Phytophthora sojae Effector PsCRN70 Suppresses Plant Defenses in Nicotiana benthamiana

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Abstract

Phytophthora sojae, an oomycete pathogen, produces a large number of effector proteins that enter into host cells. The Crinklers (Crinkling and Necrosis, CRN) are cytoplasmic effectors that are conserved in oomycete pathogens and their encoding genes are highly expressed at the infective stages in P. sojae. However, their roles in pathogenesis are largely unknown. Here, we functionally characterized an effector PsCRN70 by transiently and stably overexpressing it in Nicotiana benthamiana. We demonstrated that PsCRN70 was localized to the plant cell nucleus and suppressed cell death elicited by all the tested cell death-inducing proteins, including BAX, PsAvh241, PsCRN63, PsojNIP and R3a/Avr3a. Overexpression of the PsCRN70 gene in N. benthamiana enhanced susceptibility to P. parasitica. The H2O2 accumulation in the PsCRN70-transgenic plants was reduced compared to the GFP-lines. The transcriptional levels of the defense-associated genes, including PR1b, PR2b, ERF1 and LOX, were also down-regulated in the PsCRN70-transgenic lines. Our results suggest that PsCRN70 may function as a universal suppressor of the cell death induced by many effectors, the host H2O2 accumulation and the expression of defense-associated genes, and therefore promotes pathogen infection.

Introduction

Phytopathogens secrete a battery of effector proteins that are delivered inside host cells to promote infection [1,2,3]. Plants detect conserved microbial molecular signatures, termed pathogen-associated molecular patterns (PAMP), resulting in PAMP-triggered immunity (PTI). To counter PTI, pathogens evolve diverse effectors to suppress PTI and trigger susceptibility (effector-triggered immunity). To overcome R protein-mediated immune responses, pathogens respond by mutating or losing effectors, or by developing novel effectors that can suppress ETI [4]. To overcome R protein-mediated immune responses, pathogens secrete effectors that can suppress PTI and trigger susceptibility (effector-triggered immunity, ETI). When the effectors are recognized by the corresponding resistance (R) proteins in the host plants, trigger susceptibility, ETS). When the effectors are recognized by the corresponding resistance (R) proteins in the host plants, host cells are activated [4]. To overcome R protein-mediated immune responses, pathogens secrete effectors that can suppress ETI [5]. For example, P. infestans expressed effector SNE1 to suppress the R3a/Avr3a-mediated PCD [6]. In the absence of the R proteins, pathogen effectors exert virulence activity to interfere with plant immunity [1]. It has been shown that in planta expression of Phytophthora effectors, such as P. infestans Avrblb2 [7], RxLR effector PITG_03192 [8], CRN8 [9], and P. sojae Avh241[10], enhanced susceptibility to pathogens.

Oomycete pathogens, such as Phytophthora spp., cause a wide variety of devastating plant diseases globally [11]. For example, P. infestans, a pathogen of late blight of potato and tomato, was responsible for the Irish potato famine in the mid-nineteenth century; P. sojae causes soybean root and stem rot and leads to substantial yield losses annually [11]. Phytophthora are hemibiotrophs that initiate the infection cycle as biotrophs, during which the pathogen proliferates asymptptomatically in the host, and at this stage pathogens must employ efficient mechanisms to evade and suppress plant immune responses. At the later stage, hemibiotrophs switch to a necrotrophic lifestyle by killing the host plants, and this process is presumably modulated by the coordinated secretion of lytic enzymes and cell-death inducers. However, the mechanisms underlying regulation of the switch from biotrophs to necrotrophs are still largely unknown. It was reported that P. infestans expresses effector SNE1 at the early infective stage to suppress cell death induced by many other effectors, which may function to maintain the biotrophic phase. A high throughput functional assay for P. sojae effectors revealed that Phytophthora pathogens may produce effectors with contrasting activity to regulate the infection process [12].

The genomes of the Phytophthora species contain large repertoires of RxLRs and CRNs [13,14,15], which are two kinds of cytoplasmic effectors. RxLR effectors are defined by a conserved N-terminal motif, which enables delivery of effector proteins inside plant cells [16,17]. CRN effectors were first identified in P. infestans as proteins that resulted in a leaf-crinkling and cell-death phenotype in plants [18]. The CRN effector family showed extensive expansion in all sequenced Phytophthora species [13,14]. The average expression levels of CRNs were much higher than those of the RxLR genes, indicating that CRNs may play important roles in pathogenicity [19]. Evolutionary analyses uncovered that gene duplication and fragment recombination drive functional diversity of CRN family [19]. Analogous to RxLR...
Effectors can trigger cell death when expressed caused cell death, however, it has been shown that few CRN effectors are localized in the plant cell cytoplasmic effectors are translocated into host plant cells to interfere with plant immunity, and it has become an efficient strategy to study the virulence functions of effectors by expressing them in planta [7,8,9,10]. Here we identified a CRN effector PsCRN70 from P. sojae, and expressed it in a model plant Nicotiana benthamiana by Agrobacterium-mediated transient expression and stable transformation. We showed that PsCRN70 can suppress cell death induced by many cell-death inducers, such as the mouse BAX, P. sojae RxLR effector Avh241, CRN effector CRN63, necrosis-inducing protein PsqNIP, and the R3a/Avr3a. Overexpression of PsCRN70 gene enhanced susceptibility of N. benthamiana to P. parasitica, indicating that PsCRN70 positively contributes to virulence. Our results indicated that, in addition to causing cell death in planta, CRN effectors may also function as a suppressor of plant cell-death and defense responses.

Materials and Methods

Plant material, bacterial strain and growth condition

Nicotiana benthamiana seeds were surface sterilized by soaking in 75% ethanol for 30 s followed by 1.0% sodium hypochlorite for 5 min. The seeds were rinsed 5 times with sterile water. They were subsequently spread onto petri dishes containing solid 1/2 MS medium. Plates were kept at 18°C for 4 days in the dark and then at 22°C for 10 days in 16/8 h light/dark cycle. The seedlings about 2–3 cm long were transferred aseptically to the glass bottles in sterile filter paper and cultured on non-selective callus induction medium (CIM) which contains 1 mg/L of 6-BA at 25°C in the dark for 3 days. After 3 days the infected explants were transferred to a fresh shoot induction medium (SIM) supplemented with 1 mg/L of 6-BA, 100 mg/L of kanamycin and 500 mg/L of carbenicillin at 25°C in the light for 25-30 days for shoot regeneration. Healthy shoots that reached a length of 1–2 cm tall were excised and transferred into a jar containing the selective rooting medium (RIM) supplemented with 100 mg/L of kanamycin, 500 mg/L of carbenicillin and 0.2 mg/L of IAA for root generation. Roots were obtained after 2–3 weeks in culture and transferred to soil under growth room conditions for seed set.

Agrobacterium-mediated transient expression

PVX constructs were transformed into A. tumefaciens strain GV3101 using the electroporation method [21]. For agroinfiltration assay, Agrobacterium containing the corresponding constructs were cultured in LB media containing 50 mg/mL of kanamycin at 28°C with shaking at 220 rpm for 48 h. The culture was harvested and washed three times in 10 mM MgCl₂ and resuspended in 10 mM MgCl₂ to an OD₆₀₀ of 0.3. Infiltration was performed on six-week-old P. sojae-transgenic N. benthamiana and the GFP-control plants. Symptom development was monitored within 72 h, and photographs were taken 36 hours post-infiltration. The experiments were repeated at least three times.

Phytophthora parasitica inoculation assay

We used two approaches, transient and stable expression, to evaluate the role of PsCRN70 in suppression of plant immunity. For transient expression approach, we expressed the PsCRN70 and GFP in N. benthamiana leaves using agroinfiltration method, and inoculated with P. parasitica zoospores 2 days post infiltration. Brieﬂy, the P. parasitica zoospores were prepared as described previously [26] and N. benthamiana leaves were detached and placed in a plastic tray, then each leaf was inoculated with 20 μL of zoospore suspensions with a concentration of 100 zoospores per microliter on the abaxial surface of the leaf. Phenotype was monitored within 72 h, and photographs were taken 36 hours post-inoculation.

For the stable PsCRN70 transgenic N. benthamiana, we used the detached leaves and the whole seedlings of 5-week old to evaluate the role of PsCRN70 in plant defense. The resistant levels of the whole transgenic plants were assessed using the root-dip inoculation assay. Twenty plants for each T2 transgenic line (#1, #3, #4 and #12) were inoculated with the P. parasitica zoospore cDNA from P. sojae through PCR with the forward primer: 5′-acgctgggaATTGTTGACGATCGGCTG-3′ and the reverse primer 5′-agtgggTAAGTACGACGGAGAATTC-3′. After digesting with the SalI and XhoI restriction enzymes, the resulting PCR product was inserted into the expression vector pBinGFP2 [10]. For the PVX construct, the PsCRN70 gene was amplified and inserted into the PVX vector pGR106 [24] using the SalI and XhoI restriction sites. The recombinant plasmids were confirmed by sequencing and introduced into Agrobacterium strains by electroporation.
RNA extraction and quantitative RT-PCR

Total RNA was extracted from N. benthamiana leaves using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The cDNA was generated using the PrimeScript RT reagent Kit (Takara). Real-time quantitative PCR was performed in 20-μL reactions including 20 ng of cDNA, 0.2 μM gene-specific primers, 0.4 ul ROX Reference Dye, 10 μL of SYBR Premix Ex Taq (Takara), and 6.8 μL of deionized water. PCR was performed on an ABI PRISM 7300 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 31 s to calculate cycle threshold values, followed by a dissociation curve program of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s to obtain the melt curves. The N. benthamiana EF1α gene was used as the internal reference gene for calculating relative transcript levels. An equal volume of cDNA was used for gene analysis expression of defense-related genes, using specific primers PR1a 5’-GTGGA-CACATATACGGTG-3’/5’-TCCAACTTTGGAAT-CAAAGG-3’, PR2b 5’-AGGTGTTTGTCCATGGAATGC-3’/5’-TCTGTACCACCATCTTGCC-3’, EF1α 5’-GCTCTTAAACGGTAGGTG-3’/5’-AGCCAAACCT-TAGCTCCATT-3’, LOX 5’-AACACCTATCGCTCAAAGAC-3’/5’-ACTGCTGCAATGGCTTGG-3’ and EF1α 5’-AGAGGCCCCTCAGACAAAC-3’/5’-TAGGTCCAAAGGTCA-CAA-3’. The induction ratio of treatment/control was then calculated by the 2\(^{-\Delta\Delta C T}\) method.

Confocal microscopy

To observe subcellular localization of PsCRN70, the transgenic N. benthamiana leaves were cut into small squares. The leaf squares or plant roots were then immersed into PBS buffer containing 5 μg/ml DAPI for staining of the nuclei for 5 min. Slides carrying the samples were observed with a Zeiss LSM 710 confocal laser scanning microscope (CLSM). The excitation wavelength used for GFP was 488 nm and 405 nm for DAPI. The GFP-transgenic N. benthamiana leaves were used as the control. Images were progressed using the Zeiss 710 CLSM and Adobe Photoshop software packages.

Protein extraction and Western blot analyses

The N. benthamiana leaf tissues were ground in liquid nitrogen. The ground materials were mixed with protein extraction buffer [50 mM Hepes, 150 mM KCl, 1 mM EDTA, 0.1% Triton X-100, adjust pH to 7.5 with KOH] supplemented with 1 mM DTT, and 1 x protease inhibitor mixture (Roche). Crude protein extracts were collected by centrifuging at 12,000 x g at 4 °C for 15 min. Protein extracts were loaded on 12% SDS-polyacrylamide gels and protein gel blot analyses were performed as reported previously [10]. Briefly, proteins were transferred from the gel to an Immobilon-P (Millipore) polyvinylidene difluoride membrane after electrophoresis. The membranes were washed in PBST (PBS with 0.1 Tween 20) for 2 min and then blocked in PBSTM (PBS with 0.1% Tween 20 and 5% non-fat dry milk) for 1 h. Mouse monoclonal antibody against GFP or HA was added into PBSTM and incubated for 90 min, followed by washing with PBST for three times. The membranes were then incubated in PBSTM with a goat anti-mouse IRDye 800CW (Li-Cor) for 40 min. The membranes were washed for three times with PBST and then visualized using a LI-COR Odyssey scanner with excitation 700 and 800 nm.

DAB staining

H2O2 was visualized using the 3,3-diaminobenzeni-dine (DAB) (Sigma) staining approach as described previously [27]. Leaves were inoculated with P. parasitica as described above; infected leaves 12 hours post inoculation were soaked in the DAB aqueous solution at 1 mg/ml and maintained at 25°C for 8 h. Leaf sections were cleared by boiling in 95% ethanol for 15 min, bleaching solution was replaced and leaves were incubated until the chlorophyll was completely bleached. DAB-staining experiments were independently repeated at least three times. Duncan’s multiple range test was used for statistical analysis (P<0.01).

Results

Generation of the PsCRN70-transgenic N. benthamiana

Introduction of PsCRN70 gene into N. benthamiana were achieved by Agrobacterium-mediated leaf disc transformation. Integration of the PsCRN70 and GFP transgenes were confirmed by PCR analysis. Thirty independent transgenic lines (T0) including ten GFP-transgenic plants and twenty PsCRN70-transgenic plants were obtained, and fifteen of them were randomly selected and self-pollinated to produce T1 lines. RT-PCR analysis confirmed that the GFP:PsCRN70 fusion genes were highly expressed in four (#1, #3, #4 and #12) independent T1 transgenic lines (Figure 1A). The seedlings of the transgenic progenies (T1 or T2 generations) were screened by checking fluorescent signal of GFP for further analyses. We confirmed the expression of GFP:PsCRN70 fusion protein using Western blot in transgenic lines, and the result showed that the fusion protein was correctly expressed (Figure 1B). Observation of the GFP fluorescence also validated the expression of the transgenes (Figure 1C). No visible differences in plant growth and other phenotypes were observed between the PsCRN70-transgenic plants and the GFP-transgenic lines, indicating that expression of PsCRN70 did not affect the development of N. benthamiana under normal growth conditions.

It was reported that the majority of CRN proteins are localized in the plant cell nuclei [20,23]. To test the subcellular localization of the target proteins, we first analyzed whether PsCRN70 harbors an NLS using the cNLS Mapper software [28], and the result showed that PsCRN70 contained a typical NLS (YLARKKRRKKEE) in the C-terminal region, indicating that PsCRN70 may also target the plant cell nucleus. We then observed the distribution of the GFP fluorescent signal in the four T2 transgenic plants (#1, #3, #4 and #12) and the GFP-control lines under a confocal microscope using the leaf and root tissues, respectively. The GFP fluorescent signal in the PsCRN70-transgenic lines was dominantly distributed in the nucleus of the leaf epidermal cells (Figure 1C). The fluorescent signal in the root epidermal cells of the PsCRN70-transgenic N. benthamiana was also distributed mainly in the plant nucleus, which was further confirmed by co-localization with DAPI staining (Figure 1D). In contrast, the fluorescent signal in the GFP-transgenic lines was equally distributed in the cytoplasm and nucleus of the leaf and root epidermal cells (Figure 1D). Thus, we concluded that PsCRN70 is a plant nucleus-localized protein.

Expression of the PsCRN70 in N. benthamiana enhance susceptibility to P. parasitica

To elucidate roles of PsCRN70 in N. benthamiana resistance, we constructed PVX:PsCRN70 and PVX:GFP, and then transiently
Figure 1. Characterizations of the PsCRN70-transgenic N. benthamiana. A. RT-PCR analysis of PsCRN70 expression in independent transgenic lines. #1, #3, #4 and #12, four independent T1 transgenic lines; P, pBinGFP:PsCRN70 plasmid as a positive control; U, untransformed plant as a negative control. The upper panel represents the 624 bp fragment of PsCRN70 gene and the lower panel represents the 100 bp fragment of EF1α gene as the control. B. Western blot analysis of expression of GFP:PsCRN70 fusion protein in transgenic N. benthamiana using monoclonal antibody against GFP. Subcellular localization of PsCRN70 in the transgenic N. benthamiana leaf tissues (C) and roots (D). The pictures were taken using a confocal microscope. The scale bar indicates 50 μm.

doi:10.1371/journal.pone.0098114.g001
Figure 2. Suppression of the plant resistance by PsCRN70. A. Lesions on the N. benthamiana leaves expressing PsCRN70 or GFP. A decolorized infected leaf was photographed 4 dpi. The experiments were repeated three times and shown with a representative image. B. Average lesion diameters on N. benthamiana leaves expressing the indicated genes inoculated with P. parasitica. Averages were calculated from four lesions per construct. Error bars represent standard errors. Different letters at the top of the columns indicate significant differences (P<0.01, Duncan's multiple range test). C. Phenotypes of the PsCRN70- and GFP-transgenic lines inoculated with P. parasitica. Detached leaves from five-week old seedlings (T1 generation) of transgenic plants (#1, #3, #4, #12) were inoculated with the P. parasitica zoospores. Photographs were taken 36 hpi under a UV lamp. D. Lesion diameters on inoculated leaves measured 36 hpi. The experiments were repeated four times in all transgenic lines with similar results. Bars represent the standard deviation (SD). Different letters at the top of the columns indicate significant differences (P<0.01, Duncan’s multiple range test). E. Phenotypes of the PsCRN70-transgenic plants inoculated with P. parasitica zoospores. The transgenic lines were inoculated with zoospores using the root dip method. Mock-inoculated seedlings were inoculated with sterile distilled water. Photographs were taken 4 days post inoculation. F. Survival rates of the transgenic plants inoculated with P. parasitica zoospores. The rates were measured at 4 dpi. The experiments were repeated four times in all transgenic lines with similar results. Twenty plants were used for each treatment in each experiment. Bars represent the standard deviation (SD). Different letters at the top of the columns indicate significant differences (P<0.01, Duncan’s multiple range test). doi:10.1371/journal.pone.0098114.g002
expressed them in *N. benthamiana* leaves. Two days post-infiltration, the infiltrated leaves were challenged with the *P. parasitica* zoospores. The detached leaf tissues expressing the *PsCRN70* developed larger lesion compared to those expressing the *GFP* control (Figure 2A). On leaf areas expressing the *GFP* gene, the average diameter of the lesions was 1.0 cm 36 hours post-inoculation; however, on areas infiltrated with *PsCRN70*, the average lesion diameter was 1.4 cm (Figure 2B). Thus, transient expression of the *PsCRN70* in *N. benthamiana* reduced its resistance to *P. parasitica*.

To validate the above observations, we also did inoculation assays of leaves and roots using stable *PsCRN70*-transgenic plants of T1 generation. The average lesion diameters were significantly larger in the *PsCRN70*-transgenic leaves than that in the *GFP*-transgenic leaves (Figure 2C, D). Root inoculations resulted in both *PsCRN70-* and the *GFP*-transgenic plants (T2 generation) displaying symptoms of wilting and stunting (Figure 2E). However, the survival rate of the *PsCRN70*-transgenic plants was significantly lower than the *GFP*-transgenic plants (Figure 2F). Taken together, these results showed that *PsCRN70* enhanced susceptibility of *N. benthamiana* to *P. parasitica* infection.

To determine whether expression of *PsCRN70* enhanced susceptibility of *N. benthamiana* to nonhost pathogen, we inoculated the transgenic *N. benthamiana* leaves (T2 generation) with *P. sojae* mycelial plugs. No obvious infection was found in both *GFP* - and *PsCRN70*-transgenic plants 5 days post inoculation (Figure S1), and this indicated that expression of *PsCRN70* in *N. benthamiana* did not affect its nonhost resistance to oomycete pathogen *P. sojae*.

**PsCRN70 suppresses cell death in *N. benthamiana***

To characterize how *PsCRN70* contributes to virulence, we tested its cell-death-manipulation activity using the *Agrobacterium*-mediated transient expression in *N. benthamiana*. This approach has been widely used for analyzing cell-death manipulation [14,21]. The cell-death inducing proteins included the mouse BAX [29], expressed in *N. benthamiana* leaves. Two days post-infiltration, the infiltrated leaves were challenged with the *P. parasitica* zoospores. The detached leaf tissues expressing the *PsCRN70* developed larger lesion compared to those expressing the *GFP* control (Figure 2A). On leaf areas expressing the *GFP* gene, the average diameter of the lesions was 1.0 cm 36 hours post-inoculation; however, on areas infiltrated with *PsCRN70*, the average lesion diameter was 1.4 cm (Figure 2B). Thus, transient expression of the *PsCRN70* in *N. benthamiana* reduced its resistance to *P. parasitica*.

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the P. sojae necrosis-inducing protein PsojNIP [30], the RxLR effector PsAvh241 [10], the CRN effector PsCRN63 [21] and the R3a/Avr3a [31]. P. sojae effector Avr1k [32] can inhibit cell death induced by all the above elicitors and was used as a positive control. As expected, no cell death phenotypes were observed in the Avr1k-infiltrated leaves (Figure 3A). Expression of the PsCRN70 gene also blocked cell death triggered by these elicitors 5 days post-infiltration. As a negative control, expression of the GFP gene did not suppress the cell death (Figure 3A). Western blot result showed that the cell-death inducers were expressed at similar levels when co-expressed with GFP control or PsCRN70, indicating that suppression of cell death was due to expression of PsCRN70. We further validated the results in the stable PsCRN70-transgenic N. benthamiana. In the transgenic line expressing GFP, cell death occurred at 5 days after infiltration with the above tested elicitors. However, cell death symptoms were only occasionally and weakly observed in the four PsCRN70-transgenic lines under the same conditions (Figure 3C, D). Western blot results also showed that the cell-death inducers were expressed at similar levels in PsCRN70-transgenic and GFP lines (Figure 1E). Collectively, these results suggested that PsCRN70 may function as a broad cell death-suppressor to manipulate the plant immunity.

Expression of the PsCRN70 impairs the H2O2 accumulation in N. benthamiana

H2O2, a kind of reactive oxygen species (ROS), plays an important role in plant defense responses [27]. To examine the role of PsCRN70 on the plant H2O2 accumulation, the H2O2 levels in the transgenic plants were detected at the early infective stages of P. parasitica using the DAB staining method. Weak staining was observed in the infected area of the PsCRN70-transgenic leaves (Figure 4A). The relative staining was significantly lower in the PsCRN70-transgenic N. benthamiana leaves compared to that in the GFP-control plants (Figure 4B). This result suggested that PsCRN70 may promote Phytophthora infection by suppressing the H2O2 accumulation.

Expressions of the PsCRN70 reduces the expression levels of the plant defense-associated genes

To further assess the role of PsCRN70 in plant defense responses, we examined the transcriptional levels of the defense-associated genes in plants, including PR1a (Pathogenesis-related protein), PR2b, ERF1 (Ethylene response factor 1) and LOX (Lipoxygenase) genes, among which, the PR1a and PR2b genes are markers in the salicylate-mediated signaling pathway [33]; the ERF1 is a marker in the ethylene-mediated signaling pathway [34]; and the LOX is a marker in the jasmonate-mediated signaling pathway [35]. These genes are all involved in downstream of the defense signaling pathways. Expressional levels of all the four tested genes were significantly repressed in the N. benthamiana leaves transiently expressing PsCRN70 compared to that in leaves expressing GFP (Figure 5A). The expression levels of the four genes also exhibited significant reduction in the stable PsCRN70-transgenic lines when challenged with P. parasitica zoospores (Figure 5B). These results suggest that PsCRN70 may repress the expression of the defense-associated genes in plants.

Discussion

Phytophthora pathogens encode a large number of RxLR and CRN effectors [13,14,15], however, virulence functions of CRN effectors are largely unknown. Overexpression of the PsCRN70 in N. benthamiana enhanced susceptibility to P. parasitica, indicating that PsCRN70 contributes to pathogen virulence. DAB staining results showed that the H2O2 accumulation in the PsCRN70-transgenic plants were significantly lower than that in the control lines, indicating that PsCRN70 can promote Phytophthora infection by reducing H2O2 levels in plants. The role of H2O2 in plant defense responses has been extensively studied [36]. It has been adopted by many pathogens to promote infection by regulating H2O2 production in plants [1,37]. For example, Ustilago maydis secretes the effector Pep1 into the apoplast to suppress the H2O2 production, resulting in suppression of plant immunity [37]. Quantitative RT-PCR results showed that the marker genes from different hormone signaling pathways were significantly down-regulated in the PsCRN70-transgenic N. benthamiana compared to the control, which further confirmed that PsCRN70 significantly reduced plant defense responses. SA and JA signaling pathways usually act antagonistically in plant defense. We showed that PsCRN70 may suppress both pathways, indicating the effector protein exhibits broad suppression activities.

It was originally surmised that the expression of CRN effectors triggered cell death [18]. However, more recent studies suggest that only a few CRNs induce necrosis [19,22]. Our results showed that PsCRN70 can suppress cell death induced by many elicitors including the mouse BAX, P. sojae RxLR effector Ahv241, CRN effector PsCRN63, necrosis-inducing protein PsojNIP, PCD triggered by the resistance protein R3a and the avirulence protein Avr3a. These results indicate that, similar to the SNE1 from P. infestans [6] and Avr1k [32] from P. sojae, PsCRN70 may function as a broad cell-death suppressor to promote P. sojae infection. These broad acting cell-death suppressor proteins will be useful tools in identifying the components of protein regulatory networks in immune signaling and cell death pathways.

It has been reported that the majority of CRN effectors are localized in the plant cell nuclei [20]. PsCRN70 is also located in the plant cell nucleus, indicating that function by members of the gene.
CRN effector family may function by manipulating the host nuclear processes to suppress the plant immune signaling. Subcellular localization of several cell-death-inducing CRN effectors demonstrated that they target distinct subnuclear compartments [23], indicating that CRNs may interfere with diverse nuclear targets. Alteration of nuclear targeting signals in several CRNs blocked their cell death-inducing activity [20,21]. However, it was unclear whether the nuclear subcellular localization of CRNs can account for the increase in effector virulence due to the expression of these proteins. In conclusion, we showed that PsCRN70 may function as a broad cell-death suppressor to promote Phytophthora infection, providing insight into the role of CRN effectors in pathogenicity.

Supporting Information

Figure S1  Phenotypes of the PsCRN70-transgenic plants inoculated with P. sojae mycelial plugs. Photographs were taken 5 days post inoculation.

Author Contributions
Conceived and designed the experiments: MZ DD. Performed the experiments: NAR YR TL JX LL JMM. Analyzed the data: NAR MZ DD. Contributed reagents/materials/analysis tools: YR TL LL. Wrote the paper: NAR MZ DD.
References

1. Dou DL, Zhou JM (2012) Phytophthora effectors subverting host immunity: different foes, similar battleground. Cell Host & Microbe 12: 484–495.
2. Hamm DR, Gimenez-Iturbe S, Rathjen JP (2010) Bacterial virulence effectors and their activities. Curr Opin Plant Biol 13: 389–393.
3. Rafiqi M, Ellis JG, Ludowici VA, Hardham AR, Dodds PN (2012) Challenges and progress towards understanding the role of effectors in plant-fungal interactions. Curr Opin Plant Biol 15: 477–482.
4. Jones JDG, Dangl JL (2006) The plant immune system. Nature 444: 323–329.
5. Stergiopoulos I, de Wit PJ (2009) Fungal effector proteins. Annu Rev Phytopathol 47: 233–263.
6. Kelley BS, Lee SJ, Damacone CMB, Chakravarty S, Kim BD, et al. (2010) A secreted effector protein (SNE1) from Phytophthora infestans is a broadly acting suppressor of programmed cell death. Plant J 62: 357–366.
7. Boukurt TO, Schornack S, Win J, Shinoh T, Iyas M, et al. (2011) Phytophthora infestans effectors Avr1b and Avr3a prevent secretion of a plant immune protease at the haustorial interface. P Natl Acad Sci USA 108: 20832–20837.
8. McLellan H, Boevink PC, Armstrong MR, Pritchard L, Gomez S, et al. (2013) An RxLR effector from Phytophthora infestans prevents re-localisation of two plant NAC transcription factors from the endoplasmic reticulum to the nucleus. PLoS Pathog 9: e1003670.
9. van Damme M, Bouzkurt TO, Caikir C, Schornack S, Sibilar J, et al. (2012) The Irish potato famine pathogen Phytophthora infestans translocates the CRN8 Kinase into host plant cells. PLoS Pathog 8.
10. Yu XL, Tang JL, Wang QQ, Ye WW, Tao K, et al. (2012) The RxLR effector Avh241 from Phytophthora sojae requires plasma membrane localization to induce plant cell death. New Phyol 196: 247–260.
11. Tyler BM (2001) Genetics and genomics of the oomycete-host interface. Trends Genet 17: 611–614.
12. Wang QQ, Han CZ, Ferreira AO, Yu XL, Ye WW, et al. (2011) Transcriptional programming and functional interactions within the Phytophthora sojae RXLR effector repertoire. Plant Cell 23: 2064–2086.
13. Tyler BM, Tripathy S, Zhang XM, Dehal P, Jiang RHY, et al. (2006) Phytophthora genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science 313: 1261–1266.
14. Haas BJ, Kamoun S, Zody MC, Jiang RHY, Handsaker RE, et al. (2009) Genome sequence and analysis of the Irish potato pathogen Phytophthora infestans. Nature 461: 393–398.
15. Lamour KH, Mudge J, Gobena D, Hurtado-Gonzales OP, Marinho P (1995) Leaf disk transformation using Agrobacterium tumefaciens. In: Jones H, editor. Plant Gene Transfer and Expression. Protocols: Springer New York pp. 39–46.
16. Lu R, Makuit I, Mollett P, Ruiz MT, Peart J, et al. (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. EMBO J 22: 5690–5699.
17. Van Damme M, Bozkurt TO, Caikir C, Schornack S, Sibilar J, et al. (2012) The Irish potato famine pathogen Phytophthora infestans translocates the CRN8 Kinase into host plant cells. PLoS Pathog 8.
18. Yu XL, Tang JL, Wang QQ, Ye WW, Tao K, et al. (2012) The RxLR effector Avh241 from Phytophthora sojae requires plasma membrane localization to induce plant cell death. New Phyol 196: 247–260.
19. Tyler BM (2001) Genetics and genomics of the oomycete-host interface. Trends Genet 17: 611–614.
20. Wang QQ, Han CZ, Ferreira AO, Yu XL, Ye WW, et al. (2011) Transcriptional programming and functional interactions within the Phytophthora sojae RXLR effector repertoire. Plant Cell 23: 2064–2086.
21. Tyler BM, Tripathy S, Zhang XM, Dehal P, Jiang RHY, et al. (2006) Phytophthora genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science 313: 1261–1266.
22. Haas BJ, Kamoun S, Zody MC, Jiang RHY, Handsaker RE, et al. (2009) Genome sequence and analysis of the Irish potato pathogen Phytophthora infestans. Nature 461: 393–398.
23. Lamour KH, Mudge J, Gobena D, Hurtado-Gonzales OP, Marinho P (1995) Leaf disk transformation using Agrobacterium tumefaciens. In: Jones H, editor. Plant Gene Transfer and Expression. Protocols: Springer New York pp. 39–46.
24. Lu R, Makuit I, Mollett P, Ruiz MT, Peart J, et al. (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. EMBO J 22: 5690–5699.
25. Gallois P, Marinho P (1995) Leaf disk transformation using Agrobacterium tumefaciens-expression of heterologous genes in tobacco. In: Jones H, editor. Plant Gene Transfer and Expression. Protocols: Springer New York pp. 39–46.
26. Zhang MX, Meng YL, Wang QH, Liu DD, Quan JL, et al. (2012) PnPMA1, an atypical plasma membrane H+–ATPase, is required for zoospore development in Phytophthora parasitica. Fungal Biol 116: 1013–1023.
27. ThordarsonChristopher H, Zhang ZG, Wei YD, Collinge DB (1997) Subcellular localization of H2O2 in plants. H2O2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J 11: 1187–1194.
28. Koushi S, Hasebe M, Tomita M, Yanagawa H (2009) Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs. P Natl Acad Sci USA 106: 13142–13142.
29. Lacombe C, Cruz SS (1999) Bax-induced cell death in tobacco is similar to the hypersensitive response. P Natl Acad Sci USA 96: 7956–7961.
30. Qutob D, Kamoun S, Gijzen M (2002) Expression of a Phytophthora sojae necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. Plant J 32: 361–373.
31. Armstrong MB, Whisson SC, Pritchard L, Boon JIB, Venter E, et al. (2005) An ancestral oomycete locus contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. P Natl Acad Sci USA 102: 7766–7771.
32. Song TQ, Kafe SD, Acredondo FD, Shen D, Su LM, et al. (2013) Two RxLR avirulence genes in Phytophthora sojae determine soybean Rps1k-mediated disease resistance. Mol Plant Microbe Int 26: 711–720.
33. Lee S, Ishiga Y, Clermont K, Mysore KS (2013) Coronatine inhibits stomatal closure and delays hypersensitive response cell death induced by nonhost bacterial pathogens. PeerJ 1: e34.
34. Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate-bacterial pathogens. PeerJ 1: e34.
35. Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate-bacterial pathogens. PeerJ 1: e34.
36. Peterson V, Van Breusegem F (2012) Hydrogen peroxide—a central hub for diverse activities in the host nucleus. Front Plant Sci 4: 387.
37. Hemetsberger C, Herrberger C, Zechmann B, Hillmer M, Doehlemann G (2012) Characterization of cell death inducing Phytophthora capsici CRN effectors suggests diverse activities in the host nucleus. Front Plant Sci 4: 387.
38. Hemetsberger C, Herrberger C, Zechmann B, Hillmer M, Doehlemann G (2012) Characterization of cell death inducing Phytophthora capsici CRN effectors suggests diverse activities in the host nucleus. Front Plant Sci 4: 387.
39. Qutob D, Kamoun S, Gijzen M (2002) Expression of a Phytophthora sojae necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. Plant J 32: 361–373.
40. Armstrong MB, Whisson SC, Pritchard L, Boon JIB, Venter E, et al. (2005) An ancestral oomycete locus contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. P Natl Acad Sci USA 102: 7766–7771.
41. Song TQ, Kafe SD, Acredondo FD, Shen D, Su LM, et al. (2013) Two RxLR avirulence genes in Phytophthora sojae determine soybean Rps1k-mediated disease resistance. Mol Plant Microbe Int 26: 711–720.
42. Lee S, Ishiga Y, Clermont K, Mysore KS (2013) Coronatine inhibits stomatal closure and delays hypersensitive response cell death induced by nonhost bacterial pathogens. PeerJ 1: e34.
43. Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate-bacterial pathogens. PeerJ 1: e34.
44. Peterson V, Van Breusegem F (2012) Hydrogen peroxide—a central hub for diverse activities in the host nucleus. Front Plant Sci 4: 387.