site and in surrounding cells (Enyedi et al., 1992; Liu et al., 2016; Betsuyaku et al., 2018) through the interplay of NONEXPRESSOR OF PR (NPR) proteins (Fu et al., 2012; Liu et al., 2016; Zavaliev et al., 2020). In a study by Zavaliev et al. (2020), the authors found that many NLRs, as well as downstream signaling components, such as EDS1, are sequestered in SA-induced NPR1 condensates (SINCs) in the cytoplasm of cells surrounding the ETI-induced cell death zone. The mechanism by which SA triggers the dynamic formation of SINCs will require future research using technologies with higher resolutions.

Recent advances in single-cell RNA sequencing coupled with live cell imaging of reporter lines can facilitate an understanding of how plants dynamically respond to infection (Denyer et al., 2019) to address several questions. For example, how many distinct response clusters occur within a leaf? What is the gradient in PRR and NLR responses in cells directly targeted by pathogen effectors, and in cells proximal and distal to pathogens?

The response to pathogen infection is not uniform even within a single cell. For example, the average diameter of *P. syringae* cells is 1.2 μm , compared with 15–35 μm for the spongy mesophyll, 40–80 μm for the palisade mesophyll, and 10–200 μm for an epidermal pavement cell in fully expanded *Arabidopsis* leaves (Melaragno et al., 1993; Monier and Lindow, 2003; Wuyts et al., 2010). The visualization of *P. syringae* effector delivery using a split-GFP system revealed that membrane localized effectors are delivered in small stretches that surround the plasma membrane and rarely exhibit uniform distribution (Henry et al., 2017; Park et al., 2017). Multiple filamentous pathogens deliver effectors at the haustorial interface (Bozkurt and Kamoun,

2020). Plant organelles cluster at this interface, which may facilitate localized defense (Bozkurt and Kamoun, 2020). Selective autophagy is also deployed at the haustorial interface to counteract pathogen invasion (Dagdas et al., 2018; Bozkurt and Kamoun, 2020). As pathogen-induced modification of individual host cells is not uniform, the corresponding host response may also be compartmentalized.

There is accumulating evidence for localized plant responses. Chitin responses in *Arabidopsis* plasmodesmata require the RLKs, LYK4 and LYK5, as well as LYSM-CONTAINING GPI-ANCHORED PROTEIN2 (LYM2) (Faulkner et al., 2013; Cheval et al., 2020). Chitin-induced plasmodesmatal closure results in dynamic changes in the association, mobility, and localization of all three proteins, but only LYM2 and LYK4 are found in the plasmodesmatal plasma membrane (Cheval et al., 2020). In response to osmotic stress, the receptor-like kinases Qian Shōu kinase (QSK1), inflorescence meristem kinase 2, and CYSTEINE-RICH RECEPTOR-LIKE KINASE2 (CRK2) rapidly relocate and cluster to plasmodesmata pores (Grison et al., 2019; Hunter et al., 2019). The flagellin receptor FLS2 and the brassinosteroid receptor BRASSENOSTEROID INSENSITIVE1 (BRI1) localize to distinct membrane microdomains, despite these two receptors requiring the same co-receptor BAK1 (Bücherl et al., 2017). The differential distribution of FLS2 and BRI1 in the plasma membrane may facilitate differentiation between pathogen perception and steroid-mediated growth. Taken together, these data highlight that the reorganization of RLKs to distinct membrane domains and subcellular compartments are important layers regulating the specificity and response to the perception of pathogens and abiotic stresses. Future research investigating spatial and temporal responses in plant immune signaling in the context of pathogen localization will shed light on how plants are capable of integrating rapid, robust, and specific responses to different stimuli.

TRANSLATIONAL REGULATION IS A CRITICAL LAYER OF CONTROL IN PLANT IMMUNE INDUCTION

In addition to the knowledge gaps outlined above, another major blind spot in the plant immune signaling network is translational regulation (Figure 1). This might be due to the intrinsic complexity of the translational process as well as technical limitations in examining the multitude of players involved. However, the recent application of sequencing technology to studies of ribosomal footprinting on mRNAs (Ribo-seq) (Ingolia et al., 2009), tRNA levels (Zheng et al., 2015a), and mRNA modifications (e.g., m⁶A) (Dominissini et al., 2012; Meyer et al., 2012), as well as *in vivo* RNA secondary structures (Ding et al., 2014), have made the genome-wide investigation of how plants regulate protein production during defense a distinct possibility.

During an infection, the host and the pathogen may compete for access to the host translational machinery. The extreme case on the pathogen side is viruses that rely on host ribosomes for viral protein synthesis. Various RNA viruses carry internal ribosome entry site elements in their genomes to recruit host ribosomes and translate viral RNA, despite the absence of the 5' cap (Martinez-Salas et al., 2017) that is required for normal

translation initiation in eukaryotes. The perturbation of the host's metabolism by the pathogen (Scheideler et al., 2002; Ward et al., 2010; Aliferis et al., 2014; Misra et al., 2016; Schwachtje et al., 2018; Yoo et al., 2020) may be an early signal for mounting defense by selectively translating pre-existing mRNAs encoding defense proteins. How these defense mRNAs are chosen, whether their translation is at the expense of those involved in growth-related activities, and whether there is a correlation between transcriptional and translational regulation need to be addressed.

Examining proteomic changes during a pathogen infection would be the most direct way to tackle the aforementioned questions. However, this approach currently has significant technical limitations. Jones et al. (2006) took the challenge and performed two-dimensional gel electrophoresis on *Arabidopsis* inoculated with the virulent bacterial pathogen *PstDC3000*, the mutant version of *PstDC3000*, *hrpA*, which exclusively activates PTI, or *PstDC3000* carrying the effector *AvrRpm1*, which induces ETI. The study detected changes in proteins enriched in defense-related antioxidants and metabolic enzymes and discovered that changes in these proteins occurred prior to significant transcriptional reprogramming, indicating the presence of translational regulation in early immune responses.

Before proteomic analysis becomes more accessible, Ribo-seq has been used to estimate the translational activity of mRNAs through their association with ribosomes (Ingolia et al., 2009). Essentially, the amount of mRNA pulled down with the ribosome is compared with the total mRNA level to calculate the translation efficiency (TE). Xu et al. (2017a) performed Ribo-seq on *Arabidopsis* after treatment with the MAMP signal, elf18. This study was inspired by the earlier finding of Pajerowska-Mukhtar et al. (2012), which reported that translation of the key immune TF, TBF1, is tightly controlled by the 5'-leader sequence (5'-LS) of its mRNA. Consistent with the findings of Jones et al. (2006), Xu et al. (2017a) found no significant correlation ($r = 0.41$) between the elf18-mediated translational changes and transcriptional dynamics, indicating that translational reprogramming is a distinct regulatory step in PTI induction.

What is the regulatory mechanism of this translational reprogramming during PTI? In yeast, mammals, and plants, stresses, such as amino acid starvation, can lead to an overall decrease in translational activity but an increase in translation of stress-responsive proteins, such as GCN4 in yeast and ATF4 in mammals whose translation is normally inhibited by upstream open reading frames (uORFs) (Hinnebusch, 2005; Lageix et al., 2008; Kilberg et al., 2009). This integrated stress response is conserved across eukaryotes and is carried out by GCN2 (or other kinases in mammals)-mediated phosphorylation and the consequent inactivation of the translation initiation factor eIF2 α (P-eIF2 α), thereby allowing the ribosome to bypass the inhibition of uORFs and reach the downstream main ORFs (mORFs) (Hinnebusch, 2005; Lageix et al., 2008; Zhang et al., 2008b; Kilberg et al., 2009). Surprisingly, several plant studies found that blocking eIF2 α phosphorylation in the *gcn2* knockout mutant did not affect immunity-induced translational reprogramming (Luna et al., 2014; Xu et al., 2017a; Izquierdo et al., 2018). For example, elf18-induced TBF1 translation, which is controlled by uORFs in the 5'-LS, and pathogen resistance were appropriately induced

in the *gcn2* background (Xu et al., 2017a). Moreover, elf18 treatment did not significantly alter the overall translational capacity. Although Liu et al. (2019) found that GCN2 plays a role in stomatal immunity through ABA, the majority of the current data suggest a novel mechanism in reprogramming translome during plant immune responses.

To elucidate the translational regulatory mechanism, Xu et al. (2017a) searched for sequence features, namely consensus sequences and uORFs, associating with mRNAs whose translation is induced during PTI. This search led to the discovery of a significantly enriched consensus sequence consisting of mostly purines ("R-motif") in the 5'-LSs of these mRNAs and found that the R-motif is not only necessary but also sufficient for PTI-induced translation. One mRNA feature that Xu et al. (2017a) did not attempt to search for was the conserved secondary structure because the prediction algorithms at that time could not accurately reflect the *in vivo* RNA structural dynamics during stress responses (Ding et al., 2014). Recent developments of *in vivo* RNA structure probing technologies, such as SHAPE-MaP (Smola and Weeks, 2018), will ultimately enable visualization of RNA structural dynamics inside living cells, which has been shown to be critical in explaining how the uORF mediates translation of the mORF (Gunisova and Valasek, 2014; Corley et al., 2017).

The identification of the R-motif in the 5'-LSs of mRNAs with enhanced TE during PTI suggests that its associated protein(s) may play a role in selecting these mRNAs for translation. Poly(A)-binding proteins (PABPs) can bind to the R-motif and affect basal as well as elf18-induced translation (Xu et al., 2017a). As PABPs link the poly(A) tail of an mRNA to its 5' cap through interactions with eIF4G and eIF3 to facilitate translation initiation (Sonenberg and Hinnebusch, 2009; Jackson et al., 2010), it is plausible that the 5' R-motif serves as a cap-independent translation enhancer (CITE) (Shatsky et al., 2018). Indeed, in yeast, the cap-independent translation of mRNAs involved in invasive growth induced by nutrient deprivation was found to require recruitment of the poly(A)-binding protein 1 (Pab1) by an A-rich element in the 5' untranslated regions of these mRNAs (Gilbert et al., 2007).

In addition to the specialized mRNA features that might serve as CITEs, different components of the translational machinery may be used specifically for translating stress-responsive proteins. In a genetic study performed in *Arabidopsis*, Izquierdo et al. (2018) found a yeast GCN1 homolog named NOXY7 that acts through GCN20, instead of GCN2-P-eIF2 α , thereby mediating translation in response to mitochondrial dysfunction, high boron concentrations, and infection by *PstDC3000*. Future biochemical, proteomic, and genetic studies will identify additional stress-specific components of the translational machinery.

The recruitment of stress-specific components and/or modifications of the translational machinery is also likely to control translation during ETI that occurs when a pathogen effector is detected by an NLR. As ETI often leads to programmed cell death, it was surprising that no significant global changes in polysome profiles were observed either by Metegnier et al. (2017), who examined *Arabidopsis* 2 h after dexamethasone-induced expression of the bacterial effector *AvrRpm1* gene, or by Yoo et al.

(2020), who performed the experiment 8 h after inoculation with *P. syringae* pv. *maculicola* ES4326 carrying *avrRpt2* (*Psm* ES4326/*avrRpt2*). Although [Meteignier et al. \(2017\)](#) observed a moderate level of overlap between transcriptionally and translationally induced genes (77%), [Yoo et al. \(2020\)](#) found a significant correlation ($r = 0.92$) between the two activities. It is intuitive to conclude that during ETI, transcriptional induction is the major driving force that defines the ETI proteome. The counter argument may be that since transcription and translation occur in distinct cellular compartments, a good correlation suggests an intricate interplay between the two separately regulated processes. How plants reprogram the proteome without significantly altering the overall translational capacity will be a fascinating question that may be addressed through the identification of ETI-specific translation regulators.

What types of genes are translationally regulated during PTI and ETI? For those identified during PTI, gene ontology (GO) term analyses did not provide a clear answer, probably because the current GO terms were mostly defined by transcriptional dynamics. However, these “first responders” to pathogen challenge are most likely translated from pre-existing mRNAs without the involvement of transcriptional regulation. Although they do not have the “defense” GO term yet, many of them (e.g., TFs and enzymes) were found to be critical for PTI ([Xu et al., 2017a](#)). In contrast to the PTI transcriptome analysis, the identification of translationally regulated genes during ETI was more difficult due to the strong correlation between transcriptional and translational activities. Perhaps for this very reason, “defense” GO terms turned out to be more helpful in narrowing down the candidate gene list in ETI. The genes involved in aromatic amino acid and phenylpropanoid metabolism and those encoding NLR immune receptors were determined to be translationally regulated by [Meteignier et al. \(2017\)](#) and [Yoo et al. \(2020\)](#). As NLR expression is regulated at many stages, from mRNA nuclear export to proteasome-mediated degradation ([Johnson et al., 2012](#); [Wu et al., 2020](#); [Zavaliev et al., 2020](#)), it was not surprising that some immune receptors are translationally regulated. However, it was somewhat unexpected that knocking out these *NLR* genes partially compromised ETI triggered by RPS2, a sensor NLR that has been studied extensively, demonstrating the effectiveness of translational profiling in identifying novel players in plant defense.

There are still many unexplored post-transcriptional processes affecting the defense proteome. mRNAs and tRNAs are known to be modified at multiple positions in different nucleotides, such as methylation at *N*⁶-adenosine (*m*⁶A), which can affect mRNA transport, stability, and translational efficiency ([Roundtree et al., 2017](#)). The writers, readers, and erasers of *m*⁶A have been identified and characterized in plants ([Ruzicka et al., 2017](#); [Aribas-Hernandez and Brodersen, 2020](#)), but it is still challenging to quantitatively measure the dynamic changes in *m*⁶A to identify defense-specific modifications and distinguish the direct impact of the modification from the pleiotropic effect on plant immunity. In addition to RNA modifications, there have been several studies focusing on the various RNA degradation processes during plant immune responses ([Gloggnitzer et al., 2014](#); [Maldonado-Bonilla et al., 2014](#); [Roux et al., 2015](#); [Meteignier et al., 2016](#); [Makinen et al., 2017](#); [Li and Wang, 2018](#); [Yu et al., 2019b](#)). For example, [Gloggnitzer et al. \(2014\)](#)

found that the nonsense-mediated mRNA decay pathway (NMD) controls the turnover of a large number of NLR mRNAs. During bacterial infection, plants turn down NMD to enhance resistance. Moreover, the formation of RNA-processing bodies upon different immune responses was reported by [Yu et al. \(2019b\)](#) to destabilize PTI-downregulated transcripts and by [Meteignier et al. \(2016\)](#) to inhibit viral RNA translation during ETI. In addition to mRNA, tRNA concentrations and charging can also be affected by pathogen challenge ([Pajerowska-Mukhtar et al., 2012](#); [Luna et al., 2014](#); [Soprano et al., 2018](#)), which may link pathogen-induced metabolic changes directly to the translation of mRNAs through codon usage, instead of indirectly through the activation of GCN2 ([Hinnebusch, 2005](#)).

Understanding the regulatory mechanisms controlling the translation of plant defense proteins is not only critical for basic science but also for applications in agriculture and beyond ([Xu et al., 2017b](#)). After all, most biological activities are carried out by proteins. Transcripts encoding key regulatory proteins, such as the mRNA of *TBF1*, carry sequences, such as R-motifs and uORFs, for translation at the proper time, in the specific tissue, and of the right amount. Although the mechanisms by which these sequences control translation still need to be elucidated, they are ready-to-use because they have been optimized through evolution.

THE CIRCADIAN CLOCK IS A KEY MECHANISM THAT BREAKS THE DISEASE TRIANGLE IN PLANTS

So far, most of our studies have focused on defense against an established infection. However, the battle between the host and the pathogen begins at an even earlier stage. It is known that a successful infection requires at least three factors: a virulent pathogen, a susceptible host, and a favorable environment collectively known as “the disease triangle” ([Francl, 2001](#)). Here, we examine how the circadian clock affects each of these factors ([Figure 1](#)).

The role of the circadian clock in controlling plant defense against pathogen infection is not as obvious as in regulating plant growth and reproduction, because plants need to be able to respond to infection whenever a pathogen invades. Reinforcing this idea, a study by [Nagano et al. \(2012\)](#) of transcriptome data of rice plants grown in a paddy field with the corresponding meteorological data found that defense genes had higher stochastic variances than those involved in basic metabolic functions. However, the serendipitous discovery that a large number of defense genes against *Hyaloperonospora arabidopsidis* (*Hpa*) was directly regulated by the morning circadian clock component, CCA1, unveiled a role for the circadian clock in controlling defense against at least certain pathogens ([Wang et al., 2011](#)). The fact that the life cycles of both the plant hosts and some of their pathogens are dictated by the diurnal cycle of the Earth suggests that when infection is a predictable event, the host can anticipate it through the function of the circadian clock ([Slusarenko and Schlaich, 2003](#); [Bhardwaj et al., 2011](#); [Goodspeed et al., 2012](#); [Zhang et al., 2013](#); [Korneli et al., 2014](#); [Hevia et al., 2015](#); [Ingle et al., 2015](#); [Schumacher, 2017](#); [Lei et al., 2019](#)). For example, for the

obligate biotrophic pathogen *Hpa* to complete its life cycle, darkness is required for the formation of spores, while spore dissemination is triggered by drying of the leaf surface in the morning (Slusarenko and Schlaich, 2003). Meanwhile, the virulence of the fungal pathogen *Botrytis cinerea* (*Bc*) on *Arabidopsis* has been shown to be controlled by the fungal circadian clock regulator, BcFRQ1, resulting in the formation of bigger lesions on leaves if the pathogen is inoculated at dusk (Hevia et al., 2015). However, there are still numerous questions to be answered. Is there a general role for the circadian clock in regulating resistance against other pathogens whose life cycles are less predictable than *Hpa* and *Bc*? Besides helping plants anticipate infection, does the circadian clock play a role in modulating defense response during an infection? How do changes in environmental conditions, such as humidity, affect plant defense through the circadian clock?

A general role for the circadian clock in plant defense is supported by Goodspeed et al. (2012) who showed circadian oscillations in basal levels of the plant defense hormones SA and JA. As SA and JA have antagonistic effects in mediating resistance against biotrophic pathogens and necrotrophs/insects, respectively (Spoel and Dong, 2008), the opposite phasing of SA (peaking at subjective night) and JA (peaking at subjective day) observed by Goodspeed et al. (2012) suggests that the circadian clock may help plants avoid such a conflict. A direct link between the circadian clock and SA synthesis was made in a study by Zheng et al. (2015b), in which the clock component, *CHE*, was shown to bind to the promoter of the SA synthesis gene, *ICS1*, to regulate its transcription. In addition to the rhythmic expression of basal SA, *CHE* was also found to be required in SA synthesis in systemic tissues and disease resistance. However, the biological significance of having a clock component regulating SAR is still unknown. The study by Li et al. (2018) may shed some light on this question. The authors observed that treating a single leaf of 2-week-old *Arabidopsis* seedlings with *Pst*DC3000 or SA dampened the circadian clock in systemic tissues. They hypothesized that this might allow plants to uncouple SA synthesis from the normal clock control and facilitate the continuous production of SA for the establishment of SAR. As *CHE* has been shown to be a repressor of the core morning clock gene *CCA1* (Pruneda-Paz et al., 2009), it is plausible that *CHE* promotes SA synthesis in systemic tissues by activating *ICS1* while dampening the clock.

However, the relationship between SA and the clock appears to be “very complicated,” to use the words of Dr. Steven Kay in describing phenomena associated with the circadian clock. Zhou et al. (2015a) found that spraying mature *Arabidopsis* with SA, instead of soaking seedlings grown on plates in SA solution, increased the amplitude of the circadian clock genes, such as *LHY* and *TOC1*, in contrast to what was observed by Li et al. (2018). This increase was facilitated by the central SA signaling component NPR1 through its associated TGA TFs. Moreover, the *npr1* mutant was not only insensitive to this SA-mediated reinforcement of the clock, but also had lower-than-wild-type amplitude of the clock gene expression.

Because the phase and the period of the clock have traditionally been the main focus of chronobiology research, it is easy to overlook the importance of the clock amplitude. The clock amplitude

might also play a critical role in the integration of the environmental signals into the regulation of physiological responses. For example, Nagano et al.’s field-grown rice transcriptome data showed that the daily amplitudes of core circadian clock genes were affected by environmental stimuli such as temperature and solar radiation, while their phases and periods remained stable (Nagano et al., 2012). Furthermore, Mwimba et al. (2018) found that, in addition to light and temperature, daily humidity oscillation is a novel environmental cue that entrains the plant circadian clock (i.e., a zeitgeber). Interestingly, adding humidity oscillation to the light/dark cycle as it occurs in nature increased the amplitude of the circadian clock, leading to better plant performance such as higher total biomass and seed weight.

This ability of the plant circadian clock to sense humidity may be critical for defense against pathogen infection because high humidity in the environment is often associated with outbreaks of plant diseases (Thurston et al., 1958; Brown and Ogle, 1997; Xin et al., 2016). High humidity has been shown to induce the expression of the *HrpL* TF in *Pst*DC3000, which activates the type III secretion system for the delivery of effectors into the host (Mwimba et al., 2018). High humidity is also required for the pathogen to cause disease by maintaining the aqueous environment in the apoplastic space where the bacterium proliferates (Xin et al., 2016). Therefore, there is “a war over water” between the pathogen and its plant host (Beattie, 2016). The SA-mediated repression of water transport genes found by Zhou et al. (2015a) suggests that inhibiting genes, such as aquaporins, may be one of the defense mechanisms of this defense hormone.

As both SA treatment and daily humidity oscillation can increase the amplitude of the circadian clock, a reasonable question to ask is how this increase affects plant defense. The morning clock components *CCA1/LHY* are known positive regulators (Wang et al., 2011; Zhang et al., 2013), whereas the evening clock component *TOC1* is a negative regulator of defense genes (Zhou et al., 2015a), and an increase in the clock amplitude results in higher SA-induced defense gene expression in the morning compared to in the evening. Indeed, a striking difference in this time-of-the-day sensitivity was observed in the microarray analysis performed by Zhou et al. (2015a) on plants after treating them with SA in the subjective morning and subjective evening. These authors found that repression of water transport genes by treating plants with SA in darkness led to a significant loss of fresh weight and, ultimately, death. Therefore, besides its anticipatory function, the circadian clock also serves to gate immune responses upon infection to minimize conflict with other physiological functions, in this case, water transport (Figure 1). As in circadian medicine, which aims to administer drugs at the appropriate time of the day to increase drug efficacy and reduce side effects, the consequence of plants taking “aspirin” at the wrong time of the day (i.e., at night) might be as severe as death by desiccation.

Despite the important role played by the circadian clock in regulating plant defense, research in this area is technically challenging and financially prohibitive because of the requirement for time series data collection and analysis. The use of clock-driven luciferase reporters is limited to organisms such as *Arabidopsis* that can be readily transformed. To remedy the reliance on

time-course experiments in circadian clock studies, the ingenious “molecular timetable” method has been developed first in mice (Ueda et al., 2004) and then adapted to plants (Kerwin et al., 2011; Higashi et al., 2016; Li et al., 2019b), and it aims to extract circadian information from data taken at a single time point. Recently, Li et al. (2019b) applied this methodology to map abiotic stress inputs into the soybean circadian clock. These findings were then experimentally confirmed using RASL-seq, a multiplexed targeted RNA-sequencing technology (Li et al., 2012). Using this two-module discovery pipeline, one can make good use of all the available plant–microbe interaction transcriptome data, regardless of whether they are generated from time-course or single-time-point experiments to study the impact that biotic stresses have on the circadian clock.

FUTURE PERSPECTIVES

Research discoveries in plant sciences have the potential to guide sustainable increases in agricultural production to feed, shelter, and clothe the world’s population. Crop losses from plant pathogens and pests range from 17% to 30% on average globally for major food crops and also directly affect human health (Gregory et al., 2009; Savary et al., 2019). The identification and deployment of diverse plant resistance genes using a variety of technologies have clear potential for crop improvement. In contrast to disease-specific resistance, broad-spectrum resistance involving multiple defense mechanisms may have the advantage of increased durability and decreased guesswork involved in the deployment, that is, if it can be tightly regulated to minimize potential yield penalty. Therefore, basic research focused on understanding how plants naturally manage their immune responses is the foundation on which applied research can be built. The utility and relevance of fundamental discoveries may not be immediately noticeable, but some of the most impactful discoveries, including genome editing, occurred as the result of curiosity-driven investigations. We believe that investigating novel disease-resistance genes, their activation mechanisms, and immune signaling networks will provide a more holistic understanding of plant defense that can be harnessed for controlling some of the most devastating plant diseases.

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AUTHOR CONTRIBUTIONS

J.Z. wrote sections on extracellular immunity, which includes stomatal, rhizospheric, and apoplastic immunity. J.-M.Z. wrote sections on sensing immunogenic signals by cell surface and intracellular immune receptors, and crosstalk among different receptors and immune activation. G.C. wrote sections on noncanonical resistance genes, immune responses in vascular tissues, and heterogeneity of responses within tissues and cells. X.D. organized the preparation of the review and wrote the sections on the translational and circadian regulation of plant immunity.

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