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Quantitative Screening of Secretory Protein Genes in *Candidatus* Liberibacter 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 *Diaphorina citri* Kuwayama and *Trioza erytreae*. 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, six candidate SPGs were chosen from *Candidatus* Liberibacter asiaticus by bioinformatic analysis and were further tested by 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 cytoplasm of tobacco leaf cells. However, the pap protein is located in the cytoplasm of tobacco leaf 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

1. Introduction

Citrus Huanglongbing (HLB), or greening disease, is a devastating disease that

seriously threatens the development of the citrus industry globally [1] [2] [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] [5] [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] [9] [10].

The content of *Candidatus* Liberibacter asiaticus is low from the infected trees and unevenly distributed in different parts of diseased plants [11] [12]. Therefore, 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 immunosorbent assay (ELISA) [14] [15] [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 (types 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 at least thousands times higher than the number of its pathogen. Studies have shown that the pathogen of *Candidatus* Liberibacter asiaticus has an incomplete type III and type IV protein secretion systems but has a complete type I protein secretion system [20] [21].

In this study, six 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 qPCR and RT-qPCR methods. Furthermore, the 408 protein was located in the nuclei and cytoplasm of tobacco cells, while the pap protein was localized in the cytoplasm of tobacco leaf 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 for the early detection and prevention of citrus HLB.

2. Materials and Methods

2.1. Materials

In 2016, the QH sample was mixed from five early HLB-infected green orange leaves with asymptomatic in Qionghai County, Hainan Province, and the QZ sample was mixed from another five early HLB-infected green orange leaves with asymptomatic in Qiongzhong County, Hainan Province. Both pathogens of QH and QZ were identical with the *Candidatus* Liberibacter asiaticus psy62 isolate (GenBank accession number: CP001677.5) [22]. 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.

2.2. Primers Design, DNA, and cDNA Preparation

In order to identify one or two high-expression SPGs, six 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, 6 primer-pairs of the 408, 24*A*, *fATP*, *pap*, *msp*, and 377 genes were designed for qPCR or RT-qPCR using the online website (https://www.idtdna.com/Scitools/Applications/RealTimePCR/) (**Table 2**). 18*S* rRNA of citrus was used as an internal reference gene [23] (**Table 2**). 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).

2.3. Screening of Candidate SPGs and Establishment of Real-Time Quantitative PCR (qPCR)

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 by each pair primer in the Stratagene Mx3005 machine. The results showed that six qPCR reactions by 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 Stratagen Mx3005P instrument using the Hieff^{TM} qPCR SYBR Green Master Mix with Low Rox Plus (Yeasan, Shanghai, China) according to the instructions, and

Table 1. Characteristic summary of six candidate secretory protein genes from *Candidatus* liberibacter asiaticus.

Protein	Region name	Protein ID	Protein function	Type of 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
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).

Table 2. Primers used in this study.

Gene	Primer name	Primer sequences (5'-3')	Length (bp)	
408	408-F	CTGTACTCCAAGATGCCTACC	131	Real-time PCR assay
	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		
18S rRNA	18S rRNA-F	TCGGGTGTTTTCACGTCTCA	120	
	18S rRNA-R	TGGATGCCGCTGGGAAGC		
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.

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 μL 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.

2.4. 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 2). 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 (TransGen, 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.

3. Results

3.1. 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 (data not shown). Total RNA samples were also extracted from these two samples and were visualized on a 1% agarose gel (data not shown). 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 (1^{st} cDNA) which subsequently could be used for RT-qPCR.

3.2. Screening of Candidate SPGs from HLB-Infected Green Orange Leaves

Analysis of the real-time qPCR showed that the amplification plot of six SPGs

and the internal reference gene shown in the dissociation curve of QH sample (Figure 1(a)), similar result was observed in QZ sample. 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

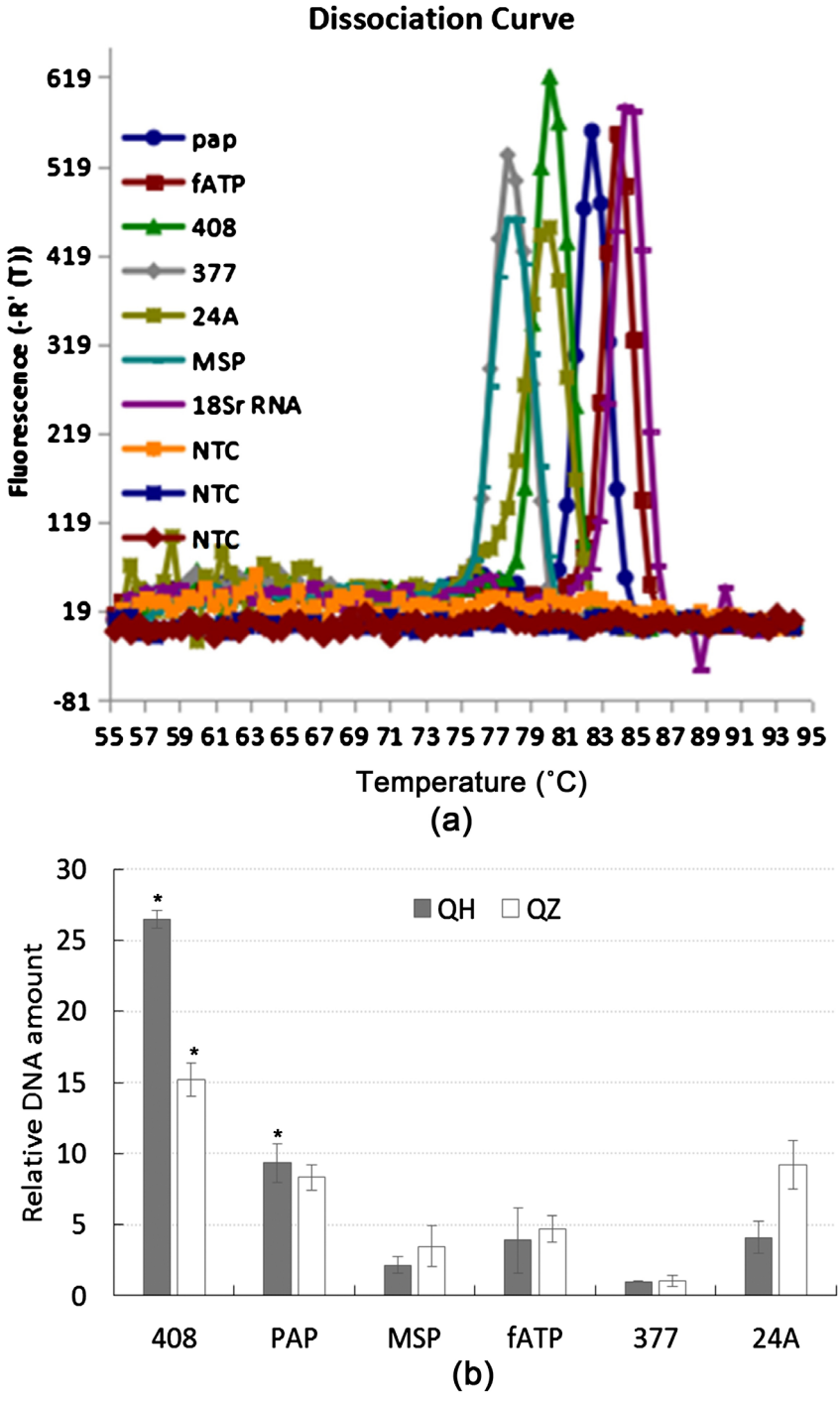

Figure 1. (a) The melt curves of six selected candidated secretory protein genes and 18S rRNA by real-time quantitative PCR; (b) Relative DNA amount of six selected candidate secretory protein genes in QH and QZ samples. Statistical analysis was performed using student's t-test. "*" represents $P < 0.05$.

377 gene in the QH sample (Figure 1(b)). In the QZ sample, the DNA contents of the 408 and *pap* genes were 15.20 and 8.35 times that of the 377 gene, while the DNA content of the 24*A* gene was also relatively high, about 9.22 times that of the 377 gene (Figure 1(b)). In summary, the relative DNA contents of the 408, *pap*, and 24*A* 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 (Very low amount or unspecific 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.

3.3. 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

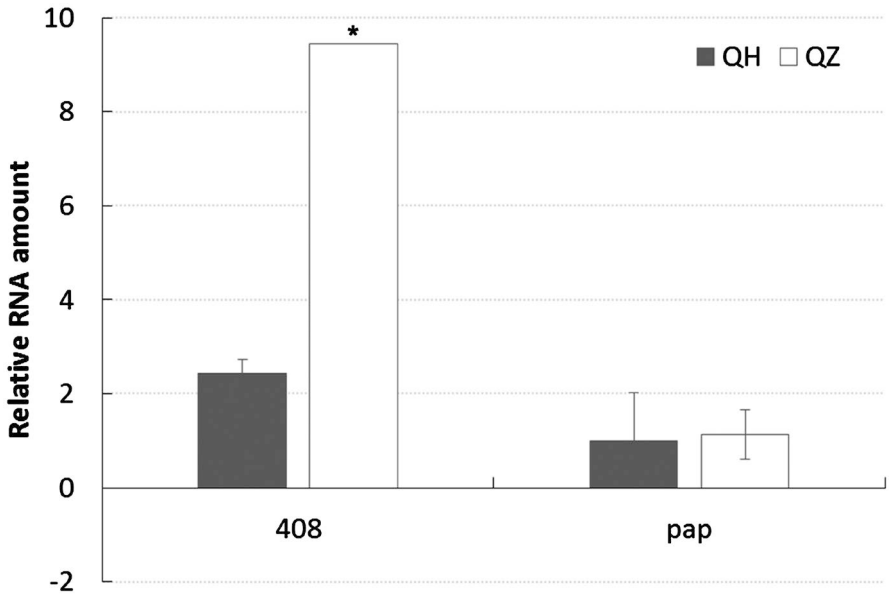

Figure 2. Relative RNA amount of 408 and *pap* candidate secretory protein genes in QH and QZ samples. Statistical analysis was performed using student's t-test. "*" represents $P < 0.05$.

by microscopy at 72 hours post inoculation (h.p.i.). The green fluorescence signal from the 408-GFP fusion protein was observed in the nuclei and cytoplasm of tobacco leaf cells, while the green fluorescence signal of the pap-GFP fusion protein was observed in the cytoplasm of tobacco leaf cells. These results indicate that the 408 protein localizes in the nucleus and cytoplasm tobacco mesophyll cells, but the pap protein localizes in the cytoplasm of tobacco leaf cells. In addition, the GFP protein is localized in the cytoplasm and the nuclei of tobacco mesophyll cells (Figure 3).

4. 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 factors extracellularly or directly to the host via these secretion systems. Currently, it is known that there are at least

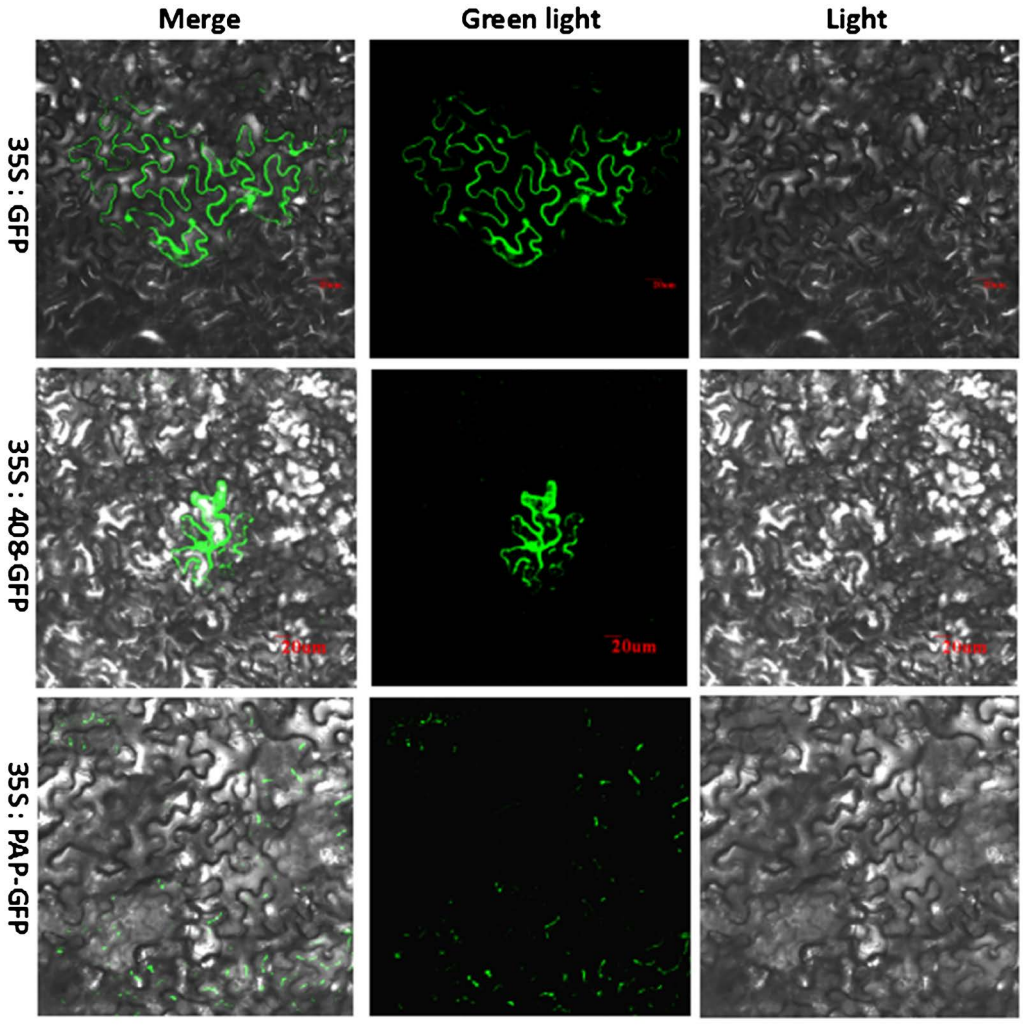

Figure 3. Subcellular localization of GFP, 408-GFP and PAP-GFP in tobacco leaf cells under the light of 488 nm and 546 nm. 35S, a constitutive promoter from the cauliflower mosaic virus; GFP, green fluorescent protein; Bar represents 20 μm.

six protein secretion systems in Gram-negative bacteria [17] [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), 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 cytoplasm 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 cytoplasm of tobacco leaf 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] [30] [31] [32] [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] [35] [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, six SPGs were selected from different protein secretion systems of *Candidatus* Liberibacter asiaticus and tested by 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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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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