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NLR diversity, helpers and integrated domains: making sense of the NLR IDentity

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Plant innate immunity relies on genetically predetermined repertoires of immune receptors to detect pathogens and trigger an effective immune response. A large proportion of these receptors are from the Nucletoide Binding Leucine Rich Repeat (NLR) gene family. As plants live longer than most pathogens, maintaining diversity of NLRs and deploying efficient 'pathogen traps' is necessary to withstand the evolutionary battle. In this review, we summarize the sources of diversity in NLR plant immune receptors giving an overview of genomic, regulatory as well as functional studies, including the latest concepts of NLR helpers and NLRs with integrated domains.

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Introduction

Henry Harold Flor first conceptualized genetics of plant–pathogen interactions in 1955 linking disease resistance (*R*-gene) loci to co-evolving pathogen virulence factors in a gene-for-gene model [1]. The first plant *R*-genes were cloned by 1994 [2,3••,4]. Soon a pattern was recognised: plant *R*-genes had a common central NB-ARC domain containing Nucleotide Binding site and ARC (present in Apaf-1, R proteins, and CED-4) subdomains followed by multiple Leucine Rich Repeats (LRR). Collectively, *R* genes and their mammalian analogs bearing such architecture were named NLRs. Subsequent genomic scans for NB-ARC-LRR domains in sequenced plant species revealed the diversity of NLR repertoires (Figure 1). Targeted sequencing of NLRs, first with PCR-based amplification [5] and now with targeted enrichment coupled to next generation sequencing [6], facilitated study of NLR evolution and diversity.

Pathogens have an armory of effectors—molecules secreted into the plant cell to subvert plant cellular function. However, effectors often betray the pathogen by alerting the plant to its presence. To overcome detection such effectors evolve rapidly by gene loss/gain and mutation. Functional analyses of plant NLRs revealed their diverse localization, activation and signaling. Pathogen recognition has been explained by multiple models, including 'direct recognition', 'indirect recognition', 'decoy/guardee' and 'integrated decoy' [7–9]. Direct detection even of a single effector is often enough to trigger immunity, however detection of individual effectors for each pathogen would be an insurmountable task. As effectors from diverse pathogens often target common plant proteins, detection of changes in such targets by NLRs is far more efficient. The most recent paradigm shift in NLR evolution and function has been identification of NLRs with exogenous domain fusions that resemble pathogen targets (integrated domains, NLR-IDs). Another recently described mechanism of plant immunity involves NLRs that safeguard and/or amplify responses of other NLRs (NLR pairs and NLR helpers). In this review, we will cover recent literature on NLR genetic and functional diversity, clarify our understanding of NLR-helpers and summarize current knowledge of NLR-IDs.

Main text of the review

Genomic and genetic diversity of NLRs

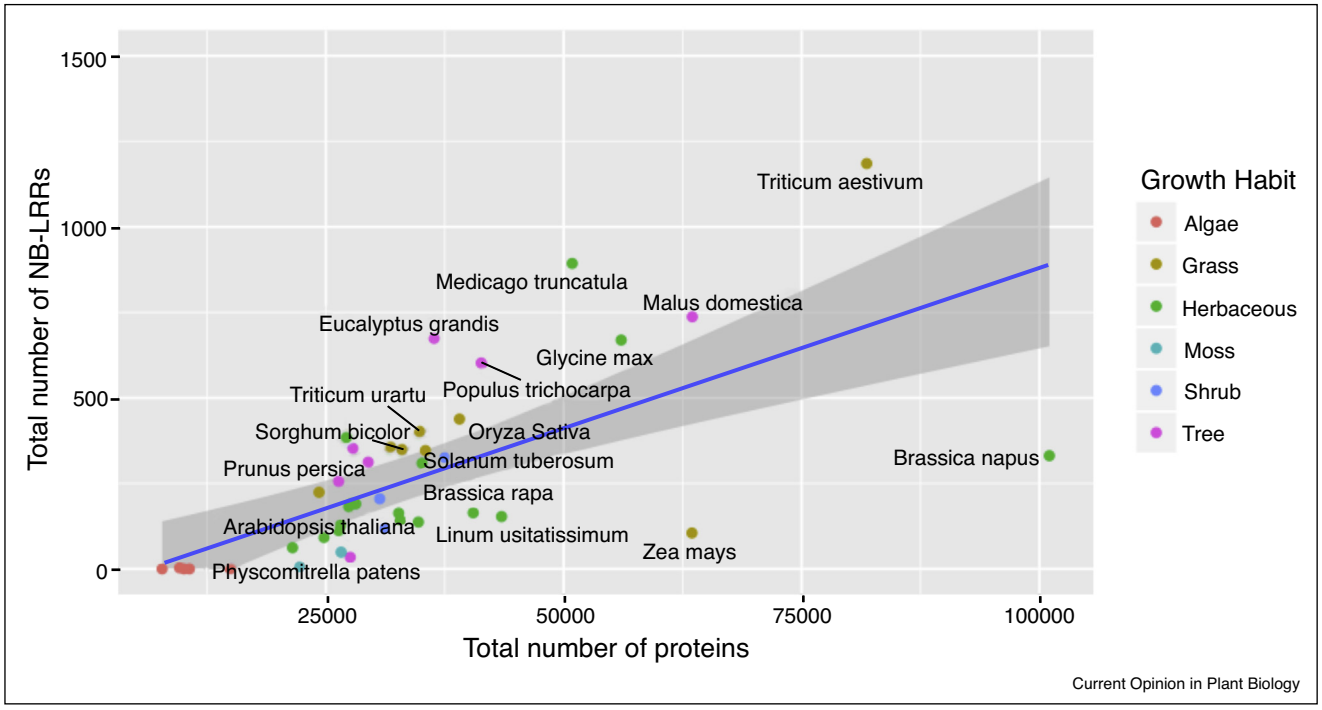
Evolution of plant immunity depends on generation of diversity at the DNA and RNA level followed by selection that primarily occurs on the expressed protein product. In the first section of this review we describe the diversity of processes that influence and safeguard the genomic vatiation of NLRs.

Attack of the clones—NLR copy number variation

Plant and animal immune related genes are associated with copy number variable (CNV) regions of the genome [10•,11–13]. NLR copy number varies by orders of magnitude across species [14•,15•] (Figure 1). The primary mechanism for increasing NLR copy number is tandem duplication, which creates complex gene clusters [16–18].

Several species have unusually high numbers of NLRs [10•,15•,17,18] (Figure 1). Elevated NLR frequency has been associated with woody plants whose longer lifespan

Figure 1



Plant genomes have variable numbers of NLRs with extreme expansions (*T. aestivum*) and contractions (*C. papaya*, *Z. mays*) normalized against the total number of proteins.

means they are behind in the evolutionary arms race due to greater exposure to pathogens and infrequent meiosis to generate novelty. Therefore more NLRs could provide broader pathogen recognition and more frequent recombination [17]. Compared to other long living species NLR copy number in apples appears elevated (Figure 1). Lineage-specific expansion of NLRs in apples has occurred rapidly, doubling since its divergence from pear 5.5–21.5 million years ago [10•]. Apple and pear trees share similar genetic and life-history traits of whole genome duplications, environmental niche, domestication and cultivation history, making NLR expansion in apples most perplexing.

Cucurbitaceae, conversely, have low NLR copy number with watermelon having only 55 NLRs; independent studies have confirmed this is not an artefact of poor annotation [10•,19]. Phylogenetic analysis identified that *Cucurbitaceae* retained NLRs from diverse NLR clades therefore their low number is likely a consequence of both scarce duplications and prevalent deletions [19]. Deletion events driving low NLR copy number are apparent in maize [20], papaya [15•] and kiwi [21]. Domestication bottlenecks offer a potential explanation for this low number of NLRs as wild relatives of domesticated species have often been shown to have elevated NLR diversity [11,19,22]. Species with low NLR copy number could provide fruitful models for functional NLR analyses due to easier generation and phenotyping of knockouts.

On the population level, CNV in NLRs has been linked to specific genomic regions [11,23•] which often contain multiple functional NLRs [22,23•,24•], therefore genomic architecture of a species may influence its NLR repertoire.

The Schrōdinger's NLR—presence/absence variation

NLRs tend to be over-represented in presence–absence variation (PAV) regions [25,26]. When a pathogen loses a virulence determining effector recognised by an NLR, the fitness cost of the NLR can become unfavorable. NLR presence is therefore likely to be determined by the frequency and severity of infection by a pathogen with the cognate effector and fluctuating frequency of effectors in pathogen populations [27]. Furthermore, for NLRs involved in indirect recognition, small changes to either NLR or its guardee can trigger auto-immunity. Such interaction would favor PAV which has been associated with lower accumulation of polymorphism [28]. For plant populations which do not encounter the effector or pathogen conditional neutrality can lead to NLR loss by drift. Resulting in PAV between accessions from different environments [25,29].

The double life of NLR allelic variation—point mutations, recombination and new domain acquisitions

Individual NLR genes have been associated with extreme allelic diversity (barley *Mla* [30], wheat *Sr33* [31], *Arabidopsis RESISTANCE TO PERONOSPORA PARASITICA 13 (RPP13)* [32] and lettuce *RESISTANCE GENE CANDIDATE 2 (RGC2)* [33]). NLR heterogeneity is a consequence of three processes: point mutations, recombination and domain fusions. Non-synonymous mutations are enriched in LRRs compared to the NB-ARC, illustrating divergent selection pressures of positive [34] and purifying selection [35] acting on the respective domains. Experimental approaches have validated that LRR polymorphisms can determine specificity to pathogen effectors [36–39], hence new pathogen recognition capabilities can be generated by LRR mutations that subsequently show signatures of positive selection.

Inter-allelic recombination plays a major role in NLR diversification. The mechanism was first proposed as the birth and death model [40]. Current evidence suggests illegitimate recombination, between LRR regions leads to shared repeats in paralogous NLRs [41]. These repeats facilitate interallelic recombination typically resulting in gene conversion [33]. Similar mechanisms might be driving *de novo* fusions of NLRs to other genes, which were recently shown to be prevalent across plant species [14•,15•]. Recombination has also been implicated in creating truncated NLRs [42••]. The pre-disposition of LRRs for forming mismatches during recombination which result in structural variability, might explain their convergent integration into immune receptors of metazoans and plants [43].

Gone with the splice—transcriptional and post-transcriptional control of NLRs

Transcriptional and post-transcriptional regulatory mechanisms act synergistically and flexibly to influence NLR variability. Mechanisms controlling NLR protein abundance include miRNA, phasiRNA, nonsense-mediated decay (NMD) as well as epigenetic regulation and alternative splicing (AS) [44,45••,46••,47].

Diversification of NLRs can have pleiotropic effects on plant fitness [46••,48••]. Several NLRs are implicated in reducing plant fitness in the absence of pathogens, due to NLR synthesis, regulation or aberrant defence activation [49,50]. In tandem with NLR expansion, transcriptional and post-transcriptional regulatory mechanisms facilitate NLR maintenance. miRNAs targeting of NLRs, through the NB-ARC p-loop, are conserved across plant species [51]. miRNA mediated degradation can be amplified by phasiRNAs; a dsRNA bi-product of miRNA directed cleavage, that perpetuates transcript degradation [52]. An inflated proportion of the NLRs targeted by miRNA originate from tandemly duplicated NLR families and large homogenous lineages [45••]. Preferential targeting of large NLR families could result from stronger selection for an miRNA with multiple NLR targets due to the associated larger reduction in energy expenditure on the NLR family [45••]. Alternatively, the genetic processes that result in high NLR tandem duplication may faciltate inverted NLR duplication which could *de novo* establish miRNAs [45••].

Post-transcriptional variation in NLRs can be enhanced through alternative splicing [53–56]. Epigenetic marks have been identified as a mechanism for regulation of the abundance of alternative NLR transcripts [57]. NLR AS transcript abundance is further modulated by nonsense mediated decay, whereby AS transcripts with premature stop codons are selectively degraded by dedicated protein complexes. NMD activity is plastic and can be repressed in response to pathogen infection promoting retention of non-canonical NLR proteins [44].

Diversity of NLR functions: sensors of non-self and modified self

In the second part of the review we examine the diversity of NLR functions and advocate that all NLRs are sensors, be it sensors of effectors, effector targets, other NLRs or signaling pathways. Importantly, NLR functions have a feedback on genomic diversity, genetic linkage and evolution.

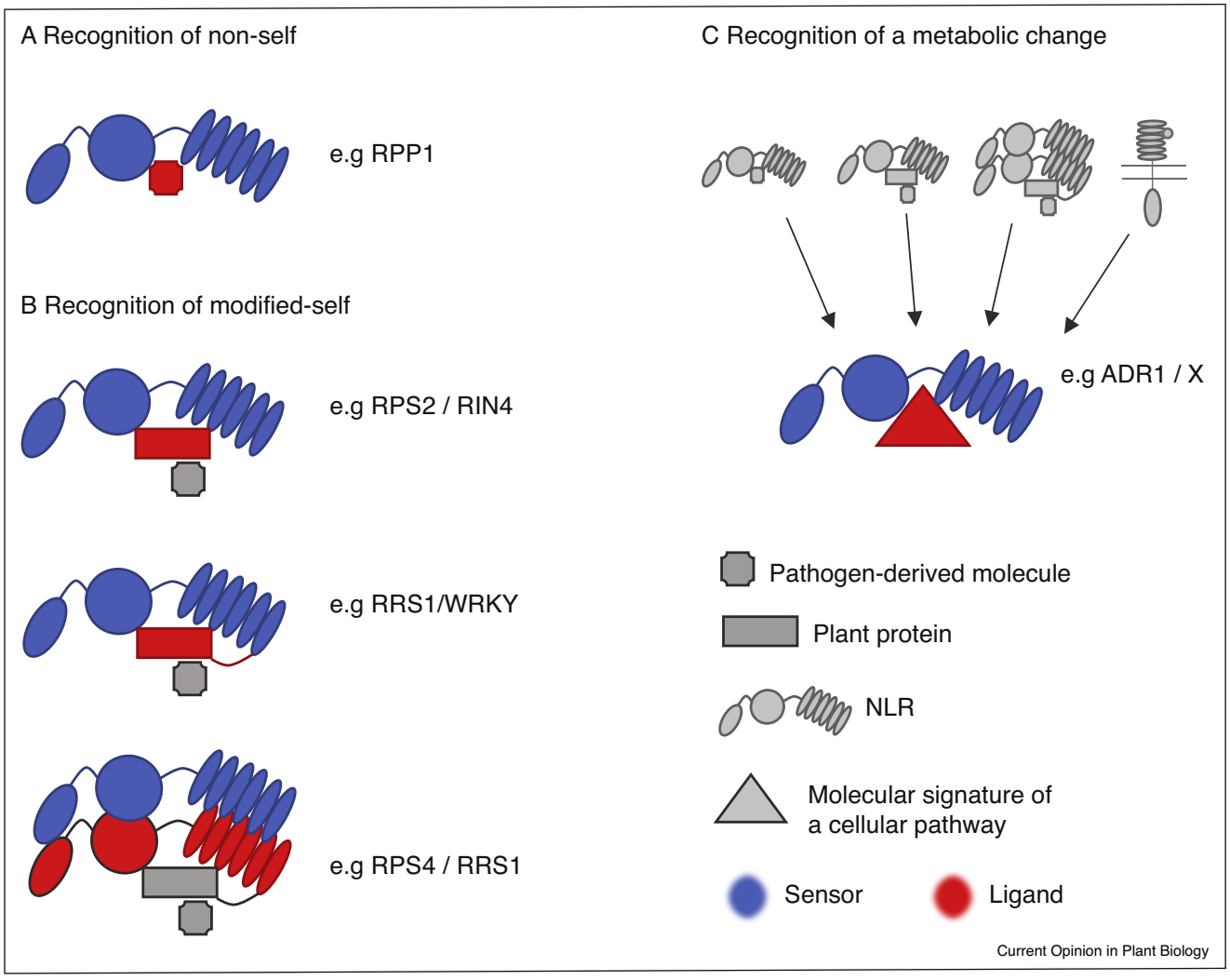
Mission impossible—NLRs directly confronting pathogen attack

Supporting Flor's original observations, receptor–ligand recognition of non-self was confirmed for several flax NLRs [36,37] as well as NLRs from other plant species [39,58,59••,60–63]. In the case of flax L/M proteins [36–38] along with *Arabidopsis* RESISTANCE TO PERONOSPORA PARASITICA 1 (RPP1) [39] recognition has been mapped predominantly to the highly variable LRR region, conversely tobacco N protein Toll/interleukin-1 receptor (TIR) domain was identified as the determinant of effector specificity [64] and a recent study implicated a TIR only protein in effector binding [42••]. The NLR involved in direct recognition of effectors are often found in an auto-inhibited state, with coordination of domains within the protein being crucial for both effector sensing and induction of signaling [65•] (Figure 2a). Reports of receptor–ligand interaction between NLRs and effectors remain sparse, and perhaps this is not surprising. If plants were to rely solely on direct recognition they would be at an evolutionary disadvantage: their germline encoded NLRs unable to diversify as fast as effectors due to pathogens' shorter lifespans. So, how do plants efficiently utilize NLR diversity to keep pace with their pathogens?

How to catch a pathogen—NLR sensors of effector targets

Recognition of modified self (also commonly referred to as indirect recognition) is a more efficient system to

Figure 2



NLRs are versatile sensors of non-self and modified self.

monitor disease-associated changes within plant cells. First, the total number of plant proteins is much smaller than that of all effectors from all pathogens. Second, diverse pathogens commonly target the same plant proteins to establish disease [66,67]. Therefore, the majority of NLRs are sensors of other plant proteins (Figure 2b). Activation of modified self recognition NLRs is regulated though an interacting partner, as exemplified by RESISTANCE TO PSEUDOMONAS SYRINGAE 2 (RPS2)/RPM1-INTERACTING PROTEIN 4 (RIN4) [68] and RESISTANCE TO PSEUDOMONAS MACULICOLA 1 (RPM1)/RIN4 [69] interactions. If the host proteins are actively contributing to immunity or other cellular processes, they are termed 'guardees', whilst if they have lost their original function serving only as effector baits, they are called 'decoys' [9]. Systems that recognize modified self have their own disadvantages including autoimmunity upon recombination combining incompatible alleles or separating an NLR from its interacting partner [70].

NLRs with additional non-canonical domains were first detected in genome-wide screens almost 15 years ago [71]. Recently, functional analyses of *Arabidopsis TIR-NBS-LRR-WRKY* gene *RESISTANCE TO RALSTONIA SOLANACEARUM 1* (*RRS1*) and two genes from rice, *CC-NBS-LRR-HMA RESISTANCE GENE ANALOG 5 (RGA5)* and *CC-HMA-NBS-LRR PYRICULARIA ORYZAE RESISTANCE K* (*Pik-1)* together with their respective paired NLRs *RESISTANCE TO PSEUDOMONAS SYRINGAE 4* (*RPS4*), *RGA4* and *Pik-2* demonstrated such exogenous domains are integral for the NLRs function [59••,72••,73••,74••]. In all three cases, the additional domain (WRKY or heavy-metal-associated binding

(HMA, also known as related to ATX1, RATX1 domain)) directly interacts with the pathogen-derived effector, inducing conformational changes in the NLR portion of the protein (Figure 2c). Conformational change is then sensed by the second genetically and physically coupled NLR leading to signaling active NLR complex [59••,72••,73••,74••]. The cascade of events is similar to the indirect recognition NLRs described above, with the exception that now the effector 'bait' and its NLR sensor are fused together. Indeed, NLRs with other integrated domains, such as RIN4, exocyst complex component 70 (exo70) and protein kinases highlight the commonality of NLR fusions and effector targets [14•,15•].

Fusion of the NLR and its 'bait' has the advantage of genetically and physically linking two interacting proteins. There is growing evidence that NLRs from particular clades form evolutionary and genomic hotspots for creating new fusions [75]. Genetic linkage prevents separation of the interactors by recombination (and potentially subsequent auto-immunity) and facilitates co-expression. Protein linkage ensures appropriate stoichiometry and creates cross-linked proteins, lowering the binding affinity threshold.

The two of us—paired NLRs

Some NLRs are self-sufficient for sensing of the pathogen and initiating signaling cascades [36,37,39,68,69]. In other cases, a pair of NLRs genetically linked together is required [76•,77–79]. There is now evidence that paired NLR proteins form heterogeneous protein complexes even during the initial signal activation steps [80]. These protein interactions are essential for suppressing NLR auto-activation (signaling in the absence of effector), initiation of pathogen perception and downstream signaling. While the first member of the pair is commonly involved in binding the 'trigger' (pathogen effector, guardee, or an integrated domain), the second member is responsible for 'sensing' the changes in the first NLR and releasing the switch to initiate signaling (Figure 2c) [72••,73••,74••]. Such separation of functions can have an evolutionary advantage, for example, increasing tolerance to point mutations in the sensor or facilitating new gene fusions. A characteristic feature of NLR pairs is that both members are neighbors in inverse orientation and share a common promoter suggesting their co-regulation.

The help—NLR helpers and what are they sensing?

Helper NLR is a relatively new term, suggested in 2011 to describe a genetic dependency of a pathogen receptor (including but not limited to NLRs) on a separate NLR that is involved in safeguarding downstream signaling pathway [81]. *ACTIVATED DISEASE RESISTANCE 1* (*ADR1*), *N REQUIREMENT GENE (NRG1)* and *NB-LRR REQUIRED FOR HYPERSENSITIVE RESPONSE-ASSOCIATED CELL DEATH 1* (*NRC1*) are the three characterized NLRs of the helper class [82••,83]. Both ADR1 and NRG1 have N-terminal regions similar to resistance to powdery mildew 8 (RPW8) domain which contains coiled coil (CC) motifs and as a subfamily are called CC_R-NLRs.

The CC_R-NLRs are conserved across all plant species suggesting they have an essential role in immunity. CC_R-NLRs form an ancestral clade relative to other NLRs [84]. A separate CC-NLR NRC helper clade to which *NRC1* helper belongs underwent a lineage specific expansion in *Solanaceae*. The expansion has been speculated to signify a divergence from function as a simple NLR helper to an NLR helper network [85••]. The biochemical determinants that trigger NLR helpers activation remain unknown and could include sensing changes in other NLRs directly (Figure 2c) or through a common signaling cascade (Figure 2d). NLRs that regulate themselves at the initial stages of pathogen perception and activation still depend on ADR1, NRG1 and NRC1 for the final signal transduction [82••,83]. Unlike the NLR pairs, 'helpers' have as yet not been found to physically interact with other NLRs. The NLR protein SUPPRESSOR OF MKK1 MKK2 2 (SUMM2) senses the disruption of an immune signaling mitogen-activated protein (MAP) kinase cascade, showing the role of NLRs in sensing activation of other immune pathways [86••]. NLR helpers can therefore act as 'hubs' to control signaling, guarding the whole immune signaling pathway rather than a specific molecule affected by an effector.

N before LR: functions of incomplete NLRs

NLRs lacking one or more of the canonical domains, commonly termed truncated NLRs, include TIR-X (TX) and TIR-NBS-X (TNX), CC-NBS-X, RPW8-X and RPW8-NBS-X, and are found in all plants [71,87]. Most of these genes show evidence of expression [87–89] and conservation across conifers, monocots and dicots, suggesting functional relevance. TX and TNX proteins can directly interact with pathogen-derived effectors [42••,88], guard plant proteins [90••] or act together with other NLRs similar to NLR-NLR pairs or helpers [91]. *Arabidopsis* TIR-only protein RESPONSE TO HOPBA1 (RBA1) directly binds bacterial effector HopBA1 [42••]. Another TNX protein guards exo70 protein, a possible effector target [90••]. The TNX gene *CHILLING SENSITIVE 1 (CHS1)* requires its paired TNL *SUPPRESSOR OF CHS1-2 3 (SOC3)* to signal temperature dependent auto-immunity [91]. Whilst, *Arabidopsis* TNX gene *RESISTANCE TO LEPTOSPHAERIA MACULANS 3* (*RLM3)* acts downstream of genetically unlinked *RLM1* gene to provide broad spectrum resistance to four necrotrophic pathogens [92], analogous to the function of NLR helpers. Global overexpression screens of *Arabidopsis* TX and TNX proteins revealed several genes capable of inducing an autoimmune phenotype and increased resistance to both bacterial and fungal pathogens [88]. Therefore, NLRs

lacking a domain, such as TX and TNX are functionally similar to canonical NLRs in recognition of effectors and amplification of immune response. The term 'adaptor' proposed to describe functions of incomplete NLRs and the term 'helper' that describes NLRs such as NRG1 and ADR1 are largely equivalent.

Conclusions

Plant NLRs show diversity both within a genome of a single organism and at the population level. It remains puzzling why there is such vast variation in the number of NLRs across flowering plants, and whether this is simply a consequence of their overall genome evolution or a result of yet unseen selection pressures. Advances in sequencing technologies are facilitating a transition from a gene centric analysis of SNPs to studies across whole NLR gene family between different plant populations as exemplified by recent analyses of NLRs in wild tomato populations [93••].

Functionally, all NLRs are sensors. They are equipped with an NB-ARC domain which is part of the well-recognized 'promiscuous' family of AAA ATPases, found in combination with second greatest diversity of other domains than any other protein module [94]. The property of NB-ARC to be controlled by a 'trigger-and-switch' mechanism make NLRs the most adaptable sensors activating signaling upon identifying a spectrum of modifications to themselves, other plant proteins or an exogenous molecule derived from a different species. With this view, we propose that NLR helpers are direct or indirect sensors and amplifiers of other NLRs or pathways induced by them. NLRs sensing a pathogen-derived molecule, a host target of it, another NLR or a conserved host pathway could lead to formation of different biochemical complexes which may be controlled by distinct activation processes.

A common theme of genetic linkage is evident upon investigation of NLR signaling pathways, exemplified by NLR-ID fusions and the genomic co-localization of pairs. One potential explanation for this trend is that without genetic linkage recombination is likely to produce incompatible combinations of alleles which can have high fitness costs if they result in auto-immunity. Furthermore, physical cross-linking of NLRs with the effector target on the protein level is advantageous as they are transcribed and translated together, therefore guaranteeing their presence in the same protein complex, same cellular location and defined stoichiometry. Cross-linked protein activation can be induced upon weaker interactions, facilitating activation of the complex by subtle signals. On the mechanistic level, fusions between NLRs and other plant proteins provide a rapid way to acquire new pathogen recognitions by sampling the plant genome.

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