NLR network mediates immunity to diverse plant pathogens

Chih-Hang Wu, Ahmed Abd-El-Haliem, Tolga O. Bozkurt, Khaoela Belhaj, Ryohue Terauchi, Jack H. Vossen, and Sophien Kamoun

*The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, United Kingdom; †Plant Breeding, Wageningen University and Research, Wageningen 6708 PB, The Netherlands; Department of Life Sciences, Imperial College London, London SW7 2AZ, United Kingdom; Division of Genomics and Breeding, Iwate Biotechnology Research Center, Iwate 024-0003, Japan; and Laboratory of Crop Evolution, Graduate School of Agriculture, Kyoto University, Kyoto 606-8501, Japan

Edited by Jeff L. Dangl, University of North Carolina at Chapel Hill, Chapel Hill, NC, and approved June 19, 2017 (received for review February 13, 2017)

Both plants and animals rely on nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins to respond to invading pathogens and activate immune responses. An emerging concept of NLR function is that “sensor” NLRs are paired with “helper” NLRs to mediate immune signaling. However, our fundamental knowledge of sensor/helper NLRs in plants remains limited. In this study, we discovered a complex NLR immune network in which helper NLRs in the NRC (NLR required for cell death) family are functionally redundant but display distinct specificities toward different sensor NLRs that confer immunity to oomycetes, bacteria, viruses, nematodes, and insects. The helper NLR NRC4 is required for the function of several sensor NLRs, including Rpi-bln2, Mi-1.2, and R1, whereas NRC2 and NRC3 are required for the function of the sensor NLR Prf. Interestingly, NRC2, NRC3, and NRC4 redundantly contribute to the immunity mediated by other sensor NLRs, including Rx, Bs2, R8, and Sw5. NRC family and NRC-dependent NLRs are phylogenetically related and cluster into a well-supported superclade. Using extensive phylogenetic analysis, we discovered that the NRC superclade probably emerged over 100 Mya from an NLR pair that diversified to constitute up to one-half of the NLRs of asterid. These findings reveal a complex genetic network of NLRs and point to a link between evolutionary history and the mechanism of immune signaling. We propose that this NLR network increases the robustness of immune signaling to counteract rapidly evolving plant pathogens.

P

Plants and animals rely on nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins to activate immune responses to invading pathogens (1–3). NLRs are among the most diverse and rapidly evolving protein families in plants (4, 5). They are modular proteins that broadly fall into two classes based on their N-terminal domain, which is either a Toll-interleukin 1 receptor or a coiled coil (CC) domain (6). Most plant disease resistance genes encode NLR receptors that detect effector proteins secreted by pathogens or indirectly binding them via effector-targeted host proteins (3, 7). An emerging model is that “sensor” NLRs dedicated to detecting pathogen effectors require “helper” NLRs to initiate immune signaling, resulting in a hypersensitive cell death response that restricts pathogen invasion (8–11). Although paired NLRs have been described across flowering plants, the degree to which plant NLRs have evolved to form higher order networks is poorly known.

Solanaceae form one of the most species-rich plant families that include major agricultural crops, such as potato, tomato, and pepper (12). The extensive breeding efforts for improving disease resistance within this family have led to the identification of many NLR-type disease resistance genes from wild relatives (13–14). To date, over 20 NLR-type disease resistance genes have been identified from different solanaceous species, which confer resistance to infection by diverse and destructive pathogens and pests, including the oomycete Phytophthora infestans, tomato spotted wilt virus (TSWV), and potato cyst and root-knot nematodes (13, 14). Several of these solanaceous NLR-type disease resistance genes have been deployed in agriculture through traditional breeding, cogenesis, or transgenesis (14, 15). For example, Rp6-blb2 has been introgressed into potato cultivars to confer broad-spectrum resistance to isolates of P. infestans (16), Mi-1.2, an ortholog of Rpi-bln2, confers resistance to root-knot nematodes, aphids, and whiteflies in cultivars of tomato (17–19). Expression of the pepper gene Bs2 in tomato confers resistance to the bacterial spot pathogen Xanthomonas campestris pv. vesicatoria (20). Sw5b, a gene from the wild tomato species Solanum pennanti, mediates resistance against TSWV in tomato (21). Furthermore, introgression of Rx and Gpa2 into potato confers resistance to potato virus X (PVX) and potato cyst nematode, respectively (22, 23).

In addition to their agricultural importance, the Solanaceae and their NLRs are a great experimental model system for understanding plant immunity. Many of the cloned solanaceous NLR genes recapitulate their effector recognition and disease resistance phenotypes when expressed into the model plant Nicotiana benthamiana. Classic examples of mechanistic studies of solanaceous NLRs in N. benthamiana include the Prf/Pto complex, which mediates resistance to Pseudomonas syringae through association with the effectors AvrPto and AvrPtoB (24–26), and the Rx/RanGAP2 complex, which confers resistance to PVX by recognizing the coat protein (23, 27–29). These studies contributed to our understanding of NLR function, particularly the role of effector-associated proteins in activating immunity.

Genome-wide annotation and cross-species comparison revealed that NLR genes are often dramatically expanded in the genomes of flowering plants, reaching hundreds of genes in diverse species like rice, soybean, grapevine, and potato (30). Across different plant species, NLR genes belonging to different phylogenetic clades may show distinct expansion and gene loss


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

*To whom correspondence should be addressed. Email: sophien.kamoun@ucl.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1702041114/-/DCSupplemental.

Significance

Plant and animal nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins often function in pairs to mediate innate immunity to pathogens. However, the degree to which NLR proteins form signaling networks across genetically linked pairs is poorly understood. In this study, we discovered that a large NLR immune signaling network with a complex genetic architecture confers immunity to oomycetes, bacteria, viruses, nematodes, and insects. The network emerged over 100 Mya from a linked NLR pair that diversified into up to one-half of the NLRs of asterid plants. We propose that this NLR network increases robustness of immune signaling to counteract rapidly evolving plant pathogens.
patterns, indicating that NLR evolution exhibits dynamic patterns of birth and death (4, 6, 30–32). Strong selection caused by pathogens is thought to drive functional diversification of NLR genes, which tend to be clustered in dynamic regions of plant genomes (32–34). However, despite the extensive knowledge generated through comparative genomics, the degree to which phylogeny correlates with mechanisms of NLR activation and signaling remains unclear.

In a previous study, we reported that the helper NLR proteins NLR required for cell death 2 (NRC2) and NRC3 are functionally redundant and are required for the function of the Prf/Pto complex in N. benthamiana (11). However, whether NRC2, NRC3, and other NRC-like genes function with other sensor NLRs remains unknown. Here, we describe another helper NLR, termed NRC4, which is unique to the NRC family. NRC4 is required for immunity triggered by Rpi-blb2, an NLR that provides resistance to P. infestans but is not required for Prf-mediated immunity. Surprisingly, NRC2, NRC3, and NRC4 turned out to be functionally redundant and essential for the activity of at least seven other NLRs that confer immunity to oomycetes, bacteria, viruses, nematodes, and insects. Remarkably, the NRC family and NRC-dependent NLRs fall into a well-supported phylogenetic superclade. Using extensive phylogenetic analyses of plant NLR sequences, we revealed that the NRC superclade probably evolved from a common ancestral NLR over 100 Mya. We conclude that NRCs and their mates form a complex genetic network that confers resistance to diverse pathogens and pests. We propose that this complex NLR network increased the evolvability and robustness of immune signaling to counteract rapidly evolving plant pathogens.

Results and Discussion

**NRC4 Is Required for Rpi-blb2-Mediated Immunity.** As part of a study performed in N. benthamiana to identify genetic components required for resistance to P. infestans conferred by the potato NLR-type gene Rpi-blb2 (35, 36), we discovered that another NLR protein, NRC4, is required for the Rpi-blb2 function (Fig. 1). Silencing of NRC4 compromised Rpi-blb2 resistance to P. infestans (Fig. 1A) and hypersensitive cell death to the P. infestans effector AVRblb2 (36) (Fig. 1B). This phenotype was rescued by a silencing-resistant synthetic NRC4 gene (Fig. 1 C and D and SI Appendix, Fig. S1 A and B), confirming that the observed phenotype was indeed caused by NRC4 silencing. Silencing of NRC4 did not affect Rpi-blb2 protein accumulation (SI Appendix, Fig. S1C).

Previous studies of NLR pairs reported contrasting findings on the role of the ATP-binding p-loop motif in immune signaling. In some cases, only one NLR in the complex requires the p-loop motif (37, 38), whereas the ADR1 helper NLR from Arabidopsis thaliana displays p-loop–independent immune activity (8). We tested the role of the p-loop in Rpi-blb2 and NRC4 functions. Mutations in either Rpi-blb2 or NRC4 p-loops abolished the hypersensitive cell death response (SI Appendix, Fig. S2). Thus, the classic sensor/helper NLR model is not sufficient to explain how the Rpi-blb2/NRC4 mediates immunity.

NRC4 defines a distinct clade within the NRC family (SI Appendix, Fig. S3A). Of the nine NRC genes in N. benthamiana, four were expressed to significant levels in leaves, but only NRC4 transcript levels were reduced in NRC4-silenced plants (SI Appendix, Figs. S1D and S3B). Among the expressed genes, NRC2 and NRC3 are required for bacterial resistance mediated by the NLR protein Prf in N. benthamiana (11, 24) but were not essential for Rpi-blb2 functions in our silencing experiments (Fig. 1A and B). In contrast, NRC4 was not essential for Prf-mediated cell death and resistance to the bacterial pathogen P. syringae (Fig. 1B and SI Appendix, Fig. S4).

**NRC Clade and Its Sister Clades Form a Signaling Network.** Phylogenetic analyses of the complete repertoire of CNL (NLR with an N-terminal CC domain) proteins from the solanaceous plants tomato, potato, and pepper and N. benthamiana revealed that the NRC family groups with the Rpi-blb2 and Prf clades in a well-supported superclade (SI Appendix, Fig. S5). Interestingly, this superclade includes additional well-known NLRs, such as Rx (23, 27), Bs2 (20), R8 (39), Sw5b (21), R1 (40), and Mi-1.2 (17), which confer resistance to diverse plant pathogens and pests (SI Appendix, Fig. S5 and Table S1). This finding prompted us to test the extent to which NRC proteins are involved in immune responses mediated by these phylogenetically related disease resistance proteins.

Silencing of NRC2 and NRC3 affected Prf and moderately reduced the hypersensitive cell death triggered by the potato late blight resistance gene R8, but did not alter the response mediated by 12 other NLR proteins (Fig. 2). In contrast, silencing of NRC4 compromised the hypersensitive cell death mediated by Mi-1.2, an Rpi-blb2 ortholog that provides resistance to nematodes and insects; CNNL1-11990**A**IV, an avirulent mutant of a CNL of unknown function; and R1, an NLR that confers resistance to P. infestans (Fig. 2 and SI Appendix, Fig. S6C). Furthermore, NRC4 silencing abolished R1-mediated disease resistance to P. infestans, and the phenotype was rescued by a silencing-resistant synthetic NRC4 gene (SI Appendix, Fig. S6 B–D).

Given that the three expressed NRC proteins share extensive sequence similarity (SI Appendix, Fig. S7), we hypothesized that NRC2, NRC3, and NRC4 are functionally redundant for...
additional NLRs in the “NRC superclade” (Fig. 2). To test our hypothesis, we simultaneously silenced the three NRC genes and discovered that triple silencing of NRC2/3/4 compromised the hypersensitive cell death mediated by Sw5b, R8, Rx, and Bs2 in addition to the five NLRs mentioned above (Fig. 2 and SI Appendix, Figs. S8 and S9). In contrast, triple silencing of NRC did not affect the hypersensitive cell death mediated by the five tested NLRs that map outside the NRC superclade (Fig. 2) and did not abolish resistance to P. infestans conferred by two of these NLR proteins (SI Appendix, Fig. S10).

We validated NRC2, NRC3, and NRC4 redundancy by complementation in the triple silencing background with silencing-resilient synthetic NRC (SI Appendix, Fig. S11). These results confirmed that the three NRC proteins display specificity to Rpi-blb2 and Prf but have redundant functions in Rx-, Bs2-, R8-, and Sw5b-mediated hypersensitive cell death (SI Appendix, Fig. S11).

The p-Loop Is Essential for the Activity of NRC4 in All of the Tested Combinations. We further tested whether the p-loop is essential for the activity of NRC homologs in different helper-sensor NLR combinations. The lysine (K) to arginine (R) mutation in the p-loops of NRC2 and NRC3 dramatically compromised steady-state protein accumulation (SI Appendix, Fig. S12), prompting us to focus on NRC4 in subsequent experiments. The p-loop mutants of NRC4 failed to rescue cell death mediated by any of the sensor NLRs we tested (SI Appendix, Figs. S12B and C), indicating that the p-loop is essential for NRC4-mediated immunity. These results challenge our understanding of helper NLR activation, in which proteins such as ADR1-L2 display p-loop-independent activity in NLR-triggered immunity (8). Phylogenetically, the ADR1/NRG1 family belongs to the RPW8 clade that is distantly related to the NRC family (CNL-14) (41, 42). This observation indicates that ADR1/NRG1 and the NRC families have independently evolved as helper NLRs, and may have acquired different mechanisms to activate immune signaling. Interestingly, activation of DM1/DM2d, an NLR complex that contributes to hybrid necrosis, was recently reported to require the p-loops of both NLRs (43), suggesting that not all genetic or physical NLR complexes are regulated through the same mechanism.

NRC2, NRC3, and NRC4 Redundantly Contribute to Rx-Mediated Resistance to PVX. To validate further that NRC2, NRC3, and NRC4 redundantly contribute to immunity, we examined the resistance mediated by Rx to PVX (23, 27) in plants silenced for single, double, or triple combinations of NRC genes. Rx-mediated resistance to PVX was only abolished in the triple silencing background, resulting in systemic spread of necrotic lesions (Fig. 3).
This phenotype, known as trailing necrosis, reflects spread of the virus when Rx-mediated extreme resistance is compromised (27). We further validated systemic spread of the virus by detecting accumulation of GFP driven by the subgenomic promoter of PVX (SI Appendix, Fig. S14). Indeed, silencing-resilient NRC2, NRC3, and NRC4 individually complemented the loss of resistance to PVX in triple NRC-silenced plants confirming their functional redundancy in disease resistance (SI Appendix, Fig. S15). This and previous results indicate that the three NRC proteins display varying degrees of redundancy and specificity toward the nine NLRs, revealing a complex immune signaling network (SI Appendix, Fig. S16).

Tomato NRCs Rescue NRC-Dependent Cell Death in N. benthamiana. Most of the sensor NLRs in the NRC network we tested here originate from wild Solanum species, and yet confer disease resistance when introduced into tomato (Solanum lycopersicum), potato (Solanum tuberosum), and N. benthamiana (SI Appendix, Table S1). This observation prompted us to test whether NRCs from tomato display the same sensor NLR spectrum as their N. benthamiana orthologs. Largely consistent with the network we proposed, expression of tomato NRCs rescued cell death when their orthologous N. benthamiana NRCs were silenced (SI Appendix, Figs. S16 and S17). However, tomato NRC3 rescued Rpi-blb2/Mi-mediated cell death in NRC4-silenced N. benthamiana (SI Appendix, Fig. S17B), and tomato NRC4 only weakly rescued Sw5-mediated cell death in NRC2/3-silenced N. benthamiana (SI Appendix, Fig. S17C). We conclude that the NRC network structure may have evolved differently in the various Solanaceae species since divergence from their last common ancestor. Further studies on sequence polymorphisms and the sensor NLR spectrum of different NRC homologs should help reveal how helper-sensor specificity is determined in an NLR signaling network.

Fig. 4. NRC superclade emerged from an NLR pair over 100 Mya. (A) Phylogeny of CNL (CC-NLR) identified from asterids (kiwifruit, coffee, monkey flower, ash tree, and tomato) and caryophyllales (sugar beet). Only sequences with complete NLR features predicted by NLR-parser were included in the analysis. Sequences identified from different species are marked with different colors as indicated. The bootstrap supports of the major nodes are indicated. The phylogenetic tree (Right) which includes only sequences from the indicated lineages (Left), shows that the NRC sequences form a well-supported superclade that occurs in asterids and caryophyllales. The scale bars indicate the evolutionary distance in amino acid substitution per site. Details of the full phylogenetic tree can be found in SI Appendix, Figs. S21 and S22. (B) Summary of phylogeny and number of NLRs identified in different plant species. A phylogenetic tree of plant species was generated using phyloT based on National Center for Biotechnology Information taxon identification numbers. Numbers of NLRs identified in each category were based on NLR-parser and the phylogenetic trees in A and SI Appendix, Figs. S18–S22. NRC, NRC superclade; NRC-H, NRC family (helper NLR); NRC-S, NRC-dependent NLR (sensor NLR). (C) Schematic representation of the NRC gene cluster on sugar beet chromosome 5. The two NRC-S paralogs are marked in blue, and the NRC-H gene is marked in red. (D) Physical map of NRC superclade genes on tomato chromosomes. The NRC-S paralogs are marked in blue, and the NRC-H paralogs are marked in red. Detailed information of the physical map is available in SI Appendix, Fig. S23.
What Forces Drive the Evolution of an NLR Pair into a Network? NRC family members appear to be a convergent signaling point for a large repertoire of NLRs. The observation that sugar beet (caryophyllales) has only three closely linked NLR genes belonging to the NRC superclade supports the hypothesis that NRC and its mates evolved from a genetically linked NLR pair. Models of NLR evolution suggest that once an NLR gene translocates to an unlinked locus, it becomes more likely to diversify into a new function than when it remains in a gene cluster (34). Thus, expansion of the NRC superclade from a genetically linked pair to a genetically unlinked network may have been a key evolutionary step that accelerated evolutionary diversification and functional diversification to confer immunity to multiple pathogens and pests. However, NLR evolution must be constrained by its mode of action. Recent studies on genetically linked NLR pairs, such as RPS4/RRS1 and RGA4/RGA5, suggested that the encoded proteins activate immune signaling through release of negative regulation (37, 38). The selective pressures shaping the evolution of NLR pairs that operate by negative regulation can be expected to limit their expansion due to the genetic load caused by autoimmunity (Fig. 5A). Autoactive NLR helpers and their negative regulators are expected to function as a single unit (supergene) and are likely to remain genetically linked over evolution. In contrast, NRC and NRC-dependent NLR proteins appear to function through a mechanism that accommodates evolutionary plasticity beyond genetically linked pairs of NLR proteins. We propose that NRC and NRC-dependent NLR proteins act through positive regulation rather than suppression of autoactivity (Fig. 5A). Such a mode of action would have enabled massive duplication and functional diversification without accumulation of deleterious effects. Interestingly, recent studies have shown that mismatched NLRs, which probably operate through positive regulation, trigger autoimmunity leading to hybrid necrosis, indicating another layer of complexity in NLR evolution (43, 45). Further studies on how NRC and NRC-dependent NLR proteins function should shed light on the mechanistic details of how this NRC network mediates immune responses and disease resistance. Of particular interest, it would be important to determine how the genetically defined sensor and helper activities of NRCs and their mates translate into biochemical models and the extent to which these proteins associate into a signaling complex.

NLR Networks Increase Robustness of the Plant Immune System. Genetic redundancy is known to enhance robustness and evolvability of biological systems (46–48). The emergence of genetic redundancy ultimately leads to a network architecture, a general feature of many complex biological processes (49). Traits under strong natural selection, such as immunity, should benefit from the increase in evolutionary plasticity and tolerance to environmental disturbance conferred by gene duplications (50, 51). Redundant helper NLRs may therefore provide a stepping stone for rapid expansion and functional diversification of their matching sensor NLRs to counteract rapidly evolving pathogens (Fig. 5B). Interestingly, a recent analysis of NLR evolutionary patterns in Solanaeae revealed that the NRC clade [termed CNL-G8 by Seo et al. (31)] stands out as having only a few recent duplications that occurred after speciation of pepper, tomato, and potato. This finding is consistent with the view that, unlike their NLR mates, NRCs may not be directly involved in detecting pathogens and are diversifying at a slower pace. NRCs may also be constrained by their central function in immune signaling as nodes in a signaling network with a bow-tie architecture (i.e., diversity of inputs converging on a few core elements). Similar bow-tie network architectures have also been described in immunity in other systems, such as animal Toll-like receptors, in which diversified receptors sense a wide variety of microbial molecules with a few core elements playing signaling roles in mediating downstream output (52). We propose that the NRC network is a powerful system to study robustness, redundancy, and specificity of an NLR immune response.
signaling network within a solid evolutionary framework. Harnessing the processes that underpin NLR network structure and function would open up new approaches for developing disease-resistant crops.

Materials and Methods

Hypersensitive Cell Death Assays. Hypersensitive cell death assays were performed using Agrobacterium-mediated transient gene expression. Detailed procedures and information on constructs used in this study are provided in SI Appendix, SI Materials and Methods.

Disease Resistance Assays. Rpi-bib2, Rpi-bib1, R3a, PtoPrf, and Rx transgenic N. benthamiana plants were used for disease resistance assays. R1 was transiently expressed on leaves of N. benthamiana for disease resistance assays. Detailed procedures on disease resistance assays on P. infestans, P. syringae, and PVX are provided in SI Appendix, SI Materials and Methods.

Virus-Induced Gene Silencing and Complementation. Virus-induced gene silencing (VIGS) was performed in N. benthamiana as described in SI Appendix, SI Materials and Methods.

Phylogenetic Analysis. Sequences of NLRs were aligned using Clustal OMEGA or MAFFT, and then manually edited in MEGA7. The sequences of the nucleotide-binding (NB) domains were used for generating a maximum-likelihood tree in MEGA7. NLR-parser was used to identify the NLR sequences from the databases of different plant species. Detailed procedures are provided in SI Appendix, SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Oliver Furer, Jonathan Jones, John Rathjen, Sebastian Schornack, Geert Smant, Brian Staskawicz, Frank Takken, Vivianne Vleeshouwers, and Cyril Zipfel for providing materials and technical support. We thank Yasin Dagdas, Ida Derenwina, Esther van der Knaap, Benjamin Petre, Silke Robatzek, and Erin Zess for helpful suggestions. This project was funded by the Gatsby Charitable Foundation, Biotechnology and Biological Sciences Research Council, and the European Research Council.