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RESEARCH ARTICLE

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Identification of *Pseudoperonospora cubensis* RxLR Effector Genes *via* Genome Sequencing

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ABSTRACT

Pseudoperonospora cubensis is a significant phytopathogen causing downy mildew disease in cucurbit crops. Understanding the molecular mechanisms underlying the interaction between *P. cubensis* and its host is essential for developing effective disease management strategies. RxLR effectors, secreted by pathogens, play a crucial role in modulating host immunity. In this study, we sequenced the genome of the *P. cubensis* isolate CNU-OTH and identified RxLR effector genes using bioinformatics tools. A total of 45 RxLR effector genes were identified from the genome of *P. cubensis*. Cloning and functional characterization of these effectors were performed through transient expression assays in *Nicotiana benthamiana* leaves. Subcellular localization of selected effectors was determined using GFP-tagged constructs. Functional characterization revealed that while most effectors did not induce a hypersensitive response (HR), a subset showed either weak or strong necrosis. Furthermore, several effectors demonstrated the ability to suppress cell death induced by BAX and INF1. Subcellular localization analysis indicated that RxLR effectors exhibited fluorescence in the nucleus and plasma membrane of *N. benthamiana* cells, suggesting diverse roles in host-pathogen interactions. This study provides insights into the genetic diversity and functional characterization of RxLR effectors in *P. cubensis*. Understanding the role of these effectors in manipulating host immunity is critical for developing strategies to combat downy mildew disease in cucurbit crops. The findings contribute to the broader understanding of plant-pathogen interactions and may facilitate the development of disease-resistant crop varieties.

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1. Introduction

Oomycetes are a group of more than 1500 microbial eukaryotes, including several significant phytopathogens [1]. They can be biotrophs (i.e., *Pseudoperonospora cubensis*, *Albugo laibachii*), hemi-biotrophs (i.e., *P. infestans*), or necrotrophs (i.e., *Pythium ultimum*). Oomycetes are known as some of the most destructive pathogens in the plant kingdom and have been linked to numerous epidemics over the years [2]. *Phytophthora*, *Pythium*, and downy mildews are well-known plant infections that seem to have descended from a common plant-parasitic ancestor [3]. Downy mildew diseases are among the most important oomycete diseases, affecting a wide range of hosts.

Numerous pathogens associated with downy mildew have emerged or reemerged, resulting in several harm to food and ornamental crops, including cucumber, basil, grapevine, rudbeckia, and impatiens [4]. *Pseudoperonospora cubensis* is an obligate biotrophic oomycete pathogen and the causal agent of downy mildew disease on cucurbit (CDM), resulting in significant yield loss. *P. cubensis* was first described by Berkeley in Cuba in 1868 [5,6]. However, it was not identified on live plants until 1903 in Moscow by Rostovzev [7,8]. The appearance of angular lesions on the adaxial leaf surface and sporangiophores on the leaf's abaxial side indicate this foliar disease [7]. Cucumber yield and quality are negatively impacted by downy mildew disease in all growing regions, causing significant financial losses [6].

Reducing the harm caused by CDM requires the adoption of effective control measures. Management of CDM is mainly achieved through cultural practices, chemical control, planting of CDM-resistant cucumber varieties, and biocontrol [6]. Although fungicide applications are the most widely used technique and necessary for adequate disease control,

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resistant varieties, and cultural techniques are important management strategy components [5,9]. To develop CDM-resistant varieties, we need to understand the resistance mechanism of cucumber to *P. cubensis* through genomic technologies. Research findings showed that effector-triggered immunity (ETI) can result from the interaction of the cucumber R-genes with the pathogen *P. cubensis*'s RxLR effectors, which are identified by their avirulence (*avr*) activity. This ETI can help the plant to develop resistance and the reaction often leads to a localized hypersensitive response (HR). To avoid the spreading of pathogens that need living tissue to colonize the host successfully, highly localized cell death or HR is generated [10]. Breeding for downy mildew resistance can be expedited by identifying and using effectors to find germplasm-carrying R genes [11,12]. Plants use disease resistance (R) gene-encoded receptors to detect the presence of effectors and respond to pathogens by inhibiting their growth and activating plant immunity [13]. Plants with durable and broad-spectrum resistance could be engineered using effectors that are highly conserved and recognized by the host [14].

The oomycete community was among the first to establish coordinated transcriptome and genome sequencing programs and use the resulting resources to propel conceptual advancements [3]. By examining genome content and structure, abundant information has been obtained, enhancing our understanding of the host-pathogen interaction and providing insight into the evolution of this interaction [15]. A search has been conducted for potential virulence determinants in *P. cubensis* draft genome sequence, such as effector proteins that could be involved in the etiology of the disease [15,16]. Similar to other oomycetes, *P. cubensis* secretes molecules called effectors during infection, which alter host cell machinery to promote colonization and infection on their hosts [13]. Hundreds of effector proteins secreted by *P. cubensis* are classified as either apoplastic effectors or cytoplasmic effectors, based on their target location in the host plant [17]. While cytoplasmic effectors, such as RxLR and Crinklers are translocated into the plant cell and target distinct subcellular compartments, apoplastic effectors, such as enzymes and enzyme inhibitors are secreted into the extracellular space of the plant [18].

Oomycete cytoplasmic effectors are modular proteins that possess conserved motifs, particularly the RxLR and EER motifs, after N-terminal signal peptides [18,19]. The Peronosporales clade which includes Phytophthora and the downy mildew pathogens has an enrichment of RXLR effector proteins [11]. The delivery of oomycete *Avr* proteins within plant cells is accomplished *via* RxLR–EER motifs [20].

Plasmodium translocation assays have demonstrated the functional nature of their RxLR-dEER motif [21], and reports have indicated that the RxLR-dEER motif is necessary for secretion and translocation of *P. infestans Avr3a* [22,23] and *P. sojae Avr1b* [24] into their plant cell. *P. infestans Avr3a* and *Avrblb2*, which correspond to the R3a and Rpiblb2 proteins, are two modular RxLR effectors that induce avirulence activity in potato plants [18,25]. In addition, research findings showed the role of RxLR effectors in the suppression of cell death triggered by *Phytophthora infestans* (INF1) elicitin and Bcl-Associated X (BAX) in *Nicotiana benthamiana* [26–28].

Here, we identified RXLR effectors from the genome of *P. cubensis* using an Illumina HiSeq X Ten sequencer. In the search for putative PscRxLR effectors from the genome of *P. cubensis*, 45 PscRxLR effectors were identified. Out of these, 42 were cloned and characterized. The findings of Savory et al. [29] showed that 271 secreted effector proteins are predicted to be encoded by *P. cubensis*, of which 67 exhibited recognizable R- or QxLR motifs, which is much higher than our finding. In this study, the potential of PscRxLR effectors to induce cell death on *N. benthamiana* as well as to suppress cell death caused by BAX and INF1 are discussed. In addition, the subcellular localization of all identified PscRxLR effectors is also presented.

2. Materials and methods

2.1. Plants used and growth conditions

Cucumber plants (*Cucumis sativus*) and tobacco plants (*N. benthamiana*) used for this study were grown in a plant growth chamber at 25 °C with a photoperiod of 14 h light/10 h darkness.

2.2. Pathogen isolation and maintenance

Infected cucumber leaves were collected from different parts of South Korea. *Pseudoperonospora cubensis* sporangia from the abaxial leaf surface were dislodged with sterilized distilled water. The suspension was filtered with Miracloth, and the concentration was adjusted to 2 x 10^4 sporangia per mL using a hemocytometer before inoculating 15 mm diameter cucumber leaf disks. Leaf disks that had been inoculated were kept at 20 °C with 12-h light/dark photoperiod to facilitate infection after being incubated for 24 h at 20 °C in a humid environment in

complete darkness. Isolates were maintained by re-inoculating weekly and incubating at 20 °C.

2.3. DNA extraction

Pseudoperonospora cubensis sporangia were collected from infected cucumber leaves by dislodging them with sterilized distilled water and centrifuged at 4000 rpm for 10 min to collect pure sporangia. DNA was extracted from all isolates of *P. cubensis* using the CTAB method. Specifically, the pure sporangia were suspended in TE buffer, and an equal amount of CTAB was added. Acid-washed glass beads were used to grind the sporangia, which were mixed at high speed for 30 min. Then, 500 µl of phenol-chloroform isoamyl alcohol was added to the ground sporangia and centrifuged for 10 min at 12,000 rpm. The aqueous phase was transferred to a new tube and incubated at 60 °C for 15 min with RNase A. After adding 500 µl of chloroform and centrifuging, the aqueous phase was transferred to a new tube, and 500 µl of isopropanol was added to precipitate the DNA. Finally, the supernatant was removed, and the pellets were washed with 70% alcohol. After drying the tube under a clean bench, the pellet was re-suspended in an elution buffer. The concentration and quality of all extracted DNA were checked using a NanoDrop spectrophotometer.

2.4. Genome sequencing of P. cubensis isolate CNU-OTH

Pure DNA of the South Korean *P. cubensis* isolates CNU-OTH was used for genome sequencing. DNA libraries were constructed using the TruSeq Nano DNA Library Kit (Macrogen, Daejeon, South Korea), and sequencing was performed using the Illumina HiSeq X Ten sequencer. The DynamicTrim and LengthSort programs from the SolexaQA (v.1.13) package were applied for quality trimming. The sequences generated from the CNU-OTH isolate were aligned to the reference genome sequence of the MSU1 isolate (NCBI Accession: GCA_000252605.1) with the BWA (Burrows-Wheeler Aligner) program. Using reference-guided assembly sequences and SRA RNA-seq data from cucumber downy mildew pathogen *P. cubensis* samples, gene prediction was performed using the Braker (version 2.1.5) program. To predict the presence or absence of signal peptides in protein sequences and the location of cleavage sites, the SignalP (version 5.0) program was applied. To explore RxLR effectors in protein sequences with signal peptides, the effectR package was used.

2.5. Gene cloning and plasmid construction

The full-length effector proteins were amplified from *P. cubensis* genomic DNA by PCR using specific primers. The band from the 0.5% agarose gel was purified using a SmartGene gel DNA clean-up kit (SJ Bioscience, Daejeon, South Korea) and cloned into a TA vector (All in one™ vector) to verify the full-length sequence of all effector genes compared with the computer analysis sequence. The matured region of candidate RxLR effectors was amplified for restriction cloning with specific primer pairs. After digesting the PCR product with the restriction enzymes 5′ClaI/AscI and 3’NotI, they were ligated into a binary Potato Virus X-based pGR106 vector. Recombinant plasmids were cloned and amplified using the *Escherichia coli* strain DH5α.

2.6. Agroinfiltration

Agrobacterium tumefaciens GV3101 carrying PGR106-*PscRxLR* (*P. cubensis* RxLR) genes were incubated in a shaking incubator for 2 days at 28 °C in YEP medium supplemented with 50 µg/mL Kanamycin and 50 µg/mL Rifampicin [18]. The recombinants were centrifuged at 3000 rpm for 20 min and re-suspended in an infiltration buffer (Sucrose, MES salt, MS without vitamins, 1 M NaOH, and Acetosyringone) [30]. The suspension was then adjusted to a final OD600 of 0.5 and incubated for 3 h in the dark at room temperature, before being infiltrated into *N. benthamiana*.

Four weeks old *N. benthamiana* plants were infiltrated with the suspension on the abaxial side by using a needleless syringe. BAX and INF1 were used as a positive control, and the pGR106 empty vector was used as a negative control. For the BAX and INF1 suppression assay, 24 h after infiltration of effectors, infiltration sites were penetrated with *A. tumefaciens* carrying INF1 and BAX at a final OD600 of 0.5, as previously described [25,31]. The pGR106 empty vector served as a negative control and PscRxLR effectors were used as a positive control.

2.7. Subcellular localization of PscRxLR effectors

Cultures of *Agrobacterium tumefaciens* carrying PCAMBIA1300-*PscRxLR*-GFP recombinants were co-inoculated with p19 protein into 4-week-old *N. benthamiana* leaves to characterize the subcellular location of candidate *P. cubensis* RxLR effectors. Images were captured 3 days after infiltration with a confocal microscope (LEICA, Germany), and the subcellular localization of PscRxLR proteins was

determined by observing the fluorescence of transiently expressed PscRxLR-GFP proteins.

3. Results

3.1. Genome sequencing of isolate CNU-OTH and identification of RxLR effector proteins

The *P. cubensis* isolate CNU-OTH, originally collected from infected cucumber leaves in Yesan, South Korea, was sequenced using the Illumina HiSeq X Ten sequencer. CNU-OTH was selected for genome sequencing due to its highly virulent phenotype, based on a pathogenicity analysis of isolates collected from downy mildew-infected cucumber leaves from different parts of South Korea. The purity of the isolate was also considered.

After removing low-quality and duplicate reads using the LengthSort program and DynamicTrim package, all processed reads were mapped to the *P. cubensis* MSU-1 reference genome [16], retrieved from the National Center for Biotechnology Information (NCBI) database, using the BWA program. The genome size of CNU-OTH is estimated to be 64.4 Mb, contained in 11,690 contigs, with a GC content of 48.88%. The longest contig is 106,007 bp, the shortest is 200 bp, and the N50 contig length for our isolate is 9309 bp. RNA-seq data of *P. cubensis* was downloaded from the NCBI Sequence Read Archive (SRA) and used for gene prediction. Spliced alignment was performed with the reference-guided assembly sequence using the HISAT2 (version 2.1.0) program, and the resulting BAM file was used for gene prediction. The number of transcripts from the genome of CNU-OTH is 12,854. In total, 12,477 protein-coding genes were identified, with an average coding sequence length of 1396 bp and an average protein sequence length of 465 aa. Among the predicted protein-coding genes, only 851 (6.82%) are secreted proteins (Table 1).

To explore RxLR effectors in protein sequences with signal peptides, the effectR package was used. The effectR package employs regular expression search (REGEX) and hidden Markov models (HMM) methods to explore oomycete RxLR effectors. Our results showed that the genome of *P. cubensis* from South Korea contains 45 validated RxLR effectors. A search for conserved motifs in these 45 PscRxLR effectors revealed that 29 of them are complete RxLR effectors, possessing both RxLR and EER motifs in their sequences, while 16 effectors contain only the RxLR motif (Table 2). The result also showed that, out of 45 RxLR effectors identified from CNU-OTH isolate, only 4 effectors have 100% sequence similarity with RxLR effectors found in MSU-1 isolate of USA. The other three effectors exhibit 98.6–99.7% similarity with effectors characterized by MSU-1 isolate, while the remaining effectors possess a unique sequence exclusive to the Korean isolate CNU-OTH.

3.2. The majority of PscRxLR effector proteins could not induce HR on N. benthamiana

To assess the potential of 45 PscRxLR effectors for inducing cell death, *Agrobacterium* transformation was used to express ORFs in *N. benthamiana* leaf cells after they were ligated into the PVX vector. BAX, the Bcl-2 protein family from mice, and INF1, a recognized *P. infestans* cell death inducer, were used as positive controls, while the empty vector served as a negative control.

Out of 45 PscRxLR effectors, 2 failed to be amplified and one contained RXLR sequence at the position of 302 aa after the signal peptide (Table 3), disqualifying it as a typical RXLR effector based on previous studies [11,13,22]. Therefore, a total of 42 PscRxLR matured proteins were successfully cloned from the *P. cubensis* CNU-OTH genome. The cloning results revealed that 38 effectors (91%) could not induce HR, while 3 showed weak necrosis, and only one *PscRxLR* could induce cell death on non-host *N. benthamiana* (Figures 1(A,B), Table 3, Supplementary Figure S1). Further characterization is needed to confirm the function of those showing weak and strong HR.

Table 1. Comparison of genome sequence information of *Pseudoperonospora cubensis* CNU-OTH isolate with the reference genome of *P. cubensis* MSU-1 isolate.

Description	CNU-OTH	MSU-1*
Estimated genome size	64.4 Mb	67.9 Mb
Number of contigs	11,690	35,546
Contig N50**	9309	3961
GC content of the genome (%)***	48.88	54.02
No. of protein-coding genes	12,477	23,519
No. of secreted protein	851	941
Total RxLR and RxLR-like	45	72

*Refs. [11,15,29].
**Contig N50: the sequence length of the shortest contig at 50% of the total assembly length.
***GC content: guanine-cytosine content.

3.3. Subcellular localization of PscRxLR effectors

To determine the subcellular localization of *P. cubensis* RxLR effectors, GFP-tagged mature PscRxLR effectors were ligated into the PCAMBIA1300 vector and transformed into *Agrobacterium tumefaciens*. The p19 protein from tomato bushy stunt virus (TBSV) was used to suppress gene silencing [32]. Three days after incubation, the p19 silencing plasmid and corresponding *Agrobacterium* strains containing the GFP constructs were mixed at a 1:10 ratio and co-infiltrated into *N. benthamiana* leaves. Three days later, confocal microscopy was used to examine the specific location of effector proteins within the cell (Figure 2(A)).

The results showed that, out of the 42 tested effectors, the majority of *P. cubensis* RxLR effectors (48%) showed green fluorescence in both the nucleus and plasma membrane, while 37% localized only in the nucleus. The remaining 5, 5, 2, and 2% were localized in the nucleus and cytoplasm, plasma membrane, plasma membrane and cytoplasm, and cytoplasm, respectively (Figure 2(B)). This result indicates that

Table 2. Identification of *PscRxLR* candidate effector genes.

No. of RxLR effectors	SP (Sec/SPI)*	Types of RxLR-EER motif**	
		RxLR-EER Motifs	Only RxLR motif
45	45	29	16

*SP (Sec/SPI): secreted signal peptide.
**RxLR: Arg-x-Leu-Arg, where x represents an any amino acid, EER: Glu-Glu-Arg).

Table 3. Description of the selected PscRxLR genes.*

Gene name	Length (AA)	Signal peptide position	RxLR sequence	RxLR position	dEER	EER position	HR cell in *Nicotiana benthamiana*	Cell death suppression	
								BAX-induced	INF1-induced
PscRxLR001	216	22–23	RSLR	54	AEER	68	0	±	+
PscRxLR002	370	22–23	RALR	54	IEER	68	0	±	+
PscRxLR003	338	22–23	RGLR	49	GEER	85	0	±	+
PscRxLR004	269	20–21	RSLR	27	TEER	41	1	±	–
PscRxLR005	149	20–21	RMLR	49	GEER	63	0	–	–
PscRxLR006	119	18–19	RHLR	40	DEER	51	0	+	+
PscRxLR007	222	23–24	RYLR	55	AEER	69	0	–	–
PscRxLR008	265	21–22	RFLR	51	AEER	68	0	±	+
PscRxLR009	242	22–23	RYLR	48	TEER	62	0	±	+
PscRxLR010	295	25–26	RFLR	57	SEER	71	0	±	+
PscRxLR011	205	22–23	RYLR	51	DEER	82	0	±	+
PscRxLR012	411	20–21	RFLR	44	VEER	55	2	–	–
PscRxLR013	244	23–24	RLLR	44	DEER	57	0	+	+
PscRxLR014	217	25–26	RLLR	57	GEER	71	0	+	+
*PscRxLR015***	244	19–20	RSLR	57	TEER	71	nd	nd	nd
PscRxLR016	398	20–21	RFLR	44	EEER	56	0	–	–
PscRxLR017	147	23–24	RMLR	49	GEER	63	0	±	±
PscRxLR018	534	19–20	RPLR	49	DEER	62	1	±	±
PscRxLR019	213	23–24	RYLR	44	DEER	57	0	±	–
PscRxLR020	290	25–26	RLLR	52	NEER	66	0	±	+
PscRxLR021	281	19–20	RSLR	43	TEER	56	0	±	+
PscRxLR022	418	25–26	RSLR	57	TEER	66	0	–	–
PscRxLR023	471	18–19	RSLR	51	IEER	65	0	–	–
PscRxLR024	485	25–26	RLLR	57	AEER	71	0	+	+
PscRxLR025	276	25–26	RSLR	57	DEER	71	0	±	+
PscRxLR026	304	20–21	RSLR	43	DEER	95	0	±	+
PscRxLR027	197	22–23	RFLR	43	HEER	71	0	–	+
PscRxLR028	306	19–20	RSLR	45	IEER	59	0	–	–
PscRxLR029	476	18–19	RFLR	49	DEER	62	0	±	±
PscRxLR030	364	20–21	RSLR	49	nd	nd	0	–	–
PscRxLR031	287	21–22	RFLR	44	nd	nd	0	±	±
*PscRxLR032***	413	20–21	RSLR	49	nd	nd	nd	nd	nd
PscRxLR033	295	19–20	RFLR	44	nd	nd	0	±	±
PscRxLR034	138	20–21	RMLR	49	nd	nd	1	±	+
*PscRxLR035***	482	24–25	RGLR	327	nd	nd	nd	nd	nd
PscRxLR036	217	28–29	RSLR	59	nd	nd	0	+	+
PscRxLR037	285	19–20	RSLR	57	nd	nd	0	±	+
PscRxLR038	88	19–20	RSLR	46	nd	nd	0	±	+
PscRxLR039	325	21–22	RFLR	47	nd	nd	0	±	±
PscRxLR040	209	25–26	RTLR	51	nd	nd	0	–	–
PscRxLR041	235	21–22	RQLR	53	nd	nd	0	+	+
PscRxLR042	323	25–26	RTLR	51	nd	nd	0	–	±
PscRxLR043	183	27–28	RSLR	43	nd	nd	0	–	–
PscRxLR044	369	24–25	RSLR	51	nd	nd	0	–	–
PscRxLR045	392	21–22	RHLR	54	nd	nd	0	+	+

*Mining of RxLR effectors in *Pseudoperonospora cubensis* resulted in 45 RxLR candidate effectors containing an RxLR-DEER (RD) motif.
**None selected, PscRxLR: *Pseudoperonospora cubensis* RxLR; AA: amino acid; nd: not determined; 0: no cell death; 1: weak cell death; 2: strong cell death in *Nicotiana benthamiana*, + Suppress cell death, ± Partially suppress cell death, – not suppress cell death in *N. benthamiana*. All candidates were cloned into the Potato virus X (PVX) expression vector pGR106 in *A. tumefaciens* enabling functional profiling for hypersensitivity and cell death suppression assay on *N. benthamiana* leaves.

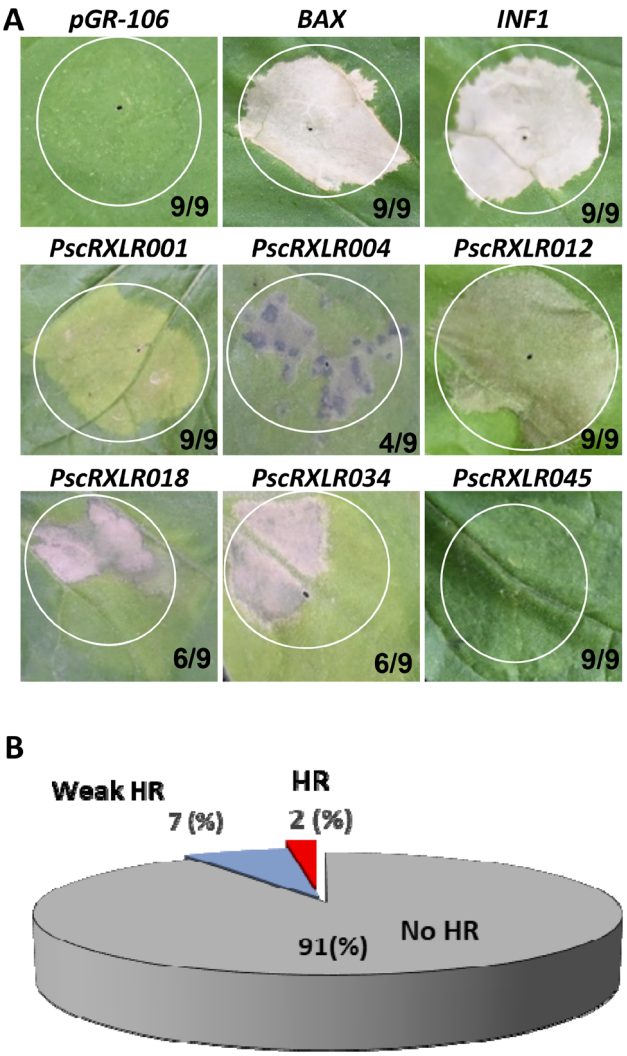

Figure 1. Cell death screening of candidate PscRxLR effectors. (A) Shows different PscRxLR effectors cell death phenotype on *Nicotiana benthamiana* leaves compared with the positive control BAX and INF1 and negative control pGR106 empty vector. The circle shows the infiltration site and the red circle shows effectors with HR phenotype. Some representative images among a total of 42 observed *PscRXLR*s. The pictures were taken 5 days after inoculation; (B) Indicates the percentage of PscRxLR effectors with HR phenotype.

RXLR effectors target either the nucleus or host membranes.

3.4. PscRxLR effectors can suppress elicitors-induced cell death (HR) in N. benthamiana

To characterize candidate PscRxLR effectors based on their ability to suppress BAX/INF1-triggered programmed cell death (PCD), an *Agrobacterium tumefaciens*-mediated transient expression test was used on *N. benthamiana* to screen all 42 PscRxLR effector proteins. *Agrobacterium* strains carrying the PGR106 expression vector, which contains each effector gene, were introduced into *N. benthamiana* leaves 24 h before the cell death inducers are infiltrated. Our results showed that out of the 42 tested PscRxLR effectors from isolate CNU-OTH, only 7 PscRxLR effectors could entirely prevent the cell death triggered by BAX, while 21 PscRxLR effectors partially inhibited cell death induced by BAX. However, 14 PscRxLR effectors were failed to suppress cell death (Table 3).

We also found that of the 42 PscRxLR effectors examined for their ability to suppress INF1-triggered PCD, the majority (22) were capable of completely suppressing INF1-induced hypersensitive response (HR). In contrast, 7 (16.6%) effectors from CNU-OTH could moderately inhibit INF1-induced cell death, while 13 effectors were unable to stop the necrosis that INF1 expression caused (Table 3).

All effectors that suppressed HR triggered by BAX (*PscRxLR006, 013, 014, 024, 036, 041, 045*) were also effective in suppressing cell death triggered by INF1 (Table 3). Furthermore, infiltration of *A. tumefaciens* cells containing these 7 *PscRxLR* alone failed to induce cell death in *N. benthamiana* leaves (Table 3). The suppression of cell death by oomycete effectors in response to various elicitors

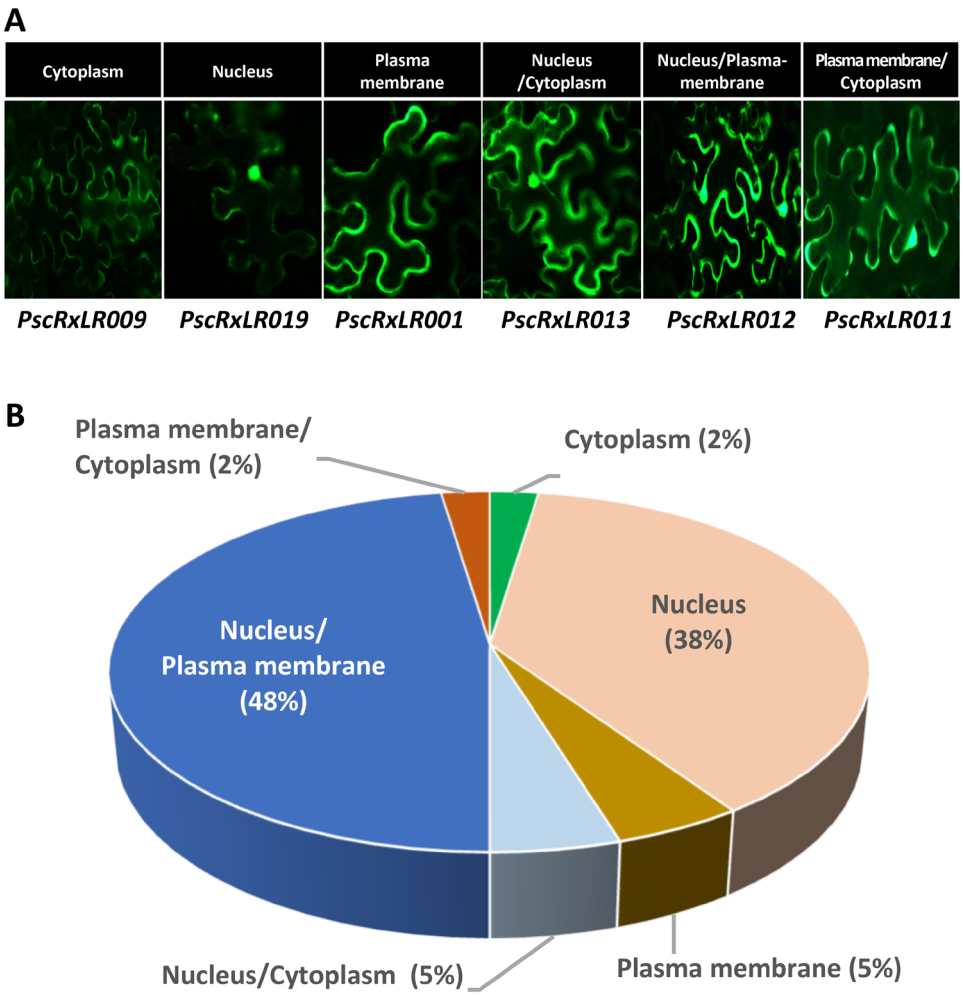

Figure 2. Subcellular localization of *Pseudoperonospora cubensis* selected RxLR effectors. (A) Effector-GFP proteins were expressed in *Nicotiana benthamiana* and the accumulation was analyzed by confocal microscopy, 3 days after infiltration; (B) Subcellular localization of effectors by percent.

indicates a sophisticated mechanism evolved by the oomycete to manipulate the host's immune response. Not only in suppression of these elicitors, but PscRxLR effectors also have partial suppressor and non-suppressor effectors in common. For example, *PscRxLR031* partially inhibited and *PscRxLR012* did not inhibit both BAX/INF1-triggered programmed cell death after co-infiltration (Figures 3(A,B)).

4. Discussion

Oomycetes that interact with their host in a biotrophic phase, like *P. cubensis*, secrete cytoplasmic effectors that are introduced into plant cells to suppress the host immunity [33]. *P. cubensis* is a challenging pathogen to examine because of its resistance to genetic alteration and required host-associated lifestyle. Separating pathogen sequences from host sequences is a challenge in establishing genomic and transcriptomic resources [34]. In this study, we analyzed the genome sequence of *P. cubensis* isolate CNU-OTH, from South Korea. The genome size of our isolate is estimated at 64.4 Mb, which is less than the size of the reference genome MSU-1 isolate estimated 67.9 Mb from the USA [29]. In addition, there are differences in the number of contigs and N50 contig size, which are 11,690 contigs with an N50 size of 9.3 Kb for our isolate CNU-OTH, while 35,546 contigs with an N50 contig size of 4.0 Kb for our reference genome MSU-1 (Table 1).

RxLR constitutes a well-studied family of phytopathogen avirulence or effector proteins. They belong to the most researched group of cytoplasmic effectors and have RxLR (Arg-Xaa-Leu-Arg) amino acid motif, characterized by conserved N-terminal domain and located within 40 amino acids downstream of the signal peptide followed by another second conserved motif Glu-Glu-Arg (EER) motif [11,13,22,35]. The presence of a functional signal peptide which is necessary for secretion from the pathogen served as a basis for predicting candidate effectors, followed by a short RxLR amino acid motif [34]. Additionally, they should contain a start codon and a stop codon. According to our genome analysis, the number of

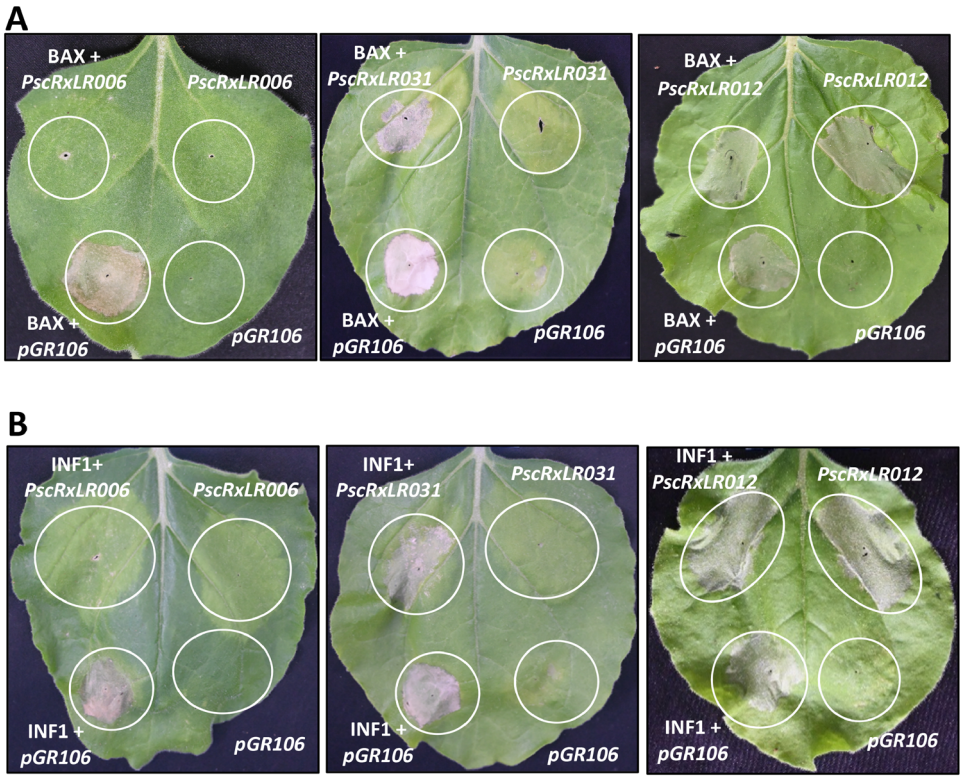

Figure 3. Ability of candidate PscRxLR effectors to inhibit programmed cell death triggered by BAX/INF1. (A) BAX-induced cell death (BCD) screening of PscRxLR candidate effectors on *Nicotiana benthamiana*: Fully suppressed BCD by *PscRxLR006*, partially suppressed BCD by *PscRxLR031*, and non-suppressed BCD by co-infiltrating with *PscRxLR012*; (B) INF1-induced cell death (ICD) screening of PscRxLR candidate effectors on *N. benthamiana*: Fully suppressed ICD by *PscRxLR006*, partially suppressed ICD by *PscRxLR031*, and non-suppressed ICD by co-infiltrating with *PscRxLR012*. The suppression of cell death was assessed 5 days after co-infiltration by examining the hypersensitive response (HR) at the infiltrated site. All experiments were replicated three times.

identified RxLR effectors is significantly lower than in the reference genome. This could be attributed to the limited genome coverage generated from our isolate. We identified 45 from CNU-OTH, whereas the total number of identified RxLR and RxLR-like effectors from MSU-1 was 61 at initial analysis with 454 pyrosequencing [16]. Subsequently, using the Illumina Genome Analyzer, a more comprehensive dataset was generated and the total number of RxLR and RxLR-like, including QxLR effectors, reached 271 [15]. These results underscore that the number of identified RxLR effectors can vary depending on genome coverage.

Out of 45 PscRxLR effectors identified from isolate CNU-OTH, 38 effectors did not induce any cell death, while only *PscRxLR012* showed strong HR phenotype when infiltrated into 4-week-old *N. benthamiana* leaves (Figure 1(A), Table 3). *PscRxLR001* from isolate MSU-1, which was not among the 45 PscRxLR effectors identified from isolate CNU-OTH, was found to induce rapid cell death in plant cells [15]. Conversely, a study by Wang et al. [36] showed that out of 169 effectors, only eleven *P. sojae* effectors are known to be capable of causing mottling, chlorosis, or cell death in *N. benthamiana* leaves, suggesting either recognition by the defense machinery of *N. benthamiana* or their role as toxins.

The subcellular localization of pathogen effectors within a plant cell upon entry can provide insights into their mechanisms of action [37]. The majority of CNU-OTH PscRxLR effectors localize in both the nucleus and plasma membrane. This finding aligns with the result of Anderson et al. [38], who reported that the majority of *H. arabidopsis* RxLR effectors targeted either the nucleus or host membranes. In planta, localization of PscRxLR1 (*P. cubensis* RxLR protein 1) showed that it localizes to the plasma membrane [15]. On the other hand, PcQNE (*P. cubensis* QxLR nuclear-localized effector) variants V1 and V6, are localized in the nucleus [16]. Effectors' subcellular localization can impact their function as well. Studies showed that effectors localized to the nucleus often interfere with gene expression and transcriptional regulation. They can modify the expression of host immune response genes, preventing the cell from mounting an effective defense as well as they may interact with transcription factors to either activate or repress specific genes that benefit the pathogen [39,40]. On the other hand, effectors in the cytoplasm target host

proteins or signaling pathways and inhibit key components of immune signaling that regulate immune responses or other cellular processes [41]. Understanding the localization of these effectors is crucial for comprehending their roles in host-pathogen interactions and manipulating plant cellular processes during infection.

The elicitors used in this study were BAX, a member of the Bcl-2 protein family from mice [42], and the PAMP elicitor INF1 from *P. infestans* [43]. *Nicotiana benthamiana* induces apoptosis-like cell death when exposed to BAX, the first known mammalian pro-apoptotic member of the Bcl-2 protein family [27]. Plants' cell death-promoting action of BAX is correlated with the increase of the defense-related protein PR-1, suggesting that BAX stimulates an endogenous cell death pathway in plants [25,42].

On the other hand, *Phytophthora infestans*' extracellular protein infestin 1 (INF1) belongs to the elicitin family of protein elicitors known to induce hypersensitive responses in some *Solanaceous* and cruciferous plants [44]. The results regarding the ability of PscRxLR effectors from the *P. cubensis* to modulate cell death responses induced by BAX and INF1 indicate that effectors suppressing cell death triggered by BAX also suppress INF1-induced cell death (Table 3). This suggests a potential common mechanism or pathway targeted by these effectors to inhibit cell death induction, demonstrating a sophisticated mechanism evolved by the pathogens to manipulate the host's immune response for their benefit.

Our results are consistent with [24], showing that when *A. tumefaciens* strains carrying both genes were infiltrated simultaneously, *P. sojae Avr1b-1* gene expression shielded *N. benthamiana* tissue from cell death caused by BAX cDNA expression. This finding supports [36], who reported that out of 49 *P. sojae* RXLR effectors, 53% could suppress INF1-triggered PCD. Additionally, *P. infestans* RXLR effector AVR3a suppresses PCD triggered by the PAMP-like elicitin INF1 [26]. Overall, this study provides valuable insights into the genomic architecture and functional characteristics of *P. cubensis* RxLR effectors, helping to comprehend host-pathogen interactions and the development of disease management strategies.

Author contributions

RDG and S-KO designed the experiments and wrote the manuscript. SHK, JM, MS, IJ, and SA carried out genome sequences analysis and HR investigation. All authors contributed to the article and approved the submitted version.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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