A New Diagnostic System for Detection of '*Candidatus Liberibacter asiaticus*' Infection in Citrus

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Abstract

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In this study, two polyclonal antibodies were produced against the Omp protein of '*Candidatus Liberibacter asiaticus*'. First, *omp* genes were sequenced to exhibit 99.9% identity among 137 isolates collected from different geographical origins. Then, two peptides containing the hydrophobic polypeptide-transport-associated (POTRA) domain and β -barrel domain, respectively, were identified on Omp protein. After that, these two peptides were overexpressed in *Escherichia coli* and purified by affinity chromatography to immunize the white rabbits. Finally, the antiserum was purified by affinity chromatography. The two Omp antibodies gave positive results (0.454 to 0.633, 1:1,600 dilution) in

enzyme-linked immunosorbent assay against '*Ca. L. asiaticus*'-infected samples collected from different geographical origins but revealed negative results against other pathogen-infected, nutrient-deficient and healthy samples. The antibody against the POTRA domain of Omp protein could detect '*Ca. L. asiaticus*' in 45.7% of the symptomatic samples compared with a 56.2% detection rate with a polymerase chain reaction assay. These new antibodies will provide a very useful supplement to the current approaches to '*Ca. L. asiaticus*' detection and also provide powerful research tools for tracking distribution of this pathogen in vivo.

Citrus greening (huanglongbing [HLB]) is one of the most destructive diseases that affect citrus husbandry in many parts of the world (Asia, North America, South America, and Africa) (5,7). The causal agents of this disease are three '*Candidatus Liberibacter* spp.', which are gram-negative, phloem-limited bacteria. The Asian form, '*Candidatus Liberibacter asiaticus*' is widespread in all HLB-affected countries (including the United States, Brazil, and several Asian countries) outside Africa (1,22,25), and its vector is a phloem-feeding citrus psyllid, *Diaphorina citri* Kuwayama (23,38). At present, there is no effective control method to manage this disease, and no cultivars resistant to the pathogen are available. Therefore, rapid diagnosis and immediate removal of the infected trees are among the most general practices used to control the HLB. Polymerase chain reaction (PCR) systems have been used for molecularly detecting HLB pathogens (35). However, PCR methods sometimes provide contradictory results, even within the same set of samples, because of the uneven distribution of the pathogen in citrus trees and because of the inherent nature of PCR assays (sensitive to contamination, nontarget amplification, and so on; 19). There-

fore, developing detection methods that improve HLB diagnosis is a pressing need in the citrus industry.

The enzyme-linked immunosorbent assay (ELISA) is an antibody-based detection method that is not only highly sensitive but also highly target specific (31). To date, several (more than 10) monoclonal antibodies (MAs) have been developed against '*Ca. L. asiaticus*' using extracts of infected periwinkle phloem tissues as immunogens. However, this has been difficult because of the non-continuously cultured nature of the '*Ca. L. asiaticus*' bacteria (14,21,25). Additionally, three more MAs were obtained using antigenic proteins extracted by affinity chromatography from infected periwinkle phloem tissues. However, these procedures are very complex and the productivity of antigens is very low (11). One other drawback with these preparation methods is that they are very susceptible to contamination with host plant proteins that can cause a lack of specificity in the antibodies developed using these antigenic preparations. What's more important, all these antibodies are too specific for the strains used for antigen preparation and often failed to react with HLB pathogen isolates from other geographical origins. This is probably because their antigenic proteins may not have a high degree of structural conservation among different isolates (13). These shortcomings are great barriers for these special antibodies to become practical HLB diagnosis reagents. Hence, antibodies which are easy to produce and have broader specificity range are needed.

Gradually, more and more new genes of HLB pathogens have been obtained (26). Also, the complete genome of '*Ca. L. asiaticus*' is now available (8). This progress has provided opportunities to explore for new candidate antigens. Omp protein is believed to be an outer membrane protein and may act as a surface antigen of the pathogen. However, outer membrane proteins usually have a very complex structure and, also, they are very difficult to express in *Escherichia coli* (30). Bioinformatics approaches to the prediction of protein structures and other means of detailed structural analysis of Omp protein may help us identify suitable sequence fragments for high-level expression in *E. coli* that can facilitate the production of antigen.

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In this study, two highly sensitive and specific polyclonal antibodies were produced against the polypeptide-transport-associated (POTRA) domain and β -barrel transmembrane domain of Omp protein with the aid of bioinformatics analysis. These Omp antibodies can be used to overcome the shortcomings of currently used antibodies. They can detect HLB pathogen strains from different geographical origins and, thus, improve the current approaches to '*Ca. L. asiaticus*' detection and HLB diagnosis.

Materials and Methods

Plant materials. Citrus shoots infected by '*Ca. L. asiaticus*' isolates with variation in Omp sequences were collected from Thailand, France, and five provinces in China. The sample descriptions are provided in Tables 1 and 2. The collected materials were grafted into healthy citrus seedlings in the greenhouse. After the inoculated seedlings showed typical HLB symptoms, '*Ca. L. asiaticus*' was transmitted to healthy periwinkles by dodders for preservation. (These plants were grown in a greenhouse at 25°C during 12-h days with illumination intensity 10,000 lux and at 20°C for 12-h nights).

The citrus plant collections from China in Tables 3 and 4 were visually examined and HLB-symptomatic samples were collected during November to April 2009 and 2010 from randomly selected commercial citrus groves located in China's Zhejiang, Guangdong, Fujian, Guangxi, and Jiangxi Provinces. Samples were stored at 4°C and processed within 48 h. Nutrient-deficient-symptomatic citrus plants were detected as described previously (10,27,28,33). We used PCR methods for detection of *Citrus tristeza virus*, *Citrus tatter leaf virus*, *Citrus exocortis viroid*, *Satsuma dwarf virus*, and '*Ca. L. asiaticus*', as described in earlier reports (12,16–18,20). Mixed infected samples were not included in our study.

PCR amplification of the omp gene. The omp gene (2,422 bp) was amplified with primers OMP5/OMP3 (5'-GATGATAGGTGC ATAAAAGTACAGAAG-3'/5'-AATACCCTTATGGGATACAAAA-3') for omp gene sequencing. The 5'-terminal POTRA domain (921-bp fragment) of the omp gene was amplified with primers OMPN5/OMP3 (5'-AGCGGATCCCAATTGAAGATGATAGTT CGC-3'/5'-AGCAAGCTTTTACACATAATCGGATACATCA-3') and the 3'-terminal β -barrel transmembrane domain (1,047-bp fragment) of the omp gene was amplified with primers OMPC5/OMPC3 (5'-AGCGGTACCAGCGCTGGTCTGTAGGTATAGC-

3'/5'-AGCAAGCTTTTACATGCGATTACCTATACG-3'). The 2,364 bp full-length omp gene was amplified with primers OMPY1/OMPY2 (5'-CGTCGACTCGTGCATAAAAGTACAGAAGAT-3'/5'-TGCGGCCGCTACATGCGATTACCTATAC-3') for Omp prokaryotic expression. The PCR reaction mixture (25 μ l) contained 10 mM Tris-HCl, 2.5 μ l of 10 $\times$ reaction buffer, 0.2 mM dNTPs, 1.5 μ l of each primer (10 μ M), 1 U of Taq DNA polymerase, and 1 μ l of template DNA. The amplification conditions included initial denaturation at 94°C for 5 min followed by 35 thermocycles, each consisting of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extending at 72°C for 2 to 3 min. Finally, an extension of 72°C for 10 min was included. PCR products were examined by electrophoresis on 1% agarose gel prestained with GelRed nucleic acid stain (Biotium).

Plasmid construction and recombinant protein preparation.

The PCR products amplified with primers OMPY1/OMPY2 were digested with *Sall*/*NotI*; then, the purified products were inserted into prokaryotic expression vector pET-32a, pGEX-4T-1, or pIGH3 (GL Biochem Ltd.). These vectors have a T7 promoter or tac promoter, and also fuse His-tag or GST-tag to the N terminal of target proteins for purification purpose. The PCR products amplified with primers OMPN5/OMP3 and OMPC5/OMPC3 were digested with *Bam*HI/*Hind*III and *Kpn*I/*Hind*III, respectively. The enzyme-digested products were purified and then inserted into a prokaryotic expression vector pIGH3 to construct pIGH3-OMP-N and pIGH3-OMP-C recombinant vectors. The recombinant vectors were transformed into *E. coli* strain BL21 (DE3) and cells were cultivated at 37°C in 300 ml of Luria-Bertani liquid medium containing ampicillin at 50 μ g/ml. At an optical density at 600 nm (OD₆₀₀) of 0.5 to 0.6, isopropyl-thio-galactopyranoside was added to induce expression of the recombinant proteins. After an additional 4 h of incubation at 37°C, cells were harvested and disrupted by ultrasound. Then, the inclusion bodies were washed three times in washing buffer I (0.5% Triton X-100, 50 mM Tris-HCl, and 10 mM EDTA, pH 8.0), then washed three times in washing buffer II (2 M urea, 50 mM Tris-HCl, and 10 mM EDTA, pH 8.0). The sediment was dissolved in buffer III (5 mM imidazole, 0.5 M sodium chloride, 20 mM Tris, and 8 M urea, pH 7.9) at 4°C for about 4 h. The insoluble materials were removed by centrifugation at 12,000 $\times$ g for 20 min at 4°C. The recombinant Omp proteins fused with His-tag were purified from

Table 1. Enzyme-linked immunosorbent assay (ELISA) detection of citrus samples infected by '*Candidatus Liberibacter asiaticus*' isolates with sequence variation in Omp proteins^a

Antibody	OD ₄₅₀ values of ' <i>Ca. L. asiaticus</i> '-infected citrus samples					
	ZJ	FJ	GX	GD	JX	CK
OMP-N	0.495 $\pm$ 0.057	0.552 $\pm$ 0.105	0.476 $\pm$ 0.076	0.538 $\pm$ 0.032	0.519 $\pm$ 0.063	0.146 $\pm$ 0.033
OMP-C	0.468 $\pm$ 0.043	0.483 $\pm$ 0.046	0.452 $\pm$ 0.092	0.527 $\pm$ 0.016	0.454 $\pm$ 0.054	0.169 $\pm$ 0.008
PA	0.692 $\pm$ 0.102	0.670 $\pm$ 0.036	0.787 $\pm$ 0.117	0.748 $\pm$ 0.032	0.640 $\pm$ 0.062	0.307 $\pm$ 0.052

^a Citrus samples in this table were infected with '*Ca. L. asiaticus*' isolates with variation in Omp sequences. They were collected from China's Zhejiang (ZJ), Fujian (FJ), Guangxi (GX), Guangdong (GD), and Jiangxi (JX) Provinces. They were tested by indirect ELISA. Antibodies used in ELISA were prepared against OMP-N and OMP-C (1:1,600 dilution) or against purified '*Ca. L. asiaticus*' (PA, 1:800 dilution). Numbers in the table are mean coefficient of optical absorption at 450 nm in ELISA with the negative control of healthy sample (CK). Optical density at 450 nm (OD₄₅₀; sample)/OD₄₅₀ (negative control) > 2.1 is considered a positive result. Results are representative of three independent experiments with similar results; $\pm$ indicates standard deviation.

Table 2. Enzyme-linked immunosorbent assay (ELISA) detection of periwinkle samples infected by '*Candidatus Liberibacter asiaticus*' isolates with sequence variation in Omp proteins^a

Antibody	OD ₄₅₀ values of ' <i>Ca. L. asiaticus</i> '-infected periwinkle samples							
	TH	FR	ZJ	FJ	GX	GD	JX	CK
OMP-N	0.683 $\pm$ 0.047	0.645 $\pm$ 0.034	0.685 $\pm$ 0.040	0.664 $\pm$ 0.039	0.618 $\pm$ 0.031	0.627 $\pm$ 0.077	0.637 $\pm$ 0.048	0.147 $\pm$ 0.013
OMP-C	0.585 $\pm$ 0.050	0.580 $\pm$ 0.027	0.62 $\pm$ 0.079	0.589 $\pm$ 0.015	0.527 $\pm$ 0.057	0.537 $\pm$ 0.029	0.521 $\pm$ 0.087	0.153 $\pm$ 0.031
PA	0.848 $\pm$ 0.034	0.704 $\pm$ 0.051	0.658 $\pm$ 0.029	0.696 $\pm$ 0.034	0.814 $\pm$ 0.053	0.799 $\pm$ 0.031	0.788 $\pm$ 0.080	0.283 $\pm$ 0.051

^a Numbers in the table are mean coefficient of optical absorption at 450 nm in ELISA. Abbreviations: Thailand (TH); France (FR); China's Zhejiang (ZJ), Fujian (FJ), Guangxi (GX), Guangdong (GD), and Jiangxi (JX) Provinces; CK, negative control of healthy sample. Optical density at 450 nm (OD₄₅₀; sample)/OD₄₅₀ (negative control) > 2.1 is considered a positive result. Results are representative of three independent experiments with similar results; $\pm$ indicates standard deviation.

the soluble phase proteins using a His-Trap FF crude Kit (GE Healthcare) following the instructions of the manufacturer. The recombinant proteins possessing the N-terminal or C-terminal region of Omp protein were dissolved in the supernatant and detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein concentration was determined by the Bradford assay using bovine serum albumen (BSA) as a standard (6).

Sequence analyses. DNA sequences at National Center for Biotechnology Information databases (<http://www.ncbi.nlm.nih.gov/BLAST>) were searched using the nucleotide BLAST program. Multiple sequence alignments were performed using the CLUSTALW (<http://www.genome.jp/tools/clustalw/>) software. The protein structure prediction was performed using the HMMer software, version 2.2 (<http://hmmer.janelia.org>). Phylogenetic trees were constructed using the MEGA4 program (34).

Purification of HLB pathogen immunogen. ‘*Ca. L. asiaticus*’-infected periwinkle midribs (30 g) were ground in liquid nitrogen, then ground with 100 ml of separation buffer (0.1 M MgCl₂, 0.5 M mannitol, and 0.6 M glycine, pH 7.4). The homogenate was then incubated with cellulase (1 mg cellulase per milliliter of homogenate) for 3 h at 35°C. Then, the mixture was filtered and centrifuged at 5,000 × *g* for 20 min at 4°C. Supernatant was further centrifuged at 35,000 × *g* for 40 min at 4°C. After that, the pellets were retained and resuspended in separation buffer. The differential centrifugation was repeated again and the pellets were resuspended in a buffer containing 0.5 M mannitol and 30 mM cysteine, pH 7.0. The suspension was kept at room temperature for 30 min and centrifuged at 5,000 × *g* at 4°C for 20 min, as described previously (21,25). The supernatant was collected and stored at 4°C for further experimentation.

Table 3. Specificity of the Omp antibody for detection of ‘*Candidatus Liberibacter asiaticus*’ by indirect enzyme-linked immunosorbent assay (ELISA)

Host	Sample types	Total number of samples	PCR ^a	ELISA ^b
<i>Citrus reticulata</i> Blanco ‘Succosa’	<i>Citrus tristeza virus</i>	10	–	0.094–0.244
<i>C. sinensis</i> Osbeck ‘Newhall’	<i>Citrus tatter leaf virus</i>	5	–	0.192–0.259
<i>C. sinensis</i> Osbeck ‘Jincheng’	<i>Citrus exocortis viroid</i>	7	–	0.147–0.173
<i>C. unshiu</i> Marc. ‘Miyagawa Wase’	<i>Satsuma dwarf virus</i>	8	–	0.193–0.132
<i>C. sinensis</i> Osbeck Newhall	<i>Xanthomonas citri</i> subsp. <i>citri</i>	12	–	0.143–0.261
<i>C. unshiu</i> Marc. Miyagawa Wase	<i>Colletotrichum gloeosporioides</i>	10	–	0.11–0.241
<i>C. reticulata</i> Blanco ‘Ponkan’	Nitrogen deficiency	12	–	0.163–0.188
<i>C. reticulata</i> Blanco Succosa	Magnesium deficiency	13	–	0.149–0.183
<i>C. sinensis</i> Osbeck ‘Hamlin’	Iron deficiency	10	–	0.131–0.161
<i>C. sinensis</i> Osbeck Hamlin	Manganese deficient	8	–	0.087–0.115
<i>C. sinensis</i> Osbeck ‘Liubencheng’	Zinc deficiency	10	–	0.108–0.173
<i>C. unshiu</i> Marc. Miyagawa Wase	Boron deficiency	7	–	0.098–0.144
<i>C. unshiu</i> Marc. Miyagawa Wase	Glyphosate phytotoxicity	15	–	0.161–0.172
<i>C. reticulata</i> Blanco Ponkan	Huanglongbing ^c	6	+	0.503–0.633
<i>C. reticulata</i> Blanco Ponkan	Healthy control ^d	6	–	0.113–0.131

^a Primers used in this column were described in Materials and Methods. Positive or negative results were denoted as + or –, respectively. Samples with no visual band displayed on an agarose gel were considered negative.

^b Optical density at 450 nm (OD₄₅₀ (sample)/OD₄₅₀ (negative control) > 2.1 would be considered to be a positive result. Values in this column represent the range of the OD₄₅₀ (sample).

^c Positive control.

^d Negative control.

Table 4. Sensitivity comparison between polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) for detection of ‘*Candidatus Liberibacter asiaticus*’ in symptomatic samples from different geographical origins and host species^a

Geographical origins	Host	Total samples	PCR	ELISA
Zhejiang Province	<i>Citrus reticulata</i> Blanco ‘Succosa’	32	15	13 (2)
	<i>C. unshiu</i> Marc. ‘Miyagawa Wase’	20	9	8
	<i>C. reticulata</i> Blanco ‘Tardiferax’	13	2	1 (1)
	<i>C. sinensis</i> Osbeck ‘Hamlin’	15	12	10 (1)
	<i>C. reticulata</i> × <i>C. sinensis</i>	7	4	4
	<i>C. sinensis</i> × <i>C. grandis</i>	12	8	7
Wenling	<i>C. grandis</i> Osbeck. ‘Yuhanyou’	25	10	10
	<i>C. reticulata</i> Blanco ‘Suavissima’	34	17	13 (2)
	<i>C. reticulata</i> Blanco ‘Ponkan’	27	20	16 (2)
Fujian Province	<i>C. grandis</i> Osbeck ‘Guanximiyu’	17	8	8
	<i>C. reticulata</i> Blanco Ponkan	15	13	11 (3)
	<i>C. reticulata</i> Blanco ‘Tangerina’	8	3	1 (1)
Jiangxi Province	<i>C. sinensis</i> Osbeck ‘Newhall’	7	5	4
	<i>C. unshiu</i> Marc. Miyagawa Wase	16	7	4
Guangxi Province	<i>C. sinensis</i> Osbeck ‘Valencia’	5	2	2
	<i>C. grandis</i> Osbeck ‘Shatianyou’	27	12	12 (1)
Guangdong Province	<i>C. reticulata</i> Blanco ‘Shatangju’	31	25	19 (1)
	<i>C. reticulata</i> Blanco ‘Gongan’	27	24	18 (3)
	<i>C. reticulata</i> Blanco Ponkan	32	12	9
	<i>C. reticulata</i> Blanco ‘Tankan’	13	2	2
	<i>C. sinensis</i> Osbeck Newhall	11	3	2
	<i>C. reticulata</i> Blanco Shatangju	24	22	17 (2)
	...	418	235	191

^a Numbers in the table are the number of total samples or positive samples. Numbers in parentheses show differences in results obtained by ELISA and PCR assays. Primer and detection methods were similar to those in Table 3.

Antisera preparation. New Zealand white rabbits received four injections with Omp recombinant proteins via the intramuscular route. The first injection consisted of 500 µg of the recombinant proteins possessing the N-terminal or C-terminal region of Omp protein in 500 µl of phosphate-buffered saline (PBS) emulsified with 500 µl of Freund's complete adjuvant. The rabbits were then immunized with 250 µg of the recombinant proteins in 500 µl of PBS emulsified with 500 µl of Freund's incomplete adjuvant in week 2, 5, and 8. Serums were collected in week 10. Prior to use, the antisera were purified, filter sterilized, and stored at -20°C. The polyclonal antibody against '*Ca. L. asiaticus*' was obtained using extracts from infected phloem tissues as immunogen with a similar procedure. However, in this case, the enriched pathogen was incubated for 30 min at 37°C with white rabbit antiserum prepared against the homogenates of healthy phloem tissues before immunization, as described previously (14).

Purification of Omp antibodies. Affinity chromatography columns were prepared by coupling Omp recombinant proteins to CNBr-Activated Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. The immune sera diluted with an equal volume of PBS with Tween 20 (PBST, including 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, and 0.05% Tween 20) were loaded into the columns and incubated for 3 h at room temperature. Then, the columns were washed with the same buffer and the bound antibodies were eluted in fractions with 100 mM glycine, pH 2.5, and neutralized with 2 M Tris/HCl, pH 8.0. The antibody solutions were dialyzed against PBS and stored at -70°C in 50% glycerol.

Indirect ELISA detection of '*Ca. L. asiaticus*' in naturally infected samples. Symptomatic leaf samples were collected in different areas by spotting trees with typical HLB symptoms. The samples were grounded with coating buffer (0.05 M carbonate buffer solution, pH 9.6) at a 1:20 (wt/vol) ratio. The optimal dilution ratio 1:20 was confirmed in serial dilution test by using the formula $1:10 \times 2^n$ ($n = 0, 1, 2, 3, \dots$). Three independent homogenates were clarified by centrifugation at $12,000 \times g$ for 1 min, and then 150 µl of supernatant was pipetted into microplate wells in triplicate and incubated overnight at 4°C. After washing three times with PBST, the microplate wells were blocked with 200 µl of 2% BSA at 37°C for 1 h. The plate was washed and serially diluted antibodies (150 µl) were added into each well and incubated at 37°C for 2 h. After washing three times, 150 µl of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) was added at a 1/10,000 dilution and incubated at 37°C for 2 h. The 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to the wells and incubated at 37°C for 15 to 30 min for color development. After the reaction was stopped by the addition of sulfuric acid solution, the OD of each well was measured at 450 nm (OD₄₅₀) in a microplate reader. Meanwhile, the PCR assay was also used to detect the pathogens in these samples (36).

Immunofluorescence microscopy. Longitudinal sections (30 µm thick) were prepared separately from healthy and infected plant midribs with a freezing microtome. Next, the sections were washed three times with 0.01 M PBS and incubated for 30 min in 0.01 M PBS with 0.3% Triton X-100, followed by washing three times with 0.01 M PBS. They were then incubated in diluted Omp antibodies for 30 min followed by incubation for 45 min at 37°C in a 100-fold dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG. The diluted IgG solution was prepared by adding 10 µl of the commercial FITC-conjugated IgG in 1 ml of PBS solution containing 0.1% Evans blue. After washing three times with PBS, the sections were mounted in 50% glycerol and observed under a fluorescence microscope, as described previously (14). More detailed information can be found in supplementary materials.

Results

Sequences variation of Omp genes among '*Ca. L. asiaticus*' isolates. To characterize sequence variation of *omp* genes among '*Ca. L. asiaticus*' isolates from different geographical origins, 137 '*Ca. L. asiaticus*'-infected citrus samples were collected from

Zhejiang, Fujian, Guangxi, Guangdong and Jiangxi, which are major citrus-producing provinces in China. The *omp* nucleotide sequences were amplified with primer set OMP5/OMP3. The *omp* sequences of isolates in other countries mentioned in the reference (3) were also included in this experiment. DNA sequences analysis revealed that these *omp* sequences exhibited more than 99.9% identity among these '*Ca. L. asiaticus*' isolates, about 73% identity with *omp* genes of '*Ca. L. africanus*' isolates (5), and nearly 69% identity with *omp* genes of '*Ca. L. solanacearum*' isolates. Protein sequence alignment revealed that the Omp sequences of '*Ca. L. asiaticus*' isolates had 99.9% identities and 99.9% similarities with each other.

Analysis of suitable Omp sequence fragments for prokaryotic expression. The full-length *omp* gene was ligated into the pET-32a, pGEX-4T-1, or pIGH3 vector for prokaryotic expression. Unfortunately, very little recombinant protein was obtained using any of these vectors and different inducing conditions. BLAST results showed that homologues of the *omp* gene widely exist among prokaryotes and eukaryotes. Most of the identified homologous genes are outer membrane proteins and contain the hydrophobic POTRA domains (4,29). Hammer analysis revealed statistically significant E values for four repeated POTRA domains in the N-terminal regions of Omp protein at 2.9×10^{-8} , 2.2×10^{-6} , 1×10^{-6} , and 2.4×10^{-9} (9). Also, the PRED-TMBB method and hydropathy plots consistently identified a β-barrel structure composed of 16 membrane-spanning β-strands on the C-terminal region of Omp (2,24). On the basis of these analyses, primers OMPN5/OMP3 and OMPC5/OMPC3 were designed to amplify the 5'-terminal POTRA and 3'-terminal β-barrel regions of the *omp* gene to ligate into a prokaryotic expression vector. Recombinant proteins of the N-terminal POTRA domain of Omp (OMP-N) or C-terminal β-barrel domain of Omp (OMP-C) were successfully obtained by expressing the proteins in *E. coli*.

Preparation of polyclonal antibodies. The OMP-N and OMP-C, fused with His-tag, were purified (to remove *E. coli* cellular proteins) using nickel column chromatography. The concentrations of recombinant Omp proteins were adjusted to working concentration (described in Materials And Methods) for use as immunogens. After four injections with antigens, rabbits were bled through cardiac puncture, and the Omp antibodies were purified with affinity chromatography columns. To determine their optimal working concentration, the antibodies were serially diluted at a ratio of $1:100 \times 2^n$ ($n = 0, 1, 2, 3, \dots$), then used to detect the pathogen in the '*Ca. L. asiaticus*'-infected citrus leaves by ELISA. The results revealed that 1:1600 dilution of the antibodies against OMP-N or OMP-C protein could produce the maximum OD₄₅₀ value of positive control/OD₄₅₀ value of negative control (P/N value). The antibody against '*Ca. L. asiaticus*' was also produced using extracts of infected phloem tissues as immunogen, and its optimal dilution ratio was 1:800.

Detection of isolates with variation in Omp sequence as well as from different geographical origins. The antibodies against OMP-N and OMP-C or against '*Ca. L. asiaticus*' were adopted to detect '*Ca. L. asiaticus*' isolates with Omp sequence variation as well as from different geographical origins. The ELISA results showed that all three antibodies could detect '*Ca. L. asiaticus*'-infected samples (Table 1). Antibodies against Omp protein had a higher P/N ratio than the antibody against '*Ca. L. asiaticus*'. The antibody against OMP-N had the highest detection sensitivity. '*Ca. L. asiaticus*'-infected periwinkle samples detected by indirect ELISA also had a similar result but these samples usually exhibited higher absorbance at 450 nm than the citrus samples (Table 2).

The specificity of Omp antibodies for detection of '*Ca. L. asiaticus*'. The specificity of antibody against OMP-N to samples infected by several viruses or viroids (*Citrus tristeza virus*, *Citrus tatter leaf virus*, *Citrus exocortis viroid*, and *Satsuma dwarf virus*) was investigated. The nutrient-deficient samples and glyphosate-damaged samples were also tested. These samples usually exhibit disease symptoms similar to those of HLB samples, which are very difficult to distinguish from one another by visual examination

(12,16–18,20,33). In addition, samples infected with *Xanthomonas citri* subsp. *citri* and *Colletotrichum gloeosporioides*, which are also common pathogens of citrus, were further tested in our study. The results of this investigation showed that all of these pathogen-infected samples as well as the nutrient-deficient samples and glyphosate-damaged samples had negative results (Table 3). The antibody against OMP-C also had a similar result.

Comparison of sensitivity between ELISA and PCR. Sensitivity of ELISA based on Omp antibody was compared with PCR assay for detection of ‘*Ca. L. asiaticus*’ in symptomatic citrus samples. Each sample represented a single tree and was randomly collected from the main cultivars from 18 different geographical origins in China. The result showed that the antibody against OMP-N could detect ‘*Ca. L. asiaticus*’ in 45.7% of the symptomatic samples, and 90.05% of the detection results were consistent with the PCR assay, which could detect ‘*Ca. L. asiaticus*’ in 56.2% of the symptomatic samples (Table 4).

In vivo detection of ‘*Ca. L. asiaticus*’ by immunofluorescence. The Omp antibodies were further tested for the possibility of tracking the distribution of ‘*Ca. L. asiaticus*’ in vivo with FITC-labeled secondary antibody. Strong green fluorescence was observed in the sieve tubes of ‘*Ca. L. asiaticus*’-infected citrus and periwinkles but not in the healthy ones, when either one of the Omp antibodies was used to treat thin sections of leaf-midribs (Fig. 1). The fluorescence was stronger on midrib sections of ‘*Ca. L. asiaticus*’-infected periwinkles than on the sections of pathogen-infected citrus.

Discussion

In the present study, we describe a new HLB diagnosis method based on Omp antibodies that improves the currently used approaches, including PCR assays, to accurately detect ‘*Ca. L. asiaticus*’. The Omp antibodies in our detection method can overcome the shortcomings of currently used antibodies. They can detect HLB pathogen strains from different geographical origins. Also, the assay procedure is more convenient and economical than earlier methods. Moreover, the Omp antibodies have potential to be used as research tools for exploring protein function and tracking distribution of HLB pathogens in citrus plants or in *D. citri* bodies.

At the beginning of this study, patterns of sequence variation of Omp proteins showed that these protein sequences had 99.9% identities and 99.9% similarities among the ‘*Ca. L. asiaticus*’ isolates collected from China and several other countries (3). This indicates that ‘*Ca. L. asiaticus*’ Omp proteins have a highly conserved protein structure in different isolates, in contrast to the former antigens that didn’t have high degree of structure conservation among different isolates (13). It also suggests that Omp antibodies have the potential to detect ‘*Ca. L. asiaticus*’ isolates from different geographical origins.

For antigen preparation, full-length Omp protein was used to express in *E. coli*. However, little or no recombinant protein was obtained from those recombinants. BLAST analyses revealed that most homologues of ‘*Ca. L. asiaticus*’ *omp* gene are outer membrane proteins (4,29). In *E. coli*, overexpressed outer membrane proteins would be degraded after triggering the σ^E stress response pathway (30). Therefore, it is probable that overexpressed full-length Omp protein might also be degraded in *E. coli*. This prompted us to analyze the detailed structure of Omp protein in order to identify suitable Omp sequence fragments for high-level expression in *E. coli*. Structure analysis identified POTRA domains on the N-terminal region and a 16-membrane-spanning β -barrel domain on the C-terminal region of Omp (5), suggesting that both of these regions possess membrane surface-exposed hydrophilic sequences that may be suitable for prokaryotic expression and antibody production (15,37). On the basis of these analyses, 5'-terminal and 3'-terminal regions of *omp* were ligated into vector to be expressed in *E. coli* and, finally, a large quantity of recombinant proteins were successfully obtained for immunization.

Identification of suitable Omp fragments that can be expressed at high levels in *E. coli* led to a more convenient antigen produc-

tion procedure. Purification of the recombinant Omp fragments from *E. coli* with an affinity chromatography column allowed us to obtain the antigen with high purity because this method eliminates mixing of citrus proteins in the purification steps. Therefore, it can be expected that the specificity and sensitivity of Omp antibodies will be enhanced. In accordance with that calculation, the antibodies against OMP-N and OMP-C in this research were found to have a higher positive/negative ratio than the antibody against ‘*Ca. L. asiaticus*’, indicating that Omp antibodies have higher sensitivity in detecting the pathogen from different geographical origins (Tables 1 and 2). ‘*Ca. L. asiaticus*’-infected periwinkle samples detected with these antibodies usually showed higher absorbance at 450 nm than citrus samples, suggesting that periwinkle may be a suitable, reasonably compatible host for this bacterium. Moreover, as the bioinformatics analysis had showed, the POTRA domains on the N-terminal region of Omp protein possess higher amounts of membrane-surface-exposed hydrophilic sequences (which are poorly conserved) than the membrane-spanning β -barrel structure on the C-terminal region of Omp (5). This most likely explains why the antibody prepared from the N-terminal region of Omp protein was more sensitive than that generated from the C-terminal region. What’s more, due to the high level of sequence conservation of ‘*Ca. L. asiaticus*’ Omp protein, it is speculated that these Omp antibodies may also be applied to detect ‘*Ca. L. asiaticus*’ isolates in other countries as well. Moreover, bioinformatics analysis indicates that Omp proteins of ‘*Ca. L. asiaticus*’ and ‘*Ca. L. africanus*’ have similar structure. Therefore, it is possible that a similar method may be used to produce the Omp antibodies of ‘*Ca. L. africanus*’.

The antibodies against OMP-N and OMP-C were specific to ‘*Ca. L. asiaticus*’. They did not show a positive signal with other common viral, bacterial, and fungal pathogens of citrus (Table 3). It is also free from interference of nutrient-deficient or glyphosate-damaged samples which also show symptoms similar to those of HLB pathogen-infected samples (12,16–18,20). The antibody against OMP-N detects ‘*Ca. L. asiaticus*’ in 45.7% of the sympto-

Fig. 1. Detection of ‘*Candidatus Liberibacter asiaticus*’ by immunofluorescence in midrib sections of healthy (A) and infected (B) periwinkles, in midrib sections of healthy (C) and infected (D) citrus. X, xylem; ST, sieve tube; bar = 50 μ m.

matic citrus samples by ELISA, which is similar to the sensitivity level obtained by traditional PCR assays (Table 4). Furthermore, ELISA is a protein-based detection method, has a different principle from PCR assays, and does not suffer from several adverse factors of citrus on DNA extraction and PCR reaction. Therefore, consensus detection results by ELISA and PCR improve the reliability of the HLB diagnosis to avoid the contradictory or ambiguous results that are prone to be provided by the single PCR detection method.

These antibodies against OMP-N and OMP-C can also be used as research tools. For example, Omp antibodies can be used in immunofluorescence to track the distribution of '*Ca. L. asiaticus*' in citrus plants (Fig. 1) or in *D. citri* bodies. They can also be used for studying protein-protein interactions to study the function of Omp proteins. Furthermore, the Omp antibodies can be used to produce an immunochromatographic colloid gold strip for the field detection of HLB pathogen (32). This method is not time intensive, as in the case of extracting DNA templates or processing then in highly advanced equipment. Thus it is a convenient and low-cost detection method and may be used extensively by farmers and quarantine officers.

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