

# 454 Genome Sequencing of *Pseudoperonospora cubensis* Reveals Effector Proteins with a QXLR Translocation Motif

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*Pseudoperonospora cubensis* is a biotrophic oomycete pathogen that causes downy mildew of cucurbits, a devastating foliar disease threatening cucurbit production worldwide. We sequenced *P. cubensis* genomic DNA using 454 pyrosequencing and obtained random genomic sequences covering approximately 14% of the genome, thus providing the first set of useful genomic sequence information for *P. cubensis*. Using bioinformatics approaches, we identified 32 putative RXLR effector proteins. Interestingly, we also identified 29 secreted peptides with high similarity to RXLR effectors at the N-terminal translocation domain, yet containing an R-to-Q substitution in the first residue of the translocation motif. Among these, a family of QXLR-containing proteins, designated as PcQNE, was confirmed to have a functional signal peptide and was further characterized as being localized in the plant nucleus. Internalization of secreted PcQNE into plant cells requires the QXLR-EER motif. This family has a large number of near-identical copies within the *P. cubensis* genome, is under diversifying selection at the C-terminal domain, and is upregulated during infection of plants, all of which are common characteristics of characterized oomycete effectors. Taken together, the data suggest that PcQNE are bona fide effector proteins with a QXLR translocation motif, and QXLR effectors are prevalent in *P. cubensis*. Furthermore, the massive duplication of PcQNE suggests that they might play pivotal roles in pathogen fitness and pathogenicity.

Plant pathogens secrete a diverse array of effector proteins during their interactions with host plants, presumably to facilitate colonization, growth and development on their hosts by suppressing host defense responses (Alfano and Collmer 2004;

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\*The e-Xtra logo stands for “electronic extra” and indicates that seven supplementary data files are published online.

Birch et al. 2009). In this sense, all effectors are virulence factors. However, some effectors can be recognized by host resistance (R) proteins and, as a result, initiate specific gene-for-gene defense responses (Alfano and Collmer 2004). In such cases, these effectors are termed avirulence (Avr) proteins (Chisholm et al. 2006; Jones and Dangl 2006). In general, based on the targeting or localization site within the host plant, effectors are broadly classified into two classes: apoplastic effectors or cytoplasmic effectors (Kamoun 2006). Whereas apoplastic effectors function in the plant extracellular space, cytoplasmic effectors are delivered inside the plant cell, where they target various subcellular compartments (Hogenhout et al. 2009; Kamoun 2006).

The mechanisms by which cytoplasmic effectors enter host cells are diverse. For example, gram-negative bacteria inject effectors into the cell through a specialized needle-like apparatus, called the type III secretion system (T3SS) (Buttner and He 2009). Similarly, pathogenic nematodes inject effectors inside the cell through a needle-like apparatus called a stylet, which is also used for feeding (Davis et al. 2008). In contrast, neither fungi nor oomycetes appear to have an injection structure similar to the T3SS or the nematode stylet. Thus, the mechanisms of delivery of oomycete and fungi effectors remain to be fully characterized. Many characterized effector proteins from fungi and oomycetes contain an endoplasmic reticulum (ER)-type signal peptide recognized by a type II secretion system (T2SS) (Dodds et al. 2009), suggesting that these proteins are secreted from the pathogen cells. How these secreted proteins cross the host plasma membrane and enter the host cell had been a mystery until the identification of an Arg-X-Leu-Arg (RXLR) translocation motif, which was identified based on its presence and conservation across all characterized Avr proteins of oomycetes (Morgan and Kamoun 2007). This motif is interchangeable with the host-targeting signal (HTS) or PEXEL motif from *Plasmodium falciparum* (Bhattacharjee et al. 2006; Grouffaud et al. 2008). The RXLR motif, together with its downstream Asp-Glu-Glu-Arg (dEER) motif, which is less conserved and comprises a stretch of acidic amino acids often ending with EER (Birch et al. 2008), was shown to be required for host cell entry for the *Phytophthora infestans* effector Avr3a (Whisson et al. 2007) as well as the *P. sojae* effector Avr1b (Dou et al. 2008). Interestingly, Avr1b was found to be able to enter the cell in the absence of the pathogen (Dou et al. 2008), suggesting a host-mediated translocation process. A further study published very recently shows that the RXLR motif and its variants in both oomycetes and fungi enable the

effectors to bind phosphatidylinositol-3-phosphate (PI3P) on the outer surface of plant and human cell plasma membranes, and this binding mediates the effector entry through lipid raft-mediated endocytosis (Kale et al. 2010). Although the detailed translocation process remains to be fully defined, this study suggests a general cell entry mechanism for cytoplasmic effectors from oomycetes and fungi.

With the completion of numerous genome sequences from a broad spectrum of plant and mammalian pathogens, bioinformatics approaches have enabled genome-wide identification of effector proteins (Arnold et al. 2009; Jiang et al. 2008; Samudrala et al. 2009; Whisson et al. 2007). These computational methods are typically developed through the identification of shared characteristics of known, characterized effector gene or protein sequences. For example, a machine-learning approach was developed for the prediction of type III secretion system effector proteins by systematically analyzing the amino acid composition and secondary structure of N termini of 100 experimentally determined effector proteins (Arnold et al. 2009). Similarly, the large-scale identification of oomycete effectors has been facilitated by computational searches for an ER-type signal peptide, followed by a downstream RXLR translocation motif and, usually, a stretch of acidic amino acids ending with an EER motif (Jiang et al. 2008; Whisson et al. 2007; Win et al. 2007). To this end, hundreds of putative effector proteins have been bioinformatically predicted to reside within each genome of the sequenced *Phytophthora* spp. (Jiang et al. 2008; Whisson et al. 2007). The identification of large repertoires of RXLR effectors provides limitless opportunities to determine how oomycetes manipulate their hosts to suppress defense responses and establish successful infection.

*Pseudoperonospora cubensis* is a biotrophic oomycete pathogen responsible for downy mildew of cucurbits, a devastating foliar disease worldwide (Savory et al. 2010). This disease is notorious for its fast development and, as such, infected cucumber leaves can become frosted within 10 days of initial infection. Since 2004, cucumber downy mildew has become an increasing concern for cucumber growers in the United States due to the emergence of new virulence within *P. cubensis* populations (Holmes et al. 2006). As a result of this increased virulence, previous downy mildew resistance, which has been bred into most of the popular cultivars in use today, has failed, leaving growers with few options to manage the disease (Holmes et al. 2006). Although the genomes of many plant pathogens have been sequenced, genomic resources for *P. cubensis* are nonexistent.

In this study, we sequenced *P. cubensis* genomic DNA using 454 pyrosequencing technology and obtained random genomic sequences covering approximately 14% of the genome. An initial search for putative RXLR effectors using the Basic Alignment Search Tool (BLAST) against RXLR effector proteins from *Hyaloperonospora arabidopsidis* and *Phytophthora infestans* identified not only RXLR-EER-containing sequences but also sequences with variations of the conserved RXLR, including Lys-X-Leu-Arg (KXLR) and Gln-X-Leu-Arg (QXLR). Interestingly, more QXLR-containing sequences were identified than RXLR- and KXLR-containing sequences. This led us to modify RXLR to QXLR strings in the RXLR effector identification pipeline (Win et al. 2007) and identify secreted RXLR-like QXLR-containing proteins. In all, 29 such sequences were found, suggesting that QXLR-containing sequences are highly represented, compared with 32 RXLR-containing sequences found in this data set. In completing and resequencing a number of QXLR-containing sequences, we identified a gene family encoding secreted and nuclear-localized proteins, with massive multiplication in the genome. We named proteins encoded by this gene family *Pseudoperono-*

*spora cubensis* QXLR nuclear-localized effectors (*PcQNE*). Variants of *PcQNE* induced chlorosis and necrosis on *Nicotiana benthamiana*, were under diversifying selection, and were upregulated during infection of plants. Internalization of secreted *PcQNE* into plant cells requires the QXLR-EER motif. Together, we conclude that *PcQNE* is a novel family of effector proteins with a functional QXLR translocation motif.

## RESULTS

### Generation of random genomic sequences of *P. cubensis*.

We performed two runs of 454 pyrosequencing with genomic DNA isolated from *P. cubensis* sporangia. In total, 605,921 reads containing 135.4 million nucleotides (nt) were generated. After removing identifiable cucumber sequence, 588,461 reads containing 131.1 million nt remained. The data were assembled using the Roche gsAssembler (version 1.1.03), and 87.8% of the reads, representing 62.9% of the nucleotides, were fully or partially assembled. In total, 42,799 contigs were generated with a total length of 12.1 million nt, accounting for approximately one-seventh (approximately 14%) of the genome, assuming that the genome size of *P. cubensis* is 88.22 Mb, as predicted by Feulgen image analysis (Voglmayr and Greilhuber 1998). Among them, there were 3,413 large ( $\geq 500$  nt) contigs, with a total length of 2.94 million nt and an average length of 863 nt. The longest contig was composed of 9,219 nt. The 454 sequencing data were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive under accession number SRX025566.1. The sequences of 3,413 large contigs are provided in Supplementary Data 1.

### Identification of putative RXLR effector proteins.

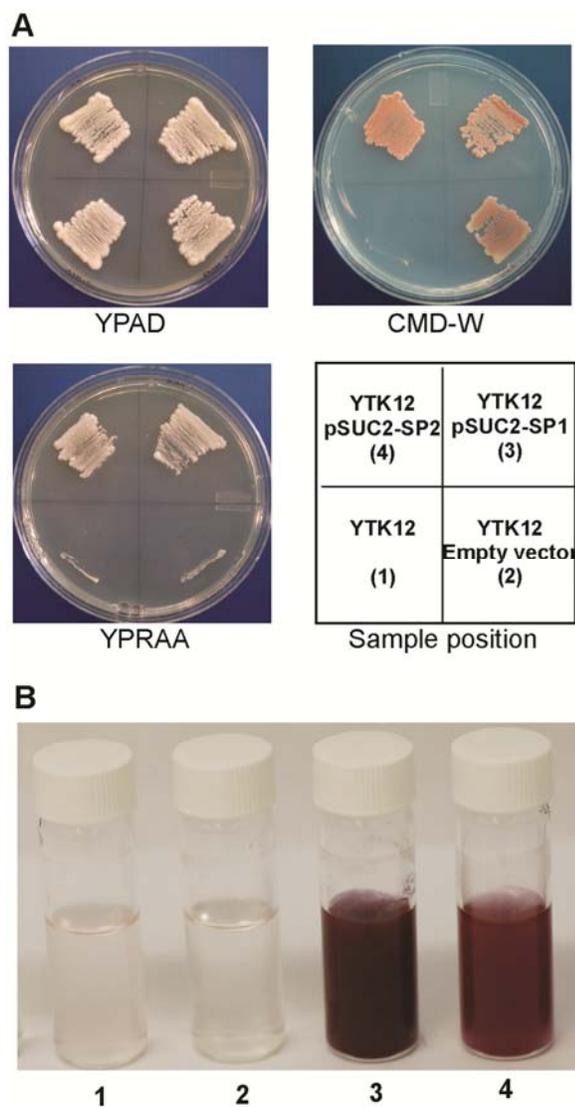
To identify potential effector proteins, the 3,413 large sequence contigs were analyzed using the RXLR effector identification pipeline (Win et al. 2007). In total, 12,131 peptides were translated from both strands. Among the 604 predicted secreted peptides, 32 nonredundant peptides (Supplementary Data 2) were found to contain an RXLR motif located downstream of the signal peptide and in position between 30 and 60 amino acids starting from the N terminus of the protein, a common feature of identified oomycete RXLR effector proteins (Win et al. 2007). We also analyzed the sequences of the 604 putative secreted peptides using a Hidden Markov Model (HMM) from an alignment of the RXLR-dEER region generated with 43 validated oomycete RXLR effector proteins (Win et al. 2007). Five sequences, which were also identified using the RXLR effector identification pipeline, were found to contain both the RXLR and EER motif.

### Discovery of a set of RXLR effector-like proteins with a QXLR motif.

In searching for putative RXLR effector proteins, we also performed bidirectional BLAST sequence similarity analyses using the 3,413 large contigs from *P. cubensis*, 149 putative RXLR effectors from *H. arabidopsidis* (Win et al. 2007), and 563 RXLR effector proteins from *Phytophthora infestans* (Haas et al. 2009). From this, a number of peptide sequences from *Pseudoperonospora cubensis* were found to align with effectors from *H. arabidopsidis* or *Phytophthora infestans* at the N-terminal RXLR-dEER regions (data not shown). Additional in silico analysis was performed to determine whether these sequences contained a putative signal peptide or an RXLR or conserved EER motif. Interestingly, we not only identified sequences encoding putative RXLR effector proteins but also sequences encoding QXLR- and KXLR-containing secreted peptides. Surprisingly, we identified more QXLR- than RXLR-containing sequences in this screen. Among 11 sequences identi-



Interestingly, all the sequences are highly related but different from each other, and were designated as V1 through V6. Whereas V1 and V3 encode a protein of 515 amino acids, V4 is 2 amino acids longer due to a 6-nt insertion in the gene, and V2, V5, and V6 are 7 amino acids shorter due to an earlier stop codon generated from a single nucleotide substitution. They all have a putative signal peptide predicted by SignalP 3.0 and QXLR that is downstream of the signal peptide cleavage sites, located between 51 and 56 amino acids, and followed by EER sequence (Fig. 2). This is consistent with identified RXLR effectors (Win et al. 2007) except the R-to-Q substitution in the translocation motif. All variants, except V1, have a putative NLS (Fig. 2).



**Fig. 3.** Functional validation of the signal peptides of *PcQNE* proteins using the yeast invertase secretion assay. **A**, Yeast growth assay on media with (yeast-peptone-glucose-adenine hemisulfate agar [YPAD] and CMD-W) or without (yeast-peptone-raffinose-Antimycin A agar [YPRAA]) glucose. Yeast YTK12 strains carrying pSUC2-SP1 (3) and pSUC2-SP2 (4), which express two different signal peptide fragments fused in frame to the mature invertase gene SUC2, are able to grow on YPRAA media with raffinose as a sole carbon source. YTK12 (1) and the YTK12 strain carrying empty vector pSUC2T7M13ORI (2) do not secrete invertase and, thus, are not able to grow on YPRAA media. Yeast growth on YPAD and CMD-W serves as an equal viability and strain integrity controls. **B**, Detection of the secreted invertase activity with 2,3,5-triphenyltetrazolium chloride (TTC). The red color indicates invertase activity. The numbers are used for labeling reactions derived from various yeast strains shown in A.

### Functional validation of signal peptides of *PcQNE* variants.

The six variants encode two distinct versions of putative signal peptides (Fig. 2). V1, V2, V3, V4, and V6 share the same signal peptide sequence, named SP1. The signal peptide from V5 is unique and was designated SP2. A genetic assay based on the requirement of invertase secretion for yeast growth on media with sucrose or raffinose as the sole carbon source was previously used for identifying secreted proteins from human peripheral blood mononuclear cells (Jacobs et al. 1997). More recently, this assay was used to validate *in silico* signal peptide predictions for *Phytophthora infestans* RXLR effector proteins (Oh et al. 2009). To test whether SP1 and SP2 were functional, we fused the predicted signal peptides SP1 and SP2 (Fig. 2) in-frame with the mature sequence of yeast invertase SUC2 in the yeast signal sequence trap vector pSUC2T7M13ORI, and the resultant constructs were transferred into a yeast SUC2-minus strain, YTK12. YTK12 and YTK12 expressing the SUC2 mature sequence did not grow on raffinose-containing yeast-peptone-raffinose-Antimycin A agar (YPRAA) medium (Fig. 3A); however, the fusion of SP1 and SP2 to the SUC2 mature sequence enabled the successful secretion of invertase, thus enabling the yeast cells to hydrolyze raffinose and grow on YPRAA media. The secretion of invertase and the hydrolysis of raffinose into monosaccharides were also confirmed with 2,3,5-triphenyltetrazolium chloride (TTC), which reacts with monosaccharides to form the insoluble (red colored) triphenylformazan (Fig. 3B). In total, these results suggest that *PcQNE* proteins are secreted from *Pseudoperonospora cubensis*.

### Variants of *PcQNE* are localized within the plant nucleus.

To confirm the *in silico* prediction of nuclear localization and to gain insight into the possible function of the *PcQNE* effectors, the subcellular localization of *PcQNE* variants was determined by transiently expressing DsRed-*PcQNE* fusion proteins in *N. benthamiana* using *Agrobacterium tumefaciens*-mediated transformation. With the exception of variant V1, all five members have a predicted NLS (Fig. 2). Based on this, we chose variants V1 and V6 for subcellular localization by laser confocal microscopy. The sequences encoding mature proteins of V1 and V6 were fused to the C-terminus of DsRed, and co-expressed with the green fluorescent protein (GFP)-tagged *Arabidopsis* fibrillar protein AtFib1, a nucleolar marker (Goodin et al. 2002). In contrast to DsRed alone, both DsRed-V1 and DsRed-V6 were localized in focal spots overlapping and surrounding the GFP-marked nucleoli (Fig. 4), suggesting that both V1 and V6 are localized in the nucleus.

### Internalization of secreted *PcQNE* requires QLLR-EER motif.

To determine whether the QXLR-EER motif plays a role in translocating effectors inside the host cell, we undertook an internalization analysis of secreted proteins in the absence of the pathogen, as described by Rafiqi and associates (2010). We used site-directed mutagenesis to make alanine (A) substitutions in the QLLR or EER motif of *PcQNE\_V1* and generated C-terminal CFP fusion expression constructs (Fig. 5), which were expressed and visualized in *N. benthamiana*. Although wild-type V1 possessed a functional signal peptide, it was localized in the nucleus (Fig. 5A), suggesting that V1 can be successfully internalized after being secreted. Mutation of EER to AAA resulted in both nuclear localization and weak fluorescence accumulation in the apoplast of *N. benthamiana* cells (Fig. 5C), suggesting that EER is involved in cell entry. Although substitution of QLLR alone with AAAA did not seem to compromise the protein internalization (Fig. 5B), altering both QLLR and EER with Ala residues almost completely

blocked cell entry, as evidenced by the strong fluorescence accumulation in the apoplast and no fluorescence observed in the nucleus (Fig. 5D). Altogether, the data suggested that both QLLR and EER function in V1 internalization after being secreted into the apoplast, implying the role of QXLR-EER motif in effector translocation during pathogen infection.

#### ***PcQNE* variants induce chlorosis and necrosis on *N. benthamiana*.**

*A. tumefaciens* transient expression in *N. benthamiana* is a rapid, informative method to gain insight into the possible mechanism of effector activity and action (Oh et al. 2009). The *Agrobacterium* strains expressing DsRed alone and DsRed-V1 or DsRed-V6 fusion constructs were infiltrated into leaves of *N. benthamiana* and the phenotypes were monitored. Leaf areas expressing DsRed-V1 started to show chlorosis at 3 days postinoculation (dpi) and weak necrosis appeared as early as 4 dpi (Fig. 6). DsRed-V6 showed similar chlorosis phenotypes, lagging DsRed-V1 by 1 to 2 days (Fig. 6). The cell death responses induced by DsRed-V1 increased following 4 dpi and, by 6 dpi, the infiltrated tissue was completely dehydrated and necrotic. In contrast, the chlorosis induced by DsRed-V6 did not change much (i.e., at 6 dpi) compared with the phenotype at 4 dpi (Fig. 6). Infiltration of *N. benthamiana* leaves with the DsRed showed no chlorosis or necrotic lesions.

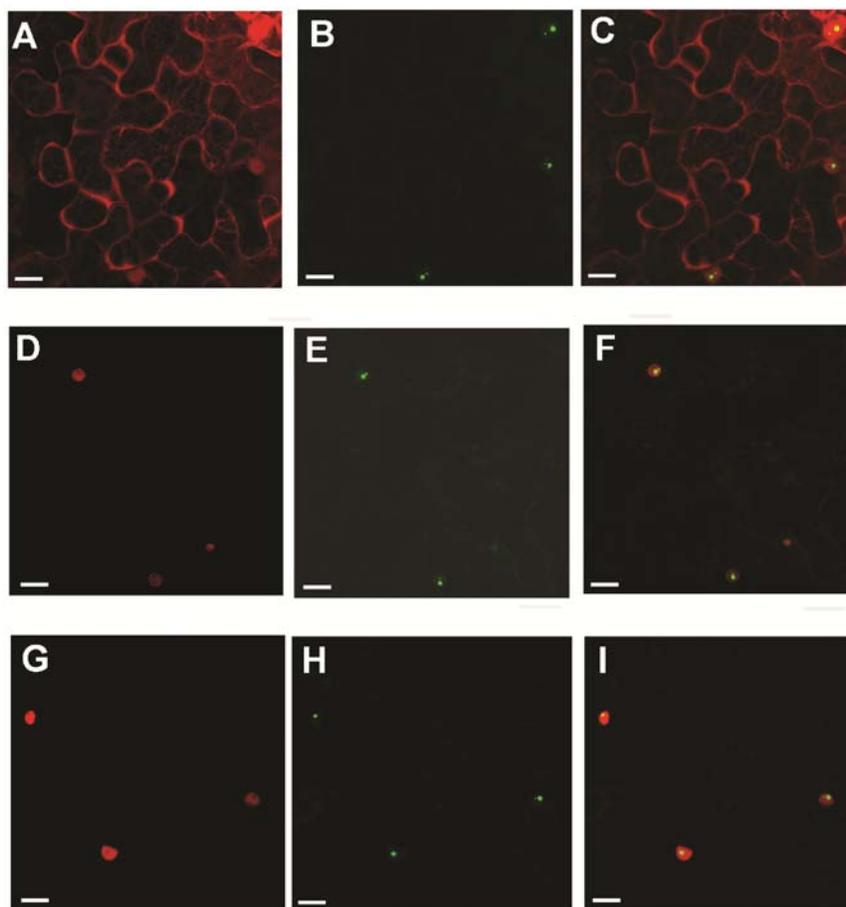
#### ***PcQNE* variants belong to a large gene family.**

The identification of six nonredundant sequences among the initially sequenced clones led us to hypothesize that *PcQNE*

variants belong to a large multigene family. Based on this hypothesis, we sequenced an additional 56 cDNA clones isolated in our original cloning of *PcQNE*. In total, 62 *PcQNE* clones were sequenced. From this, we determined that 47 highly related yet divergent sequences with at least one nonsynonymous nucleotide change were present (Supplementary Data 5). Among the 47 variants, 39 were represented as single copies, whereas the remaining eight variants were present in approximately two to four copies. Although proofreading *Pfu* Turbo DNA polymerase (Stratagene) was used for amplification to minimize changes through PCR, it is possible that some variants were simply mutated PCR products. Following the method described by Cline and associates (1996), the percentage of mutated PCR products was calculated as 6.8% based on the error rate ( $1.3 \times 10^{-6}$ ) of *Pfu* Turbo DNA polymerase, the length (approximately 1.5 Kb) of *PcQNE* genes, and number of template doublings (35, assuming all PCR cycles are effective in amplification). As a result, four to five of 62 *PcQNE* clones could contain mutations generated through PCR error. Nevertheless, there were still 42 divergent sequences present in the *P. cubensis* genome, suggesting that *PcQNE* variants belong to a large gene family.

#### **The *PcQNE* family is under diversifying selection.**

To identify the selection pressure driving sequence diversification in the *PcQNE* family, we calculated the rates of nonsynonymous ( $d_N$ ) and synonymous ( $d_S$ ) changes across the entire ORF of the 47 sequences. In all, 84% (909/1,081) pairwise comparisons were found to have greater  $d_N$  than  $d_S$  ( $\omega = d_N/d_S > 1$ )



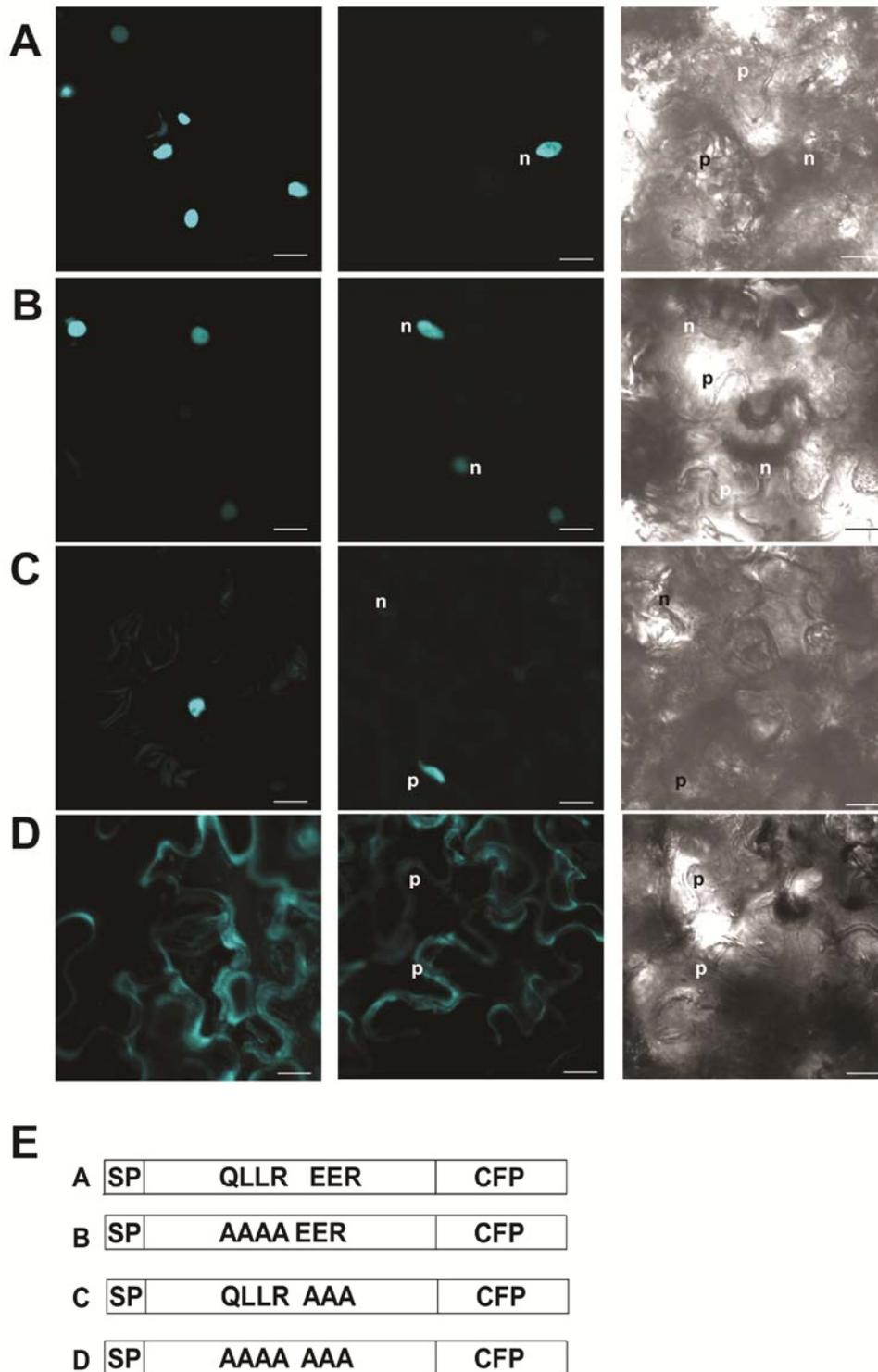
**Fig. 4.** Laser-scanning confocal micrographs showing fluorescence of leaf cells coexpressing **A, B, and C**, DsRed and AtFib1-green fluorescent protein (GFP); **D, E, and F**, DsRed-V1 and AtFib1-GFP; or **G, H, and I**, DsRed-V6 and AtFib1-GFP by transient agroinfiltration in *Nicotiana benthamiana*. The red channel shows localization of **A**, DsRed alone; **D**, DsRed-V1; or **G**, DsRed-V6. The green channel shows localization of **B, E, and H**, AtFib1-GFP. Overlays of micrographs from **A and B, D and E, and G and H**, are shown in **C, F, and I**, respectively. Bars = 20  $\mu$ m.

(Fig. 7A; Supplementary Data 6), suggesting that the *PcQNE* family is experiencing active diversifying selection. Win and associates (2007) showed that positive selection usually targets to the C-terminal domain of oomycete effectors. To determine whether this is applicable to the *PcQNE* family, we partitioned the sequences into N-terminal regions (from the start codon to QXLR-EER sequences) and C-terminal regions. For the C-terminal regions, we found that  $d_N$  was greater than  $d_S$  in approxi-

mately 90.2% (975/1,081) of comparisons (Fig. 7B). In sharp contrast, only 20.6% (223/1,081) of comparisons gave higher  $d_N$  than  $d_S$  in the N-terminal regions (Fig. 7C).

#### The *PcQNE* family is upregulated upon infection.

We used quantitative reverse-transcription (RT)-PCR to examine the expression of *PcQNE* genes during infection of cucumber by *P. cubensis*. Although the primers used were designed



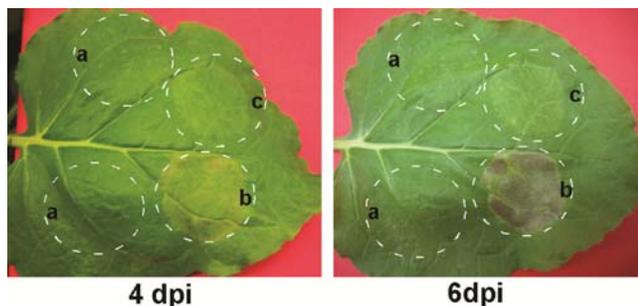
**Fig. 5.** **A** through **D**, Laser-scanning confocal micrographs showing subcellular localization of transiently expressed CFP-tagged *PcQNE\_V1* and mutants in *Nicotiana benthamiana* leaf cells and **E**, schematic representation of constructs. Images in the left panel are of fluorescence in untreated cells. The center and right panel show the fluorescence and differential interference contrast images of plasmolyzed cells, respectively; n, nucleus; p, plasma membrane separated from cell wall. Scale bars = 20  $\mu$ m.

based on the sequence of V1, it is not surprising that many other variants can be amplified as well due to their high similarity with each other. Compared with the expression level in isolated sporangia, the expression of *PcQNE* genes was induced after infection of plants and reached the highest level at 3 dpi (Fig. 8). This expression pattern is consistent with many functional effectors, which are upregulated upon infection, suggesting a role that *PcQNE* plays in facilitating pathogen infection and disease development.

## DISCUSSION

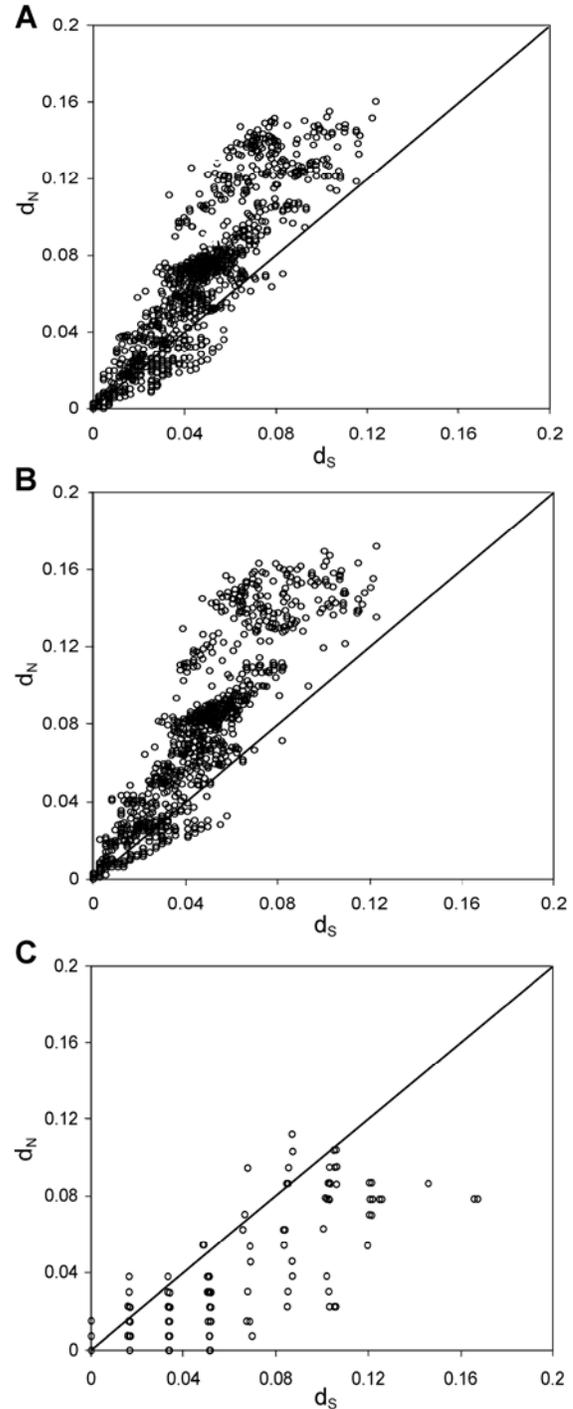
Despite the worldwide economic impact of cucurbit downy mildew, the molecular mechanisms underlying the *P. cubensis*-cucurbit interactions are unknown. The availability of the genome sequences from both pathogens and their hosts has greatly advanced our understanding of pathogen virulence and host resistance (Ebbole 2007; Quirino and Bent 2003). In 2009, Huang and associates (2009) and Ren and associates (2009) presented a genome sequence and cytogenetic blueprint, respectively, for cucumber (*Cucumis sativus*). However, a lag still exists in developing these resources for the obligate pathogen *P. cubensis*, due in large part to the difficulty in culturing and propagation of the pathogen. For example, the isolation of pure pathogen structures from infected hosts is undoubtedly more challenging than isolation from pure cultures grown on synthetic media. In the present study, we have established the protocols to propagate a pure *P. cubensis* isolate from a single sporangium, to collect highly pure sporangia from infected leaves, and to extract high-quality genomic DNA for 454 pyrosequencing. Although the sequence data we created from this study are still limited, the work presented herein represents the first description of a genomic resource for *P. cubensis* and a glimpse into its pathogenicity mechanisms.

Members of the *PcQNE* family possess a functional ER-type signal peptide, and two of the members tested are localized within the host nucleus following secretion and translocation. *PcQNE* proteins are under diversifying selection at the C-terminal domains, a common characteristic of oomycetes RXLR effectors (Liu et al. 2005; Oh et al. 2009; Win et al. 2007). They share similar primary sequence structure with a typical RXLR effector protein at the N terminus, including a signal peptide, Q/RXLR motif, followed by the conserved EER sequence. QXLR and EER are both conserved blocks present in all 47 *PcQNE* members (Fig. 2), suggesting that QXLR could be a translocation motif, like RXLR. Indeed, internalization of secreted *PcQNE\_V1* requires the QXLR-EER motif (Fig. 5). Other than RXLR, other translocation motifs, such as LXLFLAK, have been found in the CRINKLER (CRN) family of effectors of *Phytophthora* spp. and *H. arabidopsidis* (Haas



**Fig. 6.** *PcQNE* variants induce chlorosis and necrosis in *Nicotiana benthamiana*. *Agrobacterium tumefaciens* strains expressing DsRed alone (a), DsRed-V1 (b), or DsRed-V6 (c) were infiltrated into *N. benthamiana* leaves. Photos were taken at 4 and 6 days postinoculation (dpi).

et al. 2009; Win et al. 2007). The identification of additional translocation motifs undoubtedly not only will increase our understanding of the mechanisms of delivery of oomycete effectors but also may shed light into the virulence function of the effectors that do not have a canonical RXLR motif. Moreover, it will increase our awareness of the presence of cytoplasmic effectors other than the RXLR and CRN classes. Indeed, it seems that QXLR-containing secreted proteins are



**Fig. 7.** Rates of nonsynonymous ( $d_N$ ) and synonymous ( $d_S$ ) nucleotide substitutions of pairwise comparisons with 47 *PcQNE* sequences. **A**, Comparison with the entire open reading frame sequences. **B**, Comparison for the C-terminal regions. **C**, Comparison for the N-terminal regions. The diagonal line indicates  $d_N = d_S$ . Points above the line represent comparisons with  $\omega = d_N/d_S$ .

fairly common in the *P. cubensis* genome. The identification of members of the PcQNE family as bona fide effectors will certainly help to catalog a comprehensive list of effector proteins of *P. cubensis*.

Although the present study demonstrates that the QXLR motif found in *P. cubensis* is likely a functional translocation motif, previous analyses of the RXLR2 motif of *Phytophthora sojae* Avr1b showed that substitution of R to Q within the RXLR2 motif abolished the translocation of Avr1b to the host soybean cells (Dou et al. 2008; Kale et al. 2010). The simplest explanation for this apparent discrepancy is that differences in the sequences flanking the R/QXLR may alter translocation efficiency. Indeed, the surrounding sequences of RXLR motifs have been shown to be important for translocation (Dou et al. 2008). From a structure-to-function standpoint, one might also argue that mutation of R to Q within the RXLR2 motif of Avr1b could influence the native interactions of adjacent amino acids, thus altering protein fold dynamics. By extension, as a result, the mutated protein is unable to be recognized by the translocation machinery. Alternatively, the discrepancy could lie in the specificity of hosts. Recently, Kale and associates (2010) reported that the translocation of cytoplasmic effectors of oomycetes and fungi is a host-mediated process requiring RXLR (or variants)-enabled binding to the phospholipid on the host plasma membrane. Even if QXLR is not readily recognized by the phospholipids of soybean, it could well be functional in cucurbits.

Oomycete effectors often exist in multiple copies within a single genome (Dong et al. 2009; Qutob et al. 2009). For example, the *Avr1a* locus from some strains of *P. sojae* comprises a tandem array of four near-identical copies (Qutob et al. 2009). Likewise, the *P. sojae* effector *Avr3a* is present in as many as four copies, depending on the strain (Qutob et al. 2009). To determine the extent of duplication of RXLR effector proteins, Qutob and associates (2009) developed a method to estimate the copy number by counting trace file matches from the whole-genome shotgun sequence. Their study suggests that multiple, near-identical copies of RXLR effector genes are prevalent in oomycete genomes. The highest predicted copy number from *P. sojae* and *P. ramorum* is 54 for *P. sojae* *Avh426*. In the present study, we sequenced 62 PcQNE cDNA clones from a single sporangium-derived *Pseudoperonospora cubensis* isolate. Among these, there are approximately 42 highly similar yet distinct variants, suggesting that PcQNE belongs to a large gene family that is likely the target of the evolutionary forces driving the “arms race” between pathogen and host. In this regard, the massive multiplication led us to hypothesize that PcQNE proteins might play highly important roles in pathogen fitness and pathogenicity.

The biochemical activity of PcQNE is unclear. PcQNE proteins do not exhibit any similarity to sequences currently available in the public databases, which is consistent with many identified oomycete effector proteins. An extensive search for domains and motifs against various publicly available databases failed to identify any functional domain. As an attempt to predict, in silico, the biochemical function of PcQNE\_V1 by molecular modeling with the I-TASSER server, I-TASSER generated five possible three-dimensional (3-D) structural models (Supplementary Data 7) with a range of confidence scores (C-score). Although we failed to directly identify the biochemical function consistent with the nuclear localization, we used the predicted 3-D structures to address DNA-binding activity through utilization of the DNA Binding Protein Prediction Server. Interestingly, this analysis returned similar results for all five models, suggesting that, in fact, PcQNE proteins are likely DNA-binding proteins. Thus, one can speculate that PcQNE effectors likely manipulate host cellular processes

by altering host gene expression, supporting our finding that PcQNE\_V1 is nuclear localized in planta.

Although prediction by Predict NLS did not identify a putative NLS in PcQNE\_V1 protein sequence, this protein was experimentally determined to be localized in the nucleus. However, this apparent discrepancy is not contradictory. The database used for prediction is composed of experimentally known NLS and potential NLS generated through in silico mutagenesis (Cokol et al. 2000), and the list of NLS in the database is by no means complete. Thus, failure to identify a canonical NLS in a protein does not necessarily imply that there is not a functional NLS present. To this end, PcQNE\_V1 could be such an example of a protein with a novel, uncharacterized NLS. In support of this, a stretch of sequence (KRKRPEGTEIQFRWKESEIINLYTKHM) in PcQNE\_V1, which aligns very well with the putative NLS of PcQNE\_V6, with 4 amino acids divergent from PcQNE\_V6 (Fig. 2.), may represent a novel NLS absent in the Predict NLS database.

In total, the work presented herein represents the characterization of a family of secreted effector proteins from the cucurbit downy mildew pathogen *P. cubensis*. As a reference tool for further analyses of oomycete effector motifs, our data supports the idea that *P. cubensis*, compared with other agriculturally important oomycetes such as *Phytophthora sojae* and *P. infestans*, may utilize alternative translocation motifs (i.e., QXLR) in addition to the well-established RXLR. This study provides evidence supporting the need for the further characterization of additional translocation motifs from plant pathogens.

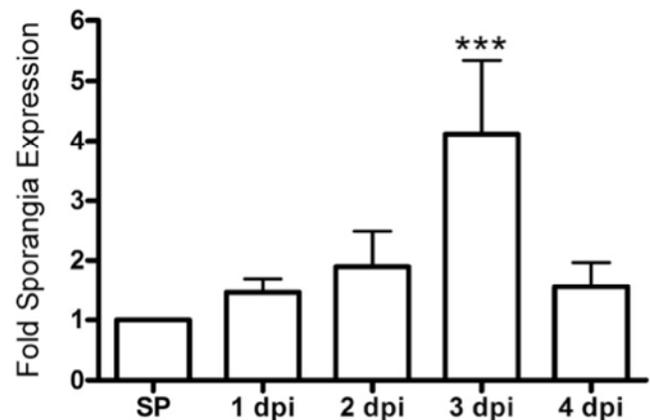
## MATERIALS AND METHODS

### Plants used and growth conditions.

Cucumber (*C. sativus*) ‘Vlasplik’ was used throughout the study and grown in a growth chamber with cycles of 25°C in light for 14 h and 20°C in darkness for 10 h. *N. benthamiana* plants were grown at 20°C under a cycle of 14 h of light and 10 h of darkness.

### Pathogen isolation, inoculation, and maintenance.

The *Pseudoperonospora cubensis* isolate used in this study was collected from infected cucumber leaves grown in Homerille, OH in 2007. Diseased leaves were incubated overnight in a moist container and washed with sterile water to collect sporan-



**Fig. 8.** Expression levels of PcQNE in sporangia (SP) and at 1, 2, 3 and 4 days postinoculation (dpi). Expression is displayed as fold sporangia expression. Error bars represent the standard error of the mean of two technical replicates from each of two biological replicates; \*\*\* indicates statistical significance compared with sporangia control ( $P < 0.001$ ) using two-way analysis of variance.

gia. Single sporangia were isolated by spreading the sporangia suspension on 1.5% water agar and cutting the agar into small pieces. The small pieces with single sporangia, observed under a microscope, were placed onto the detached cucumber leaf discs or whole leaves in a moist container for infection. At 5 to 7 dpi, propagated sporangia from the single sporangia-infected leaf tissues were collected. Subsequent pathogen propagation and maintenance were carried out by spraying a sporangia suspension ( $10^4$  sporangia/ml) on the leaves of intact plants. Inoculated plants were kept in a plastic tray with lids covered to maintain approximately 100% humidity, kept in darkness at ambient temperature for 24 h, and then moved to the growth chamber with the same settings for growing healthy cucumber plants described above. For examining the expression of *PcQNE* during the infection time course, 4-week-old plants were drop inoculated on the abaxial surface with  $10 \mu\text{l}$  of a  $1 \times 10^5$  sporangia/ml solution and then treated as described for spray inoculation.

#### Collection and purification of sporangia and isolation of genomic DNA and total RNA.

Sporangia were detached from infected leaves by submerging the leaves in sterile water and gently swirling for 5 min. Sporangia suspension was passed through eight layers of cheesecloth followed by passage through a 45- $\mu\text{m}$  nylon mesh. Sporangia were then collected by centrifugation at  $1,000 \times g$  for 5 min. The purity of sporangia was checked using light microscopy. Once a sample was confirmed as having a minimal amount (i.e., <2%) of plant tissue contamination, sporangia were processed for genomic DNA and total RNA isolation. DNA and RNA isolation from sporangia were performed with the DNeasy plant mini kit and RNeasy plant mini kit (Qiagen), respectively. Briefly, sporangia were resuspended in 400  $\mu\text{l}$  of buffer AP1 (for DNA extraction) or RLT (for RNA isolation) and then an equal volume of 525- to 600- $\mu\text{m}$  acid-washed glass beads (Sigma) was added. Suspended sporangia were disrupted by vigorously shaking for 12 s using a FastPrep FP120 beadbeater (ThermoSavant). DNA and RNA isolations were performed following the manufacturer's instructions.

#### 454 sequencing and assembly.

Sequencing of *P. cubensis* genomic DNA isolated from sporangia was performed using the 454 GS-FLX Titanium Sequencer (Roche) at the Michigan State University Research Technology Support Facility. The procedure was described previously (Quinn et al. 2008). The reads were screened for contaminated cucumber DNA using the available sequence data from the NCBI databases. After the identifiable cucumber sequences were removed, the remaining reads were assembled using the Roche gsAssembler (version 1.1.03).

#### Sequence analyses to identify putative RXLR and QXLR effector proteins.

Putative RXLR effector proteins were identified using the RXLR effector identification pipeline and the HMMsearch program using an HMM profile constructed based on 43 validated oomycete RXLR effectors, as described previously (Win et al. 2007). Putative QXLR effector proteins were identified using a modified pipeline from the RXLR effector identification pipeline. In brief, the search string in the PERL script was changed from RXLR to QXLR to modify the search for QXLR-containing secreted proteins.

#### Validation of the functionality of *PcQNE* signal peptides.

The functionality of predicted *PcQNE* signal peptides was validated using the yeast secretion system described by Jacobs and associates (1997). In brief, SP1 and SP2 were fused to the invertase gene *SUC2* lacking both its initiating methionine

codon and its signal peptide, by cloning the PCR-amplified fragments into the *EcoRI* and *XhoI* sites of the yeast signal sequence trap vector pSUC2T7M13ORI. The same forward primer (5'-GCGGAATTCatgatgccccctgcgaaactcg-3') was used for amplifying both signal peptides. The reverse primers 5'-GCGCTCGAGcgcctcagatcgcgctagaacgatagacgaagca-3' and 5'-GCGCTCGAGcgcctcagatcgcgctagaacga-3' were used for SP1 and SP2, respectively (gene-specific sequence is in lowercase and the introduced restriction sites are underlined). The plasmid constructs, named pSUC2-SP1 and pSUC2-SP2, together with the original vector, were transferred into the yeast *SUC2*-minus strain YTK12 using the Frozen-EZ Yeast Transformation IITM kit (Zymo Research, Orange, CA, U.S.A.) following the manufacturer's instructions. Transformants were selected by growing on CMD-W plates (0.67% yeast nitrogen base without amino acids, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar) for 3 days. To test whether SP1 and SP2 were functional by yeast growth assays, single colonies of YTK12 with or without expressing various plasmids were streaked onto raffinose-containing YPRAA plates (1% yeast extract, 2% peptone, 2% raffinose, Antimycin A at 2  $\mu\text{g}/\text{liter}$ , and 2% agar) (Jacobs et al. 1997). Yeast strains were replicated onto YPDA plates (1% yeast, 2% peptone, 2% glucose, 0.003% adenine hemisulfate, and 2% agar) and CMD-W plates as equal viability controls. The detection of the secreted invertase activity with TTC was performed as described by Oh and associates (2009).

#### 3' RACE-PCR, RT-PCR, DNA sequencing, and sequence analyses.

Total RNA isolated from *P. cubensis* sporangia was treated with DNA-Free (Ambion, Austin, TX, U.S.A.) to remove residual genomic DNA. Synthesis of 3'-RACE-Ready cDNA and 3' RACE-PCR were performed with total RNA using the Smart RACE cDNA amplification kit (Clontech, Mountain View, CA, U.S.A.) following the instructions of the manufacturer. A gene-specific primer was designed based on the partial sequence obtained from 454 sequencing. The resultant RACE-PCR product was cloned into the PCR2.1 TOPO vector (Invitrogen, San Diego, CA, U.S.A.) and sequenced with an ABI 3730 Genetic Analyzer. For amplification of *PcQNE* cDNA sequences encoding the ORF, 3'-RACE-Ready cDNA was used as template. PCR with gene-specific primers (forward, 5'-atgatgccccctgcgaaactcg-3'; reverse, 5'-tcattgtggagacggcaggagttc-3') was carried out for 35 cycles using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). The amplicons were cloned and sequenced as described above. Sequences were aligned and checked against chromatograms using Sequencher 4.7 (Gene Codes, Ann Arbor, MI, U.S.A.). Signal peptides were predicted by SignalP 3.0. Putative NLS were identified by PredictNLS. Multiple alignments were generated with ClustalX version 1.8.

#### Transient expression

##### in *N. benthamiana* and subcellular localization.

The plasmids for expressing mature proteins of *PcQNE\_V1* and *PcQNE\_V6* fused with DsRed at the N-terminus were constructed by cloning PCR-amplified fragments encoding V1 and V6 mature proteins into *BglIII* and *HindIII* sites of pGDR (Goodin et al. 2002). The two primers used were forward: 5'-gcgagatctAGATATGAGGCGTCTACTGATATC-3' and reverse: 5'-gccaagcttTCATGTGGAGACGGCAGGAGTTC-3' (the gene-specific sequence is in uppercase and the introduced restriction sites are underlined). For observing internalization of secreted *PcQNE\_V1* and its mutants with QXLR or EER substituted for alanine (A), C-terminal CFP fusion expression constructs were made as follows. Full-length V1 was first cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen) using gene-specific primers (forward: 5'-caccATGATGCCCC

CTGCGAA-3' and reverse: 5'-TGTGGAGACGGCAGGAGT TC-3') to make *PcQNE\_V1\_GW*. To make V1 mutants, site-directed mutagenesis was done via quick-change PCR using *PcQNE\_V1\_GW* as a template. The change from QLLR to AAAA to generate *PcQNE\_V1\_AAAA\_GW* was done in two steps: i) the QLLR motif was altered to ALAR using the primers forward: 5'-GACACGAAGGCACTCGCGGACTTCTG AC-3' and reverse: 5'-GTCAGAAAGTCCGCGGAGTGCCTT CGTGTC-3') and ii) the ALAR was further altered to AAAA using the primers forward: 5'-GACACGAAGGCAGCCGCGGCGACTTCTGAC-3' and reverse: 5'-GTCAGAAAGTCCGCGG GGCTGCCTTCGTGTC-3'. To change the EER motif to AAA generating *PcQNE\_V1\_AAA*, the primer set forward: 5'-AA GATAACGCAGCGGCATGTTCAAC-3' and reverse: 5'-GTTGAACATCGCGCTCGCTTATCCTT-3' was used. To make the mutant with both the QLLR and EER motifs converted to alanines, the *PcQNE\_V1\_AAAA\_GW* construct was used as a template for site-directed mutagenesis with the primers described to change the EER motif to AAA. For all primers, the underlined bases indicate mutations. Quick-change PCR was performed for 18 cycles (95°C, 1 min; 55°C, 3 min; and 68°C, 5 min) on a BioRad MyCycler thermal cycler (BioRad Laboratories, Hercules, CA, U.S.A.) using *Pfu* Turbo DNA polymerase (Clontech). Resultant products were treated with *DpnI* overnight at 37°C and then transformed into *Escherichia coli* DH5 $\alpha$  cells. Clones were sequenced to verify mutagenesis. C-terminal CFP fusion expression constructs were further generated from the above entry clones to the destination vector pVKH18En6gw-cCFP using recombination via LR Clonase per the manufacturer's instructions (Invitrogen).

Construction of the pVKH18En6gw-cCFP vector was performed as follows. The Gateway Reading Frame Cassette was amplified using *Pfu* Ultra II HS fusion (Stratagene) with a forward primer that introduced a unique *XbaI* site and a reverse primer that introduced a unique *XhoI* site. The amplified product was verified by agarose gel electrophoresis and purified using a MicroSpin S-400 HR column (GE Healthcare, Piscataway, NJ, U.S.A.). The purified product was digested with *XbaI* and *XhoI* and ligated into an *XbaI*- and *SalI*-restricted pVKH18En6 binary plasmid containing the Cerulean CFP gene (Batoko et al. 2000; Rizzo et al. 2004).

For transient expression in *N. benthamiana*, the expression constructs described above were transformed into *A. tumefaciens* C58-C1. Confocal microscopy was performed as described previously (Goodin et al. 2002; Tian et al. 2009) using the Olympus Fluoview. For plasmolysis analysis, cells were treated using 0.8 M mannitol for 10 to 15 min prior to imaging (Rafiqi et al. 2010).

### Diversifying selection analyses.

Diversifying selection analyses were performed as described by Oh and associates (2009). The rates of nonsynonymous nucleotide substitutions per site ( $d_N$ ) and the rates of synonymous nucleotide substitutions per site ( $d_S$ ) in all pairwise comparisons were estimated using the approximate methods of Yang and Nielsen (2000) and Nei and Gojobori (1986) implemented in the YN00 program in the PAML4.2a software package (Yang 2007).

### Quantitative RT-PCR.

Total RNA was extracted from *P. cubensis*-infected cucumber leaves at 1, 2, 3, and 4 dpi using the PrepEase plant RNA spin kit (USB, Cleveland, OH, U.S.A.) per the manufacturer's instructions. First-strand cDNA was synthesized from 1  $\mu$ g total RNA using the First-strand cDNA synthesis kit (USB). Quantitative RT-PCR was performed on a Mastercycler ep Realplex real-time PCR (Eppendorf, Hamburg, Germany) using HotStart

SYBR Green qPCR Master Mix (2 $\times$ ) (USB). For amplification of *PcQNE* transcripts, gene-specific forward (5'-TCCGACAT GACACGAACGA $\Delta$ ACTCT-3') and reverse (5'-AGTGAGACA TCGTAGATGCGCGTT-3') primers were used. Primers specific for the *P. cubensis* internal transcribed spacer region (forward: 5'-CGGGGGTTTTGTTGGCGGT-3' and reverse: 5'-CGAAGCCCAAACGCTCGCCA-3') were used as an endogenous control. Cycle time used was 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. Fold expression was calculated based on expression in sporangia and data were analyzed by two-way analysis of variance using Prism 4 (GraphPad Software, La Jolla, CA, U.S.A.).

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## LITERATURE CITED

- Alfano, J. R., and Collmer, A. 2004. Type III secretion system effector proteins: Double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* 42:385-414.
- Armstrong, M. R., Whisson, S. C., Pritchard, L., Bos, J. I., Venter, E., Avrova, A. O., Rehmany, A. P., Bohme, U., Brooks, K., Cherevach, I., Hamlin, N., White, B., Fraser, A., Lord, A., Quail, M. A., Churcher, C., Hall, N., Berriman, M., Huang, S., Kamoun, S., Beynon, J. L., and Birch, P. R. 2005. An ancestral oomycete locus contains late blight avirulence gene *Avr3a*, encoding a protein that is recognized in the host cytoplasm. *Proc. Natl. Acad. Sci. U.S.A.* 102:7766-7771.
- Arnold, R., Brandmaier, S., Kleine, F., Tischler, P., Heinz, E., Behrens, S., Niinikoski, A., Mewes, H. W., Horn, M., and Rattei, T. 2009. Sequence-based prediction of type III secreted proteins. *PLoS Pathog.* 5:e1000376. Published online.
- Batoko, H., Zheng, H.Q., Hawes, C., and Moore, I. 2000. A rab1 GTPase is required for transport between the endoplasmic reticulum and Golgi apparatus and for normal Golgi movement in plants. *Plant Cell* 12:2201-2218.
- Bhattacharjee, S., Hiller, N. L., Liolios, K., Win, J., Kanneganti, T. D., Young, C., Kamoun, S., and Haldar, K. 2006. The malarial host-targeting signal is conserved in the Irish potato famine pathogen. *PLoS Pathog.* 2:e50. Published online.
- Birch, P. R., Boevink, P. C., Gilroy, E. M., Hein, I., Pritchard, L., and Whisson, S. C. 2008. Oomycete RXLR effectors: Delivery, functional redundancy and durable disease resistance. *Curr. Opin. Plant Biol.* 11:373-379.
- Birch, P. R., Armstrong, M., Bos, J., Boevink, P., Gilroy, E. M., Taylor, R. M., Wawra, S., Pritchard, L., Conti, L., Ewan, R., Whisson, S. C., van West, P., Sadanandom, A., and Kamoun, S. 2009. Towards understanding the virulence functions of RXLR effectors of the oomycete plant pathogen *Phytophthora infestans*. *J. Exp. Bot.* 60:1133-1140.
- Buttner, D., and He, S. Y. 2009. Type III Protein secretion in plant pathogenic bacteria. *Plant Physiol.* 150:1656-1664.
- Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. 2006. Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* 124:803-814.
- Cline, J., Braman, J. C., and Hogrefe, H. H. 1996. PCR fidelity of *pfu* DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res.* 24:3546-3551.
- Cokol, M., Nair, R., and Rost, B. 2000. Finding nuclear localization signals. *EMBO (Eur. Mol. Biol. Organ.) Rep.* 1:411-415.
- Davis, E. L., Hussey, R. S., Mitchum, M. G., and Baum, T. J. 2008. Parasitism proteins in nematode-plant interactions. *Curr. Opin. Plant Biol.* 11:360-366.
- Dodds, P. N., Rafiqi, M., Gan, P. H., Hardham, A. R., Jones, D. A., and Ellis, J. G. 2009. Effectors of biotrophic fungi and oomycetes: Pathogenicity factors and triggers of host resistance. *New Phytol.* 183:993-1000.
- Dong, S., Qutob, D., Tedman-Jones, J., Kuflu, K., Wang, Y., Tyler, B. M., and Gijzen, M. 2009. The *Phytophthora sojae* avirulence locus *Avr3c*

- encodes a multi-copy RXLR effector with sequence polymorphisms among pathogen strains. *PLoS One* 4:e5556. Published online.
- Dou, D., Kale, S. D., Wang, X., Jiang, R. H., Bruce, N. A., Arredondo, F. D., Zhang, X., and Tyler, B. M. 2008. RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* 20:1930-1947.
- Ebbole, D. J. 2007. *Magnaporthe* as a model for understanding host-pathogen interactions. *Annu. Rev. Phytopathol.* 45:437-456.
- Goodin, M. M., Dietzgen, R. G., Schichnes, D., Ruzin, S., and Jackson, A. O. 2002. pGD vectors: Versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. *Plant J.* 31:375-383.
- Grouffaud, S., van West, P., Avrova, A. O., Birch, P. R., and Whisson, S. C. 2008. *Plasmodium falciparum* and *Hyaloperonospora parasitica* effector translocation motifs are functional in *Phytophthora infestans*. *Microbiology* 154:3743-3751.
- Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H., Handsaker, R. E., Cano, L. M., Grabherr, M., Kodira, C. D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T. O., Ah-Fong, A. M., Alvarado, L., Anderson, V. L., Armstrong, M.R., Avrova, A., Baxter, L., Beynon, J., Boevink, P. C., Bollmann, S. R., Bos, J. I., Bulone, V., Cai, G., Cakir, C., Carrington, J. C., Chawner, M., Conti, L., Costanzo, S., Ewan, R., Fahlgren, N., Fischbach, M. A., Fugelstad, J., Gilroy, E. M., Gnerre, S., Green, P. J., Grenville-Briggs, L. J., Griffith, J., Grünwald, N. J., Horn, K., Horner, N. R., Hu, C. H., Huitema, E., Jeong, D. H., Jones, A. M., Jones, J. D., Jones, R. W., Karlsson, E. K., Kunjeti, S. G., Lamour, K., Liu, Z., Ma, L., Maclean, D., Chibucos, M. C., McDonald, H., McWalters, J., Meijer, H. J., Morgan, W., Morris, P. F., Munro, C. A., O'Neill, K., Ospina-Giraldo, M., Pinzón, A., Pritchard, L., Ramsahoye, B., Ren, Q., Restrepo, S., Roy, S., Sadanandom, A., Savidor, A., Schornack, S., Schwartz, D. C., Schumann, U. D., Schwessinger, B., Seyer, L., Sharpe, T., Silvar, C., Song, J., Studholme, D. J., Sykes, S., Thines, M., van de Vondervoort, P. J., Phuntumart, V., Wawra, S., Weide, R., Win, J., Young, C., Zhou, S., Fry, W., Meyers, B. C., van West, P., Ristaino, J., Govers, F., Birch, P. R., Whisson, S. C., Judelson, H. S., and Nussbaum, C.. 2009. Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* 461:393-398.
- Hogenhout, S. A., Van der Hoorn, R. A., Terauchi, R., and Kamoun, S. 2009. Emerging concepts in effector biology of plant-associated organisms. *Mol. Plant-Microbe Interact.* 22:115-122.
- Holmes, G., Wehner, T., and Thornton, A. 2006. An old enemy re-emerges. *Am. Veg. Grow.* February:14-15.
- Huang, S., Li, R., Zhang, Z., Li, L., Gu, X., Fan, W., Lucas, W. J., Wang, X., Xie, B., Ni, P., Ren, Y., Zhu, H., Li, J., Lin, K., Jin, W., Fei, Z., Li, G., Staub, J., Kilian, A., van der Vossen, E. A., Wu, Y., Guo, J., He, J., Jia, Z., Ren, Y., Tian, G., Lu, Y., Ruan, J., Qian, W., Wang, M., Huang, Q., Li, B., Xuan, Z., Cao, J., Asan, Wu, Z., Zhang, J., Cai, Q., Bai, Y., Zhao, B., Han, Y., Li, Y., Li, X., Wang, S., Shi, Q., Liu, S., Cho, W. K., Kim, J. Y., Xu, Y., Heller-Uszynska, K., Miao, H., Cheng, Z., Zhang, S., Wu, J., Yang, Y., Kang, H., Li, M., Liang, H., Ren, X., Shi, Z., Wen, M., Jian, M., Yang, H., Zhang, G., Yang, Z., Chen, R., Liu, S., Li, J., Ma, L., Liu, H., Zhou, Y., Zhao, J., Fang, X., Li, G., Fang, L., Li, Y., Liu, D., Zheng, H., Zhang, Y., Qin, N., Li, Z., Yang, G., Yang, S., Bolund, L., Kristiansen, K., Zheng, H., Li, S., Zhang, X., Yang, H., Wang, J., Sun, R., Zhang, B., Jiang, S., Wang, J., Du, Y., and Li, S. 2009. The genome of the cucumber, *Cucumis sativus* L. *Nat. Genet.* 41:1275-1281.
- Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., Kriz, R., LaVallie, E. R., Merberg, D., Spaulding, V., Stover, J., Williamson, M. J., and McCoy, J. M. 1997. A genetic selection for isolating cDNAs encoding secreted proteins. *Gene* 198:289-296.
- Jiang, R. H., Tripathy, S., Govers, F., and Tyler, B. M. 2008. RXLR effector reservoir in two *Phytophthora* species is dominated by a single rapidly evolving superfamily with more than 700 members. *Proc. Natl. Acad. Sci. U.S.A.* 105:4874-4879.
- Jones, J. D., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.
- Kale, S. D., Gu, B., Capelluto, D. G., Dou, D., Feldman, E., Rumore, A., Arredondo, F. D., Hanlon, R., Fudal, I., Rouxel, T., Lawrence, C. B., Shan, W., and Tyler, B. M. 2010. External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell* 142:284-295.
- Kamoun, S. 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. *Annu. Rev. Phytopathol.* 44:41-60.
- Liu, Z., Bos, J. I., Armstrong, M., Whisson, S. C., da Cunha, L., Torto-Alalibo, T., Win, J., Avrova, A. O., Wright, F., Birch, P. R., and Kamoun, S. 2005. Patterns of diversifying selection in the phytotoxin-like scr74 gene family of *Phytophthora infestans*. *Mol. Biol. Evol.* 22:659-672.
- Morgan, W., and Kamoun, S. 2007. RXLR effectors of plant pathogenic oomycetes. *Curr. Opin. Microbiol.* 10:332-338.
- Nei, M., and Gojobori, T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* 3:418-426.
- Oh, S. K., Young, C., Lee, M., Oliva, R., Bozkurt, T. O., Cano, L. M., Win, J., Bos, J. I., Liu, H. Y., van Damme, M., Morgan, W., Choi, D., Van der Vossen, E. A., Vleeshouwers, V. G., and Kamoun, S. 2009. In planta expression screens of *Phytophthora infestans* RXLR effectors reveal diverse phenotypes, including activation of the *Solanum tuberosum* disease resistance protein Rpi-blb2. *Plant Cell* 21:2928-2947.
- Quinn, N. L., Levenkova, N., Chow, W., Bouffard, P., Borojevich, K. A., Knight, J. R., Jarvie, T. P., Lubieniecki, K. P., Desany, B. A., Koop, B. F., Harkins, T. T., and Davidson, W. S. 2008. Assessing the feasibility of GS FLX Pyrosequencing for sequencing the Atlantic salmon genome. *BMC Genomics* 9:404.
- Quirino, B. F., and Bent, A. F. 2003. Deciphering host resistance and pathogen virulence: The *Arabidopsis/Pseudomonas* interaction as a model. *Mol. Plant Pathol.* 4:517-530.
- Qutob, D., Tedman-Jones, J., Dong, S., Kufu, K., Pham, H., Wang, Y., Dou, D., Kale, S. D., Arredondo, F. D., Tyler, B. M., and Gijzen, M. 2009. Copy number variation and transcriptional polymorphisms of *Phytophthora sojae* RXLR effector genes Avr1a and Avr3a. *PLoS One* 4:e5066. Published online.
- Rafiqi, M., Gan, P. H., Ravensdale, M., Lawrence, G. J., Ellis, J. G., Jones, D. A., Hardham, A. R., and Dodds, P. N. 2010. Internalization of flax rust avirulence proteins into flax and tobacco cells can occur in the absence of the pathogen. *Plant Cell* 22:2017-2032.
- Rehmany, A. P., Gordon, A., Rose, L. E., Allen, R. L., Armstrong, M. R., Whisson, S. C., Kamoun, S., Tyler, B. M., Birch, P. R., and Beynon, J. L. 2005. Differential recognition of highly divergent downy mildew avirulence gene alleles by RPP1 resistance genes from two *Arabidopsis* lines. *Plant Cell* 17:1839-1850.
- Ren, Y., Zhang, Z., Liu, J., Staub, J. E., Han, Y., Cheng, Z., Li, X., Lu, J., Miao, H., Kang, H., Xie, B., Gu, X., Wang, X., Du, Y., Jin, W., and Huang, S. 2009. An integrated genetic and cytogenetic map of the cucumber genome. *PLoS One* 4:e5795. Published online.
- Rizzo M. A., Springer, G. H., Granada, B., and Piston, D. 2004. An improved cyan fluorescent protein variant useful for FRET. *Nat. Biotechnol.* 22:445-449.
- Samudrala, R., Heffron, F., and McDermott, J. E. 2009. Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type III secretion systems. *PLoS Pathog.* 5:e1000375. Published online.
- Savory, E. A., Granke, L. L., Quesada-Ocampo, L. M., Varbanova, M., Hausbeck, M., and Day, B. 2010. The cucurbit downy mildew pathogen *Pseudoperonospora cubensis*. *Mol. Plant Pathol.* 12:217-226.
- Shan, W., Cao, M., Leung, D., and Tyler, B. M. 2004. The Avr1b locus of *Phytophthora sojae* encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene Rps1b. *Mol. Plant-Microbe Interact.* 17:394-403.
- Tian, M., Chaudhry, F., Ruzicka, D. R., Meagher, R. B., Staiger, C. J., and Day, B. 2009. *Arabidopsis* actin-depolymerizing factor AtADF4 mediates defense signal transduction triggered by the *Pseudomonas syringae* effector AvrPphB. *Plant Physiol.* 150:815-824.
- Voglmayr, H., and Greilhuber, J. 1998. Genome size determination in *peronosporales* (Oomycota) by Feulgen image analysis. *Fungal Genet. Biol.* 25:181-195.
- Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I. K., Pritchard, L., and Birch, P. R. 2007. A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* 450:115-118.
- Win, J., Morgan, W., Bos, J., Krasileva, K. V., Cano, L. M., Chaparro-Garcia, A., Ammar, R., Staskawicz, B. J., and Kamoun, S. 2007. Adaptive evolution has targeted the C-terminal domain of the RXLR effectors of plant pathogenic oomycetes. *Plant Cell* 19:2349-2369.
- Yang, Z. 2007. PAML 4: Phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* 24:1586-1591.
- Yang, Z., and Nielsen, R. 2000. Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. *Mol. Biol. Evol.* 17:32-43.

## AUTHOR-RECOMMENDED INTERNET RESOURCES

PredictProtein database: [www.predictprotein.org](http://www.predictprotein.org)  
SignalP 3.0 server: [www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP)  
I-TASSER server: [zhanglab.cmb.med.umich.edu/I-TASSER](http://zhanglab.cmb.med.umich.edu/I-TASSER)  
DNA-binding protein prediction server:  
[www.enzim.hu/~szia/dnabind.html](http://www.enzim.hu/~szia/dnabind.html)