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Plant mitochondria and chloroplasts are targeted by the *Rhizoctonia solani* RsCRP1 effector

Georgios Tzelepis [a, *], Fredrik Dölfors [b], Louise Holmquist [c], Christina Dixelius [b]

[a] Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala Biocenter, Box 7026, SE-750 07, Uppsala, Sweden
[b] Swedish University of Agricultural Sciences, Department of Plant Biology, Uppsala BioCenter, Linnean Center for Plant Biology, P.O. Box 7080, S-75007, Uppsala, Sweden
[c] MariboHilleshög Research AB, Säbyholmsvägen 24, S-26191, Landskrona, Sweden

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ABSTRACT

The fungal species *Rhizoctonia solani* belongs to the Basidiomycota division and is a ubiquitous soil-borne pathogen. It is the main agent of the damping-off disease in seedlings and causes the root and crown rot disease in sugar beets. Plant pathogens deploy small secreted proteins, called effectors, to manipulate plant immunity in order to infect the host. Here, a gene (*RsCRP1*) encoded a putative effector cysteine-rich protein was cloned, expressed in *Cercospora beticola* and used for virulence assays. The *RsCRP1* gene was highly induced upon the early-infection stage of sugar beet seedlings and disease was promoted. Confocal microscopy demonstrated localization to the chloroplasts and mitochondria upon transient expression of RsCRP1 in leaves of *Nicotiana benthamiana*. Further, this effector was unable to induce necrosis or to suppress hypersensitive response induced by the Avr4/Cf4 complex in *N. benthamiana*. Overall, these data indicate that RsCRP1 is a novel effector targeting distinct plant cell organelles in order to facilitate a successful infection at the early stages of the disease development.

1. Introduction

Pathogens can enter plant hosts using various strategies; via openings and wounds, secretion of cell wall degrading enzymes or manipulation of a wide range plant defense mechanisms. Commonly, these strategies are combined to promote efficient colonization and proliferation in the host. Events involving pathogen growth and reproduction in host tissue require nutrients, which is the ultimate rational to cause disease by any organism. In order to establish a compatible interaction, pathogens must evade or suppress plant immunity [1]. To do that, among others, they secrete small proteins, called effectors. Effectors can have various functions such as inducing necrosis, protecting fungal hyphae from plant chitinases, suppressing hypersensitive response (HR), or helping fungal hyphae to stealth themselves, avoiding recognition by plant receptors [2]. Although effector biology is a growing field, still a majority are undiscovered and important aspects of their exact roles and functions are unknown. This is particularly the case for the understudied but important soil-borne pathogens.

Plants on the other hand, deploy different layers of defense including sophisticated signaling against pathogens [3]. The first layer induced by microbial elicitors, called pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) [4]. PAMPs can be essential components of fungal cell wall, such as chitin, or proteins with a crucial role in the formation of filament in a bacterial flagellum, such as flagellin. Recognition of PAMPs by the plant leads to a PAMP-triggered immunity (PTI) response. Next layer of defense involves recognition of effectors by intracellular plant resistance (*R*) genes, leading to induction of effector-triggered immunity (ETI) such as the hypersensitive response, HR [5]. However, the present understanding of the plant immune system is far more differentiated.

Rhizoctonia solani (teleomorph: *Thanatephorus cucumeris*) is a soil-borne pathogen, with a wide host range. Isolates are categorized in different anastomosis groups (AG) based on their hyphal anastomosis reactions [6]. *Rhizoctonia solani* AG2-2IIIB is the causal agent of crown root rot in sugar beets. During recent years the genomes of different R. *solani* AGs have been sequenced with the purpose to enhance our knowledge of the infection pathways [7–11]. To assist the work on sugar beet improvement we used the

* Corresponding author.
E-mail address: georgios.tzelepis@slu.se (G. Tzelepis).

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genome information of *R. solani* AG2-2IIIB [12] to search for 1) novel effector candidates, and 2) investigate their function. The latter is a challenge since *R. solani* is not amenable for genetic modifications as many other basidiomycetes.

2. Materials and methods

2.1. Fungal isolates and growth conditions

Rhizoctonia solani AG2-2IIIB isolate BBA 69670 (DSM 101808) was used in this study and cultured as earlier described [12]. The *Cercospora beticola* strain Ty1 (MariboHilleshög, Research AB) was cultured on potato dextrose agar (PDA, Difco) at 22 °C in darkness. To induce sporulation, *C. beticola* was grown on tomato extract medium at 25 °C with a photoperiod of 12 h.

2.2. RNA preparation and quantitative RT-qPCR

For gene expression analysis of the *RsCRP1* (*RSOLA-G22IIIB_02432*) gene, 3-week-old sensitive sugar beet plantlets (hybrid 1604511801, MariboHilleshög Research AB) replanted in soil infested with *R. solani* mycelia. Total RNA was extracted from infected plants using the RNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions, while *R. solani* mycelia grown on potato dextrose broth (PDB, Difco) were used as a control. Primers are listed in Table S1. RT-qPCR was conducted as previously described [13]. The data was normalized to the *G3PDH* expression [14] and relative transcripts were calculated according to the $2^{-\Delta\Delta Ct}$ method [15]. Statistical analysis was done using Student's t-test.

2.3. Cloning and Cercospora beticola transformation

The *RsCRP1* gene was PCR amplified from *R. solani* cDNA using high fidelity Phusion Taq polymerase (Thermo Fisher Scientific). Primers are listed in Table S1. The cDNA fragment was inserted in the pRFHUE-eGFP vector [16] using the In-Fusion HD cloning kit (Takara Bio), followed by plasmid transformation to the *Agrobacterium tumefaciens* C58C1 strain. Transformation of *C. beticola* was performed using an *A. tumefaciens*-mediated protocol [17] and three individual colonies were used for further analysis. Expression of the *RsCRP1* gene was validated using RT-PCR on hygromycin-resistant colonies (Fig. S1).

2.4. Virulence assay and fungal biomass

For the virulence assay, leaves of 3-week-old sugar beet plants (hybrid 16045118 01 MariboHilleshög Research AB) were inoculated with *C. beticola* conidia as previously described [18]. The area of disease lesions was calculated 7 days post infection (dpi). Total genomic DNA was extracted from mock (H_20) and inoculated leaves and fungal DNA was quantified using the *C. beticola* actin (*act*) gene and normalized with *B. vulgaris* elongation factor (*elf-1*), using qPCR analysis (Table S1). At least three biological replicates were used and each replicate comprised of two leaves from four inoculated plants.

2.5. Sequence analysis and confocal microscopy

Presence of conserved domains in the RsCRP1 effector was tested using the SMART 6.0 protein analysis tool [19]. Subcellular localization was investigated using the WoLF PSORT predictor [20] the ChloroP [21] and the DeepMito servers [22]

For confocal microscopy, the *RsCRP1* gene was subcloned to the pENTR/D-TOPO vector (Thermo Fisher Scientific) and inserted to the pGWB605 destination vector using the Gateway system, followed by Agro-transformation in C58C1 cells and transiently expressed in *N. benthamiana* leaves. Imaging was performed using an LSM 800 confocal microscope (Zeiss). The green fluorescence was excited/emitted at 488/516 nm and detected at 411–553 nm. The red fluorescence was excited/emitted at 633/684 and detected at 645–700 nm. For the HR suppression assay, the *RsRCP1* gene was entered to the pGWB602 binary vector and transiently expressed in *N. benthamiana* plants harboring the Cf-4 receptor protein from tomato plants. The HR was triggered 24 h after RsRCP1 Agro-infiltration with the *Cladosporium fulvum* Avr4 effector.

3. Results and discussion

3.1. The RsCRP1 gene is highly induced upon early infection stages

The current wealth of pathogen genomes led to prediction of effector proteins which in general builds on the presence of a secretion signal, size (>400 aa) and content of cysteines [2]. To narrow down the effector candidates in the *R. solani* genome we compared the data from five different strains resulting in eleven genes unique for the AG2-2IIIB strain [12]. The small cysteine-rich protein-encoding gene *RsCRP1* was chosen for further studies based on its transcription patterns. It was highly induced already 4dpi in sugar beet seedlings, followed by reducing levels at 5 dpi as compared to fungal mycelia grown in PDB (Fig. 1).

3.2. Heterologous expression of RsCRP1 in Cercospora beticola promotes disease development

To take the next step involving further gene analysis, we evaluated the options among fungal sugar beet pathogens. We finally chose the ascomycete *C. beticola* causing Cercospora leaf spot disease which per se is a serious problem particularly in countries with strict fungicide restrictions. *C. beticola* produces rich amount of conidia another feature that further simplify its use compared to *R. solani*. The *RsRCP1* gene was ligated to the pRFHUE-eGFP vector driven by the constitutively expressed PgdpA promoter from *Aspergillus nidulans,* transformed to *C. beticola* and used for sugar beet infection. Increased necrotic lesions was observed for the *RsCRP1* + strains as compared to the wild type (WT) and the strain where only the empty vector was inserted (Fig. 2a). In parallel, DNA was extracted from infected leaf regions 7dpi and fungal biomass was calculated. No significant difference in the amount of fungal DNA was observed among WT and strains where the *RsCRP1* gene was overexpressed (Fig. 2b). Taking together, these data indicate

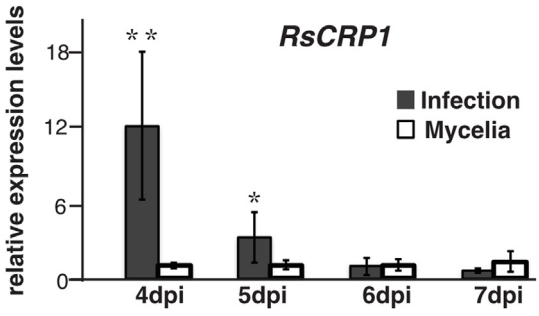

Fig. 1. The *RsCRP1* gene is highly induced upon early infection stages. Relative transcript levels were analyzed in sugar beets 4, 5, 6 and 7 dpi. *R. solani* mycelia was grown in PDB medium and used as a control. Data were normalized to the expression levels of the *G3PDH* gene according to the $2^{-\Delta\Delta Ct}$ method. Asterisks (*p < 0.05, **p < 0.01) indicate statistically significant differences between columns at the same time point according to Student's t-test. Error bars represent SD and is based on at least three biological replicates.

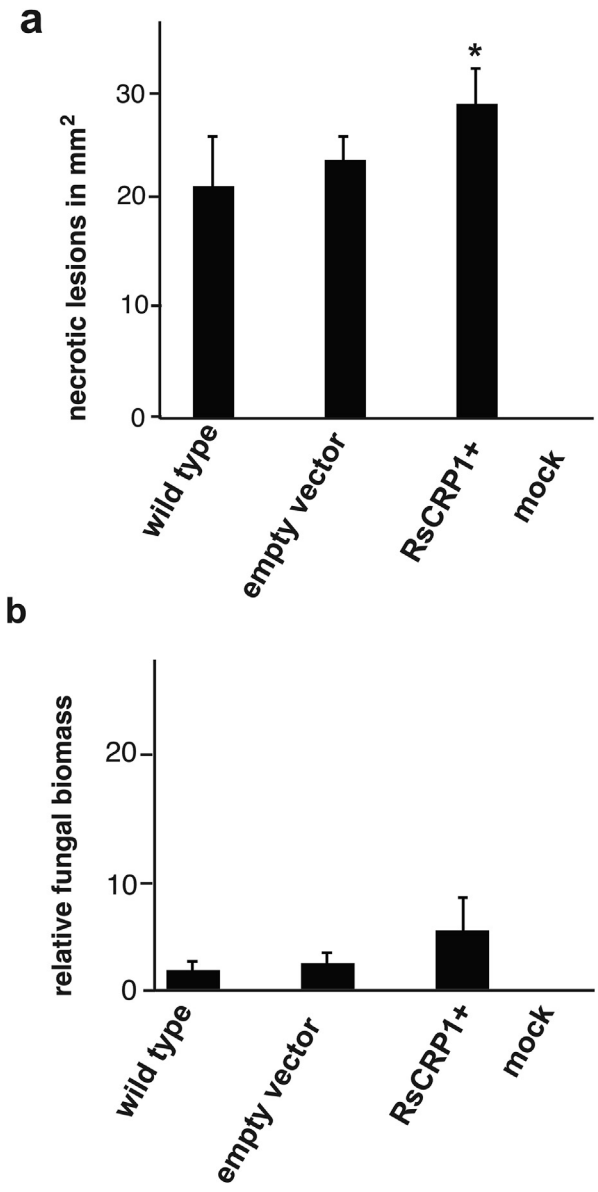

Fig. 2. Overexpression of the *RsCRP1* gene promotes *C. beticola* disease development. a) Symptoms in sugar beet leaves. b) Area (mm^2) of necrotic lesions in sugar beet leaves. c) *C. beticola* DNA biomass in infected leaves. Data show the average of three independent overexpression strains each includes three biological replicates 7dpi. Asterisk (*$p < 0.05$) indicates statistically significant differences according to Student's t-test.

that RsCRP1 is involved in disease development at the early stages of the infection process.

3.3. Transient expression of RsCRP1 does not suppress PTI-related HR

A broad variety of effectors have been found in secretomes of different *R. solani* strains. In the rice sheath blight disease pathogen, *R. solani* AG1 IA, three effectors associated with necrosis are found among other categories such as carbohydrate-active enzymes [8]. A cell death-inducing effector was later identified in this genome together with RslA_NP8 [23,24]. In *R. solani* AG8, AG1-IA and AG3 secretomes, a xylanase and a protease are involved in the cell death process as well [25]. Further, our previous data showed that *R. solani* deploys LysM effectors to suppress chitin-induced immunity similar to hemibiotrophic ascomycete pathogens [18].

To generate additional functional data on RsCRP1, it was transiently expressed in *N. benthamiana* plants using a construct where *RsCRP1* was driven by the *35S* promoter. No necrosis was observed in the Agro-infiltrated area, indicating that the RsCRP1 effector is not involved in this process (Fig. 3). It is also known that certain effectors suppress immune responses such as HR [26]. In case of *R. solani* the newly found effector RsRlpA (a rare lipoprotein A) has this feature, suggesting that *R. solani* deploys effectors to suppress basal immune responses [27]. To investigate whether RsCRP1 functions as a suppressor of programmed cell death, the Avr4/Cf4 complex was used. The Avr4 is a chitin-binding effector from the tomato pathogen *Cladosporium fulvum* and recognized by the tomato PRR Cf4 leading to a strong HR [28,29]. Our data showed that RsRlpA was not able to suppress PTI-related HR induced by this complex (Fig. 3).

3.4. RsCRP1 targets plant mitochondria and chloroplasts

Knowledge on fungal effectors has expanded over the last years and it is known that they can be localized in different parts of host cells such as apoplast, nucleus and vacuoles [2]. To get insights to the subcellular localization of RsCRP1 in host cells, it was fused with the GFP fluorescence protein at the C-terminus, keeping its signal peptide intact followed by transiently expression in *N. benthamiana* leaves. Examination under confocal microscope 48 h post infiltration showed that RsCRP1 targeted distinct cell compartments, a novel feature for this pathogen (Fig. 4). To clarify localization, RsCRP1-GFP was co-expressed with the ScCOX4-mCherry, a marker of mitochondria [30]. Co-localization was observed, indicating accumulation of RsCRP1-GFP in this organelle (Fig. 4). In addition, co-localization between RsCRP1-GFP and chlorophyll was also seen, suggesting chloroplasts targeting as well (Fig. 4). Analysis of the RsCRP1 amino acid sequence revealed presence of a chloroplast transit peptide (cTP) at the N-terminus, and prediction of localization to the mitochondrial matrix, further support organellar accumulation of this effector.

Effector localization to chloroplasts and mitochondria of host plants has been mostly reported in host-bacteria interactions [31–34]. Similar observations have also emerged from the poplar rust fungal basidiomycete *Melampsora larici-populina* [35]. This fungal pathogen is thought to use its chloroplast-targeted protein 1 (CTP1) effector to subvert host cell machinery for protein sorting [36]. CTP1 also accumulates in the mitochondria [35]. The N-terminus of CTP1 facilitate the organelle targeting. Whether targeting domains that mimics the plant transit system may have evolved via

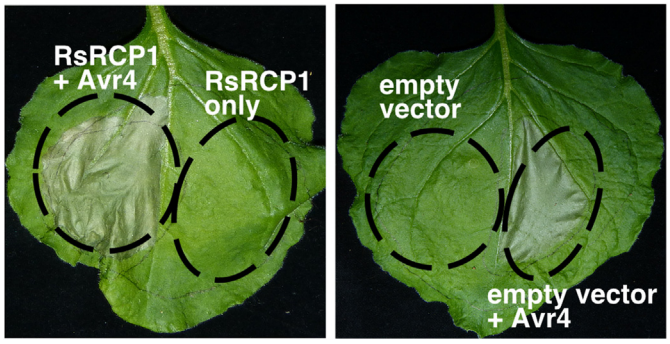

Fig. 3. RsCRP1 does not suppress PTI-induced HR. Leaves were Agro-infiltrated first with the RsCRP1 effector ligated to the pGWB602 binary vector driven by the 35S promoter, followed by HR challenge 24hpi with the Avr4 effector derived from *Cladosporium fulvum* in Cf-4 transgenic *N. benthamiana* plants. Agro-infiltration with empty vector was used as a control. Images taken 3dpi.

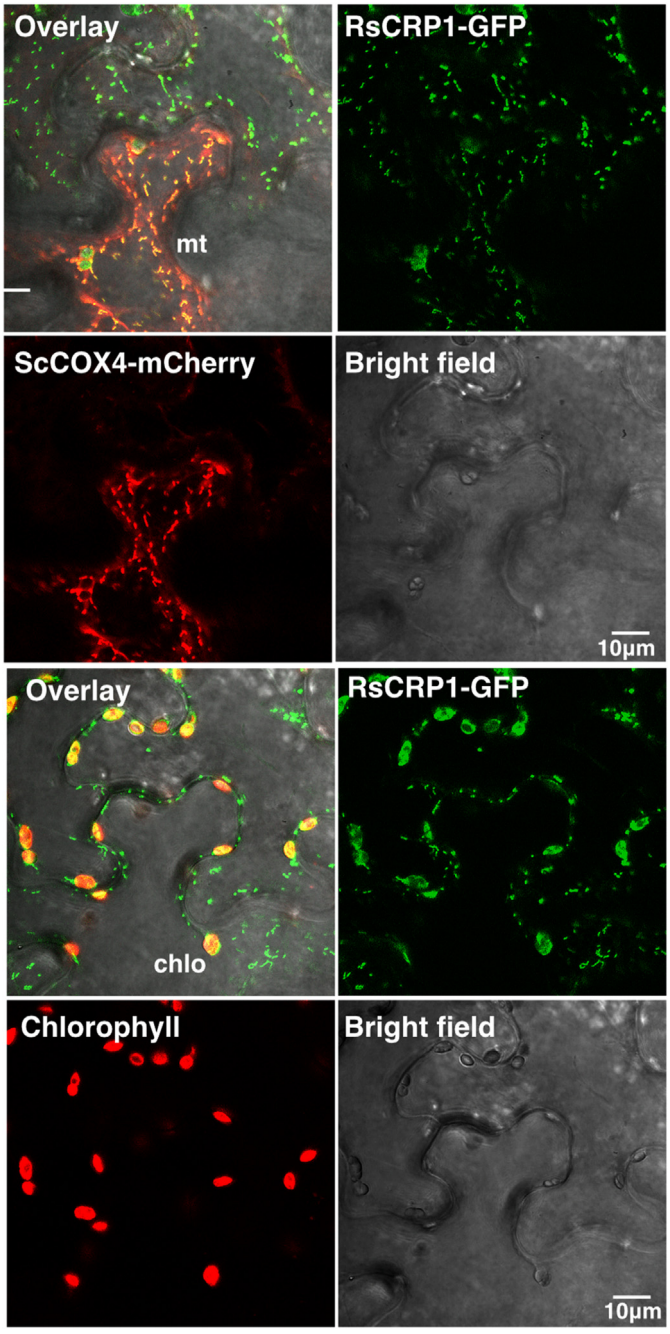

Fig. 4. RsCRP1 is localized to plant mitochondria and chloroplasts. Live-cell imaging of C-terminal GFP-tagged RsCRP1 in *N. benthamiana* leaf epidermal cells. Proteins were expressed in *N. benthamiana* leaves by Agro-infiltration. Monitoring was performed using a laser-scanning confocal microscope with a sequential scanning mode 48 h post infiltration. The GFP and the chlorophyll were excited at 488 nm. GFP (green) and chlorophyll (red) fluorescent signals were collected at 505–525 nm and 680–700 nm, respectively. Mitochondrial localization was assayed using the ScCOX4-mCherry marker (red) and excited at 561 nm and collected at 580–620 nm. (cp): chloroplasts, (mt): mitochondria.

sequence exchange with fungal mitochondrial or horizontal gene transfer process is presently unclear.

Exploitation of an endogenous plant system is an efficient strategy to abate plant defense. To this end, impact on plant cellular compartments added to the list of resistance genes, different phytohormones and gene regulatory pathways that could be targeted by pathogen effectors. Impaired photosynthesis or functions channeled via mitochondria such as production of reactive oxygen species [37] could be an important complement of the *R. solani* effector repertoire affecting other functions than those related to biotrophic or necrotrophic infection stages.

Authors contribution

CD, GT, FD and LH conceived the experiments, GT performed the transformation, confocal microscopy and transient expression in plants and FD performed the virulence assays. All authors contribute to writing of the manuscript and text revision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.01.019.

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