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Research Article

Expression of Secretory Protein Genes in *Candidatus Liber Ibacter Asiaticus*

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Abstract

Huanglongbing (HLB) is the most destructive disease of citrus worldwide. The disease is caused by *Candidatus Liberibacter spp.,* which is vectored by the *psyllids Diaphorinacitri Kuwayama* and *Triozaerytreae*. Secretory proteins are important in bacterial pathogenesis and structure components. Some of them are expressed at a high level. To obtain the highly-expressed Secretory Protein Genes (SPGs) for antiserum preparation, ten candidate SPGs were chosen from *Candidatus LiberibacterAasiaticus* by bioinformatic analysis and were further tested by PCR, qPCR, and RT-qPCR methods, respectively. The result showed that two SPGs, 408 and pap (both are Flp pilus assembly protein genes), have relative high amounts of DNA and RNA transcripts of early HLB-infected green orange leaves. The 408 and pap genes were further constructed into the plant expression vector pCAMBIA1300 (GV1300: GFP) and expressed in tobacco leaf epidermal cells for subcellular localization analysis. The transient expression results indicated that the 408 protein is located in the nuclei and cytoplasms of tobacco leaf cells. However, the pap protein is located in the radially-arranged micro-fibrils of tobacco guard cells, which may help the pathogen invade into plant cells. This research is an important foundation for the preparation of the antiserum against *Candidatus Liberibacter Asiaticus* and the early detection of HLB disease.

Keywords: *Candidatus liberibacter asiaticus*; Secretory protein; DNA amount; RNA transcription; Subcellular localization

Abbreviations

HLB: Huanglongbing; SPGs: Secretory Protein Genes; ELISA: Enzyme-Linked Immunosorbent Assay; N. benthamiana: Nicotiana benthamiana; 1st Cdna: First strand cDNA; *E.Coli*: *Escherichia Coli*; H.P.I.: Hours Post Inoculation; LAMP: Loop-Mediated Isothermal Amplification

Introduction

Citrus Huanglongbing (HLB), or greening disease, is a devastating disease that seriously threatens the development of the citrus industry globally [1-3]. Currently, the disease has been found in about 50 countries in the Asia, Africa, Oceania, South America, and North America regions. In China, 11 of the 19 major citrus-producing areas suffer from the HLB disease [1,4-6]. Citrus Huanglongbing is caused by the pathogen of *Candidatus Liberibacter Asiaticus*, africanus, and americanus, a Gram-negative bacterium, which belongs to the genus *Candidatus Liberibacter* [7,8]. There are no effective therapeutic agents or ideal resistant varieties for now. Integrated control management of HLB occurs mainly through controlling psyllids in field areas, removing HLB-infected trees, and planting healthy nursery trees. Of these three steps, the effective removal of infected trees depends on an accurate diagnosis of HLB at the early infection stage [8-10].

The content of *Candidatus Liberibacter Asiaticus* is low in infected trees and unevenly distributed in different parts of diseased plants [11,12]. Therefore, the establishment of an efficient and sensitive detection method for diagnosis of HLB at the early infection stage is a key factor for healthy development of the citrus industry. In recent years, with the fast development of the green orange industry in Hainan Province of China, the citrus Huanglongbing also spread rapidly [13]. At present, a rapid and large-scale field detection method for the pathogen mainly depends on protein technology, e.g., Enzyme-Linked Immuno Sorbent Assay (ELISA) [14-16]. However, a commercial large-scale detection method based on the protein level for HLB disease is yet to be developed.

There are six types of protein secretion system (type's I-VI) in Gram-negative bacteria [17,18]. Each type of protein secretion system consists of a series of proteins with specific structures and functions. Through these protein secretion systems, Gram-negative bacteria can release various toxic factors and effector factors to the extracellular environment or into the host cell to cause infection, which eventually leads to various diseases [18,19]. Therefore, it is an ideal gene for the preparation of the antiserum against the *Candidatus Liberibacter Asiaticus* because the content of secreted protein is often 100 to 1000 times higher than the number of its pathogen. Studies have shown that the pathogen of CandidatusLiberibacterasiaticus has an incomplete type III and type IV protein secretion system but has a complete type I protein secretion system [20,21].

In this study, 10 candidate Secretory Protein Genes (SPGs) from *Candidatus Liberibacter Asiaticus* were chosen by bioinformatics analysis and two SPGs of 408 and pap with relatively high DNA and RNA contents were identified by PCR, qPCR, and RT-qPCR methods. Furthermore, the 408 protein was located in the nuclei and

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Table 1: Characteristic summary of ten candidate secretory protein genes from *Candidatus Liberibacter Asiaticus*.

Protein	Region name	Protein ID	Protein function	Type of protein
ATPase	Sun T	ACT56858.2	ABC-type protease/lipase transport system, ATPase and permease components	Type I secretion system
mfp	Type IhlyD	ACT56859.1	Membrane-fusion protein	Type I secretion system
Serralysin	Peptidase M10C	ACT56857.1	RTX toxins and related Ca^{2+}-binding proteins	Secreted protein
408	T2SS-T3SS pilN	ACT57211.2	Flp pilus assembly protein, secretin CpaC	Secreted protein
24A	Peptidase A24	ACT57202.1	Type II secretory pathway, prepilin signal peptidase PulO and related peptidases	Type II secretory pathway
pap	CpaC	ACT57200.1	Flp pilus assembly protein, secretin CpaC	Secreted protein
MSr	fliF	ACT57168.1	Flagellar biosynthesis/type III secretory pathway lipoprotein	Type III secretory pathway
msp	FliN	ACT57161.1	Flagellar motor switch/ Predicted secreted (periplasmic) protein	Secreted protein
fATP	fliI	ACT57157.1	Flagellar biosynthesis/type III secretory pathway ATPase	Type III secretory pathway
377	COG5462	ACT57577.1	Predicted secreted (periplasmic) protein	Secreted protein

The database sources: NCBI Reference Sequence database (http://www.ncbi.nlm.nih.gov)

cytoplasms of tobacco cells, while the pap protein was localized in the microfibers of tobacco guard cells by Agrobacterium-mediated transformation in tobacco leaf cells for transient expression. The study is an important foundation for the preparation of the antiserum against *Candidatus Liberibacter Asiaticus* to be used for the early detection and prevention of citrus HLB.

Materials and Methods

Materials

In 2016, the QH sample was mixed from five early HLB-infected green orange leaves with asymptomatic disease in Qionghai County, Hainan Province, and the QZ sample was mixed from another five early HLB-infected green orange leaves with asymptomatic disease in Qiongzhong County, Hainan Province. Wild-type *Nicotiana benthamiana (N. benthamiana)* (Ferox genus) was kept in the Laboratory of Molecular Virology, Institute of Tropical Bioscience and Biotechnology (ITBB), Chinese Academy of Tropical Agricultural Sciences (CATAS). The GV1300 plasmid (pCAMBIA1300: GFP) was provided by Professor Ming Peng of ITBB, CATAS.

Primers design, DNA, and cDNA preparation

In order to identity one or two high-expression SPGs, ten SPGs were chosen from the different protein secretory systems according to the complete genome sequence of *Candidatus Liberibacter Asiaticus* [22] (Table 1). Based on the nucleotide sequences of these SPGs, ten primer pairs (Table 2) were designed for PCR assay by Primer Premier 5 software (Premier Bio soft International, Palo Alto, CA, USA). Furthermore, six primer-pairs of the 408, 24A, fATP, pap, msp, and 377 genes were also designed for qPCR or RT-qPCR using the online website (https://www.idtdna.com/Scitools/Applications/RealTimePCR/) (Table 3). 18S rRNA of citrus was used as an internal reference gene [23] (Table 3). All primers were synthesized by the Beijing Genomics Institute (BGI).

Total DNA extraction of the QH and QZ samples was performed according to the manufacturer's instructions with a Plant Genomic DNA Kit (TIANGEN BIOTECH, Beijing, China) and total RNA extraction was performed according to the Trizol Universal Regent (TIANGEN BIOTECH, Beijing, China). First-strand cDNA was synthesized from 2.0 μL of total RNA using 0.5 μL of random hexamer primer (10 μM) and the Fast Quant RT Kit (with gDNase) (TIANGEN BIOTECH, Beijing, China).

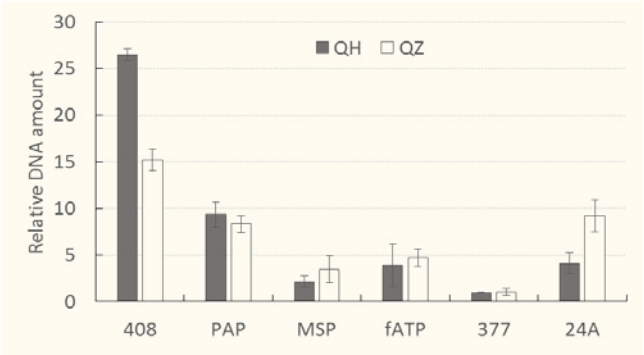

Figure 1: Relative DNA amount of six selected candidate secretory protein genes in QH and QZ samples. Statistical analysis was performed using student's t-test. "*" represents $P < 0.05$.

Screening of candidate SPGs and establishment of real-time quantitative PCR (qPCR)

PCR assay was firstly conducted to identify the possible candidate SPGs from the QH and QZ samples. PCR reactions were performed by using 2× HS™ Mix kit (Dongsheng BIOTECH, Guangzhou, China): 12.5 all of 2× HS™ Mix, 0.5 all of the forward primer (10 μM), 0.5 μL of reverse primer (10 μM), and 1 μL of DNA template, with ddH_2O added to 25 μL. The PCR program involved pre-denaturing at 94°C for 3 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, extending at 72°C for 90s, and finally, the reaction was terminated by post-extending at 72°C for 10 min.

In order to measure the efficiency and correlation coefficients of six SPG primer pairs in qPCR, the initial amplified DNA template was further diluted to $1:10^{-1}$, $1:10^{-2}$, $1:10^{-3}$, $1:10^{-4}$, and $1:10^{-5}$ by ddH_2O, and these samples were used to establish the standard curves for each pair primer in the Strata gene Mx3005 machine. The results showed that six qPCR systems with the specific primer pairs amplified SPGs highly efficiently (efficiencies of 86.8% to 92.2%) with correlation coefficients between 0.981 and 0.999.

In order to measure the relative DNA amounts and their RNA expression levels of six SPGs, qPCR, and RT-qPCR analyses were performed on an Agilent Stratagem Mx3005P instrument using the

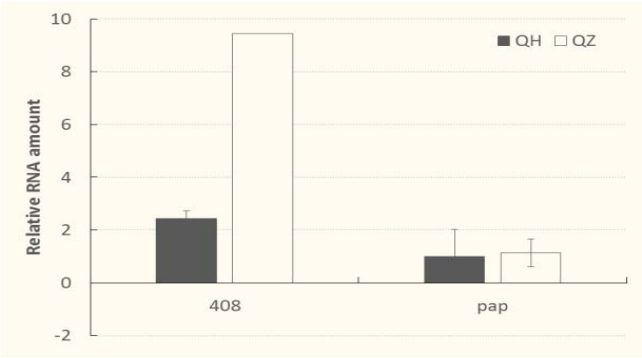

Figure 2: Relative RNA amount of 408 and pap candidate secretory protein genes in QH and QZ samples. Statistical analysis was performed using student's t-test. "*" represents $P < 0.05$.

Hieff™ qPCR SYBR Green Master Mix with Low Rox Plus (Yeasan, Shanghai, China) according to the instructions, and each gene was measured three times independently. The total DNA from healthy green orange leaves was used as a negative control. The qPCR or RT-qPCR mixture was 10 μL of Hieff™ qPCR SYBR Green Master Mix, 0.4 μL of Forward Primer (10 μM), 0.4 μL of Reverse Primer (10 μM), 1 Ml of template DNA or cDNA, and 8.2 μL of ddH_2O. The qPCR and RT-qPCR programs involved pre-denaturing at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 10 s, annealing at 55°C for 30 s, extending at 72°C for 20 s, and a dissolution curves program using the Agilent Strata Mx3005P instrument.

Recombinant plasmid construction and subcellular localization of 408 and pap proteins

The 408 gene was amplified with the 408-gF/408-gR primers, while the pap gene was amplified by using the pap-gF/pap-gR primers (Table 1). The PCR reaction was conducted using PrimeSTAR HS DNA Polymerase kit (Takara, Dalian, China): 0.5 μL of PrimeSTAR HS DNA Polymerase (2.5 U/μL), 10 μL of 5 × PrimeSTAR Buffer (Mg^{2+} plus), 4 μL of dNTP Mixture (2.5 mM each), 2 μL of F/R (5 μM) primer, and 2 μL of total DNA, and ddH_2O was added up to 50 μL. The PCR program involved pre-denaturing at 98°C for 3 min, followed by 35 cycles of denaturing at 98°C for 30 s, annealing at 55°C for 30 s, extending at 72°C for 50 s; and finally, the reaction was terminated by post-extending at 72°C for 10 min. The amplified target fragments of 408 and pap were gel-extracted by using the DNA Gel Extraction Kit (Omega Bio-Tek, Doraville, GA, USA) and subsequently, cloned into the plant expression vector GV1300 using T4 DNA ligase (Takara, Dalian, China). The recombinant plasmid was further transformed into *Escherichia coli (E. coli)* Trans 5a competent cells (Trans Gen, Beijing, China), and three positive clones were selected for bidirectional sequencing by 1300-F and 1300-R primers at Thermo Fisher (Guangzhou, China).

The recombinant plasmids of GV1300, GV1300-408, and GV1300-pap were transformed into *Agrobacterium tumefaciens* GV3101 competent cells by the freeze-thaw method, as described in Sparkes and Al [24]. The transfected tobacco leaves were cut into pieces of 1 cm × 1 cm, and fluorescence images were visualized on a microscope (FluoView FV1000D IX81; Olympus, Tokyo, Japan) to observe the subcellular localization of the fusion protein under wavelengths of 488 nm and 546 nm.

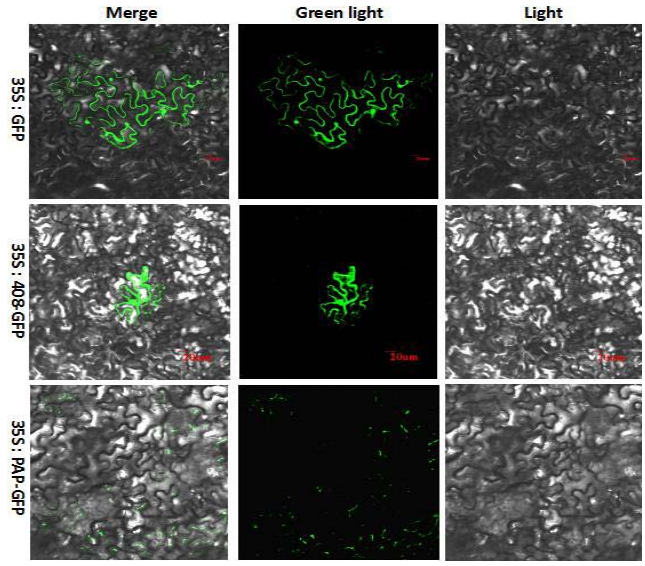

Figure 3: Subcellular localization of GFP, 408-GFP and PAP-GFP in tobacco leaf cells under the light of 488 nm and 546 nm. 35S, a constitutive promoter from the cauliflower mosaic virus; GFP, green fluorescent protein; Bar represents 20 μm.

Results

Preparation of total DNA and cDNA from diseased green orange leaves

Total DNA extracted from two mixed samples were visualized on a 1% agarose gel, and the specific DNA bands of more than 10 kbp in lengths were consistent with the predicted sizes. Total RNA samples were also extracted from these two samples and were visualized on a 1% agarose gel. The results indicated that the RNA bands of 28S, 18S, and 5S were abundant which suggests that a high quality of total RNA was obtained. The total RNA was further used to synthesize the first strand cDNA (1st cDNA) which subsequently could be used for RT-qPCR.

Screening of candidate SPGs from HLB-infected green orange leaves

PCR assayindicated that 408, 24A, fATP, pap, msp, and 377 gene candidate SPGs were amplified and the length of each gene was consistent with the expected size. However, no band occurred due to the amplification of the ATPase, Serralysin, MSr, and MFP genes (Supplemental Figure 1). The target DNA fragments of these six genes were gel-extracted with the DNA Gel Extraction Kit (Omega Bio-Tek, Doraville, GA, USA) and subsequently, cloned into a pMD19-T vector (Takara, Dalian, China) to transform them into *Escherichia coli (E. coli)* Trans 5a competent cells (TransGen, Beijing, China). These SPGs were sequenced with RV-M and M13-47 primers at Thermo Fisher (Guangzhou, China).

Analysis of the real-time qPCR showed that the amplification plot of six SPGs and the internal reference gene shown in the "S" curve of QH and QZ samples (Supplemental Figure 2). Further analysis indicated that the DNA contents of the 408 and pap genes were 26.48 and 9.36 times that of the 377 gene, while the other genes were 2.17-4.11 times that of the 377 gene in the QH sample (Figure 1). In the QZ sample, the DNA contents of the 408 and pap genes were 15.20

Table 2: Thirteen pair-primers designed from candidate secretory protein genes of *Candidates Liberibacter Asiaticus* or GV1300 for this study.

Gene	Primer name	Primer sequences (5'-3')	Length (bp)	
ATPase	ATPase-F	CCATAAAAACGCTATTGCGATGATC	1841	For PCRassay
	ATPase-R	CTTTTAAGGGATGCAGGGTGATTTC		
mfp	Mfp-F	TCAATACCGTCACTCACAATCAGA	1399	
	Mfp-R	GAGAGGATGAGATTGTTGATTGGG		
Serralysin	Serralysin-F	CTCCACATTTTATTAAAGAAGGTCTTGG	2154	
	Serralysin-R	GGCTCGGAAACCACAATGCC		
408	408-F	CCCTTGCCGCCTTTACCTCC	495	
	408-R	GCGCGTCAGGTAGATGATCAG		
24A	24A-F	GGCTTAGGGTTCTTCTATTCTTATGC	726	
	24A-R	CCACTATTCCCATCAGTCGAGTT		
pap	pap-F	CGGTAGCTAAGTTATCACTAGTATTGC	1632	
	pap-R	CCCCTTGAAACACACACCTCCG		
MSr	MSr-F	GCACAAGCTTATATCAGTAGAGACC	1810	
	MSr-R	ACCTAACCCTTGTGATATATGTGCC		
msp	msp-F	CCTGTGAATTAGGTCGTGTTGGG	582	
	msp-R	GTTCAAAACCAATACACCTTTTTATTCAG		
fATP	fATP-F	CCATGTTGGAGGCGATAAGAAATATC	1420	
	fATP-R	CCTTACCATCTCTTGTTCTATTGCTC		
377	377-F	CGTGCAGGAATGCGTACTGTAG	510	
	377-R	CTAGAAGTATAACCTCCCCACTCG		
408	408-gF	CGC**GTCGAC**TTGCATCGTAAGCGCC (Sal I)	423	Recombinant plasmids construction
	408-gR	CGC**ACTAGT**CCTGACGGGAGGAGAGGAG (Spe I)		
pap	pap-gF	CGC**GTCGAC**ATGAGGTATTTGCAACGCAC (Sal I)	1440	
	pap-gR	CGC**GGATCC**TTTATAAATAAAACCAATTGCACC (BamH I)		
GV1300	1300-F	AACTTGTGGCCGTTTACGTCG	207	Primers for GV1300
	1300-R	TTTGGAGAGAACACGGGGGAC		

Note: The bold sequences represent the restriction enzymes.

and 8.35 times that of the 377 gene, while the DNA content of the 24A gene was also relatively high, about 9.22 times that of the 377 gene (Figure 1). In summary, the relative DNA contents of the 408, pap, and 24A genes were relatively high in QH and QZ samples.

The results from the RT-qPCR quantification did not match the DNA amount shown in the qPCR reactions. Of these six SPGs, only the 408 and pap genes had amplification curves, and the Ct value was between 15 and 35. Other genes did not have an obvious amplification curve, or their Ct value was more than 35 which should be insignificant (Verylowamountorunspecific amplification). Further analysis revealed that the relative transcription level of the 408 gene was 2.43 higher than the transcription level of the pap gene in QH sample. In the QZ sample, the relative transcription level of the 408 gene was 9.45 higher than the transcription level of pap gene, and the relative transcript RNA level was much higher than that of the 408 gene in the QH sample (Figure 2). In this study, two relatively high transcription levels of SPGs were screened from the ten candidate SPGs.

Subcellular localization of 408 and pap proteins

In order to further clarify the distribution of the 408 and pap proteins in the host cells, the recombinant plasmids of GV1300-408 and GV1300-pap were transformed into *Agrobacterium tumefaciens* GV1301 competent cells. Then, the positive clones were identified by colony PCR (Single colony was used as template), as described above. After injection of GV1300-408/GV1301 and GV1300-pap/GV1301 into the tobacco leaves, fluorescence images were visualized by microscopy at 72 hours postinoculation (h.p.i.). The green fluorescence signal from the 408-GFP fusion protein was observed in the nuclei and cytoplasms of tobacco leaf cells, while the green fluorescence signal of the pap-GFP fusion protein was observed in the radially-arranged micro-fibrils of tobacco guard cells. These results indicate that the 408 protein localizes in the nucleus and cytoplasm tobacco mesophyll cells, but the pap protein localizes in the radially-arranged micro-fibrils of tobacco guard cells. In addition, the GFP protein is localized in the cytoplasms and the nuclei of tobacco mesophyll cells (Figure 3).

Discussion

Bacteria-secreted proteins play important roles in pathogenicity and infection in host cells [25,26]. Briefly, pathogenic bacteria have a number of different protein secretion systems and secrete virulence

Table 3: Primers designed from six selected candidate secretory protein genes of *Candidatus Liberibacter Asiaticus* for quantitative real-time PCR analysis.

Gene	Primer name	Primer sequences (5'-3')	Length (bp)
408	408-F	CTGTACTCCAAGATGCCTACC	131
	408-R	CGTGCCTATCATGCTTGTTTC	
PAP	PAP-F	AGCCAGTAATCGGAGTCAATG	119
	PAP-R	TCATCTTTCAATAACCCCGCC	
MSP	MSP-F	AGACATGTGCCATTTTAAGTGC	96
	MSP-R	TCTATCTGTTATGCGAATCGTGT	
377	377-F	CCAAGAGAACTGTAGAAAGGCG	147
	377-R	AGAAGTATAACCTCCCCACTCG	
24A	24A-F	GGGTGGAGGGGATGTAAAATT	113
	24A-R	GACAGATAATATTCCGCCTAAAATAGC	
fATP	fATP-F	ATAGCGGATTCTGTTCGTAGC	136
	fATP-R	ATCAGCACTCCAAGCCTTATC	
18Sr RNA	18Sr RNA-F	TCGGGTGTTTTCACGTCTCA	120
	18Sr RNA-R	TGGATGCCGCTGGGAAGC	

factors extracellularly or directly to the host *via* these secretion systems. Currently, it is known that there are at least eight protein secretion systems in Gram-negative bacteria [18]. *Candidatus Liberibacter Asiaticus* has incomplete type III and type IV protein secretion systems and a complete type I protein secretion system. In this study, the obtained 408 gene was secreted by the type III secretion system, while the pap gene was secreted by the type IV secretion system [22]. The Flp pilus, which is assembled by the proteins encoded by the flp (fimbrial low-molecular-weight protein) operon, may play an important role in bacterial adherence. Here, both of 408 and pap proteins are flp pilus assembly proteins. Pili, flagella, and other adhesive structures usually assemble at the cell surface of Gram-negative bacteria. The ability of diverse bacteria to adhere to host cell surfaces is an important property and a critical step in colonization [27]. Therefore, 408 and pap may be involved in these adhesive organelle assemblies *via* the extracellular nucleation-precipitation pathway [28]. Furthermore, subcellular localization analyses indicated that the 408 protein is located in the nuclei and cytoplasms of tobacco leaf cells. This suggests that the 408 gene may have other functions besides the formation of flagella on the bacterial surface. However, the pap protein was shown to be located in the radially-arranged micro-fibrils of tobacco guard cells and may interact with host cells to help pathogens invade into plant cells.

At present, the effective detection methods of pathogen microscopy, Loop-Mediated Isothermal Amplification (LAMP), PCR and real-time quantitative PCR were available for HLB diagnosis [3,29-33]. However, a protein detection technology for citrus Huanglongbing with convenience and high sensitivity at a large-scale needs to be developed. Although Yuan et al. and Liu et al. reported monoclonal antibodies against *Candidatus Liberibacter Asiaticus* [34-36], there are no commercial products available yet. In order to prepare an antiserum against the *Candidatus Liberibacter Asiaticus* for the early detection and prevention of citrus HLB, ten SPGs were selected from different protein secretion systems of *Candidatus Liberibacter Asiaticus* and tested by PCR, qPCR, and RT-qPCR, and two SPGs of 408 and pap with relatively high DNA contents and their transcription level were identified. This provides an important scientific basis for the preparation of an antiserum against *Candidatus Liberibacter Asiaticus* and the early detection and prevention of citrus HLB.

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