A complex resistance locus in *Solanum americanum* recognizes a conserved *Phytophthora* effector

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Abstract

Late blight caused by *Phytophthora infestans* greatly constrains potato production. Many *Resistance (R)* genes were cloned from wild *Solanum* species and/or introduced into potato cultivars by breeding. However, individual *R* genes have been overcome by *P. infestans* evolution; durable resistance remains elusive. We positionally cloned a new *R* gene, *Rpi-amr1,* from *Solanum americanum*, that encodes an NRC helper-dependent CC-NLR protein. *Rpi-amr1* confers resistance in potato to all 19 *P. infestans* isolates tested. Using association genomics and long-read RenSeq, we defined eight additional *Rpi-amr1* alleles from different *S. americanum* and related species. Despite only ~90% identity between Rpi-amr1 proteins, all confer late blight resistance but differentially recognize *Avramr1* orthologs and paralogs. We propose that *Rpi-amr1* gene family diversity facilitates detection of diverse paralogs and alleles of the recognized effector, enabling broad-spectrum and durable resistance against *P. infestans*.

35 # Introduction

36

37 Potato is the fourth most important directly-consumed food crop world-wide[1].
38 *Phytophthora infestans,* an oomycete pathogen, causes late blight disease in potato, and
39 can result in complete crop failure. Disease management is primarily based on repeated
40 fungicide applications (10-25 times per season in Europe). However, fungicide-
41 resistant races have emerged[2].

42

43 To elevate late blight resistance, *Resistance* to *Phytophthora infestans* (*Rpi*) genes were
44 identified in wild relatives of potato and used for resistance breeding[3]. More than 20
45 *Rpi* genes have been mapped and cloned from different *Solanum* species[4]. All encode
46 coiled-coil (CC), nucleotide binding (NB), leucine-rich repeat (LRR) (NLR) proteins[5]
47 and some require helper NLR proteins of the NRC family[6]. However, most cloned *Rpi*
48 genes have been broken by *P. infestans*[7]. Provision of durable late blight resistance for
49 potato remains a major challenge.

50

51 NLR-mediated immunity upon effector recognition activates "effector-triggered
52 immunity" (ETI)[8]. In oomycetes, all identified recognized effectors, or avirulence (*Avr*)
53 genes, carry a signal peptide and an RxLR motif[9]. 563 RxLR effectors were predicted
54 from the *P. infestans* genome, enabling identification of the recognized effectors[10,11].
55 Many *P. infestans* effectors show signatures of selection to evade recognition by
56 corresponding NLR proteins[12]. NLR genes also show extensive allelic and
57 presence/absence variation in wild plant populations[13,14] and known *Resistance (R)*
58 gene loci like *Mla*, *L*, *Pi9*, *RPP1* and *RPP13* from barley, flax, rice and Arabidopsis
59 show substantial allelic polymorphism[15-18]. Remarkably, different *Mla* alleles can
60 recognize sequence-unrelated effectors[19,20].

61

62 Technical advances like RenSeq (Resistance gene enrichment and Sequencing) and
63 PenSeq (Pathogen enrichment Sequencing) enable rapid definition of allelic variation
64 and mapping of plant *NLRs*, or discovery of variation in pathogen effectors[21-23].
65 Combined with single-molecule real-time (SMRT) sequencing, SMRT RenSeq enabled
66 cloning of *Rpi-amr3* from *Solanum americanum*[24]. Similarly, long read and cDNA
67 PenSeq enabled us to identify *Avramr1* from *P. infestans*[25].

In this study, we further explored the genetic diversity of *S. americanum*, and by applying sequence capture technologies, we fine-mapped and cloned *Rpi-amr1* from *S. americanum*, (usually) located on the short arm of chromosome 11. Multiple *Rpi-amr1* homologs were found in different *S. americanum* accessions and in relatives, including *Solanum nigrescens* and *Solanum nigrum*. Functional alleles show extensive allelic variation and confer strong, broad-spectrum resistance to all 19 tested diverse *P. infestans* isolates. Although differential recognition was found between different *Rpi-amr1* and *Avramr1* homologs, all *Rpi-amr1* alleles recognize the *Avramr1* homologs from *Phytophthora parasitica* and *Phytophthora cactorum*. Our study reveals unique properties of genetic variation of *R* genes from "non-host" species.

Results

Rpi-amr1 **maps to the short arm of chromosome 11**

We previously investigated *S. americanum* and isolated *Rpi-amr3* from an accession 944750095 (SP1102)[24]. To discover new *Rpi-amr* genes, we characterized additional 14 lines of *P. infestans*-resistant *S. americanum* and close relatives *S. nigrescens* and *Solanum nodiflorum* by crossing them to a susceptible (S) *S. americanum* line 954750186 (hereafter SP2271) (Table 1). To avoid self-pollination, a resistant parent was always used as a pollen donor. All the corresponding F1 plants (6-10 per cross) were resistant in a detached leaf assay (DLA) (Table 1). Around 60-100 F2 progeny derived from each self-pollinated F1 plant were phenotyped by DLA using *P. infestans* isolate 88069[26]. The F2 progenies that derived from the resistant parents with working numbers SP1032, SP1034, SP1123, SP2272, SP2273, SP2360, SP3399, SP3400, SP3406, SP3408 and SP3409 segregated in a ratio suggesting the presence of a single (semi-) dominant resistance gene (fitting 3:1 or 2:1 [likely due to segregation distortion], R:S - resistant to susceptible - ratio). Two crosses showed a 15:1 segregation (resistant parent SP2300 and SP2307), suggesting the presence of two unlinked resistance genes, while SP1101 showed no susceptible plants in 100 individuals, suggesting the presence of three or more resistance genes.

101 **Table 1. *S. americanum*, *S. nodiflorum* and *S. nigrescens* accessions used in this**
102 **study and the corresponding *Rpi-amr1* homologs**
103

Table 1

Accession	Working name	Species	Reported origin	Source	Late blight resistance	*Rpi-amr1* homolog	Similarity	Cloning method
954750186	SP2271	*S. americanum*	Brazil	RU	Susceptible			
954750184	SP2273	*S. americanum var. patulum*	unknown	RU	Resistant	*Rpi-amr1-2273*	100%	Map-based cloning
sn27	SP1032	*S. americanum sensu lato*	China	BGS	Resistant	*Rpi-amr1-1032*	92.8%	Association genomics
Veg422	SP1034	*S. americanum sensu lato*	unknown	NN	Resistant	*Rpi-arm1-2273*	100%	Association genomics
A54750014	SP1101	*S. americanum sensu lato*	unknown	RU	Resistant	*Rpi-amr1-1101*	89.4%	SMRT RenSeq
A14750006	SP1123	*S. americanum sensu lato*	unknown	RU	Resistant	*Rpi-amr1-1123*	91.8%	Association genomics
954750174	SP2272	*S. americanum*	unknown	RU	Resistant	*Rpi-amr1-2272*	89.4%	Association genomics
SOLA 226	SP2300	*S. americanum*	Cuba	IPK	Resistant	*Rpi-amr1-2300*	90.4%	SMRT RenSeq
SOLA 425	SP2307	*S. americanum*	America	IPK	Resistant	*Rpi-amr1-2307*	91.7%	Association genomics
Wang 2059	SP2360	*S. americanum*	China	NHM	Resistant	*Rpi-arm1-2273*	100%	Association genomics
A14750138	SP3399	*S. americanum*	unknown	RU	Resistant	*Rpi-amr1-2272*	89.4%	Association genomics
A14750130	SP3400	*S. nodiflorum*	unknown	RU	Resistant	*Rpi-amr1-2273*	100%	Association genomics
944750261	SP3406	*S. nigrescens*	Bolivia	RU	Resistant	*Rpi-amr1-3406*	92.5%	Association genomics
954750172	SP3408	*S. nigrescens*	Bolivia	RU	Resistant	*Rpi-amr1-3408*	92.6%	Association genomics
A14750423	SP3409	*S. nigrescens*	Mauritius	RU	Resistant	*Rpi-amr1-3409*	89.5%	SMRT RenSeq

RU - Radboud University, Nijmegen, The Netherlands

IPK - IPK Gatersleben, Germany

NHM - Natural History Museum, London, United Kingdom

BGS - Shanghai Botanical Garden, Shanghai, China

104 NN - Nicky's Nursery Ltd, Kent, United Kingdom

105

106 To identify *Rpi* genes from these resistant *S. americanum* accessions, we prioritized an
107 F2 population derived from resistant parent SP2273 and named the corresponding gene
108 *Rpi-amr1*. Using markers from RenSeq, genotyping by sequencing (RAD markers) and
109 Whole Genome Shotgun sequencing (WGS), the *Rpi-amr1* gene was mapped in a small
110 population (n=188 gametes) to the short arm of chromosome 11, between markers
111 RAD_3 and WGS_1 (Fig. 1a, Table S1, S2). We expanded the mapping population and
112 developed a PCR marker WGS_2 that co-segregated with resistance in 3,586 gametes
113 (Fig. 1b, Table S2). To generate the physical map of the target interval from SP2273, a
114 BAC library was generated. Two BAC clones (12H and 5G) covering the target interval
115 were isolated and sequenced on the PacBio RSII platform, and assembled into a single
116 contig of 212 kb (Fig. 1c). We predicted 11 potential coding sequences on the BAC_5G,
117 nine of which encode *NLR* genes (Fig. 1c). These *NLR* genes belong to the CNL class
118 and have 80-96% between-paralog identity.

To define which of these *NLR* genes are expressed, cDNA RenSeq data of the resistant parent SP2273 were generated and mapped to the BAC_5G sequence. Seven out of nine *NLR* genes were expressed. These genes - *Rpi-amr1a, b*, *c*, *d*, *e*, *g* and *h* - were tested as candidate genes for *Rpi-amr1* (Fig. 1c).

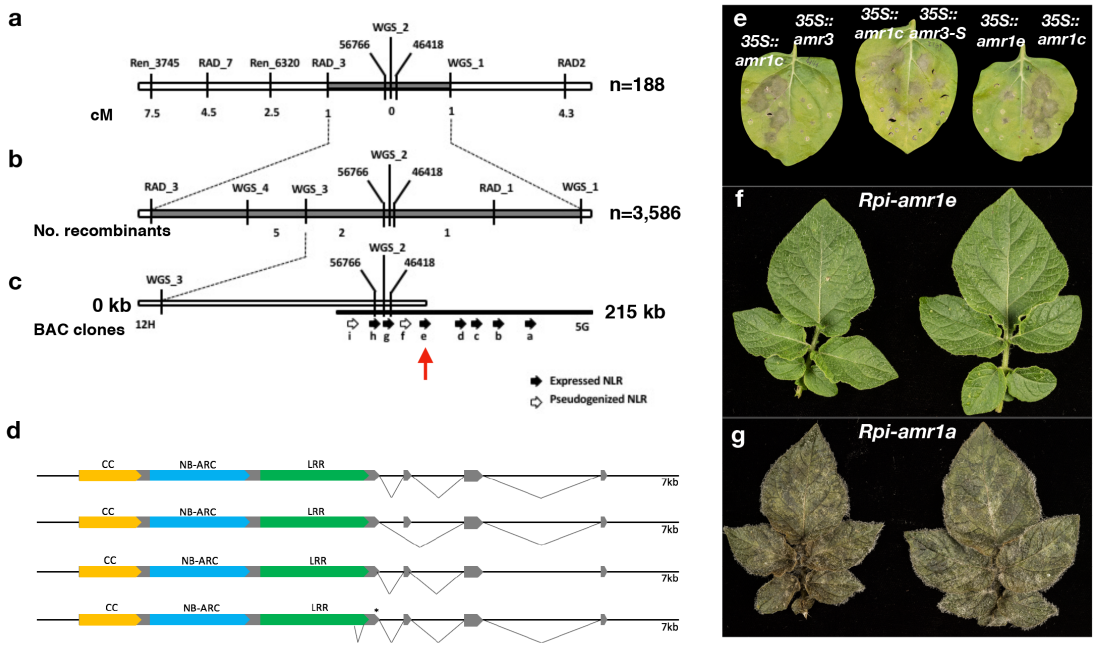

Fig. 1. Map-based cloning of *Rpi-amr1* and its resistance to *P. infestans*.

(a) Mapping of *Rpi-amr1* in a small F2 population (n=188 gametes); the names of the markers and genetic distances are shown above or below the bar.
(b) Fine mapping of *Rpi-amr1* in the F2 population of 3,586 gametes. The names of the markers and the number of recombinants are shown above or below the bar.
(c) Physical map of the target *Rpi-amr1* interval based on the assembled BAC contig. The markers present on the BAC are shown. The predicted NLR genes are depicted as black arrows (expressed NLRs) or empty arrows (pseudogenized NLRs). *Rpi-amr1* (formerly *Rpi-amr1e*) is indicated by a red arrow.
(d) Four *Rpi-amr1* transcripts detected by 3' RACE PCR.
(e) Leaves of *N. benthamiana* plants were infiltrated with the binary vector pICSLUS0003::*35S* overexpressing either the late blight resistance gene *Rpi-amr3* (positive control), one of seven *Rpi-amr1* candidates, or the non-functional *Rpi-amr3-S* (negative control). Leaves were inoculated with *P. infestans* strain 88069 24 h after infiltration. Only leaves infiltrated with *Rpi-amr3 and Rpi-amr1e* (pictured) showed reduced pathogen growth, whereas *P. infestans* grew well in the presence of the remaining *Rpi-amr1* candidates. Only *Rpi-amr1c* is shown as the phenotype of all other non-functional candidate genes was indistinguishable. Photographs were taken 9 dpi.
(f) Transgenic potato cv. Maris Piper which expresses *Rpi-amr1* under the native regulatory elements is resistant to *P. infestans* isolate 88069 (top), displaying no symptoms at the spot of inoculation. Each leaflet was inoculated with a droplet containing approximately 1,000 zoospores; photographs were taken 9 dpi.
(g) The control plants carrying the non-functional candidate *Rpi-amr1a* show large necrotic lesions and sporulation. Each leaflet was inoculated with a droplet containing approximately 1,000 zoospores; photographs were taken 9 dpi.

151

152 ***Rpi-amr1e* confers resistance in *Nicotiana benthamiana* and cultivated potato**

153

154 To test the function of the seven candidate genes, we cloned their open reading frames
155 into a binary expression vector under control of the 35S promoter. *Rpi-amr3* was used
156 as a positive control and the non-functional *Rpi-amr3-S* was used as a negative control.
157 The constructs carrying each of the seven candidate genes were transiently expressed
158 after *Agrobacterium* infiltration into *N. benthamiana* leaves, which were subsequently
159 inoculated with the *P. infestans* isolate 88069 as described[24]. *P. infestans* growth was
160 observed six days post inoculation (dpi). Only *35S::Rpi-amr1e*-infiltrated leaves
161 showed reduced pathogen growth at 6 dpi compared to other candidate genes like *Rpi-*
162 *amr1c*, or negative control *Rpi-amr3-S.* (Fig. 1e). Hence, we conclude that *Rpi-amr1e*
163 is the functional *Rpi-amr1* (hereafter) gene from *S. americanum* SP2273.

164

165 To test if *Rpi-amr1* confers late blight resistance in potato, we cloned it with its native
166 promoter and terminator, and generated transgenic potato cultivar Maris Piper plants
167 carrying *Rpi-amr1*. A non-functional paralog *Rpi-amr1a* was also transformed into
168 Maris Piper as a negative control. As in the transient assay, stably transformed *Rpi-*
169 *amr1* lines resisted *P. infestans* 88069 in potato (Fig. 1f), but *Rpi-amr1a*-transformed
170 plants did not (Fig. 1g).

171

172 ***Rpi-amr1* is a four exon CC-NLR**

173

174 To characterize the structure of *Rpi-amr1*, we mapped the cDNA RenSeq data to the
175 full length *Rpi-amr1* gene, and found four alternatively spliced forms of *Rpi-amr1*. The
176 most abundant form, supported by >80% of reads, comprises four exons encoding a
177 protein of 1,013 amino acids. This was confirmed with 3' RACE PCR (Fig. 1d). The
178 Rpi-amr1 is a typical CC-NB-LRR resistance protein, with a coiled-coil domain (CC;
179 amino acids 2-146), nucleotide binding domain (NB-ARC; amino acids 179-457) and
180 leucine-rich repeats (LRR; located between amino acids 504-900) which are all
181 positioned in the first exon (1-918 aa, Fig. 2a). The remaining three short exons (amino
182 acids 919-943, 944-1002 and 1,003-1,013) lack homology to any known domains. No
183 integrated domains[27] were found in the Rpi-amr1 protein.

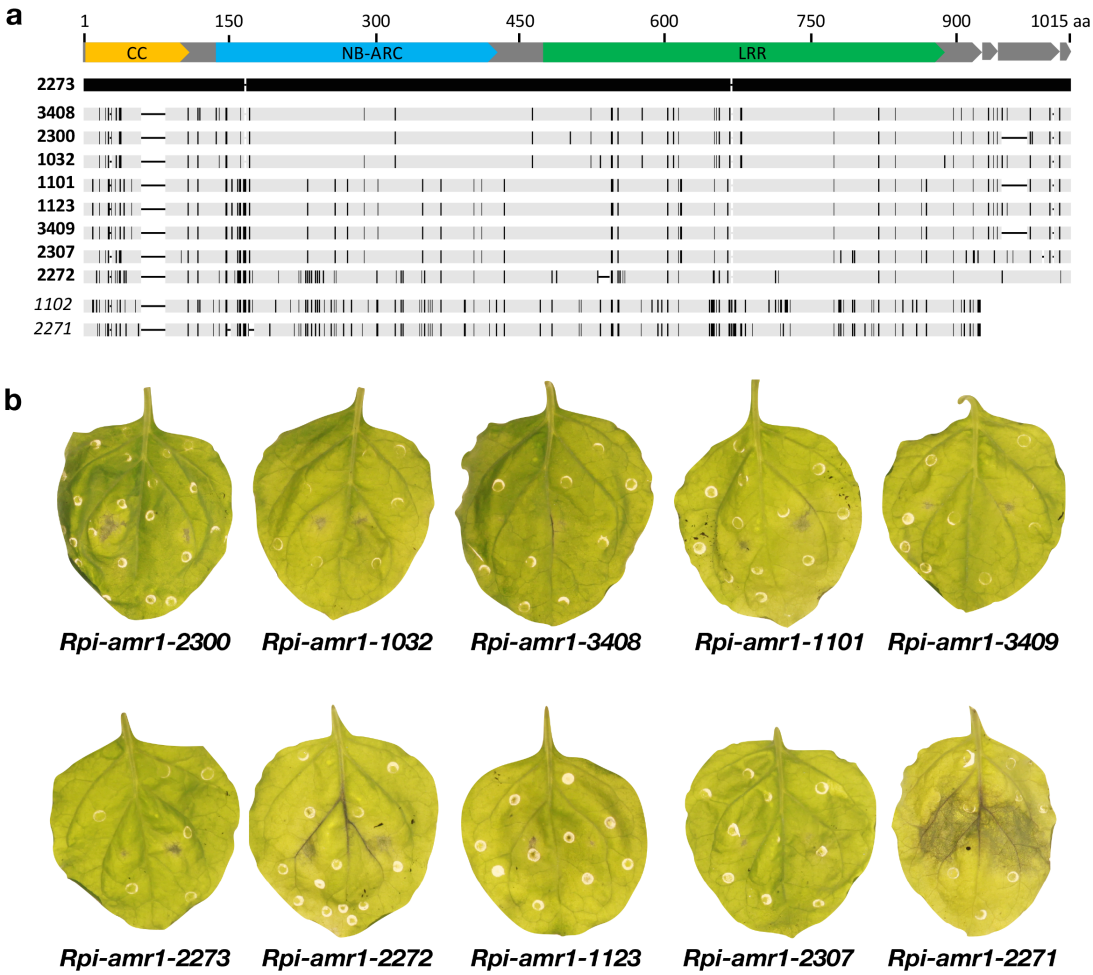

Fig. 2. Schematic representation of amino acid sequence alignment of *Rpi-amr1* homologs (a) and *P. infestans* resistance in transient assay (b).

(a) The exons and the conserved NLR domains are highlighted at the top of the alignment (exons, grey; CC, orange; NB-ARC, blue; LRR, green). Black bars in the alleles indicate the polymorphic nucleotides and indels as compared with *Rpi-amr1-2273*. The numbers next to the alleles refer to the accession working numbers (Table 1). Figure drawn to the scale.

(b) Nine *Rpi-amr1* homologs provide resistance to *P. infestans* in transient complementation assay. *Rpi-amr1* genes with native regulatory elements were infiltrated into *N. benthamiana* leaves. At 1 dpi, leaves were cut off and drop inoculated with 10 µl of zoospore suspension (50,000/mL) from *P. infestans* isolate 88069. The non-functional *Rpi-amr1-2271* homolog from susceptible accession SP2271 was used as negative control. Photographs were taken 8 dpi.

Functional *Rpi-amr1* homologs were identified from multiple resistant *S. americanum* and relatives

Previously, we found at least 14 *S. americanum* accessions and related species that resist late blight (Table 1). To test if *Rpi-amr1* contributes to late blight resistance in other resistant *S. americanum* accessions, we genotyped 10-50 susceptible F2 plants of

204 the populations derived from resistant accessions, with *Rpi-amr1* linked markers
205 (markers 3745 and 56766, Fig. 1 and Table S2). We found that in SP1032, SP1034,
206 SP1123, SP2272, SP2307, SP2360, SP3399, SP3400, SP3406 and SP3408, resistance
207 is linked to the *Rpi-amr1* locus. To test if in these accessions the resistance is conferred
208 by functional *Rpi-amr1* homologs, we performed SMRT RenSeq-based *de novo*
209 assembly of each resistant accession, and looked for homologs with the greatest identity
210 to *Rpi-amr1*. For accessions SP2307, SP3399 and SP3406, we also used cDNA RenSeq
211 to monitor their expression. We mapped *de novo* contigs to the coding sequence of *Rpi-*
212 *amr1* allowing for 15% mismatches and gaps, and selected the closest homolog as a
213 candidate *Rpi-amr1* ortholog (Table S3). In three resistant parents, namely SP1034,
214 SP2360 and SP3400, the functional alleles showed 100% identity at the amino acid
215 level to Rpi-amr1, while amino acid sequences from the remaining accessions had as
216 little as 89% identity to the functional Rpi-amr1 (Table S3). As described previously,
217 we transiently expressed the closest related candidate *Rpi-amr1* homologs in *N.*
218 *benthamiana* leaves followed by DLA with *P. infestans* isolate 88069, and verified their
219 functionality. The unique homologs of *Rpi-amr1-2273* were named as *Rpi-amr1-1032*,
220 *Rpi-amr1-1123*, *Rpi-amr1-2272*, *Rpi-amr1-2307* and *Rpi-amr1-3408.*
221
222 For some accessions, like SP1101 and SP2300, the *Rpi-amr1*-linked markers gave
223 ambiguous results, so we directly performed bulked segregant analysis (BSA) and
224 RenSeq. Additional *Rpi-amr1* co-segregating paralogs, *Rpi-amr1-1101* and *Rpi-amr1-*
225 *2300*, were identified and verified in transient assays as above (Fig. 2b).
226
227 Similarly, we inspected an F2 population derived from *S. nigrescens* accession SP3409
228 (Table 1). We applied BSA RenSeq and SMRT RenSeq to the resistant parents and F2
229 segregating population, and we found five candidate *NLRs* belonging to the same *Rpi-*
230 *amr1* clade, all of which are expressed. The five candidates were cloned, and transient
231 assays verified one of them as a functional *Rpi-amr1* homolog, *Rpi-amr1-3409*.
232 However, *Rpi-amr1-3409* does not co-segregate with *Rpi-amr1*-linked markers. We
233 used GenSeq sequence capture-based genotyping (Chen *et al.* 2018), and found that
234 *Rpi-amr1-3409* locates on chromosome 1, based on the potato DM reference genome[28].
235 This result suggests that a fragment of DNA that locates on distal end of the short arm
236 of chromosome 11 in other resistant accessions was translocated to the distal end of the
237 long arm of chromosome 1 in SP3409.

238 When the full-length amino acid sequences of nine *Rpi-amr1* homologs were aligned,
239 the polymorphisms between different functional alleles were found to be distributed
240 through all domains including the LRR region (Fig. 2a and Fig. S1).
241
242 Taken together, by using BSA RenSeq, SMRT RenSeq, cDNA RenSeq, association
243 genomics and GenSeq, we cloned eight additional functional *Rpi-amr1* homologs from
244 different resistant accessions, of which all confer resistance to *P. infestans* 88069 in
245 transient assays. The closest *Rpi-amr1* homolog from susceptible parent SP2271 does
246 not confer resistance (Fig. 2b).
247
248 ***Rpi-amr1* is present in hexaploid *S. nigrum* accessions**
249
250 Most *S. nigrum* accessions are highly resistant to *P. infestans* and *S. nigrum* has been
251 reported to be a "non-host" to *P. infestans*[29], even though rare accessions are
252 susceptible[30]. *S. americanum* may be the diploid ancestor of hexaploid *S. nigrum*[31]. To
253 test if *Rpi-amr1* also contributes to late blight resistance in *S. nigrum*, we amplified and
254 sequenced the first exon of *Rpi-amr1* from four resistant and one reported susceptible
255 *S. nigrum* accessions[30]. From three resistant accessions (SP1095, SP1088 and SP1097;
256 Table S4), we amplified sequences with >99% nucleotide identity to *S. americanum*
257 *Rpi-amr1-2273* (Fig. S2). *Rpi-amr1-1104* was more polymorphic, with 96.7%
258 nucleotide identity to *Rpi-amr1-2273*, and primers used for allele mining did not
259 amplify anything from the susceptible line SP999. These data suggest that *Rpi-amr1*
260 homologs are present in some *S. nigrum* accessions and were most likely inherited from
261 *S. americanum*.
262
263 ***Rpi-amr1* confers broad-spectrum late blight resistance in cultivated potato**
264
265 To test the scope of late blight resistance conferred by *Rpi-amr1* and its homologs, we
266 generated stably transformed transgenic potato cv Maris Piper plants carrying *Rpi-*
267 *amr1-2272* and *Rpi-amr1-2273*, the most diverged of the homologs (Table S3), and
268 inoculated them by DLA with 19 *P. infestans* isolates from UK, the Netherlands,
269 Belgium, USA, Ecuador, Mexico and Korea (Table 2). Many of the tested *P. infestans*
270 isolates can defeat multiple *Rpi* genes (Table 2). Our DLAs show that Maris Piper
271 carrying *Rpi-amr1-2272* or *Rpi-amr1-2273* resist all 19 tested *P. infestans* isolates,

272 while the wild-type Maris Piper control is susceptible to all of them. This indicates that
273 *Rpi-amr1* confers broad-spectrum resistance against diverse *P. infestans* races.
274
275 **Table 2. Phenotypes of potato plants stably transformed with *Rpi-amr1-2272* and**
276 ***Rpi-amr1-2273* after inoculation with multiple isolates of *P. infestans*.**
277

Isolate	*Rpi-amr1-2272*	*Rpi-amr1-2273*	Maris Piper	Origin	Race[e]
NL00228	R	R	S	The Netherlands	1.2.4.7
US23	R	R	S	USA	n.a.
3928A[a]	R	R	S	UK	1.2.3.4.5.6.7.10.11[f]
EC3626[b]	R	R	S	Ecuador	n.a.
NL14538[c]	R	R	S	The Netherlands	n.a.
NR47UH[d]	R	R	S	UK	1.3.4.7.10.11[f]
T30-4	R	R	S	The Netherlands	n.a.
USA618	R	R	S	USA	1.2.3.6.7.10.11
KPI15-10	R	R	S	Korea	n.a.
IPO-C	R	R	S	Belgium	1.2.3.4.5.6.7.10.11
PIC99189	R	R	S	Mexico	1.2.5.7.10.11
UK7824	R	R	S	UK	n.a.
PIC99177	R	R	S	Mexico	1.2.3.4.7.9.11
VK98014	R	R	S	The Netherlands	1.2.4.11
NL08645	R	R	S	The Netherlands	n.a.
PIC99183	R	R	S	Mexico	1.2.3.4.5.7.8.10.11
NL11179	R	R	S	The Netherlands	n.a.
EC1[b]	R	R	S	Ecuador	1.3.4.7.10.11
NL01096	R	R	S	The Netherlands	1.3.4.7.8.10.11

278 [a] Clonal lineage EU_13_A2, or "Blue13"
279 [b] Overcomes *Rpi-vnt1*
280 [c] Overcomes *Rpi-vnt1* and partially *Rpi-blb1*, *Rpi-blb2*
281 [d] Clonal lineage EU_6_A1, commonly known as "Pink6"
282 [e] Summarized in[32]
283 [f] See[33]
284
285

286 **Differential recognition by *Rpi-amr1* alleles of *Avramr1* homologs**

287

288 *Avramr1* (*PITG_07569*) was identified in *P. infestans* race T30-4 by long-read and
289 cDNA PenSeq, and multiple *Avramr1* homologs were identified in four *P. infestans*
290 isolates and classified into four subclades[25]. To investigate if all nine cloned *Rpi-amr1*
291 homologs could recognize diverse *Avramr1* homologs from different *P. infestans*
292 isolates, in addition to *Avramr1* from race T30-4 that corresponds to clade A, we
293 synthesized three *Avramr1* homologs *Avramr1-13B1*, *Avramr1-13C2* and *Avramr1-*
294 *13D1* from isolate 3928A (EU_13_A2, commonly known as "Blue 13"), corresponding
295 to clades B, C and D, respectively (Fig. 3). We also synthesized the *Avramr1* homologs
296 from *P. parasitica* and *P. cactorum*[25]. These six *Avramr1* homologs were co-expressed
297 in *N. benthamiana* by agro-infiltration in all possible combinations with nine functional
298 *Rpi-amr1* homologs and the non-functional *Rpi-amr1-2271* as a negative control (Fig.
299 3).

300

301 We found that different combinations of *Rpi-amr1* alleles and *Avramr1* homologs led
302 either to strong, weak or no HR phenotype in transient assay, but the non-functional
303 *Rpi-amr1-2271* allele failed to recognize any *Avramr1* homologs (Fig. 3). *Rpi-amr1-*
304 *2300* and *Rpi-amr1-2307* recognized one *Avramr1* homolog each, but others detected
305 *Avramr1* homologs from more than one clade. Clade C, represented here by *Avramr1-*
306 *13C2*, is usually not expressed[25], and when expressed from 35S promoter, this effector
307 was not recognized by most *Rpi-amr1* homologs, though a weak HR was observed upon
308 co-expression with *Rpi-amr1-2272*. *Avramr1-13D1* belongs to Clade D, which is
309 absent in T30-4 but present in four other sequenced isolates[25], and was recognized by
310 all but one (*Rpi-amr1-2300*) homologs in the transient assay. Surprisingly, two
311 *Avramr1* homologs from *P. parasitica* and *P. cactorum* are strongly recognized by all
312 functional *Rpi-amr1* homologs, apart from *Rpi-amr1-2272* which showed a weaker HR
313 (Fig. 3).

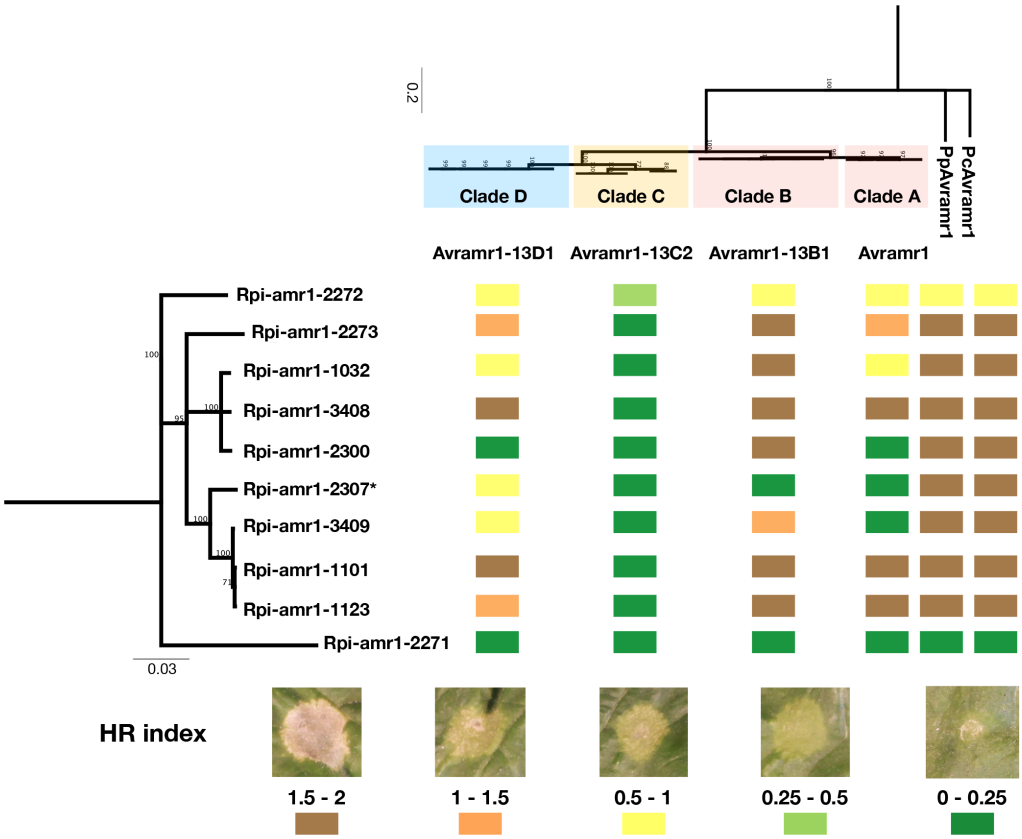

Fig. 3. Differential recognition of *Rpi-amr1* and *Avramr1* homologs.

Four *Avramr1* homologs representing clades A-D, and *P. parasitica* and *P. cactorum* homologs were co-infiltrated with ten Rpi-amr1 homologs, including a non-functional homolog *Rpi-amr1-2271*, into *N. benthamiana* leaves. Colours from green to brown represent the strength of HR scored from 0 to 2 (see bottom panel). N=3.
Left: phylogenetic tree of nine functional *Rpi-amr1* homologs and non-functional homolog *Rpi-amr1-2271*. Top: phylogenetic tree of *Avramr1* homologs from four isolates of *P. infestans*.
* Stable *Rpi-amr1-2307 N. benthamiana* transformants show HR upon transient expression of *Avramr1* and *Avramr1-13B*1.

Collectively, our data shows that *Rpi-amr1*/*Avramr1* homolog pairs provoke quantitatively and qualitatively different HRs, but all functional *Rpi-amr1* homologs detect at least one *Avramr1* homolog from *P. infestans* isolate 3928A.

Both *Rpi-amr1*-mediated resistance and effector recognition are NRC2 or NRC3 dependent

We generated a phylogenetic tree for representative *Solanaceae* NLR proteins. Rpi-amr1 is grouped with clade CNL-3, from which no functional resistance genes were previously cloned (Fig. 4a). This phylogenetic affiliation suggested that Rpi-amr1 is

335 likely to depend on the helper NRC clade because CNL-3 is among the large super-
336 clade of NRC-dependent sensors (Fig. 4a)[6].
337 To test this hypothesis, we transiently expressed *Rpi-amr1-2273* together with
338 *PpAvramr1* in NRC4, NRC2/3 or NRC2/3/4 knock out *N. benthamiana* leaves[34,35] (Fig.
339 S3). The HR phenotype was abolished in NRC2/3 and NRC2/3/4 knockout plants (Fig.
340 S4 c and b), but not in NRC4 knock-out or wild-type plants (Fig. S4 d and a). The HR
341 was recovered when NRC2 or NRC3 was co-expressed in the NRC2/3/4 or NRC2/3
342 knock out plants, but co-expression of NRC4 did not complement the loss of HR
343 phenotype in NRC2/3/4 knockout plants. (Fig. S4 b and c). We further showed that also
344 *Rpi-amr1* mediated resistance is dependent on NRC2 or NRC3 but not NRC4, as
345 transient expression of *Rpi-amr1-2273* followed by *P. infestans* infection restricted
346 pathogen growth only in NRC4 knockout *N. benthamiana* plants (Fig. 4b). These data
347 indicate that both the effector recognition and resistance conferred by *Rpi-amr1* is
348 NRC2 or NRC3 dependent.
349

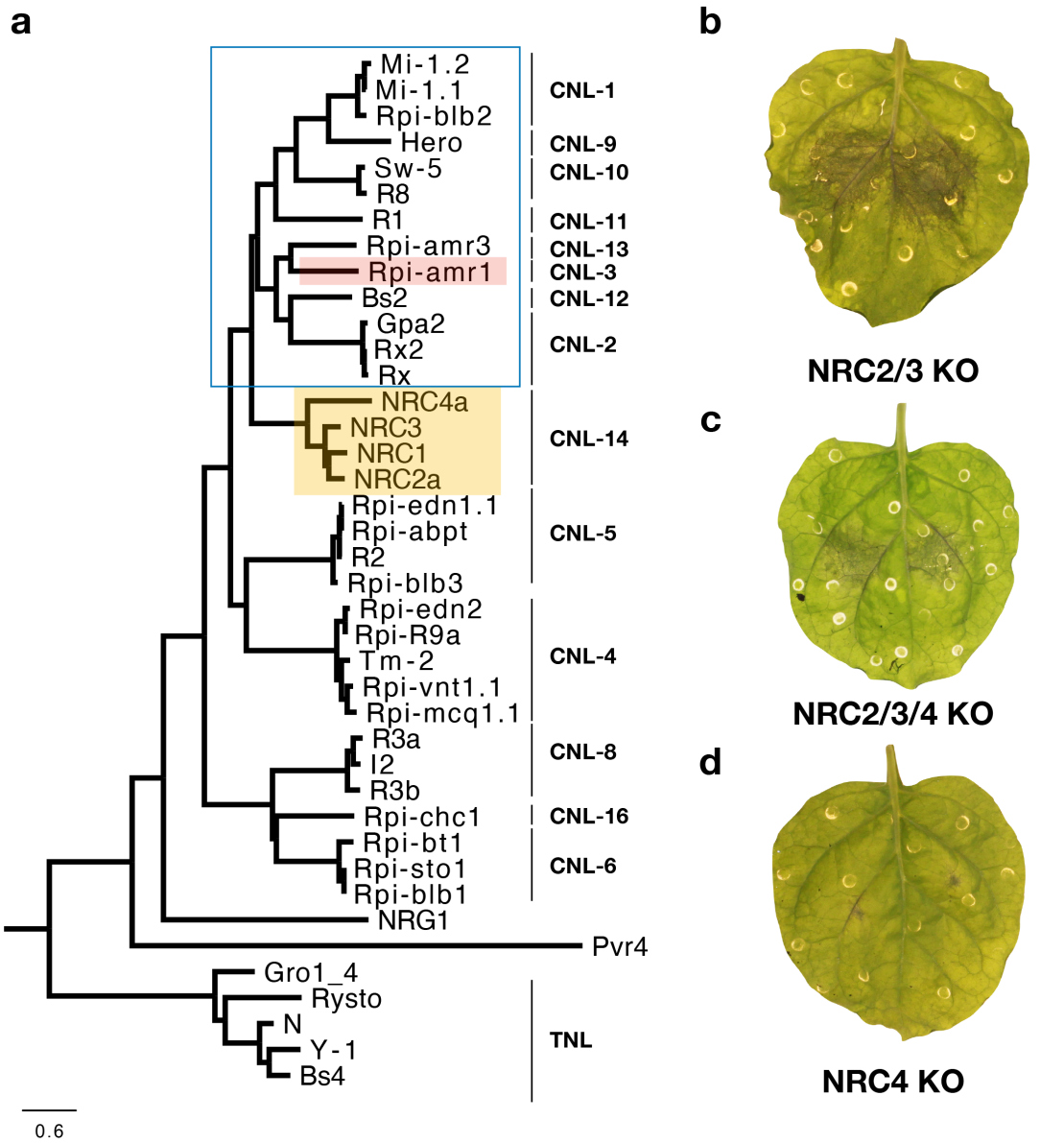

Fig. 4. Rpi-amr1 is NRC2 or NRC3 dependent.

(a) Phylogenetic analysis of Rpi-amr1 protein and other functional Solanaceae NLR proteins. The NLR clades shown here are as described previously[24], the NRC-dependent sensor clades are marked by blue box.

(b) Transient expression of *Rpi-amr1-2273* in *NRC2*/*NRC3* double knockout *N. benthamiana*, followed by zoospore inoculation of *P. infestans* isolate 88069, results in large necrotic lesions indicating lack of resistance.

(c) Transient expression of *Rpi-amr1-2273* in *NRC2*/*NRC3*/*NRC4* triple knockout *N. benthamiana*, followed by zoospore inoculation of *P. infestans* isolate 88069, results in large necrotic lesions indicating lack of the resistance.

(d) Transient expression of *Rpi-amr1-2273* in *NRC4* knockout *N. benthamiana*, followed by zoospore inoculation of *P. infestans* isolate 88069 results in small necrotic lesions indicating resistance.

366 **High allelic diversity at *Rpi-amr1* was generated through inter-paralog and**
367 **ortholog sequence exchange**
368
369 *Rpi-amr1* alleles show relatively high nucleotide diversity (π=0.04), which could be an
370 indication of balancing or diversifying selection (Table S5). In addition, *Rpi-amr1*
371 alleles differ in their recognition of the *Avramr1* homologs (Fig. 3) which is also
372 consistent with selection in a host-parasite co-evolutionary arms race. To test the
373 hypothesis that allelic polymorphism at *Rpi-amr1* results from diversifying selection,
374 we calculated diversity statistics and performed a McDonald-Kreitman test on both *Rpi-*
375 *amr1* alleles and *Avramr1* homologs. As expected, *Avramr1* homologs show a signature
376 consistent with balancing selection (Tajima's D = 2.27) (Table S5). Remarkably,
377 despite the high nucleotide diversity, no clear signals of balancing or diversifying
378 selection were detected for *Rpi-amr1* (Tajima's D = 0.09083) (Table S5). Aligning the
379 *Rpi-amr1* alleles against the reference and scrutinizing the sequences in more detail
380 provided further insights. The nucleotide similarity of alleles varies markedly across
381 the *Rpi-amr1* homologs (Fig. 2a and Table S3); this pattern is consistent with occasional
382 recombination between highly diverged alleles or paralogs.
383
384 To test whether recombination could explain the observed polymorphisms in *Rpi-amr1*
385 alleles, we predicted the possible recombination events using 3SEQ. Several
386 recombination events were detected between *Rpi-amr1* orthologs from different *S.*
387 *americanum* accessions, and *Rpi-amr1* paralogs from SP2273 (Table S6). Some
388 sequence exchanges were visualized using HybridCheck (Fig. S5)[36], and these data
389 suggest that sequence exchange occurred between functional *Rpi-amr1* alleles and
390 paralogs. To confirm these findings, we mapped all cloned *Rpi-amr1* CDS back to the
391 BAC_5G sequence from accession SP2273 (Fig. S6). As expected, some *Rpi-amr1*
392 homologs (e.g. SP2300 and SP2272) show a perfect match with the fourth NLR, and
393 show a distribution of high identity that reflects the intron-exon structure. For some
394 homologs (e.g. 2271), 5' end sequences match different NLR sequences on the
395 BAC_5G and for others (e.g. 2275) part of the sequence is highly diverged from
396 BAC_5G. Taken together, our results indicate that the polymorphism of *Rpi-amr1*
397 alleles appears to have arisen partly due to sequence exchange between highly diverged
398 alleles and paralogs, and not just through mutation accumulation.

399 **Discussion**

400

401 Achieving durable resistance is the ultimate goal of resistance breeding. Here, we report
402 significant progress towards durable resistance against potato late blight. Most cloned
403 late blight resistance genes derive from wild potatoes, and many have been overcome
404 by one or more *P. infestans* strains[37]. Conceivably, resistance to *P. infestans* in nearly
405 all *S. americanum* and *S. nigrum* accessions is due to multiple *NLR* genes, as zoospores
406 from *P. infestans* can germinate on *S. nigrum* leaves but penetration is stopped by strong
407 HR[29,38]. *Rpi* genes from plant species that only rarely support pathogen growth have
408 likely not participated, or are no longer participating, in an evolutionary arms race with
409 *P. infestans*, and hence, the pathogen's effectors have not (yet) evolved to evade
410 detection by these *Rpi* genes. Under this scenario, a pre-existing standing variation in
411 the pathogen for overcoming such *Rpi* genes is either absent or extremely rare. This
412 makes such genes promising candidates for provision of broad-spectrum and durable
413 late blight resistance, provided they are not deployed alone which facilitates one-step
414 genetic changes in the pathogen to evade them, but rather in combination with other
415 genes, as in the source plant[39].

416

417 We report here a novel, broad-spectrum *S. americanum* resistance gene, *Rpi-amr1*. We
418 also identified eight additional *Rpi-amr1* alleles from different *S. americanum*
419 accessions and relatives, including one *Rpi-amr1* allele that translocated to the long arm
420 of chromosome 1. Allele mining also suggested the presence of *Rpi-amr1* homologs in
421 *S. nigrum*. All nine cloned *Rpi-amr1* alleles confer late blight resistance in transient
422 assays in *N. benthamiana*, and both *Rpi-amr1*-2272 and *Rpi-amr1-2273* in potato cv
423 Maris Piper background confer resistance to all 19 tested *P. infestans* isolates from
424 different countries, many of which overcome other *Rpi* genes. Thus, *Rpi-amr1* is widely
425 distributed in germplasm of *S. americanum*, its relatives and *S. nigrum*, and may
426 contribute to the resistance of nearly all accessions to *P. infestans*.

427

428 Many plant *R* genes and their corresponding *Avr* genes evolved differential recognition
429 specificities with extensive allelic series for both *R* gene and *Avr* genes. Examples
430 include *ATR1* and *RPP1* or *ATR13* and *RPP13* from *Hyaloperonospora arabidopsidis*
431 and *Arabidopsis*[9], *Avr567* and *L* genes from the rust *Melampsora lini* and flax[40], and

432 multiple and diverse recognized effectors from barley powdery mildew and *Mla* from
433 barley. In the same manner, *Avramr1* and its homologs from several *P. infestans* races[25]
434 were found to be differentially recognized by high allelic variation at the *Rpi-amr1* gene.
435 Remarkably though, the nucleotide diversity of the *R* gene did not show any of the
436 hallmarks of diversifying or balancing selection.
437
438 Rather than through mutation accumulation, the high allelic variation observed at *Rpi-*
439 *amr1* appears to have been generated partly by recombination between significantly
440 diverged alleles and paralogs. The recombination events are likely to be rare relative to
441 the mutation rate, given that the alleles carry many polymorphisms. This evolutionary
442 scenario can explain the observed mosaic-like structure of high and low sequence
443 similarities when the *Rpi-amr1* alleles were mapped against the contig based on two
444 overlapping BAC clones. The deep coalescence of alleles that is implicit in this scenario
445 can be generated by balancing selection, but we did not find evidence of such selection
446 when analysing the nucleotide substitution patterns. Recombination between *Rpi-amr1*
447 alleles could have eroded this signature of selection, as has been observed also in *Rp1*
448 resistance genes in grasses[41] and in the vertebrate immune genes of the major
449 histocompatibility complex (MHC)[42,43]. Nucleotide sequence diversity across the *Rpi-*
450 *amr1* alleles is correlated with only slight differences in *Avramr1* recognition
451 specificity. *Rpi-amr1* alleles can even recognize multiple *Avramr1* paralogs from a
452 single *P. infestans* strain, a scenario that might elevate durability of resistance. Since
453 the *S. americanum* population recognizes multiple *Avramr1* alleles and paralogs, small
454 mutational changes in *Avramr1* gene are unlikely to suffice to escape detection, which
455 makes resistance-breaking less likely, thus promoting evolutionary durability of *Rpi-*
456 *amr1*. We hypothesise that this enhanced recognition capacity could be key to the
457 evolution of "non-host" resistance, offering an escape from the coevolutionary arms
458 race. Conceivably, stacking *Rpi-amr1* alleles *in cis* could extend the recognition
459 specificities, which could potentially lead to even more durable late blight resistance.
460
461 Intriguingly, two *Avramr1* homologs from *P. parasitica* and *P. cactorum* are
462 recognized by all *Rpi-amr1* homologs. Presumably, these genes have been under even
463 less selection pressure to evade *Rpi-amr1* recognition. This result indicates that *Rpi-*
464 *amr1* has the potential to provide "non-host" type resistance in *S. americanum* against
465 multiple oomycete pathogens like *P. parasitica* and *P. cactorum*, which can infect a

466 wide range of hosts. As both the resistance and effector recognition of *Rpi-amr1* are
467 *NRC2* or *NRC3* dependent, co-expression of *NRC2* or *NRC3* with *Rpi-amr1* might
468 enable it to confer resistance to other *Phytophthora* species outside the *Solanaceae*.
469
470 In summary, we cloned *Rpi-amr1,* a broad-spectrum *Rpi* gene that contributes to the
471 strong resistance of nearly all *S. americanum* accessions to late blight. The apparent
472 redundancy across the *Rpi-amr1* gene family may serve an evolutionary function by
473 broadening the scope for recognizing multiple *Avramr1* alleles and paralogs, and
474 potentially reducing the probability of evolution of resistance-breaking strains.
475 Stacking this type of *Rpi* gene with additional *Rpi* genes might help to turn host plants
476 such as potato into non-hosts for late blight, enabling broad-spectrum and durable
477 resistance.
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482 # Methods

483 Methods and associated references are in supplementary information.

484

485 # Accession codes

486 Supporting raw reads and annotated BAC sequences were deposited in European Nucleotide
487 Archive (ENA) under project number PRJEB38240.

488

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490

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Author contributions:

K.W., X.L., F.J., R.S., C.O. and J.D.G.J. designed the study. K.W., X.L., H.S.K., F.J., A.I.W., S.B., W.B., L.T. and T.S., performed the experiments. K.W., X.L., H.S.K., F.J., A.I.W., B.S., R.S., C.O., S.F., and J.M.C. analysed the data. K.W., X.L., H.S.K., F.J. and J.D.G.J. wrote the manuscript with input from all authors. V.G.A.A.V., B.B.H.W, C.-H.W., H.A. and S.K.

615 contributed resources. K.W., X.L and H.S.K. made equivalent contributions and should be
616 considered joint first authors. All authors approved the manuscript.
617

618 ## Conflict of interest:

619 K.W., H.S.K., F.G.J. and J.D.G.J. are named inventors on a patent application
620 (PCT/US2017/066691) pertaining to *Rpi-amr1* that was filed by the 2Blades Foundation on
621 behalf of the Sainsbury Laboratory.
622

623 ## Supplementary files:

624

625 **Fig. S1:** Alignment of Rpi-amr1 proteins, including non-functional homolog from SP2271.
626 **Fig. S2:** Alignment of *Rpi-amr1-2273* and *Rpi-amr1* DNA sequences from *S. nigrum*.
627 **Fig. S3:** Genotypes and phenotypes of *NRC2/3* knockout *N. benthamiana*. (a). Amplicon
628 sequencing results of the *NRC* loci of the *NRC2/3* knockout *N. benthamiana* line *nrc23*_1.3.1.
629 Sequences of the sgRNAs are marked in red; (b). The *NRC2/3* knockout line (*nrc23*_1.3.1) did
630 not exhibit any growth defects when compared to the wild type plants. Six-week-old wild type
631 and *NRC2/3* knockout *N. benthamiana* lines were used in the photograph.
632 **Fig. S4:** The effector recognition of *Rpi-amr1* is NRC2 or NRC3 dependent. The *Rpi-amr1*
633 and *Pp-Avramr1* were co-expressed by agro-infiltration on (a) wild type *N. benthamiana*; (b)
634 NRC2/3/4 knockout line; (c) NRC2/3 knockout line and (d) NRC4 knockout line. *NRC2*, *NRC3*
635 or *NRC4* were co-expressed with *Rpi-amr1* or *Pp-Avramr1* on different knockout lines. *Rpi-*
636 *amr1-2273* or *Avramr1* alone were used as negative controls.
637 **Fig. S5:** Sequence exchange between *Rpi-amr1* homologs. Sequence exchange events were
638 visually checked and highlighted (b and d) or identified by HybridCheck (a and c). For
639 HybridCheck, sequence similarity was visualised using the colours of an RGB colour triangle
640 (top); deviation from the default red, green and blue at positions with the same colour indicates
641 regions where two sequences share the same polymorphisms, which is indicative of intra- or
642 inter-locus recombination. Line plot shows the percentage of SNPs shared at informative sites
643 between sequences in each of the three pairwise combinations for the triplet.
644 **Fig. S6:** Cloned *Rpi-amr1* CDS were mapped back to BAC_5G using BLAT and visualized
645 on the BAC sequence using the Sushi package.
646

647 **Table S1:** Linked RAD markers identified based on tomato reference genome.

648 **Table S2:** Molecular markers used in this study.

649 **Table S3:** Amino acid sequence similarity between Rpi-amr1 homologs.

650 **Table S4:** *S. nigrum* accessions used in this study.

651 **Table S5**: Tajima's D analysis of *Rpi-amr1* and *Avramr1* homologs.

652 **Table S6**: Evidence of sequence exchange between *Rpi-amr1* orthologs and paralogs from
653 SP2273 using 3SEQ.

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681 # Supplementary Materials and Methods:

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683 **Development of mapping populations**

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685 14 *P. infestans* resistant diploid *Solanum americanum* and relatives were used in this study
686 (Table 1). The F1 populations were generated by crossing with a susceptible *Solanum*
687 *americanum* accession 954750186 (working name SP2271) as a female parent. Heterozygous
688 F1 progeny was allowed to self-pollinate to generate F2 segregating populations, or further
689 back-crossed to the susceptible parent and allowed to self-pollinate until resistance to *P.*
690 *infestans* co-segregated as a monogenic trait.

691

692 ***P. infestans* infection assay**

693

694 *P. infestans* isolates were cultured on rye and sucrose agar (RSA) medium at 18 °C for 10 days.
695 Sporangia were washed off with cold water and incubated at 4°C for 1-2 h to induce zoospore
696 release. Detached leaves were inoculated on the abaxial side with 10 µl droplets of zoospore
697 suspension (50-100,000 per ml). The inoculated leaves were incubated at 18°C in high
698 humidity under 16 h day/8 h night photoperiod conditions. Disease scoring was done at 5-7
699 days after infection.

700

701 **DNA and RNA extraction**

702

703 RenSeq experiments (both short- and long-reads protocols) were conducted on gDNA freshly
704 extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen) according to the
705 manufacturer's protocol. For the cDNA RenSeq experiment, RNA was extracted using TRI-
706 Reagent (Sigma-Aldrich, MO, USA) and Direct-zol RNA MiniPrep Kit (Zymo Research, CA,
707 USA), following manufacturer's recommendations.

708

709 **Mapping of *Rpi-amr1***

710

711 To map the underlying resistance gene from the resistant parent 954750184 (working name
712 SP2273), we generated an F2 segregating population which was phenotyped with *P. infestans*
713 isolates EC1_3626 and 06_3928A. Selected resistant plants were self-pollinated and up to 100

714 plants from F3 populations were screened for resistance and susceptibility with *P. infestans*
715 isolates EC1_3626 and 06_3928A. gDNA from susceptible F2 and F3 plants (BS pool), as well
716 as gDNA from the resistant (R) and susceptible parent (S) were subjected to RenSeq using
717 Solanaceae bait library[1] and sequenced with Illumina GAII 76 bp paired-end reads. Pre-
718 processing, assembly, mapping and SNP calling was performed as described earlier[1,2].
719
720 The same gDNA samples were used in a RAD-seq experiment using PstI digestion and
721 Illumina HiSeq sequencing, which was outsourced to Floragenex Inc. (OR, USA).
722 Bioinformatic analysis was also performed by Floragenex using *Solanum lycopersicum*
723 genome as a reference[3]. SNP calling resulted in sixteen polymorphic sites with eleven of them
724 locating at the top of chromosome 11 (Supplementary table 1). The remaining ones were
725 randomly distributed on chromosomes 4 and 1.
726
727 We additionally outsourced Whole Genome Shotgun sequencing (WGS) of R and S samples
728 to BGI (BGI, Shenzhen, China) for ~30 deep Illumina HiSeq sequencing with 100PE. Reads
729 from the resistant parent were assembled as described in[2] and we used our previously published
730 *in silico* trait mapping pipelines to perform SNP calling and detection of polymorphisms linked
731 to disease resistance[1,2]. Contigs polymorphic between R and S parents were further aligned to
732 the DM reference genome to identify their position.
733
734 Screening a set of markers derived from these three approaches on gDNA of 94 susceptible F2
735 and F3 plants identified 12 markers linked to resistance response that flank the *R* locus between
736 7.5 cM to one side and 4.3 cM to the other side (WGS, Table S1). Four of these markers were
737 found to co-segregate with the resistance, and two others located around 1 cM on either side,
738 CAPS marker RAD_3 to the distal side and the PCR marker WGS_1 to the proximal side
739 (Figure 1). Both 1 cM markers were subsequently used to genotype 1,793 F2 plants, and we
740 identified 228 recombinants (118 homozygous susceptible to one side and heterozygous to the
741 other, 110 homozygous resistant to one side and heterozygous to the other).
742
743 The 118 informative recombinants (homozygous susceptible / heterozygous) were further
744 genotyped using eight linked markers (Figure 1b), and tested in detached leaf assays for their
745 response to *P. infestans* isolates EC1_3626 and 06_3928A. This revealed that markers CLC_3
746 (WGS_3) and RAD1 are flanking with a single recombination event for each marker, and
747 CLC_2 (WGS_2), 56766 and 46418 are co-segregating with the resistance locus (Figure 1b).

748 Comparison of the linkage map (Figure 1) with the potato reference genome[4] identified the
749 homogeneous CNL-3 NLR gene sub-family to be within the co-segregating locus. This cluster
750 comprises 18 members on potato reference chromosome 11.
751
752 **BAC clones identification and analysis**
753
754 Construction and screening of 5x BAC library from resistant parent SP2273 was outsourced to
755 BioS&T company (Quebec, Canada). Two candidate BAC clones (5G and 12H) were
756 identified in PCR screen with WGS_2 marker-specific primers. BAC sequencing with RSII
757 PacBio platform and bioinformatic analysis was outsourced to Earlham Institute (Norwich,
758 UK); both BACs were assembled into single contigs with length of 125,327 bp (5G) and
759 144,006 bp (12H). While the co-segregating marker WGS_2 was present on both BAC clones,
760 a further co-segregating marker WGS_3 was only present on 12H. The BACs were further
761 assembled into one 212,773 contig (available in ENA under study number PRJEB38240).
762 NLRs on the contig sequence were annotated using NLR-annotator[5] and Geneious 8.1.2 build-
763 in ORF prediction tool. Gene models were annotated manually using cDNA RenSeq data
764 generated from *S. americanum* accession SP2273 as described below.
765
766 **3' RACE**
767
768 Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) and treated with RNase-Free
769 DNase (Qiagen) following manufacturer's instructions. First strand cDNA was synthesized
770 from total RNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen,
771 CA, USA) with P7-oligoDT primer. The resulting product was amplified with P7- and gene
772 specific primers by using KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, Cape
773 Town, SA) and cloned into pCR™-Blunt II-TOPO vector by using Zero Blunt® TOPO® PCR
774 Cloning Kit (Invitrogen) and transformation was performed using One Shot™ TOP10
775 Chemically Competent *E. coli* (Invitrogen). Isolation of plasmid DNA was performed with
776 NucleoSpin® Plasmid kit (MACHEREY-NAGEL, Duren, Germany).
777
778 **Resistance gene enrichment sequencing (RenSeq) and Gene enrichment sequencing**
779 **(GenSeq)**

780 SMRT RenSeq, short-read RenSeq and cDNA RenSeq were performed as described
781 previously[2] and enriched libraries were sequenced at Earlham Institute, Norwich, UK (PacBio
782 RSII, Illumina MiSeq) and Novogene, Hong Kong (Illumina HiSeq).
783
784 Illumina GenSeq was performed as described above (Illumina RenSeq), except GenSeq baits[6]
785 were used instead of RenSeq baits.
786
787 PacBio reads were processed and assembled using Geneious R8.1.8[7] as described[2]. NLR
788 coding sequences were predicted with Geneious and AUGUSTUS[8] and annotated with NLR-
789 parser[5].
790
791 To infer linked polymorphisms, the quality control for Illumina paired-end reads was
792 performed using Trimmomatic[9] with standard settings. For the RenSeq, the paired reads were
793 mapped to PacBio-assembled contigs from the resistant parent, while GenSeq reads were
794 mapped to the reference DM genome (PGSC_DM_v4.03_pseudomolecules.fasta), using BWA
795 mapper[10] with default settings. PCR duplicates and unmapped reads were removed and
796 Mpileup files to find out potential linked SNPs were created using SAMtools[11]. Mpileup files
797 were processed with VarScan[12] set to minimum read depth 20, minimum variant allele
798 frequency threshold 0.1, and minimum frequency to call homozygote 0.98. The candidate SNPs
799 were manually inspected using Savant genome browser[13]. TopHat[14] with default settings was
800 used to map cDNA Illumina reads to assembled PacBio data. All the tools used in this study
801 were embedded in The Sainsbury Laboratory (TSL) customized Galaxy instance, if not stated
802 otherwise.

803 **Transient complementation of a candidate genes in *N. benthamiana***

804

805 The candidate genes were PCR amplified from gDNA with their own promoters (1-2
806 kb upstream of start codon) and up to 1 kb terminator elements, and cloned into USER
807 vector as described[2]. Transient complementation assays followed by *P. infestans*
808 inoculation were performed as described[2].

809

810 **Stable transformation of susceptible potato cultivar Maris Piper**

811

812 Stable transgenic plants with constructs carrying *Rpi-amr1-2272*, *Rpi-amr1-2273* or
813 *Rpi-amr1a* under the control of their native regulatory elements were created in the
814 background of potato cultivar Maris Piper as described previously[15]. At least 10
815 independent transgenic lines were generated for each construct and tested for the
816 presence of the transgene using gene specific primers. All positive *Rpi-amr1-2272* and
817 *Rpi-amr1-2273* lines showed resistance in DLA with *P. infestans* isolate 88069, while
818 *Rpi-amr1a* transgenic plants were fully susceptible. Selected lines of *Rpi-amr1-2272*
819 and *Rpi-amr1-2273* were tested in DLA with 19 additional *P. infestans* isolates (Table
820 2). WT Maris Piper plants were used as a negative control.

821

822 **Generation of *NRC2/3* knockout *N. benthamiana***
823

824 *NRC4* and *NRC2*/*NRC3*/*NRC4* knockout *N. benthamiana* lines were described
825 previously[16,17]. Knocking out of *NRC2*/*NRC3* in *N. benthamiana* were performed
826 according to the methods described previously[16]. Forward primers CHW_sgNbNRCs
827 and reverse primer JC_sgrna_R[16] were used to clone sgRNA2.1-4, sgRNA3.1-4 into
828 Golden Gate level 1 vectors for different positions. Constructs of sgRNAs targeting *N.*
829 *benthamiana NRC2* and *NRC3* were assembled into level 2 vector pICSL4723 together
830 with pICSL11017 (pICH47732::NOSp::BAR, Addgene no. 51145) and
831 pICH47742::35S::Cas9[18]. Leaf discs of *N. benthamiana* were transformed with the
832 binary vector pICSL4723 containing the BAR selection marker gene, Cas9 expression
833 cassette, and sgRNAs targeting *NRC2* and *NRC3*. T0 transgenic plants were selected in
834 the medium with phosphinothricin (2 mg/L) and then transferred into the soil. The
835 progeny of the transformants were genotyped using amplicon sequencing as described
836 previously[16] (Fig. S6a). T3 populations from the selected T2 plants were used for

837 further experiments. *NRC2/3* knockout line (*nrc23*_1.3.1) did not exhibit any growth
838 defects when compared to the wild type plants (Fig. S6b).
839
840 **Phylogenetic tree construction**
841
842 Phylogenetic tree was generated from protein sequences of the cloned *Solanaceae R*
843 genes obtained from NCBI. Full-length sequences were aligned using ClustalW 1.74[19]
844 and the alignments were imported to the MEGA7[20] to build a maximum-likelihood
845 phylogenetic tree with Jones-Taylor-Thornton (JTT) substitution model and 100
846 bootstraps.
847
848 **Evolutionary analyses of *Rpi-amr1* and *Avramr1* homologs**
849
850 CDS were aligned using MUSCLE[21] as implemented in seaview[22] with and without
851 outgroup (the closest homologs from *S. lycopersicum* and *P. capsici* for *Rpi-amr1* and
852 *Avramr1*, respectively). Calculations of diversity statistics and the MacDonald-
853 Kreitmann Test were executed through DNAsp5.0[23]; DAMBE[24] was used to rule out
854 saturation. For *Rpi-amr1* homologs, the calculations were preformed separately on
855 annotated full-length sequences as well as the individual domains.
856
857 We used 3SEQ[25] to identify break points in the aligned CDS. To confirm gene
858 conversion events in *Rpi-amr1*, we mapped the CDS back to the BAC_5G sequence
859 using BLAT (minScore 1500, minMatch 93)[26]. The resulting .psl files were converted
860 into .bed files using a custom R script, prior to visualization using the R package Sushi[27].
861
862 **HybridCheck**
863
864 For each accession, FASTA files of all *Rpi-amr1e* orthologs or *Rpi-amr1* paralogs in
865 combinations of three (triplets) were generated and aligned using MUSCLE v3.8.31[21].
866 The sequence triplets were analysed using HybridCheck[28] to detect and date
867 recombination blocks between *Rpi-amr1* orthologs (sliding windows = 200bp) or
868 paralogs (sliding windows = 100 bp); non-informative sites were removed from the
869 sequence triplets. Figures showing sequence similarity were plotted (MosaicScale = 50)

870 with HybridCheck and formatted using R v3.2.0 (https://www.r-project.org). The
871 colour of each sequence window was calculated based on the proportion of SNPs shared
872 between pairwise sequences at informative sites.
873

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