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A CYTOPLASMIC EFFECTOR PSCRN161 ENHANCED DISEASE RESISTANCE IN *NICOTIANA BENTHAMIANA* AGAINST *PHYTOPHTHORA CAPSICI*

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Abstract

Phytophthora sojae, is a pathogen of soybean that deliver an extensive number of effector proteins into plant cells. The Crinkling and Necrosis (CRN) cytoplasmic effectors that are conserved in water loving pathogens and their encoding genes are extremely expressed at the infective periods in *P. sojae*. However the molecular mechanisms are mostly unknown. Here, an effector of *P. sojae* CRN161 was characterized by transiently expressed in *Nicotiana benthamiana*. We confirmed that PsCRN161 was localized to the host cell nucleus and suppressed host cell death elicited by above tested cell death-inducing proteins, such as PsAvh241, R3a/Avr3a, PsojNIP, BAX and PsCRN63. Our results showed that PsCRN161 could significantly enhanced plant resistance to the infection of *P. capsici*. Furthermore, we found that expression levels of the plant defense-associated genes were also up-regulated *in planta* expression of PsCRN161. This study indicate that PsCRN161 may function as a suppress host plant immunity and therefore enhanced plant disease resistance.

Key words: *Phytophthora sojae*, PsCRN161, suppress cell death, disease resistance

Introduction

Phytopathogen phytophthora encode a extensive number of effectors to suppress host cell death and defense responses (Dou & Zhou, 2012; Rajput *et al*., 2014). These effectors generally facilitate infection, or to establish compatible interactions and manipulate host physiology by suppression and consequently enhance susceptibility ETS (Effector-triggered susceptibility). Therefore, effector proteins are also recognized by the plant surveillance system and activate immune responses, resulting in ETI (effector-triggered immunity), in the presence of the subsequently resistance (R) proteins (Jones & Dangl, 2006; Hogenhout *et al.,* 2009). Regularly the results of ETI in a hypersensitive response that restricts the spread of pathogens from the infection site (Stergiopoulos & de Wit, 2009; Thomma *et al.,* 2011).

Oomycete plant pathogens cause a wide variety of devastating diseases on several different types of plants. Oomycetes are a different group of fungus-like organisms, which are related to algae of the stramenopiles, that cause many diseases including late blight of potato and tomato, caused by *Phytophthora infestans*, sudden oak death responsible for *Phytophthora ramorum*, and root and stem rot of soybean caused by *P. sojae*, which cause estimated annual losses about 1–2 billion dollar worldwide (Erwin & Ribeiro, 1996; Tyler, 2007). Oomycetes genome sequences of above described *Phytophthora* species have been completed (Haas *et al.,* 2009). These oomycete plant pathogens are regularly hemibiotrophs initiate the infection as biotrophs during the asymptomatic period followed by a necrotrophic phase in the post infection period. These processes are probably altered by the corresponding secretion of mechanism including lytic enzymes and cell-death elicitors (Koeck *et al.,* 2011).

Phytophthora species secrete and translocate large repertoire of cytoplasmic effectors, namely members of the RXLR and CRN Crinkler families, contain a conserved motif, RXLR and FLAK following the signal peptide with share similar delivery function (Torto *et al.,* 2003; Kamoun, 2006; Haas *et al.,* 2009). The large repertoire motif RXLR, genome sequences were discovered encode numerous genes encoding protein based on a common sequence model identified in *Phytophthora* effectors such as *P. sojae* and *P. ramorum* their avirulence proteins function (Tyler *et al.,* 2006; Jiang *et al.,* 2008). Cytoplasmic effector as of oomycete pathogen protein family named CRN (Crinkling and Necrosis) was first recognized based on their ability with ectopic expression of *P. infestans* secreted proteins in plants (Torto *et al.,* 2003). A motif, FLAK (F-Phe; L-Leu; A-Ala and K-Lys) was recognized in the C-terminus of *Phytophthora* effectors intracellular expression in plants (Torto *et al.,* 2003; Haas *et al.,* 2009) and was experimentally showed to be a host-targeting cell death and crinkling of several defense-related genes. In Oomycete pathogens, CRN protein families showed extensive expansion to all genomes sequenced (Torto *et al.,* 2003; Tyler *et al.,* 2006; Win *et al.,* 2007; Gaulin *et al.,* 2008; Haas *et al.,* 2009; Levesque *et al.,* 2010; Lamour *et al.,* 2012). The transcription levels of CRN genes were very high because these proteins faced with enormous selection pressure (Tyler, 2009). Recent report, numerous CRN effectors of *P. sojae* are expanded through a birth-and-death model (Shen *et al.,* 2013) and also showed the fragment recombination and gene duplication in *P. sojae*. Similar to RXLR motif, FLAK motif could be predicted a large collection of CRN effectors candidates and provide a good resource for CRN genes identification (Haas *et al.,* 2009). Moreover, the characterization of intracellular effector CRN proteins may

have the ability to translocate inside host cells through infiltration, resulting in a suppression and elicitation activation of many defence related genes (Torto *et al.,* 2003). CRN63 and CRN115 effectors of *P. sojae* were identified following in planta functional expression resulted in either induce cell death, while suppression of cell death (Liu *et al.,* 2011). The Kinase activity of CRN8 protein from *P. infestans* has been discovered (van Damme *et al.,* 2012), also may showed the C-terminus to localize the host nucleus and triggers cell death when overexpressed in plant transient expression assays. CRN70 of *P. sojae* suppresses host cell death, H_2O_2 accumulation and expression of plant defense-associated genes (Rajput *et al.,* 2014).

Previously, functions and molecular mechanisms of many effectors were investigated by expression in model plants, including *Arabidopsis thaliana* and *N. benthamiana*. Here we analyzed *P. sojae* CRN161 functions in *N. benthamiana* through transiently expressed. We showed that this gene can enhance resistance of *N. benthamiana* to *P. capsici* by suppression of ROS accumulation. Different signal marker genes from SA, ET and JA-mediated defense pathways were significantly up-regulated in overexpression of PsCRN161 gene. However, overexpression of PsCRN161 functions generally as a cell death inhibitor in multiple plant species, and localization to the nucleus is required to suppress cell death.

Material and Methods

Plasmid construction: The present study was performed at the laboratory of *Phytophthora*-Plant interaction, Department of Plant Pathology, Nanjing Agricultural University, Nanjing China. A *P. sojae* CRN161 gene was amplified using cDNA through PCR with forward primer 5'- CGC**GGATCC**atgGTGAAGCTGAGTTGCGT-3' containing a BamHI restriction site and reverse primer 5'-GC**TCTAGA**TCAGGGCTCAGATTCGTTCG -3' with a XbaI restriction site. After digesting with BamHI and XbaI restriction enzymes, the PCR product results were inserted into the BamHI and XbaI predigested modified expression vector pBinplus. The pBinplus derivative plasmid which contain GFP reporter gene construct caries the PsCRN161 gene cloned downstream of the Cauliflower mosaic virus CaMV35S promoter and nopaline synthase (*NOS*) terminator. The T-DNA of this binary vector including the neomycin phosphotransferase II gene (*nptII*), which is derived from the *NOS* promoter and terminator sequences. DNA containing expression cassette was confirmed by sequence analysis (Takara Biotech. Dalian) and introduced into Agrobacterium strains by electroporation for transformation.

Plant infection assays of *Phytophthora* on *N. benthamian*: *P. sojae* and *P. capsici* was cultured on V8 medium at 28°C for 3-5 days. Leaf infection with PVX infiltration was performed on 4- to 6 week old *N. benthamiana* plants. We *A. tumefaciens* cell solutions of PsCRN161 and GFP carrying the individual constructs were infiltrated into *N. benthamiana* plant leaves. After two days transiently expressing PsCRN161 and GFP leaves were inoculated with the pathogen *P. capsici* for 36 hpi, and photograph were taken after 4 days. For leaf infection with plants, detached leaves (5 wk old) were placed on moist filter paper in large square incubator plates at room temperature. Infection assays of *P. capsici* were performed by culture disk inoculation onto PsCRN161 *N. benthamiana* and GFP leaves. The experiment was repeated twice with three replications of each experiment for results. Statistical analysis was performed by Duncan's test ($P< 0.01$).

Agroinfiltration assays by PVX: For agroinfiltration assay, Plasmids purified from *E. coli* was transformed into GV3101 *A. tumefaciens* with using of the freeze thaw method (Rajput *et al.,* 2014). *A. tumefaciens* were cultured in Luria-Bertani media supplemented with 50 mg/mL kanamycin in a tube at 28°C and 220 (rpm) for 48 (h). After that harvested, three times washed in 10 mM MgCl2, resuspended in 10 mM MgCl2 to an OD_{600} of 0.3. *Agrobacterium*-mediated transient expression experiments, *A. tumefaciens* solutions carrying the individual constructs were infiltrated into *N. benthamiana* plant leaves by pressure infiltration, a small scratch was placed in every leaf with a needle and after that 30 to 50 mL of suspension was infiltrated through the scratch using a syringe lacking a needle. *A. tumefaciens* cells carrying the programmed cell death inducing genes (PsCRN63, PsojNIP, Avh241, Bax, and Avr3a+R3a) were infiltrated into the same place 12 h later. Symptom was monitored from 4-6 d after infiltration, and after 5 d photographs were taken. The experiments were repeated at three times.

Quantitative RT- PCR analysis: Total RNA was extracted from each *N. benthamiana* leaves were harvested and transferred to liquid nitrogen. The total RNA was extracted through RNeasy Mini Kit, and included an additional on-column DNase digestion step (Quiagen RNase-free DNase), as recommended by manufacturer's instructions. The RNA was quantified with usage of Thermo Scientific NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE, USA). cDNA was generated through reverse transcriptase using PrimeScript™ RT reagent Kit (TaKaRa) and random primers. The program consisted of an initial denaturation step at 95°C for 5 min, followed by 32 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1:00 min. The PCR product was fractionated by electrophoresis on a 0.8% agarose gel, detected by ethidium bromide staining and photographed under ultraviolet light. Quantitative RT PCR was performed in 20-µL reactions with 20 ng of cDNA, 0.2 µM subject-gene primers, 0.4ul ROX Reference Dye and 10ul SYBR ® Premix ExTaq (Takara), and 6.8 µL of DI water. PCR was performed on a ABI PRISM 7300 Fast RT-PCR System (Applied Biosystems) with the following conditions: 95°C for 30s, 40 cycles of 95°C for 5s and 60°C for 31s to calculate cycle threshold values followed by a dissociation program of 95°C for 15s, 60°C for 1 min and 95°C for 15s to get melt curves. The cDNA samples were tested by using three independent repetitions in the same conditions**.** The tobacco gene *EF-1a* was used as the internal target gene for calculating the transcript expression levels. The cDNA equal volume was used for analysis of gene expression of plant defense-related genes, using subject primers *PR1b* 5'- GTGGACACTATACTCAGGTG-3'/5'-TCCAACTTGGAATCAAAGGG-3', *PR2b* 5'-AGGTGTTTGCTATGGAATGC-3'/5'-TCTGTACCCACCATCTTGC-3', *ERF1* 5'-GCTCTTAACGTCGGATGGTC-3'/5'-

AGCCAAACCCTAGCTCCATT-3', *LOX* 5'-AAAACCTATGCCTCAAGAAC-3'/5'-ACTGCTGCATAGGCTTTGG-3' and *EF1-a* 5'-AGAGGCCCTCAGACAAAC-3'/5'-TAGGTCCAAAGGTCACAA-3'. The 7300 System Sequence Detection Software was used to obtain relative expression levels of every sample. The induction ratio of treatment/control was then calculated by the equation 2^ (-ΔΔCt). Gene expression was analyzed using the analysis of variance, ANOVA followed by statistical analysis was performed by Dunnett's test ($P< 0.01$).

Detection of reactive oxygen species: H_2O_2 in *N. benthamiana* leaves were visualized by 3,3-diaminobenzidine, DAB (Sigma). Detached leaves were inoculated with culture disk of *P. capsici*. Infected leaves afer inoculation were soaked in DAB solution pH 3.5 at 1 mg/ml, maintained at 25°C for 8 h. The leaf sections were cleaned by boiling with 95% ethanol at 15 min, bleaching solution was replaced and infected leaves were incubated for 12 h. Photographs were taken with EOS 600D Canon digital camera. DAB-staining were performed at least three times, with identical results from the repetitions. Statistical analysis was performed by Duncan's test ($P< 0.01$).

Confocal microscopy: The expression of pBinGFP: PsCRN161 in *N. benthamiana* plants were analyzed on a Zeiss LSM 710 confocal microscope (Zeiss Microsystems) with the following excitation wavelengths: CFP, 458 nm; GFP, 488 nm; and RFP, 561 nm. pBinGFP: PsCRN161 subcellular localization were monitored in the *N. benthamiana* leaves after transformation. pBinGFP *N. benthamiana* leaves were used as positive control. For each of the fluorescence images a positive and negative control was photographed for comparison of fluorescence intensities between samples so that the contribution from autofluorescence could be examined.

Results

***PsCRN161* expression enhanced plant resistance to *P. capsici*:** To elucidate whether PsCRN161 function to effect by *P. capsici* for the transient expression in *N. benthamiana* plants. We constructed PVX::GFP or PVX::PsCRN161, which was transiently expressed in *N. benthamiana* leaves. Detached leaves of transient PsCRN161 and GFP as a control measure plants were challenged two days after infected by culture disk of *P. capsici*. During infection we repeatedly observed better infection ratio and earlier progression at the infection sites to the stage of sporulating hyphae in *N. benthamiana* leaves (Fig. 1a). On infiltrated leaves with strains carrying a control gene (GFP), the diameter of the infection ratio of lesion length was approximately 3.2 cm at 36 hours post-infection (hpi); however, on infiltrated leaves with strains carrying PsCRN161 showed infection ratio of lesion length expanded to 1.8 cm (Fig. 1b). The infection ratio of lesion length of PsCRN161 indicates that the tested *P. capsici* isolates are highly resistanc with the low infection site diameter, while *GFP* expression showed larger infection zones. Our results transient expression of PsCRN161 showed that enhanced the resistance of *N. benthamiana* plants to *P. capsici* infection.

***PsCRN161* suppresses cell death in *N. benthamiana*:** To identify the function of PsCRN161 could induce necrosis in *N. benthamiana* using infiltration of *A. tumefaciens* cells with a potato virus X (PVX)-based vector. We independently confirm that the ability of PsCRN161 could suppress the cell death inducing protein BAX, PsojNIP, Avh241, PsCRN63 and Avr3a/R3a in plant cells. Although, we infiltrated *N. benthamiana* leaves 12 h prior with *A. tumefaciens* cells containing a PsCRN161 triggered suppress cell death, and also could suppress cell death by all the tested elicitors and green fluorescent protein (GFP) were used as a negative control, did not protect against cell death in plant (Fig. 2). However, PsCRN161 could block cell death strongly triggered by above all elicitor of cell death inducing protein in *N. benthamiana* tissue compared to gfp gene after 5 d infiltration. Western blot analysis further confirmed that all the cell death inducers were expressed at similar levels in PsCRN161 and GFP in *N. benthamiana.* These finding indicates that PsCRN161 has a strong cell death inhibitory activity and suggests that it might be in *P. sojae* infection significant impairment of host defense responses.

Expression of the PsCRN161 enhanced the H_2O_2 accumulation in *N. benthamiana*: Reactive oxygen species (ROS) production plays an important role in defense responses plant-pathogen interactions. To examined the diaminobenzidine (DAB) staining for detection of H_2O_2 accumulation at the early stage of infection by the expression of PsCRN161 compared with GFP in *N. benthamiana* (Fig. 3a). Infiltrated PsCRN161 and GFP leaves were inoculated with a culture disk of *P. capsici* and observed after 12 hpi through relative staining (Fig. 3b). We observed higher amount of H_2O_2 accumulation in PsCRN161 compared to GFP leaves. Our results suggested that expression of PsCRN161 leaves infected with *P. capsici* enhance resistance with lower accumulation of H_2O_2 compared to the GFP leaves.

Expressions of PsCRN161 up-regulated the expressional levels of the plant defense-associated genes: To investigate the defense-associated gene activation in PsCRN161 and GFP expression in *N. benthamiana* leaves by *Agrobacterium*-mediated transient using the above-introduced methods. We carried out separately quantitative RT-PCR using total RNA from 5 d infiltrated tissue with *P. capsici* infection in *N. benthamiana* plants. To examined the expression of salicylate SA-mediated signaling pathway genes, such as PR1a and PR2b (*pathogenesis-related protein*), and ethylene ET-mediated signaling pathway gene, such as ERF1 (*Ethylene response factor 1*) and jasmonic acid JA-mediated signaling pathway gene, such as LOX (*Lipoxygenase*) which are involved in signaling defense pathways, respectively (Brederode *et al.,* 1991; Niki *et al.,* 1998). The level of PsCRN161 expression in infiltrated tissues was significantly up-regulated the PR1a, PR2b, ERF1 and LOX gene expression compared to GFP (Fig. 4). We used the constitutively expressed EF1a gene as internal control. These results suggest that PsCRN161 may increase the expression of defense associated genes in plants.

Subcellular localization of the PsCRN161: To test the subcellular localization functions of PsCRN161 in *N. benthamiana* plants while the GFP alone under constitutive 35S promoter control line. We investigate the nuclear localization revealed that PsCRN161 fusion

protein and GFP control accumulated everywhere inside the leaf epidermal cells (Fig. 5). The fluorescent signal was distributed equally in the cytoplasm and nucleus, as reported previously (Schornack *et al.,* 2010). In summary, these results indicate that, whether expression of PsCRN161 fusion protein expressed in plants that may detected fluorescent signal in the cytoplasm and nucleus.

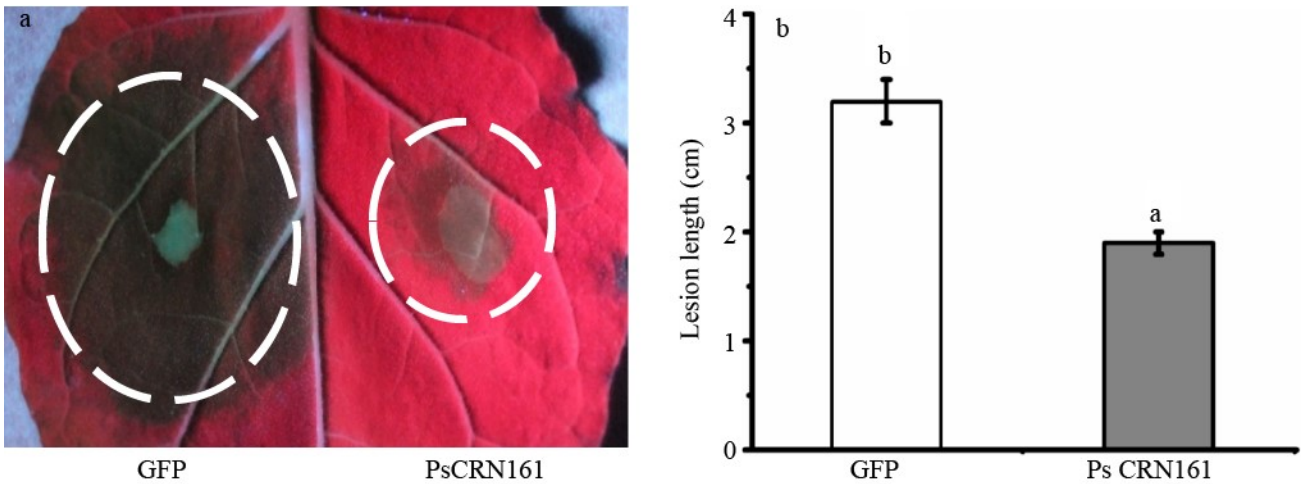

Fig. 1. PsCRN161 enhanced resistance to *P. capsici* infection. a Infiltrated GFP and PsCRN161 *N. benthamiana* leaves inoculated with *P. capsici.* b Lesion length were measured from inoculated leaves. The experiment was repeated twice with three replications for each experiment with same results. Bars represent the means ± standard deviation (SD). The top of columns with different letters indicate significant differences (P,0.01, Duncan's test).

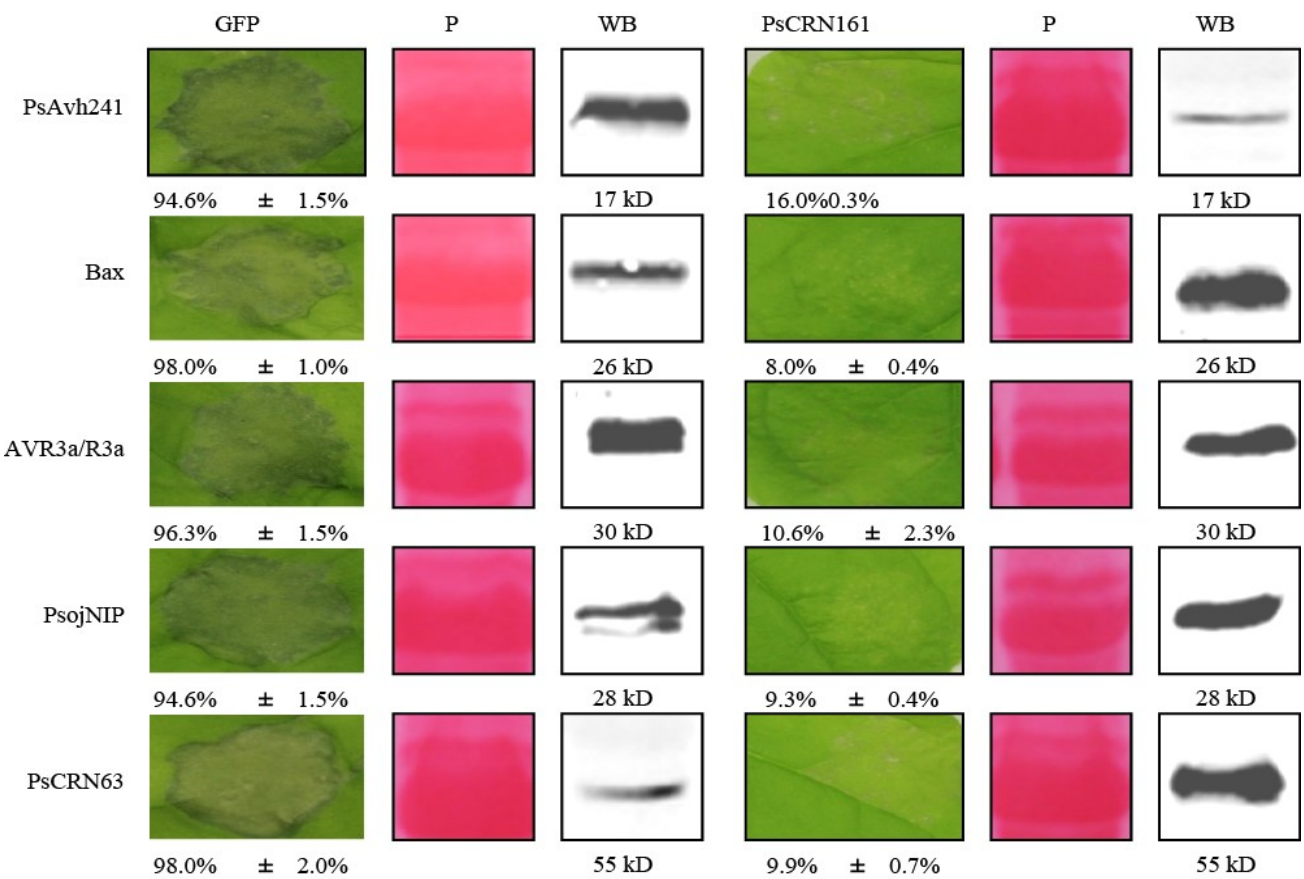

Fig. 2. PsCRN161 suppresses cell death induced by many cell-death inducers.
PsCRN161 transiently expressed in *N. benthamiana* leaves. Agroinfiltration sites in each *N. benthamiana* leaf expressing GFP and PsCRN161 were tested with *A. tumefaciens* expressing Bax, Avh241, PsCRN63, PsojNIP and Avr3a + R3a. Photographs were taken at 5 d after cell death. Western blot results of the cell-death inducers in transiently *N. benthamiana* leaves, co-expressed with GFP or PsCRN161. Antibody against the HA-epitope was used to identify the expression of PsCRN63, PsAvh241, PsojNIP, Avr3a and Bax, when the co-expressed with GFP or PsCRN161. The quantification percentage of cell death sites infiltrated with the cell death inducers. The means ± standard deviation (SD) percentages of sites on leaves showing cell death were achieved from 8 infiltrated leaves with three independent experiments.

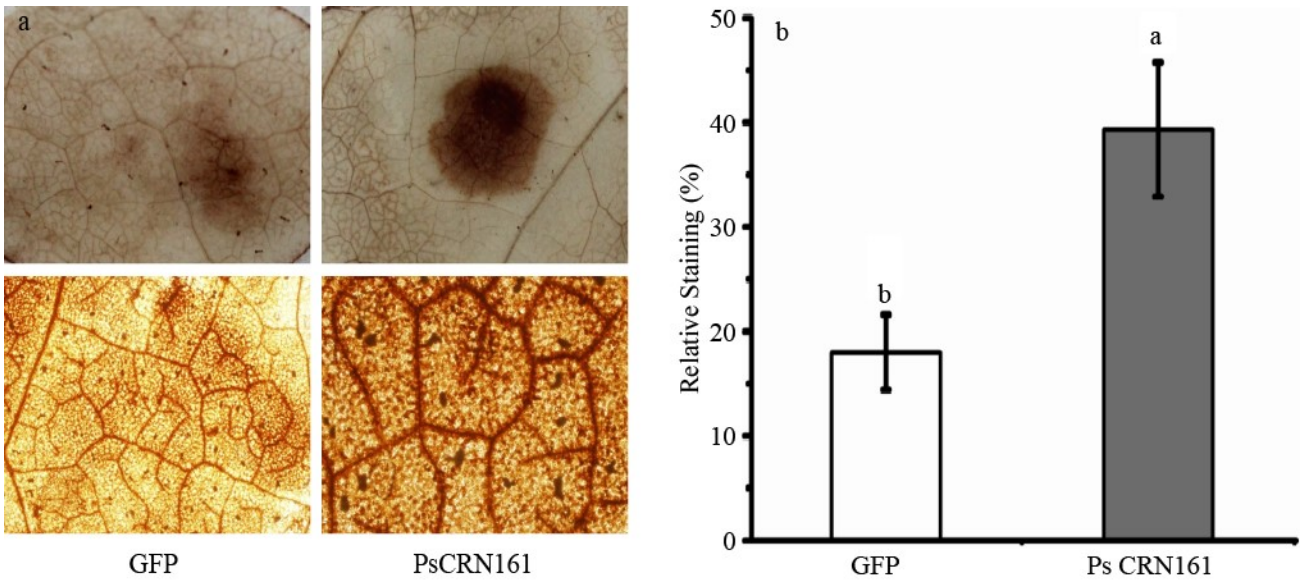

Fig. 3. Expression of PsCRN161 increased ROS accumulation.
a The accumulation of ROS in PsCRN161 and GFP expressing leaves were stained with DAB after 12 hpi to *P. capsici*. Photographs were taken after decolorization of leaves with ethanol from three independent experiments. b The relative staining for accumulation of ROS in the leaves were observed after decolorization. The experiments were repeated with three times with similar results. Four leaves were used with each treatment and each experiment. The bars represent means ± standard deviation (SD). The top of columns of different letters indicate significant differences (P,0.01, Duncan's test).

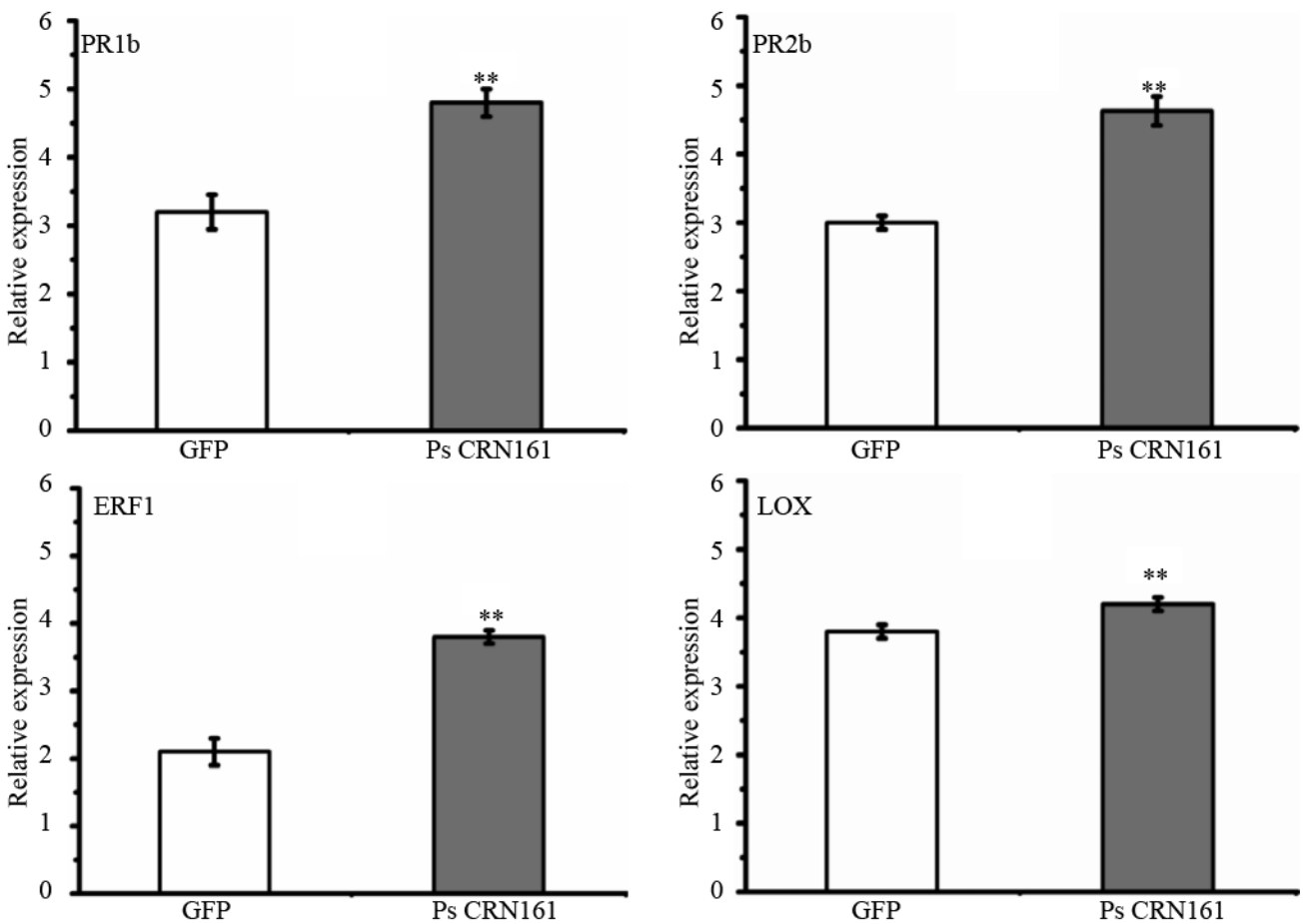

Fig. 4. Up-regulation of the defense-related genes in *N. benthamiana* by PsCRN161. A. Transiently expressed of PsCRN161 and GFP in *N. benthamiana* after five days were determined with the expression of ERF1, LOX, PR1b and PR2b genes. Relative expression results were performed using three replications, where each replicate consists with three technical replicates. The bars represent means ± standard deviation (SD), with (**) represent significantly difference (P,0.01, Dunnett's test). The qRT-PCR results were normalized to *EF1α* transcript and shown as relative target gene expressions in *N. benthamiana* leaves.

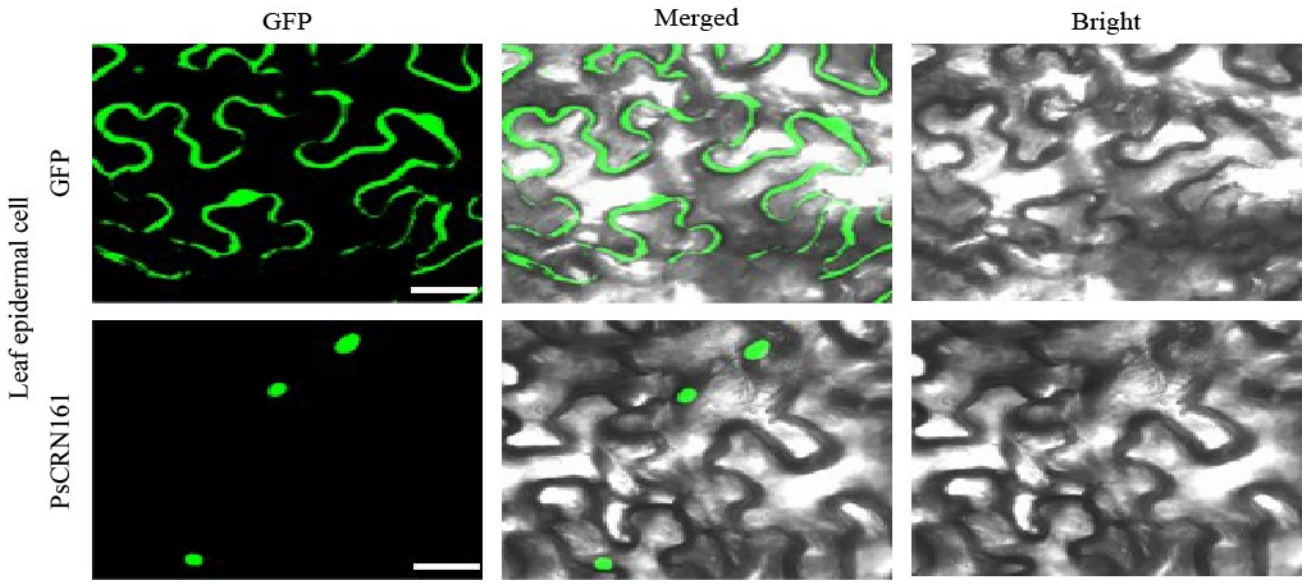

Fig. 5. Subcellular localization of the PsCRN161. Subcellular localization of PsCRN161 in *N. benthamiana* plants. Confocal microscopy images of PsCRN161 and GFP *N. benthamiana* leaves plant were showed leaf epidermal cells. The scale bar indicates 10 µm.

Discussion

Phytopathogenic oomycete effector CRN proteins were first time revealed that triggered induce necrosis and leaf crinkling in plants by transient expression assays (Torto *et al.,* 2003). In this study, we found that expression of PsCRN161 enhanced resistance to *P. capsici* infection in *N. benthamiana*. Extensive mechanism and functional analysis of the *P. sojae* effector *PsCRN161* to determine the structural requirements for cell death inducing protein suppression during infection. We performed transient expression of PsCRN161 in *N. benthamiana* plant, it showed that the tested PsCRN161 may strong ability to suppress all cell death inducing protein from CRN effectors family BAX, PsojNIP, Avh241, PsCRN63 and Avr3a/R3a in plant cells. Likewise, Liu and coworkers reported that PsCRN115 could completely suppress cell death induced by PsojNIP and PsCRN63 (Liu *et al.,* 2011). Previously several other secreted proteins have been identified to translocate into the host cells, such as elicitins *P. infestans* (Torto *et al.,* 2003; Haas *et al.,* 2009; Schornack *et al.,* 2010), *P. sojae* (Liu *et al.,* 2011; Rajput *et al.,* 2014) and *P. capsici* (Stam *et al*., 2013a) all activate programmed cell death. Our results suggested that transient expression of PsCRN161 could be significantly suppresses different inducer of cell death in plant

Regulation of biological process enhance with the accumulation of ROS, whereas oxidative stress, irreversible damage and hypersensitive response like cell death may be occur due to high accumulation of ROS (Kovtun *et al.,* 2000). On the basis of our results we suggest that expression of PsCRN161 function exhibited was significantly increased ROS accumulation in plants.

We investigated whether the role of signaling pathways mediated such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) in PsCRN161 transient expression in *N. benthamiana* plants. Quantitative RT-PCR analyses of defense-associated gene were performed and showed significantly up-regulated activation in mediate expression *N. benthamiana* plants. Further analysis revealed that PsCRN161 expression levels in plants inhibited by SA, ET and JA defense signaling pathways, whereas the expression levels of PsCRN161 could be significantly enhanced resistance to *P. capsici* infection. Evidence for a cross-talk between SA, JA and ET signaling pathways has been provide mechanism of the activation and regulation of plant defense responses (Kunkel & Brooks, 2002; Koornneef & Pieterse, 2008). Therefore, the mechanism by which PsCRN161 expression suppresses cell death and enhanced resistance appear to be concomitant activation of respective defense-associated genes in plants.

Majority of the CRN effectors are localized in host cell nuclei (Haas *et al.,* 2009; Liu *et al.,* 2011; Stam *et al.,* 2013b; Schornack *et al.,* 2010; Rajput *et al.,* 2014). PsCRN161 is also located in the host cell nucleus, indicating that the function by members of CRN effectors family may function with manipulating the host nuclear process, to suppresses the plant immune signaling. PsCRN161 expressed in *N. benthamiana* suppresses cell death by several cell death-inducing proteins via the PVX vector in a sufficient quantity. These results agree with previous findings (Liu *et al.,* 2011) that several individual effectors which induces cell death could be suppressed by PsCRN115 when expressed in plants. Furthermore, expression of PsCRN161 enhanced resistance to *P. capsici* infection in *N. benthamiana* plants and increase ROS accumulation compared with GFP plants. Our observations of this functional hypothesis was that PsCRN161 effector play an important role in *N. benthamiana* to suppression of host defenses and enhance resistance and involves in the expression of the defense-associated genes.

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