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Conserved effector families render *Phytophthora* species vulnerable to recognition by NLR receptors in nonhost plants

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NLR receptor is suggested as a component of plant nonhost resistance (NHR). However, the evolutionary process of how plants develop receptors for recognizing broad-spectrum pathogens is still elusive. Here, we observe that multiple RxLR effector families including 12 reported avirulence effectors of *Phytophthora infestans* are broadly conserved across the *Phytophthora* species. We select 69 effectors distributed into 8 families from 6 *Phytophthora* species, and confirm that 60.87% of the tested effectors are recognized by *Solanum* NLRs according to their defined families. Furthermore, we confirm that expression of *R1, R8*, and *Rpi-amr1* confer broad-spectrum resistance against multiple *Phytophthora* species. Combined results suggest that conserved effector families of *Phytophthora* species allow solanaceous plants to recognize broad-spectrum pathogens via NLRs that originally reported to recognize *P. infestans*. Thus, NLR-mediated recognition would contribute to NHR against pathogens that possess similar repertoires of effectors. Moreover, this homology-based approach would be applicable to other plant-pathogen systems and provide an alternative strategy of genetic mapping to identify functional NLRs against various crop-threatening pathogens.

In nature, while plants are surrounded by numerous pathogenic microorganisms, only a portion of those pathogens which are adapted to a given plant are able to colonize the plant. In this context, nonhost resistance (NHR) could be simply defined as the resistance of a plant species against non-adapted pathogens[1]. To date, a number of studies have investigated molecular mechanisms of NHR, and currently, NHR is thought to rely on the cooperation of multiple layers of the plant immune system[2], including pre-formed physico-chemical barriers and receptor-mediated induced immune responses[3,4]. The molecular mechanism of NHR is quite depend on context, and evolutionary distance between original host and nonhost plant of a given pathogen have been suggested as a key factor of determining which component of plant immune system majorly contribute to the each circumstance[4,5]. In particular, cytoplasmic immune receptor proteins (NLRs) are also suggested as a component of NHR[1]. Various loss-of-function approaches[6–8] and multiple cases of transferring NLRs derived from nonhost plant into host plant[9–13] support that NLR-mediated immunity is associated with NHR. However, several questions still remain regarding the NLR-mediated NHR, such as 'How and why does plant retain receptors for recognizing non-adapted pathogens?'. Considering that pattern recognition receptors (PRRs) recognize broadly conserved pathogen-associated molecular patterns

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(PAMPs), it seems quite possible that PRRs recognize PAMPs from both evolutionarily distant adapted and non-adapted pathogens of a given plant[14–16]. However, NLRs recognize effectors that are relatively less conserved and generally known to have lineage-specific sequence diversity[17,18]. Thus, it is relatively hard to imagine how NLRs contribute to recognition of a wide range of pathogen species including non-adapted pathogens of a given plant.

Several recent studies have provided clues on the mechanism by which NLRs contribute to the recognition of effectors from a wide range of pathogens. In a bacterial system, a screen of 529 effectors distributed into 70 families of multiple *Pseudomonas syringae* pathovars revealed that multiple *Arabidopsis* NLRs are able to recognize orthologous effectors of closely related *Pseudomonas syringae* pathovars[19]. Similarly, in oomycetes, *Rpi-amr3* (resistance gene against *P. infestans*) is reported to recognize *Avramr3* orthologs of multiple *Phytophthora* species[20]. These studies suggest that certain effectors that are conserved across both adapted and non-adapted pathogens of a given plant could be similarly recognized by NLRs and may contribute to broad-spectrum resistance.

The *Phytophthora* genus is composed of multiple subclades and more than one hundred species[21]. Each *Phytophthora* species is adapted to infect its host plant species. Thus, *Phytophthora* genus threaten a wide range of plants, including vegetables (Solanaceae, Cucurbitaceae, Fabaceae), fruits, orchids, and even trees[22–24]. Since various *Phytophthora* isolates that tolerant to fungicide have been continuously reported[24,25], there has always been a demand for breeding resistant crops against *Phytophthora* pathogens. Indeed, approximately 2–30 NLR type resistance genes against *P. infestans* have been identified in the wild *Solanum* species[26,27]. However, only a few resistance genes against most other *Phytophthora* species have been reported.

In this context, we assumed that not only *Avramr3*[20], but also other effectors might be conserved across the evolutionarily related *Phytophthora* species, and some of these conserved effectors could also be similarly recognized by the previously reported *Solanum* NLRs that originally known to recognize *P. infestans* effectors. To test this hypothesis, we focused on the RxLR (conserved N-terminal Arg-Xaa-Leu-Arg motif) effectors of multiple *Phytophthora* species[21,28]. We discovered that multiple effector families, including 12 well-characterized *P. infestans* avirulence effectors are conserved across *Phytophthora* species. In addition, 42 out of the 69 tested homologous effectors were recognized by *Solanum* NLRs which were originally reported to recognize avirulence effectors of *P. infestans* according to their defined families. Finally, we confirmed that three *Solanum* NLRs, *R1*, *R8*, and *Rpi-amr1* are capable of conferring broad-spectrum resistance against multiple *Phytophthora* species in addition to *P. infestans*. These results reveal the landscape of how effectors are conserved across the *Phytophthora* genus and provide evidence supporting the role of NLRs in resistance against a certain range of pathogens by recognizing those effectors.

Breeding disease resistant crops through classical approaches requires a number of genetic resources and enormous costs. Since resistance genes against most *Phytophthora* species that threaten various crop species, such as soybean, chili pepper, pumpkin, cacao, apple, strawberry, orchid, and tobacco, have never been identified, the NLRs identified in this study support that homology-based reverse genetic screening could be an alternative strategy of genetic mapping to identify functional NLRs against a wide range of pathogens. In addition, these resources could also be exploited for improving crop resistance against multiple *Phytophthora* species.

Results

Multiple RxLR effector families are conserved across *Phytophthora* species

To investigate the conservation of RxLR effectors across plant pathogenic oomycetes, we searched for homologs of 12 reported *P. infestans* avirulence effectors, including Avr1[29,30], Avr2[31], Avr3a[32], Avr3b, Avr4[33], Avr8[34], Avr10, Avramr1[35], Avramr3[20], Avrblb1[36], Avrblb2[37], and Avrvnt1[38] in the protein database of 12 oomycete species (Supplementary Table 1, 2). We clustered 243,558 proteins from 12 oomycetes into 19,039 orthogroups, and obtained 647 homologous effector candidates from 34 orthogroups (Fig. 1a, Supplementary Fig. 1, 2, and Supplementary Table 2). Candidate effectors were categorized through motif and domain analyses to determine the structural features of RxLR effector (Fig. 1b), and these effectors were further classified into each family through phylogenetic analyses (Fig. 1c). Subsequently, 514 homologous effector candidates of *Phytophthora* species remained after excluding non-homologous candidates from the phylogenetic analysis (Supplementary Fig. 1, Supplementary Data 1). The majority of investigated *P. infestans* effectors were conserved within the closely related *Phytophthora* species, and some families such as *Avr3a* and *Avramr1* were conserved beyond *Hyaloperonospora arabidopsidis* (Fig. 1b). Moreover, by comparing the conservation of effector families based on phylogenetic (patristic) distances between the reference *P. infestans* avirulence effectors and their closest homologs in each *Phytophthora* species, *Avr1*, *Avr3a*, Avr8, *Avramr1*, and *Avramr3* were found to be more conserved than other families in multiple *Phytophthora* species (Supplementary Fig. 3).

We also performed protein structure prediction and structure-based clustering of 514 homologous effector candidates to strengthen our phylogeny-based classification of effector families. The accuracy of the predicted protein structure was evaluated using the predicted local distance difference test (pLDDT) score, and most effectors with WY domain structures had a high pLDDT scores (Supplementary Fig. 4). Structure-based clustering revealed that most effector family members, except for *Avrblb2*, were structurally conserved within multiple *Phytophthora* species (Supplementary Fig. 5). In most cases, a positive correlation was observed between the protein sequence similarity and the structural homology of each homologous effector candidate relative to the corresponding reference *P. infestans* effector (Supplementary Fig. 6). The combined results suggest that multiple effector families are conserved among *Phytophthora* species.

Homologous effectors of multiple *Phytophthora* species are recognized by *Solanum R* genes

Given that *Avramr3* and its orthologs in multiple *Phytophthora* species were recognized by *Rpi-amr3*[20], we expected that the other conserved effector families of multiple *Phytophthora* species could also be similarly recognized by their putative corresponding *Solanum* NLRs according to their defined families (Fig. 1a, Supplementary Fig. 2, 3, 5). To test whether the homologous effectors of multiple *Phytophthora* species could be similarly recognized by their cognate *Solanum* NLRs, we minimized the candidate pool to the proper size for the experimental validation. We selected effectors that exhibited higher sequence and structural similarity against each reference avirulence effector of *P. infestans* (*Avr1, Avr2, Avr3a, Avr8, Avr-amr1, Avr-blb1, Avr-blb2*, and *Avr-vnt1*) by comparing bit-scores and pTM scores (Supplementary Fig. 6). Candidate effectors were obtained from *P. parasitica*, *P. palmivora*, *P. cactorum*, *P. capsici*, and *P. sojae* which are able to colonize the Solanaceae model plant *N. benthamiana*, except for *P. sojae* (Supplementary Table 3).

Among the 514 candidates, we successfully cloned 69 effectors distributed into 8 families (Supplementary Data 2) into an expression vector (Supplementary Data 3). Cloned effectors were co-expressed with their putative corresponding *Solanum* NLRs (*R1*[39], *R2*[40], *R3a*[41], *R8*[42], *Rpi-amr1*[43], *Rpi-blb1*[36,44], *Rpi-blb2*[45], and *Rpi-vnt1*[38,46]) in *N. benthamiana*. As a result, 60.87% (42/69) of the tested effectors induced cell death upon being co-expressed with their putative corresponding *Solanum* NLR (Fig. 2a, Supplementary Table 4, Supplementary Data 2). To our surprise, all the tested *Solanum* NLRs induced cell death against multiple homologs derived from multiple *Phytophthora* species, with the

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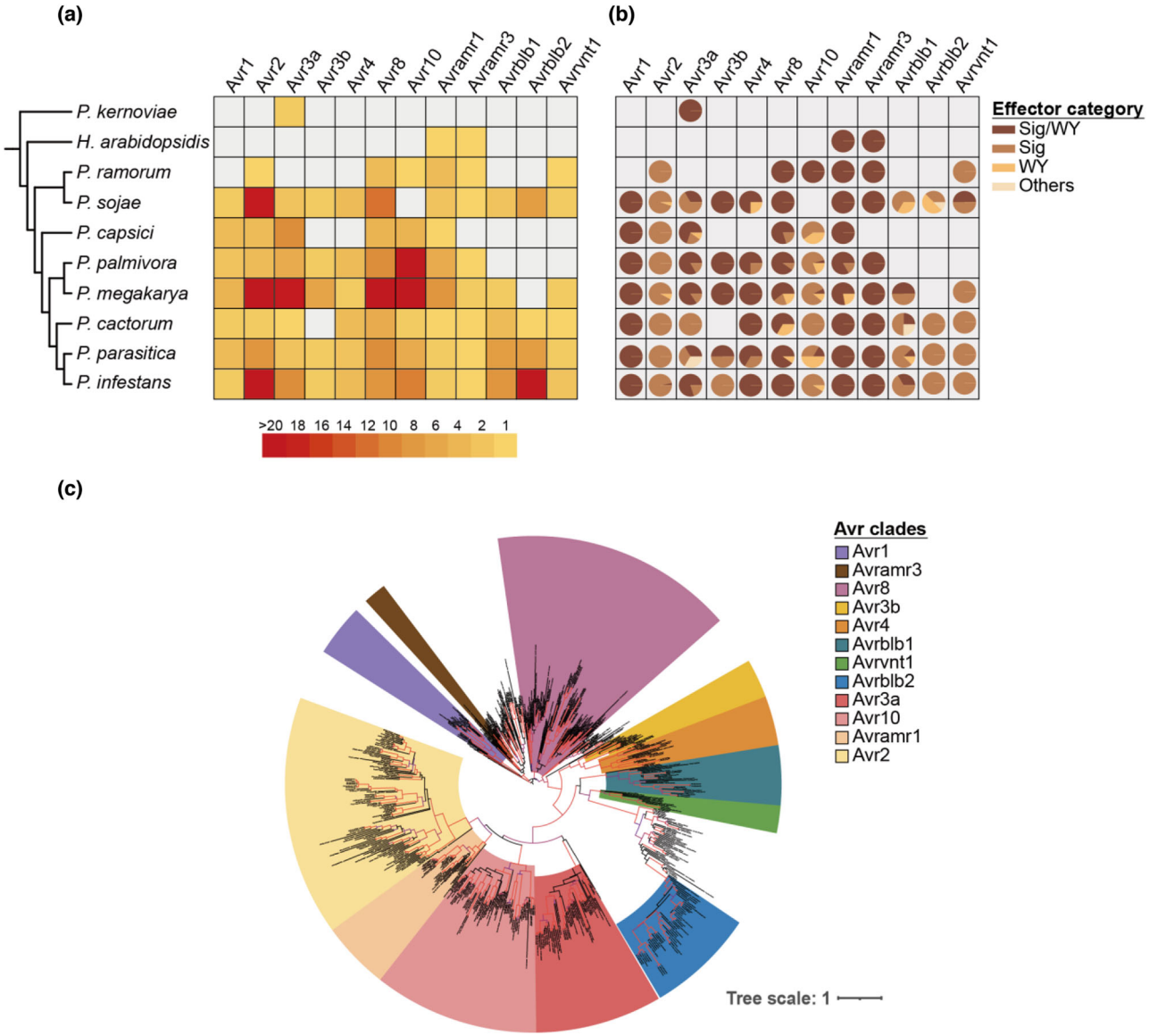

Fig. 1 | Multiple avirulence effector families are conserved across *Phytophthora* species. a The number of homologs of each reference avirulence effector of *P. infestans* in multiple Oomycetes species. Light gray boxes indicate absence of homologous effector. Full species names are described in Supplementary Table 2. **b** The proportion of categories based on the signal peptide (Sig) and WY domain (WY) of homologous effectors. Others represent effector candidates without Sig and WY. The detailed domain and motif information of homologous effectors is provided in Supplementary Data 1. **c** The phylogenetic relationships of homologous effectors against reference avirulence effectors. The range of colors indicates the clade of each reference avirulence effector. The red branches represent ultra-fast bootstrap value above 95.

exception of *Rpi-blb2* (Fig. 2a, Supplementary Data 2). Considering that *Rpi-amr1/3* are reported to recognize *Avramr1/3* orthologs of multiple *Phytophthora* species[20,43], eight of the nine investigated effector families (89%) of *Phytophthora* species are conserved enough to be recognized by the same *Solanum* NLRs (Fig. 2a, Supplementary Fig. 7b, 9b, 10b, 12b, Supplementary Data 2). Based on these results, NLR-mediated recognition of conserved effectors of *Phytophthora* species may also be applicable to other effector families that were not functionally validated in our investigation due to the lack of identified cognate NLRs.

Solanum NLRs confer resistance against multiple *Phytophthora* species by recognizing conserved effectors

To determine whether the *Solanum* NLR-mediated cell death phenotypes against conserved effector families of multiple *Phytophthora* species are also associated with resistance against multiple *Phytophthora* species, we transiently expressed each *Solanum* NLR in *N. benthamiana* and inoculated four *Phytophthora* species (*P. parasitica* or *Pp*, *P. cactorum* or *Pcac*, *P. palmivora* or *Ppal*, and *P. capsici* or *Pc*). As a result, although not all the *Solanum* NLRs that induce HR cell death against effectors of certain *Phytophthora* species could reduce lesion size of each pathogen, we observed that the expression of *R1*, *R8*, or *Rpi-amr1* reduced lesion size of multiple *Phytophthora* species in addition to *P. infestans* (Fig. 2b, c, Supplementary Figs. 7–12, Supplementary Data 2).

To validate *Solanum* NLR-mediated resistance against multiple *Phytophthora* species using transgenic plants, we generated transgenic *N. benthamiana* expressing *R1*, *R8*, or *Rpi-amr1*, through Agrobacterium-mediated transformation. We obtained 30, 15, and 21 T_0 plants that survived on kanamycin media, which were thought to express *R1*, *R8*, or *Rpi-amr1*. To select T_0 plants that properly expressed *R1*, *R8*, or *Rpi-amr1* by observing effector-triggered cell death phenotypes, *Avr1*, *Avr8*, or *Avramr1* was transiently expressed in the leaves of each T_0 plant (Supplementary Fig. 13a, 13b). T_1 seeds were harvested

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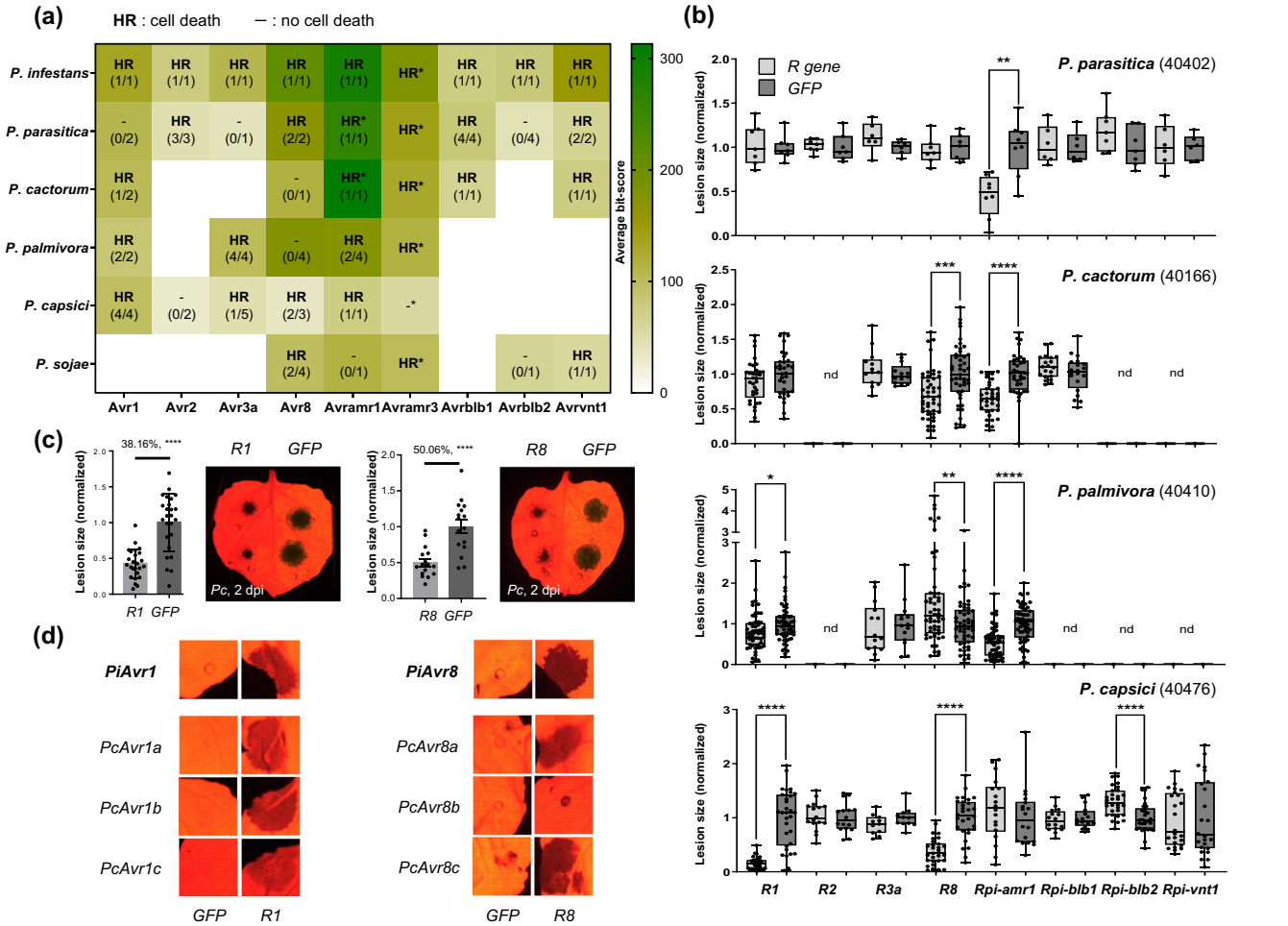

Fig. 2 | *Solanum* NLRs are able to recognize conserved effectors of multiple *Phytophthora* species and confer resistance in *Nicotiana benthamiana*. **a** Eight *Solanum* NLRs, except for *Rpi-amr3* (results presented with asterisk mark as HR*/-* were previously reported in Witek et al.[43]; Lin et al.[20]), were co-expressed with their putative corresponding effectors of multiple *Phytophthora* species in *N. benthamiana* leaves. The number of HR positive/tested effectors are presented as fraction for each case (blank space means not tested). Average bit-scores obtained by comparing 69 cloned effectors against each reference avirulence effector of *P. infestans* are presented as heatmap. **b** Average lesion size of four *Phytophthora* species on *N. benthamiana* leaves expressing one of the eight *Solanum* NLRs and GFP (used as negative control, normalized to 1.0) are presented as box plot, the dots show individual values, while the boxes display the first quartile, median, and third quartile, with whiskers extending to the smallest and largest values. Statistical significances were calculated from unpaired *t*-test (two-tailed $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; nd: non-determined). The number of replicates is presented in the corresponding Supplementary Figs. 7–12. **c** Representative image of reduced lesion of *P. capsici* (40476) on *R1*-expressed half compared to the *GFP*-expressed area in *N. benthamiana* leaf and bar graph presenting average lesion size (left), image and graph for the case of *R8* (right). Statistical significances were calculated from unpaired *t*-test (two-tailed $^{****}P < 0.0001$) and presented with the error bands (percentiles indicate average lesion size of R1 or R8/GFP-expressed leaves). **d** Representative images of *R1*-mediated cell death when co-expressed with *Avr1* homologs of *P. capsici* (left), and *R8*-mediated cell death against *Avr8* homologs of *P. capsici* (right).

from the confirmed lines that induced cell death against transient expression of *Avr1*, *Avr8*, or *Avramr1*, respectively (Supplementary Fig. 13c).

The roots of T_1 transgenic plants expressing *R1*, *R8*, or *Rpi-amr1* were inoculated with *Pc* zoospores for the whole plant disease resistance assay (Fig. 3a, Supplementary Figs. 14, 15, 16) and detached leaves of each plant were inoculated with zoospores of *Pp*, *Pcac*, *Ppal*, or *Pc* (Fig. 3c–f). From the detached leaves assay (DLA), similar results were observed compared to the transient expression-based DLA result (Figs. 2b, 3). Using transgenic *N. benthamiana*, we additionally found that expression of *R1* slightly reduced lesion size of *Pcac* (Fig. 3d). Indeed, the results obtained using transgenic plants were more reliable with HR screening test, because *R1* induced cell death in response to the transient expression of *PcacAvr1* (Fig. 2a, Supplementary Fig. 9a). Similar to the DLA results for *Pc* (Figs. 2b, c, and 3f), *R1* and *R8* transgenic plants exhibited significant resistance against root infection caused by soil drenching of *Pc* zoospores, while wild type and *Rpi-amr1* transgenic plants exhibited complete wilt and shrivel phenotypes (Fig. 3a, b, Supplementary Figs. 14, 15, 16).

In conclusion, four of the nine investigated *Solanum* NLRs, including *Rpi-amr3*, which has previously been reported to confer resistance against *P. infestans, P. parasitica*, and *P. palmivora*[20], conferred broad-spectrum resistance against multiple *Phytophthora* species (Figs. 2, 3). These results suggest that effectors which are conserved across multiple *Phytophthora* species would enable plants to recognize and fend off a wide range of pathogens through NLRs.

Sequence- and structure-based identification of homologous effectors enable to search broadly conserved effector families across the *Phytophthora* species

The combined results of *Solanum* NLR-mediated HR cell death against homologous *Phytophthora* effectors and resistance against multiple *Phytophthora* species stimulated us to determine the correlation between the sequence or structural similarities of the homologous effectors and the possibility of being recognized by corresponding

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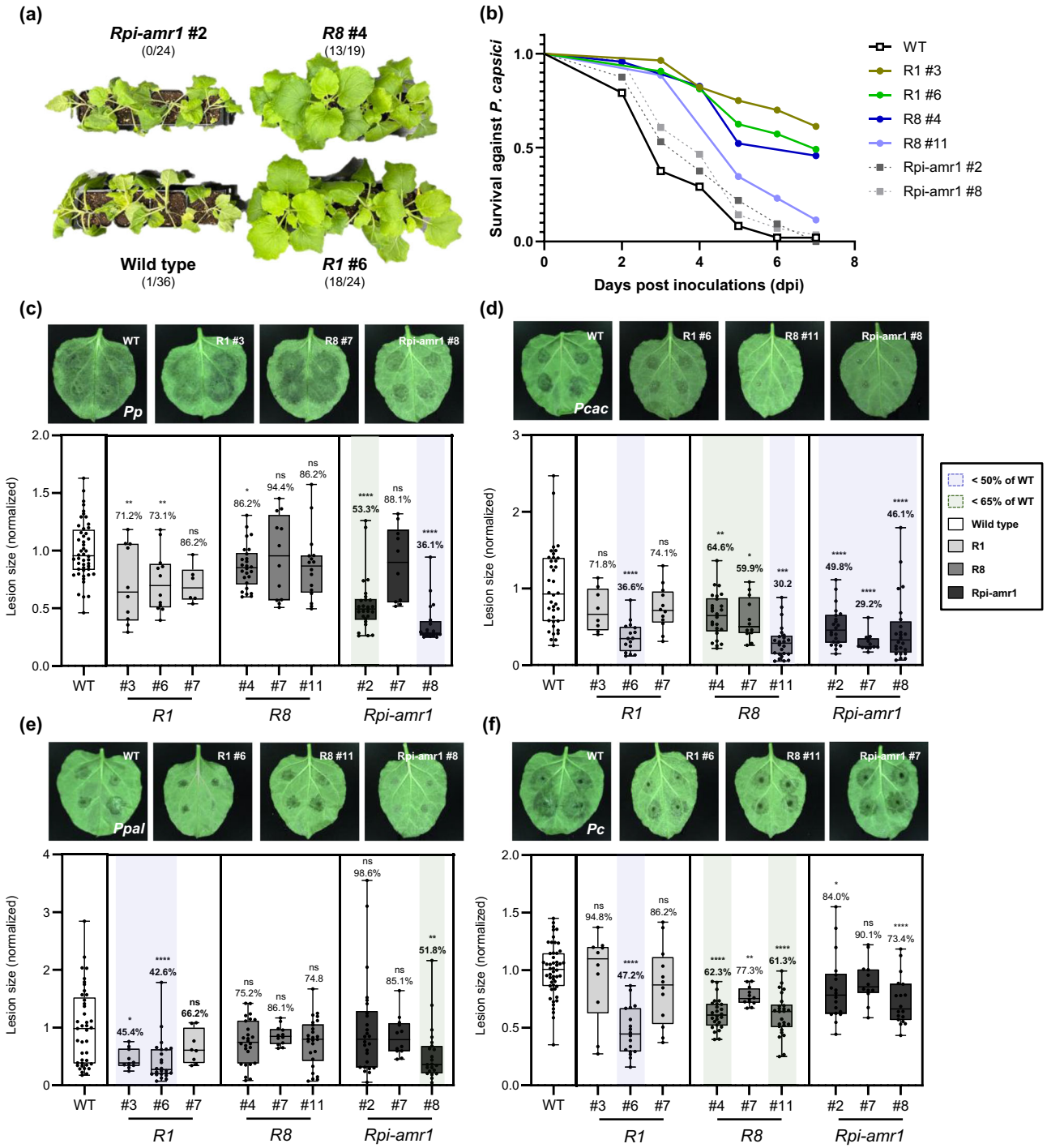

Fig. 3 | Transgenic *N. benthamiana* expressing *R1*, *R8*, or *Rpi-amr1* conferred resistance against multiple *Phytophthora* species. a Representative images for the *P. capsici* root infection assay. T_1 plants expressing *R1* or *R8* exhibited significant resistance against *P. capsici* compared to the wild type or T_1 *Rpi-amr1* plants. The number of healthy/tested plants are described as fraction. **b** Survival rate (healthy/tested plants) of each transgenic line in *P. capsici* root infection assay (combined results obtained from three independent trials) is presented as line graph. Phenotypes were observed until all wild type plants were completely wilt. **c**–**f** Representative images of (**c**) *P. patasitica* 40402 *(Pp)*; (**d**) *P. cactorum* 40166 *(Pcac)*; (**e**) *P. palmivora* 40410 *(Ppal)*; and (**f**) *P. capsici* 40476 *(Pc)* lesions inoculated on wild type (WT), *R1*, *R8*, or *Rpi-amr1* transgenic plants (upper) and the average lesion size of each case is presented with box plot, the dots show individual values, while the boxes display the first quartile, median, and third quartile, with whiskers extending to the smallest and largest values. Average lesion size of each pathogen on the WT plant were normalized to 1.0, and the ratio of average lesion size on transgenic/WT plant are marked as percentage above each box (for the cases of average lesion size of transgenic/WT plant < 50% and < 65%, are marked with blue and green shades, respectively). Statistical significances were calculated from unpaired *t*-test (two-tailed $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

Solanum NLRs. Specifically, we tried to identify the critical sequence or structural features of effectors that are recognized by the same *Solanum* NLRs.

To determine common threshold of sequence and structural similarities in multiple effector families recognized by *Solanum* NLRs, we compared the normalized bit-scores and pTM scores of HR-positive and negative effectors to their corresponding reference effectors of *P. infestans* (Fig. 4a, b, Supplementary Fig. 17). We found that the overall structural conservation of candidate effectors (average pTM score: HR-positive = 0.66, HR-negative = 0.64) was higher than the sequence conservation (average normalized bit-scores: HR-positive = 0.43, HR-negative = 0.32), however, a statistically significant difference was observed only in sequence similarity between the HR-positive and HR-negative effectors (Fig. 4a, b). The average normalized bit-score of HR-positive effectors was significantly higher than the average of HR-negative effectors. Notably, 20 of 26 (76.92%) tested effectors with a 0.4 normalized bit-score were HR positive (Fig. 4a). However, the average pTM score of HR-positive and negative effectors were not significantly different (Fig. 4b).

After observing the overall trends, we focused on several specific effectors which were not recognized by their putative corresponding *Solanum* NLR despite exhibiting high normalized bit-scores or pTM scores compared to reference *P. infestans* effectors. In particular, we compared the sequences and structures of the HR-positive and negative homologs of Avr1, Avr8, and Avramr1 families (Fig. 4c–e, and Supplementary Figs. 18, 19). Since the WY domain is known to be functionally critical and structurally conserved through effectors of Peronosporales species[47], we predicted WY domain structure of HR-positive/negative effectors and observed that all the defined members of Avr1, Avr8, and Avramr1 families possess 2, 2, and 3 conserved WY domains, respectively (Fig. 4c–e, and Supplementary Fig. 18). However, structural comparison showed no significant differences between the WY domain of HR positive (Supplementary Fig. 18a, c, e) and negative (Supplementary Fig. 18b, d, f) effectors compared to the reference *P. infestans* effectors. But contrary, protein sequence alignment revealed that several residues were differently conserved between the HR-positive and negative homologs (Supplementary Fig. 19). In particular, PpalAvramr1a_1 and PpalAvramr1a_2 exhibited only several amino acid differences (94.25% identical) within the WY domains, despite of their opposite responses to *Rpi-amr1* (Supplementary Data 2). These results suggest that several residual changes in the WY domain of homologous effectors of *Phytophthora* species may enable effectors to avoid from *Solanum* NLR-mediated recognition without significant changes in the overall structure of the effectors.

In conclusion, the combined results indicate that both sequence- and structure-based methods for defining homologous effector could be used to identify candidate *Phytophthora* effectors that could be similarly recognized by the same *Solanum* NLRs. However, in our dataset, a sequence similarity-based method would be better for identifying candidates with a higher probability of being recognized by NLRs because significant structural differences are hardly observed between the homologous effectors of *Phytophthora* species which were already defined through phylogenetic analysis and sequence-based clustering. Thus, additional functional validations and sequence/structure-based comparisons using less homologous (compared to the tested effectors used in this study) effector candidates could provide more accurate thresholds to be similarly recognized by *Solanum* NLRs. We expect the criteria used in this study to identify homologous effectors would contribute to search for functionally homologous effectors and identification of additional NLR-effector combinations in other plant-pathogen systems as well.

Discussion

This study suggests that multiple homologous effectors recognized by similar NLRs, could be identified through sequence-based comparisons and homology-based screening in evolutionarily related pathogens such as *Phytophthora* species. These evolutionarily conserved effectors of *Phytophthora* species enable plants to recognize a wide range of pathogens and would contribute to NHR. Furthermore, this study suggest that homology-based reverse genetic approach could be

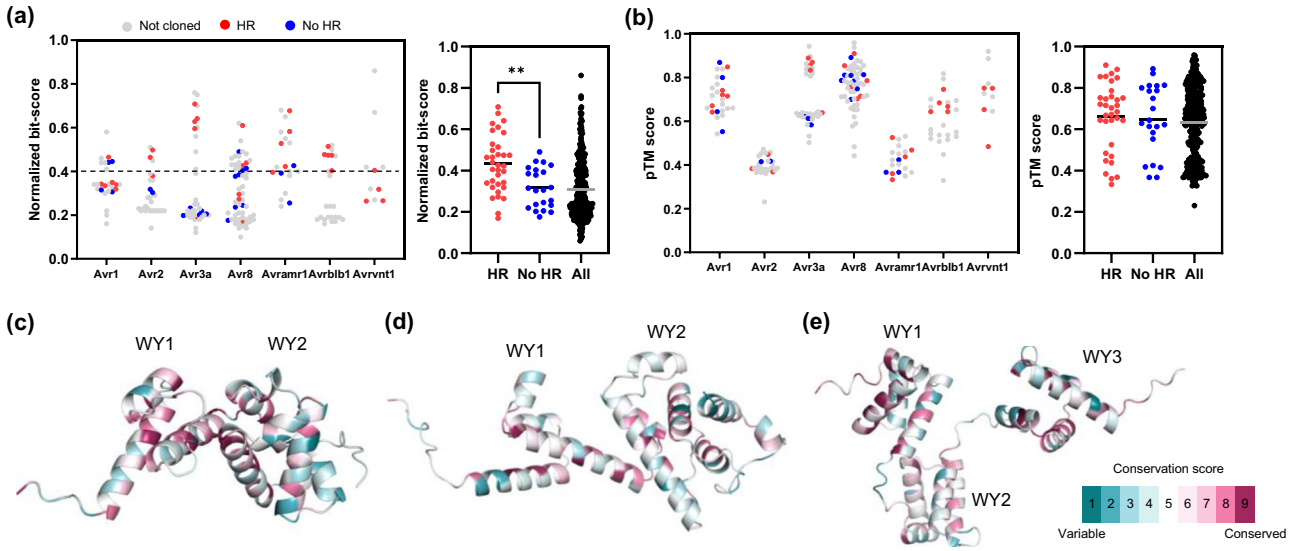

Fig. 4 | Sequence and structure-based identification of homologous effectors enable to search broadly conserved effector families across the *Phytophthora* species. a Normalized bit-score of 316 homologous effectors (of *P. infestans*, *P. parasitica*, *P. cactorum*, *P. palmivora*, and *P. sojae*) against corresponding reference *P. infestans* effectors are plotted (left). Red dots, blue dots, and gray dots represent HR positive, negative, and not cloned effectors, respectively. Average normalized bit-score of HR positive, negative, and 316 homologous effectors presented as black and gray bar (right). Average normalized bit-score of HR positive effectors ($n = 34$) was significantly higher than HR negative effectors ($n = 22$). Statistically significance is analyzed with unpaired *t*-test (two-tailed $^{**}P < 0.01$). **b** pTM score of 316 homologous effectors against corresponding reference *P. infestans* effectors are plotted (left). Average pTM score of HR positive, negative, and 316 homologous effectors presented as black and gray bar (right). Average pTM score of HR positive effectors was not significantly different from HR negative effectors. Statistically significance is analyzed with unpaired *t*-test. **c**–**e** Predicted effector domain structures of PiAvr1 (**c**), PiAvr8 (**d**), and PiAvramr1 (**e**). The WY domain is represented by four α-helix units. The amino acid conservation scores of structures were calculated in ConSurf server using sequence alignment of HR-positive effectors.

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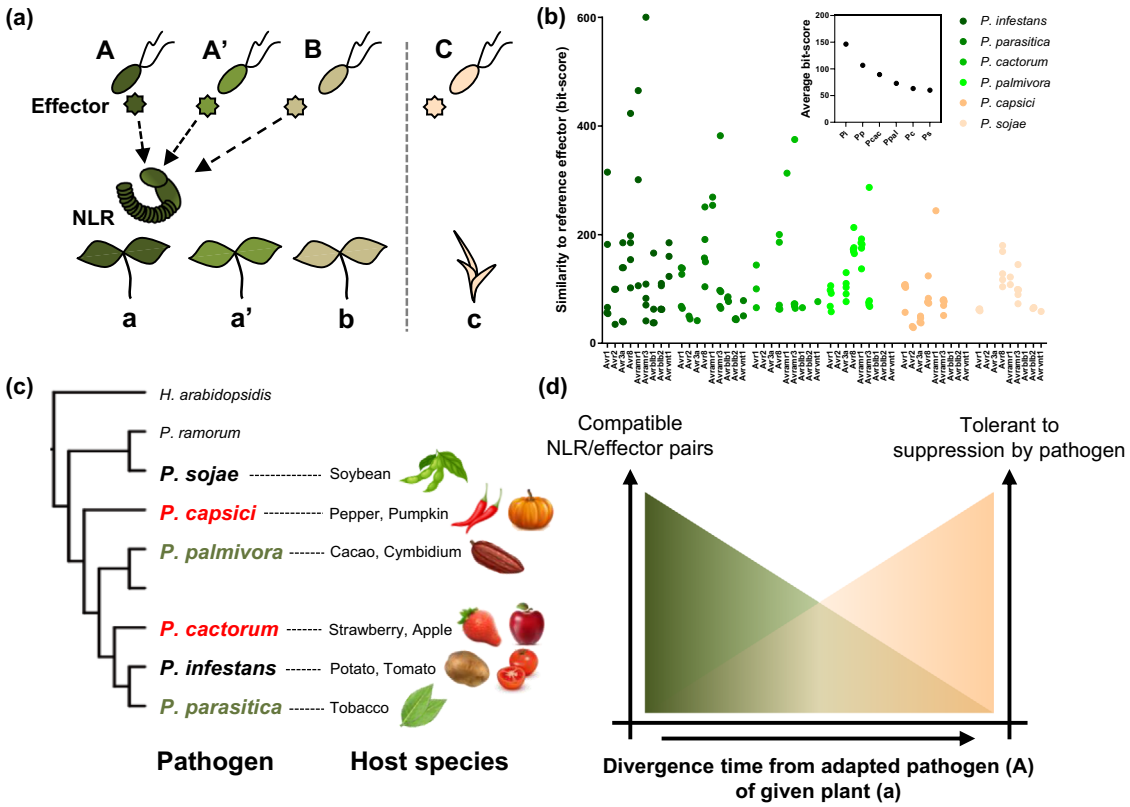

Fig. 5 | NLR-mediated recognitions of conserved effectors would contribute to resistance against a wide range of pathogens. a Pathogens diverged from common ancestral species adapt to its host plant species (with the same color) and this adaptation is accompanied with the changes in repertoire and sequence of effectors. However, effectors are conserved between the recently diverged pathogen species. Thus, a portion of conserved effectors could be recognized by same NLRs. **b** Bit-scores obtained by comparing homologous effectors (69 cloned effectors, *Avramr3* and its homologs are included) against each reference avirulence effector of *P. infestans* are presented as dot graph, and average bit-score of whole tested effectors of each species is presented in a small boxed graph (above). Conservation of *P. infestans* reference avirulence effectors across the *Phytophthora* species gradually decreases with increasing divergence time from *P. infestans*. **c** Resistance genes against most *Phytophthora* species have never been identified (red: no NLRs were reported; green: few NLRs were reported) though they cause serious disease in various crops. **d** Considering that conservation of effectors decreased with increasing of divergence time between pathogen species, the number of compatible NLR/effector pairs would also similarly decrease. Simultaneously, pathogens are less-likely to suppress NLRs derived from evolutionarily distant plants from its host species.

an alternative strategy for identifying functional plant resistance genes against pathogens that pose a threat to various crops, instead of the forward genetics breeding program that requires diverse genetic resources and long-term planning[27]. In addition, it is worth noting that resistance genes against most *Phytophthora* species, including *P. cactorum* and *P. capsici*, have not yet been identified.

NLRs recognizing effectors of broad-spectrum pathogens have a potential to be exploited to confer durable resistance in crops

The conserved NLRs and effectors are thought to have originated from common ancestral plant and pathogen species that have struggled with each other in the past. As plant species diverge, each pathogen species also diverges and adapts to each host plant species. This evolutionary process is accompanied by changes in repertoires of NLRs and effectors (Fig. 5a). A previous study suggested that the relative contribution of NLRs to 'how NHRs are mediated' depends on the evolutionary distance between host and nonhost plants of the given pathogen species[5]. Similarly, this study showed that plants are possibly recognize effectors of pathogens which are recently diverged from the adapted pathogen of a given plant (pathogen A in Fig. 5a), because a similar repertoire of effectors are conserved in recently diverged pathogen species (Fig. 5a–c). Moreover, the number of functional NLRs that recognize effectors of evolutionarily distant pathogens (pathogen B or C in Fig. 5a) would decrease proportionally with the divergence time between pathogen species.

Simultaneously, the NLRs that function against pathogen species that are more divergent from the adapted pathogen of a given plant species might be less possibly to be overcome by the pathogen (Fig. 5a and d). Indeed, among the tested *Solanum* NLRs, only *Rpi-amr1*[43] and *Rpi-amr3*[20] conferred significant resistance against *P. parasitica*, although six of the nine tested *Solanum* NLRs induced cell death against the cognate effector homologs of *P. parasitica* (Figs. 2, 3). Considering that *S. americanum* is evolutionarily more distant from potato compared to the other wild *Solanum* species such as *S. demissum* or *S. bulbocastanum*, we could assume that *P. parasitica* is relatively well adapted to potato and its closely related species but not to *S. americanum*. Thus, it may enable *P. parasitica* to colonize *N. benthamiana* expressing NLRs derived from closely related wild *Solanum* species, despite possessing multiple effectors that could be recognized by corresponding *Solanum* NLRs. Moreover, we observed a similar phenomenon that *P. infestans* was able to suppress *Rpi-blb2* of *S. bulbocastanum* but could not suppress *Rpi-blb2* homolog (*CaRpi-blb2a*) derived from chili pepper[48], which plant is more diverged from potato compared to *S. bulbocastanum*. In this context, introducing NLRs from the evolutionarily more distant plant species could be a promising strategy for conferring durable resistance against pathogens adapted to a target crop.

Disparity between HR cell death against effectors and resistance against pathogens

All the tested *Solanum* NLRs, except for *Rpi-blb2*, were able to recognize effectors of multiple *Phytophthora* species (Fig. 2a). However, only *R1*, *R8*, and *Rpi-amr1* conferred resistance to multiple *Phytophthora* pathogens (Figs. 2b, 3). These disparities between HR cell death and resistance phenotype may be due to the complexity of the virulence mechanisms of pathogen. When pathogens infect plants, they secrete hundreds of effectors into plant cells. Hence, while certain *Solanum* NLRs could induce HR cell death against *Phytophthora* species by recognizing the conserved effectors of pathogens, they may fail to confer resistance against the particular pathogen if the pathogen is able to suppress immune response of *N. benthamiana* by using numerous other effectors that target the essential components for NLR-mediated immune signaling[49], such as NRC, or by directly suppressing the transferred sensor NLR. Furthermore, we cannot exclude the possibility that pathogens would transcriptionally regulate recognizable effectors or suppress them from being secreted, because the experiments performed in this study were mostly based on Agrobacterium-based overexpression. In addition, we observed increased lesions of *Phytophthora* pathogen in NLR-expressed *N. benthamiana* leaves in some cases (Fig. 2b). We assume that both NLRs (*R8* and *Rpi-blb2*) cannot function as resistance gene (because *R8* were not able to induce cell death against *PpalAvr8* and no *Avrblb2* in *Pc* as shown in Fig. 2a) against each pathogen but the unknown physiological changes occurred by over-expressing those NLRs provide more infectious environment in *N. benthamiana* leaves to each pathogen.

Therefore, obtaining a more comprehensive understanding of the detailed molecular mechanisms and host proteins that are associated with each NLR-mediated resistance and pathogen effectors would provide a strategy to harness the potential of these HR-inducing *Solanum* NLRs that cannot confer resistance in this study.

Transferring NLRs to evolutionarily distant plant species

In order to transfer functional NLRs to distantly related plant species, genetic compatibility between NLRs and their signaling components, such as NLRs and guardee/decoy proteins or helper and sensor NLRs, should be considered. Indeed, several studies have reported that each component of EDS1-PAD4-ADR1/EDS1-SAG101-NRG1 signaling complex is not fully compatible between tomato and Arabidopsis[50]. Similarly, *CaRpi-blb2a* is compatible with NRC8 of pepper but not with NRC4 of *N. benthamiana*[48].

Considering that *R1*, *R8*, and *Rpi-amr1* are NRC-dependent sensor NLRs[43,51], transferring these NLRs beyond the solanaceous plants would be a complex task. However, transferring these NLRs into other solanaceous plants could be regarded as a feasible strategy. Thus, we expect that generating *R1*/*R8*-expressing pepper/tomato or *Rpi-amr1*-expressing tobacco/eggplant would be promising strategies to fend off *P. capsici* and *P. parasitica*, respectively.

Beyond *Phytophthora* species and solanaceous plants

Similar to this study, several cases of conserved effectors recognized by similar NLRs have been reported in different kingdoms of pathogens. For instance, various CCG effectors conserved in *Albugo candida* races are recognized by *Arabidopsis WRR4A* and *WRR4B*[52], and *Avramr1/3* homologs of multiple *Phytophthora* species are recognized by *Rpi-amr1/3*[20,43]. Additionally, multiple effector families of *Pseudomonas syringae*, a bacterial pathogen, are also recognized by corresponding Arabidopsis NLRs[19]. In this context, we anticipate that the NLR-mediated recognition of conserved effectors between plant and pathogen species would also be applied beyond the Solanaceae-*Phytophthora* interaction. Therefore, identification of more NLRs recognizing broadly conserved effectors would contribute to reducing damage caused by a wide range of continuously evolving plant pathogenic microorganisms beyond *Phytophthora* species.

Methods

Plant materials and growth conditions

N. benthamiana were grown in a controlled chamber at 24–26 °C and 40–60% relative humidity with a 16-h light/8-h dark cycle. Commercial topsoil for general/horticultural (product of Seoulbio®, http://www.seoulbio.co.kr/product/product_04_01.asp) was used and additional nutrients were not added.

Maintenance conditions for pathogenic oomycete materials and sporulation methods

P. infestans (T30-4) were grown on rye sucrose agar plate media in a dark chamber at 17–19 °C for 7–9 days. *P. parasitica* (40164, 40402, 40906 strains were provided from RDA, Korea), *P. cactorum* (40166, 40183), *P. palmivora* (40402, 40410), *P. capsici* (40476) and *P. sojae* (48989, 40468, 40412) were grown on V8 juice plate media in dark chamber at 23–25 °C for 7–9 days. Except for *P. infestans*, 7 days old plate were flooded with 4 ml of TDW (triple distilled water) and placed under continuous fluorescent light during 2 days (1 days for *P. capsici*) for the sporulation (in case of *P. infestans*, 7 ~ 9 days old pate were flooded with chilled 8 ml of TDW and incubated at 4 °C for an hour and additional 30 min without sporulation step). Zoospores were harvested from flooded (pouring 6–8 ml of chilled TDW and rubbing mycelia) plates after being incubated at 4 °C for an hour and additional 30 min at 25 °C before used for inoculation.

Extraction of effector homologs against functional avirulence effectors

Protein sequences of the 12 oomycete genomes were downloaded from the NCBI GenBank database (*P. cactorum* [GCA_016864655.1], *P. megakarya* [GCA_002215365.1], and *P. palmivora* var. *palmivora* [GCA_002911725.1]) and the Oomycete Gene Order Browser (OGOB, https://ogob.ie/v1/gob/data.html)[53]. The full list of species information used in this study was described in Supplementary Table 2. The longest primary protein sequences were clustered into orthogroups by OrthoFinder v2.5.4 with -S diamond_ultra_sens parameter[54]. The known functional RXLR effectors in *P. infestans* were collected from a literature survey and UniProtKB/Swiss-Prot database[55] using an "Avr" keyword search. To identify known effector orthogroups, a similarity search was performed between the 12 known effectors as query and the 12 oomycetes proteins as subject using BLASTp with a cutoff value of query coverage above 80%. Orthogroups with proteins matched in the similarity search were used for downstream analysis. The effector signature was identified based on a previously reported study[56]. Briefly, the signal peptide was predicted using SignalP v4.1[57] with sensitive mode (-u 0.34 -U 0.34). The RXLR, highly degenerate RXLR (hdRXLR), and EER motifs were searched in the first 100 amino acids of protein sequence as the following regular expressions: [RQGH]XLR for RXLR, [RKHGQ][X] {0,1} [LMYFIV][RNK] for hdRXLR, and [DE][DE][KR] for EER[56]. The WY domain was predicted using HMMer v3.1b[58] with the best 1 domain score above 0 based on an HMM file constructed by 721 protein sequences obtained from previous study[59].

Phylogenetic analyses and classification of *Phytophthora* RxLR effectors

To identify effector homologs, protein sequences of orthogroups with similarity to known effectors were aligned using MAFFT v7.407 with --maxiterate 1000 --globalpair[60], and the positions containing gaps in >30% were trimmed using trimAl[61] v1.4.rev22 with -gt 0.3 parameter. A maximum-likelihood phylogenetic tree was reconstructed using IQ-TREE[62] v1.6.12 with -bb 1000 -alrt 1000 -safe parameters. The best-fit substitution model JTT + F + R7 was selected by ModelFinder[63] implemented in IQ-TREE. The avirulence effector clades were classified according to ultra-fast bootstrap value[64] over 95% and taxonomic distribution. The phylognetic distances from each reference avirulence effector in *P. infestans* to its nearest-neighbor homologs in other

Phytophthora species were calculated and visualized by a previously reported Python script (https://github.com/slt666666/Phylogenetic_distance_plot2)[65].

Structure-based clustering of homologs of *P. infestans* avirulence effectors

Effector domains were extracted from a defined alignment for each phylogenetic-based avirulence effector clade. The effector domain region was defined as the sequence following the RxLR or RxLR dEER motifs based on the reference avirulence protein of each clade. Proteins with a miss rate exceeding 80% in the alignment of the effector domain region were filtered out for downstream analysis. Effector domain structures were predicted using Colabfold_batch v1.5.2[66] with --num-recycle 12 --num-seeds 3 parameters. For each protein, the best model defined by the pLDDT score was selected, and structures with a pLDDT score of < 50 were considered inaccurate and filtered out. Out of a total of 514 effector homologs, 473 successfully predicted the structures of effector domain. Using 473 predicted protein structures, structure-based clustering was performed in three main steps: (1) all-against-all alignment, (2) network construction, and (3) community detection. First, all predicted structures were aligned using US-align v20230609[67] with default parameter, and the TM-score and alignment length were calculated for each pair. Next, a protein structure network was constructed based on the pairwise structural similarities. We connected two protein structures in the network if the average of their reciprocal TM-score and alignment coverage were both > 0.5. Finally, the structure clustering was performed using Louvain community detection algorithm[68] implemented in networkx v3.1[69]. Cytoscape v3.9.1[70] was used for network visualization.

Comparison of sequence and structure similarity between avirulence effectors and their homologs

We calculated the sequence and structural similarity of the effector domain between avirulence effectors of *P. infestans* and their homologs in each avirulence effector clade. For sequence similarity analysis, bit-scores were calculated using blastp 2.15.0+ and normalized them by dividing by the bit-score of each reference avirulence effector. For structure similarity analysis, we used the average score of the reciprocal TM-scores calculated from structure clustering.

Selection of sequence- and structure-based homologous effectors for cloning

Eight avirulence effectors of *P. infestans* including, Avr1, Avr2, Avr3a, Avr8, Avramr1, Avrblb1, Avrblb2, and Avrvnt1 were queried to protein database of *P. parasitica, P. cactorum, P. palmivora, P. capsici*, and *P. sojae* reference strains. We selected effectors with higher similarity against each reference avirulence effector by sorting bit-score and pTM score (cut off was normalized bit-score > 0.2; pTM > 0.35). At least one and max five of each family were targeted.

Cloning of homologous avirulence effectors into expression vector

Effector domains (after RxLR dEER motif, in case of no dEER, just after RxLR) were defined based on protein sequence database of each *Phytophthora* species (*P. infestans, P. parasitica, P. cactorum, P. palmivora, P. capsici, and P. sojae*). Then, predicted coding sequence (from start to end codons) of each effector domain were amplified using Primestar GXL (TAKARA®) enzyme from genomic DNA of each *Phytophthora* species. Among the initial candidates, 69 were successfully amplified from the genomic DNA of each *Phytophthora* species. Each amplicon was cloned into the pICH31160 vector (pKW, PVX virus coat protein promoter) using the ligation-independent cloning (LIC) method and the cloned sequences were validated by bi-directional sequencing[71,72]. Total 69 effectors were successfully cloned into expression vector.

Screening of *R* / *Avr* interactions through agroinfiltration

Agrobacterium strain GV3101 (pMP90) containing each construct was cultured in YEP medium for 1 day. Pellets were collected by centrifugation (10 min, 3000 rpm), resuspended into agroinfiltration buffer (10 mM MES, 10 mM $MgCl_2$, 150 µM acetosyringone, pH 5.6). *Agrobacterium* expressing *Solanum R* genes and candidate effectors were mixed into 1:1 ratio after being adjusted to OD_{600} at 0.7 and 0.3 respectively. 4 ~ 5 weeks old *N. benthamiana* leaves were used for agroinfiltration. We observed infiltrated leaves until the 5 dpi, and took images using Fluorescence in vivo imaging system (FOBI, CELLGENTEK). Cell death phenotypes were simply recorded as positive or negative with picture.

Detached leaf assay using *Solanum* NLRs against *Phytophthora* species

For the detached leaf assay, the abaxial side of the detached leaves of 4 ~ 5 weeks old *N. benthamiana* were inoculated with 10 ~ 11 µl of zoospore solution ($1.0 \sim 2.0 \times 10^5$ zoospores/ml) and placed in SPL® rectangular plates with wet tissue paper to maintain 100% relative humidity. Inoculated leaves were incubated at 23–25 °C in dark chamber until further examination. We observed until 2 ~ 4 dpi, and took images using FOBI machine.

Generation of transgenic plants

Thirty days old *N. benthamiana* leaves were cut into 3 cm x 3 cm pieces and sterilized with NaClO (0.1%) solution for 5 min. *Agrobacterium* strain GV3101 (pMP90) containing *R1*, *R8*, and *Rpi-amr1* were cultured in YM media (D-mannitol: 10 g/L, yeast extract: 0.4 g/L, K_2HPO_4: 0.328 g/L, $MgSO_4$ $7H_2O$: 0.2 g/L, NaCl: 0.1 g/L) for 24 h, respectively, and co-incubated with cut leaves for 20 min in shaking incubator. Incubated leaves were wiped and moved to MS media (with acetosyringon) and co-cultured for 2 days in controlled chamber (23–25 °C, dark condition). Incubated leaves were washed out for 3 times with washing buffer (liquid MS media with benzyladenine and cefotaxime, pH 5.7) and moved to MS media (3% sucrose, kanamycin: 100 mg/L, BAP cytokinin 1.5 mg/L) for 4 weeks under the continuous light. Shoots were cut from the callus and moved to MS media (without cytokinin) and cultured until the root generation.

Root infection assay using *Solanum* NLRs against *Phytophthora* species

For the root infection assay, 3 ~ 4 weeks old *N. benthamiana* plants expressing *Solanum R1*, *R8*, or *Rpi-amr1* were inoculated with 500 = ul of zoospore solution ($1.0 \sim 2.0 \times 10^5$ zoospores/ml) of *Phytophthora* species and placed in controlled chamber (23–25 °C,16 h/8 h of light/dark cycle) until the further examination. Phenotypes were categorized into three groups (healthy; wilt: wilting leaves; dead: shrink, and main stem bends) until the wild type plants were completely dead (takes 4 ~ 9 days). Unsterilized commercial topsoil for general/horticultural (product of Seoulbio®, http://www.seoulbio.co.kr/product/product_04_01.asp) were used and additional nutrition was not added.

Sequence and structural comparisons of cloned homologous avirulence effectors

Sequences and structures of cloned effectors from the Avr1, Avr8, and Avramr1 clades were aligned based on their respective reference avirulence proteins. Sequence alignment was performed using MAFFT v7.407 with --maxiterate 1000 --globalpair[60]. Aligned sequences were visualized by Jalview v2.11.3.2[73]. To confirm the evolutionary conservation of the sequence, a conservation score was calculated based on the aligned sequences of HR-positive effectors and each reference Avirulence protein using the ConSurf server[74]. Comparison of protein structures was performed using the jFATCAT[75]-flexible mode of pairwise structure alignment in the RCSB PDB database and visualized using PyMol v2.5.5[76].

Statistical information

Statistics in this paper were performed on the all replicates for Figs. 2b, c, 3c–f, 4a, b, and Supplementary Figs. 7–12. For the all cases, we performed unpaired *t*-test, using a *P*-value threshold for a significance of two-tailed $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Individual *P*-values are presented in Supplementary Data 4.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Data supporting the findings of this work are available within the paper and its Supplementary Information files. All the resources described are available from the corresponding authors upon request. Source data are deposited in Figshare (https://doi.org/10.6084/m9.figshare.26421439).

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Author contributions

S.O., M.K., and D.C. designed the research. S.O., H.K., and T.K. performed the experiments. S. O., M.K., J.K. analyzed the data. S.O., and M.K. draw the figures. S.O., M.K., and D.C. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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