NLR network mediates immunity to diverse plant pathogens

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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" NLR proteins 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-blb2, 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 clustering into a well-supported superclade. Using extensive phylogenetic analysis, we discovered that the NRC-superclade has probably emerged over 100 million years ago from an NLR pair that diversified to constitute up to one half of the NLRs of asterids. These results reveal a complex genetic network of NLRs by linking evolutionary history to immune signaling. We propose that this NLR network increases robustness of immune signaling to counteract rapidly evolving plant pathogens.

immunity | host-microbe interactions | evolution

Text:

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 (TIR) or a coiled coil (CC) domain (6). Most plant disease resistance genes encode NLR receptors that detect effector proteins secreted by pathogens either by directly binding them or indirectly 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 (8-12). 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.

The Solanaceae forms one of the most species-rich plant families that includes major agricultural crops, such as potato, tomato and pepper (13). The extensive breeding efforts for improving disease resistance within this family has led to the identification of many NLR-type disease resistance genes from wild Solanaceae species (14, 15). 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 the potato cyst and root-knot nematodes (14, 15). Several of these solanaceous NLR-type disease resistance genes have been deployed in agriculture through either traditional breeding, cisgenesis or transgenesis (15, 16). For example, *Rpi-blb2* has been introgressed into potato cultivars to confer broad-spectrum resistance to isolates of *P. infestans* (17). *Mi-1.2*, an ortholog of *Rpi-blb2*, confers resistance to root-knot nematodes, aphids and whiteflies in cultivars of tomato (18-20). Expression of the pepper NLR gene *Bs2* in tomato confers resistance to the bacterial spot pathogen *Xanthomonas campestris* pv. *vesicatoria* (21). *Sw5b*, a NLR gene originated from the wild tomato species *Solanum peruvianum*, mediates the resistance against TSWV in tomato (22). Furthermore, introgressions of NLR genes *Rx* and *Gpa2* into potato cultivars confer resistance to *Potato virus X* (PVX) and potato cyst nematode, respectively (23, 24).

In addition to their agricultural importance, the solanaceous plants 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 *N. benthamiana*, one of the most widely used model plant species for laboratory-based research (25). Classic examples of mechanistic studies of solanaceous NLRs in *N. benthamiana* include Prf/Pto complex which provides resistance to *Pseudomonas syringae* through association with the effectors AvrPto and AvrPtoB (26-30), and Rx/RanGAP2 complex which confers resistance to PVX by recognizing the coat protein (CP) (24, 31-33). These studies contributed to our understanding of NLR function, particularly the role of effector recognition and interacting partners in activating immunity.

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 beyond 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 million years ago 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.

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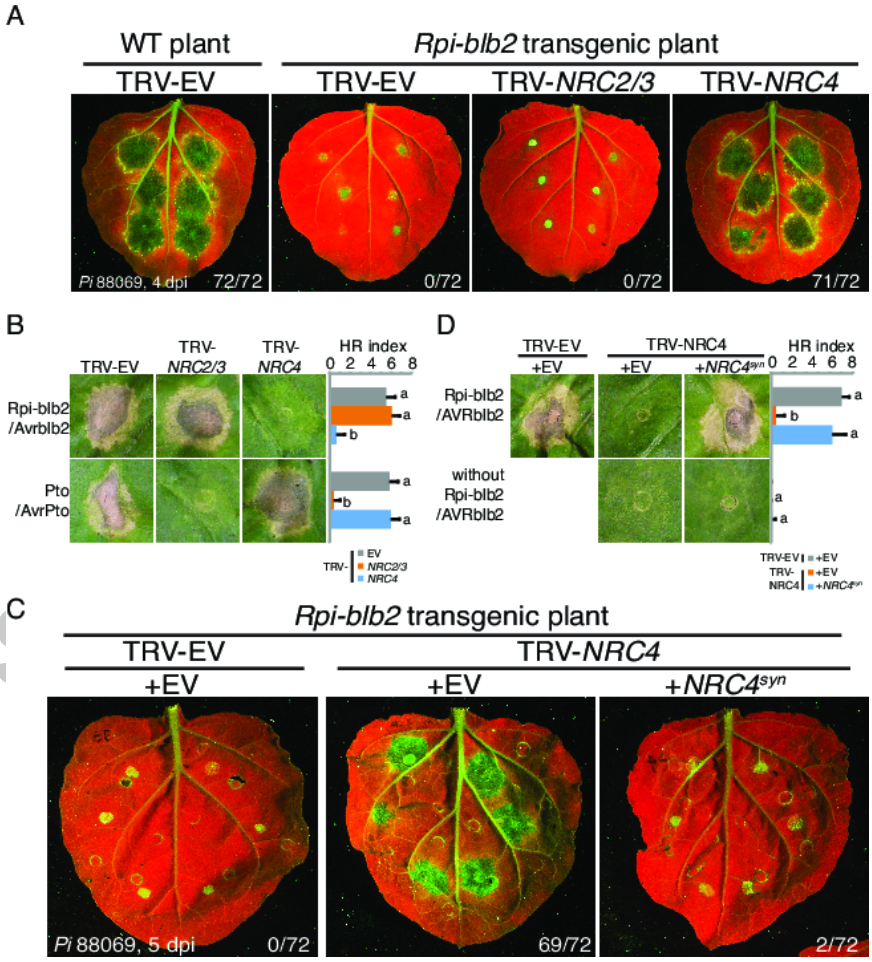

Fig. 1. . ***NRC4* isrequiredforRpi-blb2-mediatedimmunity(A)** Silencing of *NRC4* compromises Rpi-blb2-mediated resistance. *Phytophthora infestans* strain 88069 (*Pi* 88069) was inoculated on *Rpi-blb2* transgenic *Nicotiana benthamiana* pre-infected with *Tobacco rattle virus* (TRV) to silence *NRC2/3* or *NRC4*. Wild type (WT) plant with TRV empty vector (TRV-EV) was used as a susceptible control. Experiments were repeated 3 times with 24 inoculation sites each time. The numbers on the right bottom indicate the sum of spreading lesions/total inoculation sites from the three replicates. Images were taken under UV light at 4 days post inoculation (dpi). **(B)** Silencing of *NRC4* compromises Rpi-blb2- but not Prf-mediated hypersensitive cell death. Rpi-blb2/AVRblb2 or Pto/AvrPto (cell death mediated by Prf) were co-expressed in *NRC2/3*- or *NRC4*-silenced plants by agroinfiltration. Hypersensitive response (HR) was scored at 7 days after agroinfiltration. Bars represent mean + SD of 24 infiltration sites. Statistical differences among the samples were analyzed with ANOVA and Tukey's HSD test (p-value < 0.001). **(C)** Expression of silencing-resilient synthetic *NRC4* (*NRC4^{syn}*) rescues Rpi-blb2-mediated resistance in *NRC4*-silenced plants. Experiments were repeated 3 times with 24 inoculation sites each time. The numbers on the right bottom indicate the sum of spreading lesion/total inoculation sites from the three replicates. Images were taken under UV light at 5 days post inoculation (dpi). **(D)** Expression of silencing-resilient synthetic *NRC4* (*NRC4^{syn}*) rescues Rpi-blb2-mediated cell death in *NRC4*-silenced plants. Hypersensitive response (HR) was scored at 7 days after agroinfiltration. Bars represent mean + SD of 24 infiltrations sites. Statistical differences among the samples were analyzed with ANOVA and Tukey's HSD test (p-value < 0.001).

Genome-wide annotation and cross-species comparison revealed that the number of 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 (34). Across different plant species, NLR genes belonging to different phylogenetic clades may show distinct expansion and gene-loss patterns, indicating that NLR evolution exhibits dynamic patterns of birth and death (4, 6, 34-36). 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 (36-38). 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 helper NLR proteins NRC2 and NRC3 are functionally redundant and are required for the function of Prf/Pto complex in *N. benthamiana* (9). However, whether NRC2 and NRC3 are essential for other sensor NLRs remained an open question. Here, we discovered another helper NLR, termed NRC4, which also belongs to the NRC family. NRC4 is required for immunity triggered by Rpi-blb2, a NLR that provide resistance to *P. infestans*, but it is not required for Prf-mediated immunity. Surprisingly, in addition to their roles in Rpi-blb2 and Prf mediated resistance, NRC2, NRC3, and NRC4 are functionally redundant and essential for the activity of at least 7 other NLRs that confer immunity to oomycetes, bacteria, viruses, nematodes, and insects. Remarkably, both 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 which constitutes up to one half of the NLRs of asterid species has probably evolved from a common ancestral NLR pair over 100 million years ago. We conclude that NRC and their mates form a complex genetic network that confers resistance to diverse pathogens and pests. We propose that this complex NLR network

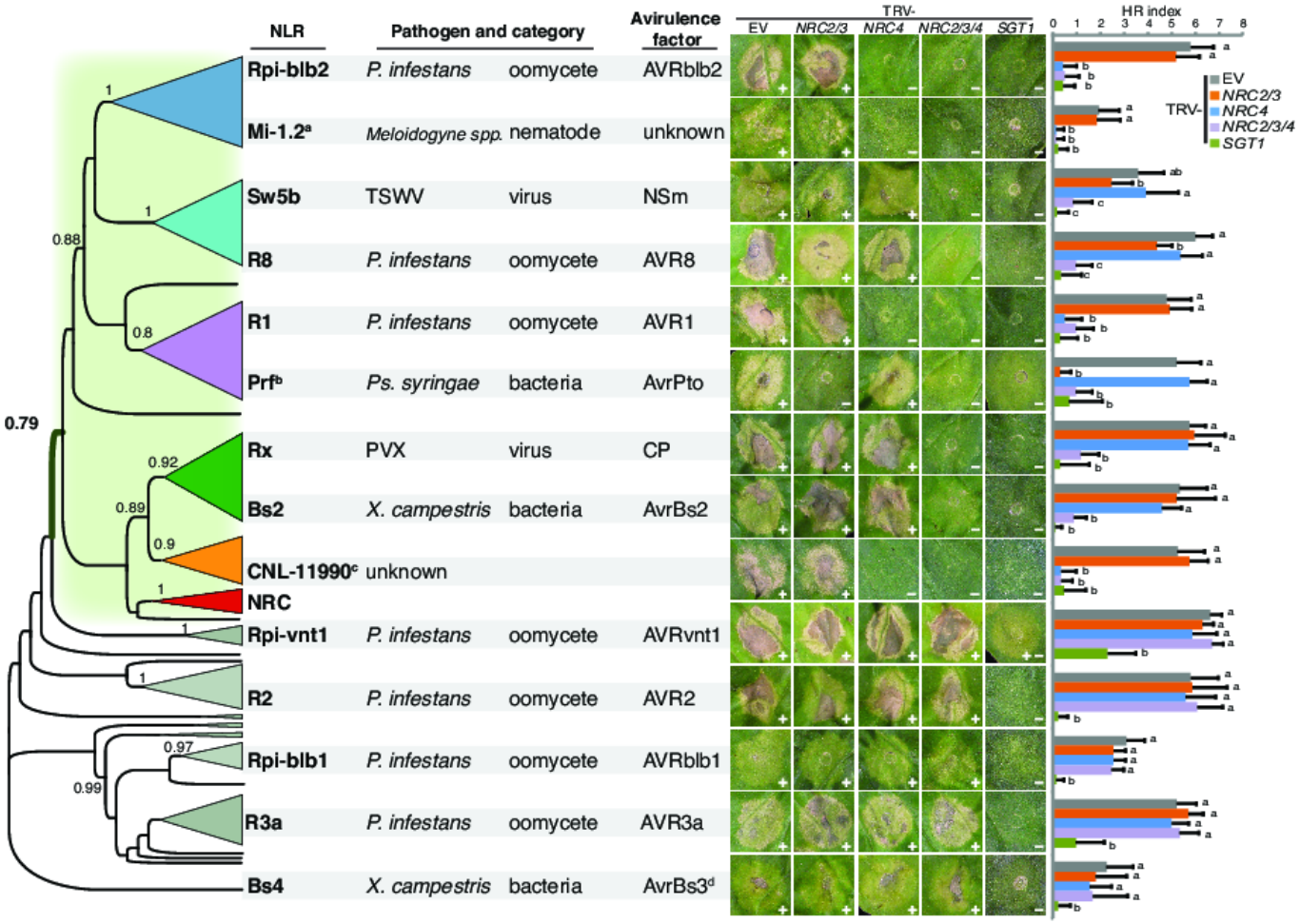

Fig. 2. NRC clade and its sister clades form a complex signaling networkLeft panel: phylogenetic tree of CNL proteins identified from genomes of solanaceous plants, simplified from Fig. S5. **Middle panel**: list of pathogens and avirulence effectors (AVR) sensed by the corresponding NLR immune receptors. TSWV, tomato spotted wilt virus; *Ps.*, *Pseudomonas*; PVX, *Potato virus X*; *X.*, *Xanthomonas*. **Right panel**: analysis of hypersensitive cell death mediated by different solanaceous NLR proteins in *NRC*-silenced plants. Different NLR and AVR effector combinations were expressed in control (EV), *NRC2/3-*, *NRC4-*, *NRC2/3/4-* and *SGT1*-silenced plants by agroinfiltration. "+" indicates cell death phenotype was observed. "-" indicates cell death phenotype was compromised. Hypersensitive response (HR) was scored at 7 days after agroinfiltration. Bars represent mean + SD of 24 infiltration sites. Statistical differences among the samples were analyzed with ANOVA and Tukey's HSD test (P-value < 0.001). [a]Pathogen proteins sensed by Mi-1.2 have not been identified yet. Hence, the autoactive mutant Mi-1.2^{T557S} was used here. [b]Co-expression of Pto and AvrPto was used for testing Prf-mediated cell death. [c]CNL-11990, a CNL cloned from tomato, has no assigned function. The autoactive mutant CNL-11990^{D474V} was used here. [d]Bs4 senses both AvrBs3 and AvrBs4 from *X. campestris*. AvrBs3 was used here. Silencing of *SGT1*, a co-chaperone that is required for steady-state accumulation of NLR proteins (62), was used as a control that compromises cell death mediated by all the NLRs tested here.

increased evolvability and robustness of immune signaling pathways to counteract rapidly evolving plant pathogens.

Results and Discussion

***NRC4* is required for Rpi-blb2-mediated immunity.** As part of a study performed in *Nicotiana benthamiana* to identify genetic components required for resistance to *P. infestans* conferred by the potato NLR-type gene *Rpi-blb2* (39, 40), we discovered that another NLR protein, NRC4 (NLR required for cell death 4), is required for 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* AVRblb2 effector (Fig. 1B) (40). This phenotype was rescued by a silencing-resilient synthetic *NRC4* gene (Fig. 1C-D, Fig. S1A-B), showing that the observed phenotype was indeed caused by the silencing of *NRC4*. *NRC4*-silencing did not affect Rpi-blb2 accumulation (Fig. S1C).

The finding that Rpi-blb2 requires NRC4 for its function reminds of previously studied NLR pair, in which only one NLR in the complex requires ATP binding p-loop motif (41, 42), as well as the ADR1 helper NLR from *Arabidopsis thaliana* which displays p-loop independent activity in immunity triggered by other NLRs (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 (Fig. S2). Thus, the classic helper/sensor NLR model is not sufficient to explain how the Rpi-blb2/NRC4 mediates immunity.

NRC4 defines a distinct clade within the NRC family (Fig. S3A). Of the 9 *NRC* genes in *N. benthamiana*, four were expressed to significant levels in leaves but only *NRC4* transcript levels were reduced in *NRC4*-silenced plants (Fig. S1D, Fig. S3B). Among the expressed genes, *NRC2* and *NRC3* are required for bacterial resistance mediated by the NLR protein Prf in *N. benthamiana* (9, 26) but were not essential for Rpi-blb2 functions in our silencing experiments (Fig. 1A-B). In contrast, *NRC4* was not essential for Prf-mediated cell death and resistance to the bacterial pathogen *Pseudomonas syringae* (Fig. 1B, Fig. S4).

NRC clade and its sister clades form a signaling network. Phylogenetic analyses of the complete repertoire of CNL (NLR

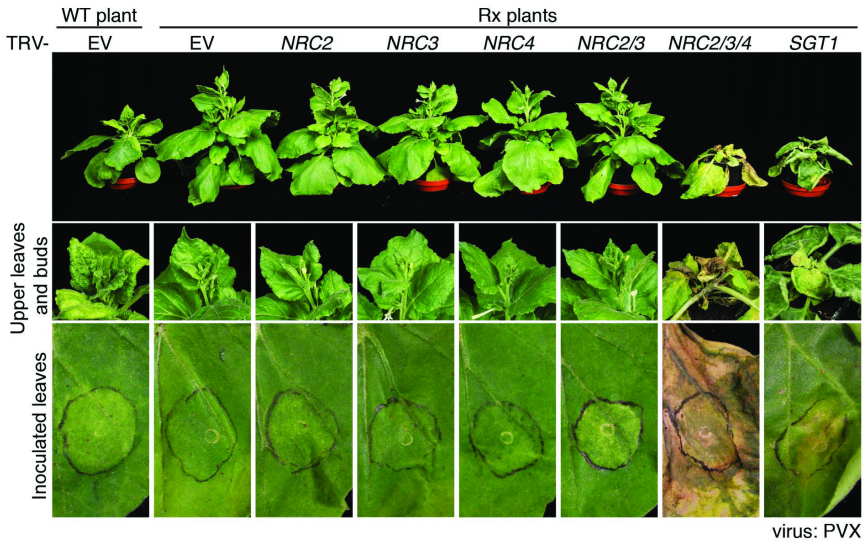

Fig. 3. Triple silencing of *NRC2, NRC3* and *NRC4* compromised Rx-mediated extreme resistance to PVX *NRC2, NRC3,* or *NRC4* were silenced individually or in combination in *Rx* transgenic plants by TRV. *SGT1* silencing, which compromises Rx-mediated resistance (62), was used as a control. The circles on the inoculated leaves indicate the area of PVX inoculation by agroinfection. Pictures were taken 2 weeks after PVX inoculation.

with a N-terminal coiled-coil domain) proteins from the solanaceous plants tomato, potato, pepper and *N. benthamiana* revealed that the NRC family groups with the Rpi-blb2 and Prf clades in a well-supported superclade (Fig. S5). Interestingly, this superclade includes additional well-known NLRs, such as Rx (24, 31), Bs2 (21), R8 (43), Sw5b (22), R1(44) and Mi-1.2 (18), which confer resistance to diverse plant pathogens and pests (Fig. S5, Table S1). This 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 blight resistance gene R8 (43), 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 (18), an Rpi-blb2 ortholog that provides resistance to nematodes and insects; CNL-11990^{D474V}, an autoactive mutant of a CNL of unknown function, and R1 (44), an NLR that confers resistance to *P. infestans* (Fig. 2, Fig. S6A). Further, *NRC4* silencing abolished R1-mediated disease resistance to *P. infestans* and the phenotype was rescued by a silencing-resilient synthetic *NRC4* gene (Fig. S6B-D).

Given that the three expressed NRC proteins share extensive sequence similarity (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 hypersensitive cell death mediated by Sw5b, R8, Rx and Bs2 in addition to the 5 NLRs mentioned above (Fig. 2, Fig. S8, Fig. S9). In contrast, the triple *NRC* silencing did not affect hypersensitive cell death mediated by the 5 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 (Fig. S10).

We validated NRC2, NRC3 and NRC4 redundancy by complementation in the triple silencing background with silencing-resilient synthetic *NRC* (Fig. S11). This 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 (Fig. S11).

P-loop is essential for the activity of NRC4 in all the tested combinations. We further tested whether p-loop is essential for the activity of NRC homologs in different helper-sensor NLR combinations. Since the lysine (K) to arginine (R) mutation in the p-loops of NRC2 and NRC3 dramatically compromised the steady-state protein accumulation (Fig. S12A), we focused on NRC4 for the subsequent experiments. P-loop mutants of NRC4 failed to rescue cell death mediated by any of the sensor NLRs we tested here (Fig. S12B-C), indicating that p-loop is essential for NRC4-mediated immunity. These results challenge our understanding of helper NLR activation, in which proteins such as ADR1-L2 displays p-loop independent activity in NLR-triggered immunity (8). Phylogenetically, ADR1/NRG1 family belongs to RPW8 clade that is distantly related to the NRC family (CNL-14) (45, 46). Therefore, despite having different evolutionary paths to become components that are genetically downstream of other NLRs, the mechanisms by which the ADR1/NRG1 family and the NRC family activate immune signaling could be different. Interestingly, a recent report indicated that activation of DM1/DM2d, a TNL complex that contribute to hybrid necrosis, also requires the p-loops of both NLRs (47), 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 *Potato virus X*. To further validate that NRC2, NRC3 and NRC4 redundantly contribute to immunity, we examined the resistance mediated by Rx to *Potato virus X* (PVX) (24, 31) 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, Fig. S13). This phenotype, known as trailing necrosis, reflects spread of the virus when Rx-mediated extreme resistance is compromised (31). We further validated systemic spread of the virus by detecting accumulation of GFP driven by the subgenomic promoter of PVX (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 (Fig. S15). This and previous results indicate that the three NRC proteins display varying degrees of redundancy and specificity towards the 9 NLRs revealing a complex immune signaling network (Fig. S16).

Tomato NRC homologs rescue NRC-dependent cell death in *N. benthamiana*. Most of the sensor NLRs in the NRC network we tested here originated from wild *Solanum* species, and yet they conferred disease resistance when introduced into tomato (*S. lycopersicum*), potato (*S. tuberosum*), and *N. benthamiana* (Table S1). This prompted us to test whether NRC homologs

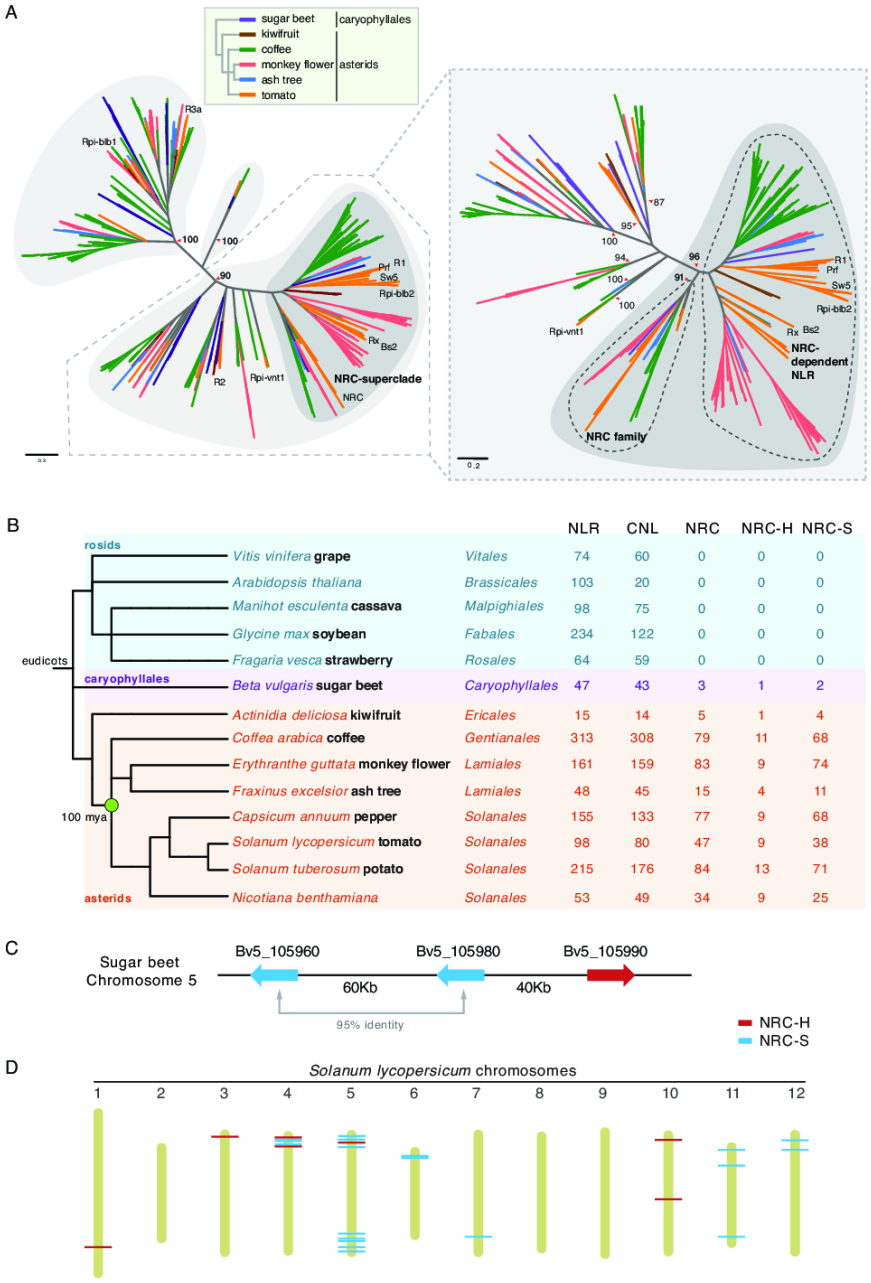

Fig. 4. NRC-superclade emerged from a NLR pair over 100 million years ago(A) Phylogeny of CNL (CC-NLR) identified from asterids (kiwifruit, coffee, monkey flower, ash tree and tomato) and caryophyllales (sugar beet). Only sequences with complete NLR features predicted by NLR-parser were included in the analysis. Sequences identified from different species are marked with different color as indicated. The bootstrap supports of the major nodes are shown. The phylogenetic tree in the right panel, which includes only sequences from the indicated lineages in the left panel, 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 Fig. S21-22. -(**B**) Summary of phylogeny and number of NLR identified in the different plant species. Phylogenetic tree of plant species was generated by using phyloT based on National center for Biotechnology Information (NCBI) taxon identification numbers. Numbers of NLR identified in each category were based on NLR-parser and the phylogenetic trees in (**A**) and Fig. S18-22. Mya, million years ago; CNL, CC-NLR; 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. The detail information of the physical map can be found in Fig. S23.

from tomato display the same sensor NLR spectrum as their *N. benthamiana* orthologs. Largely consistent with the model we proposed, expression of tomato *NRC* homologs rescued cell death when the corresponding *N. benthamiana NRC* homologs were silenced (Fig. S16, Fig. S17). However, tomato *NRC3* rescued Rpi-blb2/Mi-mediated cell death in *NRC4*-silenced *N. benthamiana* unlike *N. benthamiana* NRC3 (Fig. S17A, Fig. S11). Furthermore, tomato *NRC2* only weakly rescued Prf-mediated cell death in *NRC2/3*-silenced *N. benthamiana* (Fig. S17B), and tomato *NRC4* only weakly rescued Sw5-mediated cell death in *NRC2/3/4*-silenced *N. benthamiana* (Fig. S17C). Given that distantly related solanaceous species may have encountered distinct selection pressures during evolution, NRC network structure may have evolved differently in each species since divergence from their last common ancestor. Further studies on sequence polymorphisms and sensor NLR spectrum of different NRC homologs may help reveal how helper-sensor specificity is determined in a NLR signaling network.

NRC-superclade emerged from a NLR pair over 100 million years ago. Our observation that NRC proteins and their NLR mates are related in the phylogeny of solanaceous CNL proteins (Fig. S5) prompted us to reconstruct the evolutionary history of the NRC-superclade. Higher order phylogenetic analyses of complete CNL repertoires from representative plant taxa revealed that the NRC-superclade is missing in rosids but present in the examined representatives of caryophyllales (sugar beet) and asterids (kiwifruit, coffee, monkey flower, ash tree and Solanaceae species) (Fig. S18, Fig. 4A-B, Fig. S19-22). Interestingly, sugar beet and kiwifruit, the early branching species, have only a single protein that groups with the NRC family (referred to as NRC-H), along with 2 and 4 NLRs that cluster with the NRC-dependent NLRs (referred to as NRC-S) (Fig. 4A-B, Fig. S22). The dramatic

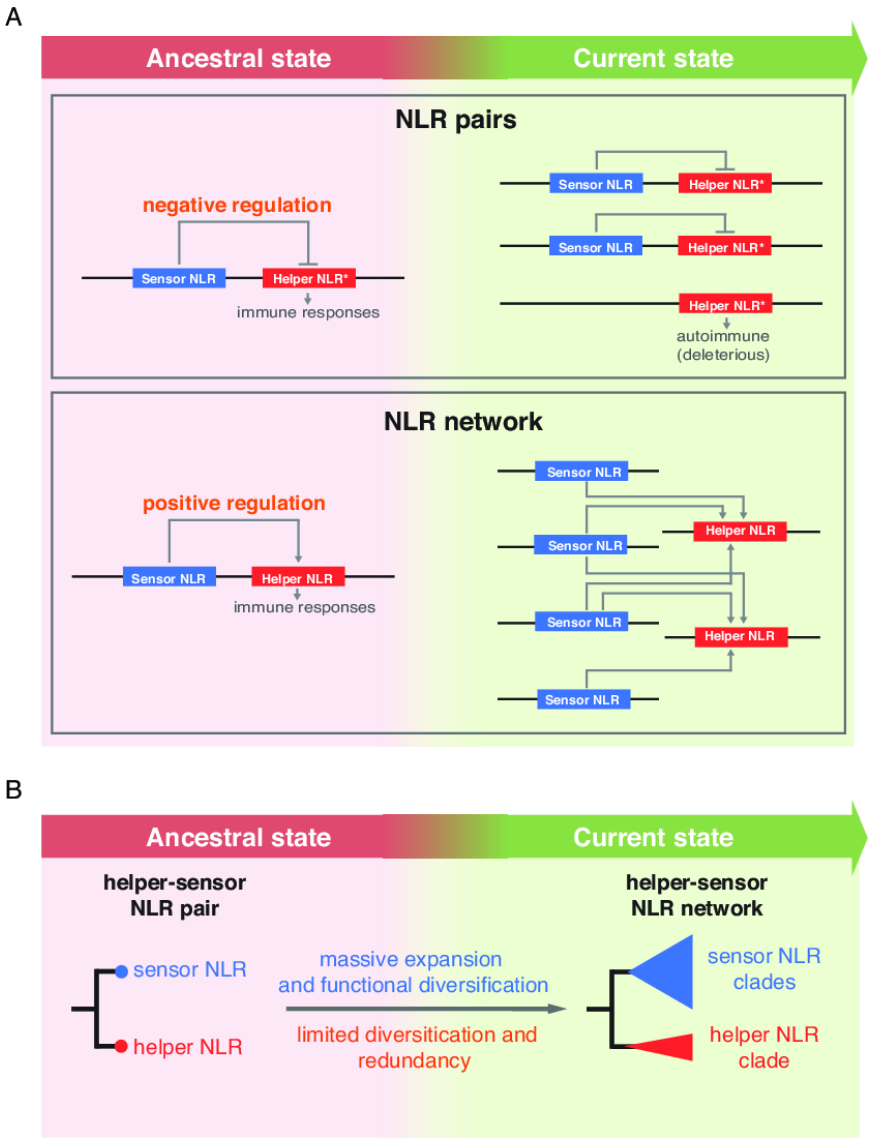

Fig. 5. Constraints and plasticity in plant NLR evolution(A) NLR evolution must be constrained by their mode of action. Some NLR pairs are known to operate by negative regulation with the helper NLR exhibiting autoimmunity (NLR*) and the sensor NLR acting as a helper inhibitor. In such cases, expansion of the pair will be constrained throughout evolution due to the genetic load caused by autoimmunity. In contrast, NLR that function through a different mechanism, e.g. positive regulation of the NLR helper by the sensor, will be less constrained to evolve into networks beyond genetically linked pairs of NLR. (B) A model of the expansion of the NRC superclade from an ancestral pair of NLR. The NRC-helper clade has expanded to create genetic redundancy and thus flexibility for the sensor NLR to evolve rapidly. However, due to the constraints for mediating a conserved downstream signaling the diversification of the helper clade is likely to remain limited. In contrast, the NRC-sensor homologs have evolved into several diversified clades to detect proteins from a diversity of pathogens. This network system with redundant helper NLR may provide a framework for rapid evolution of plant NLR-triggered immunity in order to counteract fast evolving pathogens.

expansion of the NRC superclade started prior to the divergence of Gentianales (coffee) from other asterids around 100 million years ago (48, 49) to account for over one half of all NLRs in some of the species (Fig. 4B). We postulate that the NRC superclade has probably evolved from an ancestral pair of genetically linked NLR genes, as in sugar beet, to duplicate and expand throughout the genomes of asterid species into a complex genetic network that confers immunity to a diversity of plant pathogens (Fig. 4C-D, Fig. S23).

What forces modulate the evolution of NLR pairs 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. Studies on mechanisms of NLR evolution have suggested that once a NLR gene has duplicated or translocated to an unlinked locus, it becomes more likely to diversify into a new function than by remaining in a gene cluster (38). Thus, the expansion of the NRC-superclade from a genetically linked pair to a genetically unlinked network may have been a key evolutionary step that accelerated functional diversification to confer immunity to multiple pathogens and pests. However, NLR evolution must be constrained by their 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 (41, 42). 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. A recent study revealed that two NLR genes with antagonistic function in resistance and yield evolved in a single cluster, and these immune inhibitory effects may be selected for in the process of crop domestication (50). In contrast, NRC and NRC-dependent NLR proteins appear to function through a mechanism that accommodates evolutionary plasticity beyond genetically linked pairs of NLR. We propose that NRC and NRC-dependent NLR proteins act through positive regulation rather than suppression of autoactivity (Fig. 5A). Such mode of action would have enabled massive duplication and functional diversification without accumulation of deleterious effects. Interestingly, some recent studies showed that mismatched

NLRs probably operating through positive regulation trigger autoimmunity leading to hybrid necrosis, adding another layer of complexity in NLR evolution (47, 51). Future studies on how NRC and NRC-dependent NLR proteins function should shed light on the mechanistic detail of how this NRC-network mediate immune responses and disease resistance.

NLR network increase robustness of plant immune system. Genetic redundancy is known to enhance robustness and evolvability of biological systems (52-55). The emergence of genetic redundancy ultimately leads to network architecture, a general feature of many complex biological processes (56). 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 (57, 58). 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 Solanaceae revealed that the NRC clade (termed CNL-G8 by Seo *et al*. 2016) stands out as having only a few duplications that occurred recently after speciation of pepper, tomato and potato (35). This is consistent with the view that, unlike their NLR mates, NRCs may not be directly co-evolving with pathogens and are constrained by their function in immune signaling thus acting as nodes in a signaling network with bow-tie architecture, although the signaling output downstream of NRCs have not been identified yet. Similar bow-tie network architectures have also been described in immunity of other systems, such as animal TLR receptors, in which diversified receptors sense a wide variety of microbial molecules with few core elements play important roles in mediating downstream output (59, 60). We propose that the NRC network is a powerful system to study robustness, redundancy and specificity of an NLR immune 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. The cell death (HR index) was scored at 7 days post infiltration. Detail procedures and information of constructs used in this study are provided in *SI Materials and Methods*.

Disease resistance assays. Rpi-blb2, Rpi-blb1, R3a, Pto/Prf and Rx transgenic *N. benthamiana* plants were used for disease resistance assays. R1 was transiently expressed on leaves of *N. benthamiana* for disease resistance assay. Inoculation of *P. infestans* was performed by applying droplets of zoospore suspension on detached leaves and imaged under UV light at indicated days post inoculation. *Ps. syringae* pv. *tomato* DC3000 $^{\triangle}$*hopQ1-1* was infiltrated into *N. benthamiana* leaves using needleless syringe. Bacterial growth assays were performed to evaluated the extent of resistance mediated by Pto/Prf. *A. tumefaciens* stains harboring expression vector pGR106 (or pGR106-GPF) were used for inoculating PVX on *N. benthamiana*. Trailing necrotic lesions and accumulation of GFP were used as indications of systemic spread of the virus. Detail procedures are provided in *SI Materials and Methods*.

Virus-induced gene silencing (VIGS) and complementation. VIGS was performed in *N. benthamiana* as described previously (61). For complementation, silencing-resilient *NRC* variants were generated by introducing synonymous substitutions into the targeted codons. Detail procedures for VIGS, construction of VIGS vectors, RT-PCR, and design of complementation are described in *SI Materials and Methods*.

Phylogenetic analysis. Sequences of NLR were aligned using Clustal OMEGA or MAFFT, and then manually edited in MEGA7. The sequences of the NB-ARC domains were used for generating maximum-likelihood tree in MEGA7. NLR-parser was used to identify the NLR sequences from the databases of different plant species. Detail procedures are provided in *SI Materials and Methods*.

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