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

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Abstract

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 RXLR effector libraries and used long read and cDNA pathogen-enrichment sequencing (PenSeq) on four *P. infestans* isolates to explore the untested effectors. Using single-molecule real-time sequencing (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 coexpressed 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*.

KEYWORDS

Avramr1, late blight, oomycete, PenSeq, *Phytophthora infestans*, *Rpi-amr1*, RXLR effector

1 | INTRODUCTION

Potato late blight, caused by the hemibiotrophic oomycete pathogen *Phytophthora infestans*, triggered the Irish and European famine in the late 1840s, and still causes severe losses to world potato production.

To reduce losses, breeders sought resistance genes in wild relatives of potato. Early in the 20th century, *Solanum demissum*, a highly

resistant hexaploid ($2n = 72$) wild potato, was found to be a useful source of resistance to *P. infestans* (*Rpi*) genes (Salaman, 1937). Since then, many resistance traits have been transferred to cultivated potatoes by introgression breeding (Toxopeus, 1956), and many *Rpi* genes have been cloned from wild potatoes, for example *R1*, *R3a*, *R8*, *Rpi-blb1*, and *Rpi-vnt1* (Ballvora *et al.*, 2002; van der Vossen *et al.*, 2003; Huang *et al.*, 2005; Foster *et al.*, 2009; Pel *et al.*, 2009; Vossen *et al.*,

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2016). Unlike wild potatoes, *Solanum nigrum* and *Solanum americanum* have been reported to be nonhosts for *P. infestans* (Colon *et al.*, 1993). Two *Rpi* genes encoding NLR proteins, *Rpi-amr3* and *Rpi-amr1*, were cloned from *S. americanum* and confer late blight resistance in potato (Witek *et al.*, 2016, 2020).

Identification of the recognized effectors for *Rpi-amr3* and *Rpi-amr1* would open the way to investigate their virulence function and distribution in *P. infestans* populations. Moreover, it could also help to diagnose *Rpi* gene repertoires in resistant plants, and individually confirm their activity in genetically modified potatoes carrying multiple *Rpi* genes. In oomycetes, all the cloned Avr proteins contain a signal peptide and RXLR motif (Rehmany *et al.*, 2005), and the genomic sequencing of *P. infestans* revealed 563 RXLR effectors in the T30-4 reference genome (Haas *et al.*, 2009). This enabled a high-throughput effectoromics approach for functional screening of the candidate effectors in plants (Vleeshouwers *et al.*, 2008, 2011), and many Avr genes were identified by this approach, including *Avrblb1*, *Avrblb2*, and *Avrvnt1* (Vleeshouwers *et al.*, 2008; Oh *et al.*, 2009; Pel, 2010).

However, available RXLR effector libraries do not contain recombinant clones of all *P. infestans* RXLR effectors, and the effector candidates were defined on the basis of expression profile, motif analysis, and distribution between *P. infestans* races (Vleeshouwers *et al.*, 2008; Haas *et al.*, 2009; Oh *et al.*, 2009). In total, c.300/563 RXLR effectors were previously cloned into expression vectors for functional screening (Rietman, 2011).

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.*, 2019; Thilliez *et al.*, 2019). 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 adapted and improved PenSeq with long read (PacBio) and cDNA sequencing, 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*.

2 | RESULTS

2.1 | Available recombinant RXLR effector libraries do not contain *Avramr1*

To identify *Avramr1*, we tested 278 available RXLR effectors (Table S1) by coexpressing them with *Rpi-amr1-2273* in *Nicotiana benthamiana* (Rietman, 2011). *Rpi-amr1-2273* (hereafter *Rpi-amr1*) is

a functional *Rpi-amr1* homolog cloned from *S. americanum* SP2273 (Witek *et al.*, 2020). However, we did not identify an effector that activated *Rpi-amr1*-dependent hypersensitive response (HR) from the available RXLR effector libraries and concluded these libraries are incomplete. Notably, *Avr8* was not originally included in the core effector selection because *Avr8* expression goes up earlier than 2 days postinoculation (dpi) (Jo, 2013), showing that the criteria adopted to define core effectors do not reveal all recognized effectors.

To find *Avramr1*, we proposed three hypotheses: (a) *Avramr1* is an RXLR effector but the recognized allele is not present in the assembled version of *P. infestans* T30-4 reference genome, (b) *Avramr1* is an RXLR effector but it has not yet been tested in previous functional studies/libraries, and (c) *Avramr1* is not a typical RXLR effector. To address the first hypothesis, we performed PacBio PenSeq to sequence the effector alleles in the four diverse *P. infestans* isolates, EU_13_A2 (2006_3928A), EC1_A1 (EC1_3626), EU_6_A1 (2006_3920A), and US23, all of them avirulent on potato plants carrying *Rpi-amr1*. To address the second hypothesis, we performed cDNA PenSeq to try to identify other RXLR effectors that are expressed during infection but not reported or defined in previous functional studies.

2.2 | PacBio PenSeq of four *P. infestans* isolates, EU_13_A2, EC1_A1, EU_6_A1, and US23

PacBio gDNA PenSeq was performed on four *P. infestans* isolates of genotypes EU_13_A2, EC1_A1, EU_6_A1, and US23 (Figure 1a). To evaluate the enrichment efficiency, quantitative polymerase chain reaction (qPCR) was performed with the DNA pre- and postcapture. In general, the targeted genes of different length were well enriched at concentration \times time (Cot) value <20 , while the untargeted genes were almost undetectable, with Cot >27 (Peterson *et al.*, 2002) (Figure S1). Furthermore, we found that the capture efficiency was increased by including a 10-fold molar excess of nonadaptor-ligated fragmented *P. infestans* DNA (500–1,000 bp) in the reannealing reaction to reduce the extent to which sequences were recovered due to concatenation of transposon-containing sequences adjacent to RXLR genes. After sequence capture, enrichment of most effector genes was more efficient when nonadaptor-ligated *P. infestans* DNA was included (Figure S1).

Following the enrichment sequencing, circular consensus sequencing reads were assembled (Figure 1a) and contigs with fewer than 10 reads were removed. The average length of the contigs of coverage >10 reads was 7 kb (Table 1), and the size of the largest contig was over 50 kb. This suggests that the PacBio PenSeq successfully captured the target effector genes and the adjacent flanking DNA sequences. In total, 1,137, 1,054, 1,283, and 925 contigs were obtained from EU_13_A2, EC1_A1, EU_6_A1, and US23, respectively, of which 687, 650, 741, and 571 contigs contain RXLR effectors (Table 1 and Notes S1–S4). The remaining contigs contained non-RXLR effectors, which were included in the bait library design for other purposes (Thilliez *et al.*, 2019).

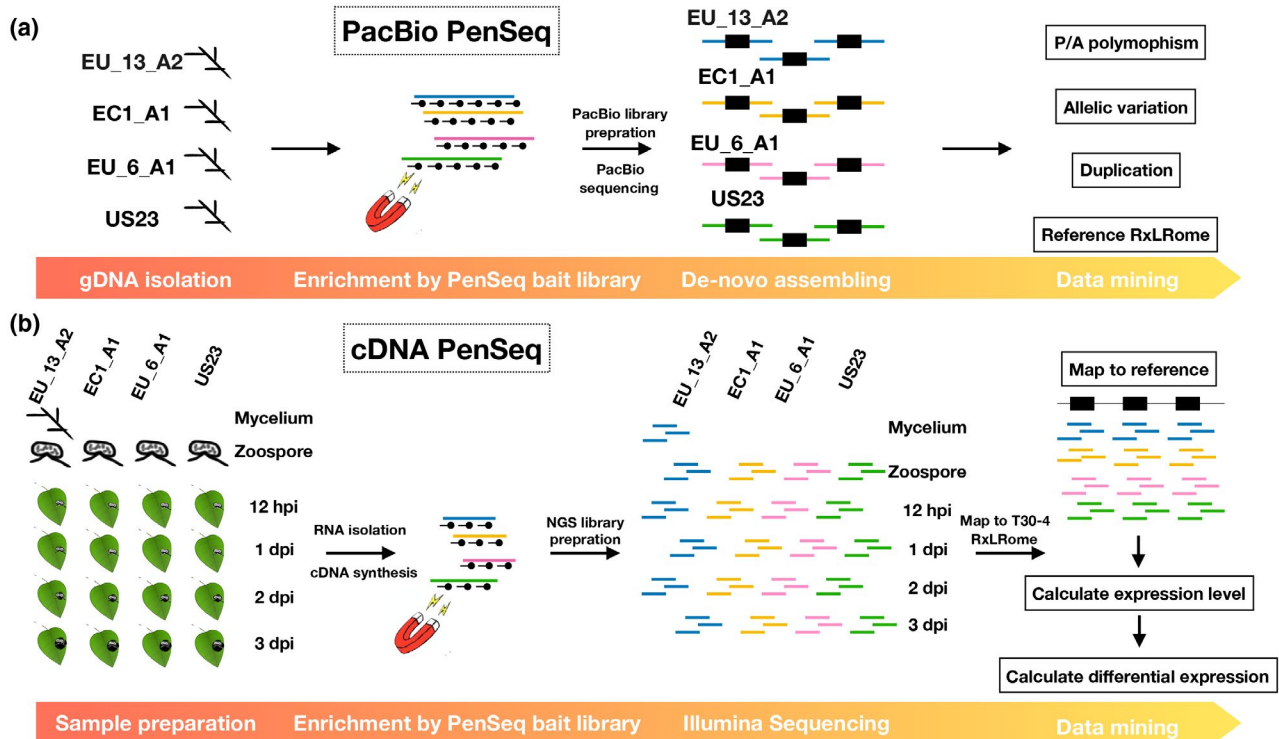


FIGURE 1 The pipelines of PacBio and cDNA pathogen-enrichment sequencing (PenSeq). (a) The pipeline of PacBio gDNA PenSeq. Briefly, the gDNA isolated from various *Phytophthora infestans* was enriched for RXLR effectors, sequenced by PacBio and de novo assembled for data mining. (b) The pipeline of cDNA PenSeq. The cDNA was synthesized using RNA sampled from various *P. infestans* at different stages (mycelium, zoospore, 12 hr postinoculation, 1, 2, and 3 days postinoculation). The libraries enriched for RXLR effectors were sequenced, reads were mapped to the RxLRome of the reference *P. infestans* genome T30-4 and the expression levels of samples were calculated and compared. Black lines with dots represent the baits, the enriched fragments are depicted in blue (EU_13_A2), yellow (EC1_A1), pink (EU_6_A1), and green (US23). The black boxes indicate RXLR effectors. EU_13_A2, EC1_A1, EU_6_A1 and US23 are *P. infestans* isolates.

TABLE 1 PacBio PenSeq for EU_13_A2, EC1_A1, EU_6_A1, and US23

<i>Phytophthora infestans</i> isolate	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

The PacBio PenSeq data allowed us to detect new RXLR effector alleles from different haplotypes of various *P. infestans* isolates and even in polyploid genotypes like EU_13_A2 (Li *et al.*, 2017). This data set can also be used to extensively study allelic variation, presence/absence (P/A) polymorphism, and effector evolution. For example, *Avr1* (PITG_16663) and a paralogous *Avr1-like* gene (PITG_06432) are located on supercontigs 1.51 and 1.8 of the reference T30-4 genome, respectively. The R1-breaking clonal lineage EU_13_A2 was reported to have an 18 kb deletion comprising the *Avr1* locus (Cooke *et al.*, 2012). Also, the Illumina PenSeq data showed that the *Avr1* locus is missing in EU_13_A2, EC1_A1, and US23 (Thilliez *et al.*, 2019). We mapped the four *Avr1* contigs from EU_13_A2 (contigs 192, 261, 296, and 329) to supercontig 1.51 and 1.8, and found that all four contigs map to the *Avr1-like* supercontig 1.8. Two contigs

(contig 261 and 286) mapped to the *Avr1-like* locus, and two other contigs (contig 192 and 329) mapped to a locus next to *Avr1-like* that was not previously annotated (Figure S2), although the genes in those two contigs might be pseudogenes as the signal peptide is missing in both of them. Additionally, in EU_6_A1 and US23, two *Avr1* contigs did not map to *Avr1* or *Avr1-like* loci of T30-4. Thus, our PacBio PenSeq data set can provide the means to detect novel RXLR effector paralogs absent from the reference genome.

As another example, our data set carries in total 504 of the 563 predicted RXLR effectors from the reference genome T30-4 (Haas *et al.*, 2009). To investigate P/A polymorphism of RXLR effectors in the four sequenced isolates, we performed a basic local alignment search (BLAST) of the 504 effectors against the PacBio contigs, with hits with <50% coverage defined as absent (Table S2). We found

that 17, 28, 15, and 33 RXLR effectors out of the 504 are missing in EU_13_A2, EC1_A1, EU_6_A1, and US23, respectively.

Taken together, we have generated a rich data set that could help to define full-length RXLR effector genes, deliver robust information on alleles and paralogs, and reveal conserved or race-specific effectors from different isolates. The data set is available in full in Notes S1–S4.

2.3 | cDNA PenSeq enables effector expression detection in early stages of infection

To clarify whether the untested effectors might be putative *Avr* genes, we performed cDNA PenSeq for the four *P. infestans* isolates EU_13_A2,

EC1_A1, EU_6_A1, and US23, at different time points after infection (12 hr postinoculation [hpi], 1, 2, and 3 dpi) and in mycelium and zoospores (Figure 1b). To analyse and visualize the cDNA PenSeq data, we built an artificial DNA sequence contig (“RXLRome”) for the RXLR effectors. In addition, nine non-RXLR genes from the bait library were included as controls (Jouet *et al.*, 2019; Figure 2). The cDNA PenSeq reads were mapped to the RXLRome and gene expression compared over time (Figure 1b).

Most of the RXLR effectors that were included in previous effector libraries show an up-regulation of expression in the early stages of infection (Figure 2). Eleven known *Avr* genes from *P. infestans*, namely *Avr4*, *Avr-smira1*, *Avr8*, *Avr10*, *Avr3a*, *Avrvnt1*, *Avr1*, *Avr3b*, *Avrblb2*, *Avrblb1*, and *Avr2*, are shown in Figure 2. Some of the untested RXLR effectors show a similar pattern of expression and

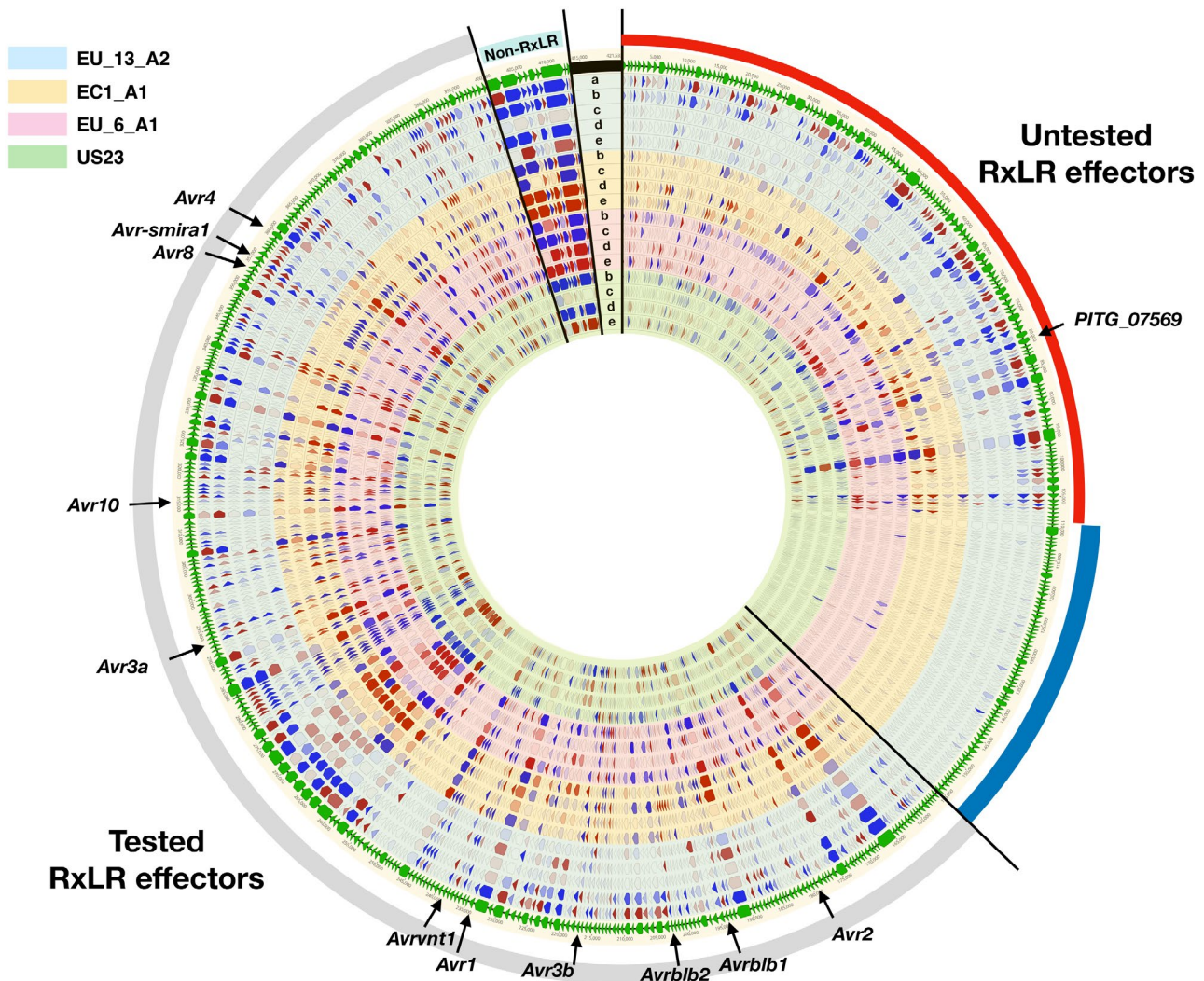


FIGURE 2 cDNA pathogen-enrichment sequencing (PenSeq) of RXLR effectors from EU_13_A2, EC1_A1, EU_6_A1, and US23. The cDNA PenSeq data for the RXLR effectors from four *Phytophthora infestans* at different stages were mapped to an artificial contig (RXLRome) of 499 RXLR effectors and nine non-RXLR genes, 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 hr postinoculation (hpi); c, 12 hpi vs. 1 day postinoculation (dpi); d, 1 dpi vs. 2 dpi; e, 2 dpi vs. 3 dpi. Eleven known *Avr* genes, namely *Avr4*, *AvrSmira1*, *Avr8*, *Avr10*, *Avr3a*, *Avrvnt1*, *Avr1*, *Avr3b*, *Avrblb2*, *Avrblb1*, and *Avr2*, are indicated by black arrows. *PITG_07569* is indicated by a black arrow

might also represent additional potential Avr genes, while others are poorly expressed in some isolates. The details of the cDNA PenSeq are available in Table S3.

2.4 | Identification of *Avramr1*

To test if *Avramr1* is among the untested effectors, we selected 47 highly expressed RXLR effectors (Figure 3) present in all tested lineages that had not previously been investigated. The effectors were synthesized, cloned into an expression vector with CaMV 35S promoter, and transformed into *Agrobacterium* GV3101-pMP90 for agroinfiltration in *N. benthamiana* (Figure 4a). All the effectors were infiltrated alone or coinfiltrated with *Rpi-amr1* (Witek *et al.*, 2020). Among the 47 effectors, PITG_07569 was the only effector that triggered an HR when

coexpressed with *Rpi-amr1*. Hence, we concluded PITG_07569 is *Avramr1*. To verify if both proteins were expressed in planta, we cloned *Avramr1* and *Rpi-amr1* with C-terminal green fluorescent protein (GFP) and His-FLAG (HF), respectively. Both recombinant proteins were expressed and detected in *N. benthamiana* by western blot (Figure 4c). The same constructs were used for agroinfiltration in *N. benthamiana*, and HR was observed specifically after coexpression of *Avramr1*-GFP and *Rpi-amr1*-HF (Figure 4b).

2.5 | *Avramr1* homologs in different *P. infestans* isolates and other *Phytophthora* species

Avramr1 is a canonical RXLR effector with RYLR and EER motifs and an N-terminal signal peptide (Figure 5b). *Avramr1* locates on supercontig

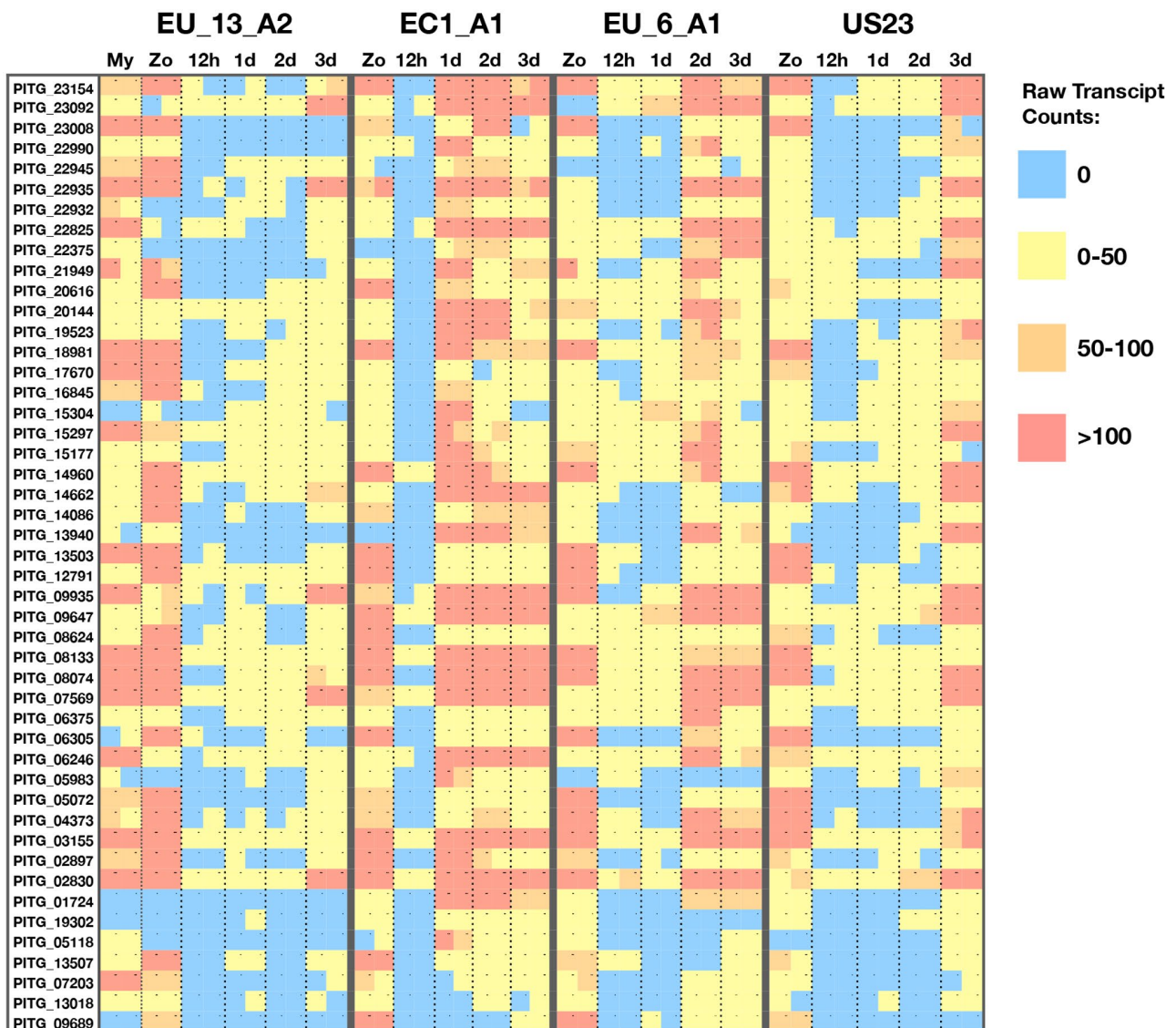


FIGURE 3 Raw transcript counts for the new candidate RXLR effectors. The 47 most differentially expressed RXLR effectors from the previously untested set were selected, and the raw transcript counts were visualized as a heat map across time points and treatments. Each square indicates a single data point derived from two independent biological replicates. The colours red, orange, yellow, and blue represent >100, 50–100, 0–50, or 0 raw transcripts, respectively

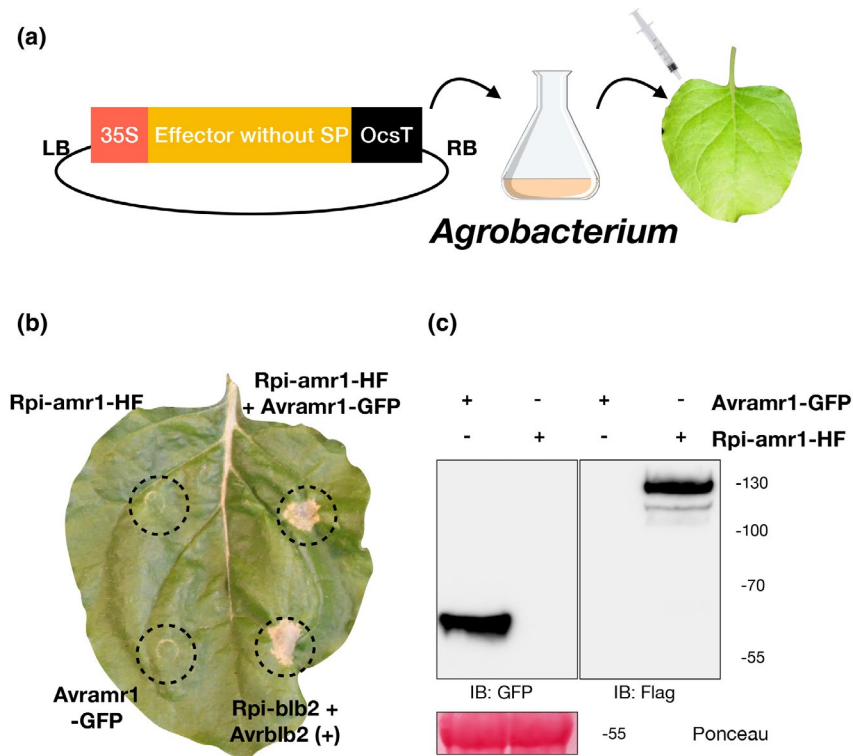


FIGURE 4 Identification of *AvrAmr1*. (a) All 47 selected effectors without signal peptides (SP) were synthesized and cloned into an expression vector under a CaMV35S promoter for *Agrobacterium*-mediated transient expression. (b) Transient expression of *AvrAmr1*-green fluorescent protein (GFP), *Rpi-amr1*-His-FLAG (HF) on their own coexpression in *Nicotiana benthamiana*. Dashed circles demarcate the infiltration sites. Hypersensitive response (HR) was observed when coexpressed *AvrAmr1*-GFP with *Rpi-amr1*-HF. *Rpi-blb2* and *Avrblb2* were used as positive control (+). This experiment was repeated three times with the same results. (c) Expression of *AvrAmr1*-GFP and *Rpi-amr1*-HF fusion proteins in *N. benthamiana* leaves. The expression of *AvrAmr1*-GFP was confirmed by immunoblot (IB) using anti-GFP antibody, and expression of *Rpi-amr1*-HF was confirmed by anti-FLAG antibody. Size markers are indicated in kDa and Ponceau stain was used to show the protein loading

1.11 of the *P. infestans* reference genome T30-4. *AvrAmr1*-like (hereafter *AvrAmr1L*), a truncated paralog (PITG_07566), maps adjacent to *AvrAmr1* (Figure 5a,b). Two known *Avr* effectors, *Avr8* (PITG_07558) and *Avrsmira1* (PITG_07550), are physically close to the *AvrAmr1* locus in the T30-4 genome (Figure 5a) (Rietman *et al.*, 2012).

To study the sequence polymorphism of *AvrAmr1* homologs in *P. infestans*, we used BLAST to search for *AvrAmr1* homologs in the PacBio PenSeq assemblies generated in this study. This revealed that EU_13_A2, EC1_A1, EU_6_A1, and US23 carry six, four, three, and six *AvrAmr1* homologs, respectively (Note S5). Next, we aligned the corresponding *AvrAmr1* amino acid sequences and generated a neighbour-joining (NJ) tree for phylogenetic analysis (Figure 6a). Two *AvrAmr1* homologs from *Phytophthora parasitica* and *Phytophthora cactorum* were identified from public databases, and they were used as an out-group (Figures 5b and 6a). Based on the phylogenetic tree, we distinguished four *AvrAmr1* clades, clade A (containing *AvrAmr1* from T30-4) and clade C (with *AvrAmr1L* from T30-4), and two more clades, B and D (Figure 5a). For a more detailed analysis, we selected one *AvrAmr1* homolog from clade B and one from D (*AvrAmr1*-13B1 and *AvrAmr1*-13D1 from EU_13_A2) and aligned them with *AvrAmr1* homologs from clade A and C, and with *P. parasitica* and *P. cactorum* homologs. Significant sequence polymorphisms were observed between effectors from

different clades (Figure 5b). Meanwhile, the *AvrAmr1* homologs within the same clade were almost identical (Figure 6a).

2.6 | Differential expression of *AvrAmr1* homologs in different *P. infestans* isolates

To investigate the expression patterns of *AvrAmr1* homologs defined in the PacBio PenSeq data, we mapped the corresponding cDNA PenSeq reads to the PacBio PenSeq contigs from EU_13_A2, EC1_A1, EU_6_A1, and US23. The transcripts per kilobase per million (TPM) values for each time point are visualized in Figure 6b. The clade A homologs *AvrAmr1*-23A1, *AvrAmr1*-13A1, and *AvrAmr1*-13A2 were highly expressed at almost all stages, and the *AvrAmr1* homologs from clade B showed a similar expression pattern. For clade C, some homologs, like *AvrAmr1*-13C, *AvrAmr1*-6C1 or *AvrAmr1L* gene from T30-4, were weakly expressed at all stages. However, two other *AvrAmr1L* homologs, *AvrAmr1*-23C1 and *AvrAmr1*-13C2, showed moderately elevated expression in zoospores, and at 1 and 3 dpi. Interestingly, the *AvrAmr1* homologs from clade D, which are missing in the reference genome T30-4, showed an intermediate expression level compared to clades A, B,

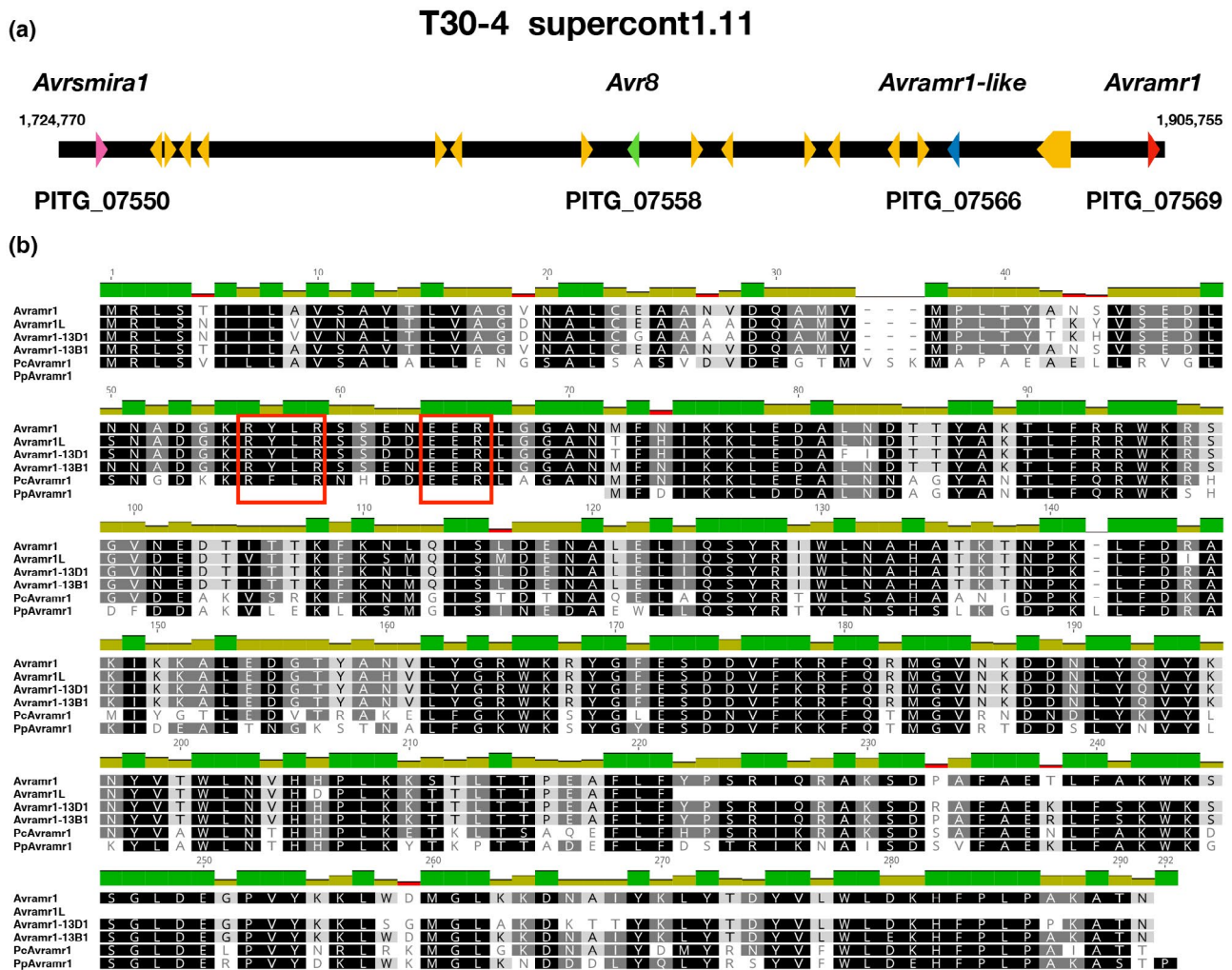


FIGURE 5 Genomic localization and amino acid alignment of *Avrsmr1*. (a) The localization of *Avrsmr1* (PITG_07569, red arrow) on supercontig 1.11 of the reference *Phytophthora infestans* T30-4 genome. A paralog *Avrsmr1L* gene (PITG_07566, blue arrow) is located close to *Avrsmr1*. The supercontig contains two other known *Avr* genes, *Avrsmira1* (PITG_07550, pink arrow) and *Avr8* (PITG_07558, green arrow). (b) The alignment of protein sequences of *Avrsmr1* and selected homologs from *P. infestans*, *Phytophthora cactorum* (Pc), and *Phytophthora parasitica* (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

and C, and most *Avrsmr1* homologs in clade D showed an increase in expression at the zoospore stage, and at 1, 2, and 3 dpi.

In summary, our PacBio PenSeq analysis created a rich data set to reveal new *Avr* variants from different *P. infestans* isolates, and to quantify their expression profile individually. This facilitates the analysis of the polymorphism of pathogen effectors and their potential differential recognition patterns with the corresponding *Rpi* genes (Witek *et al.*, 2020).

3 | DISCUSSION

The availability of the *P. infestans* genome sequence enabled a step-change in the rate of investigation of this pathogen, accelerating the discovery of recognized effectors, and of new *Rpi* genes (Vleeshouwers *et al.*, 2008, 2011; Haas *et al.*, 2009). However, some questions remain open. For example, how different are the effector

repertoires in different *P. infestans* isolates? To what extent do they show differential expression between races? The study of plant NLR gene repertoires faces similar challenges, and sequence capture, combined with long-read sequencing technologies, has enabled the refinement of tools to cost-effectively investigate diversity, such as RenSeq, SMRT RenSeq, RLP/KSeq, and AgRenSeq (Jupe *et al.*, 2013; Witek *et al.*, 2016; Arora *et al.*, 2019; Lin *et al.*, 2020). Recently, the pan-NLRome of 65 diverse *Arabidopsis thaliana* accessions was determined by a similar strategy, revealing that any one accession lacks many of the NLRs found in the species pan-NLRome (Van de Weyer *et al.*, 2019).

PenSeq was developed to facilitate cost-effective investigation of pathogen diversity on infected plants, and polymorphism of pathogen effectors (Jouet *et al.*, 2019; Thilliez *et al.*, 2019). The first PenSeq studies, however, were conducted using Illumina short reads. This significantly limited their resolving power as many oomycete genomes are highly heterozygous, and some

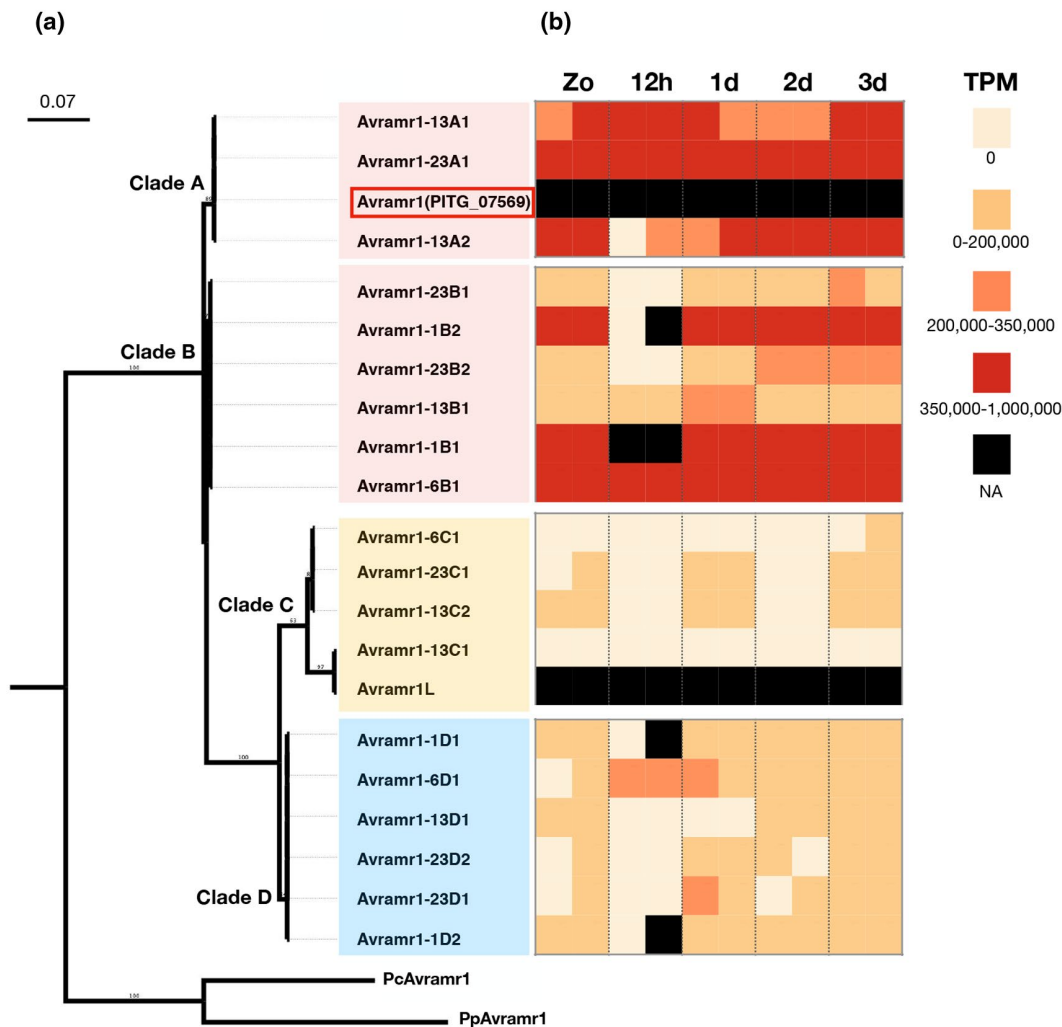


FIGURE 6 Phylogeny and expression profile of *AvrAmr1* homologs from EU_13_A2, EC1_A1, EU_6_A1, and US23. (a) Maximum-likelihood phylogeny of the protein sequences of the *AvrAmr1* homologs were made by IQ-TREE (Minh *et al.*, 2020). PcAvrAmr1 and PpAvrAmr1 are *AvrAmr1* homologs from *Phytophthora cactorum* and *Phytophthora parasitica*, and they were used as out-groups. (b) The expression profile of *AvrAmr1* homologs at different stages and time points (zoospores, 12 hr postinoculation, 1, 2, and 3 days postinoculation). 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. *AvrAmr1* and *AvrAmr1L* are from the reference genome T30-4, which was not included in the cDNA PenSeq

effectors belong to large gene families with multiple sequence-related paralogs that can lead to false assemblies (Gilroy *et al.*, 2011; Oliva *et al.*, 2015).

In this study, we combined long read and cDNA PenSeq, enabling a detailed analysis of the RXLR genes and their expression patterns in different *P. infestans* isolates. The cDNA PenSeq data set allowed us to define an additional set of 47 RXLR genes expressed during infection that were not previously investigated. Amongst these, we identified *AvrAmr1*, which encodes the cognate recognized effector for *Rpi-amr1* from *S. americanum* (Witek *et al.*, 2020). It is noteworthy that PITG_07569 (*AvrAmr1*) was identified by an alternative splicing reporter system as a splicing regulatory effector; furthermore, it was shown to promote the colonization of *P. infestans* (Huang *et al.*, 2020).

The long read PenSeq data helped us to obtain full-length RXLR effector haplotypes with their flanking sequences. This allowed us to

distinguish individual alleles from polyploid isolates like EU_13_A2, and also distinct effector paralogs. The sequences flanking the RXLR genes enabled us to understand the possible translocation events and identify new RXLR loci. We were also able to identify multiple new *AvrAmr1* homologs from different isolates, and identified a new *AvrAmr1* clade D that is not present in T30-4. This data set allows us to study the differential recognition pattern of *Rpi-amr1* and *AvrAmr1* homologs. Indeed, different *Rpi-amr1* homologs could recognize different sets of *AvrAmr1* homologs, including the *AvrAmr1* homolog from the newly identified clade D (Witek *et al.*, 2020). So far, no *Rpi-amr1*-breaking *P. infestans* isolates have been found (Witek *et al.*, 2020), and therefore we propose that *AvrAmr1* might be crucial for the virulence of *P. infestans*. The identification of *AvrAmr1* will enable us to study its virulence function and its polymorphism in the *P. infestans* population, and study the effector-triggered immunity mediated by *Rpi-amr1*.

Collectively, the PenSeq data set constitutes a valuable community resource for investigating the allelic and expression diversity of multiple recognized effectors. The long reads and cDNA PenSeq methods will contribute to understanding this fast-evolving and destructive oomycete pathogen, and to achieving durable late blight resistance in potato.

4 | EXPERIMENTAL PROCEDURES

4.1 | Sample preparation

To collect the mycelium of *P. infestans* for DNA extraction, *P. infestans* strains were grown on rye sucrose agar (RSA) for 7 days and then moved to Plich liquid medium 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. Grown mycelia were covered with cold (4°C) sterile water and then incubated at $4-6^{\circ}\text{C}$ for 2–3 hr. 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 were incubated at 20°C in high humidity for a required time after inoculation. Leaf discs of the infection area were collected and stored at -80°C until DNA or RNA extraction.

4.2 | DNA and RNA extraction

DNA was extracted using phenol/chloroform. *P. infestans* mycelium samples or infected leaf discs were ground into powder in liquid nitrogen. Ground material was resuspended in 500 μl of Shorty buffer (0.2 M Tris.HCl pH 9, 0.4 M LiCl, 25 mM EDTA, 1% SDS) and one volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The upper aqueous phase containing DNA was mixed with one volume of 100% ice-cold isopropanol to precipitate DNA. The pellet was washed twice using 70% ethanol, heated at 70°C for 2–5 min to completely remove ethanol, and resuspended in sterile water. Resuspended DNA was then heated at 65°C for 20 min to inactivate DNases before RNase treatment was performed (2 μl of 10 mg/ml RNase A, 37°C , 1 hr) and RNase A removed by chloroform precipitation. Genomic DNA was resuspended in water and sheared into 3–5 kb fragments using the S220 Focused-ultrasonicator (Covaris Inc.).

RNA samples were extracted with Direct-zol RNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions.

4.3 | PacBio and Illumina PenSeq capture

The biotinylated RNA bait library of 120 nucleotides (nt) was designed for enriching RXLR effectors from *P. infestans* and some other

genes of interest. The library contains 18,348 baits, as described previously (Jouet *et al.*, 2019; Thilliez *et al.*, 2019).

PacBio library was constructed with DNA samples from the mycelium of four *P. infestans* strains, EU_13_A2 (2006_3928A), EC1_A1 (EC1_3626), EU_6_A1 (2006_3920A), and US23. The library construction and target DNA sequence capture were performed according to Witek *et al.* (2016), with minor modifications. A Qubit Fluorometer (ThermoFisher) was used to quantify the barcoded DNA library from each isolate. Equimolar amounts of DNA from the four individually barcoded samples were pooled to obtain 250 ng of total DNA and then subjected to sequence capture. A $10\times$ excess of nonadaptor-ligated *P. infestans* DNA at about 500–1,000 bp was added for the hybridization. The final mixture of the amplicons of the captured library was further size selected by SageELF electrophoresis system (Sage Science) according to the manufacturer's instructions.

The Illumina library was constructed with RNA samples from zoospores of the four *P. infestans* strains, from the corresponding infected leaf discs harvested at 12 hpi, 1, 2, and 3 dpi, and from mycelium of EU_13_A2. An Illumina library for each sample was constructed with KAPA mRNA HyperPrep Kit for Illumina Platforms (KR1352 v. 5.17) following the manufacturer's instructions. mRNA was fragmented to 300–400 bp. The barcoded libraries were mixed together at a ratio of 16:8:4:1:1 for 12 hpi, 1, 2, and 3 dpi and zoospores samples, respectively.

Before and after sequence capture, quantitative PCR was performed on a Bio-Rad CFX96 real-time detection system with an input of 1 ng DNA to assess the efficiency of capture.

4.4 | Sequencing

PacBio PenSeq libraries were sequenced at the Earlham Institute (Norwich, UK) using the Sequel platform. Illumina PenSeq cDNA libraries were sequenced at Novogene (Hong Kong, China) using HiSeq, PE250.

4.5 | gDNA PenSeq assembly

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

4.6 | Analysis of cDNA PenSeq

All RXLR effectors from the *P. infestans* reference genome T30-4 were used to generate an artificial "RXLRome" contig, where RXLR effectors' sequences were separated by stretches of 500 "Ns". The contig also contained nine non-RXLR control genes (Jouet *et al.*, 2019). The cDNA PenSeq reads from all treatments were mapped to the T30-4 RXLRome, and the expression analyses were performed and visualized using Geneious R10 (Kearse *et al.*, 2012).

4.7 | New candidate RXLR effectors

For the previously untested RXLR effectors, we first selected the effectors showing differential expression at different stages and ranked them based on the raw transcript counts. Next, local alignment searches (BLAST) were performed against the 563 predicted RXLR effectors (Haas *et al.*, 2009) to remove the previously tested effectors. This analysis revealed 47 candidate RXLR effectors that were not included in the previous functional study. The 47 RXLR effectors were synthesized by Twist Bioscience. The signal peptides were removed, the sequences were domesticated for Golden Gate cloning, and overhangs containing *Bsa*I restriction sites were added to both ends of all effector sequences.

All the effectors were cloned into vector pICSL86977 (TSL SynBio) with CaMV 35S promoter and OCS terminator. To further verify the expression Avramr1 and Rpi-amr1, they were fused with C-terminal GFP and His-FLAG (HF) tags, respectively. *Agrobacterium* strain GV3101-pMP90 was transformed with the constructs for agroinfiltration.

4.8 | Cell death assay

Transient expression of RXLR effectors and *Rpi-amr1* in *N. benthamiana* was performed as described previously (Bos *et al.*, 2006). *Agrobacterium* was infiltrated at OD₆₀₀ = 1, and each effector was coinfiltrated with *Rpi-amr1* (Witek *et al.*, 2020). The cell death phenotype was observed at 4 dpi. p35S-Rpi-blb2 and p35S-Avrblb2 were coexpressed as positive control in the HR assay (Oh *et al.*, 2009).

4.9 | Protein extraction and immunoblot analysis

The p35S-Rpi-amr1-HF and p35S-Avramr1-GFP constructs were used to transiently express the fusion proteins in *N. benthamiana*. The leaf tissue was harvested 2 days after infiltration and proteins were extracted as described in Guo *et al.* (2020). The expression of recombinant Avramr1-GFP and Rpi-amr1-HF was determined by SDS-PAGE as described in Guo *et al.* (2020). Horseradish peroxidase-conjugated antibodies (anti-FLAG M2, 1:10,000 dilution, Sigma; anti-GFP, 1:10,000 dilution, Santa Cruz Biotechnology) were used for the immunoblot. The chemiluminescence was detected by ImageQuant LAS 4,000 (Life Sciences) after chemiluminescent substrate incubation (SuperSignal West Pico & West Femto).

4.10 | Sequence and phylogenetic analysis

All sequences were analysed in Geneious R10 (Kearse *et al.*, 2012), MAFFT was used for sequence alignment (Katoh and Standley, 2013), and the signal peptides of Avramr1 homologs were removed manually for the phylogenetic analysis. IQ-TREE was used for the phylogenetic analysis and the JTTDCMut model was selected as best-fit model by IQ-TREE (Minh *et al.*, 2020).

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DATA AVAILABILITY STATEMENT

Raw PacBio and cDNA PenSeq read sequences have been deposited in the Sequence Read Archive (SRA) at <https://www.ncbi.nlm.nih.gov/sra> under BioProject IDs PRJNA623167 and PRJNA598824.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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