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Review

The plant immune system: From discovery to deployment

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SUMMARY

Plant diseases cause famines, drive human migration, and present challenges to agricultural sustainability as pathogen ranges shift under climate change. Plant breeders discovered Mendelian genetic loci conferring disease resistance to specific pathogen isolates over 100 years ago. Subsequent breeding for disease resistance underpins modern agriculture and, along with the emergence and focus on model plants for genetics and genomics research, has provided rich resources for molecular biological exploration over the last 50 years. These studies led to the identification of extracellular and intracellular receptors that convert recognition of extracellular microbe-encoded molecular patterns or intracellular pathogen-delivered virulence effectors into defense activation. These receptor systems, and downstream responses, define plant immune systems that have evolved since the migration of plants to land ~500 million years ago. Our current understanding of plant immune systems provides the platform for development of rational resistance enhancement to control the many diseases that continue to plague crop production.

PLANT DISEASES IMPACT HUMAN AND ENVIRONMENTAL HEALTH

In 1974, as the first issue of *Cell* hit libraries around the world, the mechanisms underpinning plant-microbe interactions were poorly understood, but a better understanding was clearly required to enable effective control of crop diseases. Plant disease pandemics have impacted societies and food security since antiquity. Among many examples, potato late blight, caused by the oomycete *Phytophthora infestans*, caused the Irish potato famine in the 1840s, driving the emigration of millions that resulted in a large Irish community in the USA.[1] English tea drinking habits are the consequence of an epidemic of fungal rust disease that destroyed Sri Lanka's coffee crops, forcing the switch to tea.[2] The wind-borne stem rust disease can devastate wheat yields and in North America, huge grain losses occurred in 1903 and 1905 and from 1950–1954.[3] Despite many advances since 1974, crop diseases have not gone away. Florida orange production has dropped by over 80% in the last 5 years, driven by an insect-borne disease caused by *Candidatus liberibacter* bacteria. Cacao (a tree crop and the source of chocolate), coffee, cassava and banana are afflicted by poorly controlled fungal, oomycete, bacterial and viral diseases that greatly reduce yield and increase the cost of cultivation. These wake-up calls encouraged recruitment in the 1970s to the emerging field of molecular plant-microbe interactions (MPMI).

In 2019, approximately 2 million tons of agrichemicals were applied in crop production, of which 47.5% were herbicides, 29.5% insecticides, and 17.5% fungicides.[4] More than $20B are spent annually on fungicides alone.[5] Climate change is exacerbating crop production challenges, including shifting pathogen growth ranges around the world. However, genetic solutions remain controversial in many jurisdictions, despite their obvious preferability to chemical solutions.

Biological discovery is driven by judicious choices of model systems. An enduring challenge for MPMI investigators has been the diversity of pathogens (fungi, bacteria, oomycetes, viruses, or invertebrates) and host plant species. Economics traditionally drove funding to support studies on diseases with the most impact on crop yields, even if they were less suitable for revealing novel biological insights. Early MPMI research also prioritized symbiotic interactions between leguminous plants and the bacterial nitrogen-fixing symbiont *Rhizobium* and the mechanism by which the crown gall-forming Agrobacterium (a close relative of Rhizobium) delivers DNA into plant cells. This was likely because the molecular techniques of the 1970s and 1980s enabled discoveries with bacteria, but less so with plants. At the first MPMI meeting in Bielefeld, Germany in 1982, nearly all presentations were on *Rhizobium* or *Agrobacterium*, with only one of the six sessions on virulence mechanisms of the bacterial pathogens *Pseudomonas*, *Xanthomonas*, and *Erwinia*. There were no sessions on eukaryotic pathogens or on molecular mechanisms of disease resistance.
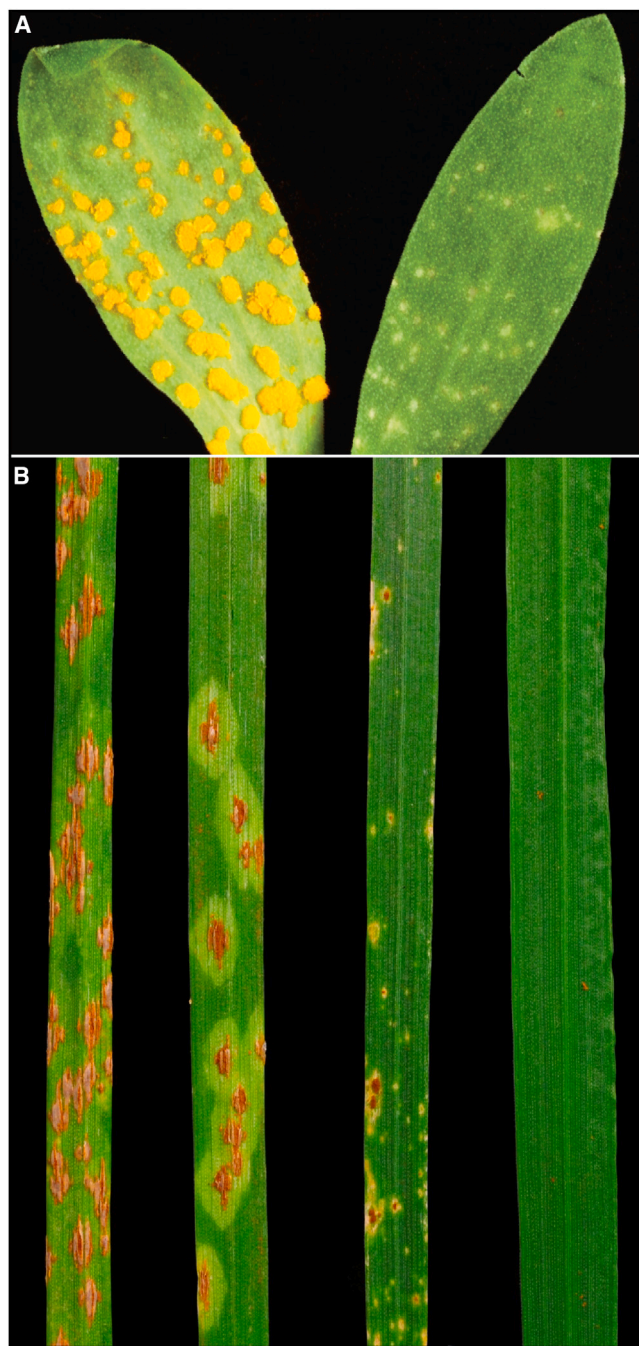
Figure 1. Important diseases caused by two basidiomycete rust pathogens

(A) Copious urediniospore production by flax rust on a susceptible (left) compared to a resistant (right) flax genotype infected by the same rust genotype. Note white flecks on right leaf where pathogen ingress has been resisted.

(B) Differing interactions between wheat host genotype and the stem rust isolate JRCQC. Abundant sporulation on left leaf contrasts with varying degrees of resistance of other three leaves; note some sporulation even on the most resistant right leaf which carries the stem rust resistance gene *Sr35* (see Figure 5).

STATE-OF-THE-ART IN PLANT IMMUNITY FROM PRE-1974 TO 1994

Genetic analyses of plant disease resistance began soon after the rediscovery of Mendel's Laws of inheritance with the report that resistance to rust fungal pathogens in wheat was conferred by single semi-dominant disease *Resistance* (*R*) genes.[6] The phenotype of disease resistance or susceptibility depended not only on the host but also on the genotype of the pathogen; some but not all races of a wheat rust fungus could overcome particular *R* genes and cause disease.[7] In genetic studies on the interaction of flax (*Linum usitatissimum*) and its rust fungus *Melampsora lini*, (Figure 1) H.H. Flor investigated the inheritance of the capacity of a rust race to be resisted by a particular *R* gene and demonstrated that "recognizability" ("Avirulence") is conferred by a dominant gene in the pathogen that was matched by a corresponding dominant *R* gene to that race in the host.[8,9] The resulting concept became known as the "gene-for-gene" model, according to which each disease *Resistance* gene confers recognition of a pathogen that carries a corresponding Avirulence (Avr) gene. This of course begs the question: why would a pathogen encode a product that enables it to be resisted? The apparent paradox has now been resolved; pathogen *Avr* genes can contribute to pathogen virulence, provided the host has not evolved an immune receptor that detects it (detailed below). Pathogens typically encode and deploy into the plant cell scores to hundreds of such virulence factors, usually referred to as virulence "effectors". If, however, one of these is recognized by a host immune receptor, sufficient defense is usually activated to thwart the pathogen. Pathogens need to deploy sufficient functionally redundant virulence effectors to infect a host, but hosts need only to detect one effector to be resistant. In genetically diverse populations of hosts, each individual carries different sets of detection capacities and frequency-dependent selection constrains pathogen success since a pathogen race that evades detection on one plant in the population may be unable to do so on most plants.

In parallel, another line of early enquiry identified pathogen-derived molecules ("elicitors") that activate defense and define the complex set of plant defense responses that they activate. Elicitors were initially defined as pathogen molecules that activate biosynthesis of phytoalexins, the name assigned to a diverse set of antimicrobial secondary metabolites, many of which derive from phenylalanine or tryptophan. This spurred investigations into the phenylalanine lyase (PAL) gene and its elicitation in various systems.[10] Notably, in cell cultures of parsley, a secreted elicitor from *Phytophthora megasperma* leads to PAL transcriptional induction. These investigations culminated in a landmark *Cell* paper defining a minimal 13 amino acid peptide (Pep13) derived from this transglutaminase elicitor, its high-affinity binding to a presumed receptor in parsley cell cultures and the resulting repertoire of elicited defense responses.[11] In addition to phytoalexin accumulation, elicitation also triggers production of reactive oxygen species (ROS) via a plasma membrane NADPH oxidase, Ca^{2+} influxes, activation of mitogen-activated protein (MAP) kinases and other protein kinases, cell wall fortification and defense-related gene

activation.[12] Defense responses of this sort, directed upon recognition of microbial patterns, came to be known as Pattern-Triggered Immunity (PTI).[13,14]

The conceptual framework of PTI was pioneered by the late Charles Janeway, Jr., who suggested that relatively conserved pathogen patterns such as flagellin or lipopoysaccharide were specific metazoan immune stimulants. This, he noted, was the likely basis for "immunology's dirty little secret" that immune responses to injected antigens often required a mix of bacterial products known as "adjuvants".[15] A contrasting, but not mutually exclusive, concept was articulated by Polly Matzinger, who suggested that the differentiation of self from non-self involved recognition of "damaged" host components that she termed "danger signals".[16] We now accept that plant cell-derived modified self-components and generic danger signals like reactive oxygen species also play key roles in plant immune systems. Early attempts to consolidate thinking across metazoan and plant immune systems were instrumental in driving the MPMI field forward.[17]

A third parallel thread involved the investigation of mutant phenotypes in which defense appears to be activated in the apparent absence of any pathogen.[18] Such lesion mimic mutants, sometimes dubbed "paranoid plants" (that activate defense even when not being attacked) can result either from recessive mutations in negative regulators of defense,[19–21] or from semidominant mutations in which an immune receptor allele has emerged, via mutation or unequal crossing over at a locus carrying multiple paralogs of an immune receptor gene, that is constitutively active. A classic early example of the latter is the *Rp1-D21* allele of the maize *Rp1* locus that confers resistance to the rust pathogen *Puccinia sorghi*.[22–24]

REVIEWING THE REVIEWS

Our current understanding of MPMI and of plant immunity would have been unimaginable in 1974. The gene-for-gene model was key to providing a conceptual framework to begin gene isolation using various methods that emerged in the 1980s. A focus on emerging model systems resulted in immune receptor genes and the effector genes being cloned, as detailed below. The discovery that most *R* genes encode nucleotide-binding, leucine-rich repeat (NLR) proteins provided a crucial advance, as detailed below. By contrast, effector genes encode proteins with diverse biochemical functions. Mechanisms of direct or indirect recognition were defined for the NLR and cell surface immune receptors, as was the interplay of defense mechanisms initiated by microbial ligands binding to cell-surface receptors and the activation of intracellular NLR receptors. Genomics accelerated the discovery and analysis of both plant immune receptor repertoires and pathogen effector complements. Important advances were accompanied by influential reviews.[13,14,25–33] The field has become too vast for a comprehensive description of every discovery, so we attempt to highlight the most significant conceptual advances in our field from the last 50 years. A timeline of important advances is shown in Figure 2.

AGROBACTERIUM, PLANT TUMORS, AND THE TUMOR-INDUCING (TI) PLASMID AS A VIRULENCE MACHINE AND RESEARCH AND ENGINEERING TOOL

How the Gram-negative soil bacterium *Agrobacterium tumefaciens* causes plant disease and enables gene transfer into plant chromosomal DNA provides a spectacular example of how curiosity-driven science can enable a game-changing technology.[34] The closely related *A. tumefaciens* and *A. rhizogenes* cause tumor-like crown gall and hairy root diseases, respectively, on many plants. In a landmark paper in *Cell*, Chilton et al. showed that part of the tumour-inducing (Ti) plasmid of Agrobacterium becomes incorporated into host genomic DNA.[35] The transferred DNA (T-DNA) carries genes encoding enzymes involved in the synthesis of plant growth hormones and for synthesis of opines (amino acid-sugar conjugates) that Agrobacteria can use as a carbon and nitrogen source. Understanding the transfer of T-DNA from Agrobacterium to plants was an early triumph for the MPMI field and a pioneering accomplishment that underpins crop genetic modification and created an indispensable tool for investigating plant gene function. The T-DNA transfer process evolved from a type 4 secretion system involved in bacterial conjugation and T-DNA integrates into host DNA at random locations, a discovery that enabled the generation of saturation mutant collections in the reference plant *Arabidopsis thaliana*.[36,37] Infiltration of Agrobacterial strains that deliver genes of interest into leaves of susceptible plants such as *Nicotiana benthamiana* results in transient gene expression that greatly facilitates investigations of defense mechanisms, protein-protein interactions, and protein preparation for structural biology on plant immune receptors.

"Arabidopsis DOESNT HAVE PATHOGENS!" AND THE EARLY DAYS OF BACTERIAL EFFECTOROMICS

The gene-for-gene model and its counter-intuitive prediction of "recognizability" genes in pathogens, prompted a search for avirulence (*Avr*) genes. The critical observations of Klement that all pathogenic strains of *Pseudomonas* can trigger a rapid 'Hypersensitive cell death Response' (HR) at the inoculation site of tobacco leaves, led him to suggest that similar molecular processes regulate virulence and resistance.[38] Recombinant DNA technology developed in the 1970s and 1980s revolutionized the field. A crucial advance was the development of broad host range cloning vectors for investigating *Rhizobium* biology[39] and the realization these could also be used to study *Pseudomonas* and *Agrobacterium* genes. The key conceptual insight was that the dominant Avr genes defined genetically by Flor in a fungal pathogen could also be isolated from pathogenic bacteria by identifying in a library of clones made from DNA of a resisted pathogen race, those clones that confer gain of HR-inducing phenotypes following delivery into virulent (non-HR-inducing) *Pseudomonas* strains. The first Avr gene was cloned from a *Pseudomonas* that caused disease on some, but not all, soybean cultivars.[40] Many others followed.[41]

The cloning of Avirulence genes provided important insights into effector biology even before the mechanisms of their delivery into host cells were elucidated. *AvrBs2* from a *Xanthomonas*

Advances in MPMI	Timeline
Agrobacterium T-DNA transfer solved	1974-1980
Transgenic plants	1981-1985
Bacterial recognized effectors cloned	1981-1985
Methods for Mendelian gene isolation	1986-1990
Resistance gene cloning, first NLR	1991-1995
Guard hypothesis	1996-2000
Decoys	2011-2015
Paired NLRs	2000-2005
Integrated decoys in paired NLRs	2011-2015
Helper NLRs	2000-2005
EDS1 SAG101 PAD4	1996-2000
FLS2 and other PTI receptors cloned	1996-2000
PTI and ETI, zigzagzig model	2006-2010
The genomics explosion: pathogenomics	2006-2010
Oomycete & fungal effectoromics, Avrs	2011-2015
The genomics explosion: NLR diversity	2016-2020
Resistosomes	2016-2020
PTI + ETI mutual potentiation	
Synthetic designer R genes	2021-2024
Alphafold Multimer NLRs and effectors	2021-2024

Figure 2. Timelines of some key advances in MPMI over the last 50 years, highlighting advances in how immune receptors recognize their ligands and in pathogen effector modes of action. Dates signify the time period in years when each advance was first achieved, with extension to later dates signifying continuing progress and deployment to the present day.

euvesicatoria strain pathogenic on pepper was the first recognized effector shown to be required for full virulence and fitness. This suggested that the corresponding pepper *R* gene, *Bs2*, would likely be "durable" because loss of AvrBs2 function compromises pathogen fitness.[42] Importantly, AvrBs2 amino acids required for recognizability are also required for virulence.[43] Pepper *Bs2* does indeed confer strong resistance in transgenic tomato[44] and protects against *Xanthomonas*, a severe pathogen in hot, humid environments.[45] These and similar findings[46,47] resolved the paradox of why pathogens encode genes that have a large fitness cost on some plant hosts; these genes encode virulence functions on other genotypes of the same host or other host species that cannot recognize them. Several papers subsequently demonstrated that the Avr proteins are recognized inside the host cell.[48–51] This suggested that there must be a delivery mechanism and that the *R* gene-encoded proteins are likely intracellular. Finally, the fact that unrelated genes from a pathogen of one plant host can be recognized as Avr genes by a second species of plant host provided the first clue that "gene-for-gene" interactions were likely to contribute to host range restriction and "non-host resistance".[52,53]

In parallel, transposon mutagenesis revealed *Pseudomonas* mutants that lost the ability to cause HR on tobacco. Consistent with Klement's hypothesis, these mutants also lost virulence on

their nominal host plant due to failure to deliver effectors required to cause disease. Simultaneous discoveries were made in bacterial pathogens of animals and sequencing of these clusters defined the Type III secretion systems that are widely distributed among gram-negative pathogens of plants and animals. Type III secretion substrates, including Avr proteins, were renamed "effectors".[54] Many bacterial effectors were subsequently shown to encode enzymes that modify host protein targets that contribute to immune responses.[54,55] The term "effector" was adopted to refer to any pathogen proteins delivered to the apoplast or the plant cell interior. Dissection of effector function and evolution, and the consequent definition of host cellular targets, has illuminated the field over the last 30 years.

When researchers converged on the model plant *Arabidopsis thaliana* in the mid-1980s, progress in MPMI research accelerated (Figure 3). Two *Pseudomonas syringae* strains (DC3000, a pathogen of tomato, and ES4326 a pathogen of Brassicas) were identified that cause disease on the reference Arabidopsis genotype Columbia (Col-0). Once cloned Avr genes were identified (see above), they could be conjugated into DC3000 or ES4326 and resulting strains tested for acquisition of recognizability (avirulence) on diverse Arabidopsis genotypes. Once accessions with differential recognition were identified, either in natural variation or from mutagenized plant populations, plant
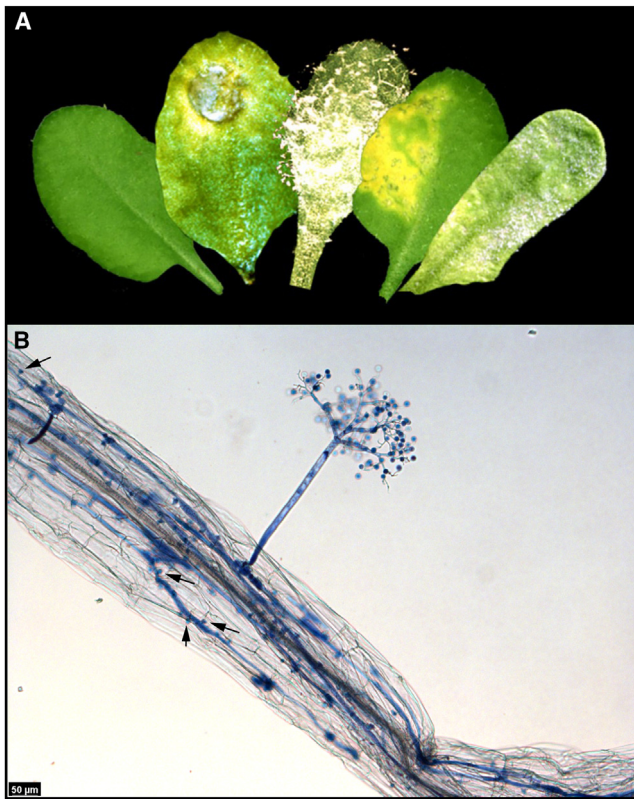
Figure 3. Arabidopsis pathogens

(A) A healthy Arabidopsis leaf at left, followed by a compilation of Arabidopsis foliar diseases: gray mold (the ascomycete *Botrytis cinerea*), downy mildew caused by the oomycete *Hyaloperonospora arabidopsidis (Hpa)*, bacterial speck caused by *Pseudomonas syringae* and powdery mildew caused by *Golovinomyces orontii*.

(B) Growth and sporulation of *Hpa* on an Arabidopsis hypocotyl. Note the trypan blue-stained hyphae growing between plant cells, the sporangiophore carrying conidia emerging from the hypocotyl and the small round blue invaginations of haustoria into host cells from the hyphae (some of which are arrowed).

genetics could be initiated to identify the corresponding immune receptor gene.

Furthermore, despite early assertions that "Arabidopsis does not have pathogens", natural pathosystems were identified and developed, notably the Arabidopsis downy mildew pathogen *Hyaloperonospora arabidopsidis* (*Hpa*; previously *Peronospora parasitica*, a fungus-like oomycete filamentous pathogen related to the potato late blight pathogen *Phytophthora infestans*), *Albugo* spp (*Ac*) that cause white blister rust on Arabidopsis and related Brassicas[56,57] and powdery mildews.[58]

These discoveries enabled the next major advance in MPMI, the identification of disease resistance *R* genes that encode intracellular innate immune receptors.

REVEALING IMMUNE RECEPTORS, 1994–2004

The focus on Arabidopsis, with its rapidly expanding resources for genome analysis and map-based cloning, enabled identification of multiple immune receptors that detect *Pseudomonas* ef-

fectors (for example, *RPS2*, *RPM1*, *RPS5*, and the *RPS4-RRS1* pair[27]). Extensive natural variation between Arabidopsis accessions in their response to specific races of *Hpa* was exploited to facilitate the isolation of a bevy of *RPP* resistance genes during this period.[59] All encoded NLR proteins (detailed below).

While tobacco, tomato, and flax were less amenable than Arabidopsis to map-based cloning, the first gene-for-gene *R* gene cloned was *Pto* from tomato. Pto is a protein kinase and recognizes the Pseudomonas effector AvrPto. The tobacco *N* gene that recognizes tobacco mosaic virus, tomato *Cf-9* conferring resistance to the fungus *Cladosporium fulvum* and the flax rust resistance gene allele *L6* that recognizes specific races of flax rust were all cloned by transforming plants carrying these genes with the maize transposon *Activator* or its non-autonomous derivative *Dissociation*. The resulting plant populations were screened for mutants that lost immune receptor function and the locus of transposon insertion was identified and analyzed, enabling isolation of the corresponding *R* gene.

Comparison of the protein sequences encoded by the *R* genes described above allowed the definition of conserved features of the NLR proteins that are recognized today as hallmarks of this superfamily.[25] All but Pto carried leucine-rich repeats (LRRs). Cf-9 and Xa21 are cell surface receptors with extracellular LRRs either with (in rice Xa21 for *Xanthomonas* resistance) or without (Cf-9) an intracellular protein kinase domain. The others encoded modular proteins with a nucleotide binding (NB) domain and C-terminal LRRs which at their N termini carry either a Toll/Interleukin-1/Resistance protein (TIR) domain (for N, L6, RPP1, RPP5, RPS4, and RRS1) or a coiled-coil (CC) domain (for RPS2, RPM1, RPS5, and RPP13). The intracellular immune receptors resemble mammalian receptors such as NOD1, NOD2, and NLRC4,[29] and this class of protein was named NLRs (either for NOD-like receptors or nucleotide-binding leucine-rich repeat receptors). Similar modular structures are apparent in apoptotic proteins (APAF1 and CED4), in fungal somatic compatibility proteins[60] and in proteins implicated in bacterial resistance to bacteriophages.[61] The immune mechanisms initiated upon pathogen detection by intracellular receptors came to be known as "Effector-triggered Immunity" (ETI).[14]

These observations set the stage for our current understanding of plant immunity based on processes initiated upon detection of pathogen ligands by either cell surface receptors, intracellular receptors, or both.

SURFACE RECEPTORS AND PATTERN-TRIGGERED IMMUNITY (PTI)

Many pathogen-derived microbial- or pathogen-associated molecular patterns (MAMPS or PAMPS) activate pattern-triggered immunity (PTI) via cell surface Pattern Recognition Receptors (PRRs), as in animal innate immunity, often by recognition of the same P/MAMPs. Bacterial flagellin, Pep13 from *P. megasperma,* the fungal polymer chitin (primarily its partial hydrolysis products into oligomers of N-acetyl glucosamine), oligogalacturonides from plant cell walls (a damage-associated molecular pattern, or DAMP) and many other ligands trigger similar responses in cells that perceive them. These include induction of similar gene sets in response to each ligand,[62] ROS production via NADPH

oxidases and the activation of multiple protein kinases including at least two sets of mitogen-activated protein kinases, or MAPKs[63]. Despite determined biochemistry efforts in several labs, no PAMP/MAMP receptor was identified until the Boller lab used genetics to identify the Arabidopsis flagellin-sensing 2 (FLS2) gene.[64] FLS2 is the genetic locus encoding the capacity to respond to bacterial flagellin or its truncated surrogate, flg22. FLS2 encodes an LRR protein kinase receptor at the plant cell surface.[65] FLS2 function requires the BAK1 co-receptor, another LRR protein kinase originally defined as required for the function of BRI1, an LRR kinase that detects and enables responses to the plant hormone brassinolide.[66] The tomato Cf-9 receptor-like protein, upon recognition of the *C. fulvum* apoplastic protein Avr9, a tightly folded secreted cystine knot peptide of 28 amino acids with 3 disulfide bridges, also activates a PTI-like set of responses and requires BAK1 to function. Avr9 was the first fungal avirulence protein to be defined.[67] Cf-9 enables Avr9 perception in tobacco and *N. benthamiana*, facilitating detailed investigation by expression profiling to reveal induced genes. Notably, these analyses revealed ACIK1, the first of many members of the receptor-like cytoplasmic kinase (RLCK) family to be implicated in surface-receptor mediated immunity.[63,68,69] Arabidopsis BIK1 is the most studied RLCK and defining its targets continues to reveal new components of the plant defense machinery.[70] Activation of the NADPH oxidases that generate ROS requires the concerted action of RLCKs[71,72] and calcium-dependent protein kinases, the latter likely activated via Ca^{2+} influx after calcium channel activation. Amongst many other targets, RLCKs activate calcium channels which play crucial roles in plant defense.[70]

PTI signaling has amplification loops mediated by various phytocytokines, which are recognized by PRR-like proteins that detect plant-derived peptides that are induced or released upon PTI initiation.[73,74] Other LRR kinases, RLCKs, calcium-dependent protein kinases and protein phosphatases serve to attenuate or modulate PTI, enabling exquisite fine-tuning of its strength and duration.

Many additional extracellular receptors have been defined that recognize bacterial, fungal or other ligands.[32,63] Most express LRR ectodomains, though several other ectodomains are found in PRRs. An enduring paradox is that homologs of the LysM receptor kinases that activate defense in response to fungal chitin can also activate symbiotic developmental programs upon detection of N-acetyl glucosamine-containing oligomers or derivatives.[75]

GUARDS, GUARDEES AND DECOYS—HOW TO MAXIMIZE A LIMITED RECEPTOR REPERTOIRE

The isolation of *Pto* provided a puzzle-how does a protein kinase provide perception capacity? This was resolved in a landmark *Cell* paper that showed Pto function (and the function of the linked *Pto* paralog, *Fen*, conferring sensitivity to the insecticide fenthion) requires an intracellular NLR immune receptor, Prf.[76] Thus, in this instance, evolution had favored the duplication and divergence of a protein monitored by an NLR rather the evolution of an NLR to perceive independent ligands acting through Pto or Fen. This prompted the proposal of the "guard hypothesis" wherein some NLRs, rather than directly recognizing path-

ogen effectors, instead recognize the modification of a host protein by a pathogen effector.[27,77] Indirect receptor-mediated recognition of a modified-self molecule that has an important defense function, and thus may be targeted repeatedly by pathogen virulence effectors, can extend a limited germline-encoded immune receptor repertoire, reducing the need to evolve many independent recognition specificities solely via an NLR repertoire.

Many NLRs function by guarding host components.[27] Arabidopsis RPS2 and RPM1 both monitor the status of the RIN4 protein, which is targeted by seven unrelated bacterial type III effectors. RPS2 activates immunity upon cleavage of RIN4 by the AvrRpt2 protease and RPM1 activates immunity upon detecting RIN4's AvrB- or AvrRpm1-mediated post-translational modifications that ultimately perturb RIN4 phospho-switch status.[78,79] RPS5 monitors the status of the RLCK PBS1; when PBS1 is cleaved by the AvrPphB protease, RPS5 activates immunity.[80] ZAR1 monitors the status of several RLCK-like proteins via intermediate pseudokinases such as ZED1 or RKS1. ZAR1 is remarkable for being one of the few NLRs that shows homologs in distantly related plant species.[81,82] Importantly, some mammalian NLRs also likely respond to the action of pathogen effectors.[83] If a guarded component is mutated, the result can be autoimmunity due to NLR derepression.[84,85]

Not all genetically defined *R* genes encode NLR proteins. An intriguing and novel class of *R* genes encodes protein kinase fusion proteins,[86] including tandem protein kinases or protein kinases fused to other domains. In wheat and barley, several *R* genes encode tandem kinase genes comprising a functional kinase and a pseudokinase. Conceivably, these polymorphic kinases might be guarded by a relatively conserved NLR, analogous to Pto-Prf or RKS1-ZAR1 detailed above.

Receptors guarding host targets are also found on the cell surface. For example, the tomato cell surface receptor Cf-2 that, like Cf-9, confers *C. fulvum* resistance, requires a secreted cysteine protease, Rcr3, to confer disease resistance. Rcr3 binds to, and is inhibited by, the *C. fulvum* apoplastic effector Avr2 (which also inhibits other secreted host cysteine proteases). Thus, Cf-2 guards Rcr3 and activates defense when Avr2 inhibits it.[87,88] Rcr3 family cysteine proteases contribute to disease resistance,[89] providing an evolutionary rationale for their targeting by pathogen-encoded inhibitors.

Are all monitored proteins defense components? The decoy model[31] points out that hosts can evolve alleles or paralogs that no longer provide indispensable defense functions themselves but instead act as mimics of authentic pathogen targets.

STRUCTURAL BIOLOGY OF IMMUNE RECEPTORS— RESISTOSOMES REVEALED

The last five years have seen spectacular advances in understanding NLR protein mechanisms, thanks to improvements in structural biology enabled by cryoEM methods (Figure 4).[90] In most examples, the NB domain of NLR proteins binds ADP in the inactive state and upon recognition of a pathogen effector, a conformational change enables exchange of ADP for ATP. When bound to ATP, the NB domains can associate with each other, driving formation of oligomers that impose induced
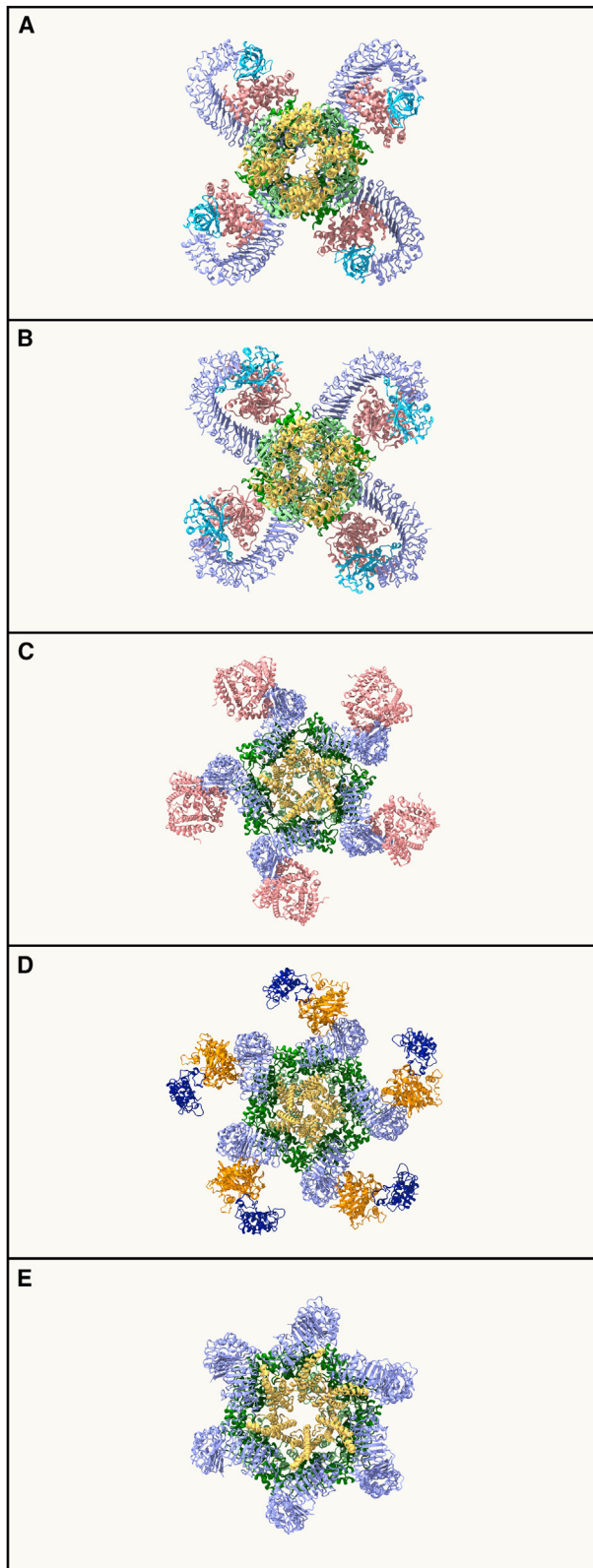
proximity on the N-terminal signaling domain and initiate signaling processes.

Arabidopsis RPP1 encodes a TIR-NLR that directly recognizes the *Hpa* effector *Arabidopsis thaliana* Recognized 1 (ATR1). *RPP1* exhibits extensive allelic diversity between different Arabidopsis accessions, and *ATR1* exhibits allelic diversity between *Hpa* races. Each *RPP1* allele confers recognition of a subset of pathogen races that carry a cognate *ATR1* allele. Recognition of specific ATR1 variants by RPP1 variants involves direct physical association. Upon binding ATR1, RPP1 oligomerizes to form a tetramer.[91] This conformational change brings together the N-terminal TIR domains of the proteins, thus activating an intrinsic NADase activity in the TIR domain that hydrolyses NAD+ into small signaling molecules that are detected by EDS1/PAD4 or EDS1/SAG101 heterodimers; see below. Roq1, an *N. benthamiana* TIR-NLR that recognizes the *Xanthomonas* effector XopQ, also forms a tetramer that activates its TIR domain NADase activity, suggesting a general model for activated TIR-NLRs.[92]

ZAR1 recognizes the activities of bacterial effector proteins that act on RLCKs required for PTI or decoys thereof. One such effector is AvrAC produced by *Xanthomonas campestris*, cause of black rot in members of the Brassica family. AvrAC uridylylates [adds a uridine monophosphate group (UMP) to] RLCKs including BIK1 and PBL2, thereby inactivating them and compromising PTI. ZAR1 constitutively forms a heterodimer with one of several pseudokinases – for example RKS1. Uridylylation of PBL2 by AvrAC forms PBL2^{UMP} that associates with RKS1 bound to ZAR1. Formation of ZAR1-RKS1-PBL2^{UMP} complexes drives a conformational change in ZAR1, leading to exchange of its ADP for ATP and oligomerization of the complex into a pentamer. The pentamer adopts a funnel shape that creates cation channels in the plasma membrane, enabling Ca^{2+} influx.[93,94] Signaling triggered by elevation of Ca^{2+} concentration induces expression of genes important for ETI and can result in cell death. Wheat Sr35, upon direct recognition of AvrSr35, also oligomerizes to a pentamer with an N-terminus that resembles that of oligomerized ZAR1.[95]

WHAT HAPPENS NEXT? SIGNALING COMPONENTS BETWEEN PERCEPTION AND DEFENSE

Genetic analysis revealed important components for defense signaling (Figure 5). Enhanced disease susceptibility 1 (EDS1) and non-specific disease resistance 1 (NDR1) genes are required respectively in Arabidopsis for full function of TNL and CNL *R* genes. Mutants were also identified that made susceptible plants even more susceptible.[96] These screens defined phytoalexin-deficient 4 (PAD4) and genes involved in biosynthesis and responsiveness to the defense hormone salicylic acid (SA).

Figure 4. Structures of activated forms of plant NLRs solved to date
(A) RPP1 bound to ATR1.
(B) ROQ1 bound to XopQ.
(C) Sr35 bound to AvrSr35.
(D) Zar1 bound to RKS1 and PBL2^{UMP}.
(E) the activated NRC4 homohexamer. The N-terminal domains, either CC (Zar1, Sr35, NRC4) or TIR (RPP1, ROQ1) are colored in yellow. NB-ARC NDB, HD1, and WHD, light green, green, and dark green, respectively; LRR, violet; C-JID (RPP1, ROQ1), light blue; and pathogen effectors ATR1 (RPP1), XopQ (ROQ1), and AvrSr35 (Sr35) are colored in salmon; Zar1 associated factors RKS1 and PBL2^{UMP} are in orange and dark blue, respectively. PDB IDs: 7CRC, 7JLX, 7JLV, 7JLU, 6J5T, 7XC2 (PDB ID for NRC4 not yet released).
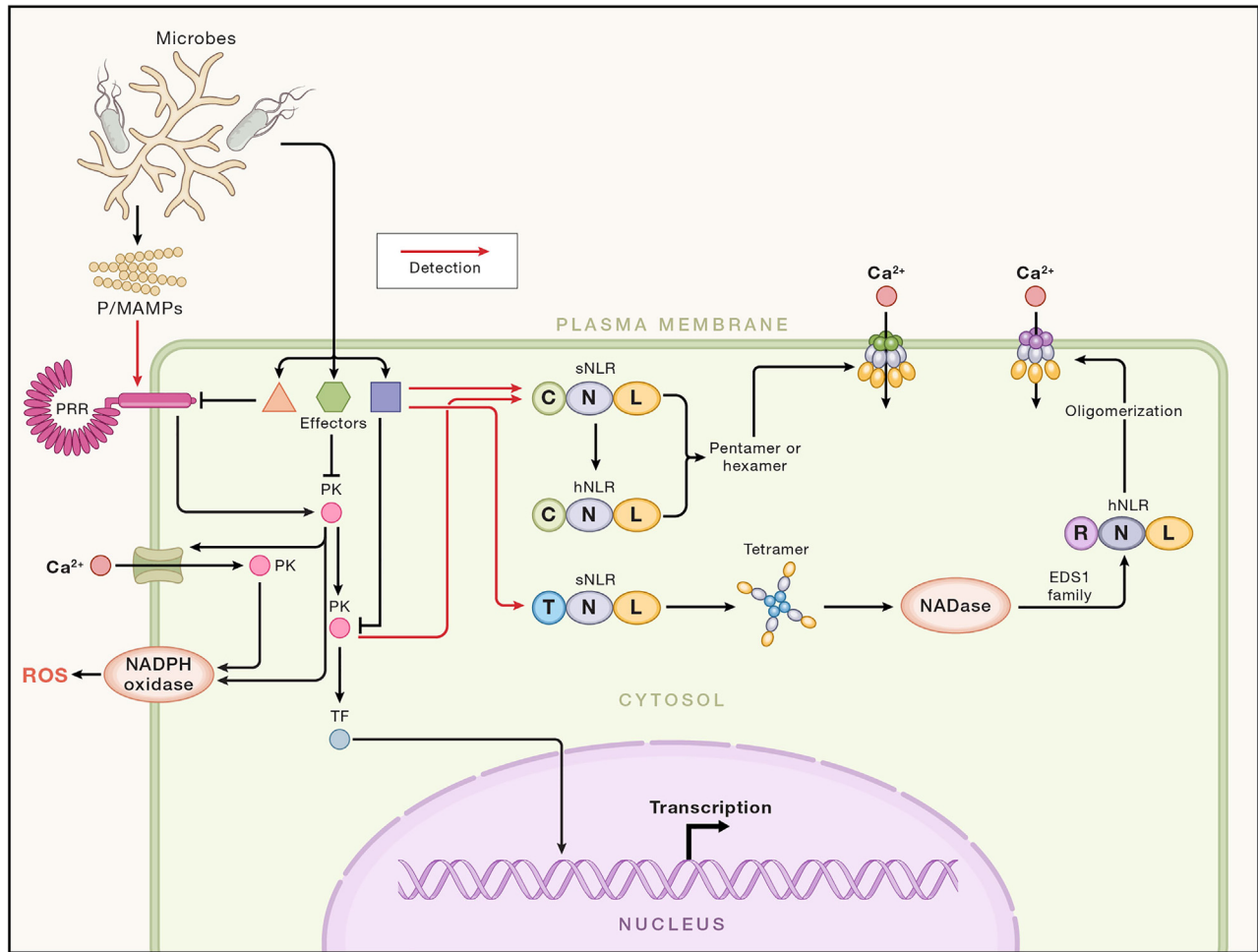
Figure 5. Simplified cartoon overview of the most important features of plant innate immunity via cell surface pattern recognition receptors (PRRs) and intracellular NLR receptors (CNLs or TNLs), and their helper NLRs (either CNLs or RNLs)
PRRs (usually a protein complex with at least one protein with a protein kinase domain) activate a series of intracellular protein kinases (PK), which can activate transcription factors (TF). Some of the PKs activate NADPH oxidases that produce ROS and also activate calcium channels that promote additional signaling. Pathogen effectors can interfere with function of PRRs or other PKs, with other host components (not shown) and also with TFs. These effectors can be detected (indicated by red arrows) either directly or indirectly by CNLs or TNLs, resulting in NLR oligomerization. TNL oligomerization to a tetramer creates an NADase enzyme activity that produces small molecules that activate EDS1 family proteins to engage with RNLs enabling RNL oligomerization (the structure of RNL oligomers, depicted here as a tetramer, is as yet unknown). RNL interactions with EDS1 family proteins can also activate transcription. CNL and RNL oligomerization lead directly to the generation of plasma membrane channels. Thus, NLR activation and oligomerization culminate by diverse paths in creation of calcium channels that promote immune activation. To avoid extra complexity in the cartoon, the mutual potentiation mechanisms between surface- and intracellular-receptor initiated signaling are not shown.

NPR1, required for SA perception, and isochorismate synthase (ICS1), required for SA biosynthesis, emerged from such a screen (see below). From protein interaction studies, EDS1 interacts with PAD4 or with a third protein of similar structure, SAG101. Comparative analysis of EDS1, PAD4 and SAG101 show that they share a lipase-like domain (though with no apparent lipase activity) and an additional shared "EP" domain.[97] Their functions are detailed below.

A different search for NLR signaling components involved a pioneering reverse genetics study using virus-induced gene silencing (VIGS). A tobacco cDNA library was constructed in a viral vector and then tested for clones that when silenced compromised function of the tobacco TNL N that confers resis-tance to tobacco mosaic virus (TMV). Remarkably, this revealed the N-requirement gene (NRG1) that encodes a CNL protein required for a TNL to function.[98] Its N-terminal CC domain shows pronounced homology to RPW8, an enigmatic protein identified as required for powdery mildew resistance.[99,100] NRG1 is now seen as a pioneer member of a class of "helper NLRs"—NLRs whose role is to support the function of "sensor NLRs" involved in pathogen detection. NRG1 is closely related to the ADR1 class of helper NLRs that also carries an N-terminal RPW8-like N ter-minus; these proteins are referred to as RNLs.[101–104]

In Arabidopsis and other dicots, EDS1 and PAD4 function with ADR1 class helper NLRs to activate transcription-dependent de-fense and EDS1 and SAG101 function with the NRG1 class of

helper NLRs to promote cell death during defense.[105] There are two NRG1 paralogs and three ADR1 paralogs in Arabidopsis Col-0, explaining why they were not identified in forward genetic screens. NRG1 and ADR1 functions are unequally redundant.[106] RNLs described to date are not activated by pathogen-encoded molecules. Rather, they are activated by the activation of upstream sensor NLRs. The NADase activity of TNL tetramers generates small nucleotide-derived molecules that act on heterodimers of EDS1 with PAD4 or SAG101 and stimulate their engagement with ADR1 or NRG1 respectively, prior to subsequent signaling events.[107–110] Current work focuses on the EDS1-PAD4-ADR1 and EDS1-SAG101-NRG1 heterotrimers and their structures. Conceivably, the rotation of the PAD4 or SAG101 EP domain outward from the body of the heterodimer upon ligand binding[108,109,111] creates a novel interface for interaction with ADR1 or NRG1, respectively. Important questions remain concerning subsequent events that ultimately lead to the formation of ADR1 or NRG1 oligomers, potentially at distinct subcellular locations with distinct functions.[112–114] A critical open question is how the specialization of the EDS1-PAD4-ADR1 and EDS1-SAG101-NRG1 signaling modules preferentially promote transcriptional output and cell death, respectively.[105,106,115]

Calcium influx into the cell has long been known to be a very early marker of defense and NLR activation.[116] Many Ca^{2+} channels were identified as candidates for this early calcium influx, but none were shown to be required.[117,118] It is now apparent that activated NLRs can function as Ca^{2+} channels. Both ZAR1 and the NRG1/ADR1 RNL proteins can function as autonomous Ca^{2+} channels in vitro or in vivo, respectively.[93,113] The Arabidopsis RNLs act downstream of, and are activated by, TIR-generated, NADase-dependent, nucleotide-derived small molecules that in turn activate EDS1-PAD4 or EDS-SAG101 heterodimers (see above). Thus, both direct activation of CNLs and indirect activation of RNLs by TNL-dependent small molecules result in Ca^{2+} channel activity. The combined data provide a mechanistic explanation for how TNL and CNL innate immune receptors initiate cell death triggered by pathogen recognition.[113]

In Solanaceae, another class of helper NLRs, called NRCs, support the function of almost half the sensor NLRs. NRCs are found throughout asterid plants.[119] Originally defined as required for function of Cf-9, multiple NRCs redundantly support the function of incompletely overlapping sets of upstream sensor NLRs. For example, some sensor NLR functions require NRC2, NRC3 and NRC4, while others require NRC2 and NRC3, or just NRC4. The N-termini of NRCs carry a conserved and functionally equivalent N-terminal motif—the MADA domain,[120] which is functionally exchangeable with that of ZAR1. NRC activation upon sensor NLR ligand detection provokes formation of an NRC oligomer from which the sensor NLR is excluded.[121,122] Recent data suggest the NRCs from a dimer in the pre-activation state[123] and the activated oligomer (surprisingly a hexamer, not a pentamer) forms a Ca^{2+} channel (Figure 4).[124]

IMMUNE RECEPTOR PAIRS AND "INTEGRATED DECOY" DOMAINS

Some NLRs work in pairs. The isolation of Arabidopsis *RPP2* revealed the first example of two NLR-encoding genes that are both required to confer perception capacity,[125] but it remains unclear how this pair acts. Around 5–10% of NLRs in many species are encoded in head-to-head genes and co-transcribed.[126] The Arabidopsis RPS4 and RRS1 TIR-NLRs comprise a well-characterized pair, respectively defined as required for recognition of *Pseudomonas* AvrRps4 and *Ralstonia* PopP2, an acetyltransferase.[127,128] PopP2-responding accessions carry the RRS1-R allele, and Col-0 carries a shorter RRS1-S allele. RRS1-R also confers resistance to fungi in the genus *Colletotrichum* through recognition of a yet-uncharacterized effector. RRS1 TIR-NLRs contain near their C-terminus an integrated WRKY transcription factor domain, and WRKY domain proteins are strongly implicated in plant defense. Remarkably, RPS4 is required for RRS1-R to recognize PopP2, and either RRS1-S- or RRS1-R are required for RPS4 to recognize AvrRps4.[129] The RPS4 and RRS1 genes are adjacent and divergently transcribed. RPS4 and RRS1 proteins constitutively associate and form an immune receptor complex in which the RRS1 WRKY domain senses pathogen effectors and defense signaling is transduced via RPS4.[130–132] The RRS1 TIR domain is enzymatically inactive. Sensors like RRS1 and its paralogs can be regarded as guarded by their respective executor partners, exemplified by RPS4 and its paralogs.

Many examples of this arrangement have been found, and the sensor detection domain has been termed an integrated decoy (ID) domain.[133,134] In rice, many adjacently encoded paired NLRs were defined, such as RGA4 and RGA5, or Pik-1 and Pik-2 that confer resistance to the rice blast pathogen *Magnaporthe oryzae*. For both RGA5 and Pik-2, the ID is a heavy-metal associated (HMA) protein domain that interacts directly with effectors from *M. oryzae*. Intriguingly, a recessive resistance gene, *pi21*[135] encodes an HMA domain protein, a class of protein targeted by many rice blast effectors. HMA ID-containing immune receptor pairs have been selected that detect the interaction of effectors with HMA domain proteins. In a remarkable advance, the HMA domain of Pik-2, which lies between the CC and the NB domain, was replaced with nanobodies that recognize GFP or RFP and condition resistance to GFP or RFP tagged viruses, bringing nearer the dream of being able to design immune receptors that can recognize any effector.[136]

IMMUNE RECEPTOR DIVERSITY

NLR proteins either guard plant molecules and respond when they have been modified by the action of pathogen effectors or directly recognize an effector protein. Immune receptors that directly interact with pathogen effectors are encoded by genes that show much greater genetic variability than immune receptors that guard host components.[126,137]

Crop monocultures are prone to disease epidemics. Even though any crop variety carries 100s–1000s of immune-receptor encoding genes, if every plant in a population is genetically identical, a pathogen isolate that can evade detection by the NLR repertoire of that variety can colonize the entire plant population. In contrast, genetically diverse natural plant populations are less prone to epidemics from co-evolved pathogens, though imported pathogens can cause disease on most hosts.[138] Analyses of pan-genomes verified that NLR-encoding gene repertoires

show extensive diversity compared to other genes.[126,137] Hamilton et al. proposed that the main selective advantage of retaining sexual reproduction and outcrossing is that polymorphism at loci contributing to parasite recognition restrict disease, and that "shuffling the deck" of pathogen recognition capacity every generation creates new combinations for a pathogen to surmount.[139] According to this frequency-dependent selection model, if a host population is extremely heterogeneous in its recognition capacity, then most isolates of the parasite will be unable to grow on most host individuals in the population. Recombination ensures that the immune receptor repertoire in each progeny individual is non-identical to the repertoire in either of its parents. Such polymorphism is more likely to be lost in self-fertilizing populations unless it is maintained by selection. Furthermore, if sexual recombination between parasites leads to exchange of dominant recognized effector genes, then most progeny of most parasites will not be able to find a host that cannot recognize them. The restriction of parasite success in plant varietal mixtures is consistent with this overall concept,[140] and the approach deserves further exploration as a strategy to provide more durable disease resistance.[141] This thinking also emphasizes the challenge of recruiting immune receptor repertoires that confer durable resistance in monocultures.

THE EXTRAORDINARY DIVERSITY OF PROKARYOTIC VIRULENCE FACTORS

A common function of bacterial type III secreted effectors (T3Es) is to disrupt plant immune responses initiated by either cell surface PRRs[142] or intracellular NLR receptors.[55] Many T3Es are enzymes, enzyme mimics or enzyme inhibitors, and often target common eukaryotic signaling modules. Mutation of any single effector usually fails to reduce virulence on a susceptible plant. Many T3E enzyme effectors are unable to lose the function that allows them to be recognized, resulting in frequent presence/absence polymorphisms[143] driven by variation in host immune receptors.

Recognition of T3E virulence functions is often indirect, as detailed above. Because indirect recognition drives selection for loss of the offending T3E, bacterial pathogens often evolve independent functions to target the same host protein.[144] For example, at least seven independently evolved effectors target the intrinsically disordered plasma membrane protein RIN4 to disrupt its function in vesicle trafficking.[78] These effectors modify RIN4 either by (i) proteolysis, which activates the RPS2 NLR or (ii) ADP-ribosylation, which alters a nearby host phosphorylation site and activates the RPM1 NLR or (iii) by acetylation of components of the RIN4-RPM1 protein complex. Other commonly targeted host defense proteins include the kinase domains of important PRRs, like the flagellin receptor FLS2 and its coreceptor BAK1, and RLCKs. Systems of indirect recognition are subject to recurrent change by selection against effector action, loss of NLR effectiveness, evolution of a new effector targeting the same host defense target, evolution and recruitment of a new NLR that recognizes the new target modification, and so on.

*Pseudomonas syringae* strains carry a pangenome of around 70 effector families.[145] These suites provide redundancy and some intra-family variation that guarantees evolutionary flexibility. Host range is typically determined by the parallel consequences of NLR-mediated recognition events. Thus, switching hosts might require near simultaneous loss of multiple effectors, an unlikely scenario. Only HopM, AvrE, HopB and HopAA are encoded in >95% of strains, suggesting their fundamental importance. In fact, three of these four effectors are physically encoded in a locus linked to the Type III secretion system itself. Similarly, recognition by hosts of single effectors, at least across *Pseudomonas syringae*, can have weak effects and many effectors converge onto single NLR genes, likely via shared targeting of a guarded host protein.[145] Thus, virulence is the sum of the functions of that set of the total delivered effectors that find a host target in the cells of a given plant species, and even weak recognition can be effective.[145] Effectors can be conceptually organized into 'guilds' that target related host cellular processes. There is evolutionary pressure to maintain a suite of effectors that covers each guild required to overcome the host defense response.[143] One critical function is to provide a high humidity apoplastic environment to support bacterial growth. HopM1 and AvrE have vital redundant functions for this phenotype. HopM1 helps establish an aqueous extracellular environment likely by manipulating host vesicle trafficking mediated by the MIN7 protein, a HopM1 target.[146] AvrE family members encode water or solute channels and small molecule inhibitors of AvrE block virulence in a HopM1 mutant.[147]

Phytopathogenic *Xanthomonas* (and also many *Ralstonia* strains) use type III secretion to deliver TAL (transcription activator–like) effectors that bind host DNA and activate specific host susceptibility genes.[148] For example, PthXo1, a TAL effector of the rice pathogen *Xanthomonas oryzae*, activates expression of a rice membrane-localized sugar transporter, the Os8N3 SWEET protein.[149] The susceptibility provided by TAL-dependent inappropriate expression of Os8N3 has selected for rice variants carrying recessive promoter mutations that prevent PthXo1 binding. Gene editing methods have been used to alter the promoter of Os8N3 and other SWEET genes that are activated by other TAL effectors, so that these genes are no longer induced upon infection and resistance is elevated.[150] The discovery of TAL effectors also enabled engineering of a new type of programmable DNA-binding protein. DNA binding by each TAL effector is specified by sequences in the variable repeats of 34 amino acids in each TAL protein. The greatest polymorphism occurs at positions 12 and 13 in each repeat, the so-called repeat-variable di-residue (RVD). The amino acid residues representing the RVDs of each TAL effector correspond to the nucleotides in the target site in the plant DNA.[151–153] TAL effectors can be designed to bind any DNA sequence and can be linked to nuclease or other domains.

Other prokaryotes cause important diseases. Virulence effectors from the mycoplasma-related *Phytoplasma*s revealed new mechanisms that promote pathogen success and attenuate defense signaling by interfering with host transcription factor abundance or function.[154–156]

EUKARYOTIC MICROBIAL PATHOGENS – GENOMICS MEETS STRUCTURAL BIOLOGY AND ALPHAFOLD

Filamentous eukaryotic microbes – including Ascomycete and Basidiomycete fungi and the phylogenetically distinct
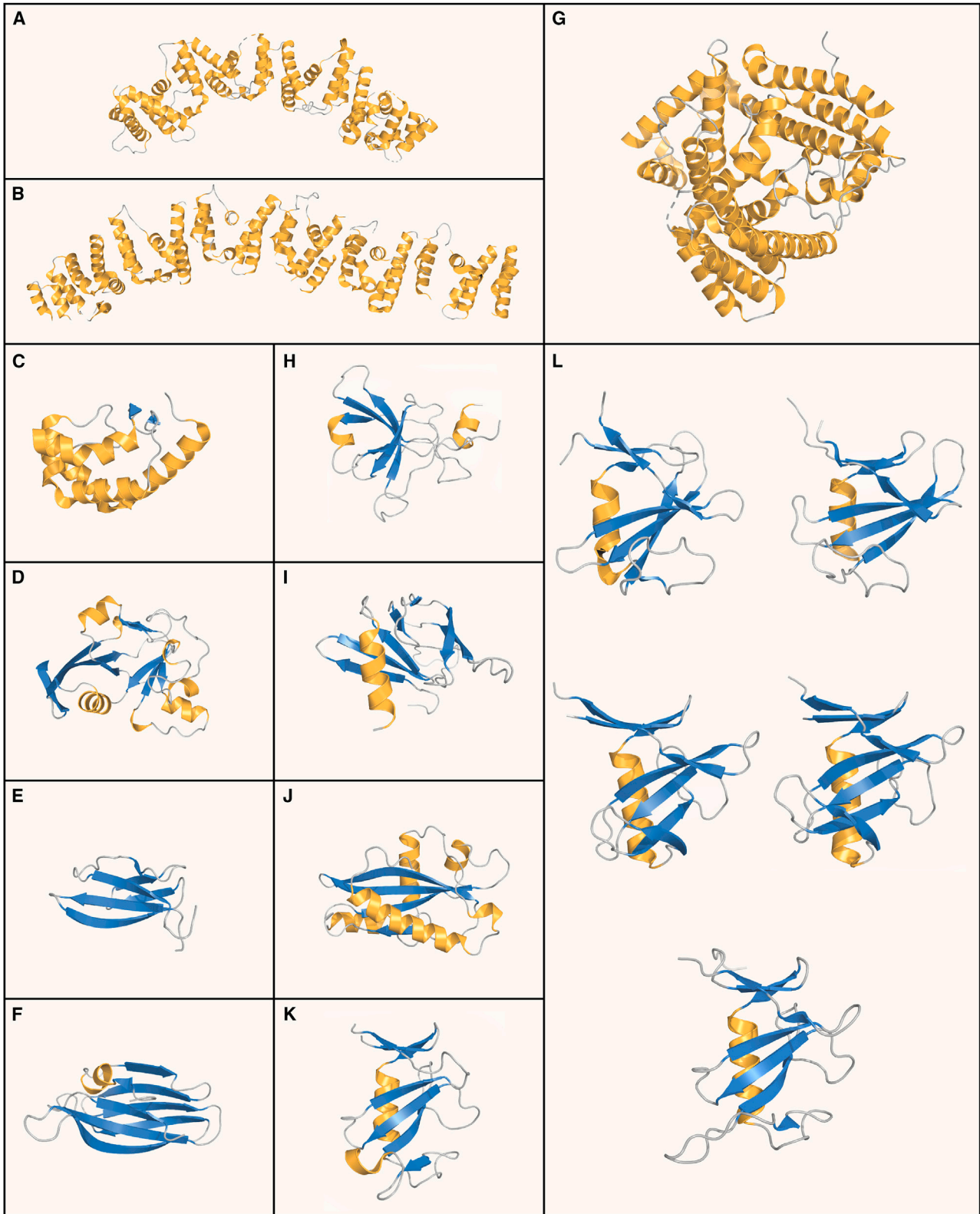
Stramenopile oomycetes[157] - cause some of the most devastating crop diseases. They share the properties of prolific spore production and dispersal by wind, germination on a leaf followed by colonization and rapid conversion of host nutrients into further rounds of sporulation and colonization. Many are obligate biotrophs that require a living host to thrive and reproduce and express hundreds of candidate virulence effector genes. Some are hemi-biotrophs, starting biotrophic and later switching to a necrotrophic lifestyle. Many biotrophs and hemi-biotrophs form haustoria, an invagination of the host plasma membrane that enables intimate contact for nutrient import and effector delivery between parasite and host.[158] Others are purely necrotrophic; these deploy toxins to kill plant cells and feed on the contents. Despite the importance and interest of toxin-dependent virulence mechanisms, some of which activate NLRs to promote host cell death,[159,160] space restrictions preclude a lengthy account of toxin-dependent virulence. Instead, we introduce a few important diseases and their causal agents and comment on eukaryotic effector evolution and delivery into host cells. For all these diseases, the acquisition of comprehensive pangenome sequences, gene expression and polymorphism data over the last 15 years has been pivotal to discovery.

Basidiomycete rust fungi colonize most plants (though not Arabidopsis). Flor's pioneering studies were conducted on flax rust and this system revealed a wealth of insights, enabling isolation of many alleles from four intracellular NLR receptor loci from flax and recognized effectors from the rust.[161] Soybean rust necessitates application of $2B of fungicides per year; vigorous efforts are underway to identify and deploy new genetic sources of resistance.[162] Wheat is afflicted by three rusts from the genus *Puccinia*; *P. graminis* (stem rust), *P. striiformis* (yellow or stripe rust) and *P. triticina* (leaf rust) and in many locations, wheat varieties are exposed to all three. Many *R* genes against wheat powdery mildew and rust strains have been identified; most encode NLR proteins and often exhibit extensive and interesting allelic series such as barley *Mla*, while others encode the tandem kinases introduced above, whose mechanism of immune signaling remains unknown.[86,163]

The most notorious oomycete pathogen is *Phytophthora infestans*, the causal agent of potato and tomato late blight. Many other *Phytophthora* species exist and cause severe diseases on other plants. Many *R* genes encoding NLRs were cloned against *P. infestans* and against its relative, the Arabidopsis downy mildew pathogen *Hpa* (see above). Other oomycetes cause significant losses in other crops, for example downy mildews of spinach, lettuce and grapevine.[164] Both *P. infestans* and *Hpa* effectors carry a signal peptide for export from the pathogen and either or both of an "RxLR motif" and a "DEER motif" in the secreted effector. Genomics revealed hundreds of such effectors in these genomes (Figure 6).[165] Host targets of some of these effectors were defined, providing novel insights into defense mechanisms, verifying important roles for protein kinase signaling, channel function, autophagy and gene silencing.[166] Many carry tandem repeats of WY domains that combine to interact with host proteins. In a remarkable recent report, a *Phytophthora* WY effector (Figure 6B) interacts with protein phosphatases in a manner that results in suppression of host gene silencing mechanisms.[167] Libraries of such effectors facilitate searches for those recognized by NLR proteins.[168] Effectors of the oomycete white rust pathogen *Albugo* share a "CCG" motif near the N-terminus of the secreted portion of the protein.[169]

Powdery mildews are Ascomycete haploid biotrophs that colonize just the leaf epidermal cells of both monocot and dicot plants. Wheat and barley coevolution with their respective host-specific powdery mildews led breeders to select many different semi-dominant *R* genes that often encode NLR immune receptors. In contrast, powdery mildews of the dicots tomato, cucurbits and Arabidopsis show less host specialization and few powdery mildew *R* genes have been identified in these taxonomic groups. For powdery mildews, the first recognized effectors revealed a class of secreted protein that often carries a CxY motif post signal peptide and likely evolved from an RNase protein.[170] Remarkably, sequence unrelated CxY mildew effectors recognized by different *Mla* NLR alleles all share a common fold (Figures 6K and 6L).[171] Similarly, multiple recognized effectors from the rice blast pathogen *Magnaporthe oryzae*, many of which interact with host HMA domain proteins, share the "MAX" effector fold (Figures 6E and 6F).[172]

These discoveries suggested that conserved effector motifs can be correlated with delivery into the plant cell, an idea originally supported by expression of rust fungi effectors inside plant cells[173] and from immunohistochemistry of a rust effector inside plant cells.[174] Filamentous fungi likely deliver intracellular effectors into plant cells via endocytosis.[175] To date, the best studied delivery system is that used by *Magnaporthe oryzae*,[176] where apoplastic effectors are secreted via conventional Golgi-dependent secretion. In contrast, effectors ultimately targeted to the host cytoplasm are first delivered to vesicles in a specialized structure on the surface of growing invasive fungal hyphae called the biotrophic-interfacial complex (BIC) via a Golgi-independent

Figure 6. The diversity of eukaryotic effector protein families
(A) Experimentally determined structure of the RxLR effector PcRxLR12 from *Phytophthora capsici* (PDB: 5ZC3).
(B) Experimentally determined structure of the WY domain effector PSR2 from *Phytophthora sojae* (PDB:5GNC).
(C) Experimentally determined structure of the beta-Cinnamomin Elicitin from *Phytophthora cinnamomi* (PDB:1LJP).
(D) Predicted structure of putative ADP-ribose transferases-like effector from *Magnaporthe oryzae* (MGG_16989).
(E) Experimentally determined structure of the MAX effector AVR1-CO39 from *Magnaporthe oryzae* (PDB:5ZNG).
(F) Experimentally determined structure of ToxA from *Pyrenophora tritici-repentis* (PDB:1ZLD).
(G) Experimentally determined structure of AvrSr35 from *Puccinia graminis* f. sp. *tritici* (PDB:7XC2).
(H) Predicted structure of putative hydrophobin-like effector from *Puccinia graminis* f. sp. *Tritici* (Pgt_Ug99_A1|8112).
(I) Experimentally determined structure of AvrSr50 QCMJC from *Puccinia graminis* f. sp. *tritici* (PDB:7MQQ).
(J) Predicted structure of putative Tin2-like effector from *Ustilago maydis* (UMAG_05930).
(K) Experimentally determined structure of RNAse-like effector BEC1054 from *Blumeria graminis f. sp. hordei* (PDB: 6FMB).
(L) Experimentally determined structure of *Blumeria graminis* RNAse-like effectors AvrA6 (PDB:8OXH), AvrA7 (PDB:8OXL), AvrA10 (PDB:8OXK), AvrA22 (PDB:8OXJ), and AvrPM2 (PDB:8PHY).

route.[177] The BIC forms after successful penetration of the first colonized plant cell. All cytoplasmic effectors analyzed to date localize to the BIC. Plant clathrin and actin co-localize with fungal effector-containing vesicles and reduction of their function impedes effector delivery. Gene silencing and inhibitor experiments targeted to plant clathrin and clathrin endocytic vesicle components compromise the generation of effector-containing vesicles and consequent rice blast symptoms, whereas silencing clathrin-independent endocytosis did not.[178] Similar results were demonstrated studying effector delivery from the host cell-invaginating haustoria of the oomycete pathogen *Phytophthora infestans*, suggesting that these processes might be conserved. Thus, different mechanisms deliver effectors to the apoplast or the host cytoplasm, and clathrin-mediated endocytosis emerges as a likely mechanism for the latter.[175] However, further diversity in delivery of fungal effectors to plant cells can be expected since the pathogens that cause smut diseases, like *Ustilago maydis*, appear to express a conserved set of genes that likely encode a translocon structure to deliver effectors into the host cell.[179]

Several recognized effectors from rust fungi have been defined.[180,181] However, no diagnostic sequence motifs have yet been defined to inspect the secretome of sequenced races. Additionally, the remarkable computational advances that enabled Alphafold can now be applied applied to investigating effector families based on structural prediction and this is opening new doors to understanding effector repertoires.[182] Finally, many invertebrates, such as nematodes and aphids, enter into protracted interactions with their host plants, and like microbes, they deliver effectors that influence host biology.[183] Further work will continue to reveal new insights into eukaryotic pathogen effectors and their mechanisms of defense inhibition.

INTEGRATING CONCEPTUAL FRAMEWORKS-MUTUAL POTENTIATION OF PTI AND ETI

PTI, the chain of events that ensues upon ligand detection by cell surface PRRs, is well documented.[63,184] However, no such analysis of ETI-specific events was available till recently because ETI assays were usually conducted in the presence of PTI. Effectors were typically delivered to plant cells using microbial delivery systems that inevitably also trigger PTI. Inducible expression of recognized effectors in the absence of PTI enables investigation of ETI alone. Upon NLR immune receptor activation by induction of a recognized effector without PTI, the abundance of proteins that contribute to PTI is elevated[185,186] and immune response genes and defense hormone biosynthesis genes are rapidly co-induced. In the absence of PTI, ETI activation results in less cell death than in the presence of PTI. This mutual potentiation of PTI and ETI is indispensable for robust defense activation. Further, the EDS1-PAD4-ADR1 node is a convergence point for defense signaling cascades activated by both surface-resident and intracellular LRR receptors[187] and ETI signaling boosts PTI outputs.[188] PTI is required for helper NLR NRG1 to oligomerize after TIR-NLR activation.[112] These findings are consistent with the fact that most NLR-encoding *R* genes are semi-dominant, which implies that the quantitative amount of defense activation is gated via the abundance of the immune receptor. A key conclusion of this work is that (1) it is PTI mechanisms that thwart pathogen success; (2) ETI restores and reboots PTI after its attenuation by pathogen effectors or during autoregulation; and (3) more immune receptors, conferred by *R* gene stacks, should enable stronger defense activation, in addition to rendering it more difficult for pathogens to evade detection via shedding multiple recognized effectors.

THE DEEP EVOLUTIONARY ORIGINS OF ANGIOSPERM IMMUNITY

Plants first colonized land ~500 million years ago and fossilized plant remains suggest the presence of haustorial structures consistent with fungal colonization (likely by symbiotic mycorrhizal fungi) of these early colonizers. Genome analysis of relatives of the earliest land plants such as mosses and liverworts revealed homologs of many angiosperm immune receptor components and functional studies show RLCKs and LysM domain PRRs contribute to pathogen resistance. Multiple NLR N-terminal architectures were also identified, including a widespread alpha/beta hydrolase N terminal domain that is not found in angiosperm NLRs. Broadly, surface receptor- and intracellular receptor-mediated immunity have both long contributed to pathogen resistance in land plants.[189–191] Immune receptor repertoire sizes vary enormously, with very few in aquatic plants, and the repertoire sizes of surface and intracellular receptors vary coordinately.[192] The motifs crucial for co-receptor interaction in LRR-RLPs are closely related to those of the LRR-receptor-like kinase (RLK) subgroup Xb, which perceives phytohormones and primarily governs growth and development. These data suggest that the cell-surface receptors involved in immunity and development share a common origin.[193]

WHAT IS THE BASIS FOR "NON-HOST" RESISTANCE?

Despite the vulnerability of monocultures to disease (see earlier), pathogen specialization means that monocultures of one species rarely succumb to pathogens that colonize a closely related species. Thus, wheat monocultures are resistant to races of powdery mildew or stripe rust that infect barley, and vice versa. What is the basis for this non-host resistance and does it involve the same mechanisms as those which underpin variation in resistance to adapted pathogens (PTI and ETI), or does it involve additional and distinct mechanisms? In a prescient review, Panstruga and Schulze-Lefert predicted that resistance via NLR-dependent processes was likely to explain why non-host species are resistant to pathogens from closely related species, but differences in surface receptors and other mechanisms may explain resistance to pathogens of more distantly related species.[194] Broadly, this prediction has been validated.[195,196] For example, there are no *Xanthomonas* pathogens of tobacco, but *Xanthomonas euvesicatoria* can grow on *N. benthamiana* if recognition of effector XopQ by the cognate Roq1 TNL NLR is lost via mutation in host or pathogen.[197] Races of *Albugo candida* that cause disease on Brassicas but cannot infect Arabidopsis are resisted by multiple TIR-NLR immune receptors in Arabidopsis that were dissected via transgressive segregation.[198] Resistance in barley to wheat stripe rust involves multiple loci, at least one of which encodes an NLR immune receptor.[199] Cell surface receptors can

also restrict host range; the EFR PRR from Arabidopsis that recognizes the bacterial EF-Tu protein can confer elevated *Ralstonia* resistance in the distantly related solanaceous species tomato that lacks this recognition capacity.[200]

STRATEGIES FOR DURABLE RESISTANCE

It is wisely said "never bet against the pathogen". Pathogen population numbers vastly exceed those of host plants, and recognition-dependent resistance against pathogens carrying hundreds of effector genes with redundant functions is likely to be easily evaded with enough mutation and recombination. And yet, most plants are resistant to most pathogens; disease susceptibility is the exception. What are the prospects for converting crop plants from hosts to non-hosts for their important diseases? Since many *R* genes are semidominant, and PTI and ETI mutually potentiate, simultaneous recognition of pathogen effector repertoires by multiple NLRs should enable quantitatively stronger resistance than a single NLR. Thus, stacks of functionally validated NLRs that recognize distinct effectors should confer a physiological strengthening of defense activation as well as a genetic benefit resulting from a requirement for multiple mutations in a pathogen to evade detection by multiple immune receptors.[74] The stacking of a five-transgene cassette which confers broad spectrum resistance to a fungal rust pathogen in wheat shows great promise[201] as does a triple stack of NLR genes for late blight resistance in potato.[202] A distinct approach is to plant mixed crop genotypes that mimic the population diversity of natural populations. However, plant varieties sold to farmers must fulfill requirements for distinctness, uniformity and stability. Conceivably, in a common genetic background, multiple distinct NLR genes could be provided and varieties carrying mixtures of such lines would slow the evolution of virulence that overcomes any such combination of distinct recognition capacities in a population.[141]

To safeguard durable disease resistance, it is important to develop technologies to define all of the effectors in a field population of a pathogen.[203] This information it is crucial for defining which *R* genes to incorporate into gene stacks. Furthermore, the combination of *R* gene stacks with mutations in disease susceptibility (S) genes may provide an additional layer of durability.[204] Such mutations in the host would lower pathogen populations and reduce the probability that spontaneous mutations can occur in pathogen effector genes that would overcome the cognate *R* genes. Conceivably, one could insert the R gene stack into a disease susceptibility gene. This might provide a single locus conferring multiple mechanisms of resistance that could be easily bred into different cultivars.

SYSTEMIC SIGNALING

Ross demonstrated that viral infections were restricted by prior infections.[205] This resistance was also broadly effective against additional pathogens. Ross coined the term "systemic acquired resistance" (SAR) for inducible systemic resistance[206] and "localized acquired resistance" for induced resistance in inoculated leaves adjacent to the inoculation site.[207] SAR correlates with accumulation of salicylic acid (SA) and is compromised if SA accumulation is prevented. SA treatment induces a set of

genes including those encoding secreted pathogenesis-related (PR) proteins, some of which are credibly implicated in restricting pathogen growth. In Arabidopsis, a screen for SA non-responsive mutants revealed the NPR1 gene and its several paralogs. In a series of elegant experiments, the Dong lab shed light on how NPR1 engages with SA and with TGA transcription factors to activate SA-responsive genes,[208] on how redox changes release NPR1 monomers that can enter the nucleus after disruption of a cytoplasmic NPR1 oligomer and how NPR1 in the cytoplasm can direct ubiquitination-dependent degradation of proteins that negatively regulate defense.[209] An additional defense signaling molecule, N-hydroxy pipecolic acid (NHP), often synthesized with a similar time course to SA, has been implicated in disease resistance. Its receptor is still unknown and the ways in which NHP and SA signaling intersect remains an active research topic.[210] With the right choice of promoter and translational regulation to minimize inappropriate expression, NPR1 can also be used to elevate crop disease resistance without incurring a yield penalty.[211]

THE GAME-CHANGING CONTRIBUTIONS OF CRISPR TO REVEALING AND DEPLOYING DISEASE RESISTANCE

The ease with which RNA programmable CRISPR nucleases can be constructed and delivered has accelerated plant gene editing. CRISPR enables researchers to make a precise gene knock-out to validate candidate genes implicated in disease resistance.[212] For instance, candidate genes for major QTLs can be validated by mutating them and scoring their phenotype. Mutations whose function was identified in one plant species can be made in orthologous genes in any plant species. The most notable example is the *mlo* gene in barley where orthologous gene knocks out in wheat and tomato result in powdery mildew resistant plants.[213,214] Importantly CRISPR gene editing allows one to make useful mutations in polyploid plants in the T0 generation. A similar example is the *dmr6* gene that was discovered in *Arabidopsis thaliana,* and enabled construction of orthologous mutations in tomato, banana, rice and citrus.[215] Eventually, one can envisage the insertion of cisgenic gene stacks into precise locations in the genome via homology directed repair (HDR). The implementation of this technology into elite cultivars already bred for enhanced yield will abolish linkage drag and reduce the time needed to introduce new traits.

OPEN QUESTIONS AND THE ROAD AHEAD

In this commentary we emphasized immune receptor recognition-dependent resistance; space did not permit extending our commentary to many other important topics such as necrotrophic diseases and toxins. Despite spectacular progress, many open questions remain. Others might make a different list, but we define here some of the questions that we hope to see solved in the next 20 years.

(1) How can we improve our ability to identify the *R* genes for every recognized effector gene and vice versa? A recent preprint suggests a path.[216] Alpha Fold multimer combined with genomics will undoubtedly be helpful.[217]

(2) How do the small molecule ligands of the EDS1 hetero-dimer complexes drive selective ADR1 and NRG1 recruitment and activation? Do different EDS1-containing signalosomes localize to, and function in, different subcellular compartments?

(3) We need to better understand the subcellular location of signaling components, particularly of the helper NLRs. How does ADR1 activation predominantly control transcriptional responses and NRG1 predominantly control HR, when the proteins appear so similar?

(4) How does creation of Ca^{2+} channels by ADR1 or NRG1 activate transcription and does this involve calcium-dependent transcription factors such as the CBP60 or CAMTA families[218,219]?

(5) What combination of ROS and DAMP and other signaling controls cell non-autonomous responses in cells neighboring the infected cell and are there cascades of NLR induction downstream of helper NLR activation in the infected cell.[220]

(6) Are there more helper NLRs? What helper NLRs are there in monocots? And what is the function of TIR domain proteins and EDS1 homologs in monocots?

(7) How do the functional few (of many) RPW8 paralogs contribute to powdery mildew resistance in Arabidopsis and what is the significance of RPW8's homology to the N terminal domains of the hNLRs NRG1 and ADR1?

(8) In many studies, the HR is used as a surrogate for defense activation, but we still have much to learn about how resistance mechanisms arrest pathogen proliferation. Experiments with the Arabidopsis RPP13 CNL showed that ATR13 effector protein delivery by either an oomycete, bacterial or viral pathogen results in pathogen inhibition. However, an HR is only observed with the oomycete and bacterial pathogen but not the viral pathogen.[221]

(9) Some virus resistance genes show "extreme resistance", where viral replication is inhibited without an HR,[222] while others permit more viral replication and an HR before viral replication ceases; why?

(10) Given the requirement for mutual potentiation of surface receptor and intracellular receptor-initiated immunity, how does virus resistance thwart the pathogen with ETI alone? Is the RNA silencing machinery recruited by viral ETI to shut down viral RNA accumulation?

(11) There is much to learn about cell biology of virulence and resistance and in particular about the role of the chloroplast and other organelles in resistance in the leaf.[223]

(12) How different are immune responses in the shoot and the root?

(13) How does the immune system reach détente with plant tissues that are in intimate contact with symbiotic or commensal microbes and how do the microbiome and abiotic stresses interface with immune system function[224]?

Progress in the MPMI field shows no sign of slowing down. The next few years will see more structural biology of immune receptors and AI-based prediction of protein/protein interactions, combined with genomics, to dramatically enrich our understanding of how plants resist disease and how pathogens circumvent host immune responses. We envisage the protein engineering of NLRs to expand recognition of novel effector proteins. Our safest prediction is for continued surprising and exciting new insights!

DECLARATION OF INTERESTS

The authors declare no competing interests.

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