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# Cloning, overexpression, and purification of a gene of unknown function of prophage loci from '*Candidatus* Liberibacter asiaticus,' the destructive bacterial pathogen of huanglongbing disease in citrus plants

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ABSTRACT

Citrus Huanglongbing (HLB) or citrus greening is one of the most destructive diseases affecting citrus industry worldwide. The causal agent in Asia is a phloem-limited, Gram-negative bacterium, '*Candidatus* Liberibacter asiaticus' (CLas). Within the genome of CLas lies prophage regions, classified as Type-A, B, C, and D. In particular, Type-D has been indicated to correlate with the blotchy-mottle symptoms of citrus trees. Here we reported the cloning, overexpression, and purification of the ORF1, an open reading frame from the partial Type-D region of CLas obtained from an infected lime tree (*Citrus aurantifolia* Swingle). Overexpression of the ORF1 was toxic to the *E. coli* BL21(DE3), and the transient expression of ORF1 in *Arabidopsis* seedlings by *Agrobacterium*-mediated transformation exhibited rapid and total chlorosis of the seedlings within two days post-transformation. The native-PAGE of the purified protein showed multiple bands, indicative of various conformations in solution. The ESI-TOF mass spectrum confirmed the molecular weight of the purified ORF1 to be 15,364.3150 Da, corresponding to the $[M+1]^+$ of the ORF1 without an N-terminal methionine. The protein predominantly consisted of α-helix as evidenced by circular dichroism (CD), and the transition toward random coil structure upon heating was reversible. The template-based modeling (I-TASSER) of the ORF1 indicated eight α-helices connected through variable loops. The simulated CD spectrum, generated from the atomic coordinates of the I-TASSER model, was notably similar to the experimental spectrum. Our report offers the basis for understanding the contributions of genes within Type-D prophage region toward the disease pathogenicity of citrus HLB.

## 1. Introduction

Citrus Huanglongbing (HLB) is a significant plant disease devastating all species of *Citrus* genus in Thailand and worldwide [1,2]. The disease spreads quickly via psyllids (*Diaphorina citri* Kuwayama) and grafting. Symptoms of huanglongbing disease include blotchy-mottling, yellowing, vein corking, chlorotic shoots, and die-back on susceptible citrus trees. Once infected, the quality of the fruit dramatically deteriorates, and the infected tree dies within 3–5 years. The causal agent of the HLB disease in Asia is '*Candidatus* Liberibacter asiaticus' (CLas), a phloem-limited, Gram-negative bacterium belonging to the alpha subdivision of proteobacteria [3]. Since the culture of CLas bacterium has not been successfully maintained outside the plant host, the detailed characterization of this important pathogen is highly limited. In 2009, Duan and co-workers reported the first complete genome sequence of the CLas strain Psy62 using metagenomic method [4]. The CLas bacterium has a significantly reduced genome of 1.23 Mb.

In 2011, the first report on the HLB phage infection in CLas strain UF506 revealed two homology prophage SC1 (40,048 bp) and SC2 (38,997 bp) insertions [5]. Since then, several reports on the presence of prophage regions in the CLas genomes have emerged [6–10]. It is well known that the prophage DNA plays an essential role in the

*Abbreviations:* CLas, '*Candidatus* Liberibacter asiaticus'; *E. coli*, *Escherichia coli*; *A. tumefaciens*, *Agrobacterium tumefaciens*; *A. thaliana*, *Arabidopsis thaliana*; *C. roseus*, *Catharanthus roseus*; *N. benthamiana*, *Nicotiana benthamiana*; $OD_{600}$, optical density at 600 nm; LB, Luria-Bertani; IPTG, isopropyl-β-D-1-thiogalactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; PVP, polyvinylpyrrolidone; MES, 2-(*N*-morpholino)ethanesulfonic acid; MS, Murashige and Skoog medium; PMSF, phenylmethanesulfonyl fluoride

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evolution of bacterial pathogenicity [11] as previously demonstrated in *Ralstonia solanacearum*, a causal agent of the bacterial wilt disease in many essential crops [12]. In CLas, two hypervariable prophage genes with intragenic tandem repeats, $lasA_I$ and $lasA_{II}$, were characterized as virulent extracellular proteins [13]. Besides, dynamic populations of CLas were analyzed based on prophage regions, and the correlations between the HLB symptoms and prophage diversity were established [10]. The prophage/phage-mediated dynamics of CLas population were classified as Type-A, B, C, and D. Particularly; the Type-D region revealed a third prophage (iFP3, 11,121 bp), which was correlated with the blotchy mottle symptoms and was absent in CLas infected psyllid vector. This prompt us to investigate the role of genes in Type-D prophage region on the HLB disease.

Herein, we have reported the cloning, overexpression, and purification of ORF1, an open reading frame in the partial Type-D region of CLas Thailand strain. The protein was stable and existed in multiple conformations in solution. The heterologous overexpression of ORF1 was toxic to the *E. coli* host cells, and the *Agrobacterium tumefaciens*-mediated transient expression of ORF1 in *Arabidopsis thaliana* resulted in a rapid and total chlorosis of the seedlings. The protein consisted of mostly α-helix with the apparent melting temperature of approximately 40 °C as evidenced by CD spectroscopy. The template-based model generated via I-TASSER web server indicated the helix structure, and the simulated CD spectrum of the model was similar to the recorded spectrum. This preliminary work will facilitate further investigation of the role of Type-D prophage genes toward the HLB disease.

## 2. Materials and methods

### 2.1. Bacterial strains, plants, plasmids, media, and chemicals

Cloning experiments were conducted using *E. coli* strain DH5α. Overexpression of the recombinant proteins was conducted using *E. coli* strain BL21(DE3). The transient expression of the protein in *A. thaliana* was performed using *Arabidopsis efr-1* mutant [14] and *A. tumefaciens* strain GV2660. Plasmids pTDORF1 and pTDORF2 were derived from the pET28b expression vector (Novagen), while plasmids pAGORF1 and pAGORF2 were created using the pCXSN-HA. Host cells were grown in LB (Luria Bertani) medium containing 1% tryptone, 0.5% yeast extract, and 1% sodium chloride supplied with kanamycin to a final concentration of 50 μg/mL. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich, Merck, and Amersham Biosciences (Currently GE Healthcare Life Sciences). Restriction endonucleases were obtained from New England Biolabs. Plasmid purification was conducted using Presto™ Mini Plasmid Kit (Geneaid). Affinity chromatography was executed using Ni-NTA agarose (Qiagen). Vivaspin™ sample concentrators were purchased from GE Healthcare Life Sciences. DNA sequencing was completed by MACROGEN (Macrogen Inc., Seoul, South Korea). All primers used in this study were listed in Table 1.

### 2.2. Sample collection and total DNA extraction

The symptomatic leaves of *Citrus aurantifolia* Swingle were collected from Trang province, Thailand (7°39′22.6″N 99°19′50.0″E). Total DNA was isolated using 0.2 g of the CLas-infected leaf midribs, according to the previously reported protocol [9]. Briefly, the midribs were ground in liquid nitrogen to a fine powder and mixed with an extraction buffer (100 mM Tris-base, 50 mM EDTA, 500 mM NaCl, 2.5% PVP, 1.5% SDS and 10 mM β-mercaptoethanol), before incubating at 65 °C for 30 min. Subsequently, 400 μL of 5 M potassium acetate was added, and the sample was placed on ice for 30 min, centrifuged at 20,290 g at 4 °C for 5 min. The supernatant was transferred to a new 1.5 mL centrifuge tube. The total DNA was precipitated using 600 μL of cold isopropanol, washed twice with 75% ethanol, resuspended in 100 μL of sterile water, and kept at −20 °C until use.

**Table 1**
Primers used in this study.

| Primer | Sequence | Source |
|---|---|---|
| **Primers for CLas and Type-D detection** | | |
| **CGO3F** | 5′-RGGGAAAGATTTTATTGGAG-3′ | [15] |
| **CGO5R** | 5′-GAAAATAYCATCTCTGATATCGT-3′ | [15] |
| **LJ759** | 5′-AAACATCCACCCCCGAACC-3′ | [10] |
| **Primers for protein expression** | | |
| **ORF1f** | 5′-CTTTAAGAAGGAGATATACCATGGGCCATCACCATCA CCATCACATGGGAGCATTAAAGAAT-3′ | This study |
| **ORF1r** | 5′-GTGGTGGTGGTGGTGCTCGAGTTAAATCTGATTG GCATA-3′ | This study |
| **ORF2f** | 5′-CTTTAAGAAGGAGATATACCAATGGGCCATCACCAT CACCATCACATGGGACAATTAAAGCGA-3′ | This study |
| **ORF2r** | 5′-GTGGTGGTGGTGGTGCTCGAGTTAAATCTGACT GGCGTA-3′ | This study |
| **Primers for transient expression in *A. thaliana*** | | |
| **Agrof** | 5′-TGTTCCAGATTACGCTCCAATACTTATGGGCCATCA CCATCACCATCAC-3′ | This study |
| **Agror** | 5′-AAATTCGCTAGTGGATCCCCAATACATCTCAGTGGTG GTGGTGGTGGTG-3′ | This study |

### 2.3. PCR detection of CLas and Type-D

The 16s rDNA specific primers for CLas, CGO3F and CGO5R, were used in the conventional PCR detection of CLas using the total DNA extract obtained from the symptomatic lime leaf midribs as a template [15]. The PCR cycles started with denaturation at 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 4 min, and the final extension at 72 °C for 10 min. The PCR products were analyzed using agarose gel electrophoresis. A similar protocol was conducted to confirm the presence of Type-D region using an inverted repeat primer LJ759 [10].

### 2.4. Plasmid construction

The PCR product of the partial Type-D region amplified using an inverted repeat primer LJ759 was cloned into pGEM®-T Easy (Promega) to afford pDSPCTD-I, which was then used as a template for the amplification of genes encoding ORF1 and ORF2. The forward primers, ORF1f and ORF2f, allowed the introduction of flanking *NcoI* site with an N-terminal six-histidine affinity tag, whereas the reverse primers, ORF1r and ORF2r, contained the *XhoI* site to facilitate cloning into the pET28b expression vector. The chemically competent *E. coli* cells (DH5α) were transformed with each ligated product, and positive transformants were selected on LB agar plates containing 50 μg/mL kanamycin. The resulting constructs, pTDORF1 and pTDORF2, were used as templates for the amplification of genes encoding ORF1 and ORF2 using Agrof and Agror primers. The subsequent PCR products were used to construct pAGORF1 and pAGORF2 via the ligation-independent cloning (LIC). The identity of the construct was confirmed by DNA sequencing of the entire open reading frame. All vector maps related to this report can be found in the Supporting Information.

### 2.5. Growth studies of E. coli

The growth of *E. coli* strain BL21(DE3) overexpressing the ORF1 and ORF2 were evaluated. The 5 mL overnight culture of a single colony in an LB broth supplied with 50 μg/mL kanamycin was used to inoculate 300 mL culture of the same medium at 37 °C to an initial $OD_{600}$ of 0.05. The culture was then incubated at 37 °C with agitation until the $OD_{600}$ reached 0.2. Then, protein expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The growth was monitored for 8 h. No expression control for each protein was performed using cultures supplemented with 50 μg/mL kanamycin and 0.5% glucose. The experiments were run in triplicate and the average values of $OD_{600}$, along with the standard

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