Identification of *Avramr1* from *Phytophthora infestans* using long read and cDNA pathogen-enrichment sequencing (PenSeq)

Xiao Lin[1], Tianqiao Song[1], Sebastian Fairhead[1], Kamil Witek[1], Agathe Jouet[1], Florian Jupe[1], Agnieszka I. Witek[1], Hari S. Karki[1], Vivianne G. A. A. Vleeshouwers[2], Ingo Hein[3] and Jonathan D. G. Jones[1*]

[1]The Sainsbury Laboratory, Norwich Research Park, Norwich, NR4 7UH, UK; [2]Plant Breeding, Wageningen University and Research, Droevendaalsesteeg 1, 6708 PB. Wageningen, The Netherlands; [3]School of Life Sciences, Division of Plant Sciences, University of Dundee and The James Hutton Institute, Dundee, DD2 5DA, UK

*Corresponding author:

Jonathan D. G. Jones

Tel: +44 (0)1603 450400

Email: jonathan.jones@tsl.ac.uk

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Summary

- Potato late blight, caused by the oomycete pathogen *Phytophthora infestans,* significantly hampers potato production. Recently, a new *Resistance to Phytophthora infestans* (*Rpi*) gene, *Rpi-amr1*, was cloned from a wild *Solanum* species, *Solanum americanum*. Identification of the corresponding recognized effector (*Avirulence*, or *Avr)* genes from *P. infestans* is key to elucidating their naturally occurring sequence variation, which in turn informs the potential durability of the cognate late blight resistance.
- To identify the *P. infestans* effector recognized by *Rpi-amr1*, we screened available effector libraries and used long read and cDNA pathogen-enrichment sequencing (PenSeq) on four *P. infestans* isolates to explore the untested effectors.
- By using SMRT and cDNA PenSeq, we identified 47 highly expressed effectors from *P. infestans*, including PITG_07569 which triggers a highly specific cell death response when transiently co-expressed with *Rpi-amr1* in *Nicotiana benthamiana*, suggesting that *PITG_07569* is *Avramr1*.
- Here we demonstrate that long read and cDNA PenSeq enables the identification of full-length RxLR effector families, and their expression profile. This study has revealed key insights into the evolution and polymorphism of a complex RxLR effector family that is associated with the recognition by *Rpi-amr1*.

40 # Introduction
41
42 Potato late blight, caused by the hemi-biotrophic oomycete pathogen *Phytophthora infestans,*
43 triggered the Irish and European famine in the late 1840s, and still causes severe losses to world
44 potato production.
45
46 To reduce losses, breeders sought resistance genes in wild relatives of potato. Early in the 20th
47 century, *Solanum demissum*, a highly resistant hexaploid (2n=72) wild potato was found to be
48 a useful source of Resistance to *P. infestans (Rpi)* genes (Salaman, 1937). Since then, many
49 resistance traits have been transferred to cultivated potatoes by introgression breeding
50 (Toxopeus, 1956), and many *Rpi* genes have been cloned from wild potatoes, e.g. *R1*, *R3a*, *R8*,
51 *Rpi-blb1* and *Rpi-vnt1* (Ballvora *et al.*, 2002; van der Vossen *et al.*, 2003; Huang *et al.*, 2005;
52 Foster *et al.*, 2009; Pel *et al.*, 2009; Vossen *et al.*, 2016). Unlike wild potatoes, *Solanum nigrum*
53 and *Solanum americanum* have been reported to be non-hosts for *P. infestans* (Colon *et al.*,
54 1993). Two *Rpi* genes encoding NLR proteins, *Rpi-amr3* and *Rpi-amr1,* were cloned from *S.*
55 *americanum* and confer late blight resistance in potato (Witek *et al.*, 2016; 2020).
56
57 Identification of the recognized effectors for *Rpi-amr3* and *Rpi-amr1* would open the way to
58 investigate their virulence function and distribution in *P. infestans* populations. Moreover, it
59 could also help to diagnose *Rpi* gene repertoires in resistant plants, and individually confirm
60 their activity in genetically modified potatoes carrying multiple *Rpi* genes. In oomycetes, all
61 the cloned Avr proteins contain a signal peptide and RxLR motif (Rehmany *et al.*, 2005), and
62 the genomic sequencing of *P. infestans* revealed 563 RxLR effectors in the T30-4 reference
63 genome (Haas *et al.*, 2009). This enabled a high-throughput effectoromics approach for
64 functional screening of the candidate effectors in plants (Vleeshouwers *et al.*, 2008; 2011), and
65 many *Avr* genes were identified by this approach, including *Avrblb1*, *Avrblb2* and *Avrvnt1*
66 (Vleeshouwers *et al.*, 2008; Oh *et al.*, 2009; Pel, 2010).
67
68 However, available RxLR effector libraries do not contain recombinant clones of all *P.*
69 *infestans* RxLR effectors, the effector candidates were defined on the basis of expression
70 profile, motif analysis and distribution between *P. infestans* races (Vleeshouwers *et al.*, 2008;
71 Oh *et al.*, 2009; Haas *et al.*, 2009). In total, ~300/563 RxLR effectors were previously cloned
72 into expression vectors for functional screening (Rietman, 2011).
73

To further explore the diversity of RxLR effectors from *P. infestans*, a pathogen enrichment sequencing (PenSeq) approach was adopted to study allelic variation of RxLR effectors and population genomics of oomycetes. A bait library of RxLR effectors and some other pathogen-related genes was synthesized and used for enrichment prior to sequencing (Jouet *et al.*, 2018; Thilliez *et al.*, 2018). However, the previous PenSeq analyses used Illumina reads and genomic DNA (gDNA), making it difficult to differentiate individual effector alleles and closely related paralogs, or to find out which effectors are expressed.

Here, to identify the recognized effector of the newly-cloned Rpi-amr1 protein from *S. americanum* (Witek *et al.*, 2020), we screened all currently available RxLR effectors for recognition but without success. Therefore, we used PenSeq with long read (PacBio) and cDNA methods, and extended the list of candidate effectors that could be screened. Amongst these additional candidate RXLR genes, we identified *Avramr1* and defined orthologs and paralogs from four different isolates of *P. infestans*.

Materials and Methods:

Sample preparation

To collect the mycelium of *Phytophthora infestans* for DNA extraction, *P. infestans* strains were grown on RSA solid medium for 7 days and then moved to Plich liquid media for 14 days. Mycelia were washed and harvested, freeze-dried using a vacuum pump and stored at -80°C until DNA or RNA extraction.

To collect the infection samples or zoospores for RNA extraction, *P. infestans* strains were cultured for 10 days on RSA medium. Grown mycelia were covered with cold (4 °C) sterile water and then incubated at 4-6 °C for 2-3h. The concentration of the inoculum was adjusted to about 50,000 zoospores/mL and 10 µL drops of inoculum were placed on the detached leaves of potato plants. Detached leaf assays (DLA) were incubated at 20 °C in high humidity for a required time post inoculation. Leaf discs of the infection area were collected and stored at -80°C until DNA or RNA extraction.

DNA and RNA extraction

110 DNA was extracted using phenol/chloroform. *P. infestans* mycelium samples or infected leaf
111 discs were ground into powder in liquid nitrogen. Ground material was resuspended in 500 µl
112 of Shorty buffer (20% 1M Tris HCl pH 9, 20% 2M LiCl, 5% 0.5M EDTA, 10% SDS 10%, 45%
113 dH2O) and one volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added. The
114 upper aqueous phase containing DNA was mixed with one volume of 100% ice-cold
115 isopropanol to precipitate DNA. The pellet was washed twice using 70% ethanol, heated at
116 70°C for 2-5 minutes to completely remove ethanol and resuspended in sterile water.
117 Resuspended DNA was then heated at 65°C for 20 minutes to inactivate DNases before RNase
118 treatment was performed (2 µl of 10 mg/ml^{-1} RNase A, 37°C, 1h) and RNase A removed by
119 chlorophorm precipitation. Genomic DNA was resuspended in water and sheared into 3-5 kb
120 fragments using the S220 Focused-ultrasonicator (Covaris Inc., MA, USA).
121
122 RNA samples were extracted with Direct-zol™ RNA MiniPrep kit (Zymo Research, Tustin,
123 CA, USA) according to the manufacturer's instructions.
124
125 **PacBio and Illumina PenSeq capture**
126
127 PacBio library was constructed with DNA samples from the mycelium of four *P. infestans*
128 strains, EU_13_A2 (2006_3928A), EC1_A1 (EC1_3626), EU_6_A1 (2006_3920A) and US23.
129 The library construction and target DNA sequence capture were performed according to (Witek
130 *et al.*, 2016), with minor modifications. Qubit Fluorometer (ThermoFisher, Dubuque, IA, USA)
131 was used to quantify the barcoded DNA library from each isolate. Equimolar amounts of DNA
132 from the four individually barcoded samples were pooled to obtain 250 ng of total DNA and
133 then subjected to sequence capture. A 10x excess of non-adaptor-ligated *P. infestans* DNA at
134 about 500-1,000 bp was added for the hybridization. The final mixture of the amplicons of the
135 captured library was further size selected by SageELF electrophoresis system (Sage Science,
136 MA, USA) according to the instructions of the manufacturer.
137
138 Illumina library was constructed with RNA samples from zoospores of the four *P. infestans*
139 strains, from the corresponding infected leaf discs harvested at 12 hours post infection (hpi), 1,
140 2 and 3 days post infection (dpi), and from mycelium of EU_13_A2. An Illumina library for
141 each sample was constructed with KAPA mRNA HyperPrep Kit for Illumina® Platforms
142 (KR1352 – v5.17) following the manufacturer's instructions. mRNA was fragmented to 300-
143 400 bp. The barcoded libraries were mixed together at a ratio of 16: 8: 4: 1: 1 for 12 hpi, 1 dpi,
144 2 dpi, 3 dpi, zoospores and mycelium samples, respectively.

145 Both types of libraries were subjected to sequence capture using the bait library as described
146 previously (Jouet *et al*., 2018, Thilliez *et al.*, 2018). Before and after sequence capture, qPCR
147 was performed on Bio-Rad CFX96 real-time detection system with an input of 1 ng DNA to
148 assess the efficiency of capture.
149
150 **Sequencing**
151
152 PacBio PenSeq libraries were sequenced at the Earlham Institute (Norwich, UK) using Sequel
153 platform. Illumina PenSeq cDNA libraries were sequenced at Novogene (Hong Kong, China)
154 using HiSeq, PE250.
155
156 **gDNA PenSeq assembly**

157 PacBio raw reads were processed as described in (Witek et al., 2016) to generate ROI reads
158 and demultiplexed using custom script (Van de Weyer et al., 2019). Demultiplexed ROI were
159 assembled using Geneious R8 (http://www.geneious.com/) using settings as in (Witek et al.,
160 2016).

161

162 **Analysis of cDNA PenSeq**
163 All RxLR effectors from the *P. infestans* reference genome T30-4 were used to generate an
164 artificial "RxLRome" contig, where RxLR effectors' sequences were separated by stretches of
165 500 "Ns". The contig also contained nine non-RxLR control genes (Jouet et al., 2018). The
166 cDNA PenSeq reads from all treatments were mapped to the T30-4 RxLRome, and the
167 expression analyses were performed and visualized using Geneious R10 (Kearse et al., 2012).
168
169 **New candidate RxLR effectors**
170
171 For the previously untested RxLR effectors, we first selected the effectors showing differential
172 expression at different stages and ranked them based on the raw transcript counts. Next, local
173 alignment searches (BLAST) were performed against the 563 predicted RxLR effectors (Haas
174 *et al.*, 2009) to remove the previously tested effectors. This analysis revealed 47 candidate
175 RxLR effectors which were not included in previous functional study. The 47 RxLR effectors
176 were synthesized by Twist Bioscience (San Francisco, CA, USA). The signal peptides were
177 removed, the sequences were domesticated for Golden Gate cloning, and overhangs containing
178 BsaI restriction sites were added to both ends of all effector sequences.
179

180 All the effectors were cloned into vector pICSL86977 (TSL SynBio) with CaMV 35S promoter
181 and OCS terminator. The constructs were transformed to *Agrobacterium* strain GV3101 for
182 agro-infiltration.
183
184
185 **Cell death assay**
186
187 Transient expression of RxLR effectors and *Rpi-amr1* in *Nicotiana benthamiana* was
188 performed as described previously (Bos *et al.*, 2006). *Agrobacterium* was infiltrated at
189 OD600=1, and each effector was co-infiltrated with *Rpi-amr1-2273* (Witek *et al.*, 2020). The
190 cell death phenotype was observed at 4 dpi.
191
192 **Data availability**
193 Raw PacBio and cDNA PenSeq read sequences have been deposited in the Sequence Read
194 Archive (SRA) under BioProject IDs PRJNA623167 and PRJNA598824.
195
196
197
198 # Results
199
200
201 **Available recombinant RxLR effector libraries do not contain *Avramr1***
202 To identify *Avramr1*, we tested 278 available RxLR effectors (Table S1) by co-expressing
203 them with *Rpi-amr1-2273 i*n *N. benthamiana* (Rietman, 2011; Witek *et al.*, 2020). However,
204 no effector activated *Rpi-amr1*-dependent HR, so we concluded that *Avramr1* was absent from
205 the available RxLR effector libraries. Notably, *Avr8* was not originally included in the core
206 effector selection, because *Avr8* expression goes up earlier then 2 dpi (Jo, 2013), showing that
207 the criteria adopted to define core effectors did not reveal all recognized effectors.
208
209 To find *Avramr1*, we proposed three hypotheses: 1) *Avramr1* is an RxLR effector but it is not
210 present in the assembled version of *P. infestans* T30-4 reference genome 2) *Avramr1* is an
211 RxLR effector but it was not yet tested in previous functional studies/libraries; 3) *Avramr1* is
212 not a typical RxLR effector. To address hypothesis 1, we performed PacBio PenSeq to
213 sequence the effector alleles in the four diverse *P. infestans* isolates, EU_13_A2 (2006_3928A),
214 EC1_A1 (EC1_3626), EU_6_A1 (2006_3920A) and US23, all of them avirulent on potato
215 plants carrying *Rpi-amr1*, it indicates they all carry the recognized effector. To address

216 hypothesis 2, we performed cDNA PenSeq to try to identify other RxLR effectors that are
217 expressed during infection but not reported or defined in previous functional studies.
218
219 **PacBio PenSeq of four *P. infestans* isolates EU_13_A2, EC1_A1, EU_6_A1 and US23**
220
221 PacBio gDNA PenSeq was performed on four *P. infestans* isolates of genotypes EU_13_A2,
222 EC1_A1, EU_6_A1 and US23 (Fig. 1a). To evaluate the enrichment efficiency, qPCR was
223 performed with the DNA pre- and post- capture. In general, the targeted genes of different
224 length were well enriched at Concentration x time (Cot) value <20, while the untargeted genes
225 were almost undetectable, with Cot value >27 (Peterson *et al.*, 2002) (Fig. S1). Furthermore,
226 we found that the capture efficiency was increased by including a 10-fold molar excess of non-
227 adaptor-ligated fragmented *P. infestans* DNA (500-1,000 bp) in the reannealing reaction, to
228 reduce the extent to which sequences were recovered due to concatenation of transposon-
229 containing sequences adjacent to *RxLR* genes. After sequence capture, enrichment of most
230 effector genes was more efficient when non-adaptor-ligated *P. infestans* DNA was included
231 (Fig. S1).
232
233 Following the enrichment sequencing, circular consensus sequencing (CCS) reads were
234 assembled (Fig. 1a) and contigs with fewer than 10 reads were removed. The average length
235 of the contigs of coverage >10 reads was 7 kb (Table 1), and the size of the largest contig was
236 over 50 kb. This suggests that the PacBio PenSeq successfully captured the target effector
237 genes and the adjacent flanking DNA sequences. In total, 1,137, 1,054, 1,283 and 925 contigs
238 were obtained from EU_13_A2, EC1_A1, EU_6_A1 and US23 respectively, of which 687,
239 650, 741 and 571 contigs contain RxLR effectors. (Table 1, Notes S1-S4). The remaining
240 contigs contained non-RxLR effectors which were included in the bait library design for other
241 purposes (Thilliez *et al*., 2018).
242
243 The PacBio PenSeq data allowed us to detect new RxLR effector alleles from different
244 haplotypes of various *P. infestans* isolates, and even in polyploid genotypes like EU_13_A2
245 (Li *et al.*, 2017). This dataset can also be used to extensively study allelic variation,
246 presence/absence (P/A) polymorphism and effector evolution. For example, *Avr1*
247 (PITG_16663) and a paralogous *Avr1-like* gene (PITG_06432) are located on supercontigs
248 1.51 and 1.8 of the reference T30-4 genome, respectively. The *R1*-breaking clonal lineage
249 EU_13_A2 was reported to have an 18 kb deletion comprising the *Avr1* locus (Cooke *et al.*,

250 2012). Also, the Illumina PenSeq data showed that the *Avr1* locus is missing in EU_13_A2,
251 EC1_A1 and US23 (Thilliez *et al.*, 2018). We mapped the four *Avr1* contigs from EU_13_A2
252 (contig 192, 261, 296 and 329) to supercontig 1.51 and 1.8, and found that all four contigs map
253 to the *Avr1-like* supercontig 1.8. Two contigs (contig 261 and 286) mapped to the *Avr1-like*
254 locus, and two other contigs (contig 192 and 329) mapped to a locus next to *Avr1-like* that was
255 not previously annotated (Fig. S2), though the genes in those two contigs might be pseudogenes
256 as the signal peptide is missing in both of them. Additionally, in EU_6_A1 and US23, two
257 *Avr1* contigs did not map to *Avr1* or *Avr1-like* loci of T30-4. Thus, our PacBio PenSeq dataset
258 can provide means to detect novel RxLR effector paralogs absent from the reference genome.
259
260 As another example, our dataset carries in total 504 of the 563 predicted RxLR effectors from
261 the reference genome T30-4 (Haas *et al.*, 2009). To investigate P/A polymorphism of RxLR
262 effectors in the four sequenced isolates, we performed a basic local alignment search (BLAST)
263 of the 504 effectors against the PacBio contigs, with hits with < 50% coverage defined as absent
264 (Table S2). We found that 17, 28, 15 and 33 RxLR effectors out of the 504 are missing in
265 EU_13_A2, EC1_A1, EU_6_A1 and US23, respectively.
266
267 Taken together, we have generated a rich dataset that could help to define full length RxLR
268 effector genes, deliver robust information on alleles and paralogs, and reveal conserved or race-
269 specific effectors from different isolates. It is available in full in Notes S1-S4.
270
271 **cDNA PenSeq enables effector expression detection in early stages of infection**
272
273 To clarify whether the untested effectors might be putative *Avr* genes, we performed cDNA
274 PenSeq for the four *P. infestans* isolates EU_13_A2, EC1_A1, EU_6_A1 and US23, at
275 different time points post-infection (12 hpi, 1, 2 and 3 dpi) and in mycelium and zoospores
276 (Fig. 1b). To analyse and visualize the cDNA PenSeq data, we built an artificial DNA sequence
277 contig ("RxLRome") for the RxLR effectors. In addition, nine non-RxLR genes from the bait
278 library were included as controls (Jouet et al., 2018; Fig. 2). The cDNA PenSeq reads were
279 mapped to the RxLRome and gene expression compared over time (Fig. 1b).
280
281 Most of the RxLR effectors which were included in previous effector libraries show an up-
282 regulation of expression in the early stages of infection (Fig. 2). Some of the untested RxLR
283 effectors show a similar pattern of expression, and might also represent potential *Avr* genes,

284 while others are poorly expressed in some isolates. The details of the cDNA PenSeq are
285 available in Table S3.
286
287 **Identification of *Avramr1***
288
289 To test if *Avramr1* is among the untested effectors, we selected 47 highly expressed effectors
290 (Fig. 3) present in all tested lineages that had not previously been investigated. The effectors
291 were synthesized, cloned into an expression vector with 35S promoter and transformed into
292 *Agrobacterium* GV3101 for agro-infiltration in *N. benthamiana* (Fig. 4a). All the effectors
293 were infiltrated alone or co-infiltrated with *Rpi-amr1-2273* (Witek *et al.*, 2020). Among the 47
294 effectors, PITG_07569 was the only effector which triggered an HR when co-expressed with
295 *Rpi-amr1-2273* (Fig. 4b). Hence, we concluded PITG_07569 is *Avramr1*.
296
297 ***Avramr1* homologs in different *P. infestans* isolates and other *Phytophthora* species**
298
299 *Avramr1* is a canonical RxLR effector with RYLR and EER motifs and an N-terminal signal
300 peptide (Fig. 5b). *Avramr1* locates on supercontig 1.11 of the *P. infestans* reference genome
301 T30-4. *Avramr1-like* (hereafter *Avramr1L*), a truncated paralog (PITG_07566) maps adjacent
302 to *Avramr1* (Fig. 5a and 5b). Two known *Avr* effectors, *Avr8* (PITG_07558) and *Avrsmira1*
303 (PITG_07550), are physically close to the *Avramr1* locus in the T30-4 genome (Fig. 5a)
304 (Rietman *et al.*, 2012).
305
306 To study the sequence polymorphism of *Avramr1* homologs in *P. infestans*, we used BLAST
307 to search for *Avramr1* homologs in the PacBio PenSeq assemblies generated in this study. It
308 revealed that EU_13_A2, EC1_A1, EU_6_A1 and US23 carry six, four, three and six *Avramr1*
309 homologs, respectively. Next, we aligned the corresponding Avramr1 amino acid sequences
310 and generated a neighbourhood joining (NJ) tree for phylogenetic analysis (Fig. 6a). Two
311 *Avramr1* homologs from *Phytophthora parasitica* and *Phytophthora cactorum* were identified
312 from public database, and they were used as an outgroup (Fig. 5b and 6a). Based on the
313 phylogenetic tree, we distinguished four Avramr1 clades, clade A (containing Avramr1 from
314 T30-4) and clade C (with Avramr1L from T30-4), and two more clades, B and D (Fig. 5a). For
315 a more detailed analysis, we selected one *Avramr1* homolog from clade B and one from D
316 (*Avramr1-13B1* and *Avramr1-13D1* from EU_13_A2) and aligned them with *Avramr1*
317 homologs from clade A and C, and with *P. parasitica* and *P. cactorum* homologs. Significant

318 sequence polymorphisms are observed between effectors from different clades (Fig. 5b).
319 Meanwhile, the *Avramr1* homologs within the same clade are almost identical (Fig. 6a).
320
321 **Differential expression of *Avramr1* homologs in different *P. infestans* isolates**
322
323 To investigate the expression patterns of *Avramr1* homologs defined in the PacBio PenSeq
324 data, we mapped the corresponding cDNA PenSeq reads to the PacBio PenSeq contigs from
325 EU_13_A2, EC1_A1, EU_6_A1 and US23. The transcript per million (TPM) values for each
326 time point are visualized in Fig. 6b. The clade A homologs *Avramr1-23A1*, *Avramr1-13A1* and
327 *Avramr1-13A2* are highly expressed at almost all stages, and the *Avramr1* homologs from clade
328 B show a similar expression pattern. For clade C, some homologs, like *Avramr1-13C*,
329 *Avramr1-6C1* or *Avramr1L* gene from T30-4, are weakly expressed at all stages. However, two
330 other *Avramr1L* homologs, *Avramr1-23C1* and *Avramr1-13C2,* show moderately elevated
331 expression in zoospores, and at 1 dpi and 3 dpi. Interestingly, the *Avramr1* homologs from
332 clade D, which are missing in the reference genome T30-4, show an intermediate expression
333 level compared to Clade A, B and Clade C, and most *Avramr1* homologs in Clade D show an
334 increase in expression at the zoospore stage, and at 1, 2, 3 dpi.
335
336 In summary, our PacBio PenSeq analysis created a rich dataset to reveal new *Avr* variants from
337 different *P. infestans* isolates, and to quantify their expression profile individually. This
338 facilitates the analysis of the polymorphism of pathogen effectors and their potential
339 differential recognition patterns with the corresponding *Rpi* genes (Witek *et al.*, 2020).
340
341
342 **Discussion**
343
344 The availability of the *P. infestans* genome sequence enabled a step-change in the rate of
345 investigation of this pathogen, accelerating the discovery of recognized effectors, and of new
346 *Rpi* genes (Haas et al., 2009; Vleeshouwers et al., 2008; 2011). However, some questions
347 remain open. For example, how different are the effector repertoires in different *P. infestans*
348 isolates? To what extent do they show differential expression between races? The study of
349 plant *NLR* gene repertoires faces similar challenges, and sequence capture, combined with
350 long-read sequencing technologies, has enabled the refinement of tools to cost-effectively
351 investigate diversity, such as RenSeq, SMRT RenSeq, RLP/KSeq and AgRenSeq (Arora et al.,

352 2019; Lin *et al.*, 2020; Jupe et al., 2013; Witek et al., 2016). Recently, the pan-NLRome of 65
353 diverse *Arabidopsis thaliana* accessions was determined by a similar strategy, revealing that
354 any one accession lacks many of the NLRs found in the species pan-NLRome (Van de Weyer
355 *et al.*, 2019).
356
357 Pathogen-enrichment sequencing (PenSeq) was developed to facilitate cost-effective
358 investigation of pathogen diversity on infected plants, and polymorphism of pathogen effectors
359 (Jouet et al., 2018; Thilliez et al., 2018). The first PenSeq studies, however, were conducted
360 using Illumina short reads. This significantly limited their resolving power as many oomycete
361 genomes are highly heterozygous, and some effectors belong to large gene families with
362 multiple sequence-related paralogs that can lead to false assemblies (Gilroy et al., 2011; Oliva
363 et al., 2015).
364
365 In this study, we combined long read PenSeq and cDNA Penseq, enabling a detailed analysis
366 of the RxLR genes and their expression patterns in different *P. infestans* isolates. The cDNA
367 PenSeq dataset allowed us to define an additional set of 47 RxLR genes expressed during
368 infection that were not previously investigated. Amongst these, we identified *Avramr1*, which
369 encodes the cognate recognized effector for *Rpi-amr1* from *S. americanum* (Witek *et al.*, 2020).
370
371 The long read PenSeq data helped us to obtain full-length RxLR effector haplotypes with their
372 flanking sequences. This allowed us to distinguish individual alleles from polyploid isolates
373 like EU_13_A2, and also distinct effector paralogs. The sequences flanking the *RxLR* genes
374 enabled us to understand the possible translocation events and identify new *RxLR* loci. We
375 were also able to identify multiple new *Avramr1* homologs from different isolates, and
376 identified a new *Avramr1* Clade D which is not present in T30-4. The PenSeq dataset
377 constitutes a valuable community resource for investigating the allelic and expression diversity
378 of multiple recognized effectors.
379
380 So far, no *Rpi-amr1*-breaking *P. infestans* isolates have been found (Witek *et al.*, 2020), and
381 therefore we propose that *Avramr1* might be crucial for the virulence of *P. infestans*. The
382 identification of *Avramr1* will enable us to study its virulence function, its polymorphism in
383 the *P. infestans* population and its recognition by *Rpi-amr1*. Collectively, these data and
384 methods will contribute to understanding this fast-evolving and destructive oomycete pathogen,
385 and to achieving durable late blight resistance in potato.

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505 # Table:

Table 1. PacBio PenSeq for EU_13_A2, EC1_A1, EU_6_A1 and US23

	Total contigs	RxLR effector	Non_RxLR	Average length (bp)	Minimum length (bp)	Maximum length (bp)
EU_13_A2	1,137	687	450	7,732	2,617	30,571
EC1_A1	1,054	650	404	7,663	2,696	33,203
EU_6_A1	1,283	741	542	7,463	3,052	50,275
US23	925	571	354	7,598	2,648	29,901

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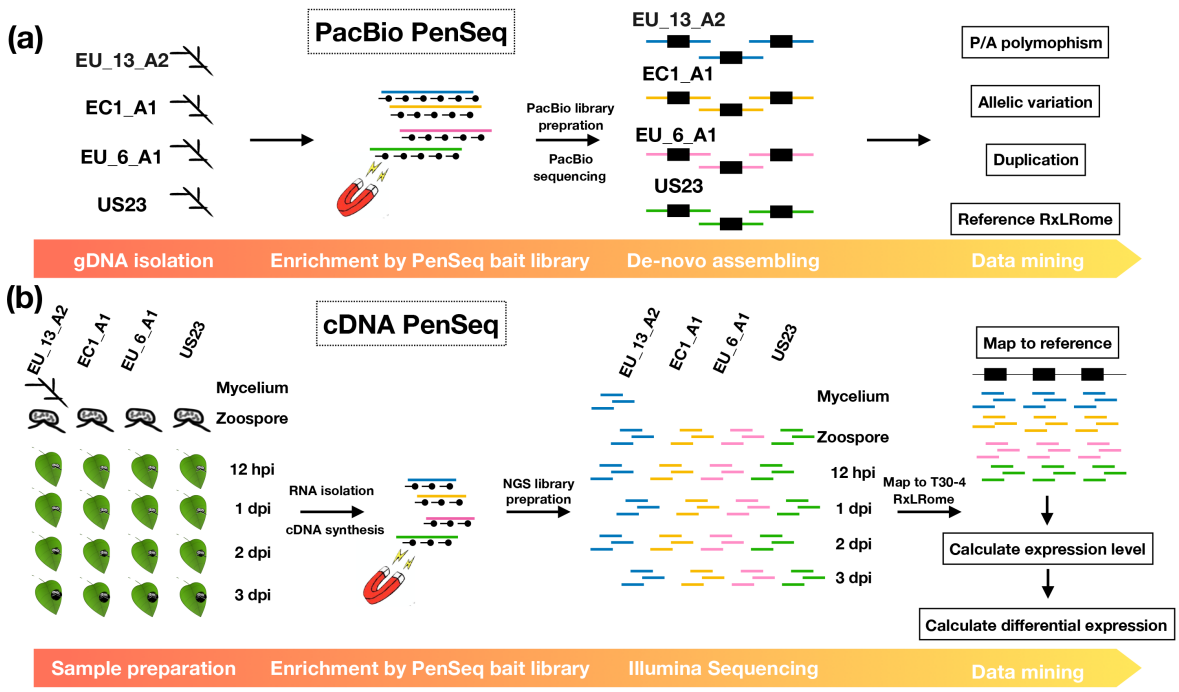
526 **Figures:**



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528 **Fig. 1 The pipelines of PacBio and cDNA PenSeq.**

529 (a) The pipeline of PacBio gDNA PenSeq. Briefly, the gDNA isolated from various
530 *Phytophthora infestans* was enriched for RxLR effectors, sequenced by PacBio and de-
531 novo assembled for data mining.

532 (b) The pipeline of cDNA PenSeq. The cDNA were synthetized using RNA sampled from
533 various *P. infestans* at different stages (mycelium, zoospore, 12 hpi, 1, 2 and 3 dpi).
534 The libraries enriched for RxLR effectors were sequenced, reads were mapped to the
535 RxLRome of the reference *P. infestans* genome T30-4 and the expression levels of
536 samples were calculated and compared.

537 Black lines with dots represent the baits, the enriched fragments are depicted in blue
538 (EU_13_A2), yellow (EC1_A1), pink (EU_6_A1) and green (US23). The black boxes
539 indicate RxLR effectors. EU_13_A2, EC1_A1, EU_6_A1, US23, *P. infestans* genotypes.

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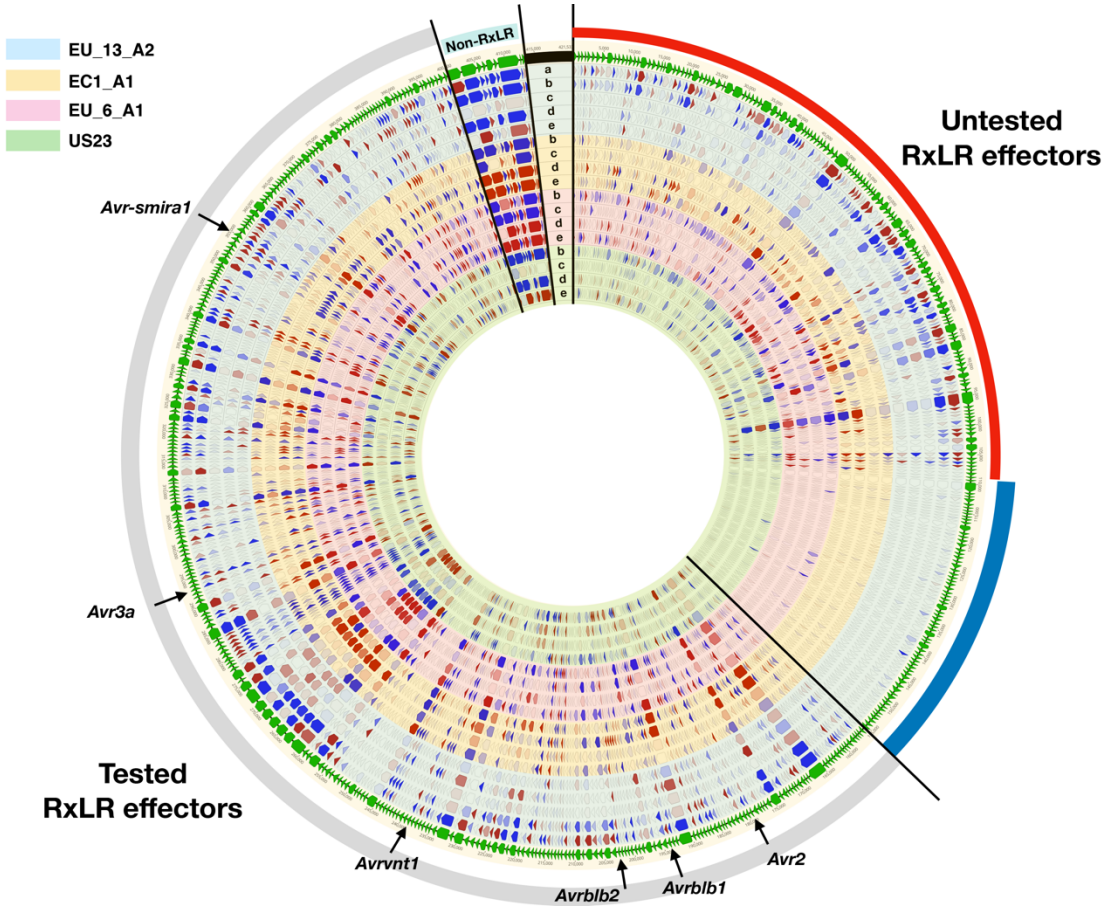


Fig. 2 cDNA PenSeq of RxLR effectors from EU_13_A2, EC1_A1, EU_6_A1 and US23

The cDNA PenSeq data for the RxLR effectors from four *Phytophthora infestans* at different stages were mapped to an artificial contig (RxLRome) of 499 RxLR effectors and nine non-RxLR genes, demarcated by bright green arrows on the outer edge of the diagram. Black lines separate the previously tested RxLR effectors (grey bar), new effector candidates with differential expression (red bar), unexpressed effectors (blue bar) and non-RxLR controls (cyan). The concentric circles in blue, yellow, pink and green represent data from *P. infestans* EU_13_A2, EC1_A1, EU_6_A1 and US23, respectively. The arrows on them indicate differential expression (red, up-regulation, blue, down-regulation; no fill, no difference), where the more intense the colour, the bigger the difference. The data are plotted as follows: a, mycelium vs zoospores (for EU_13_A2 only); b, zoospores vs 12 hpi; c, 12 hpi vs 1 dpi; d, 1 dpi vs 2 dpi; e, 2 dpi vs 3 dpi. Six known *Avr* genes, *AvrSmira1*, *Avr3a*, *Avrvnt1*, *Avrblb2*, *Avrblb1* and *Avr2* are indicated by black arrows.

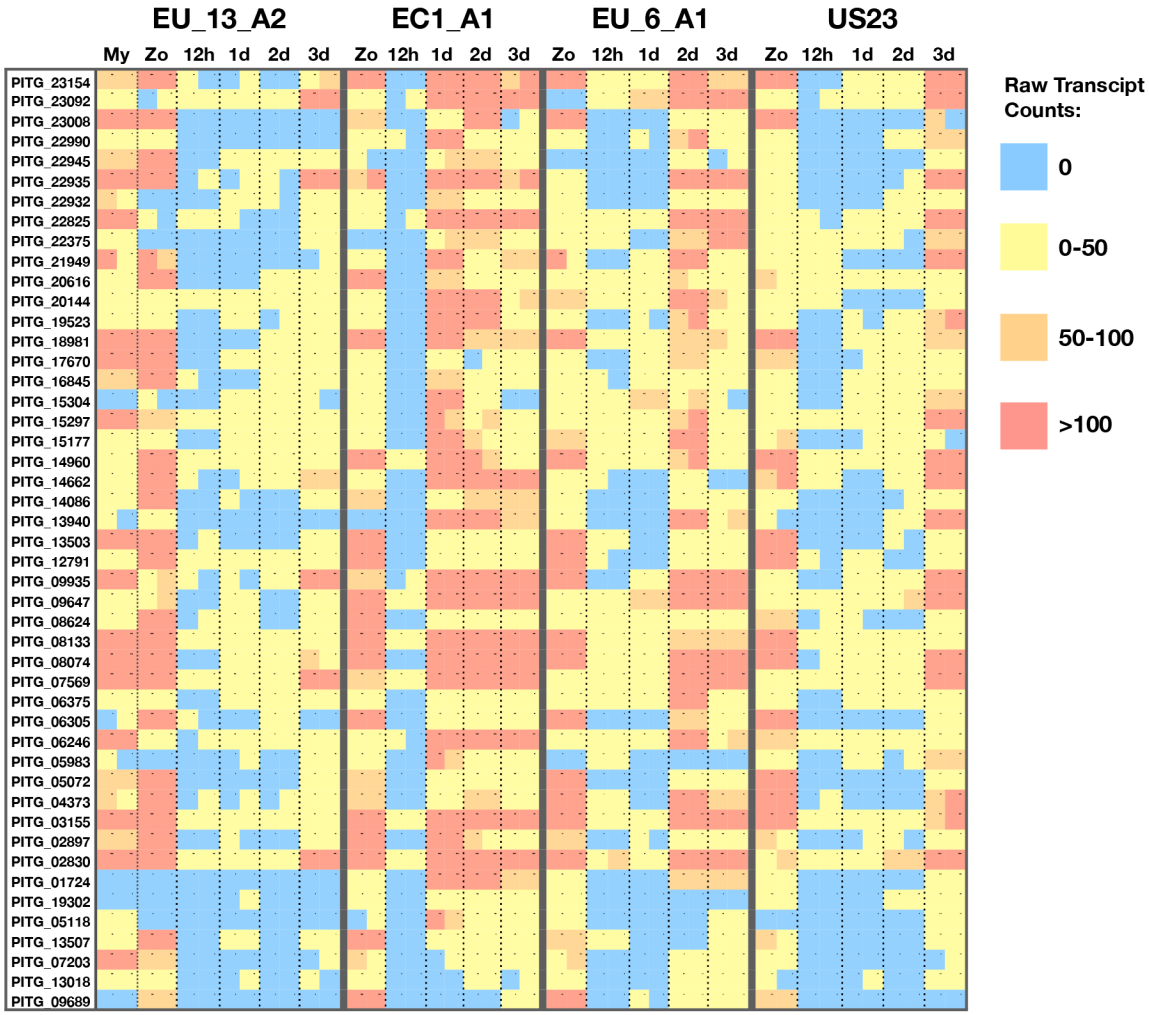
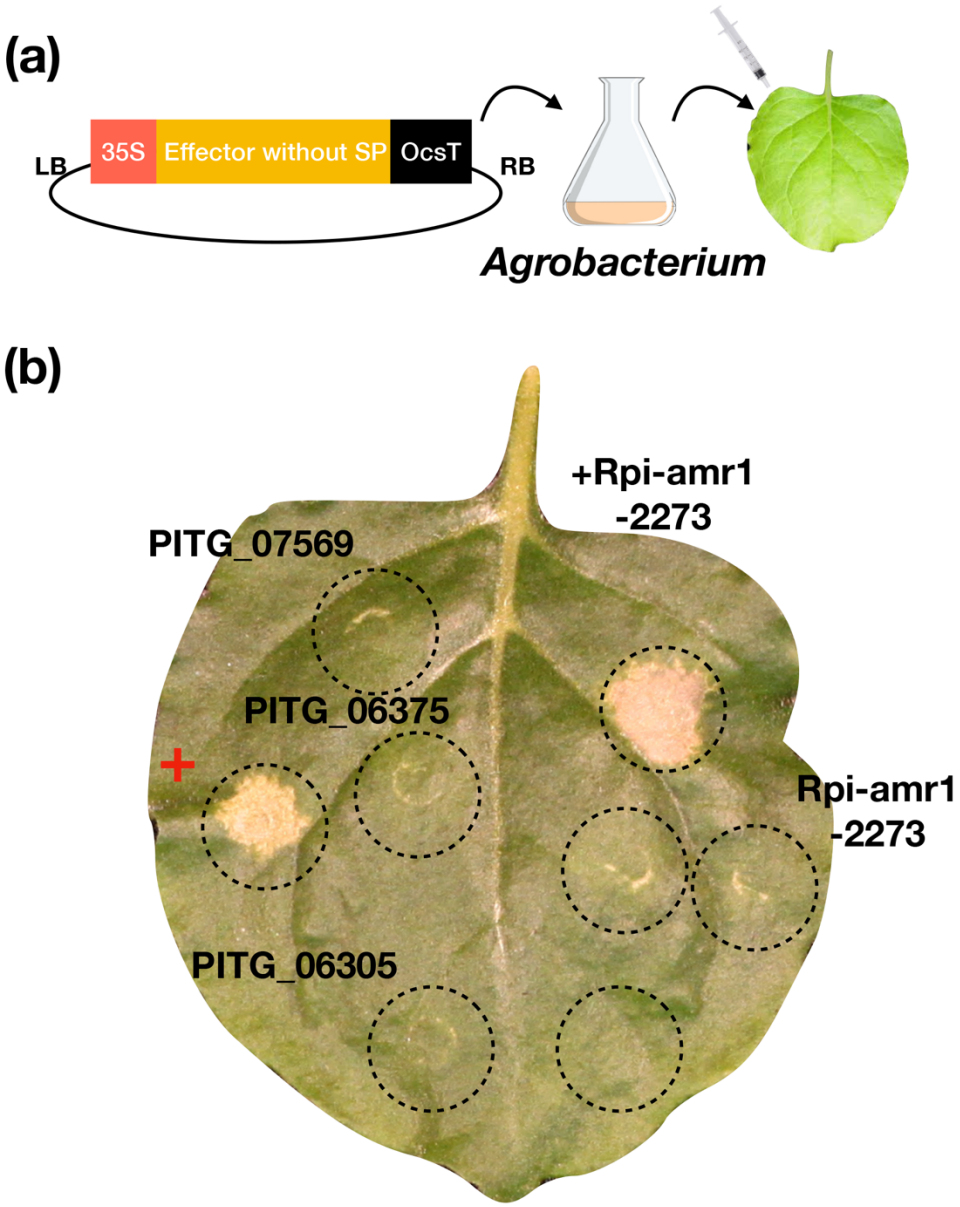


Fig. 3 Raw transcript counts for the new candidate RxLR effectors.

47 most differentially expressed RxLR effectors from the previously untested set were selected, and the raw transcript counts were visualized as a heat map across time points and treatments. Each square indicates a single data point derived from two independent biological replicates. The colours red, orange, yellow and blue represent >100, 50-100, 0-50, or 0 raw transcripts, respectively.



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579 **Fig. 4 Identification of *Avramr1*.**

580 (a) All 47 selected effectors without signal peptides (SP) were synthesized and cloned into
581 an expression vector under CaMV-35S promoter for *Agrobacterium*-mediated transient
582 expression.

583 (b) Transient expression of candidate effectors on their own or with *Rpi-amr1-2273* in
584 *Nicotiana benthamiana*. Dashed circles demarcate the infiltration sites. Only
585 PITG_07569 triggers HR when co-expressed with *Rpi-amr1-2273*. All other effectors
586 that did not trigger HR are represented by PITG_06375 and PITG_06305. A known
587 *R*/*Avr* gene pair was used as positive control (+). This experiment was repeated more
588 than 10 times with the same results.

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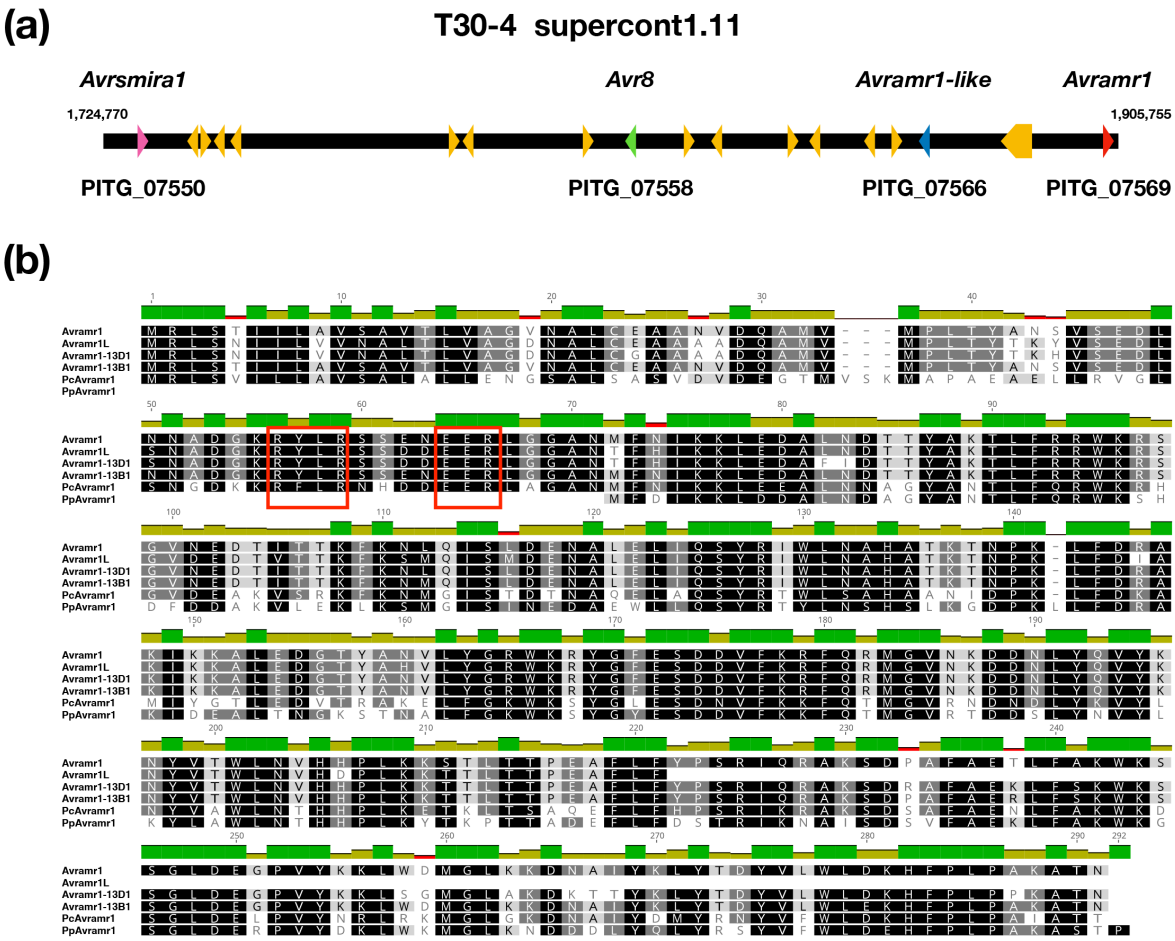

Fig. 5 Genomic localization and amino-acid alignment of *Avramr1*.

(a) The localization of *Avramr1* (*PITG_07569*, red arrow) on supercontig 1.11 of the reference *Phytophthora infestans* T30-4 genome. A paralog *Avramr1L* gene (*PITG_07566*, blue arrow) is located close to *Avramr1*. The supercontig contains another two known *Avr* genes, *Avrsmira1* (*PITG_07550*, pink arrow) and *Avr8* (*PITG_07558*, green arrow).

(b) The alignment of protein sequences of Avramr1 and selected homologs and paralogs from *P. infestans*, *P. capsici* (Pc) and *P. palmivora* (Pp). The dark green bars on top of the alignment indicate 100 % identity while olive green and red bars indicate various degrees of polymorphism between the sequences. RxLR and EER motifs are highlighted by red boxes.

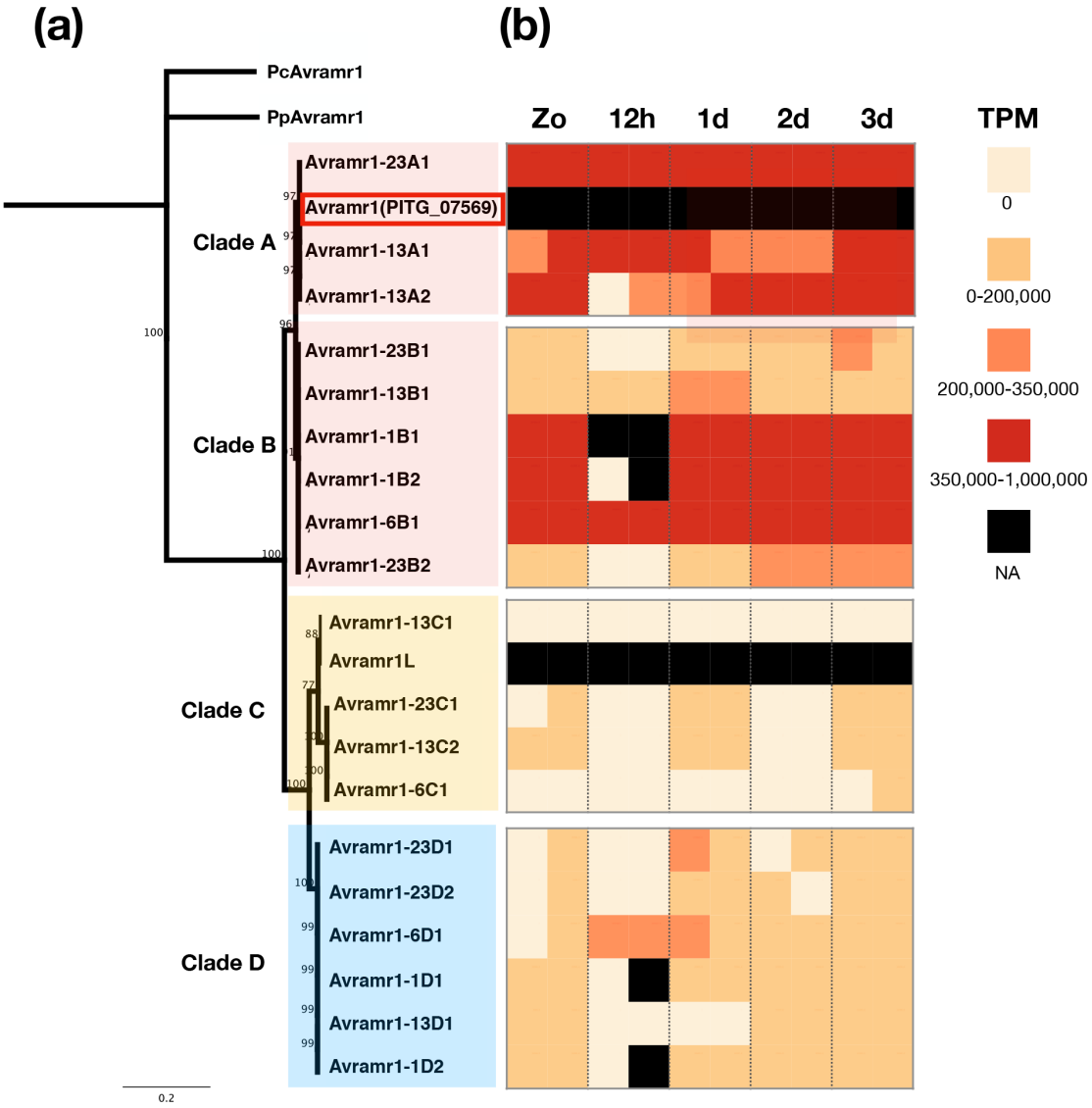

Fig. 6 Phylogeny and expression profile of *Avramr1* homologs from EU_13_A2, EC1_A1, EU_6_A1 and US23.

(a) Neighbor joining tree of the protein sequences of the *Avramr1* homologs. Pc-Avramr1 and Pp-Avramr1 were used as outgroups.

(b) The expression profile of *Avramr1* homologs at different stages and time points (zoospores, 12hpi, 1, 2 and 3 dpi). Transcripts per kilobase million (TPM) for each effector homologs were visualized as follows: black, data not available; red, 350,000-1,000,000 TPM; orange, 200,000-350,000 TPM; yellow, 0-200,000 TPM; beige, 0 TPM.

620 ## Supporting Information:

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622 **Notes S1:** PacBio PenSeq contigs of EU_13_A2.

623 **Notes S2:** PacBio PenSeq contigs of EC1_A1.

624 **Notes S3:** PacBio PenSeq contigs of EU_6_A1.

625 **Notes S4:** PacBio PenSeq contigs of US23.

626 **Table S1:** 278 RxLR effectors from previously available effector libraries.

627 **Table S2:** P/A polymorphism of RxLR effectors from EU_13_A2, EC1_A1, EU_6_A1 and

628 US23.

629 **Table S3: cDNA PenSeq for** EU_13_A2, EC1_A1, EU_6_A1 and US23.

630 **Fig. S1:** Enrichment efficiency with/ without non-adaptor-ligated DNA.

631 **Fig. S2:** Compare EU_13_A2 *Avr1* contigs and the T30-4 reference genome.

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