A *Phytophthora palmivora* Extracellular Cystatin-Like Protease Inhibitor Targets Papain to Contribute to Virulence on Papaya

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Papaya fruits, stems, and leaves are rich in papain, a cysteine protease that has been shown to mediate plant defense against pathogens and insects. Yet the oomycete *Phytophthora palmivora* is a destructive pathogen that infects all parts of papaya plants, suggesting that it has evolved cysteine protease inhibitors to inhibit papain to enable successful infection. Out of five putative extracellular cystatin-like cysteine protease inhibitors (Ppa-IEPICs) from *P. palmivora* transcriptomic sequence data, PpaIEPIC8 appeared to be unique to *P. palmivora* and was highly induced during infection of papaya. Purified recombinant PpaIEPIC8 strongly inhibited papain enzyme activity, suggesting that it is a functional cysteine protease inhibitor. Homozygous PpaIEPIC8 mutants were generated using CRISPR/Cas9-mediated gene editing via *Agrobacterium*-mediated transformation (AMT). Increased papain sensitivity of in-vitro growth and reduced pathogenicity during infection of papaya fruits were observed for the mutants compared with the wild-type strain, suggesting that PpaIEPIC8, indeed, plays a role in *P. palmivora* virulence by inhibiting papain. This study provided genetic evidence demonstrating that plant-pathogenic oomycetes secrete cystatins as important weapons to invade plants. It also established an effective gene-editing system for *P. palmivora* by the combined use of CRISPR/Cas9 and AMT, which is expected to be applicable to other oomycetes.

Oomycetes are a unique group of fungus-like eukaryotes that belong to phylum Oomycota within the kingdom Straminipila (Kamoun 2003; Margulis and Schwartz 2000). They include many destructive pathogens of plant and animals (Kamoun et al. 2015; Phillips et al. 2008). The genus *Phytophthora* contains more than 100 species, with many of them causing severe damage and huge losses to natural ecosystems and agricultural production (Erwin and Ribeiro 1996; Kamoun et al. 2015). Although knowledge is limited, it is widely accepted that oomycete plant pathogens secrete a large number of effector proteins into host tissues to either suppress host defense responses, subvert host metabolisms, or both to promote effective colonization of their hosts (Ali et al. 2017; Baxter et al. 2010; Haas et al. 2009; Jiang et al. 2008). These effectors are generally classified into apoplastic effectors and

cytoplasmic effectors, depending on whether they act in the extracellular space or function inside the host cells (Kamoun 2006, 2007). So far, only a limited number of these effectors have been characterized using genetic approaches. Due to inherent characteristics of oomycetes, such as being diploid (or polyploid) and many being heterothallic (Judelson 1997; Lamour et al. 2007; Sansome 1977), and the difficulties in manipulation of the sexual stage (Lamour et al. 2007), genetic identification of oomycete virulence genes had been very challenging until the recent emergence of CRISPR/Cas9-mediated gene-editing technology (Fang and Tyler 2016). As CRISPR/Cas9 gene editing can generate homozygous mutations in diploid (polyploid) species as early as the first generation of transformants (Pan et al. 2016; Wang et al. 2016), this technology is especially desirable for oomycete studies for which obtaining homozygous mutants by sexual reproduction is either impossible or difficult (Lamour et al. 2007). Recently, Fang and Tyler (2016) adapted CRISPR/Cas9 technology for use in *Phytophthora sojae*. By expressing all components required for gene editing in *P. sojae* using PEG (polyethylene glycol)/CaCl₂-mediated protoplast transformation, *P. sojae* genes were successfully mutated and homozygous mutants were obtained (Fang and Tyler 2016). The adapted *P. sojae* gene-editing system is expected to be applicable to other oomycetes and thus accelerate the dissection of the pathogenicity mechanisms of these extremely destructive pathogens.

Phytophthora palmivora is an oomycete plant pathogen with a very wide range of hosts, including many economically important hosts such as papaya, cacao, rubber tree, and oil palm (Ekchaweng et al. 2017; Erwin and Ribeiro 1996; Nelson 2008; Thevenin et al. 2012; Torres et al. 2016). It is omnipresent in the tropics and propagates very rapidly in wet weather conditions, causing destructive diseases (Drenth and Guest 2016). On papaya, it infects all plant parts, causing root rot, foot rot, stem canker, fruit rot, leaf blight, and seedling damping off (Nelson 2008).

Accumulating evidence suggests that various plant pathogens promote colonization by secreting protease inhibitors to target plant defense-related proteases. Two families of apoplastic effectors of the oomycete pathogen *Phytophthora infestans*, including extracellular Kazal-like serine protease inhibitors EPI1 and EPI10 and cystatin-like cysteine protease inhibitors EPIC1 and EPIC2B, were found to target host immune proteases. Both EPI1 and EPI10 target tomato pathogenesis-related subtilase P69B (Tian et al. 2004, 2005). EPIC1 and EPIC2B target host papain-like cysteine proteases RCR3 and C14, both of which are integral components of plant immunity (Kaschani et al. 2010; Shabab et al. 2008; Song et al. 2009). EPIC2B also targets another plant immune cysteine protease PIP1 (Tian et al. 2007). Interestingly, RCR3, C14, and PIP1 are also targets of protease inhibitors

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from plant pathogens of other taxonomic groups. AVR2 effector of the tomato fungal pathogen *Cladosporium fulvum* targets PIP1 and RCR3 (Rooney et al. 2005; Shabab et al. 2008). The bacterium *Pseudomonas syringae* pv. tomato DC3000 virulence factor Cip1, which contains motifs of the chagasin-like protease inhibitors, inhibits C14, PIP1, and, also, RCR3 with a lower affinity (Shindo et al. 2016). The fungal pathogen *Ustilago maydis* apoplastic effector Pit2, which is required for compatibility on maize, functions as an inhibitor of several maize apoplastic cysteine proteases that are directly linked with salicylic acid-associated immunity (Mueller et al. 2013). The importance of apoplastic protease inhibitors in pathogen virulence is also supported by an oomycete effector evolution study, in which *Phytophthora mirabilis* PmEPIC1, an ortholog of *P. infestans* EPIC1, was found to adapt to its host protease target MRP2 after *P. mirabilis* split from its sister species *P. infestans* and jumped to its host four o'clock (*Mirabilis jalapa*) (Dong et al. 2014).

It is well-known that papaya bark, leaves, and fruits, especially green fruits, are rich in papain, a cysteine protease with strong antifungal and anti-insect activities (Konno et al. 2004; Souza et al. 2011). Yet *P. palmivora* is a destructive pathogen of papaya that is capable to infect all plant parts (Nelson 2008), prompting us to think that *P. palmivora* is likely armed with cysteine protease inhibitors to inhibit papain enzyme activities in order to protect itself from the harsh protease-rich environment. Of five putative extracellular cystatin-like cysteine protease inhibitors (PpalEPICs) from *P. palmivora* transcriptomic sequence data, we identified one, named PpalEPIC8, to be unique to *P. palmivora* and highly induced during infection of papaya. PpalEPIC8 was shown to be a functional cystatin that strongly inhibited papain enzyme activity. To identify the role of PpalEPIC8 in pathogen virulence, PpalEPIC8 mutant strains were generated using CRISPR/Cas9-mediated gene editing via *Agrobacterium*-mediated transformation (AMT). Increased papain sensitivity of in-vitro growth and reduced

pathogenicity during infection of papaya fruits were observed for PpalEPIC8 mutants compared with the wild-type (WT) strain, suggesting that PpalEPIC8 plays a role in promoting *P. palmivora* colonization of papaya by inhibiting papain. This study not only presents the biochemical and genetic characterization of PpalEPIC8 but, also, an easy and effective CRISPR/Cas9-based genetic modification system for *P. palmivora* and possibly for other oomycetes.

RESULTS

Identification of putative PpalEPICs from *P. palmivora*.

From transcriptome sequences generated from *P. palmivora* during infection of *Nicotiana benthamiana* (Evangelisti et al. 2017), five PpalEPICs, homologs of EPICs from other *Phytophthora* species, were identified. These include PLTG_01864|PEX_0002, PLTG_02101|PEX_1552, PLTG_02360.2|m.2441, PLTG_07851|PEX_1251, and PLTG_09209|PEX_1797 (Evangelisti et al. 2017). The length of PpalEPICs ranged from 112 to 155 amino acids (Fig. 1). They were predicted to have a signal peptide, using SignalP 4.1 (Petersen et al. 2011). They all possessed the signature sequences that define cystatin-like cysteine protease inhibitors, including the conserved Gly (G) residue in the N-terminal trunk, the highly conserved QxVxG (Q, Gln; V, Val; x, any amino acid) motif in the first binding loop and the conserved Trp (W) in the second binding loop (Fig. 1) (Nagata et al. 2000; Turk and Bode 1991).

To assess the phylogenetic relationships of PpalEPICs with EPICs from other *Phytophthora* species, a maximum likelihood phylogenetic tree was constructed using amino-acid sequences of five PpalEPICs and 19 previously characterized or annotated EPICs from *P. infestans*, *P. sojae*, *P. ramorum*, *P. mirabilis*, *P. ipomoeae*, and *P. phaseoli* (Dong et al. 2014; Tian et al. 2007) (Supplementary Fig. S1). PLTG_09209|PEX_1797 was clustered with *P. infestans* EPIC4, *P. sojae* EPIC4 (PsEPIC4), and

Fig. 1. Sequence alignment of five *Phytophthora palmivora* extracellular cystatin-like cysteine extracellular protease inhibitors (PpalEPICs) with *P. infestans* EPIC1 and EPIC2B. EPIC1 and EPIC2B were described previously (Tian et al. 2007). CHKCYs, chicken egg white cystatin (UniProtKB: P01038), a type member of cystatin-like cysteine protease inhibitors. The predicted signal peptides are shown in boxes. The putative active sites of cystatins, including the N-terminal trunk (NT) and binding loops 1 and 2 (L1 and L2), are shown. The conserved amino acids that define cystatins are marked with asterisks.

P. ramorum EPIC4 (PrEPIC4) and was designated as PpalEPIC4. PLTG_02360.2|m.2441 was grouped with *P. ramorum* EPIC5 (PrEPIC5) and *P. sojae* EPIC5 (PsEPIC5) and was designated as PpalEPIC5. PLTG_07851|PEX_1251 and PLTG_02101|PEX_1552 were closely related to *P. sojae* EPIC6 (PsEPIC6) and *P. ramorum* EPIC7 (PrEPIC7), respectively, and therefore, were named PpalEPIC6 and PpalEPIC7. Very interestingly, PLTG_01864|PEX_0002 appeared to be unique to *P. palmivora* and was not closely related to previously characterized or named *Phytophthora*

EPICs and was designated as PpalEPIC8. The distant relationship of PpalEPIC8 with EPICs from other *Phytophthora* species may suggest that PpalEPIC8 has evolved and adapted to protease targets unique to the host plants of *P. palmivora*.

PpalEPIC8 is upregulated during infection of papaya.

To investigate the potential roles of PpalEPICs in plant infection, we determined the expression patterns of PpalEPICs during infection of papaya leaves and in mycelium grown in vitro by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). All five *PpalEPIC* genes were expressed during papaya leaf colonization and in-vitro growth (Fig. 2). *PpalEPIC8* was strongly upregulated during infection, in comparison to mycelium grown in vitro, and induced by 8.6- and 4.3-fold at 1 and 2 days postinoculation (dpi), respectively (Fig. 2). The expression of *PpalEPIC4* was slightly induced at 2 dpi, with an expression at 1.8-fold relative to the expression in mycelium. In contrast, the expression of *PpalEPIC5* and *PpalEPIC7* genes was downregulated during infection. These results suggest that *PpalEPIC8* may play a role in *P. palmivora* colonization of papaya. Because of its uniqueness among other *PpalEPICs*, *PpalEPIC8* was subjected to further detailed characterization.

PpalEPIC8 is a functional protease inhibitor that inhibits papain enzymatic activity.

To test whether PpalEPIC8 encodes a functional cystatin-like cysteine protease inhibitor, we cloned the DNA fragment corresponding to the mature sequence of PpalEPIC8 to pHIS-ATS, a vector that allows secreted expression of His-tagged protein in *Escherichia coli* (Tian and Kamoun 2017), to express N-terminal His-tagged fusion protein. We sequenced multiple recombinant plasmids and identified two versions of *PpalEPIC8*, *PpalEPIC8A* and *PpalEPIC8B*, which may represent two different alleles at the same locus on homologous chromosomes or paralogs

Fig. 2. Expression patterns of *PpalEPIC* genes during *Phytophthora palmivora* infection of papaya leaves and in-vitro growth determined by reverse transcription quantitative polymerase chain reaction. Expression levels at 1 and 2 days postinoculation (dpi) were normalized, using *P. palmivora* β -tubulin gene, and relative to mycelium grown in vitro with the expression level in mycelium calculated as 1. The error bars represent standard deviations from three technical replicates. The experiment was repeated twice with similar results.

PpalEPIC8A	ATGGCTTTCACCTCGCTCCGTCGCTCTTCTTGCTGGCTTGGCGCTAGCTTCCTCCTCTGTT
PpalEPIC8B	-----
	M A F T R S V A L L A G L A L A S S S V
PpalEPIC8A	CAGGGTCAAACGGCTCTCGGTAGACAAACAGTTCTCGGCGGCTACACACAGAAGAACGCC
PpalEPIC8B	-----T-----
	Q G Q T A L G R Q T V L G G Y T Q K N A
	*
PpalEPIC8A	ACCAGCGAAGACATTGCAGTTCTCACACAGGCAGCAGGCAACGCTAGCGCCTACAACGAG
PpalEPIC8B	---A-----
	T S E D I A V L T Q A A G N A S A Y N E
	3' -GGACTAACGATAACTCCTGGAGG-5' (sgRNA target)
PpalEPIC8A	GAAGTTACCGCGAGAATCTGCCTGATTGCTATTGAGGACCTCCAGACGCAGACTGTAGCT
PpalEPIC8B	-----
	E V T A R I C L I A I E D L Q T Q T V A
	* *
PpalEPIC8A	GGCACCAACTACAAGTTTCAAGTGGCAGGCTGCGTCGTGGAGTCGGACGACGAGCTGGGT
PpalEPIC8B	-----
	G T N Y K F Q V A G C V V E S D D E L G
	*
PpalEPIC8A	GCCTGTGATGACCGCAACTGCGACTATTCCAGCTACGACGTTGTGATCTACTCTCAGCCA
PpalEPIC8B	-----A-----
	A C D D R N C D Y S S Y D V V I Y S Q P
	I
PpalEPIC8A	TGGACTGACACGCTCGAAGTGACGTCCATCACCTTGGCGGATTAA
PpalEPIC8B	-----
	W T D T L E V T S I T L A D
	*

Fig. 3. The nucleotide and amino-acid sequences of PpalEPIC8A and PpalEPIC8B with the single guide RNA (sgRNA) target sequence used for CRISPR/Cas9-mediated gene editing. The dashed lines represent the same nucleotides in *PpalEPIC8B* as in *PpalEPIC8A*. The single nucleotide polymorphisms (SNPs) are shown with the SNP leading to valine (V) to isoleucine (I) substitution in the box. The sgRNA target sequence together with its downstream protospacer adjacent motif, AGG, is shown. The conserved residues that define cystatins are marked with asterisks.

at different loci. There were four single nucleotide polymorphisms (SNPs) between them, with only one SNP (G to A substitution) at the 340th nucleotide of the open reading frame leading to a valine (V) to isoleucine (I) residue change (Fig. 3). To include a negative control for testing their papain inhibition activity, we generated a PpalEPIC8A mutant (PpalEPIC8Am) with the conserved Q, V, and G amino acids in the first binding loop (Fig. 1) mutated to alanine (A). His-tagged recombinant PpalEPIC8A, PpalEPIC8B, and PpalEPIC8Am were expressed and purified by affinity chromatography. The purified proteins appeared as a single band on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Fig. 4A), indicating their high purity.

Using papain isolated from papaya latex and casein as a substrate, we tested whether recombinant PpalEPIC8 (rPpalEPIC8) proteins inhibit the enzymatic activities of papain. Cleavage of casein substrate by papain released short peptides that stayed in the soluble fractions after trichloroacetic acid (TCA) precipitation of the reaction mixtures. Therefore, the absorbance of the soluble fractions at 280 nm was measured as papain enzyme activity. Both rPpalEPIC8A and rPpalEPIC8B inhibited papain enzyme activity in a dose-dependent manner with more than 90% reduction of enzyme activity at a concentration of 150 nM or higher (Fig. 5B). In contrast, rPpalEPIC8Am did not exhibit clear inhibition even at 200 nM. These results suggest that PpalEPIC8 is a functional inhibitor that strongly inhibits the cysteine protease papain.

Generation of PpalEPIC8 mutant strains by CRISPR/Cas9 gene editing.

To determine whether PpalEPIC8 contributes to pathogen virulence using a genetic approach, we adopted CRISPR/Cas9-mediated gene-editing technology to generate PpalEPIC8 mutants. Recently, Fang and colleagues (Fang and Tyler 2016; Fang et al. 2017) developed a CRISPR/Cas9-mediated gene-editing system to edit genes in the *P. sojae* genome, using PEG/CaCl₂-mediated protoplast transformation. As AMT has many advantages over PEG/CaCl₂-mediated protoplast transformation, such as easy handling and mainly generating low-copy number integration events in the genome, we recently developed a simple and efficient AMT system for *P. palmivora* (Wu et al. 2016). To generate PpalEPIC8 mutants using CRISPR/

Cas9 via AMT, we constructed an “all-in-one” plasmid, pCB301TOR-CRISPR (Fig. 5A), which contains all elements necessary for CRISPR/Cas9-mediated gene editing via AMT (Fig. 5A). For generating the plasmid to edit a gene of interest, the exact procedures described by Fang et al. (2017) can be followed.

To generate mutants with both variants of *PpalEPIC8* mutated, we selected a 20-nt single guide RNA (sgRNA) target sequence (5'-GGAGGTCCTCAATAGCAATC-3') that matches 100% to the complementary strand of both *Ppal-EPIC8A* and *Ppal-EPIC8B* in the central region of the protein-encoding sequences (Fig. 3). This target sequence together with the HH ribozyme sequence (Fang et al. 2017) was cloned to pCB301TOR-CRISPR upstream of the sgRNA scaffold to generate the construct pCB301TOR-CRISPR-PpalEPIC8, which was used to transform *P. palmivora* via AMT. A total of 33 G418-resistant transformants were obtained. We isolated single zoospore-derived transformants from 16 initial transformants, with two to three single zoospore-derived lines for each. *PpalEPIC8* DNA fragments were amplified by PCR from single zoospore-derived lines and were sequenced, using primers that amplify both variants. Five single zoospore lines T1-3, T4-2, T11-1, T11-2, and T15-1 originating from four initial transformants T1, T4, T11, and T15 were shown to contain homozygous mutations (Fig. 5B). No mixed peaks in the sequence chromatograms were observed in the regions surrounding the sgRNA target site (Fig. 5B), while mixed peaks were observed for the polymorphic sites of *PpalEPIC8A* and *PpalEPIC8B* (Supplementary Fig. S2), demonstrating that both *PpalEPIC8A* and *PpalEPIC8B* were mutated and the mutations were homozygous. All mutations were deletions and occurred near the Cas9 cleavage site (Fig. 5C), which is usually 3 nt upstream of NGG, the protospacer adjacent motif required for the cleavage. T1-3 and T15-1 had the same 8-bp deletion leading to frame shift and a premature stop codon at 3 bp after the deletion site (Fig. 5C). T4-2 had a 4-bp deletion leading to frame shift and a premature stop codon at 27 bp after the deletion site (Fig. 5C). T11-1 and T11-2 had a 6-bp deletion leading to an in-frame deletion with a loss of two amino acids (Fig. 5C). These mutations caused either the complete loss of the QxVxG motif in the first binding loop or the loss of two residues that were 6 amino acids preceding the QxVxG motif (Fig. 5C). As QxVxG were shown to be the residues in cystatins that directly interact with the targeted cysteine protease (Kaschani and

Fig. 4. PpalEPIC8 inhibits papain enzymatic activity. **A**, Purified His-tagged rPpalEPIC8A (1), rPpalEPIC8B (2), and rPpalEPIC8Am (3) visualized on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained with Coomassie brilliant blue. Lane M represents protein markers, with the numbers on the left showing the molecular weight. **B**, Papain enzymatic activity assays were conducted with papain at 400 nM and casein as a substrate in the absence (0 nM) or the presence of rPpalEPIC8A (dashed line, diamond), rPpalEPIC8B (circle), and rPpalEPIC8Am (triangle) at indicated concentrations, measured as absorbance at 280 nm. The error bars represent standard deviations calculated from three replicates. The experiment was repeated three times with similar results.

Van der Hoorn 2011), the functionality of PpalEPIC8 was very likely to be compromised in these mutants.

***PpalEPIC8* mutants exhibit reduced virulence on papaya fruits and are more sensitive to papain.**

To test whether PpalEPIC8 contributes to *P. palmivora* virulence, we did papaya infection assays with WT *P. palmivora* and *PpalEPIC8*-edited mutants T1-3, T4-2, T11-1, and T15-1.

Mature green Sunrise papaya fruits were used for the assays as the fruits at this stage are rich in papain and yet successful infection can be consistently achieved in almost all inoculated sites. Although fruits of similar size and mature level were selected for inoculation, a high level of lesion size variation was observed from fruit to fruit and at different locations of the same fruit, especially between the longitudinal locations. To better compare the virulence between WT and mutants, the

Fig. 5. Generation of *PpalEPIC8* mutants via CRISPR/Cas9 gene editing. **A**, Schematic representation of pCB301TOR-CRISPR. Three expression cassettes, including PsNLS-hSpCas9 under the control of the HAM34 promoter (HAM34_p) and HAM34 terminator (HAM34_t), NPTII under the control of the HSP70 promoter (HSP70_p) and HSP70 terminator (HSP70_t), and the whole single guide RNA (sgRNA) expression cassette from pYF2.3G-Ribo-sgRNA (Fang et al. 2017), which contains the RPL41 promoter (RPL41_p), restriction sites *NheI* and *BsaI*, the sgRNA scaffold, HDV ribozyme sequence, and HSP70 terminator, are shown within the T-DNA left border (LB) and right border (RB) of pCB301 (Genbank AF139061.1). **B**, Chromatograms showing the mutations of *PpalEPIC8* in the mutant lines indicated. **C**, *PpalEPIC8* nucleotide mutations and the resultant amino-acid changes in the mutant lines. The sgRNA target sequence is underlined. The nucleotides corresponding to the protospacer adjacent motif are in red. The putative Cas9 cleavage site is shown by an arrow. The dashed lines indicate deleted nucleotides. The premature stop codons are shown in dots. The conserved residues of QxVxG motif in the first binding loop are marked with asterisks.

mutants were inoculated with WT side-by-side and the pairwise lesion diameter ratios were calculated. In general, all mutants produced smaller lesions than WT (Fig. 6A). The average lesion diameter ratios (mutant/WT) were 0.696, 0.593, 0.779, and 0.822 for T15-1, T1-3, T4-2, and T11-1, respectively (Fig. 6B). For T1-3 and T11-1, at all inoculated sites, the mutants produced smaller lesions than WT (Fig. 6B). For T15-1 and T4-2, at most of the inoculated sites, the mutants produced smaller lesions than WT, although opposite results were observed at a small percentage of inoculated sites (Fig. 6B). The *P* values from paired *t* test analyses using pairs (mutant/WT) of lesion diameter data were much less than 0.01 (Fig. 6B), suggesting that the lesion sizes of WT and mutants were significantly different. Overall, the data indicated that the mutations of *PpaIEPIC8* reduced *P. palmivora* virulence.

To investigate whether the reduced virulence of *PpaIEPIC8* mutants is due to increased sensitivity to papain as a result of compromised PpaIEPIC8, we did in-vitro growth assays of WT and mutant strains in liquid Plich media with or without papain. This assay was initially performed in wells of 96-well plates and the mycelium growth was observed visually. The presence of 50 and 100 µg per milliliter of papain in the media reduced the mycelium growth of both WT and the mutant strains (Fig. 7A), suggesting the damaging effect of papain on *P. palmivora*. The level of growth inhibition by papain to the mutant strains was visually higher than to WT, as evidenced by the mycelial clumps of the mutants compared with the continuous hyphal

growth of WT in the presence of papain (Fig. 7A). To quantify inhibition, the strains were grown in 10 ml of liquid Plich media in the absence or presence of 75 µg of papain per milliliter in 60-mm petri dishes for 1 week, and the mycelium dry weight was measured. The quantitative results also demonstrated that the level of inhibition by papain to the mutant strains was higher than to WT. Both assays demonstrated that *PpaIEPIC8* mutants were more sensitive to papain inhibition than WT (Fig. 7B), suggesting the role of PpaIEPIC8 in protecting *P. palmivora* from the damaging effects of papain by inhibiting its enzymatic activity.

DISCUSSION

Multiple studies suggest that oomycete pathogens utilize apoplastic protease inhibitors as an important counter-defense mechanism based on their interaction with and inhibition of plant defense-related proteases (Ekchaweng et al. 2017; Song et al. 2009; Tian et al. 2004, 2005, 2007), however, direct genetic evidence was lacking due to the difficulty in genetic modification of oomycetes. This study took advantage of the newly developed CRISPR/Cas9-mediated gene-editing technology and successfully generated *PpaIEPIC8* homozygous mutants, which exhibited reduced pathogen virulence and were more sensitive to papain compared with the WT strain. These results indicated that *PpaIEPIC8* plays a role in protecting the pathogen and facilitating infection by combating the plant defense mediated by abundant papain in papaya. As such, this study provides genetic evidence

Fig. 6. Infection assays of papaya fruits by the *Phytophthora palmivora* wild-type (WT) strain and *PpaIEPIC8* mutants. **A**, Symptoms of representative fruits infected by WT and mutant strains at 4 days postinoculation. **B**, Box plots of pairwise lesion diameter ratios (mutant/WT) and *P* values for paired *t* test analyses of side-by-side lesion diameter data. Data from three independent experiments with nine fruits for each mutant were analyzed. The medians of pairwise lesion diameter ratios are shown in thick lines within the boxes, and the dots represent the averages.

demonstrating that oomycete pathogens secrete apoplastic protease inhibitors as important weapons to invade plants.

Although PpalEPIC8 mutants exhibited reduced virulence and were more sensitive to papain inhibition compared with WT, they were able to infect papaya and grow in papain-containing media reasonably well. This is not surprising as four other PpalEPICs were also expressed during infection of papaya and PpalEPIC4 mutants were induced during infection, although the induction level was low (Fig. 2). It is very likely that other PpalEPICs play redundant roles in papain inhibition. Further biochemical characterization and simultaneous mutation of multiple PpalEPICs by gene editing would confirm this.

Compared with oomycete gene editing via PEG/CaCl₂-mediated transformation and other transformation methods, gene editing via AMT is more desirable, as the construct for gene editing is large and AMT introduces low-copy number integrations (Vijn and Govers 2003; Wu et al. 2016). In contrast, other transformation methods often generate multicopy integrations in the genome (Ah-Fong et al. 2008). There is a high possibility that the random insertions of many copies of the gene-editing construct might disrupt the functions of other genes and, therefore, complicate the inference of the function of the gene under study. In addition, the procedure of AMT is easy and cost effective (Wu et al. 2016). The establishment of CRISPR/Cas9 gene-editing system via AMT for *P. palmivora* will generate profound impact on understanding and control of this destructive pathogen. As *P. palmivora* is tetraploid and heterothallic (Ali et al. 2017; Ko 1988), genetic studies used to be extremely challenging. The CRISPR/cas9 gene-editing system can be considered as a game changer, as it allows the generation of homozygous mutants in a short period of time without sexual reproduction. With the recent release of the genome and transcriptome sequences of *P. palmivora* (Ali et al. 2017; Evangelisti et al. 2017), this gene-editing system is expected to greatly speed up dissecting its pathogenesis mechanisms.

In summary, we functionally characterized PpalEPIC8 from *P. palmivora*, using both biochemical and genetic approaches. PpalEPIC8 was shown to contribute to virulence on papaya by protecting the pathogen in papain-rich harsh conditions, providing additional evidence demonstrating that apoplastic protease inhibitors serve as a common counter-defense mechanism of plant pathogens. The genetic modification system via the combined use of CRISPR/Cas9 and AMT established in this study will greatly facilitate functional genomic studies of not only *P. palmivora* but, also, other oomycetes.

MATERIALS AND METHODS

P. palmivora strain and plant materials.

P. palmivora papaya isolate P1 (Wu et al. 2016) was used throughout this study and routinely cultured on 10% unclarified V8 agar under a 12-h light and 12-h dark cycle at room temperature (around 22°C). Sunrise papaya plants were grown under greenhouse conditions at 28°C with a 14-h light and 10-h dark cycle for 6 weeks before infection for gene expression analyses. For infection assays with papaya fruits, mature green Sunrise papaya fruits were harvested in the Poamoho Research Station, University of Hawaii at Manoa.

Sequence and phylogenetic analyses.

Four predicted peptides, PLTG_01864|PEX_0002, PLTG_02101|PEX_1552, PLTG_07851|PEX_1251, and PLTG_09209|PEX_1797, were previously annotated as putative EPICs in the secretome of *P. palmivora* during infection of *N. benthamiana* (Evangelisti et al. 2017). To identify any additional PpalEPICs from this transcriptome sequence dataset (Evangelisti et al. 2017), we used the amino-acid sequences of these four

PpalEPICs as the query to perform a TBLASTN search against *P. palmivora* transcriptome (Evangelisti et al. 2017) and retrieved all the sequences with an E-value lower than 1e-10. The amino-acid sequences of all retrieved sequences were subjected to signal peptide prediction, using SignalP4.0 (Petersen et al. 2011), and a functional domain search, using InterProScan (Jones et al. 2014), to detect the presence of a putative signal peptide and a cystatin-like domain. In addition to the above mentioned four peptides, one additional peptide, PLTG_02360.2|m.2441 (Evangelisti et al. 2017), was also identified as a putative PpalEPIC.

Phylogenetic analyses of *Phytophthora* EPICs were performed as described previously (Condon et al. 2013). The amino-acid sequences of five putative PpalEPICs were aligned with 19 previously characterized or annotated EPICs from other *Phytophthora* species (Dong et al. 2014; Tian et al. 2007) and chicken egg-white cystatin (UniProtKB: P01038), using ClustalX 2.1 (Larkin et al. 2007). The alignment was used to determine the best-fit model to be used for phylogenetic tree construction with ProtTest (Abascal et al. 2005). The WAG model was determined as the best-fit model. The phylogenetic tree was inferred using CIPRES Science Gateway V. 3.3 (Miller et al. 2010). RAxML-HPC BlackBox (8.2.9) was used to infer the tree, using maximum likelihood and rapid bootstrapping with 1,000 bootstrap replicates. WAG was selected as the

Fig. 7. Papain inhibition of in-vitro growth of the *Phytophthora palmivora* wild-type (WT) strain and *PpalEPIC8* mutants. **A**, The growth of WT and mutants in liquid Plich media in the absence (0 µg/ml) or the presence of papain (50 or 100 µg/ml), performed in 96-well plates. **B**, Percentage of growth inhibition by 75 µg of papain per milliliter compared with the growth in the absence of papain. The assays were conducted using 60-mm petri dishes and mycelium dry weights were measured. Error bars represent standard deviations of three replicates. The asterisks (*) indicate statistically significant differences ($P < 0.05$) between the mutants and WT, determined by one-tailed *t* test. Experiments were repeated three times with similar results.

protein substitution matrix model and chicken egg-white cystatin as an outgroup. The tree was viewed by opening the generated RAxML_bipartitions.result file using FigTree v1.4.3.

Gene expression analyses of PpalEPICs.

Leaves of 6-week-old Sunrise papaya plants were inoculated with droplets of 10 µl of a 5×10^4 zoospore/ml suspension. Plants were kept in a translucent high humidity box. Leaf disks of similar sizes from the inoculated regions were sampled each day for 2 dpi. Noninoculated papaya leaves were sampled for Day 0 as a negative control. The disks were rapidly frozen in liquid nitrogen and were stored, at -80°C , for later use in RNA extraction. For total RNA isolation, the frozen leaf discs were ground to fine powder in liquid nitrogen using mortars and pestles. Tissue powder (100 mg) was used for RNA isolation using Qiagen RNeasy plant mini kit. Contaminating genomic DNA was removed using a DNA-free kit (Ambion). Synthesis of first-strand cDNAs from 1 µg of total RNA was performed using Invitrogen SuperScript II reverse transcription according to the manufacturer's instructions. Primers for qPCR were designed to yield a product of 90 to 150 bp and are listed in Table 1. The primers for *PpalEPIC8* amplify both variants. Constitutively expressed *P. palmivora* β -tubulin gene was used as an internal control to normalize the expression of *PpalEPIC* genes. qPCR reactions were set up using SsoAdvanced Universal SYBR green supermix (Bio-Rad), following the manufacturer's instructions. The reactions were run with CFX96 Touch real-time PCR detection system. The fold change of expression during infection relative to in vitro-grown mycelium was calculated using the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak 2008).

Expression and purification of recombinant PpalEPIC8.

E. coli BL21 strains transformed with the plasmids pHis-PpalEPIC8A, pHis-PpalEPIC8B, and pHis-PalEPIC8Am were used to express His-tagged proteins. pHis-PpalEPIC8A and PpalEPIC8B were constructed by cloning the PCR-amplified DNA fragment corresponding to the mature sequence of PpalEPIC8 into the *HindIII* and *KpnI* sites of pHis-ATS, a vector that allows secreted expression of His-tagged proteins in *E. coli* and was modified from pFLAG-ATS (Sigma) (Tian and Kamoun 2017). The oligonucleotides PpalEPIC8-FHindIII (5'-gcgaagctt CAAACGATCGTTGGAACCTGGAC-3') and PpalEPIC8-RKpnI (5'-gcggtaccTCAATCTCCGTTTGAGCTGTGTTG-3') were used for PCR. The introduced restriction sites are underlined and the gene-specific sequences are in upper case. Sanger sequencing of the resultant plasmids was performed to confirm the sequence of the insert. pHis-PpalEPIC8Am was generated by site-directed mutagenesis of pHis-PpalEPIC8A following the protocols described by Zheng et al. (2004). Two oligonucleotides pHis-PalEPIC8A-mutantF (5'-GACGGCGACTGCAGCTGCCACCAACTACA AGTTTCAAGTG-3') and pHis-PalEPIC8A-mutantR (5'-

TGGTGGCAGCTGCAGTCGCCGTCTGGAGGTCCTCAATAG-3') were used to mutate Q, V, and G amino acids to A. The codons encoding A are underlined.

The expression of His-tagged PpalEPIC8 proteins was conducted as described previously (Tian et al. 2004). The *E. coli* culture supernatant was collected by centrifugation at $6,000 \times g$ for 15 min and were further filtered with a polyvinylidene difluoride Durapore syringe-driven filter unit (Millipore). Stock solutions of 5 M NaCl and 1 M imidazole were slowly added to the culture filtrate to reach a final concentration of 500 mM NaCl and 5 mM imidazole. The solution was loaded into a column packed with 1 ml of the Complete His-Tag purification resin (Roche) that was pre-equilibrated with wash buffer (25 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM imidazole, 10% glycerol). After the resin was washed with 20 ml of wash buffer, the bound proteins were eluted multiple times with 1 ml of elution buffers (25 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol, and different concentrations of 50 to 250 mM imidazole). The 1-ml fractions were collected and were analyzed on 15% SDS-PAGE, followed by Coomassie brilliant blue staining to determine the presence and purity of the protein. The fractions containing the purified protein were combined and were concentrated to 2.5 ml, using Amicon ultra-15 centrifugal filters (Millipore), were desalted with a PD-10 column (GE Healthcare) using protein storage buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol), and were further concentrated using Amicon ultra-15 centrifugal filters. The concentration of the purified proteins was measured using the Bradford protein assay kit (Bio-Rad).

Assays of papain enzyme activity and inhibition by His-PpalEPIC8.

Papain (Sigma P4762) derived from papaya latex was dissolved in sterile MilliQ water to make a 10-mg/ml stock solution and was stored at 4°C . Papain enzyme activity assay was modified from a plant proteolytic analytical method (FCC-PU) (Enzyme Development Corporation, New York). A 0.05-mg/ml papain solution was prepared on the day of assay by diluting the stock solution with freshly made phosphate-cysteine-EDTA solution (50 mM Na_2HPO_4 , 50 mM L-cysteine, 40 mM EDTA, pH 6.0). For papain enzyme activity and inhibition assays, 160 µl of papain enzyme (0.05 mg/ml) was preincubated with various amounts of His-PpalEPIC8 proteins in a total volume of 200 µl, at room temperature, for 30 min. After that, 0.5 ml of casein substrate (10 mg/ml) was added and the hydrolysis reactions were conducted at room temperature for 1 h. The final concentration of papain in the reactions was 400 nM. To stop the reactions and precipitate the unhydrolyzed casein substrate and other proteins, 300 µl of 30% TCA was then added. The tubes were vortexed vigorously and were incubated in a 42°C water bath for 30 min, followed by centrifugation at $16,000 \times g$ for 10 min to completely pellet the precipitated proteins while leaving the hydrolyzed small peptides in the supernatant. The papain enzyme activities were measured as the absorbance of the supernatant at 280 nm. A higher absorbance indicates a higher degree of papain enzyme activity. As a blank, the highest amount of His-PpalEPIC8 used for the inhibition assay was preincubated with papain, followed by the addition of 30% TCA before the addition of casein substrate, and the following procedures were performed as described above.

Construction of an "all-in-one" plasmid for CRISPR/Cas9-mediated gene editing in oomycetes via AMT.

PsNLS-hSpCas9 (Fang and Tyler 2016) was cloned to pCB301TOR, a binary vector previously constructed for oomycete transformation via AMT (Wu et al. 2016), using *Clai* and *EcoRI* restriction sites. The PsNLS-hSpCas9 was amplified

Table 1. Primers used to determine the expression patterns of putative cystatin-like extracellular protease inhibitor genes (*PpalEPICs*) by reverse transcription-quantitative polymerase chain reaction

Genes	Primers
<i>PpalEPIC4</i>	F: 5'-CACTATCGGCCTGTGAACCT-3' R: 5'-GCTGCTGGTCTGAGACTCCT-3'
<i>PpalEPIC5</i>	F: 5'-CAGAAACAGAACCCGTCGAT-3' R: 5'-ACATGGTAGCGGAAGTGGAT-3'
<i>PpalEPIC6</i>	F: 5'-CGAGGAAGCTACGAAGATGG-3' R: 5'-GTCCTCGTTGTACGGGAAGA-3'
<i>PpalEPIC7</i>	F: 5'-GAACCGCGAATGTACCAAT-3' R: 5'-ATCTCCGTTTGAGCTGTGTT-3'
<i>PpalEPIC8</i>	F: 5'-AGCTGGGTGCCTGTGATGA-3' R: 5'-ACGTCACCTTCGAGCGTGTCA-3'
<i>Phytophthora palmivora</i> β -tubulin	F: 5'-GTGGATCCCCAACAAACATCA-3' R: 5'-ACATCTCCTGGATGGCAGT-3'

by PCR, using the plasmid pYF2-PsNLS-hSpCas9 (Fang and Tyler 2016) as template. The primers used are hSpCas9-FClal (5'-gcatgcatATGCACAAGCGCAAGCGCGAGGA-3') and hSpCas9-REcoRI (5'-gcggaattcTTAGTCGCCTCCAGCTGAGAC-3'). The restriction sites are underlined and the gene-specific sequences are in upper case. The generated plasmid was designated as pCB301TOR-hSpCas9. The entire sgRNA expression cassette in the plasmid pYF2.3G-Ribo-sgRNA (Fang et al. 2017), including RPL41 promoter, the sgRNA target cloning sites *NheI* and *BsaI*, the sgRNA scaffold, the HDV ribozyme sequence, and the HSP70 terminator, was cloned to pCB301TOR-hSpCas9 via the *KpnI* site, which is located between HSP70 terminator of the NPTII gene expression cassette and the T-DNA left border (LB). The sgRNA expression cassette was amplified with primers RPL41p-FKpnI (5'-gctgggtaccGTTCCGTCATTTCTCTCGCAGCAAC-3') and Thsp70-RKpnI (5'-gcgggtaccAAGACAATAGGCCAGACTC-3'). The restriction sites are underlined and the specific sequences are in upper case. The orientation of the sgRNA cassette was determined by sequencing the resultant plasmids, using primer T-DNA_LB-R1 (5'-tggctgctgtatcgatgg-3'), which is located within the T-DNA LB. Thus, a plasmid containing all elements necessary for CRISPR/cas9-mediated gene editing and suitable for oomycete AMT was generated and designated as pCB301TOR-CRISPER (Fig. 5A).

sgRNA design and cloning.

sgRNA design and cloning were performed essentially as described by Fang and Tyler (2016). sgRNA target sites of *PpalePIC8* were identified using the eukaryotic pathogen CRISPR guide RNA design tool (Peng and Tarleton 2015). The whole genome sequence of a *P. palmivora* strain isolated from cacao (Ali et al. 2017) was used as the custom genome to identify potential off-targets. Both *PpalePIC8A* and *PpalePIC8B* sequences were used to select target sites. Candidate target sites located near or upstream of the central region of the *PpalePIC8* encoding sequence and matching 100% to both variants were chosen for further analysis of RNA secondary structures, using the RNAstructure web server (Reuter and Mathews 2010). The target sites with the least-severe secondary structures (with three or fewer hydrogen bonds) were chosen. The target sequence C223R (5'-GGAGGTCCTCAATAGCAATC-3'), which is on the reverse complementary strand and immediately upstream 5'-AGG-3' (Fig. 3), was selected for this study. C223R matches the encoding sequence of both variants of *PpalePIC8* and its potential off-targets were not found in the *P. palmivora* genome. Cloning of the 20-nt sgRNA target sequence together with the flanking HH-ribozyme sequence to the *NheI* and *BsaI* sites of pCB301TOR-CRISPER was performed as previously described (Fang et al. 2017). Two oligos, *PpalePIC8*-Crisper_F1 (5'-ctagcACCTCCCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCGGAGGCTCTCAATAGCAATC-3') and *PpalePIC8*-Crisper_R1 (5'-aacGATTGCTATTGAGGACCTCCGACGAGCTTACTCGTTTCGTCTCACGGACTCATCAGGGAGGTg-3') were annealed to assemble the insert. The specific target sequence is underlined. The HH ribozyme sequence is shaded, with the first six nucleotides, which are the reverse complement of the first six nucleotides of the target sequence, shown in bold italic. The nucleotides necessary for the cloning are in lower case. The resultant plasmid pCB301TOR-CRISPR-PpalePIC8 was transformed into *Agrobacterium tumefaciens* EHA105 using electroporation (Mersereau et al. 1990).

Transformation of *P. palmivora* and identification of *PpalePIC8* mutants.

P. palmivora P1 (Wu et al. 2016), a papaya isolate, was transformed with *A. tumefaciens* EHA105 containing pCB301TOR-CRISPR-PpalePIC8, following the method described previously

(Wu et al. 2016). The initial G418-resistant transformants were purified by generating single zoospore-derived transformants as described previously (Ho and Ko 1997). Single zoospore-derived transformants were grown on 10% V8 agar plates supplemented with 30 µg of G418 per milliliter, under 12 h of light and 12 h of dark at room temperature for 5 to 7 days. Mycelia and the sporangia mixture were scraped from the culture plates with a 200-µl pipette tip and were suspended in 500 µl of MilliQ water in a 1.5-ml microcentrifuge tube. The suspension was boiled for 5 min at 100°C and was centrifuged at 16,000 × g for 1 min. The supernatant was transferred to a new 1.5-ml microcentrifuge tube and was used as the templates for PCR to confirm the integration of PsNLS-hSpCas9 and the sgRNA expression cassette in the genome and to detect mutations in *PpalePIC8*. Primers HspCas9-FClal and HspCas9-REcoRI were used to confirm the presence of PsNLS-hSpCas9. Primers RPL4/Pro-SeqF1 (5'-GAACATCGTTCGGGGTAAGTG-3') and sgRNA-scaffold-R (5'-TGTCCCATTGCGCATGCCGAAG-3') were used to confirm the presence of the sgRNA expression cassette. Primers *PpalePIC8*-FBamHI (5'-gctgggtaccCAAACGGCTCTCGGTAGACAAACAG-3') and *PpalePIC8*-RSall (5'-gctgctgacTTAATCCGCCAAGGTGATGGACGT-3') were used to amplify the *PpalePIC8* gene from the WT and transformants, using Phusion high-fidelity DNA polymerase (New England Biolabs). The PCR products were gel-purified using QiaQuick gel extraction kit (Qiagen) and were sequenced using the same primers.

Virulence assays of *PpalePIC8* mutants on papaya fruits.

Green mature Sunrise papaya fruits harvested from the Waimanalo Research Station, University of Hawaii, were washed and were surface-dried with paper towels. The WT and mutant strains were grown on 10% V8 agar plates under 12 h of light and 12 h of dark at room temperature for 7 days. Zoospores were released by flooding the plates with 15 ml of cold sterile water and incubating at 4°C for 15 min, followed by incubation at room temperature under light for another 15 min. Fifteen microliters of zoospores (10⁴ per milliliter) were drop-inoculated on papaya fruits. To reduce position effects, two drops of WT were inoculated on one side of the longitudinal line of the fruit and two drops of one mutant strain were inoculated on the other side. The inoculated papaya fruits were arranged randomly in trays covered with clear plastic domes to maintain high humidity. The domes were removed 2 dpi. Photos were taken and lesion diameters were measured at 4 dpi. Mutant/WT pairwise diameter ratios were calculated for each pair of side-by-side inoculations. The experiments were repeated three times with three fruits used for each mutant for each experiment. Data collected from all three experiments were used to perform paired *t* test analyses using pairs (mutant/WT) of lesion diameter data and to generate the box plot using pairwise diameter ratios with R.

Inhibition assays of *in-vitro* *P. palmivora* growth by papain.

Inhibition assays of *P. palmivora* growth were performed using both 96-well plates and 60-mm diameter petri dishes. For assays with 96-well plates, 20 µl of zoospores (10⁴ per milliliter) from each strain were inoculated into 96-well plates containing 180 µl of liquid Plich medium (Kamoun et al. 1993) without or with papain (50 or 100 µg/ml). The plates were kept in the dark at room temperature for 5 days and were photographed. For the assays with 60-mm diameter petri dishes, 1 ml of zoospores (10⁴ per milliliter) were added to 9 ml of liquid Plich medium (Kamoun et al. 1993) in the absence or presence of papain at a final concentration of 75 µg/ml. The plates were kept in the dark at room temperature for 7 days. The mycelia were collected on preweighed filter papers by vacuum filtration. The filter papers together with mycelia were dried by baking at 70°C overnight

and were then weighed. The mycelium dry weight was calculated by subtracting the weight of the filter paper from the total weight. Three replicates were set up for each treatment. The percentage of growth inhibition by papain was calculated for WT and mutant strains by dividing the dry weight difference of mycelia grown in the absence and the presence of papain by the dry weight of mycelia grown in the absence of papain. One-tailed *t* test was performed using R program to evaluate whether the inhibition to each mutant was significantly greater than that to WT.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- CIPRES Science Gateway v. 3.3: <http://www.phylo.org>
- Eukaryotic pathogen CRISPR guide RNA design tool: <http://grna.ctegd.uga.edu>
- FigTree v1.4.3: <http://tree.bio.ed.ac.uk/software/figtree>
- InterProScan: <http://www.ebi.ac.uk/interpro/search/sequence-search>
- ProtTest: http://darwin.uvigo.es/software/prottest2_server.html
- RNAstructure web server: <http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html>
- SignalP 4.1 server: <http://www.cbs.dtu.dk/services/SignalP>