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Article

UVI_02019870, a Puptive Effector from *Ustilaginoidea virens*, Interacts with a Chloroplastic-Like Protein OsCPL1

Shuai Li, Jianming Zhou, Shibo Xiang and Songhong Wei*

Department of Plant Pathology, College of Plant Protection, Shenyang Agricultural University, Shenyang, 110866, China
*Corresponding Author: Songhong Wei. Email: shw@syau.edu.cn
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Abstract: *Ustilaginoidea virens*, which causes rice false smut (RFS), is one of the most detrimental rice fungal diseases and poses a severe threat to rice production and quality. Effectors in *U. virens* often act as a group of essential virulence factors that play crucial roles in the interaction between host and the pathogen. Thus, the functions of individual effectors in *U. virens* need to be further explored. Here, we found a small secreted hypersensitive response-inducing protein UVI_02019870 was highly conserved in fungi. Furthermore, we performed Y2H and BiFC assay to demonstrated UVI_02019870 interacted with OsCPL1, which was predicted as a chloroplast precursor to regulate chloroplast signaling pathways. Our data provide a theory for gaining an insight into the molecular mechanisms underlying the UVI_02019870 virulence function.

Keywords: Effector; *Ustilaginoidea virens*; chloroplast; interaction; pathogenicity

1 Introduction

Rice false smut (RFS) caused by the ascomycetous fungus *Ustilaginoidea virens* (Cooke) Takah (teleomorph *Villosiclava virens*) is one of the most important rice fungal diseases [1–3]. With heavy losses in rice production worldwide, focus on RFS control methods has grown recently. *U. virens* infects the rice florets and forms false smut balls, which is covered by chlamydospore on the infected spikelets (Fig. 1), causing a significant yield loss of up to 50% around the world [4,5]. The false smut balls also contain a variety of mycotoxins, such as ustilaginoidins and ustiloxins. 26 ustilaginoidins derivatives and 7 ustiloxins have been isolated and identified so far. It has been reported that these secondary metabolites, which inhibit assembly of tubulin and mitosis of cells in eukaryotes, are toxic to human and animals [6–10].

When a pathogen and host plant come in contact with each other, several elicitors are released by the pathogen, and plant defense mechanisms are also activated to combat the infection [11,12]. The pathogen contains a pathogen-associated molecules pattern (PAMP), which is recognized by pathogen recognition receptors (PRR) of plant cells, and active defense signals are sent to trigger the PAMP-triggered immunity (PTI) [13,14]. Adapted pathogens also secrete a vast array of effectors into the plant cell to hijack the plant immune system [15]. Evolutionarily, plant cells have developed R genes that express R proteins termed as resistance protein, which detect and recognize pathogen effectors specifically. Such

Figure 1: Disease symptoms of rice false smut. Natural infection of *Ustilaginoidea virens* to rice that cause the fungal diseases. The red arrows indicated false smut balls, which is covered by chlamydospore on the infected spikelets

interaction triggers rapid and robust defense responses, usually accompanied by the hypersensitive response (HR), called effector-triggered immunity (ETI) [16–18].

More and more effectors have been found in plant pathogens which manipulate plant innate immunity through different molecular strategies [19]. For example, SCREs in *U. virens* significantly inhibit defense gene expression and oxidative burst triggered by PAMP, and contributes to full virulence of *U. virens* to rice [20,21]. Slp1 in *Magnaporthe oryzae* and Ecp6 in *Cladosporium fulvum* competitively binds chitin through its LysM domain and host chitin receptors CEBiP and OsCERK, respectively, thereby drastically perturbing the host immune response triggered by chitin and promoting fungal infection [22,23]. Pit2 secreted by *Ustilago maydis* inhibit the activity of apoplastic maize cysteine proteases (CP2), and the *pit2* knockout mutant significantly attenuated *U. maydis* virulence in the host [24]; The core effector Pep1 suppresses oxidative burst driven by peroxidase POX12 and promotes the infection of *U. maydis* in maize [25,26]. A lipase domain-containing protein FGL1 decreases callose formation during *Fusarium graminearum* infection by releasing free fatty acids to inhibit the activity of callose synthase, and therefore, plays an essential role in *F. graminearum* virulence [27]. Furthermore, the effector LysM and AGLIP1, secreted by necrotrophic pathogen *Rhizoctonia solani*, inhibit chitin-induced immunity and promote pathogen infection in the host [28,29].

With the help of the recently-discovered genome, the molecular mechanism of pathogenicity in *U. virens* has been further excavated. *U. virens* encodes at least 628 potential secreted proteins, and 193 of them, characterized with small amounts of amino acids (<400) and cysteine-rich (≥4), have been considered as putative effectors. The cell death inhibition assays in *Nicotiana benthamiana* leaves, together with transcriptome analysis at different periods after pathogen infection, suggest that most effectors could manipulate the plant immune responses and promote the successful colonization of pathogens in the host [2]. Furthermore, many putative effectors induce both host rice and non-host *N. benthamiana* cell death or defense responses. The signal peptides of these proteins are critical to their ability to cause cell death [30]. Collectively, many putative effectors have the ability to suppress or activate plant immunity and play a key role in *U. virens* infection. However, the functions of most effector proteins are still unknown and need to be further explored.

In this study, a putative secreted protein UVI_02019870 was found to be conserved in fungi. UVI_02019870 interacted with chloroplastic-like protein OsCPL1. Our results indicated UVI_02019870 might be required in the virulence of *U. virens*. The research provides new information about the putative

host target of a *Ustilaginoidea virensa* effector, facilitating further study in better understanding of molecular mechanisms of the rice-*Ustilaginoidea virens* interaction.

2 Materials and Methods

2.1 Plant Materials, Pathogen Strains and Growth Conditions

Ustilaginoidea virens isolate strains were cultured using PSA medium (200 g peeled potato extract boiled in water, 20 g sucrose and 16 g agar/L). *N. benthamiana* was growth in an artificial climate chamber at 14 h light (25°C)/12 h dark (23°C). *Agrobacterium* EHA105 for transient expression were cultured using LB medium (10 g tryptone, 5 g yeast extract and 10 g NaCl/L). Yeast strain Gold was cultured using YPDA medium (10 g yeast extract, 20 g peptone, 20 g glucose, 0.03 g adenine hemisulfate/L). In this study, the concentrations of antibiotics were used as followed (μg/ml): rifampin, 25; kanamycin, 50, ampicillin, 50. All data were repeated at least three times, and the results were similar. Strains and plasmids used in this study were listed in Tab. 1.

Table 1: Strains and plasmids used in this study

Strains/plasmids	Characteristics	References or source
Escherichia coli		
DH5a	High efficiency transformation	Lab collection
Yeast strain		
Gold		Clontech Co., Ltd.
Plasmids		
pGADT7	Expression vector in Yeast for Y2H assay, $Amp^{R,\ 1}$	Clontech Co., Ltd.
pGADT7-*OsCPL1*		This study
pGBKT7	Expression vector in Yeast for Y2H assay, $Ka^{R,\ 2}$	Clontech Co., Ltd.
pGBKT7-*UVI_02019870*		This study
pSPYCE	Expression vector in *Nicotiana benthamiana* for BiFC assay, Ka^R	[33]
pSPYCE-*UVI_02019870*		This study
pSPYNE	Expression vector in *Nicotiana benthamiana* for BiFC assay, Ka^R	[33]
pSPYNE-*OsCPL1*		This study

[1] Amp^R means ampicillin resistance.
[2] Ka^R means kanamycin resistance.

2.2 Plasmids Construction

The total RNA of *U. virens* was extracted using the RNA extraction kit (TaKaRa), and the concentration and quality of that were determined by NanoDrop 2000. The complementary DNA (cDNA) synthesis was performed using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa). The full-length of UVI_02019870 coding sequence amplified by *Phanta* Max ultra-fidelity DNA polymerase using the cDNA as a template. Primers used in this study are listed in Tab. 2.

2.3 Yeast Two-Hybrid Screening

The Matchmaker™ Gold yeast two-hybrid system (Clontech) was used for protein-protein interaction screening in this study [31,32]. The coding sequence of *UVI_02019870* was cloned into pGBKT7 to generate

Table 2: The designed primers used in this study

Purpose of use	Primer name	DNA sequence
Y2H	OsCPL1-pGADT7-NdeI-F	GATTACGCTCATATGATGGCCGCTCTTTCCTCTG
	OsCPL1-pGADT7-EcoRIR	TTAGTTGACGGTGACCTTGCCACCCGGGTGGAATTC
	UVI_02019870-pGBKT7-EcoRI-F	ATGGCCATGGAGGCCGAATTCATGAAGACCTCTGTTGTCGCTCTC
	UVI_02019870-pGBKT7-PstI-R	CTAGTTATGCGGCCGCTGCAGGTTACTTGCAGAGCTGGGCAGT
BiFC	UVI_02019870-CE-BamHI-F	GCCACTAGTGGATCCATGAAGACCTCTGTTGTCGC
	UVI_02019870-CE-XhoI-R	AGCGGTACCCTCGAGCTTGCAGAGCTGGGC
	OsCPL1-NE-BamHI-F	GCCACTAGTGGATCCATGGCCGCTCTTTCCTCTG
	OsCPL1-NE-XhoI-R	TTAGTTGACGGTGACCTTGAGCGGTACCCTCGAG

bait for screening in rice cDNA library. The cDNA was synthesized by OE-Biotech Co., Ltd. (Shanghai, China). For one-to-one validation, The coding sequence of *UVI_02019870* and *OsCPL1* were cloned into pGBKT7 and pGADT7, respectively. Preparation of yeast competent cells and transformation were performed using a Frozen-EZ Yeast Transformation II Kit™ (ZYMO Research) following the manufacturer's instructions. The constructed pGBKT7 and pGADT7 plasmids were pairwise co-transformed into the yeast strain Gold. The protein-protein interaction in yeast was analyzed on the SD double dropout (DDO, SD/-Trp-Leu) medium and SD quadruple dropout (QDO, SD/-Trp-Leu-His-Ade) medium plates.

2.4 Bimolecular Fluorescence Complementation Assays

The full-length of *UVI_02019870* were in frame fused with the 5'-end of coding sequence of YFP in pSPYNE and *OsCPL1* was cloned into pSPYCE using the respective specific primers (Tab. 2) [33]. The constructs were transformed into *Agrobacterium* strain EHA105 using the freeze-thaw method [34]. Overnight-cultured *Agrobacterium* strains were collected and re-suspended in induction medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$ and 150 μM acetosyringone) to a final concentration of OD600 = 0.5. After incubating at room temperature for 2 h, *Agrobacterium* cultures with the pSPYNE and pSYPCE constructs were co-infiltrated into leaves of 4–5 week-old *N. benthamiana* plants. YFP or green fluorescence in the infiltrated *N. benthamiana* leaves was monitored using confocal microscopy (Olympus FV3000).

3 Results

3.1 UVI_02019870 Is Conserved in Fungi

Though BLAST searches against the EMBL-EBI database (https://www.ebi.ac.uk/), UVI_02019870 was found to encodes a small protein consisting of 151 amino acids with a signal peptide (SP) at the first

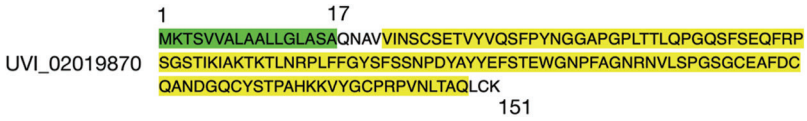

Figure 2: Sequence analysis of UVI_02019870. The predicted structures of UVI_02019870 with 151 amino acids. Green shadow indicates signal peptide with the first 17 amino acid. Yellow shadow indicates hypersensitive response-inducing protein elicitor

Table 3: The hypersensitive response-inducing protein elicitor was conserved in fungi

Fungi	Characteristics	Protein ID (NCBI)	Identity with UVI_02019870 (%)
Ustilaginoidea virens	UVI_02019870	UVI_02019870	100
Magnaporthe oryzae	Chain A, elicitor protein Hrip2	5FID_A	66.91
Diaporthe ampelina	Putative alkaline foam protein b	KKY29658.1	67.76
Xylaria longipes	Hypothetical protein	RYC59563.1	69.39
Rosellinia necatrix	Putative 16 kDa allergen	GAP83028.1	65.13
Eutypa lata	Putative bys1 domain protein	EMR70558.1	68.24
Monosporascus ibericus	Hypothetical protein	RYP04607.1	69.08
Daldinia sp. EC12	Hypothetical protein	OTB13205.1	62.59
Hypoxylon sp. EC38	Hypothetical protein	OTA65356.1	66.00
Chaetomium globosum	Hypothetical protein	XP_001223586.1	69.08
Phialemoniopsis curvata	Uncharacterized protein	XP_030999288.1	69.59
Fusarium oxysporum f. sp. *narcissi*	Hypothetical protein	RYC97243.1	63.16
Claviceps purpurea	Uncharacterized protein	CCE28551.1	75.19
Penicillium griseofulvum	Hypothetical protein	KXG52779.1	76.82
Aspergillus udagawae	Hypothetical protein	GFF53674.1	77.33
Metarhizium anisopliae	Hypothetical protein	KFG81213.1	80.79
Pochonia chlamydosporia	Allergen	XP_018140533.1	80.79
Gaeumannomyces tritici	Hypothetical protein	XP_009228976.1	65.65

17 residues of N-terminal. UVI_02019870 was also predicted to be a hypersensitive response-inducing protein elicitor, which is conserved in fungi (Fig. 2 and Tab. 3).

3.2 UVI_02019870 Interacts with Chloroplastic-Like Protein OsCPL1 in Yeast

To investigate the molecular mechanism underlying the virulence of UVI_02019870 in rice, the yeast two-hybrid (Y2H) system has been performed to preliminarily screen host proteins interacting with UVI_02019870. With the UVI_02019870 as the bait, a chloroplastic-like protein, named as OsCPL1 (LOC_Os06g01210), was identified from a rice cDNA library (Fig. 3). The result indicated UVI_02019870 interacts with OsCPL1 *in vitro*.

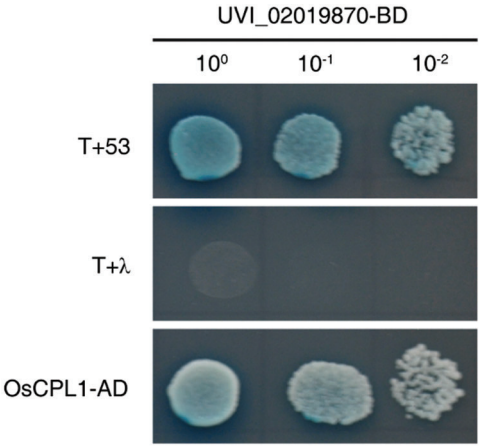

Figure 3: UVI_02019870 interacts with OsCPL1 *in vitro*. The interaction between UVI_02019870 and OsCPL1 was revealed by yeast two-hybrid assays. The yeast strain Gold was co-transformed with pGBKT-*UVI_02019870* and pGADT7-*OsCPL1* and grown on the QDO medium plates. The pGADT7-T plasmid was transformed into yeast with pGBKT7-53 or pGBKT7-Lam for positive and negative controls, respectively. T, pGADT7-T; 53, pGBKT7-53; λ, pGBKT7-Lam

3.3 UVI_02019870 Interacts with Chloroplastic-Like Protein OsCPL1 in N. benthamiana

The *in vivo* interaction between UVI_02019870 and OsCPL1 was further investigated by bimolecular fluorescence complementation (BiFC) in *N. benthamiana* leaves. OsCPL1 and UVI_02019870 were fused in frame with the N-terminal domain (nYFP) and C-terminal domain of yellow fluorescence protein (cYFP), respectively. When the two fusion proteins were co-expressed in *N. benthamiana* leaves, the fluorescence signal was observed in the plant cells. By contrast, no fluorescence was detected when either of the fusions was expressed with empty vectors (Fig. 4). The result demonstrated that UVI_02019870 interacts with OsCPL1 *in vivo*.

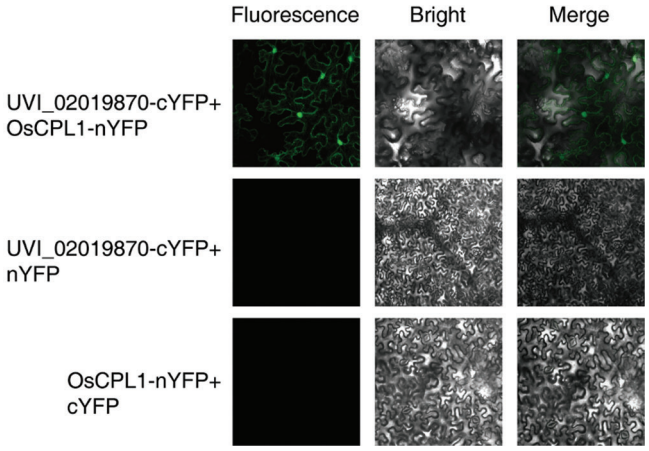

Figure 4: UVI_02019870 interacts with OsCPL1 *in vivo*. The *in planta* interaction between UVI_02019870 and OsCPL1 was indicated by bimolecular fluorescence complementation (BiFC). Strong green fluorescence was observed in the *N. benthamiana* cells co-expressing UVI_02019870-cYFP and OsCPL1-nYFP, but not in the *N. benthamiana* leaves infiltrated with *UVI_02019870-cYFP* and *nYFP* empty vector or with *OsCPL1-nYFP* and *cYFP* empty vector under confocal microscopy at 3 days post agroinfiltration

4 Discussion

Rice false smut, caused by *U. virens*, occurs at the late stage of rice development which reduces grain yield and quality. The disease has been reported in most rice-growing regions of China and emerged as one of the major rice diseases [1,4]. Many studies have been carried out to reduce the yield loss caused by RFS. However, little is known about the molecular mechanism underlying the interaction between rice and *U. virens*. Phytopathogenic microbes secrete the majority of effectors to regulate plant immunity by targeting different host key components [19,35]. More than 600 secreted proteins have been predicted in *U. virens* genome, 193 of which are identified as candidate effectors. The genes encoding many putative effectors are transcriptionally induced during *U. viren* infection in rice via expression profiling analyses, indicating they may be involved in inhibiting immunity-associated responses [2]. In this study, we demonstrated that UVI_02019870 as an effector regulates plant defense responses during pathogen infection.

The core effector shows a similar sequence and conserved motif across species [25,29]. BLAST searches against the EMBL-EBI database indicated UVI_02019870 is a hypersensitive response-inducing protein (Hrip) elicitor, which is similar to MoHrip2 in *M. oryzae* [36], and highly conserved in fungi. Alignment analysis demonstrated that the full length of UVI_02019870 and MoHrip2 showed 66.91% identities (Fig. 2 and Tab. 3). The Hrip-elicitors have been identified to improve plant resistance to pathogen, such as Hrip1 from *Alternaria tenuissima* [37], PaNie from *Pythium aphanidermatum* [38], and MoHrip1 from *M. oryzae* [36]. The defense responses are often accompanied by HR, ion influx, accumulation of NO and production ROS [39]. However, no cell death symptoms were monitored within 3 days after UvHirp1-expressing *Agrobacterium* was inoculated into *N. benthamiana*. Possibly, UvHirp1 induces cell death in a later time after *Agrobacterium* inoculation, or perceived by specific R protein as an avirulence protein to trigger HR in the host. Therefore, the precise function of UvHirp1 will be confirmed by further experiments in rice.

The effectors have been reported to disable the plant immune system using multiple biochemical strategies and by targeting a variety of host proteins [19]. Here, the host target of UVI_02019870 was screened to gain insights to the molecular mechanisms underlying the UVI_02019870 virulence function. OsCPL1 was initially identified to interact with UVI_02019870 through the Y2H system. The *in vivo* interaction of UVI_02019870 and OsCPL1 was subsequently confirmed through BiFC in *N. benthamiana* leaves (Figs. 3 and 4). OsCPL1 is predicted as a chloroplast precursor, which participates in electron transfer between P700 and the cytochrome b6-f complex in photosystem I [40]. Hence, whether UVI_02019870 regulates the chloroplast and immune response of rice by interacting with OsCPL1 remains to be further investigated.

5 Conclusion

In summary, we identified a novel conserved protein UVI_02019870 in *U. virens* as an effector interacts with OsCPL1, which is a putative regulator in chloroplast signaling pathways. However, the precise molecular mechanism of UVI_02019870's role in the interaction between rice and *U. virens* remains to be further invesitaged.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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