Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2013) **14**(4), 391–404 DOI: 10.1111/mpp.12015

# Global gene expression changes in *Candidatus* Liberibacter asiaticus during the transmission in distinct hosts between plant and insect

QING YAN[1,†], ASWATHY SREEDHARAN[1,†], SHIPING WEI[1], JIHUA WANG[1,2], KIRSTEN PELZ-STELINSKI[1], SVETLANA FOLIMONOVA[1] AND NIAN WANG[1,*]

[1]*Department of Microbiology and Cell Science, Citrus Research and Education Center, IFAS, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA*
[2]*Crops Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China*

## SUMMARY

Huanglongbing (HLB) or citrus greening disease is a destructive disease of citrus worldwide, which is associated with *Candidatus* Liberibacter asiaticus. This phloem-limited fastidious pathogen is transmitted by the Asian citrus psyllid, *Diaphorina citri*, and appears to be an intracellular pathogen that maintains an intimate association with the psyllid or the plant throughout its life cycle. The molecular basis of the interaction of this pathogen with its hosts is not well understood. We hypothesized that, during infection, *Ca*. L. asiaticus differentially expresses the genes critical for its survival and/or pathogenicity in either host. To test this hypothesis, quantitative reverse transcription-polymerase chain reaction was performed to compare the gene expression of *Ca*. L. asiaticus *in planta* and in psyllid. Overall, 381 genes were analysed for their gene expression *in planta* and in psyllid. Among them, 182 genes were up-regulated *in planta* compared with in psyllid ($P < 0.05$), 16 genes were up-regulated in psyllid ($P < 0.05$) and 183 genes showed no statistically significant difference ($P \geq 0.05$) in expression between *in planta* and in psyllid. Our study indicates that the expression of the *Ca*. L. asiaticus genes involved in transcriptional regulation, transport system, secretion system, flagella assembly, metabolic pathway and stress resistance are changed significantly in a host-specific manner to adapt to the distinct environments of plant and insect. To our knowledge, this is the first large-scale study to evaluate the differential expression of *Ca*. L. asiaticus genes in a plant host and its insect vector.

## INTRODUCTION

In order to adapt to diverse environments, bacteria alter their gene regulation. Bacteria are known to sense and respond to changes in nutrients, pH, temperature, oxygen tension, redox potential and osmolality for optimal growth and survival in different environmental niches (Mekalanos, 1992). Bacterial gene expression is altered by the activation of specific genes whose products assist in survival and by the deactivation of those whose products are not necessary in a particular environment (Chowdhury *et al*., 1996). Plant pathogenic bacteria are known to up-regulate genes which are implicated in bacterial adaptation to the host environment during infection. As reported by Okinaka *et al*. (2002), *Erwinia chrysanthemi* genes encoding products involved in anaerobiosis, iron uptake, transporters, chemotaxis and stress responses to reactive oxygen species (ROS) and heat are induced *in planta*. It has been suggested that virulence factors are expressed at different stages of the infection process dictated by the changing microenvironment of the host (Chowdhury *et al*., 1996).

Interestingly, insect-transmitted bacterial plant pathogens switch hosts belonging to two distinct kingdoms, namely plants (Plantae) and insects (Animalia). These different environments seem to have a dramatic effect on the gene regulation of bacterial plant pathogens. Oshima *et al*. (2011) have carried out pioneering work comparing the gene expression of *Candidatus* Phytoplasma asteris OY-M in garland chrysanthemum (*Chrysanthemum coronarium*) and its leafhopper vector, *Macrosteles striifrons*. Approximately 33% of Phytoplasma genes showed differential expression on switching between plant and insect hosts, suggesting that Phytoplasma regulates genes encoding transporters, secreted proteins and metabolic enzymes in a host-specific manner. The elucidation of the mechanism underlying the adaptation of a pathogen to different hosts may contribute to the development of novel methods for disease control tactics.

Among the insect-transmitted bacterial plant pathogens, *Candidatus* Liberibacter asiaticus is associated with citrus huanglongbing (HLB), which is the most devastating disease of citrus worldwide. This disease is widespread in the citrus-growing regions of Asia, Africa and the Americas, resulting in a substantial reduction in the lifespan of infected trees and fruit production (Bové, 2006). *Candidatus* L. asiaticus is capable of infecting all known commercial varieties of citrus and several close relatives, although symptom expression varies depending on the host genotype (Folimonova *et al*., 2009; Sagaram *et al*., 2009; Tyler *et al*.,

**Correspondence*: Email: nianwang@ufl.edu
†These authors contributed equally to this work.

2009). Characteristic symptoms associated with HLB include blotchy mottle and the yellowing and thickening of leaves, together with enlarged midribs and lateral veins (Bové, 2006). The infected leaves grow upright and, eventually, defoliation followed by dieback may occur. HLB also affects fruit quality and appearance, and interferes with sucrose transport, resulting in heavy starch accumulation, phloem damage and eventual phloem collapse (Etxeberria *et al*., 2009; Folimonova and Achor, 2010; Kim *et al*., 2009).

*Candidatus* L. asiaticus is transmitted by the Asian citrus psyllid *Diaphorina citri*. The insect transmits the bacterium via salivary secretion in a persistent manner (Hung *et al*., 2004; Xu *et al*., 1988). *Diaphorina citri* ingests the bacteria whilst feeding on the phloem of infected plants, and the bacteria circulate inside its vector, passing from the midgut into the haemolymph, and then migrating to the salivary glands, from which they are inoculated into the plant phloem with saliva during subsequent probing (Ammar *et al*., 2011). Although the psyllid can acquire the pathogen during nymphal and adult stages (Inoue *et al*., 2009; Xu *et al*., 1988), the adults are more successful in transmitting the bacteria when they acquire them as a nymph (Pelz-Stelinski *et al*., 2010). Once infected, *D. citri* is capable of inoculating the host plant with *Ca*. L. asiaticus throughout its life (Hung *et al*., 2004), although the level of pathogen transmission decreases over time (Pelz-Stelinski *et al*., 2010).

*Candidatus* L. asiaticus maintains an intimate association with the psyllid or plant throughout its life cycle. Being a fastidious intracellular pathogen, the chromosome of *Ca*. L. asiaticus has undergone significant reduction compared with other members of the Rhiozobiaceae family. *Candidatus* L. asiaticus lacks the common virulence determinants, including the type III (T3SS) and type II (T2SS) secretion systems (Duan *et al*., 2009). Therefore, information regarding the virulence determinants of this pathogen and the mechanism(s) of interaction of *Ca*. L. asiaticus with its hosts remains limited. A clear understanding of the molecular basis of the interaction between *Ca*. L. asiaticus and its hosts is essential to devise control measures against HLB.

In this study, we compared the gene expression of *Ca*. L. asiaticus in its plant host, sweet orange (*Citrus sinensis*) 'Valencia', and its insect host, the psyllid *D. citri*. The implication of the gene expression pattern to the pathogen adaptation of *Ca*. L. asiaticus to its distinct hosts is discussed.

## RESULTS AND DISCUSSION

### Global gene expression profiling of *Ca*. L. asiaticus *in planta* and in psyllid

To compare the gene expression of *Ca*. L. asiaticus in its plant host, sweet orange (*C. sinensis*) 'Valencia', and its insect host, psyllid *D. citri*, quantitative reverse transcription-polymerase chain reaction (QRT-PCR) was conducted. QRT-PCR is sensitive and reliable for the measurement of gene expression (Erickson *et al*., 2009). It is possible to measure the gene expression of *Ca*. L. asiaticus using QRT-PCR because of its small genome size of 1.23 Mb. It contains 1136 predicted open reading frames (ORFs), approximately 74% of which have homologues with known and putative functions, whereas the other 26% represent hypothetical proteins (Duan *et al*., 2009). To further narrow down the genes being tested, we neglected most housekeeping genes, and focused on putative virulence genes, such as genes with domains and motifs found in known virulence factors, and genes involved in transcriptional regulation, the metabolic pathway, secretion system, transportation, motility and signal transduction. In total, 523 genes were selected (Table S1, see Supporting Information) for QRT-PCR analysis, and gene-specific primers were designed (Table S2, see Supporting Information). Among them, 43 genes were discarded because of lack of amplification and 99 as a result of nonspecific amplification (data not shown). We were able to optimize the primers and to perform specific amplification for 381 genes, that were subsequently analysed for their gene expression *in planta* and in psyllid.

It should be noted that, unlike the *Ca*. L. asiaticus-infected plant samples, which were collected according to visible symptoms, no visible difference was observed between infected and noninfected psyllid samples. Thus, two methods were used to collect psyllid RNA samples subjected to QRT-PCR in this work: (i) 15–20 psyllids were pooled before RNA extraction; (ii) RNA was extracted from a single psyllid and only *Ca*. L. asiaticus-infected psyllids (Fig. S1, see Supporting Information) were used for further analysis. To validate the gene expression profiles of *Ca*. L. asiaticus, the expression of 21 randomly selected genes was compared by QRT-PCR using the two differently isolated psyllid RNA samples. The results showed a high degree of concordance (correlation coefficient $R^2 = 0.73$) between the data generated by the two methods (Fig. 1). This result indicates that both methods could reliably detect the gene expression of *Ca*. L. asiaticus in psyllid.

To identify genes with statistically significant changes in expression, only those with a $P$ value for a $t$-test of less than 0.05 are discussed further in this work. Among the genes selected for the expression test, 198 showed a statistically significant expression change in the host plant or insect. Specifically, 182 genes were up-regulated significantly *in planta* compared with in psyllid; 16 genes were up-regulated significantly in psyllid. Using the Clusters of Orthologous Groups (COG) Database, the 198 genes were categorized into 20 diverse functional groups plus one group of genes not classified in the COG Database (Fig. 2). In addition to the hypothetical genes, genes involved in cell motility constituted a major portion of the genes up-regulated in psyllid (Fig. 2). Large numbers of genes involved in transcriptional regulation, metabolism, transport and cell membrane biosynthesis were up-regulated *in planta* (Fig. 2).

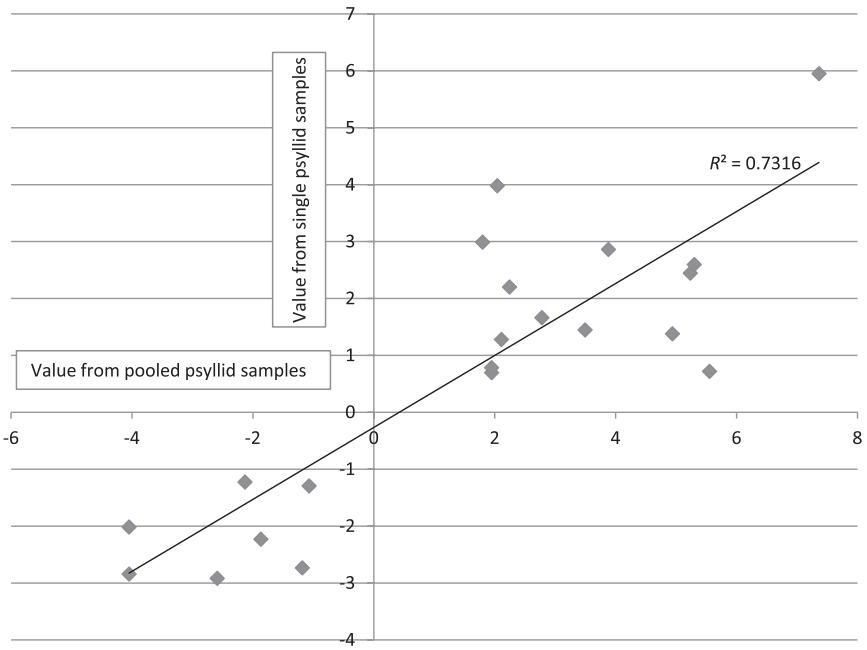


**Fig. 1** Correlation of gene expression profile of *Candidatus* Liberibacter asiaticus *in planta* (*Citrus sinensis*) and in psyllid (*Diaphorina citri*) using two methods to collect psyllid RNA samples. The relative expression values (*in planta* versus in psyllid, $\log_2$ ratio) of 21 genes were plotted. $R^2$, correlation coefficient.

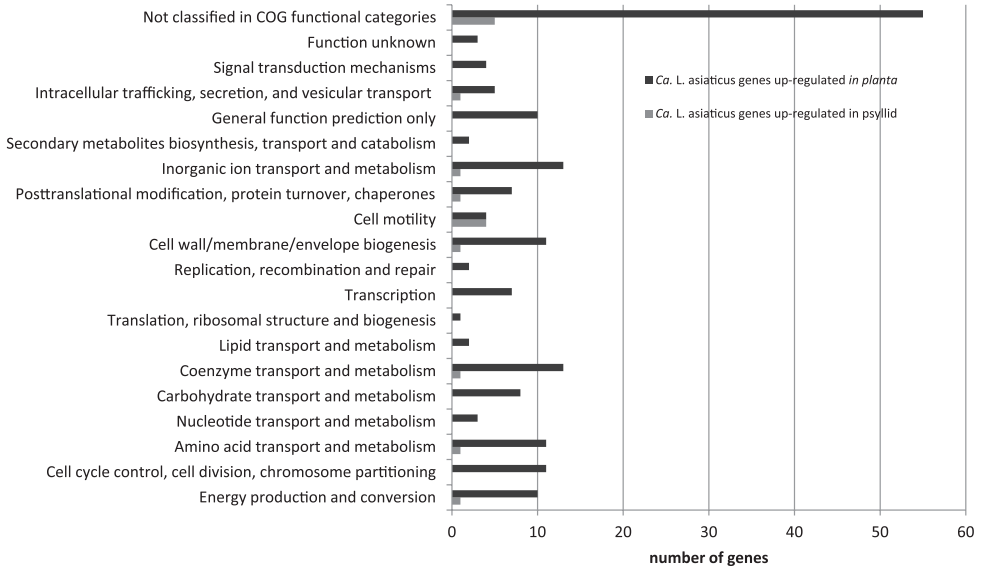


**Fig. 2** Functional classification of differentially expressed genes of *Candidatus* Liberibacter asiaticus. A total of 198 genes of *Ca*. L. asiaticus showed significant expression changes ($P < 0.05$) *in planta* (*Citrus sinensis*) and in psyllid (*Diaphorina citri*) hosts. Using the Clusters of Orthologous Groups (COG) Database, the 198 genes were classified into 20 functional groups plus one group of genes that were not classified in COG. Red represents genes up-regulated *in planta* compared with in psyllid; blue represents genes up-regulated in psyllid compared with *in planta*.

## Transcriptional factors

Consistent with the large numbers of genes of *Ca*. L. asiaticus up-regulated in the host plant, 11 genes encoding (putative) transcriptional regulators were up-regulated *in planta* (Table 1). Sigma factors are transcriptional regulators that interact with the core RNA polymerase and direct the initiation of transcription at cognate promoter sites. Two sigma factors, RpoD and RpoH, were annotated in the genome of *Ca*. L. asiaticus. In this work, we found that the expression of *rpoH* (CLIBASIA_02490) was up-regulated significantly in the host plant. RpoH, also called alternative sigma factor 32, plays an important role in response to diverse environmental stresses, including heat shock, pH and oxidative stress. In addition, RpoH has also been reported to be involved in virulence and growth in the hosts of the human pathogens *Brucella melitensis* and *Vibrio cholera* (Delory *et al*., 2006; Slamti *et al*., 2007). The significant up-regulation of *rpoH* indicates that this sigma factor may be important in the gene regulation of *Ca*. L. asiaticus during

its adaptation to environmental changes between insect and plant.

The expression of two putative LuxR family transcriptional regulators (CLIBASIA_02900, CLIBASIA_02905) was up-regulated in the host plant (Table 1). Regulators of the LuxR family are known to regulate gene expression in the process of quorum sensing. Quorum sensing is an intraspecies or interspecies cell–cell communication system widely distributed in bacteria, and controls multiple bacterial behaviours, including symbiosis, motility, biofilm and virulence (Waters and Bassler, 2005). For example, mutation of the quorum sensing (*las* or *rhl*) in *Pseudomonas aeruginosa* PAO1 reduced rhamnolipid production and elastase activity, which are known virulence factors in this pathogen (Pearson *et al*., 1997). Quorum sensing is also required for virulence and insect transmission of *Xylella fastidiosa* (Chatterjee *et al*., 2008b), an insect-vectored pathogenic bacterium causing Pierce's disease on grape and variegated chlorosis on citrus (Chatterjee *et al*., 2008a). However, no gene(s) encoding the AHL synthase (*luxI*) homologue was observed in *Ca*. L. asiaticus (Duan *et al*., 2009). How *Ca*. L. asiaticus coordinates its gene expression using LuxR remains to be determined.

Another interesting result was the up-regulation of an iron response regulator-encoding gene, *rirA* (CLIBASIA_02535), *in planta* (Table 1). Iron is an essential micronutrient for almost all known organisms. Highly efficient iron acquisition systems have been evolved by bacteria to scavenge iron from living niches (Andrews *et al*., 2006). However, the overloading of iron is deleterious to bacteria, mainly because of the formation of hydroxyl radicals that strongly react with all kinds of biomolecules (Braun, 1997). RirA (rhizobial iron regulator) was initially identified in *Rhizobium leguminosarum* as a negative regulator of iron uptake (Todd *et al*., 2002). A similar function of RirA homologues was also found in *Sinorhizobium meliloti* and *Agrobacterium tumefaciens* (Chao *et al*., 2005; Ngok-Ngam *et al*., 2009). In this work, two genes encoding the ABC transport system involved in iron uptake and four genes involved in haem biosynthesis were dramatically up-regulated *in planta* (Tables 2 and 3). The up-regulation of *rirA* might be a protective measure for *Ca*. L. asiaticus to prevent the deleterious consequences caused by potential iron overload.

## Transport systems

A large number of genes involved in active transport were overexpressed *in planta* (Table 2). ABC transporters are known to be involved in the virulence of various bacteria, and the virulence is associated with the uptake of nutrients (Darwin and Miller, 1999), uptake of metal ions (Boyer *et al*., 2002) or cell attachment (Tamura *et al*., 2002). *Candidatus* L. asiaticus encodes a much larger number of ABC transporters compared with other intracellular bacteria, which suggests that they may be involved in virulence or the elicitation of symptoms (Duan *et al*., 2009). In our study, the expression of 16 ABC transporter genes was up-regulated *in planta*. In psyllid, however, only two ABC transporter genes were up-regulated compared with *in planta* (Table 2).

Many of the ABC transporter genes up-regulated *in planta* were involved in the uptake or efflux of essential micronutrients, in order to maintain appropriate levels of these nutrients in the cell (Table 2). The genes CLIBASIA_02120 and CLIBASIA_02125, encoding homologous components of the SitABCD system responsible for high-affinity uptake of $Mn^{2+}$ and $Fe^{2+}$ (Zhou *et al*., 1999), were up-regulated *in planta*. The SitABCD transport system has been shown to be essential for the virulence of various pathogenic bacteria, including *Salmonella enterica* serovar Typhimurium and

**Table 1** Expression profile of genes encoding transcriptional factors of *Candidatus* Liberibacter asiaticus *in planta* compared with in psyllid.

| Locus tag | Gene name† | Gene product‡ | Relative expression§ |
|---|---|---|---|
| CLIBASIA_00835* | NA | LysR family transcriptional regulator | 2.23 |
| CLIBASIA_00985* | NA | Two-component response regulator CpdR | 1.61 |
| CLIBASIA_01180 | NA | MarR family transcriptional regulator | 5.23 |
| CLIBASIA_01510* | NA | CarD family transcriptional regulator | 1.24 |
| CLIBASIA_01805* | NA | Two-component response regulator. CheY-like receiver domain | 1.08 |
| CLIBASIA_02490* | *rpoH* | Alternative sigma factor RpoH (sigma factor 32) | 1.37 |
| CLIBASIA_02535* | *rirA* | Iron-responsive transcriptional regulator | 1.04 |
| CLIBASIA_02900 | NA | Putative LuxR family transcriptional regulator | 2.11 |
| CLIBASIA_02905 | NA | Putative LuxR family transcriptional regulator | 2.75 |
| CLIBASIA_02950 | *phoU* | Putative phosphate transport system protein | 3.49 |
| CLIBASIA_03950* | *ctrA* | Two-component response regulator | 1.74 |

*Psyllid RNA samples were extracted from a single psyllid (see Experimental procedures).

†NA, gene name was not assigned.

‡Information on gene product was based on the annotation of the genome of *Ca*. L. asiaticus strain psy62 and definition in the KEGG database.

§Fold change ($log_2$ ratio) is the relative gene expression (*in planta* versus in psyllid) of *Ca*. L. asiaticus. Positive value indicates that gene was overexpressed *in planta* compared with in psyllid; negative value indicates that gene was overexpressed in psyllid compared with *in planta*.

**Table 2** Expression profile of genes encoding transport systems of *Candidatus* Liberibacter asiaticus *in planta* compared with in psyllid.

| Locus tag | Gene name† | Gene product‡ | Relative expression§ |
|---|---|---|---|
| CLIBASIA_00090 | NA | Putative ATP-binding component of ABC-type transport system involved in resistance to organic solvents | 7.60 |
| CLIBASIA_00095 | NA | Putative substrate-binding protein of ABC-type transport system involved in resistance to organic solvents | 2.43 |
| CLIBASIA_00265 | NA | Periplasmic binding protein of ABC transporter involved in general L-amino acid transport | −1.29 |
| CLIBASIA_00275 | *aapM* | General L-amino acid transport system permease | 3.64 |
| CLIBASIA_00540 | NA | ABC transporter permease | 2.52 |
| CLIBASIA_00790 | NA | Putative ATPase components of ABC transporters with duplicated ATPase domains | 4.52 |
| CLIBASIA_01135 | *proX* | Glycine betaine/proline ABC transporter | 1.84 |
| CLIBASIA_01140 | NA | Predicted Co/Zn/Cd cation transporters | 3.88 |
| CLIBASIA_02120 | NA | Iron(II)/manganese ABC transporter (homologue of SitA) | 7.92 |
| CLIBASIA_02135 | NA | Iron(II)/manganese ABC transporter (homologue of SitD) | 5.72 |
| CLIBASIA_02415 | NA | ABC transporter nucleotide binding/ATPase protein. Sulphonate/nitrate/taurine transporter | 2.41 |
| CLIBASIA_02420 | NA | ABC transporter permease. Sulphonate/nitrate/taurine transporter | 7.24 |
| CLIBASIA_02955 | *pstB* | ABC transporter, nucleotide binding/ATPase protein. Phosphate transporter | 7.28 |
| CLIBASIA_02960 | *pstA* | ABC transporter, permease. Phosphate transporter | 2.23 |
| CLIBASIA_02965 | *pstC* | ABC transporter, membrane spanning protein. Phosphate transporter | 3.28 |
| CLIBASIA_02970 | *pstS* | Putative phosphate-binding periplasmic protein. Phosphate transporter | 1.28 |
| CLIBASIA_03155 | *lptB* | ABC transporter nucleotide binding/ATPase protein. Lipopolysaccharide ABC transporter | 7.46 |
| CLIBASIA_03625 | *kup* | Putative potassium uptake transport system protein | 1.42 |
| CLIBASIA_04115 | NA | Kef-type $K^+$ transport system, predicted NAD-binding component | 3.62 |
| CLIBASIA_04145* | *nodT* | Nodulation outer membrane efflux protein | 1.54 |
| CLIBASIA_04415 | NA | Putative threonine efflux protein | 2.09 |
| CLIBASIA_04810 | NA | ABC transporter nucleotide binding/ATPase | −1.26 |
| CLIBASIA_05070 | NA | ABC-type amino acid transport/signal transduction systems, periplasmic component/domain | 2.97 |
| CLIBASIA_05075 | NA | ABC-type amino acid transport system, permease component | 7.96 |
| CLIBASIA_05125 | NA | ATPase components of ABC transporters with duplicated ATPase domains | 4.80 |

*Psyllid RNA samples were extracted from a single psyllid (see Experimental procedures).

†NA, gene name was not assigned.

‡Information on gene product was based on the annotation of the genome of *Ca*. L. asiaticus strain psy62 and definition in the KEGG database.

§Fold change ($\log_2$ ratio) is the relative gene expression (*in planta* versus in psyllid) of *Ca*. L. asiaticus. Positive value indicates that gene was overexpressed *in planta* compared with in psyllid; negative value indicates that gene was overexpressed in psyllid compared with *in planta*.

*Escherichia coli* (Boyer *et al*., 2002; Sabri *et al*., 2008). Iron is essential to almost all organisms, including bacteria. It participates in many major biological processes, including respiration, the trichloroacetic acid cycle, oxygen transport, gene regulation and DNA biosynthesis (Andrews *et al*., 2006). Iron uptake is important in the plant–bacterium interaction in the symbiont *S. meliloti* and the pathogen *A. tumefaciens* (Chao *et al*., 2005; Kitphati *et al*., 2007). The ability to acquire $Mn^{2+}$ plays a major role in virulence, and also contributes to protection against oxidative stress in various plant and animal pathogens (Li *et al*., 2011; Papp-Wallace and Maguire, 2006). Studies have also shown that *sitABCD* genes are induced specifically during the systemic infection of *Salmonella enterica* serovar Typhimurium. During infection, the host has been reported to actively reduce the availability of extracellular iron, as a nonspecific defence mechanism, prompting successful pathogens to evolve efficient strategies to acquire iron from this iron-limiting environment (Wooldridge and Williams, 1993), including the utilization of low-iron-induced genes, such as *sitABCD* (Janakiraman and Slauch, 2000).

Our results also showed the up-regulation of the *pstSCAB-phoU* operon (CLIBASIA_02950, CLIBASIA_02955, CLIBASIA_02960, CLIBASIA_02965, CLIBASIA_02970) encoding an ABC-type transporter system for phosphate uptake into the bacterial cell of *Ca*. L. asiaticus in the host plant (Tables 1 and 2). Phosphate plays a major role in the conversion and transfer of energy in the tricarboxylic acid cycle and in glycolysis. Several studies have associated the *pst-phoU* system with the survival and virulence of bacteria, with mutations causing reduced virulence, sensitivity to the bactericidal effect of serum, reduction in the amount of capsular antigen at the cell surface, impaired colonization ability and attachment, and reduced capacity to multiply within phagocytes and serum, collectively suggesting that bacterial cell surface modifications occur in the mutants, and implicating the *pst-phoU* system in the regulation of bacterial pathogenicity (Lamarche *et al*., 2008). In addition to the role in phosphate uptake, PhoU has also been found to be a master regulator in a process of persistence involved in bacterial survival to antibiotic treatment in *E. coli* and *Mycobacterium tuberculosis*. Mutation of *phoU* leads to higher susceptibility to diverse stresses, including antibiotics, starvation, weak acid, heat and energy inhibitors (Li and Zhang, 2007; Shi and Zhang, 2010).

The gene *proX* (CLIBASIA_01135), involved in glycine betaine/proline transport, was up-regulated *in planta* compared with in psyllid (Table 2). The induction of *proX in planta* might contribute to the adaptation of *Ca*. L. asiaticus in the two diverse environments of the plant and insect systems. After inoculation into the

**Table 3** Expression profile of genes involved in metabolism pathway of *Candidatus* Liberibacter asiaticus *in planta* compared with in psyllid.

| Locus tag | Gene name† | Gene product‡ | KEGG pathway | Relative expression§ |
|---|---|---|---|---|
| Carbohydrate metabolism | | | | |
| CLIBASIA_00375 | *fumC* | Fumarate hydratase | Citrate cycle | 3.39 |
| CLIBASIA_00825 | *glk* | Glucokinase | Glycolysis | 2.78 |
| CLIBASIA_01065 | NA | UDP-glucose 4-epimerase | Galactose | 3.44 |
| CLIBASIA_01165 | NA | 5-Amino-6-(5-phosphoribosylamino)uracil reductase/diaminohydroxyphosphoribosylaminopyrimidine | Riboflavin metabolism | 3.88 |
| CLIBASIA_01680 | *acnA* | Aconitate hydratase | Glyoxylate, dicarboxylate metabolism/citrate cycle | 5.27 |
| CLIBASIA_02695 | NA | Fructose-bisphosphate aldolase | Glycolysis | 3.4 |
| CLIBASIA_02700 | *pgk* | Phosphoglycerate kinase | Glycolysis | 2.1 |
| CLIBASIA_02705 | NA | Glyceraldehyde 3-phosphate dehydrogenase | Glycolysis | 2.77 |
| CLIBASIA_02710 | *tkt* | Transketolase | Pentose phosphate pathway | 6.24 |
| CLIBASIA_02785 | *eno* | Phosphopyruvate hydratase | Glycolysis | 2.46 |
| CLIBASIA_02810 | *lpdA* | Dihydrolipoamide dehydrogenase | Glycolysis | 1.49 |
| CLIBASIA_03595* | *pckA* | Phosphoenolpyruvate carboxykinase | Glycolysis | 0.79 |
| CLIBASIA_04750 | *mdh* | Malate dehydrogenase | Glyoxylate,dicarboxylate metabolism | 2.32 |
| CLIBASIA_05045 | NA | Phosphoglucomutase | Glycolysis | 3.47 |
| Energy metabolism | | | | |
| CLIBASIA_00340 | *glnA* | Glutamine synthetase protein | Alanine, aspartate and glutamate metabolism | 4.76 |
| CLIBASIA_00345 | *glsA* | Glutaminase | D-glutamine and D-glutamate metabolism | 1.76 |
| CLIBASIA_00585* | NA | Inorganic pyrophosphatase | Oxidative phosphorylation | 2.2 |
| CLIBASIA_02750 | NA | Carbonate dehydratase | Nitrogen metabolism | 1.33 |
| CLIBASIA_03735 | *nuoA* | NADH dehydrogenase subunit A | Oxidative phosphorylation | −2.09 |
| CLIBASIA_04725 | *sdhA* | Succinate dehydrogenase flavoprotein subunit | Oxidative phosphorylation | 2.47 |
| Nucleotide metabolism | | | | |
| CLIBASIA_00055 | *gmk* | Guanylate kinase | Purine metabolism | 2.41 |
| CLIBASIA_00400 | *pyrG* | CTP synthetase | | 1.51 |
| CLIBASIA_00515 | *tmk* | Thymidylate kinase | Pyrimidine metabolism | 3.87 |
| Metabolism of terpenoids and polyketides | | | | |
| CLIBASIA_03210 | *ispB* | Octaprenyl-diphosphate synthase protein | Terpenoid backbone biosynthesis | 2.23 |
| CLIBASIA_04105 | *rfbA* | Glucose-1-phosphate thymidylyltransferase | Polyketide sugar unit biosynthesis | 1.82 |
| CLIBASIA_05065 | NA | Geranyltranstransferase protein | Terpenoid backbone biosynthesis | 3.65 |
| Metabolism of cofactors and vitamins | | | | |
| CLIBASIA_00425 | *hemH* | Ferrochelatase | Porphyrin and chlorophyll metabolism | 3.29 |
| CLIBASIA_01160 | NA | Riboflavin synthase subunit α | Riboflavin metabolism | −2.99 |
| CLIBASIA_01965 | *folD* | Methylenetetrahydrofolate dehydrogenase/cyclohydrolase protein | One-carbon pool by folate | 1.9 |
| CLIBASIA_02815 | NA | Lipoyl synthase | Lipoic acid metabolism | 2.84 |
| CLIBASIA_03515 | *hemE* | Uroporphyrinogen decarboxylase | Porphyrin and chlorophyll metabolism | 3.93 |
| CLIBASIA_03560 | *folC* | FolC bifunctional protein | Folate biosynthesis | 2.49 |
| CLIBASIA_04185 | *acpS* | 4′-Phosphopantetheinyl transferase | Pantothenate and CoA biosynthesis | 1.84 |
| CLIBASIA_04670 | NA | Hypothetical protein | Folate biosynthesis | 2.65 |
| CLIBASIA_04685 | *hemD* | Uroporphyrinogen-III synthase | Porphyrin and chlorophyll metabolism | 3.03 |
| CLIBASIA_04695 | *hemC* | Porphobilinogen deaminase | Porphyrin and chlorophyll metabolism | 4.1 |
| CLIBASIA_04875 | NA | Coproporphyrinogen III oxidase | Porphyrin and chlorophyll metabolism | 2.16 |
| Amino acid metabolism | | | | |
| CLIBASIA_04835 | *kamA* | L-lysine 2,3-aminomutase protein | Lysine degradation | 6.39 |
| CLIBASIA_02600 | NA | Creatinine amidohydrolase | Arginine/proline metabolism | 8.43 |
| CLIBASIA_00220 | NA | Phytoene synthase protein | Biosynthesis of secondary metabolites | 8.56 |
| CLIBASIA_03995 | *thrA* | Homoserine dehydrogenase | Glycine, serine and threonine metabolism | 2.30 |
| CLIBASIA_01170 | *glyA* | Serine hydroxymethyltransferase | Glycine, serine and threonine metabolism | 2.32 |
| CLIBASIA_04210 | *glmS* | Glucosamine-fructose-6-phosphate aminotransferase | Alanine, aspartate and glutamate metabolism | −1.71 |
| Metabolism of other amino acids | | | | |
| CLIBASIA_02780 | *kdsA* | 2-Dehydro-3-deoxyphosphooctonate aldolase | Lipopolysaccharide biosynthesis | 1.82 |
| CLIBASIA_02915 | *gshB* | Glutathione synthetase | Glutathione metabolism | 1.59 |
| CLIBASIA_03015 | *gnd* | 6-Phosphogluconate dehydrogenase | Glutathione metabolism | 7.01 |
| CLIBASIA_03280 | *kdsB* | 3-Deoxy-manno-octulosonate cytidylyltransferase | Lipopolysaccharide biosynthesis | 3.29 |
| CLIBASIA_03290 | *lpxB* | Lipid-A-disaccharide synthase | Lipopolysaccharide biosynthesis | 6.12 |
| CLIBASIA_03420 | *mviN* | Integral membrane protein MviN | Peptidoglycan biosynthesis | 3.62 |
| CLIBASIA_04785 | *gor* | Glutathione reductase | Glutathione metabolism | 4.17 |
| Genetic information processing | | | | |
| *Folding, sorting and degradation* | | | | |
| CLIBASIA_03720 | NA | Chaperonin GroEL | RNA degradation | 1.85 |
| *Replication and repair* | | | | |
| CLIBASIA_02530 | NA | Primosome assembly protein PriA | Homologous recombination | 4.97 |

*Psyllid RNA samples were extracted from a single psyllid (see Experimental procedures).

†NA, gene name was not assigned.

‡Information on gene product was based on the annotation of the genome of *Ca*. L. asiaticus strain psy62 and definition in the KEGG database.

§Fold change ($\log_2$ ratio) is the relative gene expression (*in planta* versus in psyllid) of *Ca*. L. asiaticus. Positive value indicates that gene was overexpressed *in planta* compared with in psyllid; negative value indicates that gene was overexpressed in psyllid compared with *in planta*.

plant phloem by the insect, the pathogen encounters a change in osmolarity and must protect itself from dehydration and loss of turgor. Many prokaryotes deal with a change in osmolarity through the uptake of compatible solutes, which do not interfere with the metabolism of the organism even at high concentrations (Roebler and Muller, 2001). One of the most common osmoprotectants is glycine betaine, utilized by many bacteria, including *E. coli* (Perroud and Le Rudulier, 1985). In addition to being an osmoprotectant, glycine betaine and proline betaine can also function as carbon, nitrogen and energy sources supporting growth in certain bacteria, such as *Rhizobium* sp. (Bernard *et al*., 1986).

### Metabolic pathway

In our study, eight genes (CLIBASIA_00825, CLIBASIA_02695, CLIBASIA_02700, CLIBASIA_02705, CLIBASIA_02785, CLIBASIA_02810, CLIBASIA_03595 and CLIBASIA_05045), encoding enzymes involved in glycolysis, were up-regulated in the host plant (Table 3). *Candidatus* L. asiaticus is known to survive exclusively in the citrus phloem where glucose and other nutrients, derived from photosynthesis, are transported. Furthermore, elevated glucose was observed in *Ca*. L. asiaticus-infected citrus (Fan *et al*., 2010). The high expression of glycolysis-associated genes *in planta* indicates that *Ca*. L. asiaticus can use glucose acquired from the host plant for the generation of energy to support the intracellular growth of the pathogen in the plant. In addition, the glycolysis pathway is also up-regulated during infection and is important to the virulence of *Yersinia pseudotuberculosis* and *Salmonella enterica* serovar Typhimurium (Chaudhuri *et al*., 2009; Rosso *et al*., 2008).

*kdsA* (CLIBASIA_02780), *kdsB* (CLIBASIA_03280) and *lpxB* (CLIBASIA_03290), involved in lipopolysaccharide (LPS) biosynthesis, together with one LPS ABC transporter gene, *lptB* (CLIBASIA_03155), were overexpressed *in planta* (Tables 2 and 3). The up-regulation of LPS genes might be important for *Ca*. L. asiaticus to survive *in planta*, as LPS is the major component of the outer membrane in Gram-negative bacteria which protects bacterial cells from unfavourable plant environments (Dow *et al*., 1995). In plant–pathogen interactions, LPS also functions as a pathogen-associated molecular pattern (PAMP) by eliciting basal defence-related responses (Parker, 2003). The infection of *Ca*. L. asiaticus was observed to induce the expression of many pathogenesis-related genes, including WRKY4, WRKY6, ERF-1 and ERF-2, in host citrus (sweet orange, *C. sinensis*), indicating an activation of defence mechanisms (Kim *et al*., 2009). Phytopathogenic bacteria have evolved the ability to deliver effector molecules inside the host cell via T3SS to suppress plant defences (Jones and Dangl, 2006). However, no T3SS was found in the genome of *Ca*. L. asiaticus. How *Ca*. L. asiaticus suppresses plant defence remains to be determined.

In this study, genes involved in haem biosynthesis, including *hemH* (CLIBASIA_00425), *hemE* (CLIBASIA_03515), *hemC* (CLIBASIA_04695) and *hemD* (CLIBASIA_04685), were up-regulated *in planta* (Table 3). Previous studies have shown that physiological factors, such as oxygen, nitrate and carbon sources, act as signals for the regulation of haem biosynthesis (Schobert and Jahn, 2002). Haem, a biological catalyst synthesized by bacteria, functions not only as a source of iron for bacterial growth, but also as a regulator involved in virulence regulation (Wandersman and Delepelaire, 2004). Haem is also involved in various aspects of oxidative metabolism, including oxidative stress responses, oxygenation reactions and detoxification (Panek and O'Brian, 2002). *hemH* encodes a ferrochelatase involved in the last step of the haem metabolic pathway and catalyses the insertion of a ferrous iron atom into the porphyrin ring. Ferrochelatase has been shown previously to be essential for intracellular survival and virulence (Almiron *et al*., 2001). The genes *hemE*, *hemD* and *hemC*, encoding uroporphyrinogen decarboxylase, uroporphyrinogen-III synthase and porphobilinogen deaminase, respectively, are also involved in bacterial haem biosynthesis (Frankenberg *et al*., 2003). In *P. aeruginosa*, these three genes have been shown to be involved indirectly in virulence by regulating the secretion of a known virulence factor, the exopolysaccharide, alginate (Mohr *et al*., 1994). Similarly, the induction of haem biosynthesis genes *in planta* may be important in the survival and virulence of *Ca*. L. asiaticus.

### Secretion system

The majority of bacterial secretory proteins and membrane proteins are translocated in a Sec-dependent secretion system. Genome sequencing has revealed that *Ca*. L. asiaticus harbours all of the basic components of the Sec machinery (Duan *et al*., 2009). In this work, the expression of *secE* (CLIBASIA_00140) and *secD/F* (CLIBASIA_04120) was found to be up-regulated *in planta* compared with in psyllid (Table 4). SecE is the component of the SecYEG translocase complex which facilitates the translocation and membrane insertion of the majority of inner membrane proteins (Dalbey and Chen, 2004). SecD/F is one of the translocase subunits, functionally associated with the SecYEG complex by optimizing the secretion of substrate proteins (Duong and Wickner, 1997). The up-regulation of Sec-associated genes *in planta* is interesting because the Sec-dependent secretory pathway is involved in the virulence of various intracellular pathogenic bacteria via the secretion of various proteins (including virulence factors) that play important roles in the host–pathogen interaction (Van Gijsegem *et al*., 1993). The absence of other well-known effector secretion systems, including T3SS, makes the Sec-dependent secretion pathway more important for the virulence of *Ca*. L. asiaticus, as it may play a major role in the translocation of virulence factors.

Proteins secreted by the Sec-dependent pathway are characterized by the presence of an N-terminal signal peptide, indicating

**Table 4** Expression profile of genes encoding secretion system of *Candidatus* Liberibacter asiaticus *in planta* compared with in psyllid.

| Locus tag | Gene name† | Gene product‡ | Relative expression§ |
|---|---|---|---|
| CLIBASIA_00070* | NA | Protein with a putative signal peptide | −1.37 |
| CLIBASIA_00140 | *secE* | Preprotein translocase subunit involved in bacterial secretion system | 4.84 |
| CLIBASIA_00215 | NA | Protein with a putative signal peptide | 1.15 |
| CLIBASIA_00470 | NA | Protein with a putative signal peptide | 7.36 |
| CLIBASIA_00525* | NA | Protein with a putative signal peptide | −2.40 |
| CLIBASIA_00530* | NA | Protein with a putative signal peptide | −0.84 |
| CLIBASIA_01345 | NA | Serralysin. RTX toxins and related $Ca^{2+}$-binding proteins | 5.55 |
| CLIBASIA_02145* | NA | Protein with a putative signal peptide | 1.86 |
| CLIBASIA_02215* | NA | Protein with a putative signal peptide | 2.59 |
| CLIBASIA_02275 | NA | Protein with a putative signal peptide | 1.77 |
| CLIBASIA_02610* | NA | Protein with a putative signal peptide | −2.75 |
| CLIBASIA_02845 | NA | Protein with a putative signal peptide | 4.17 |
| CLIBASIA_02990 | *ffh* | Signal recognition particle protein involved in bacterial secretion system | 1.81 |
| CLIBASIA_03085 | NA | Protein with a putative signal peptide | 4.94 |
| CLIBASIA_03180 | *lspA* | Lipoprotein signal peptidase involved in bacterial secretion system | 1.46 |
| CLIBASIA_03230 | NA | Protein with a putative signal peptide | 3.52 |
| CLIBASIA_03915* | NA | Protein with a putative signal peptide | 2.75 |
| CLIBASIA_04025* | NA | Protein with a putative signal peptide | 3.40 |
| CLIBASIA_04030* | NA | Protein with a putative signal peptide | 0.83 |
| CLIBASIA_04040 | NA | Protein with a putative signal peptide | 2.43 |
| CLIBASIA_04120 | *secD/F* | Bifunctional preprotein translocase subunit involved in bacterial secretion system | 4.33 |
| CLIBASIA_04190 | *lepB* | Type I signal peptidase involved in bacterial secretion system | 1.46 |
| CLIBASIA_04330* | NA | Protein with a putative signal peptide | 1.00 |
| CLIBASIA_04515 | NA | Protein with a putative signal peptide | 4.03 |
| CLIBASIA_04530* | NA | Protein with a putative signal peptide | 2.67 |
| CLIBASIA_04540* | NA | Protein with a putative signal peptide | −0.91 |
| CLIBASIA_04560* | NA | Protein with a putative signal peptide | 2.04 |
| CLIBASIA_04580 | NA | Protein with a putative signal peptide | 2.04 |
| CLIBASIA_05150* | NA | Protein with a putative signal peptide | 1.70 |
| CLIBASIA_05320* | NA | Protein with a putative signal peptide | 4.39 |
| CLIBASIA_05480 | NA | Protein with a putative signal peptide | 7.67 |
| CLIBASIA_05570 | NA | Protein with a putative signal peptide | 6.79 |

*Psyllid RNA samples were extracted from a single psyllid (see Experimental procedures).

†NA, gene name was not assigned.

‡Information on gene product was based on the annotation of the genome of *Ca*. L. asiaticus strain psy62 and definition in the KEGG database. Signal peptide was predicted using signal V4.0 program.

§Fold change ($log_2$ ratio) is the relative gene expression (*in planta* versus in psyllid) of *Ca*. L. asiaticus. Positive value indicates that gene was overexpressed *in planta* compared with in psyllid; negative value indicates that gene was overexpressed in psyllid compared with *in planta*.

potential translocation into the host. In this study, 26 hypothetical genes with a predicted N-terminal signal peptide showed altered expression in the host plant or insect. Among the 26 genes, 21 were up-regulated *in planta* (Table 4). Specifically, the expression of CLIBASIA_05480, CLIBASIA_00470 and CLIBASIA_05570 was increased dramatically by a factor of more than six ($log_2$ value, equal to 64 times fold change) *in planta* compared with in psyllid. Although no known functional domain was found, the significant induction of these secreted proteins indicates that they may be important in the plant–pathogen interaction between *Ca*. L. asiaticus and the host plant.

The gene product of CLIBASIA_05150 harbours an 'OMP_b-brl' domain (amino acids 52–225, *e* value = 6.70E-5) and a transmembrane segment predicted by the TMHMM2 program. The OMP_b-brl domain assumes a membrane-bound β-barrel which is conserved in a wide range of outer membrane proteins, such as OmpA, OmpX and NspA. Proteins with this domain have been suggested to be involved in diverse biological functions, including virulence, membrane stability and resistance to environmental stresses (Kim *et al*., 2010; Ried and Henning, 1987; Wang, 2002). In particular, many of the outer membrane proteins with a barrel structure are involved in the initial interaction between pathogenic bacteria and their hosts (McClean, 2012). For example, OmpA was found to be the major protein of *Cronobacter sakazakii*, binding to fibronectin of human cells, which is the first step in the invasion of the host by the pathogen (Mohan Nair *et al*., 2009).

Five of the 26 putative secreted proteins were up-regulated in the psyllid host. One, the gene CLIBASIA_02610, encoding a hypothetical protein, harbours an imelysin domain (amino acids 37–391, *e* value = 3.2E-75) (Table 4). Two biological functions have been assigned to imelysin: (i) peptide cleavage as a metal-

loproteinase; (ii) iron uptake. Imelysin was first named in *P. aeruginosa* as a zinc peptidase involved in insulin cleavage (Fricke *et al*., 1999). A homologous peptidase was found in the nematode pathogen *Xenorhabdus nematophila*, in which the imelysin homologue was indicated to be involved in insect immunosuppression by destroying antibacterial factors present in insect haemolymph (Caldas *et al*., 2002). In addition, imelysin is important in the support of bacterial growth of *Synechococcus* sp. in iron-limited conditions and is involved in iron uptake or metabolism in *P. aeruginosa* and *V. cholera* (Reddy *et al*., 1988; Xu *et al*., 2011). Thus, the up-regulation of the gene CLIBASIA_02610 in psyllid may indicate that this gene is important in the survival and propagation of *Ca*. L. asiaticus in the insect host.

In addition to the Sec-dependent pathway, *Ca*. L. asiaticus also contains an intact type I secretion system (T1SS) (Duan *et al*., 2009). It is known that offensive enzymes and effectors can be secreted via T1SS in plant and animal pathogenic bacteria (Van Gijsegem *et al*., 1993). A putative T1SS effector, a serralysin, encoded by CLIBASIA_01345, which is located next to the T1SS locus in the genome, has been identified recently by computational analysis of *Ca*. L. asiaticus (Cong *et al*., 2012). In this work, we found that the expression of CLIBASIA_01345 was up-regulated *in planta* compared with in psyllid (Table 4). Serralysin is a secreted metalloprotease produced by a wide range of microorganisms, including plant and human pathogenic bacteria, such as *Serratia marcescens*, *P. aeruginosa*, *Erwinia chrysanthemi*, *Proteus mirabilis* and *Caulobacter crecentus* (Dahler *et al*., 1990; Maeda and Morihara, 1995). It has been shown that serralysin inactivates diverse antimicrobial proteins and peptides (Schmidtchen *et al*., 2002). For example, serralysin produced by *P. mirabilis* has been reported to degrade host immunoglobulins and cleave antimicrobial peptides, including human β-defensin and LL-37 (Belas *et al*., 2004). These allow *P. mirabilis* to modify the host immune response. The production of antimicrobial proteins and peptides is one of the major defence strategies utilized by plants in response to infection by pathogenic organisms (Castro and Fontes, 2005). The up-regulation of the serralysin biosynthesis gene *in planta* indicates that *Ca*. L. asiaticus may also utilize serralysin to modify plant defence, possibly by degrading host antimicrobial peptides. It has also been suggested that serralysin might aid in the acquisition of carbon and nitrogen for bacterial growth and metabolism through the proteolysis of host proteins and nutrient uptake (Basu and Apte, 2008; Belas *et al*., 2004). Serralysin may further help *Ca*. L. asiaticus to survive in its hosts. In addition, the introduction of exogenous antimicrobial peptides into citrus plants, by various transgenic approaches, is being used to control HLB. The presence of serralysin poses a potential challenge for the selection of efficient antimicrobial peptides against *Ca*. L. asiaticus. Thus, the serralysin of *Ca*. L. asiaticus could be a potential target for the screening of antimicrobial compounds for the control of HLB.

## Flagellar assembly

The expression of genes involved in flagellar assembly, including *fliF* (CLIBASIA_02910), *flgI* (CLIBASIA_01305) and *flgD* (CLIBASIA_02035), and the *motB* (CLIBASIA_02080) gene involved in motor function, was up-regulated *in planta*. In contrast, *flgL* (CLIBASIA_02050), *flgK* (CLIBASIA_02055) and *fliE* (CLIBASIA_01320) were overexpressed in psyllid (Table 5). In spite of the small genome size, *Ca*. L. asiaticus has retained most of its flagellar genes (Duan *et al*., 2009), although electron microscopy studies have failed to detect the presence of flagella associated with the bacterium in the phloem (Bové, 2006). Studies have shown that, in intracellular pathogens, the presence of flagella confers a growth disadvantage (Macnab, 1996) and is energetically expensive for the bacteria, unless it serves another essential purpose in the plant

**Table 5** Expression profile of motility-related genes of *Candidatus* Liberibacter asiaticus *in planta* compared with in psyllid.

| Locus tag | Gene name† | Gene product‡ | Relative expression§ |
|---|---|---|---|
| CLIBASIA_02910 | *fliF* | Flagellar MS-ring protein involved in flagellar assembly | 3.39 |
| CLIBASIA_02035 | *flgD* | Flagellar basal body rod modification protein involved in flagellar assembly | 1.96 |
| CLIBASIA_02055 | *flgK* | Flagellar hook-associated protein FlgK | −2.03 |
| CLIBASIA_02080 | *motB* | Flagellar motor protein MotB involved in flagellar assembly | 4.86 |
| CLIBASIA_02090 | NA | Flagellin domain-containing protein | −2.59 |
| CLIBASIA_02050* | *flgL* | Flagellar hook-associated protein FlgL | −1.28 |
| CLIBASIA_03105 | NA | Flp/Fap pilin component | −4.05 |
| CLIBASIA_03945* | *cheY* | Probable two-component response regulator protein involved in bacterial chemotaxis | 2.99 |
| CLIBASIA_01305 | *flgI* | Flagellar basal body P-ring protein involved in flagellar assembly | 3.42 |
| CLIBASIA_01320* | *fliE* | Flagellar hook-basal body protein FliE | −2.29 |

*Psyllid RNA samples were extracted from a single psyllid (see Experimental procedures).

†NA, gene name was not assigned.

‡Information on gene product was based on the annotation of the genome of *Ca*. L. asiaticus strain psy62 and definition in the KEGG database.

§Fold change ($\log_2$ ratio) is the relative gene expression (*in planta* versus in psyllid) of *Ca*. L. asiaticus. Positive value indicates that gene was overexpressed *in planta* compared with in psyllid; negative value indicates that gene was overexpressed in psyllid compared with *in planta*.

**Table 6** Expression profile of genes not classified in the KEGG pathway or other functional category of *Candidatus* Liberibacter asiaticus *in planta* compared with in psyllid.

| Locus tag | Gene† | Gene product‡ | Relative expression§ |
|---|---|---|---|
| CLIBASIA_00495 | NA | Metal-dependent hydrolases of the β-lactamase superfamily I | 6.12 |
| CLIBASIA_00780 | *clpX* | ATP-dependent protease ATP-binding subunit | 3.05 |
| CLIBASIA_00970 | NA | ComF family protein. Predicted amidophosphoribosyltransferases | 2.98 |
| CLIBASIA_01240 | *fpr* | Flavodoxin reductases (ferredoxin-NADPH reductases) family | 2.02 |
| CLIBASIA_01265 | *etfB* | Electron transfer flavoprotein, β subunit | 2.13 |
| CLIBASIA_01480 | NA | Prolipoprotein diacylglyceryltransferase | 4.17 |
| CLIBASIA_02160 | *ftsH* | Metalloprotease. ATP-dependent Zn proteases | 3.93 |
| CLIBASIA_02605 | NA | tRNA and rRNA cytosine-C5-methylases. NOL1/NOP2/SUN family signature protein | 1.59 |
| CLIBASIA_02625 | *dnaJ* | DnaJ-class molecular chaperone with C-terminal Zn finger domain | 2.80 |
| CLIBASIA_02850 | NA | Membrane proteins related to metalloendopeptidases | 2.04 |
| CLIBASIA_02945 | *grpE* | Molecular chaperone GrpE (heat shock protein) | 1.45 |
| CLIBASIA_03135 | NA | Uncharacterized protein, similar to the N-terminal domain of Lon protease | 7.59 |
| CLIBASIA_03170 | NA | Periplasmic serine proteases (ClpP class) | 2.62 |
| CLIBASIA_03175 | NA | Bacterial nucleoid DNA-binding protein. Integration host factor, β subunit | 1.60 |
| CLIBASIA_03315 | *omp* | Surface antigen (D15).Outer membrane protein/protective antigen OMA87 | 2.24 |
| CLIBASIA_03490 | *gidB* | Glucose-inhibited division protein B | 1.82 |
| CLIBASIA_04070 | NA | Oligoendopeptidase F | 2.93 |
| CLIBASIA_04290 | NA | Putative hydrolase serine protease transmembrane protein | 3.80 |
| CLIBASIA_04830 | NA | FeS assembly scaffold SufA | 1.74 |
| CLIBASIA_04885 | NA | Alanyl-tRNA synthetase | 4.12 |
| CLIBASIA_05000 | NA | Cell division protein FtsW | 5.46 |
| CLIBASIA_05405 | NA | Outer membrane assembly lipoprotein YfiO | 3.69 |

†NA, gene name was not assigned.

‡Information on gene product was based on the annotation of the genome of *Ca*. L. asiaticus strain psy62 and definition in the KEGG database.

§Fold change ($\log_2$ ratio) is the relative gene expression (*in planta* versus in psyllid) of *Ca*. L. asiaticus. Positive value indicates that gene was overexpressed *in planta* compared with in psyllid; negative value indicates that gene was overexpressed in psyllid compared with *in planta*.

or insect, such as the export of proteins including virulence factors (Young *et al*., 1999). The absence of flagellar genes is common in many nonmotile obligate intracellular endosymbionts, such as *Blochmannia floridanus* and *Baumannia* (Gil *et al*., 2003; Wu *et al*., 2006). Furthermore, *Ca*. L. asiaticus lacks a functional T3SS, and hence it is possible that the flagellar system may be involved in the delivery of virulence determinants inside the host cell (Duan *et al*., 2009). This was observed in *Buchnera aphidicola*, a nonmotile endosymbiont which has lost most of the flagellar assembly genes, whereas numerous basal bodies (lacking the filament part of the flagellum) covered the cell surface in this pathogen, possibly involved in protein export to the host (Maezawa *et al*., 2006). Interestingly, the expression of the *Ca*. L. asiaticus flagellin gene, CLIBASIA_02090, was significantly lower *in planta* compared with in psyllid (Table 5), which might be a tactic used by the pathogen to avoid the elicitation of plant defence responses. Flagellin has been shown to trigger the host response and to induce basal defence *in planta* as a PAMP factor (Felix *et al*., 1999).

## Environmental stress

In addition to providing the nutrients for the pathogen, plants also produce toxic chemicals and pathogen-degrading enzymes, and undergo deliberate cell suicide in response to invasion by a pathogen (Freeman and Beattie, 2008). Interestingly, the expression of ATP-dependent protease genes, including *clpX* (CLIBASIA_00780) and *ftsH* (CLIBASIA_02160), which are involved in the adaptation of the bacterium to environmental stresses, and are implicated in the virulence of some pathogens (Lithgow *et al*., 2004), was up-regulated *in planta* (Table 6). In addition, known stress response-related genes *dnaJ* (CLIBASIA_02625), *grpE* (CLIBASIA_02945) and *groEL* (CLIBASIA_03720) (Farr and Kogoma, 1991; Gomes and Simão, 2009) were also overexpressed *in planta* (Tables 1 and 6). In *E. coli*, the genes *clpX*, *ftsH*, *grpE*, *groEL* and *dnaJ* belong to the heat-shock regulon controlled by alternative sigma factor RpoH (Nonaka *et al*., 2006), which is required for stress resistance and environment fitness. The up-regulation of *clpX*, *ftsH*, *grpE*, *groEL* and *dnaJ* was consistent with the induction of *rpoH* in the host plant (Table 1). In addition, the expression of *gshB* (CLIBASIA_02915), *gor* (CLIBASIA_04785) and *gnd* (CLIBASIA_03015), encoding enzymes involved in the metabolism of glutathione, was also up-regulated in the host plant (Table 3). Glutathione peroxidase is a common antioxidant of bacteria for efficient protection against oxidative damage (Arenas *et al*., 2011). The up-regulation of these stress resistance-related genes is believed to protect *Ca*. L. asiaticus from harmful plant environments and to contribute to successful colonization of the pathogen in the host plant.

### Hypothetical proteins

Large numbers of hypothetical genes of *Ca*. L. asiaticus were found in this work whose expression was changed significantly *in planta* or in psyllid (Table S3, see Supporting Information). Information on the biological function played by these hypothetical genes is limited. Domain analysis revealed that 10 genes encoded a protein with a transmembrane helix domain, indicating a putative role in membrane-associated function. Specifically, the genes CLIBASIA_01365, CLIBASIA_04165 and CLIBASIA_05050, harbouring a von Willebrand factor type A (vWFA) domain, were up-regulated by a factor of more than three ($\log_2$ value, equal to eight times fold change) *in planta* compared with in psyllid. vWFA is widely distributed in eukaryotes and prokaryotes. It functions as an adhesive glycoprotein on the surface of blood cells in mammals and is involved in multiple processes, including adhesion, migration and signal transduction, by interacting with a large array of ligands (Colombatti *et al*., 1993). In contrast, the functions of the majority of bacterial vWFA are still unknown, with only a few exceptions. For example, the TerY protein of *E. coli* has been found to protect bacterial cells from the toxic effects of heavy metals (Whelan *et al*., 1997), implying a binding role to metal ions (Ponting *et al*., 1999). The vWFA domain-containing D subunit of the enzyme magnesium-protoporphyrin IX chelatase interacts with the enzyme's I subunit in *Synechocystis* PCC6803 (Jensen *et al*., 1998). Similarly, the vWFA proteins of *Ca*. L. asiaticus might contribute to the compatible interactions between the pathogen and host plant by interacting with putative ligands of the plant.

### Why are so many genes up-regulated *in planta* compared with in psyllid?

The gene expression profiles revealed that 198 genes of *Ca*. L. asiaticus showed a significant change in expression *in planta* compared with in psyllid. It is interesting that most (182 of 198 genes, 92%) were up-regulated *in planta* compared with in psyllid. One of the reasons could be the putative bias in the choice of the target genes in our study. As described above, the 381 genes tested in this work were mainly associated with virulence and/or survival, which only represents 32% of the total predicted genes in the whole genome of *Ca*. L. asiaticus. It is possible that some of the genes activated in psyllid were not included in this assay. Another possible reason is that plants are more favourable than psyllids for the gene expression of *Ca*. L. asiaticus. This probably results from the co-evolution among *Ca*. L. asiaticus, citrus and psyllids. It is probable that *Ca*. Liberibacter spp. evolved from an ancestor in the Rhizobiaceae family through adaptive, diversifying and reductive evolutionary processes that occurred during host adaptation (Toft and Andersson, 2010). This is possibly a result of the intimate relationship between rhizobia and plant roots (Gage, 2004). The intimate associations of *Ca*. Liberibacter spp. with plants as endophytes predispose them to frequent encounters with herbivorous insects, providing *Ca*. Liberibacter spp. with ample opportunity to colonize and eventually evolve alternative associations with insects (Nadarasah and Stavrinides, 2011). The genetic contents and regulation of *Ca*. L. asiaticus are thus more suitable for its interaction with plants than with insects, e.g. suppression of plant defence and acquisition of nutrients. As a successful pathogen, *Ca*. L. asiaticus has gained the ability to overcome the plant defence mechanism. It is consistent with our results that the genes of *Ca*. L. asiaticus up-regulated *in planta* are mainly involved in the virulence and/or survival of the pathogen.

## CONCLUSIONS

In this study, transcriptional profiling of the genes involved in the survival and virulence of *Ca*. L. asiaticus was investigated in two of its hosts, sweet orange 'Valencia' and the psyllid vector *D. citri*. Our study indicated that *Ca*. L. asiaticus alters its expression of the genes involved in transcriptional regulation, transport systems, metabolic pathways, secretion systems and stress resistance in a host-specific manner to adapt to the distinct environment of plant and insect. To our knowledge, this is the first large-scale study to evaluate the differential expression of *Ca*. L. asiaticus genes in a plant host and its insect vector.

## EXPERIMENTAL PROCEDURES

### Plant materials

Nine-month-old seedlings of sweet orange (*C. sinensis*) 'Valencia' infected with *Ca*. L. asiaticus were used to evaluate the differential gene expression of *Ca*. L. asiaticus in infected plants. The plants were graft inoculated with two pieces of *Ca*. L. asiaticus-infected budwood from PCR-positive HLB source trees. Inoculated plants were kept in a US Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS)-approved secure glasshouse, with a temperature ranging from 26 to 32 °C at the Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA. After 3 months of graft inoculation, infection was verified by PCR assays using the specific primers A2-J5 and CQULA04F-CQULA04R as described previously (Hocquellet *et al*., 1999; Wang *et al*., 2006) (data not shown).

### Psyllid materials

Healthy psyllids (*D. citri*) were obtained from a healthy culture of psyllids which was established from field populations in Polk Co., FL, USA (28.0′N, 81.9′W) during 2000, prior to the discovery of HLB in Florida. Psyllids were maintained at 27 $\pm$ 1 °C, 80% $\pm$ 2% relative humidity and a 16 h : 8 h light : dark photoperiod without exposure to insecticides. *Candidatus* L. asiaticus-infected psyllids were maintained on confirmed *Ca*. L. asiaticus-infected sweet orange in secure, insect-proof enclosures at the Citrus Research and Education Center, Lake Alfred, FL, USA.

### Extraction of total RNA from infected plant and psyllid

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. For the extraction of RNA from *Ca*. L. asiaticus-infected plants, the midrib of symptomatic leaves was collected and ground in liquid nitrogen to a fine powder before RNA isolation. Two methods were used for RNA isolation from putative *Ca*. L. asiaticus-infected psyllids. As a validation of the gene expression profile, the psyllid RNA samples isolated by these two methods were used for QRT-PCR analysis. The first method was performed by pooling psyllids before RNA extraction. As a result of the lack of visible differences between *Ca*. L. asiaticus-infected and uninfected psyllids, and the low titre of *Ca*. L. asiaticus in psyllids (Benyon *et al*., 2008), 15–20 psyllids were pooled and ground in liquid nitrogen before RNA isolation. The other method involved the extraction of RNA from a single psyllid. The single psyllid was homogenized in 600 μL of lysis buffer using a TissueRuptor (Qiagen). Only the clear supernatant from the lysis was used for RNA extraction following the manufacturer's instructions. In this work, we extracted RNA from a total of 210 psyllids individually. These 210 psyllid RNA samples were subjected to QRT-PCR assay to test for infection of *Ca*. L. asiaticus using primers CQULA04F-CQULA04R, which specifically target the β-operon of *Ca*. L. asiaticus (Wang *et al*., 2006). Forty-eight psyllid RNA samples positive for *Ca*. L. asiaticus were collected for further assay (Fig. S1). Contamination of genomic DNA was removed by RNA treatment with a TURBO-DNA free kit (Ambion, Austin, TX, USA), and the RNA was eluted in 30 μL of water. Checking for DNA contamination in the plant or psyllid RNA samples was performed by a normal PCR test with primers targeting the 16S rDNA of *Ca*. L. asiaticus (Table S2). No detectable DNA contamination was observed in either plant or psyllid RNA samples (data not shown). RNA quantification was performed using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and stored at −80 °C.

### Selection of *Ca*. L. asiaticus genes for QRT-PCR analysis

To narrow down the genes, we decided to neglect most housekeeping genes, and focused on putative virulence genes, genes with putative domains and motifs found in virulence factors and genes encoding metabolic pathways, transporters, motility and signal transduction. The list of genes containing a signal peptide was accessed from Integrated Microbial Genomes (http://img.jgi.doe.gov/), predicted using the SignalP v3.0 program (http://www.cbs.dtu.dk/services/SignalP/). The transmembrane domain was predicted by the SMART program (http://smart.embl-heidelberg.de/). The remaining proteins encoded by *Ca*. L. asiaticus were analysed based on their annotations in the GenBank and KEGG databases.

### QRT-PCR analysis

All QRT-PCRs were performed using an Applied Biosystems 7500 Fast Real-time PCR system (Foster City, CA, USA) with a QuantiTect SYBR Green RT-PCR kit (Qiagen). The primers were designed from the sequence of the *Ca*. L. asiaticus genome using DNASTAR software. The total reaction volume of one-step QRT-PCR was 25 μL and contained 2 × QuantiTect SYBR Green RT-PCR Master Mix (12.5 μL), 10 μM gene-specific primers (1.25 μL), QuantiTect RT Mix (0.5 μL) and 50 ng of RNA template (1 μL). 16S rRNA was used as the endogenous control. Reactions were incubated at 50 °C for 30 min, and at 95 °C for 15 min, cycled (40 times) at 94 °C for 15 s, 54–56 °C for 30 s and 72 °C for 30 s. Melting curve analysis was conducted to verify the specificity of the QRT-PCR products. The products were also run on a 2% agarose gel to confirm the presence of only a single band. Two technical replicates and three biological replicates were used for each of the genes. The relative fold change in target gene expression was calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Statistical analysis of all data was conducted by Student's *t*-test (SAS v9.2).

## ACKNOWLEDGEMENTS

This work was supported by the Citrus Research and Development Foundation.

## REFERENCES

**Almiron, M., Martinez, M., Sanjuan, N. and Ugalde, A.R.** (2001) Ferrochelatase is present in *Burcella abortus* and is critical for its intracellular survival and virulence. *Infect. Immun.* **69**, 6225–6230.

**Ammar, E.D., Shatters, R.G. Jr and Hall, D.G.** (2011) Detection and relative titer of *Candidatus* Liberibacter asiaticus in the salivary glands and alimentary canal of *Diaphorina citri* (Hemiptera: Psyllidae) vector of citrus huanglongbing disease. *Ann. Entomol. Soc. Am.* **104**, 526–533.

**Andrews, S.C., Robinson, A.K. and Rodríguez-Quiñones, F.** (2006) Bacterial iron homeostasis. *FEMS Microbiol. Rev.* **27**, 215–237.

**Arenas, F.A., Covarrubias, P.C., Sandoval, J.M., Perez-Donoso, J.M., Imlay, J.A. and Vasquez, C.C.** (2011) The *E. coli* BtuE protein functions as a resistance determinant against reactive oxygen species. *PLoS ONE*, **6**, e15979.

**Basu, B. and Apte, S.K.** (2008) A novel serralysin metalloprotease from *Deinococcus radiodurans*. *Biochim. Biophys. Acta*, **1784**, 1256–1264.

**Belas, R., Manos, J. and Suvanasuthi, R.** (2004) *Proteus mirabilis* ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides. *Infect. Immun.* **72**, 5159–5167.

**Benyon, L., Zhou, L., Powell, C., Gottwald, T. and Duan, Y.** (2008) Improved detection of low-titer, non-lethal, seed transmitted Candidatus Liberibacter asiaticus in citrus, periwinkle and dodder using nested PCR. In: *Proceedings of the International Research Conference on Huanglongbing*, (Burns, J.K., Graham, J.H., and Gottwald, T.R., eds) pp. 120–123. Orlando, FL: US Department of Agriculture.

**Bernard, T., Pocard, J.-A., Perroud, B. and Le Rudulier, D.** (1986) Variations in the response of salt-stressed *Rhizobium* strains to betaines. *Arch. Microbiol.* **143**, 359–364.

**Bové, J.M.** (2006) Huanglongbing: a destructive, newly emerging, century-old disease of citrus. *J. Plant Pathol.* **88**, 7–37.

**Boyer, E., Bergevin, I., Malo, D., Gros, P. and Cellier, M.F.M.** (2002) Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* **70**, 6032–6042.

**Braun, V.** (1997) Avoidance of iron toxicity through regulation of bacterial iron transport. *Biol. Chem.* **378**, 779–786.

**Caldas, C., Cherqui, A., Pereira, A. and Simoes, N.** (2002) Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immunosuppression. *Appl. Environ. Microbiol.* **68**, 1297–1304.

**Castro, M.S. and Fontes, W.** (2005) Plant defense and antimicrobial peptides. *Protein Peptide Lett.* **12**, 11–16.

**Chao, T.C., Buhrmester, J., Hansmeier, N., Pühler, A. and Weidner, S.** (2005) Role of the regulatory gene *rirA* in the transcriptional response of *Sinorhizobium meliloti* to iron limitation. *Appl. Environ. Microbiol.* **71**, 5969–5982.

**Chatterjee, S., Almeida, R.P.P. and Lindow, S.** (2008a) Living in two worlds: the plant and insect lifestyles of *Xylella fastidiosa*. *Annu. Rev. Phytopathol.* **46**, 243–271.

**Chatterjee, S., Wistrom, C. and Lindow, S.E.** (2008b) A cell–cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa*. *Proc. Natl. Acad. Sci. USA*, **105**, 2670–2675.

**Chaudhuri, R.R., Peters, S.E., Pleasance, S.J., Northen, H., Willers, C., Paterson, G.K., Cone, D.B., Allen, A.G., Owen, P.J. and Shalom, G.** (2009) Comprehensive identification of *Salmonella enterica* serovar Typhimurium genes required for infection of BALB/c mice. *PLoS Pathog.* **5**, e1000529.

**Chowdhury, R., Sahu, G.K. and Das, J.** (1996) Stress response in pathogenic bacteria. *J. Biosci.* **21**, 149–160.

**Colombatti, A., Bonaldo, P. and Doliana, R.** (1993) Type A modules: interacting domains found in several non-fibrillar collagens and in other extracellular matrix proteins. *Matrix*, **13**, 297–306.

**Cong, Q., Kinch, L.N., Kim, B.H. and Grishin, N.V.** (2012) Predictive sequence analysis of the *Candidatus* Liberibacter asiaticus proteome. *PLoS ONE*, **7**, e41071.

**Dahler, G.S., Barras, F. and Keen, N.T.** (1990) Cloning of genes encoding extracellular metalloproteases from *Erwinia chrysanthemi* EC16. *J. Bacteriol.* **172**, 5803–5815.

**Dalbey, R.E. and Chen, M.** (2004) Sec-translocase mediated membrane protein biogenesis. *Biochim. Biophys. Acta, Mol. Cell Res.* **1694**, 37–53.

**Darwin, A.J. and Miller, V.L.** (1999) Identification of *Yersinia enterocolitica* genes affecting survival in an animal host using signature-tagged transposon mutagenesis. *Mol. Microbiol.* **32**, 51–62.

**Delory, M., Hallez, R., Letesson, J.J. and De Bolle, X.** (2006) An RpoH-like heat shock sigma factor is involved in stress response and virulence in *Brucella melitensis* 16M. *J. Bacteriol.* **188**, 7707–7710.

**Dow, J.M., Osbourn, A.E., Wilson, T.J.G. and Daniels, M.J.** (1995) A locus determining pathogenicity of *Xanthomonas campestris* is involved in lipopolysaccharide biosynthesis. *Mol. Plant–Microbe Interact.* **8**, 768–777.

**Duan, Y., Zhou, L., Hall, D.G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, M., Gabriel, D.W., Williams, K.P., Dickerman, A., Sun, Y. and Gottwald, T.** (2009) Complete genome sequence of citrus huanglongbing bacterium, '*Candidatus* Liberibacter asiaticus' obtained through metagenomics. *Mol. Plant–Microbe Interact.* **22**, 1011–1020.

**Duong, F. and Wickner, W.** (1997) Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme. *EMBO J.* **16**, 2756–2768.

**Erickson, H.S., Albert, P.S., Gillespie, J.W., Rodriguez-Canales, J., Marston Linehan, W., Pinto, P.A., Chuaqui, R.F. and Emmert-Buck, M.R.** (2009) Quantitative RT-PCR gene expression analysis of laser microdissected tissue samples. *Nat. Protoc.* **4**, 902–922.

**Etxeberria, E., Gonzalez, P., Achor, D. and Albrigo, G.** (2009) Anatomical distribution of abnormally high levels of starch in HLB-affected Valencia orange trees. *Physiol. Mol. Plant Pathol.* **74**, 76–83.

**Fan, J., Chen, C., Brlansky, R., Gmitter Jr, F. and Li, Z.G.** (2010) Changes in carbohydrate metabolism in *Citrus sinensis* infected with '*Candidatus* Liberibacter asiaticus'. *Plant Pathol.* **59**, 1037–1043.

**Farr, S.B. and Kogoma, T.** (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.* **55**, 561–585.

**Felix, G., Duran, J.D., Volko, S. and Boller, T.** (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18**, 265–276.

**Folimonova, S.Y. and Achor, D.S.** (2010) Early events of citrus greening (Huanglongbing) disease development at the ultrastructural level. *Phytopathology*, **100**, 949–958.

**Folimonova, S.Y., Robertson, C.J., Garnsey, S.M., Gowda, S. and Dawson, W.O.** (2009) Examination of the responses of different genotypes of citrus to Huanglongbing (Citrus Greening) under different conditions. *Phytopathology*, **99**, 1346–1354.

**Frankenberg, N., Moser, J. and Jahn, D.** (2003) Bacterial heme biosynthesis and its biotechnological application. *Appl. Microbiol. Biotechnol.* **63**, 115–127.

**Freeman, B.C. and Beattie, G.A.** (2008) An overview of plant defenses against pathogens and herbivores. *Plant Health Instructor*. doi: 10.1094/PHI-I-2008-0226-01.

**Fricke, B., Parchmann, O., Kruse, K., Rücknagel, P., Schierhorn, A. and Menge, S.** (1999) Characterization and purification of an outer membrane metalloproteinase from *Pseudomonas aeruginosa* with fibrinogenolytic activity. *Biochim. Biophys. Acta, Mol. Basis Dis.* **1454**, 236–250.

**Gage, D.J.** (2004) Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.* **68**, 280–300.

**Gil, R., Silva, F.J., Zientz, E., Delmotte, F., Gonza'lez-Candelas, F., Latorre, A., Rausell, C., Kamerbeek, J., Gadau, J., Hölldobler, B., van Ham, R.C.H.J., Gross, R. and Moya, A.** (2003) The genome sequence of *Blochmannia floridanus*: comparative analysis of reduced genomes. *Proc. Natl. Acad. Sci. USA*, **100**, 9388–9393.

**Gomes, S.L. and Simão, R.C.G.** (2009) Stress response: heat. In: *Encyclopedia of Microbiology* (Schaechter, M., ed.), pp. 464–474. Oxford: Elsevier Academic Press.

**Hocquellet, A., Toorawa, P., Bove, J.M. and Garnier, M.** (1999) Detection and identification of the two *Candidatus* Liberibacter species associated with citrus huanglongbing by PCR amplification of ribosomal protein genes of the β-operon. *Mol. Cell. Probes*, **13**, 373–379.

**Hung, T.H., Hung, S.C., Chen, C.N., Hsu, M.H. and Su, H.J.** (2004) Detection by PCR of *Candidatus* Liberibacter asiaticus, the bacterium causing citrus huanglongbing in vector psyllids: application to the study of vector–pathogen relationships. *Plant Pathol.* **53**, 96–102.

**Inoue, H., Ohnishi, J., Ito, T., Tomimura, K., Miyata, S., Iwanami, T. and Ashihara, W.** (2009) Enhanced proliferation and efficient transmission of *Candidatus* Liberibacter asiaticus by adult *Diaphorina citri* after acquisition feeding in the nymphal stage. *Ann. Appl. Biol.* **155**, 29–36.

**Janakiraman, A. and Slauch, J.M.** (2000) The putative iron transport system *sitABCD* encoded on *SPI1* is required for full virulence of *Salmonella typhimurium*. *Mol. Microbiol.* **35**, 1146–1155.

**Jensen, P.E., Gibson, L.C. and Hunter, C.N.** (1998) Determinants of catalytic activity with the use of purified I, D and H subunits of the magnesium protoporphyrin IX chelatase from *Synechocystis* PCC6803. *Biochem. J.* **334**, 335–344.

**Jones, J.D. and Dangl, J.L.** (2006) The plant immune system. *Nature*, **444**, 323–329.

**Kim, J., Sagaram, U., Burns, J., Li, J. and Wang, N.** (2009) Response of sweet orange (*Citrus sinensis*) to '*Candidatus* Liberibacter asiaticus' infection: microscopy and microarray analyses. *Phytopathology*, **99**, 50–57.

**Kim, K., Kim, K.P., Choi, J., Lim, J.A., Lee, J., Hwang, S. and Ryu, S.** (2010) Outer membrane proteins A (OmpA) and X (OmpX) are essential for basolateral invasion of *Cronobacter sakazakii*. *Appl. Environ. Microbiol.* **76**, 5188–5198.

**Kitphati, W., Ngok-ngam, P., Suwanmaneerat, S., Sukchawalit, R. and Mongkolsuk, S.** (2007) *Agrobacterium tumefaciens* fur has important physiological roles in iron and manganese homeostasis, the oxidative stress response, and full virulence. *Appl. Environ. Microbiol.* **73**, 4760–4768.

**Lamarche, M.G., Wanner, B.L., Crepin, S. and Harel, J.** (2008) The Phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiol. Rev.* **32**, 461–473.

**Li, C., Tao, J., Mao, D. and He, C.** (2011) A novel manganese efflux system, YebN, is required for virulence by *Xanthomonas oryzae* pv. *oryzae*. *PLoS ONE*, **6**, e21983.

**Li, Y. and Zhang, Y.** (2007) PhoU is a persistence switch involved in persister formation and tolerance to multiple antibiotics and stresses in *Escherichia coli*. *Antimicrob. Agents Chemother.* **51**, 2092–2099.

**Lithgow, J.K., Ingham, E. and Foster, S.J.** (2004) Role of the *hprT–ftsH* locus in *Staphylococcus aureus*. *Microbiology*, **150**, 373–381.

**Livak, K.J. and Schmittgen, T.D.** (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method. *Methods*, **25**, 402–408.

**Macnab, R.M.** (1996) Flagella and motility. In: Escherichia coli *and* Salmonella*: Cellular and Molecular Biology*, 2nd edn. (Neidhardt, F.C., Curtis, R. III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M. and Umbarger, H.E., eds), pp. 123–145. Washington, DC: American Society for Microbiology Press.

**Maeda, H. and Morihara, K.** (1995) Serralysin and related bacterial proteinases. *Methods Enzymol.* **248**, 395–413.

**Maezawa, K., Shigenobu, S., Taniguchi, H., Kubo, T., Aizawa, S. and Morioka, M.** (2006) Hundreds of flagellar basal bodies cover the cell surface of the endosymbiotic bacterium *Buchnera aphidicola* sp. strain APS. *J. Bacteriol.* **188**, 6539–6543.

**McClean, S.** (2012) Eight stranded-barrel and related outer membrane proteins: role in bacterial pathogenesis. *Protein Peptide Lett.* **19**, 1013–1025.

**Mekalanos, J.J.** (1992) Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* **174**, 1–7.

**Mohan Nair, M.K., Venkitanarayanan, K., Silbart, L.K. and Kim, K.S.** (2009) Outer membrane protein A (OmpA) of *Cronobacter sakazakii* binds fibronectin and contributes to invasion of human brain microvascular endothelial cells. *Foodborne Pathog. Dis.* **6**, 495–501.

**Mohr, C.D., Sonsteby, S.K. and Deretic, V.** (1994) The *Pseudomonas aeruginosa* homologs of *hemC* and *hemD* are linked to the gene encoding the regulator of mucoidy AlgR. *Mol. Gen. Genet.* **242**, 177–184.

**Nadarasah, G. and Stavrinides, J.** (2011) Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol. Rev.* **35**, 555–575.

**Ngok-Ngam, P., Ruangkiattikul, N., Mahavihakanont, A., Virgem, S.S., Sukchawalit, R. and Mongkolsuk, S.** (2009) Roles of *Agrobacterium tumefaciens* RirA in iron regulation, oxidative stress response, and virulence. *J. Bacteriol.* **191**, 2083–2090.

**Nonaka, G., Blankschien, M., Herman, C., Gross, C.A. and Rhodius, V.A.** (2006) Regulon and promoter analysis of the *E. coli* heat-shock factor, σ32, reveals a multifaceted cellular response to heat stress. *Genes Dev.* **20**, 1776–1789.

**Okinaka, Y., Yang, C.-H., Perna, N.T. and Keen, N.T.** (2002) Microarray profiling of *Erwinia chrysanthemi* 3937 genes that are regulated during plant infection. *Mol. Plant–Microbe Interact.* **15**, 619–629.

**Oshima, K., Ishii, Y., Kakizawa, S., Sugawara, K., Neriya, Y., Himeno, M., Minato, N., Miura, C., Shiraishi, T., Yamaji, Y. and Namba, S.** (2011) Dramatic transcriptional changes in an intracellular parasite enable host switching between plant and insect. *PLoS ONE*, **6**, e23242.

**Panek, H. and O'Brian, R.M.** (2002) A whole genome view of prokaryotic haem biosynthesis. *Microbiology*, **148**, 2273–2282.

**Papp-Wallace, K.M. and Maguire, M.E.** (2006) Manganese transport and the role of manganese in virulence. *Annu. Rev. Microbiol.* **60**, 187–209.

**Parker, J.E.** (2003) Plant recognition of microbial patterns. *Trends Plant Sci.* **8**, 245–247.

**Pearson, J.P., Pesci, E.C. and Iglewski, B.H.** (1997) Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J. Bacteriol.* **179**, 5756–5767.

**Pelz-Stelinski, K.S., Brlansky, R.H., Ebert, T.A. and Rogers, M.E.** (2010) Transmission parameters for *Candidatus* Liberibacter asiaticus by Asian citrus psyllid (Hemipera: Psyllidae). *J. Econ. Entomol.* **103**, 1531–1541.

**Perroud, B. and Le Rudulier, D.** (1985) Glycine betaine transport in *Escherichia coli*: osmotic modulation. *J. Bacteriol.* **161**, 393–401.

**Ponting, C., Aravind, L., Schultz, J., Bork, P. and Koonin, E.** (1999) Eukaryotic signalling domain homologues in archaea and bacteria. Ancient ancestry and horizontal gene transfer. *J. Mol. Biol.* **289**, 729–745.

**Reddy, K., Bullerjahn, G.S., Sherman, D. and Sherman, L.A.** (1988) Cloning, nucleotide sequence, and mutagenesis of a gene (*irpA*) involved in iron-deficient growth of the cyanobacterium *Synechococcus* sp. strain PCC7942. *J. Bacteriol.* **170**, 4466–4476.

**Ried, G. and Henning, U.** (1987) A unique amino acid substitution in the outer membrane protein OmpA causes conjugation deficiency in *Escherichia coli* K-12. *FEBS Lett.* **223**, 387–390.

**Roebler, M. and Muller, V.** (2001) Osmoadaptation in bacteria and archaea: common principles and differences. *Environ. Microbiol.* **3**, 743–754.

**Rosso, M.L., Chauvaux, S., Dessein, R., Laurans, C., Frangeul, L., Lacroix, C., Schiavo, A., Dillies, M.A., Foulon, J. and Coppée, J.** (2008) Growth of *Yersinia pseudotuberculosis* in human plasma: impacts on virulence and metabolic gene expression. *BMC Microbiol.* **8**, 211.

**Sabri, M., Caza, M., Proulx, J., Lymberopoulos, M.H., Bre'e, A., Moulin-Schouleur, M., Curtiss, R. and Dozois, C.M.** (2008) Contribution of the SitABCD, MntH, and FeoB metal transporters to the virulence of avian pathogenic *Escherichia coli* O78 strain _7122. *Infect. Immun.* **76**, 601–611.

**Sagaram, U.S., DeAngelis, K.M., Trivedi, P., Andersen, G.L., Lu, S. and Wang, N.** (2009) Bacterial diversity analysis of Huanglongbing pathogen-infected citrus using PhyloChips and 16S rDNA clone library sequencing. *Appl. Environ. Microbiol.* **75**, 1566–1574.

**Schmidtchen, A., Frick, I.M., Andersson, E., Tapper, H. and Bjorck, L.** (2002) Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol. Microbiol.* **46**, 157–168.

**Schobert, M. and Jahn, D.** (2002) Regulation of heme biosynthesis in nonphototrophic bacteria. *J. Mol. Microbiol.* **4**, 287–294.

**Shi, W. and Zhang, Y.** (2010) PhoY2 but not PhoY1 is the PhoU homologue involved in persisters in *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* **65**, 1237–1242.

**Slamti, L., Livny, J. and Waldor, M.K.** (2007) Global gene expression and phenotypic analysis of a *Vibrio cholerae rpoH* deletion mutant. *J. Bacteriol.* **189**, 351–362.

**Tamura, G.S., Nittayajarn, A. and Schoentag, D.L.** (2002) A glutamine transport gene, *glnQ*, is required for fibronectin adherence and virulence of group B *streptococci*. *Infect. Immun.* **70**, 2877–2885.

**Todd, J.D., Wexler, M., Sawers, G., Yeoman, K.H., Poole, P.S. and Johnston, A.W.B.** (2002) RirA, an iron-responsive regulator in the symbiotic bacterium *Rhizobium leguminosarum*. *Microbiology*, **148**, 4059–4071.

**Toft, C. and Andersson, S.G.** (2010) Evolutionary microbial genomics: insights into bacterial host adaptation. *Nat. Rev. Genet.* **11**, 465–475.

**Tyler, H.L., Roesch, L.F., Gowda, S., Dawson, W.O. and Triplett, E.W.** (2009) Confirmation of the sequence of '*Candidatus* Liberibacter asiaticus ' and assessment of microbial diversity in huanglongbing-infected citrus phloem using a metagenomic approach. *Mol. Plant–Microbe Interact.* **22**, 1624–1634.

**Van Gijsegem, F., Genin, S. and Boucher, C.** (1993) Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. *Trends Microbiol.* **1**, 175–180.

**Wandersman, C. and Delepelaire, P.** (2004) Bacterial iron sources: from siderophores to hemophores. *Annu. Rev. Microbiol.* **58**, 611–647.

**Wang, Y.** (2002) The Function of OmpA in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **292**, 396–401.

**Wang, Z., Yin, Y., Hu, H., Yuan, Q., Peng, G. and Xia, Y.** (2006) Development and application of molecular-based diagnosis for '*Candidatus* Liberibacter asiaticus', the causal pathogen of citrus huanglongbing. *Plant Pathol.* **55**, 630–638.

**Waters, C.M. and Bassler, B.L.** (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* **21**, 319–346.

**Whelan, K.F., Sherburne, R.K. and Taylor, D.E.** (1997) Characterization of a region of the IncHI2 plasmid R478 which protects *Escherichia coli* from toxic effects specified by components of the tellurite, phage, and colicin resistance cluster. *J. Bacteriol.* **179**, 63–71.

**Wooldridge, K.G. and Williams, P.H.** (1993) Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiol. Rev.* **12**, 325–348.

**Wu, D., Daugherty, S.C., Van Aken, S.E., Pai, G.H., Watkins, K.L., Khouri, H., Tallon, L.J., Zaborsky, J.M., Dunbar, H.E., Tran, P.L., Moran, N.A. and Eisen, J.A.** (2006) Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. *PLoS Biol.* **4**, e188.

**Xu, C.F., Xia, Y.H., Li, K.B. and Ke, C.** (1988) Further study of the transmission of citrus huanglongbing by a psyllid, *Diaphorina citri* Kuwayama. *Proceedings of the 10th Conference of the International Organization of Citrus Virologists, 17–21 November 1986, Valencia, Spain* (Timmer, L.W., Garnsey, S.M. and Navarro, L., eds), pp. 243–248. Riverside, CA: University of California.

**Xu, Q., Rawlings, N.D., Farr, C.L., Chiu, H.J., Grant, J.C., Jaroszewski, L., Klock, H.E., Knuth, M.W., Miller, M.D. and Weekes, D.** (2011) Structural and sequence analysis of imelysin-like proteins implicated in bacterial iron uptake. *PLoS ONE*, **6**, e21875.

**Young, G.M., Schmiel, D.H. and Miller, V.L.** (1999) A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc. Natl. Acad. Sci. USA*, **96**, 6456–6461.

**Zhou, D., Hardt, W.D. and Galan, J.E.** (1999) *Salmonella typhimurium* encodes a putative iron transport system within the centisome 63 pathogenicity island. *Infect. Immun.* **67**, 1974–1981.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1** Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) test of RNA extracted from a single psyllid by targeting of the β-operon of *Candidatus* Liberibacter asiaticus. RNA extracted from a single psyllid (*Diaphorina citri*) was subjected to a QRT-PCR test using primers CQULA04F-CQULA04R that specifically target the β-operon of *Ca*. L. asiaticus (Wang *et al*., 2006). Six microlitres of the PCR product were loaded and visualized in a 1% agarose gel. All samples, including positive and negative controls, were performed in triplicate; only one representative result of each sample is shown. C–, negative control using water as template; C+, positive control using RNA-extracted huanglongbing (HLB) symptomatic citrus leaves as template; S1–S11, single psyllid RNA samples; M, 100-bp DNA ladder.

**Table S1** List of genes selected for quantitative reverse transcription-polymerase chain reaction (QRT-PCR) analysis of *Candidatus* Liberibacter asiaticus genes in *Ca*. L. asiaticus-infected 'Valencia' sweet orange plants and psyllids.

**Table S2** Gene-specific forward and reverse primers used for quantitative reverse transcription-polymerase chain reaction (QRT-PCR) analysis of gene expression of *Candidatus* Liberibacter asiaticus *in planta* and in psyllid.

**Table S3** Expression profile of hypothetical genes of *Candidatus* Liberibacter asiaticus *in planta* compared with in psyllid.