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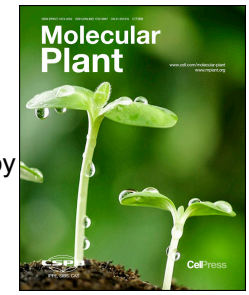

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A potato late blight resistance gene protects against multiple *Phytophthora* species by recognizing a broadly conserved RXLR-WY effector

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Running Title: *Rpi-amr3* against multiple *Phytophthora* diseases

Short Summary: *Rpi-amr3* was cloned from *Solanum americanum*, it confers potato late blight resistance against multiple *Phytophthora infestans* isolates. Here we identified its corresponding Avr effector AVRamr3. AVRamr3 is widely conserved in *P. infestans* isolates as well as other *Phytophthora* species. The recognition of this conserved effector by Rpi-amr3 led to resistance against other *Phytophtora* pathogens like *P. parasitica* and *P. palmivora*.

Abstract

Species of the genus *Phytophthora* - the plant killer - cause disease and reduce yields in many crop plants. Although many *Resistance to Phytophthora infestans* (*Rpi*) genes effective against potato late blight have been cloned, few have been cloned against other *Phytophthora* species. Most *Rpi* genes encode nucleotide-binding, leucine-rich repeat- containing (NLR) immune receptor proteins, that recognize RXLR effectors. However, whether NLR proteins can recognize RXLR effectors from multiple *Phytophthora* species has rarely been investigated. Here, we identified a new RXLR-WY effector AVRamr3 from *P. infestans* that is recognized by *Rpi-amr3* from a wild Solanaceae species *Solanum americanum*. Rpi-amr3 associates with AVRamr3 *in planta*. AVRamr3 is broadly conserved in many different *Phytophthora* species, and the recognition of AVRamr3 homologs by Rpi-amr3 activates resistance against multiple *Phytophthora* pathogens, including the tobacco black shank disease and cacao black pod disease pathogens *P. parasitica* and *P. palmivora*. *Rpi-amr3* is thus the first characterized resistance gene that acts against *P. parasitica* or *P. palmivora*. These findings suggest a novel path to redeploy known *R* genes against different important plant pathogens.

Key words: Rpi-amr3, AVRamr3, potato late blight, *Phytophthora* disease, RXLR-WY effector, *Solanum americanum*

Introduction

Species in the oomycete genus *Phytophthora* cause many devastating plant diseases. For example, *P. infestans*, *P. parasitica*, *P. cactorum*, *P. ramorum*, *P. sojae*, *P. palmivora* and *P. megakarya* cause potato and tomato late blight, tobacco black shank disease, strawberry crown and leather rot, sudden oak death, soybean root and stem rot, and cacao black pod disease, respectively. *P. infestans* and *P. sojae* infect few plant species, while others like *P. parasitica*, *P. ramorum* and *P. palmivora* have a broad host range (Kamoun et al., 2015).

Plant immunity involves detection of pathogen-derived molecules by either cell-surface pattern recognition immune receptors (PRRs) or intracellular nucleotide-binding domain, leucine-rich repeat-containing (NLR) immune receptors, that activate either pattern-triggered immunity (PTI) or effector-triggered immunity (ETI), respectively (Jones and Dangl, 2006). So far, more than 20 *Resistance to P. infestans* (*Rpi*) genes were cloned from wild *Solanum* species that confer resistance against potato late blight (Vleeshouwers et al., 2011). Several Resistance genes against *P. sojae* (*Rps)* have also been mapped in different soybean accessions, and a few were cloned [4,5]. In tobacco, the black shank resistance genes *Phl*, *Php*, and *Ph* were genetically mapped but not yet cloned; these confer race-specific resistance to *P. parasitica* (aka *P. nicotianae*) isolates (Gallup and Shew, 2010; Bao et al., 2019). For *P. palmivora*, some resistant cacao (*Theobroma cacao*) accessions were identified, but no dominant *R* genes have been defined or cloned (Thevenin et al., 2012). In summary, apart from *Rpi* genes, very few *R* genes against *Phytophthora* pathogens have been cloned.

Solanum americanum and *Solanum nigrum* are wild Solanaceae species and are highly resistant to *P. infestans* (Witek et al., 2016; Witek et al., 2021). Two *Rpi* genes of coiled-coil (CC) type,

Rpi-amr3 and *Rpi-amr1,* were cloned from different *S. americanum* accessions; both confer late blight resistance in cultivated potato (Witek et al., 2016; Witek et al., 2021). *S. nigrum* is a hexaploid species, that was thought to be a "non-host" plant of *P. infestans*. No *Rpi* gene had been cloned from *S. nigrum* until we reported the functional *Rpi-amr1* homolog *Rpi-nig1* (Witek et al., 2021).

In oomycetes, the recognized effectors are usually secreted RXLR (Arg-X-Leu-Arg, X represents any amino acid)-EER (Glu-Glu-Arg) proteins that are translocated into plant cells (Rehmany et al., 2005; Wang et al., 2019). Dozens of *Avirulence* (*Avr*) genes encoding recognized effectors from *Phytophthora* species have been identified and they are typically fast-evolving and lineage-specific molecules (Jiang et al., 2008). Recently, AVRamr1 (PITG_07569), the recognized effector of Rpi-amr1 was identified by a cDNA pathogen enrichment sequencing (PenSeq) approach (Lin et al., 2020). Surprisingly, AVRamr1 homologs were identified from *P. parasitica* and *P. cactorum* genomes and both are recognized by all Rpi-amr1 variants (Witek et al., 2021). Similarly, AVR3a-like effectors were found in different *Phytophthora* species, including *P. capsici* and *P. sojae*, and the recognition of AVR3a homologs correlates with *P. capsici* or *P. sojae* resistance in *Nicotiana* species and soybean (Shan et al., 2004; Vega-Arreguín et al., 2014). Additionally, AVRblb2 homologs from *P. andina* and *P. mirabilis* trigger HR with Rpi-blb2 (Oliva et al., 2015). Remarkably, a single N336Y mutation in R3a expands its recognition specificity to a *P. capsici* AVR3a homolog (Segretin et al., 2014). These reports raise intriguing questions. Could RXLR effectors be widely conserved molecules among different *Phytophthora* species? Could these effectors be recognized by the same plant immune receptor? Of particular interest, could such effector recognition capacity enables disease resistance?

Here we show *Rpi-amr3* confers resistance to all tested late blight isolates in both field and lab conditions. We also identified AVRamr3, a novel AVR protein from *P. infestans*, by screening an RXLR effector library. AVRamr3 is a broadly conserved effector found in thirteen different *Phytophthora* species. We also show functional *Rpi-amr3* genes are widely distributed among *S. americanum* and *S. nigrum* accessions. The recognition of AVRamr3 not only enables resistance to a wide range of *P. infestans* isolates, but also to other economically important *Phytophthora* pathogens such as tobacco black shank disease and cacao black pod disease pathogens *P. parasitica* and *P. palmivora*. *Rpi-amr3* is the first reported *R* gene that confers resistance against *P. parasitica* and *P. palmivora*.

Results:

Rpi-amr3 confers late blight resistance in lab and field

Rpi-amr3 was cloned by SMRT-RenSeq and reported to confer resistance against two *Phytophthora infestans* isolates 88069 and 06_3928A in a diploid potato line (Line 26, Solynta B.V.) (Witek et al., 2016) in lab conditions. However, whether *Rpi-amr3* confers broad-spectrum and field resistance to late blight was not reported.

To address this, we transformed *Rpi-amr3* with its native promoter and terminator into a favoured UK potato cultivar *cv.* Maris Piper. Two lines (SLJ24895-5C and SLJ24895-9A) (Figure S1B) were selected for a field experiment in 2017, and SLJ24895-5C was further tested in the field in 2018 (Figure 1A). These field trials indicate that *Rpi-amr3* confers protection against potato late blight in field conditions, while the wild-type Maris Piper control lines were infected completely within ~3 weeks once disease symptoms appeared (Figure 1A and 1B). As a result, the tuber yield of the *Rpi-amr3* transgenic lines was significantly higher than the wild-type Maris Piper lines (Figure 1C and 1D). To determine the *P. infestans* genotypes present in the field trial, the infected leaves were sampled and genotyped by SSR markers, most of the isolates corresponded to a dominant UK strain 6_A1 (aka Pink6) (Table S1).

To further evaluate the resistance spectrum of *Rpi-amr3*, we performed detached leaf assay (DLA) on SLJ24895-5C, with wild-type Maris Piper and *Rpi-amr1* transgenic Maris Piper as controls. Seventeen *P. infestans* isolates with different origins and races were tested (Figure 1E and Table S2). Our results show that *Rpi-amr3* confers resistance against all tested isolates in potato in lab conditions but with different efficacy, and *Rpi-amr3* mediated resistance is weaker than *Rpi-amr1* in potato (Figure 1E). We also generated *Rpi-amr3* stably transformed *N. benthamiana* lines. Two homozygous T2 lines #13.3 and #16.5 were tested with nine *P. infestans* isolates. Both *Rpi-amr3* transgenic *N. benthamiana* lines confer complete resistance to all tested *P. infestans* isolates (Table S2).

These data show that *Rpi-amr3* confers potato late blight resistance in both lab and field conditions.

Avramr3 encodes a conserved RXLR-WY effector protein

To identify the effector recognized by Rpi-amr3, we screened an RXLR effector library (Rietman, 2011; Lin et al., 2020) of 311 RXLR effectors by *Agrobacterium tumefaciens*-mediated co-expression with Rpi-amr3 in *N. benthamiana*. Most of these effectors (296/311) do not induce a hypersensitive response (HR) when expressed alone or co-expressed with Rpi-amr3; Fourteen effectors are auto-active in *N. benthamiana*, and we found PITG_21190 specifically induces an HR with *Rpi-amr3* (Figure 2A and Table S3), therefore we concluded that PITG_21190 is *Avramr3*. *Avramr3* encodes a 339-aa protein with a signal peptide followed by RXLR, EER motifs and four predicted WY motifs (Win et al., 2012) (Figure 2C). To characterize the expression profile of *Avramr3*, eight *P. infestans* isolates (T30-4, 88069, NL01096, 06_3928A, 6_A1, EC1, US23 and 99183) were used to inoculate a susceptible potato cultivar Maris Piper, RNA was isolated 2 days after the infection for RT-PCR. Our data shows that *Avramr3* are expressed in all eight isolates at 2 days days after infection (Figure S1A).

Many RXLR effectors are encoded by fast-evolving, multiple-member family genes with extensive sequence polymorphism, such as the *Avr2* and *Avrblb2* families (Gilroy et al., 2011; Oliva et al., 2015). To study the sequence polymorphism of *Avramr3*, seventeen additional *Avramr3* homologs from eleven isolates were identified from published databases (KR_1, 3928A, EC1, 6_A1 and US23) (Lee et al., 2020; Lin et al., 2020) or cloned by PCR (EC1, NL01096, NL14538, 88069, PIC99183 and PIC99177) (Fig. S2). The sequence alignment shows *Avramr3* is a highly conserved RXLR effector among *P. infestans* isolates, with only two polymorphic amino acids found among the eighteen AVRamr3 homologs (Figure S2).

To define the domain responsible for recognition by Rpi-amr3, ten truncated *Avramr3* fragments were fused with HIS-FLAG tags and cloned into an expression vector with 35S promoter (T1 to T10, Figure 2C, Figure S3), and transiently co-expressed with *Rpi-amr3* in *N. benthamiana*. Six AVRamr3 truncations (T1, T2, T5, T6, T7 and T9) cannot be recognized by Rpi-amr3 (Figure 2B). The protein levels of HIS-FLAG tagged T6 and T7 are lower than others (Figure S3A), therefore we generated GFP-tagged constructs. The expression of T6-GFP is comparable with AVRamr3-GFP, but T7-GFP is not stable (Figure S3B and C). We found four AVRamr3 truncations (T3, T4, T8 and T10) can be recognized by Rpi-amr3. T10 (111-240 aa) which carries the 2nd and 3rd WY motifs is the minimal region to be recognized by Rpi-amr3 but not the adjacent T9 protein (130-258 aa) (Figure 2B). This suggests these 130 amino-acids of AVRamr3 T10 are sufficient for recognition by Rpi-amr3 and initiation of HR.

Rpi-amr3 is dependent on the helper NLRs NRC2, NRC3 and NRC4

In Solanaceae, the functionality of many CC-NLR proteins requires helper NLR proteins of the NRC class (Wu et al., 2017). To test if *Rpi-amr3* is NRC-dependent, we co-expressed *Rpi-*

amr3 and *Avramr3* in NRC knockout *N. benthamiana* lines (nrc2/3_1.3.1, nrc4_185.9.1.3, nrc2/3/4_210.4.3)(Adachi et al., 2019; Wu et al., 2020; Witek et al., 2021), as with wild-type *N. benthamiana*. We found HR on the nrc2/3_1.3.1 and nrc4_185.9.1.3 knockout lines, but not the nrc2/3/4_210.4.3 knockout lines. Similarly, only nrc2/3/4_210.4.3 knockout lines show susceptibility to *P. infestans* after *Rpi-amr3* transient expression (Figure S4). These data suggest both *Rpi-amr3*-mediated effector recognition and resistance is supported by either NRC2, NRC3 or NRC4.

Rpi-amr3 associates with AVRamr3 *in planta*

To date, most Rpi proteins recognize their cognate effectors in an indirect manner, apart from the RB and IPI-O effectors (Chen et al., 2012; Zhao and Song, 2021). To test the interaction between Rpi-amr3 and AVRamr3, Rpi-amr3::HA and AVRamr3::HIS-FLAG epitope-tagged constructs were generated and transiently co-expressed in nrc2/3/4 knockout *N. benthamiana* leaves to avoid cell death. Protein was then extracted, and bi-directional co-immunoprecipitation (Co-IPs) were performed. These co-IPs indicate that Rpi-amr3 associates with AVRamr3 bidirectionally (Figure 2D). We also tested their interaction using a split-luciferase assay. Rpi-amr3::Cluc and AVRamr3::Nluc constructs were generated and transiently expressed in the nrc2/3/4 knockout *N. benthamiana*. Luciferase signal was only detected when Rpi-amr3::Cluc and AVRamr3::Nluc were co-expressed (Figure 2E), but not in the negative controls. These data suggest Rpi-amr3 associates with AVRamr3 *in-planta*, though do not exclude the possible involvement of additional proteins.

***Avramr3* orthologs occur in multiple *Phytophthora* species**

To study the evolution of *Avramr3* in *Phytophthora* species, we searched for *Avramr3* homologs from published *Phytophthora* and *Hyaloperonospora arabidopsidis* (Hpa) genomes. Surprisingly, we found *Avramr3* homologs in many *Phytophthora* genomes, including *P. parasitica*, *P. cactorum*, *P. palmivora*, *P. pluvialis*, *P. megakarya*, *P. lichii*, *P. ramorum*, *P. lateralis*, *P. sojae*, *P. capsici*, *P. cinnamomi*, and in *H. arabidopsidis*. Most of the *Avramr3* homologs are located at a syntenic locus (Figure 3A). Notably, the *P. infestans Avramr3*-containing contig was not fully assembled, it lacks sequences on the 5' side of *Avramr3* (Fig. 3a).

To test if those AVRamr3 homologs from different *Phytophthora* species are also recognized by Rpi-amr3, we synthesized and cloned them into an expression vector with the 35S promoter, and performed transient expression assays in *N. benthamiana*. Expressing the effectors alone does not trigger HR in *N. benthamiana* (Figure 3B), but AVRamr3 homologs from *P. parasitica*, *P. cactorum*, *P. palmivora*, *P. megakarya*, *P. lichii*, *P. sojae*, *P. lateralis* and *P. pluvialis* can induce HR when co-expressed with Rpi-amr3 respectively. The AVRamr3 homolog from *P. cinnamomi* triggers a weaker HR compared to other recognized AVRamr3 homologs, and the AVRamr3 homologs from *P. ramorum*, *P. capsici*, and *H. arabidopsidis* (Figure 3C) do not trigger Rpi-amr3-dependent HR. All these AVRamr3 homologs carry multiple WY motifs, but many polymorphic amino acids are present among these homologs. We then predicted the structures of all AVRamr3 homologs by AlphaFold, and compared their T10 regions with AVRamr3. We found that the T10 regions of most recognized AVRamr3 homologs fold into a structure similar to AVRamr3 from *P. infestans* (Figure S5). These data suggest that Rpi-amr3 recognizes a conserved fold of these AVRamr3 homologs from different *Phytophthora* species.

To test if other recognized AVRamr3 homologs also directly interact with Rpi-amr3, we performed co-immunoprecipitation and split-luciferase assays in nrc2/3/4_210.4.3 knockout lines. We found all the recognized AVRamr3 homologs associate with Rpi-amr3 by co-immunoprecipitation, although with varied affinity. Two unrecognized AVRamr3 homologs from *P. capsici* and *H. arabidopsidis* do not associate with Rpi-amr3. However, two unrecognized or weakly recognized AVRamr3 homologs from *P. ramorum* and *P. cinnamomi* also associate with Rpi-amr3, and the unrecognized AVRamr3-T9 truncation shows a weak association (Figure S6). In contrast, the output of split-luciferase assay is fully consistent with the HR assay (Figure S7). These data indicate that an *in-planta* receptor-ligand association is necessary but might not be sufficient for the activation of Rpi-amr3 and triggering of HR.

Rpi-amr3 confers resistance to multiple _P. parasitica_ and _P. palmivora_ strains in _N. benthamiana_

Previously, we showed that *Rpi-amr3* confers resistance against potato late blight caused by multiple *P. infestans* isolates (Figure 1 and Table S2). Its broad effector recognition capacity suggested *Rpi-amr3* might confer resistance against additional *Phytophthora* pathogens.

To test this hypothesis, two *Rpi-amr3* stable transformed *N. benthamiana* T2 lines #13.3 and #16.5 were used to evaluate *P. parasitica* and *P. palmivora* resistance. Both these pathogens have a wide host range, including the model plant *N. benthamiana*.

Six *P. parasitica* isolates (R0, R1, 310, 666, 329 and 721) were tested on *N. benthamiana* carrying *Rpi-amr3*, and on wild-type *N. benthamiana* plants as negative control. These plants were phenotyped for wilting symptoms. A suspension of zoospores was used for root

209 inoculation. We found both *N. benthamiana – Rpi-amr3* lines were resistant to three *P.*
210 *parasitica* isolates R1, 666 and 721, but were susceptible to R0, 310 and 329 (Figure 4). In
211 summary, *Rpi-amr3* confers resistance against three out of six tested *P. parasitica* isolates in
212 *N. benthamiana*.
213
214 To study the *PpAvramr3* polymorphism in different *P. parasitica* isolates, the *PpAvramr3*
215 homologs from the six *P. parasitica* isolates were PCR amplified, sub-cloned and sequenced.
216 *PpAvramr3* homologs were identified from R0, R1 and 310, 666 and 721, but not from 329
217 (Figure S8). To test if Rpi-amr3 can recognize other PpAVRamr3 alleles, the T10 region of
218 Pp666-c2 was synthesized and cloned into an expression vector. The Pp666-c2 induces HR
219 when co-expressed with Rpi-amr3 (Figure S8 and S9B). These data suggest the presence of
220 recognized AVRamr3 homologs from *Phytophthora* pathogens is necessary but not sufficient
221 to induce Rpi-amr3 mediated resistance.
222
223 We tested an additional broad host range *Phytophthora* pathogen, *P. palmivora*, which causes
224 major losses on many tropical tree crops like papaya, mango, cacao, coconut and palm tree.
225 We tested seven *P. palmivora* isolates on the two *Rpi-amr3* transgenic *N. benthamiana* lines
226 by root inoculation followed by phenotyping for wilting. Wild-type *N. benthamiana* was used
227 as a control. We found *Rpi-amr3* confers resistance to three out of seven tested *P. palmivora*
228 isolates, including 7551, 7547, 7545, but not to 3914, 7548. For two other isolates 0113 and
229 3738, inconsistent results were obtained from the two *Rpi-amr3* transgenic lines (Figure 5). To
230 verify the presence of *Avramr3* homologs in these tested *P. palmivora* isolates, we PCR-
231 amplified the *Avramr3* homologs from genomic DNA of the seven *P. palmivora* isolates. All
232 the tested *P. palmivora* strains carry *PpalAvramr3* variants (Figure S9), and we found Rpi-
233 amr3 can recognize the T10 region from all these PpalAVRamr3 variants (Figure S9). Taken

234 together, *Rpi-amr3* confers resistance to at least 3/7 tested *P. palmivora* isolates in the root
235 inoculation assay.
236
237 **_Rpi-amr3_ is widely distributed in _S. americanum_ and _S. nigrum_**
238
239 Though susceptible accessions can be identified in detached leaf assays, most *S. americanum*
240 and *S. nigrum* accessions show complete resistance in the field to *P. infestans*. Previously,
241 many functional *Rpi-amr1* alleles were cloned from different *S. americanum* and *S. nigrum*
242 accessions (Witek et al., 2021).
243
244 The identification of AVRamr3 allows us to investigate the distribution of *Rpi-amr*3 from all
245 *S. americanum* and *S. nigrum* accessions. In total, 54 *S. americanum* accessions and 26 *S.*
246 *nigrum* accessions were tested by agro-infiltration with AVRamr3 for detecting functional *Rpi-*
247 *amr3*. We found 43/54 tested *S. americanum* accessions show HR after AVRamr3 agro-
248 infiltration (Figure 6A). Similarly, 21/26 tested *S. nigrum* accessions recognize AVRamr3
249 (Figure 6B).
250
251 To further investigate the sequence polymorphism of *Rpi-amr3* from different accessions, the
252 *Rpi-amr3* homologs from 15 additional accessions were extracted from PacBio RenSeq dataset
253 (Witek et al., 2021), including 12 accessions (SP2300, SP1101, SP1123, SP2273, SP3409,
254 SP2307, SP3406, SP3408, SP2272, SP3399, SP2360 and SP3400) which respond to AVRamr3
255 and 3 accessions (SP1032, SP2271 and SP2275) that do not respond to AVRamr3.
256
257 To test the functionality of *Rpi-amr3* from *S. americanum* and *S. nigrum*, *Rpi-amr3* homologs
258 were PCR amplified from gDNA of three *S. americanum* accessions SP2272, SP2273 and

SP3406, and from gDNA of two *S. nigrum* accessions SP1088 and SP1084. *Rpi-amr3* alleles (*Rpi-nig3* hereafter) were amplified from each of these two *S. nigrum* accessions and cloned into an expression vector with 35S promoter. We found all the seven *Rpi-amr3*/*Rpi-nig3* genes can recognize AVRamr3 in transient assays (Figure 6D), but not the negative control AVRamr1. Compared to Rpi-amr3 from SP1102, the amino-acid identity ranges from 82.2% to 95.7% (Figure 6C). Premature stop codons were found in *Rpi-amr3* homologs from SP2271 and SP2275 (Figure S10), which result in loss of *Rpi-amr3* function.

Taken together, these data suggest *Rpi-amr3* gene is widely distributed in diploid *S. americanum* and hexaploid *S. nigrum*, it contributes to their resistance to *P. infestans* and perhaps other *Phytophthora* pathogens.

Discussion

We show here that *Rpi-amr3* from *S. americanum* can protect potato against late blight disease in the field and confers resistance to all tested *Phytophthora infestans* isolates in lab condition. Furthermore, by screening an effector library of 311 RXLR effectors, we identified and characterized a novel effector AVRamr3 (PITG_21190) that is recognized by Rpi-amr3. Although the effector library covers nearly all expressed RXLR effectors in *P. infestans* (Lin et al., 2020), conceivably Rpi-amr3 could recognize additional RXLR effectors. AVRamr3 is highly conserved and expressed in all tested *P. infestans* isolates during infection. These findings indicate that *Rpi-amr3* might confer broad-spectrum late blight resistance.

Using AVRamr3 as a probe, we found *Rpi-amr3* is widely distributed in *S. americanum* and *S. nigrum* species (Witek et al., 2021) (Figure 6A and 6B). We noticed that PITG_21190 (AVRamr3) was found to trigger HR in many *S. nigrum* accessions in a large-scale effector screening study (Dong, 2016), consistent with our finding, and went on to clone and verify functional *Rpi-amr3* homologs from *S. nigrum* and *S. americanum* accessions (Figure 6D). The wide distribution of *Rpi-amr3* in these species suggests that *Rpi-amr3*, perhaps with other *Rpi* genes like *Rpi-amr1*, underpin their strong resistance against late blight.

In the “arms race” between plants and pathogens, the RXLR effectors are usually considered to be fast-evolving molecules (Dong et al., 2015). However, *Avramr3* homologs were identified in twelve additional *Phytophthora* and *Hyaloperonospora arabidopsidis* genomes. There are extensive sequence variations among these AVRamr3 homologs, but surprisingly, nine out of thirteen tested AVRamr3 homologs are recognized by Rpi-amr3 and lead to strong HR in *N. benthamiana*. All the AVRamr3 homologs carry multiple WY motifs, which were proposed to

be the functional units of RXLR effectors (He et al., 2019). These effectors might fold into similar structures despite high sequence diversity (Outram et al., 2022). Here we show that the predicted AVRamr3 structures from different *Phytophthora* species indeed share a common fold, although this structure cannot fully explain their recognition specificity, and sequence polymorphisms might also determine their recognizability by Rpi-amr3.

Some plant NLRs that recognize widely conserved effectors/effector epitopes are reported to confer broad broad-spectrum resistance. Sw-5b from tomato confers broad-spectrum tospovirus resistance by recognizing a conserved, 21-amino acid epitope NSm ^{21} which derives from the viral movement protein NSm (Zhu et al., 2017). Similarly, the ETI mediated by two conserved NLRs CAR1 and ZAR1 from *Arabidopsis thaliana* confer resistance to 94.7% *Pseudomonas syringae* strains (Laflamme et al., 2020). To connect the effector recognition and disease resistance of Rpi-amr3, we tested *P. parasitica* and *P. palmivora* on *Rpi-amr3* transgenic *N. benthamiana*. These pathogens cause dramatic yield losses of many crops from different plant families (Meng et al., 2014; Ali et al., 2017), like tobacco black shank disease and cacao black pod disease. Importantly, we found *Rpi-amr3* confers resistance against some but not all *P. parasitica* and *P. palmivora* isolates (Figure 4 and Figure 5). Although many resistance resources have been identified or genetically mapped, this is the first report of cloned *R* genes against *P. parasitica* and *P. palmivora*, and their cognate Avr effectors (Kourelis et al., 2021). However, we cannot rule out the possibility that Rpi-amr3 also recognizes additional effectors in *P. parasitica* and *P. palmivora*, and this should be tested in future investigations. *P. parasitica* is becoming a more severe pathogen in many crops, correlated with global climate change. For example, it can cause potato tuber rot and foliar disease at high temperatures. Identification of *R* genes that confer resistance to both *P. infestans* and *P. parasitica* could therefore restrict losses to these pathogens in a warmer world (Panabières et al., 2016).

Additionally, in nature, many *Phytophthora* pathogens can co-inoculate the host and interspecific hybridization might occur (Goss et al., 2011), and natural hybrids of *P. parasitica* and *P. cactorum* were also found on infected loquat trees (Hurtado-Gonzales et al., 2017). An *R* gene that provides protection against both foliar and root *Phytophthora* pathogens of different species would be extremely valuable. However, some *Rpi-amr3*-breaking *P. parasitica* and *P. palmivora* strains were also identified in this study, although most of them carry the recognized AVRamr3 homologs. This might be caused by silencing of the recognized effector gene, as in the case of the silencing of *Avrvnt1* to evade recognition by Rpi-vnt1, or due to other suppressors or regulators like AVRcap1b or splicing regulatory (SRE) effectors (Pais et al., 2018; Huang et al., 2020; Derevnina et al., 2021).

Thus, *Rpi-amr3* could be deployed in Solanaceae crops like potato, tomato and tobacco against different *Phytophthora* diseases. However, whether *Rpi-amr3* could be used in other crops of other plant families remain unclear. Interfamily transfer of *NLR* genes remains a challenge as some *NLR* genes show "restricted taxonomic functionality" (Tai et al., 1999). Therefore, to investigate the mechanism of AVRamr3 recognition and Rpi-amr3 activation, we showed that Rpi-amr3 is a "sensor" NLR that requires "helper" NLRs NRC2, NRC3 and NRC4 in *N. benthamiana* (Figure S4). This enabled us to reveal the association between Rpi-amr3 and AVRamr3 homologs in *planta*. Interaction between Rpi protein and their recognized RXLR effector was rarely reported, except for RB and IPI-O effectors (Chen et al., 2012; Zhao and Song, 2021). Surprisingly, the association has not led to accelerated evolution of AVRamr3 to evade detection, as we also observed for Rpi-amr1 and AVRamr1 (Lin et al., 2020; Witek et al., 2021). This could predispose *Rpi-amr3* to function in different plant species. In a companion paper, we show that Rpi-amr3 activates NRC2 to form a high-molecular weight, resistosome-like complex upon AVRamr3 recognition (Ahn et al., 2022). Consistent with our

pathogen assay of *P. parasitica*, PpAVRamr3 recognition also leads to NRC2 oligomerization, but not the un-recognized AVRamr3 homolog from *P. capsici* (Ahn et al., 2022). These findings indicate that co-delivery of *Rpi-amr3* and *NRC* genes might be required to elevate resistance to these *Phytophthora* diseases in plant families that lack *NRC* genes.

In summary, this study reveals that *Rpi-amr3* is a conserved *R* gene from *S. americanum* and its relatives. The recognition of the conserved AVRamr3 effectors enables resistance against several different *Phytophthora* pathogens. This finding shows great potential for resistance enhancement in many crop plants such as tobacco, cacao, soybean and strawberry against different *Phytophthora* diseases.

Methods

RXLR effector libraries

The list of RXLR effector libraries is shown in Table S3. *Rpi-amr3* with its native promoter and terminator (Witek et al., 2016) was co-expressed with individual effectors in *N. benthamiana* by agro-infiltration. The HR phenotype was scored three days after the agro-infiltration. OD_{600} = 0.5.

Plant materials

The plant materials used in this study are listed in Table S4, the *Nicotiana benthamiana* NRC2/3, NRC4, and NRC2/3/4 knockout lines are described previously (Adachi et al., 2019; Wu et al., 2020; Witek et al., 2021). *Rpi-amr3* under native promoter were transformed into potato cv. Maris Piper, the protocol was described previously (Witek et al., 2016). Two transgenic lines (SLJ24895-5C and SLJ24895-9A) were selected for the field trials. *N. benthamiana-Rpi-amr3* transgenic lines were generated, full-length *Rpi-amr3* gene with its native promoter and terminator was cloned into a binary vector, and used for the *N. benthamiana* transformation (Witek et al., 2016), two homozygous T2 lines *Rpi-amr3*#13.3 and *Rpi-amr3*#16.5 were selected by detached leaves assays (DLA), 15 T2 plants of each *Rpi-amr3*#13.3 and *Rpi-amr3*#16.5 lines were tested, and all are resistant to *P. infestans* isolate 88069. The wild-type, knockout and transgenic *N. benthamiana* were propagated in a glasshouse, for the experiments, the plants were grown in a controlled environment room (CER) with 22 °C, 45-65% humidity, and 16 hours photoperiod.

The *S. americanum* and *S. nigrum* accessions were collected from different seed banks (Table S4), and the seeds were sowed and grown in a containment glasshouse for agro-infiltration experiments.

Potato late blight field trials

The field trials were performed in Norwich Research Park (NR4 7UH, Norwich, UK) from April to October 2017 and 2018. For the 2017 field trial, six plants (clones) were planted per

genotype per block. The trial included three blocks and the location of the genotypes was randomized within each block following a randomized complete block design. In total, eighteen plants were used for each line. The guard plants (Potato cv. Desiree) were planted in April, the transgenic and control lines were propagated from tissue culture, and grown in a glasshouse, the plantlets were transplanted to the field in July. In August, natural infections were observed and we also inoculated the plants with infected material from a nearby allotment. The scoring started when the control plants began to show late blight symptoms. The scorings were taken twice a week until the control lines reached 100% severity. The scoring of disease severity was described previously (Cruickshank et al., 1982). In October, the tubers were harvested from each block and the tuber numbers and yields were measured.

To genotype the field isolates, samples were taken and genotyped by David Cooke's group at James Hutton Institute. Most isolates from the field including 2017_NR47UK and 2017_NR94HH were 6_A1 (aka Pink6), but 36_A2 was also detected in one sample. Both isolates are prevalent in Europe.

In 2018, SLJ24895-5C was tested again in the field, following the same randomized complete block design. In this case, the plantlets taken to the field were grown from tubers in the glasshouse, instead of being propagated from tissue culture. Due to weeks of hot and dry weather, the natural infection did not occur as expected. Therefore, artificial inoculations were performed three times (3rd August, 9th August, and 10th August) with isolate 2017_NR94HH (6_A1). This isolate was sampled in 2017 from the infected material used to inoculate the trial. The artificial inoculations were performed by spraying the guard plants with 34 mL-84 mL inoculum of 50,000-70,000 zoospores/mL. The first clear symptom of late blight appeared in the guard plants on 14th August 2018.

Pathogens and disease test

The pathogens used in this study are listed in Table S5. *Phytophthora infestans* isolates were used for the *P. infestans* disease test, they were propagated and maintained on rye sucrose agar (RSA) medium in an 18°C incubator, ice-cold water was used to induce zoospores from 7-14 days old plates. The plate was then incubated at 4 °C for an hour then the zoospores suspension was collected and 10 µL inoculum was used for detached leaf assay (10,000/mL - 20,000/mL

zoospores). For the detached leaf assay of *Rpi-amr3* transgenic potatoes, leaves from 8-10 weeks old potato plants were used for the detached leaf assay (DLA), the lesion diameter was measured by a caliper, and the data was visualized and analyzed in R (4.1.1). One-way ANOVA and Tukey's HST test were used for examining the statistical differences.

Both *Phytophthora parasitica* and *Phytophthora palmivora* isolates were propagated and maintained in V8 plates in a 25°C incubator. To produce the zoospore from *P. palmivora*, 7-10 days old plates were flooded with 4 °C water and incubated at 4 °C for 1h, then moved to room temperature for another 1h, the released zoospores were counted by a hemocytometer, 30,000/mL - 50,000/mL zoospores were used for the root inoculation, 1 mL zoospore suspension was added to the root. For *P. parasitica*, 10 day old plate was flooded with 0.1% KNO_3 solution, incubated at 25°C for 2 days in the dark, then incubated at 4 °C for 1 hour and 25 °C for another 1h to release the zoospores. 3 - 4 weeks of wild-type or *Rpi-amr3* transgenic *N. benthamiana* plants were used in the disease test, they were grown in a CER, and the inoculated plants were grown in a Sanyo cabinet with 25 °C and 16 h photoperiod, the root inoculation experiment takes 4-7 days, the scoring was taken when the wild-type *N. benthamiana* plants were infected completely.

RT-PCR

The *P. infestans* zoospores were used to infect leaves from a potato cultivar Maris Piper, the infected tissues were samples 2 and 3 days after inoculation. RNA was isolated from these tissues by RNeasy mini Kit (QIAGEN, Cat:74104), and the gDNA was removed by TURBO DNA-free kit (ThermoFisher, Cat:AM1907). cDNA was synthesized by Superscript IV Transcriptase kit (ThermoFisher, Cat: 18090010) for RT-PCR (40 cycles). The primers are shown in Table S8.

Genomic and sequence analysis

The *Phytophthora* genomes used in this study are listed in Table S6. The genomes were imported into Geneious R10 (Kearse et al., 2012), and local BLAST databases were generated. The *Avramr3* homologs containing contigs were identified by BLAST, then *Avramr3* loci were extracted with flanking sequences. All the *Avramr3* loci were re-annotated by the gene prediction tool in EumicobeDB (http://www.eumicrobedb.org/eumicrobedb/gene_predict.php). Then the GenBank (.gb) file was exported and visualized by Clinker (https://github.com/gamcil/clinker) (Gilchrist and Chooi, 2021). All other sequences were analyzed in Geneious R10. All sequence alignments were performed by MAFFT (Katoh and Standley, 2013). The phylogenetic tree was generated by IQ-tree (v1.6.12) (Minh et al., 2020), 546 protein models were tested and JTT+G4 was selected as the best-fit model, 1000 samples were generated for the ultrafast bootstrap analysis.

Molecular cloning and constructs used in this study

All constructs, primers, and synthesized fragments that were used in this study are listed in Table S7, S8 and S9. In brief, the *Rpi-amr3* CDS were cloned into golden gate level 0 entry vector pICSL01005, then fused with C-HA (pICSL50009), C-GFP (pICSL50008) or C-Flag-Nluc (pICSL500047) tags and recombined into level 1 vector pICSL86977OD with 35S promoter and Ocs Terminator. All the *Avramr3* homologs from different *Phytophthora* species, were synthesized based on their reference genomes, the signal peptides were removed, and BsaI and BpiI sites were domesticated to facilitate the golden gate cloning, the sequences are listed in Table S9. All the *Avramr3* homologs and truncated *Avramr3* were cloned into level 0 entry vector pICSL01005, then fused with C-HIS-FLAG (pICSL50001) or C-Flag-Cluc (pICSL500048) tags. The *Rpi-amr3/Rpi-nig3* homologs from *S. americanum* and *S. nigrum*

were amplified by nested PCR and cloned into level 1 vector pICSL86922OD containing a 35S promoter and Ocs Terminator. GenBank accession numbers: OP020886-OP020892.

For potato transformation, *Rpi-amr3* with its native promoter and terminator were cloned into vector pAGM31195 and transformed into *Agrobacterium* strain AGL1 for plant transformation. For generating the *Rpi-amr3* transgenic *N. benthamiana* lines, *Rpi-amr3* with its native promoter and terminator was cloned into USER vector pICSLUS0001OD (Witek et al., 2021), and shuffled into *Agrobacterium* strain AGL1 for plant transformation. The *N. benthamiana* plants were propagated in a glasshouse, two homozygous T2 lines were selected for the *Phytophthora* disease test.

Agro-infiltration

All the over-expression constructs were shuffled into *Agrobacterium* strain GV3101-pMP90, and they are stored in a -80 °C freezer with 20% glycerol. The *Agrobacterium* were streaked out on solid L medium with antibiotics and incubated at 28 °C for two days, then the *Agrobacterium* were re-suspended into infiltration buffer ($MgCl_2$-MES, 10mM $MgCl_2$, and 10mM MES, pH 5.6) with 1 mM acetosyringone and used for agro-infiltration, OD_{600} = 0.5.

Western blot and co-immunoprecipitation

The Western blot and co-immunoprecipitation protocols were described previously (Guo et al., 2020). In brief, 35S::*Rpi-amr3*::HA or 35S::*Rpi-amr3*::GFP, 35S:: *Avramr3*::HIS-FLAG or other *Avramr3* homologs with C-HIS-FLAG tag were transiently co-expressed in *N. benthamiana* nrc2/3/4 knockout line, OD_{600} = 0.5. The leaves were sampled at 3 dpi and total protein was extracted by GTAN buffer for co-immunoprecipitation. EZview™ Red Anti-HA Affinity Gel (Sigma-Aldrich, Cat: E6779), Anti-Flag® M2 affinity gel (Sigma-Aldrich, Cat:

A2220), GFP-Trap Agarose (ChromoTek, Planegg-Martinsried, Germany) were used for the immunoprecipitation, HRP conjugated HA antibodies (Sigma-Aldrich, Cat: H6533), HRP conjugated anti-FLAG antibodies (Sigma, Cat: A8592) and HRP conjugated anti-GFP antibodies (Santa Cruzm Cat: sc-9996HRP) were used for the Western blot. NuPage 4-12% Bis-Tris protein gels (ThermoFisher, Cat: NP0302BOX) and MOPs SDS Running Buffer (ThermoFisher, Cat: NP0001) were used for separating the protein.

Split-luciferase assay

The split-luciferase system was described previously (Chen et al., 2008). *Rpi-amr3* was fused with 1x Flag::C-luciferase (Cluc) tag and *Avramr3* homologs were fused with 1x Flag::N-luciferase (Nluc), the *Rpi-amr3*::Cluc and *Avramr3*::Nluc were transiently co-expressed in *N. benthamiana* nrc2/3/4 knockout line by agro-infiltration. Three days after infiltration, 0.4 mM luciferin on 100mM sodium citrate buffer (pH 5.6) was infiltrated into the leaves, then the leaves were detached for imaging (NightOWL II LB 983 in Vivo Imaging System with WinLight Software, BERTHOLD TECHNOLOGIES GmbH & Co KG, Germany). Two leaves were used for each experiment, and three independent biological repeats were performed. Western blots with HRP-FLAG antibodies were used for detecting the presence of the recombinant protein.

Protein Structure prediction

The structure of AVRamr3 homologs was predicted by AlphaFold and ColabFold (Jumper et al., 2021; Mirdita et al., 2022). The structures were visualized and aligned by PyMOL (The PyMOL Molecular Graphics System, Version 2.5.1 Schrödinger, LLC.) (Schrödinger, L. and DeLano).

Data availability

517 *Avramr3* (GenBank: XM_002895186.1). All other sequence data will be submitted to NCBI
518 before publishing. All data and materials will be available from the corresponding author upon
519 request.
520

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Author contributions:

X.L. and J.D.G.J. designed the study. X.L., A.C.O.A., R.H., M.P., K.W., H.K.A, S.B., H.S.K., T.S., C.-H.W. and H.A. performed the experiments. X.L., A.C.O.A., R.H., M.P., and K.W., H.K.A, H.Z and S.B analysed the data. X.L. and J.D.G.J. wrote the manuscript with inputs from all authors. S.K. and V.G.A.A.V. contributed resources. All authors approved the manuscript.

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Competing interests:

K. W. and J.D.G.J. are named inventors on a patent application (PCT/US2016/031119) pertaining to *Rpi-amr3* that was filed by the 2Blades Foundation on behalf of the Sainsbury Laboratory. The other authors declare no competing interests.

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Figures legends:

Figure 1. *Rpi-amr3* confers late blight resistance in field and lab conditions.

(A). Field trials of *Rpi-amr3* transgenic potato cultivar Maris Piper in 2017 (solid line) and 2018 (dotted line). Two wild-type Maris Piper lines (Maris Piper-A and Maris Piper-B) are shown by dark and light blue lines, and two *Rpi-amr3* transformants SLJ24895-5C and 9A are shown by orange and yellow lines. The *X*-axis indicates the days after planting, the first scoring was taken when the late blight symptoms were observed in the wild-type potatoes. The *Y*-axis indicates the severity of the late blight symptom. (B). The relative area under the disease progress curve (rAUDPC) is shown, and the colour codes are the same as in Fig. 1a. Data are mean ± s.d., data were analyzed by one-way ANOVA with Tukey's test ($P < 0.001$). (C). Total tuber weight (Kg) per block. Data are mean ± s.d., and the data were analyzed by one-way ANOVA with Tukey's test ($P < 0.001$). (D). Total tuber number per block. Data are mean ± s.d., and data were analyzed by one-way ANOVA with Tukey's test ($P < 0.01$). the colour codes are the same as Fig. 1a. (E). Detached leaf analysis (DLA) for *Rpi-amr3* transgenic potato cv. Maris Piper (SLJ24895-5C), wild-type Maris Piper and *Rpi-amr1* transgenic Maris Piper (SLJ25029) were used as controls. 100-200 zoospores from different *P. infestans* isolates were used for inoculation. The lesion diameter (mm) was scored by a calliper at 4 days post-inoculation (dpi). Two replicated were performed with similar results (red and blue dots), 24 data points were collected in total, and the outliers are indicated by black dots. The visualization and statistical analysis were performed in R. Statistical differences among the lines were analyzed one-way ANOVA with Tukey's HSD test ($P < 0.001$).

Figure 2. Identification and characterization of AVRamr3. (A). Co-expression of Rpi-amr3::HA and AVRamr3::HIS-FLAG trigger cell death on *N. benthamiana*, Rpi-amr1 and AVRamr1 were used as controls. The photos were taken 3 days after infiltration, *Agrobacterium* strain GV3101(pMP90) carrying Rpi-amr3:HA or AVRamr3::HIS-FLAG constructs were used in this experiment. OD_{600}=0.5. Three biological replicates were performed with the same results. **(B).** Co-expression of Rpi-amr3::HA and AVRamr3 truncations. All truncations are tagged with a C-terminal HIS-FLAG tag. T3, T4, T8, and T10 trigger cell death when co-expressed with Rpi-amr3, but not T1, T2, T5, T6, T7, and T9. Full-length AVRamr3::HIS-FLAG was used as control. OD_{600}=0.5. Three biological replicates were performed with the same results. **(C).** Cartoon of AVRamr3 (PITG_21190), a protein with 339 amino acids with a signal peptide (lemon), RXLR-EER motif (green), and an effector domain (red) with four predicted WY motifs (Details are shown in Fig. S4). T1-T10 indicates the AVRamr3 truncations used in HR assays. Those that induce HR after co-expression with Rpi-amr3 are marked by orange bars, otherwise by blue. **(D).** Rpi-amr3::HA and AVRamr3::HIS-FLAG constructs were used for a bidirectional co-immunoprecipitation experiment, with Rpi-amr1-HA and AVRamr1::HIS-FLAG used as control. After HA pull-down of Rpi-amr3::HA or Rpi-amr1::HA, only AVRamr3::HIS-FLAG is associated with Rpi-amr3::HA. After Flag pull-down of AVRamr3::HIS-FLAG or AVRamr1-HIS-FLAG, only Rpi-amr3::HA is associated with AVRamr3::HIS-FLAG. *Agrobacterium* strain GV3101(pMP90) carrying different constructs was used for transient expression in the nrc2/3/4 knockout *Nicotiana benthamiana* line (210.4.3) to abolish the cell death phenotype. OD_{600}=0.5. Three biological replicates were performed with the same results. **(E).** Rpi-amr3::Cluc and AVRamr3::Nluc constructs were used to test their interaction *in planta*, Rpi-amr1::Cluc and AVRamr1::Nluc

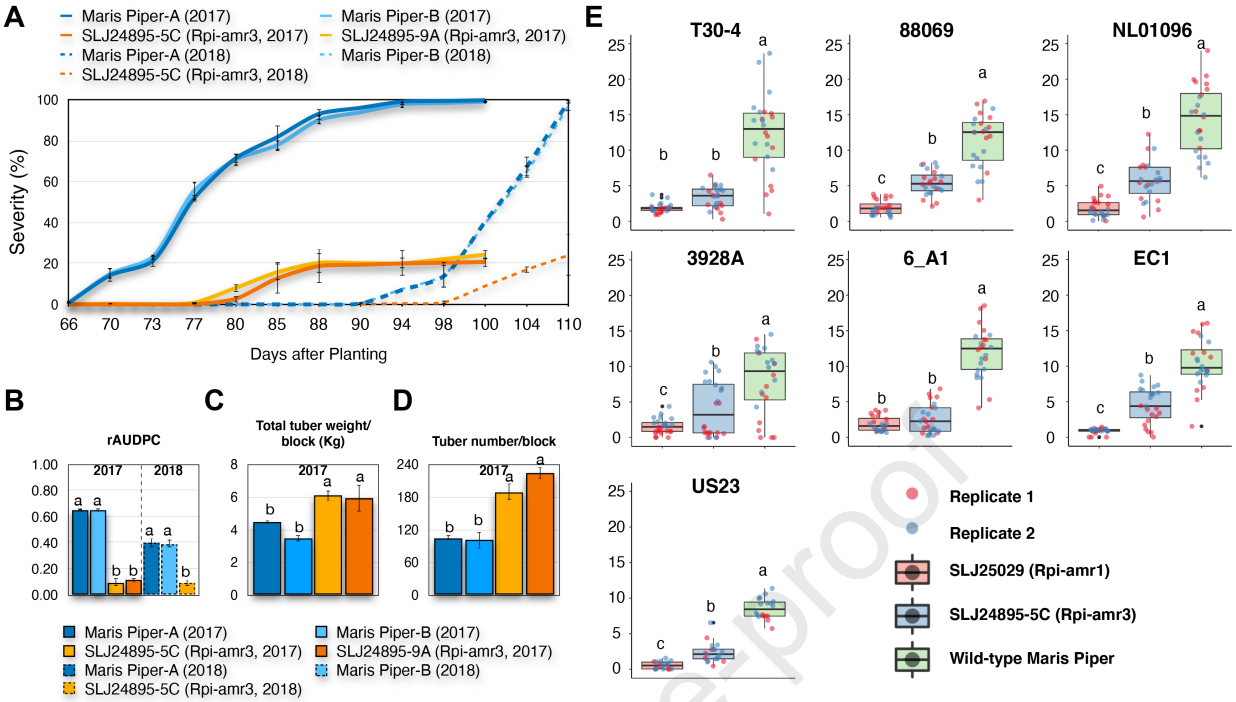
were used as controls. The luciferase signal can only be detected upon Rpi-amr3::Cluc and AVRamr3::Nluc co-expression. The nrc2/3/4 knockout *Nicotiana benthamiana* line (210.4.3) was used to abolish the cell death phenotype.

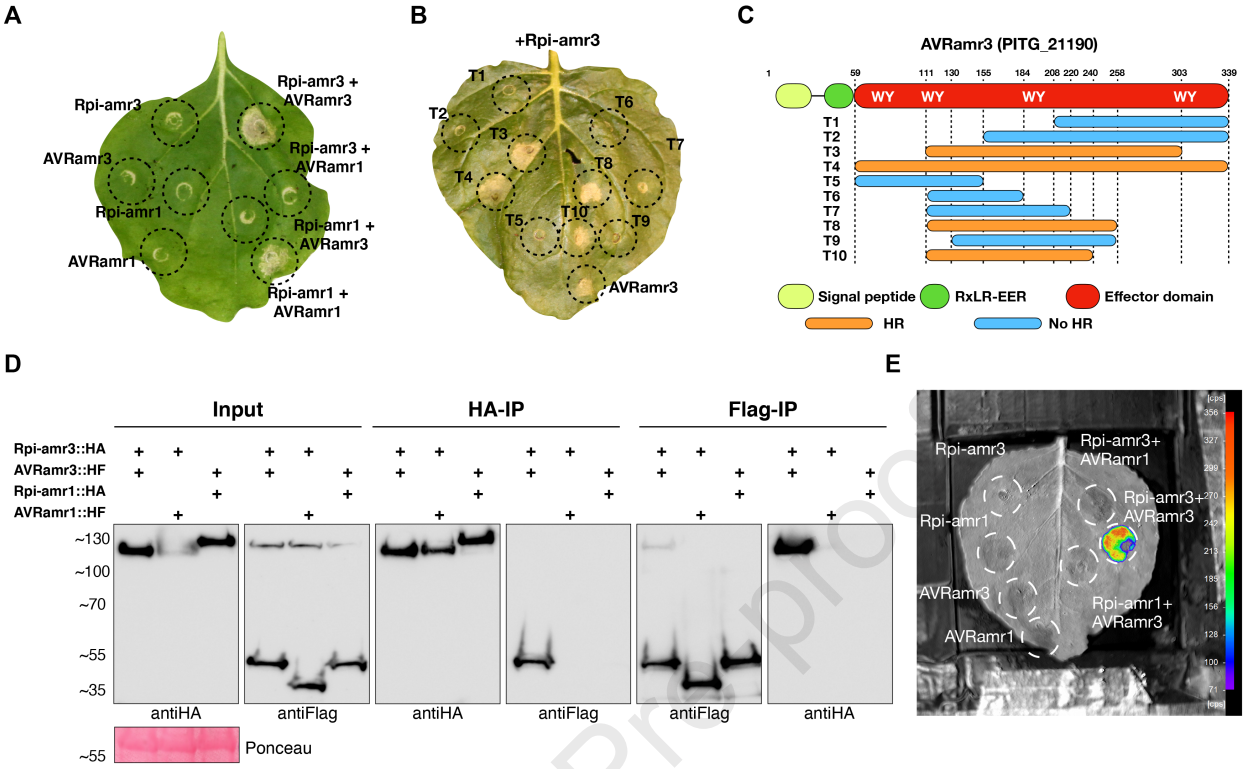
Figure 3. AVRamr3 is a conserved effector among different *Phytophthora* species. (A). The synteny map of *Avramr3* loci from twelve different *Phytophthora* genomes. The *Avramr3* loci were extracted from different genomes, annotated by the gene prediction tool in EumicrobeDB, then analyzed and visualized by Clinker. *Avramr3* homologs are shown by purple triangles and indicated by a black arrow, the flanking genes with homology are represented by the corresponding colours. The *Phytophthora* clades are adapted from the *Phytophthora* database (Rahman et al., 2014). **(B)**. Expression of AVRamr3 homologs with HIS-FLAG tag alone does not trigger cell death on *Nicotiana benthamiana*. *Agrobacterium* strain GV3101(pMP90) carrying different constructs was used in this experiment. OD_{600}=0.5. Three biological replicates were performed with the same results. **(C)**. Co-expression of HIS-FLAG tagged AVRamr3 homologs with Rpi-amr3::GFP in *N. benthamiana*. The AVRamr3 homologs from *Phytophthora infestans* (Pi), *P. parasitica* (Pp), *P.cactorum* (Pc), *P. palmivora* (Ppal), *P. megakarya* (Pmeg), *P. litchi* (Plit), *P. sojae* (Ps), *P. lateralis* (Plat) and *P. pluvialis* (Pplu) induce cell death after co-expression with Rpi-amr3::GFP, but not AVRamr3 homologs from *P. ramorum* (Pr), *P. capsici* (Pcap) and *Hyaloperonospora arabidopsidis* (Hpa). The AVRamr3 homolog from *P. cinnamomi* (Pcin) shows an intermediate cell death. *Agrobacterium* strain GV3101(pMP90) carrying different constructs was used in this experiment. OD_{600}=0.5. Three biological replicates were performed with the same results. The protein expression of the AVRamr3 homologs with HIS-FLAG tag was shown in Figure S6.

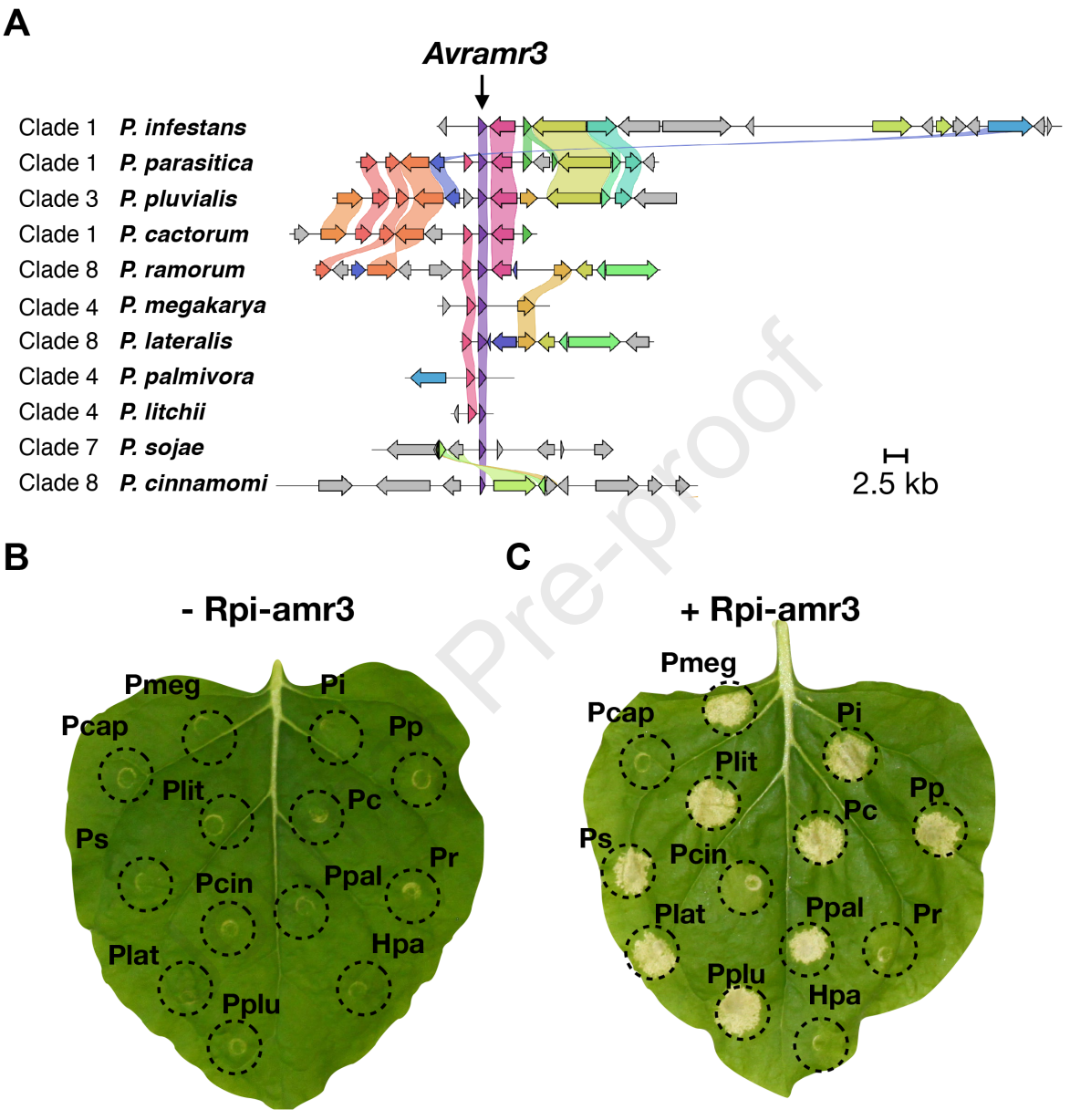
Figure 4. Root inoculation of six *Phytophthora parasitica* isolates on *Rpi-amr3* transgenic *Nicotiana benthamiana* lines. Representative photos for the *P. parasitica* root inoculation tests are shown. Two homozygous *N. benthamiana* - *Rpi-amr3* lines #13.3 and #16.5 were used in this experiment. Wild-type *N. benthamiana* plants were used as control. Six *P. parasitica* isolates were used for root inoculation, *Rpi-amr3* confers resistance against R1, 666 and 721, but not R0, 310 and 329. Three to four-week-old *N. benthamiana* were used for the root inoculation, three plants/lines were used for each experiment and at least three biological replicates were performed with similar results. The numbers indicate susceptible plants/total tested plants.

Figure 5. Root inoculation of 7 *Phytophthora palmivora* isolates on *Rpi-amr3* transgenic *Nicotiana benthamiana* lines. Two homozygous *N. benthamiana* - *Rpi-amr3* lines #13.3 and #16.5 were used in this experiment, and wild-type *N. benthamiana* were used as control. Seven *P. parasitica* isolates were used for root inoculation, *Rpi-amr3* confers resistance against isolates 7547, 7551 and 7545, but not 3914 and 7548. For isolates 0113 and 3738, we obtained some variable results for the two transgenic lines. 3-4 week-old *N. benthamiana* were used for the root inoculation, 3 plants/lines were used for each experiment and three or more biological replicates were performed with similar results.

Figure 6. Screen for AVRamr3 recognition on *S. americanum* and *S. nigrum* accessions. **(A).** 54 *S. americanum* accessions were screened with *Agrobacterium* strain GV3101(pMP90) carrying 35S::AVRamr3. The accessions with cell death upon agro-infiltration are marked by red, otherwise blue. 35S::HpaAVRamr3 was used as a negative control. **(B).** 26 *S. nigrum* accessions were screened with *Agrobacterium* strain GV3101(pMP90) carrying 35S::AVRamr3. The accessions with cell death upon agro-infiltration are marked by red, otherwise blue.35S::HpaAVRamr3 was used as a negative control. **(C).** The maximum likelihood (ML) tree of Rpi-amr3 and Rpi-nig3 proteins was made by iqtree with GTT+G4 model. The *Rpi-amr3* homologs from *S. americanum* were extracted from PacBio RenSeq assemblies (Witek et al., 2021). The four *Rpi-nig3* genes were PCR amplified from *S. nigrum* accession SP1088 and SP1084 (red). The non-functional *Rpi-amr3* homologs were marked by blue. Rpi-amr3b from SP1102 is a paralogue of Rpi-amr3, which was used as an outgroup of the phylogenetic analysis. The scale bar indicates the number of amino acid substitutions per site. The protein identities of each homolog compared to Rpi-amr3 (Rpi-amr3-1102) are shown by %. **(D).** Selected *Rpi-amr3* homologs (*Rpi-amr3-2272*, *Rpi-amr3-2273* and *Rpi-amr3-3406*) were cloned from three *S. americanum accessions* SP2272, SP2273, and SP3406. Four *Rpi-nig3* homologs (*Rpi-nig3-1088a*, *Rpi-nig3-1088b*, *Rpi-nig3-1084a* and *Rpi-nig3-1084b*) were cloned from *S. nigrum* accessions SP1088 and SP1084, they were co-expressed with AVRamr3 or AVRamr1 (negative control) in *N. benthamiana*. All of them can recognize AVRamr3 in the transient assay but not AVRamr1.



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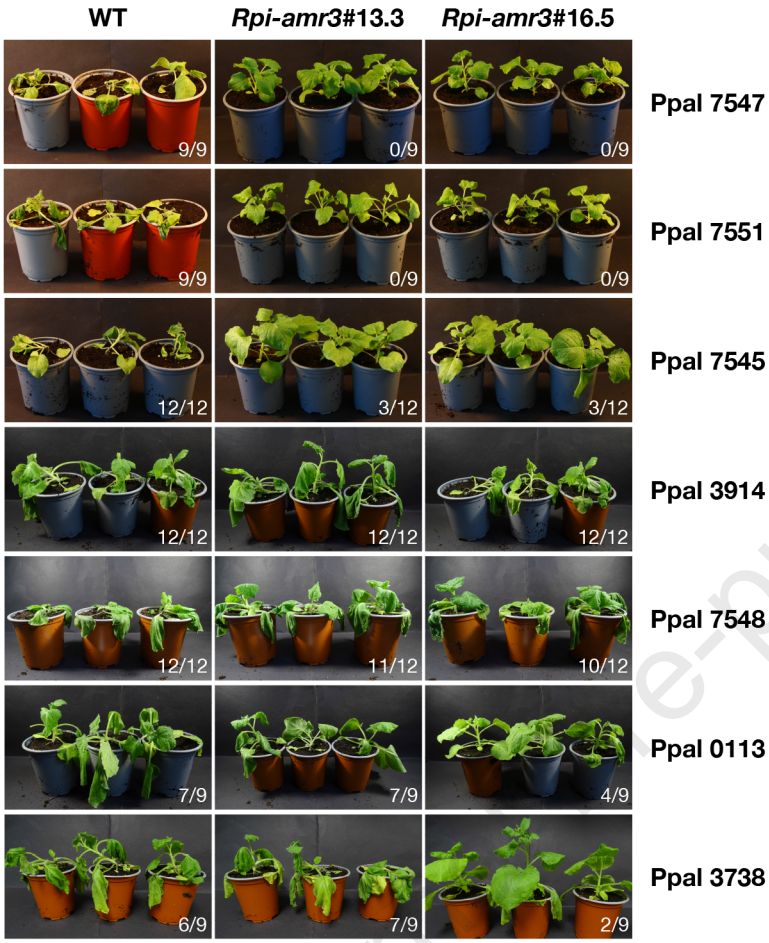
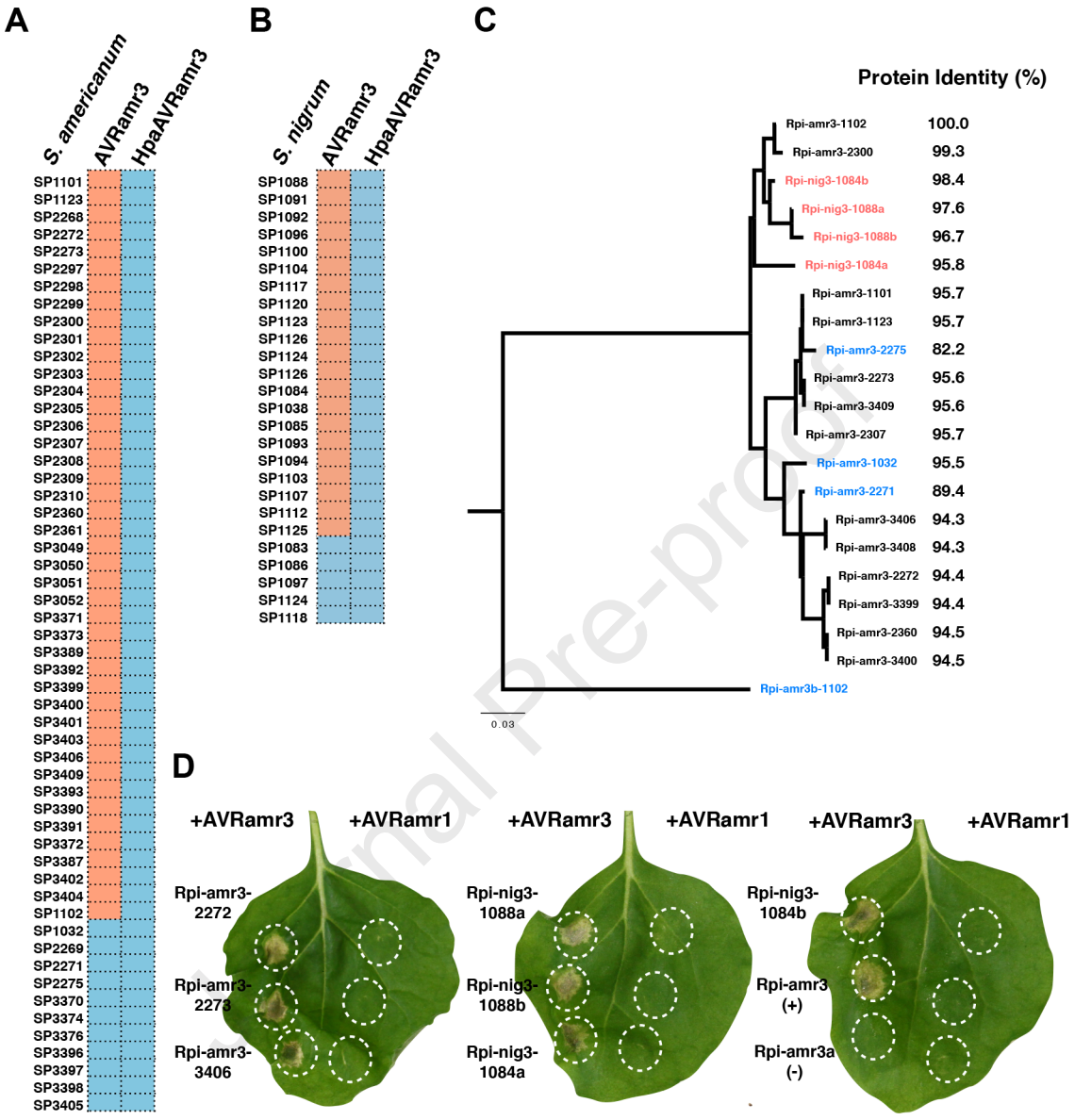
Avramr3
Clade 1 P. infestans
Clade 1 P. parasitica
Clade 3 P. pluvialis
Clade 1 P. cactorum
Clade 8 P. ramorum
Clade 4 P. megakarya
Clade 8 P. lateralis
Clade 4 P. palmivora
Clade 4 P. litchii
Clade 7 P. sojae
Clade 8 P. cinnamomi
2.5 kb
B
- Rpi-amr3
Pmeg
Pi
Pcap
Pp
Plit
Pc
Ps
Pr
Pcin
Ppal
Hpa
Plat
Pplu
C
+ Rpi-amr3
Pmeg
Pcap
Pi
Plit
Pp
Pc
Ps
Pcin
Plat
Ppal
Pr
Pplu
Hpa


WT
Rpi-amr3#13.3
Rpi-amr3#16.5
18/21
1/21
0/12
Pp R1
29/29
1/28
1/12
Pp 666
19/20
0/20
2/12
Pp 721
15/15
10/15
6/9
Pp R0
12/12
10/12
8/9
Pp 310
14/15
10/15
8/9
Pp 329




A
S. americanum
AVRamr3
HpaAVRamr3
SP1101
SP1123
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SP2272
SP2273
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SP2299
SP2300
SP2301
SP2302
SP2303
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SP1032
SP2269
SP2271
SP2275
SP3370
SP3374
SP3376
SP3396
SP3397
SP3398
SP3405
B
S. nigrum
AVRamr3
HpaAVRamr3
SP1088
SP1091
SP1092
SP1096
SP1100
SP1104
SP1117
SP1120
SP1123
SP1126
SP1124
SP1126
SP1084
SP1038
SP1085
SP1093
SP1094
SP1103
SP1107
SP1112
SP1125
SP1083
SP1086
SP1097
SP1124
SP1118
C
Protein Identity (%)
Rpi-amr3-1102 100.0
Rpi-amr3-2300 99.3
Rpi-nig3-1084b 98.4
Rpi-nig3-1088a 97.6
Rpi-nig3-1088b 96.7
Rpi-nig3-1084a 95.8
Rpi-amr3-1101 95.7
Rpi-amr3-1123 95.7
Rpi-amr3-2275 82.2
Rpi-amr3-2273 95.6
Rpi-amr3-3409 95.6
Rpi-amr3-2307 95.7
Rpi-amr3-1032 95.5
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Rpi-amr3-3406 94.3
Rpi-amr3-3408 94.3
Rpi-amr3-2272 94.4
Rpi-amr3-3399 94.4
Rpi-amr3-2360 94.5
Rpi-amr3-3400 94.5
Rpi-amr3b-1102
0.03
D
+AVRamr3
+AVRamr1
Rpi-amr3-2272
Rpi-amr3-2273
Rpi-amr3-3406
+AVRamr3
+AVRamr1
Rpi-nig3-1088a
Rpi-nig3-1088b
Rpi-nig3-1084a
+AVRamr3
+AVRamr1
Rpi-nig3-1084b
Rpi-amr3 (+)
Rpi-amr3a (-)