Proteomic analyses of Citrus petiole responses to early Huanglongbing Disease

Bo Li
Chinese Academy of Agricultural Sciences Institute of Plant Protection
https://orcid.org/0000-0003-0035-221X

Shuangchao Wang
Chinese Academy of Agricultural Sciences Institute of Plant Protection

Yi Zhang
Chinese Academy of Agricultural Sciences Institute of Plant Protection

Dewen Qiu (qiudewen@caas.cn)

Research article

Keywords: Citrus greening, Huanglongbing, Candidatus liberibacter, Proteomics, Defense response, Phloem-restricted

Posted Date: September 24th, 2019

DOI: https://doi.org/10.21203/rs.2.14879/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Huanglongbing (HLB) is currently one of the most destructive citrus disease worldwide. It is caused by Candidatus Liberibacter asiaticus (CLas), a nonculturable alpha-proteobacterium, which it resides exclusively in the phloem tissues. Therefore, understanding the early CLas-responsive proteins in citrus petiole where pathogenic bacteria colonized will help to investigate plant resistance to the pathogen. Results In this study, a comparative proteomic approach was applied to identify the petiole proteins associated with the response to CLas infection. A total of 777 proteins were differentially expressed in response to CLas. Among them, 499 proteins were up-regulated and 278 were down-regulated. Among the most highly up-regulated differentially expressed proteins (DEPs) were salicylate carboxymethyltransferase, ubiquitin carboxyl-terminal hydrolase 13, trans-resveratrol di-O-methyltransferase, linoleate 13S-lipoxygenase 2-1, granule-bound starch synthase 1 and thaumatin-like proteins. While the most highly down-regulated DEPs were oxygen-evolving enhancer proteins, ribulose bisphosphate carboxylase/oxygenase activase, peroxidases and photosystem reaction center subunits. The results of qPCR analysis of a number of indicated DEPs and western blotting further validated four representative DEPs, including salicylate carboxymethyltransferase, linoleate 13S-lipoxygenase 2-1, granule-bound starch synthase 1 and photosystem I reaction center subunit showed that most of detected DEPs were positively correlated with their mRNA and protein levels. Conclusions Our comparative proteomic analysis first profiling reveals early and primary proteome alterations in CLas-infected citrus petiole, where pathogens reside in. The DEPs results demonstrate that CLas infection could promote the carbohydrate metabolism, depress the photosystem and activate/inhibit defense responses.

Background

Huanglongbing (HLB), which is also called citrus greening disease, is a destructive citrus disease worldwide[1]. It is caused by a gram-negative and phloem-inhabiting bacterium of the genus Candidatus Liberibacter, including three known species, "Candidatus Liberibacter asiaticus (CLas)”, "Candidatus Liberibacter americanus” and "Candidatus Liberibacter africanus”. All known citrus species and citrus relatives can be infected with the pathogens. HLB disease is first reported in Asia and is widely distributed in more than 40 countries and regions in Asia, Oceania, Africa, and the Americas[2–5]. The HLB pathogens could be transmitted by the citrus psyllid Diaphorina citri[6, 7]. After colonizing citrus trees, the pathogen spreads to all tissues quickly, and leads to plant death after a period of several months to a few years[8]. The pathogen blocks the phloem of citrus trees, resulting in symptoms such as vein yellowing and hardening, and blotchy mottling and chlorosis that are sometimes mistaken for element deficiency. The fruits of infected trees are small, with a bitter and acidic flavor as well as a smooth but lackluster pericarp[8–10].

It is important to clarify the host responses to CLas infection. Previous studies have been reported to identify CLas-affected genes and proteins in leaf, root and fruit[11, 12]. These studies suggested that CLas could mediate a lot of significant biological processes in plants, including carbohydrate metabolism, photosynthesis, multiple hormone pathways and secondary metabolites. Previous studies
also have showed that there were remarkable differences between diseased and healthy fruit in the concentrations of sugars, limonin glucoside and some other organic compounds[13]. These differences explained the bitter and acidic flavor of the fruit from diseased trees. CLas-infection depressed the absorption of elements in roots and leaves[12], this is one reason why CLas-infected leaves show several symptoms of element deficiency. Remarkably, the carbohydrate transport system is largely disturbed by CLas. Studies suggested that phloem blockage in infected trees caused starch accumulation in plastids and phloem[14–16]. Excessively accumulated starches results in disruption of chloroplast inner grana structure[17].

Transcriptomic works have been demonstrated that genes related to starch metabolism and photosynthesis were down-regulated in all detected tissues[11, 18, 19], however, photosynthesis-related genes were up-regulated in fruit[20]. Additionally, CLas was able to enhance transcription of genes involved in photosynthesis and ATP synthesis[21]. These transcriptomics data are to some extent consistent with several reported proteome results. Photosynthesis-related proteins, such as oxygen-evolving enhancer (OEE) proteins and a photosystem I 9 kDa protein, were down-regulated in CLas-infected plant leaf tissues, however, several proteins related to starches metabolism were found to be up-regulated[22]. Although many proteins related to carbohydrate metabolism and photosynthesis were identified in a up/down-regulation manner, in addition to validation in transcriptional levels, no further confirmation was performed.

Most interestingly, CLas infection could induce defense responses in plants[12, 22]. Phloem protein 2 (PP2) is one of the most abundant proteins in the phloem[23] and shows insecticidal activities[24, 25]. The PP2-like genes were observed to be significantly up-regulated in CLas-infected roots[12], implying a possible defense response induced by CLas against psyllids, although no observation of regulated PP2 in leaf and other tissues. The most interesting point is the plant innate immune system mediated by hormonal crosstalk, particularly between salicylic acid (SA) and jasmonic acid (JA) pathways. SA and JA signaling pathways were activated in CLas-infected plants as reported previously[12, 26], and CLas also mediates ethylene, auxin and brassinosteroid signaling pathways[27].

Previous studies generally focused on the differentially expressed genes or proteins (DEPs) mediated by CLAs in leaves, fruits and roots[26, 28–32]. Leaves infected with CLas display yellowing and blotchy mottled appearances. However, the pathogens colonize in the phloem. The global analysis of infected source tissues is an appropriate tool to understand the fundamental effects of CLas in plants. Here, a systematic analysis of the citrus petiole proteome was performed through the use of a comprehensive proteomic method to explore global changes in protein expression in response to CLas infection. Furthermore, qPCR and western blotting were performed to validate the reliability of proteome data. To best of our knowledge, this is the first study focusing on the proteome of CLas-infected citrus petiole, where pathogens reside in. The results provide a new insight into knowing fundamental and initial responses of citrus to CLas infection.

Results
Characterization of the proteome of the CLas-affected and control citrus petiole

HLB symptoms appeared at 4 months post CLas-inoculation, containing yellowing, blotchy mottle and/or variegated chlorosis of leaves (Fig. 1A). The presence of CLas was confirmed by qPCR. Ct values of CLas-infected petioles were less than 30 while control samples showed no amplified product (Fig. 1B). To examine the variation in the proteome of citrus petioles infected with CLas, a proteomic method based on LC–MS/MS was applied and tandem mass spectra were searched against the UniProt_Citrus sinensis database. Total protein was extracted from petioles of mock-inoculated (without symptoms, qPCR-negative) and CLas-inoculated (with symptoms, qPCR-positive) sweet oranges as described[33]. In our study, a large number of peptides were identified based on the MS data. The length of most peptides distributed was between 6 and 14 amino acids, which agrees with the property of tryptic peptides (Fig. 2A). The distribution of peptide delta mass in each replicate is between −1.2–2.2 ppm (Additional file 1: Figure S1), and the number of spectrum for each protein in different replicates showed a reasonable consistency (Additional file 2: Figure S2), which means the mass accuracy of the MS data fit the requirement. The analysis of LC-MS/MS data showed the identification of 7321 proteins (False Discovery Rate (FDR) was set as 1.0%, at least 2 unique peptide for one protein) (Additional file 3: Table S1), of which 5270 proteins were identified in each biological replicate (Fig. 2B). Further analysis identified 777 proteins as DEPs at a cutoff value of >|±1.3|-fold (p value <0.005), which included 499 up-regulated and 278 down-regulated proteins (Fig. 2C and Additional file 3: Table S1). Of the DEPs, a salicylate carboxymethyltransferase (CsSAMT, A0A067FUD3) was significantly up-regulated by more than 3-fold. The ubiquitin carboxyl-terminal hydrolase (A0A067DGD4) was increased by 2.95-fold. A trans-resveratrol di-O-methyltransferase (A0A067DIU3) and linoleate 13S-lipoxygenase (CsLOX2.1, A0A067ERJ6) showed a 2.43 and 2.23-fold increases, respectively. In addition, a histone deacetylase HDT1 and two oxygen-evolving enhancer proteins were found to be down-regulated by more than 2-fold.

Gene ontology (GO) analysis results

To further study the proteomics data, a GO analysis was performed to classify the DEPs to account for their biological processes, molecular functions and cellular component (Fig. 3A and Additional file 4: Table S2). In the biological process category, there were 145 GO terms, the protein groups associated with response to stimulus (GO:0050896), single-organism metabolic process (GO:0044710) and response to stress (GO:0006950) accounted for 17.25%, 14.67% and 11.58%, respectively, indicating that response to stimulus and single-organism metabolic process are likely to be affected significant by HLB-infection. The organism’s response to stimulus suggested that some proteins participated in the perception and transmission of extrinsic stimuli signals or that the proteins may be conducive for plants to perceive external stimuli. At molecular function GO level, 60 total GO terms were assigned, the protein groups connected with catalytic (GO:0003824), hydrolase activity (GO:0016787) and cation binding (GO:0043169) accounted for 27.67%, 10.42% and 9.91%, respectively. The other important protein groups
on molecular function were oxidoreductase activity (GO:0016491) and transporter activity (GO:0005215). In the cellular component category, there were 81 GO terms, corresponding to 35.39% of proteins in the cells (GO: 0005623), 32.56% of proteins in the intracellular (GO:0005622), 29.73% of proteins in the cytoplasm (GO:0005737) and 28.44% of proteins in the organelle (GO:0043226). The metabolic processes in biological processes and the catalytic activity in molecular functions showed that DEPs are associated with enzymatic proteins involved in metabolism.

**KEGG pathway analysis**

To determine the involvement of these DEPs in response to CLas-infection, a pathway analysis was carried out to identify the potential target proteins. The DEPs have been identified to be involved in 5 main pathways (Fig. 3B and Additional file 4: Table S2), including photosynthesis (cit00195), protein processing in endoplasmic reticulum (cit04141), phenylpropanoid biosynthesis (cit00940), galactose metabolism (cit00052) and monobactam biosynthesis (cit00261). Among them, the largest number of DEPs were enriched in protein processing in endoplasmic reticulum, followed by the phenylpropanoid biosynthesis and photosynthesis, respectively. These data clearly indicate that HLB has significant regulatory effects in citrus petiole cells.

**Analysis and verification of carbohydrate synthesize related DEPs**

In CLas-inoculated plants, there was a lot of CLas-mediated down-regulation DEPs. Remarkably, 21 DEPs were involved in carbohydrate metabolism, most of which were up-regulated in CLas-inoculated plants compared to mock-inoculated plants (Additional file 5: Table S3), especially in D-glucose and starch synthesis pathways (Fig. 4A). Additionally, to explore the regulation in transcript levels, 10 representative DEPs were chosen to perform qPCR to validate the gene expression patterns. The transcript level in the Mock-inoculated plant was set to 1.0 (black square) and the CLas-inoculated fold-regulation ratios were shown in Table 1. The results indicated that the expression profile of all detected genes was in agreement with the proteome data. In addition, an indicated fragment of the protein (Granule-bound starch synthase 1, CsGBSS1, A0A067H3M6) was first expressed in *E. coli* (Fig. 4B), the purified protein was then used to prepare its antibodies in rabbit. Western blotting was then carried out to confirm the translational levels of CsGBSS1 with its antibodies. The result exhibited a similar mediation with proteome result in response to CLas (Fig. 4C). These data indicate that CLas-infection indeed induced an up-regulated expression of starch and sucrose metabolism-related proteins.

**Table 1. Representatives of differentially expressed proteins on starch and sucrose metabolism identified in CLas-infected leaf petioles.**

*false*
According to analysis of KEGG enrichment, there were total 18 DEPs involved in citrus photosynthesis in response to CLas-infection (Additional file 5: Table S3). CLas-inoculated plants used in this study showed typical blotchy mottling and chlorosis symptoms. We observed that these DEPs were involved in photosystem I (4 proteins, A0A067FZ61, A8C1A6, A8C172 (CsPSI) and A0A067ESN4), photosystem II (7 proteins, A0A067F2K2, A0A067GIE1, A0A067DL0, A0A067FX28, A0A067DU68, A0A067GJW2 and A0A067EDZ9), photosynthetic electron transport (3 proteins, A0A067HB98, A0A067DS64 and A0A067E882) and F-type ATPase (A0A067H8B2) (Fig. 5A), which are the components of the photosystem (Additional file 6: Figure S3, http://www.genome.jp/kegg-bin/show_pathway?cit00195). Additionally, a Rubisco (A0A067EBB3), carbonic anhydrase (A0A067FFU3) and protein disulfide-isomerase (A0A067HAZ0) were observed in a decrease manner in CLas-infection plants compared to mock plants.

To validate the proteome data, 10 proteins were confirmed using the qPCR in transcript levels. As shown in Table 2, the expression profiles of 7 tested genes were consistent with the proteome results, showing a down-regulation in mRNA levels. While the transcriptional levels of carbonic anhydrase 2 (A0A067FFU3), protein disulfide-isomerase (A0A067HAZ0) and ATP synthase delta chain (A0A067H8B2) indicated a not significant regulation. At the meantime, the antibodies of CsPSI was prepared to perform western blotting analysis, the result showed that CLas decreased the level of CsPSI in plant (Fig. 5B,C). In addition, we found that the expression of chlorophyllase(A0A067DGV8) was significantly increased by 1.67 times(Additional file 5: Table S3).

Table 2. Representatives of differentially expressed proteins on photosystem identified in CLas-infected leaf petioles.

falseAnalysis and verification of defense responses related DEPs

CLas-infection resulted in a series of variation of the plant defense-related proteins (Additional file 5: Table S3). Several hormone-related proteins were differentially expressed in CLas-infected plants, including a CsSAMT and a CsLOX2.1, which were significantly up-regulated 3.46 and 2.23-fold, respectively. In addition, SA-mediated defense-related proteins, CsPR1 (A0A067DC18) and CsPR5s (A0A067FP42 and A0A067DCM7) (also called thaumatin-like protein), were all up-regulated (Table 3 and Additional file 5: Table S3). Interestingly, most of the DEPs involved in phenylpropanoid biosynthesis were down-regulated by CLas-inoculation (Fig. 6A). The expression of 14 genes in citrus petioles was validated by qPCR analysis to corroborate the proteome data. The results were shown in Table 3, revealing a consistency with proteome and transcript data. As the expression of SA and JA-related proteins (CsSAMT and CsLOX2.1, respectively) was elevated in a high level in response to CLas infection, we further confirm their expression level via western blot analysis (Fig. 6B,C), the results suggested that CLas caused the accumulation of CsSAMT and CsLOX2.1. Furthermore, the content of SA and JA in both CLas-inoculated and mock-inoculated plants were detected, the data showed that the SA and JA contents were indeed elevated by CLas-infection (Fig. 6D).
Table 3. Representatives of differentially expressed proteins on plant defense response identified in CLas-infected leaf petioles.

Discussion

HLB is a devastating citrus disease that causes tremendous economic losses to the citrus industries worldwide. It is of difficulty to control, one of the reasons is that the pathogen is a phloem-restricted bacterium. The first global analysis of citrus petiole protein profiles using TMT-labeled technology was carried out in this study. A total of 777 proteins were differentially expressed in response to CLas infection. The CLas-mediated DEPs involved in synthesis of carbohydrate, photosystem and defense responses of plants mediated by CLas-infection were mainly discussed based on our findings.

CLas-infection enhances the synthesis of carbohydrate

The accumulation of starch in CLas-affeted plant tissues has been previously reported[15, 16]. A study has been shown that the accumulation of starch is assumed to be resulted from phloem plugging associated with not only the bacteria growing inside but also callose deposition and accumulation of phloem proteins[17]. We found that the up-regulation of starch synthesis-related proteins may be direct reason for its accumulation. These results correspond to the previous studies that showed an up-regulation of starch metabolism-related genes/proteins[12, 22], although starch metabolism genes were also down-regulated in several examined tissues[21, 34].

In general, the surplus carbohydrates produced during photosynthesis would be stored in form of starch in plants. Starch is a polymeric carbohydrate consisting of a large number of glucose units joined by glycosidic bonds. It consists of two types of molecules: the linear and helical amylose and the branched amylopectin. Plants produce starch by first converting glucose 1-phosphate to ADP-glucose using the enzyme glucose–1-phosphate adenylyltransferase. The starch synthase then adds the ADP-glucose via a 1,4-alpha glycosidic bond to a growing chain of glucose residues, liberating ADP and creating amylose. Starch branching enzyme introduces 1,6-alpha glycosidic bonds between these chains, creating the branched amylopectin[35]. A ADPase (glucose–1-phosphate adenylyltransferase, A0A067DG91), 3 starch synthases (A0A067H3M6 (CsGBSS1), A0A067ESR0 and A0A067GDC7), 2 glucan-branching enzymes (A0A067F4L8 and A0A067HB75) were up-regulated in CLas-inoculated plants, and the CsGBSS1 was up-regulated to 2.23-fold (Table 1 and Additional file 5: Table S3). However, ADPase, which is rate-limiting enzyme for starch biosynthesis, has been shown to be repressed in CLas-infected plants[26]. Glucose–6-phosphate/phosphate translocator 2 (GPT2) is involved in the formation of starch by transporting glucose into chloroplast[36], and has been reported to be decreased by CLas[21]. We identified a significant up-regulation (1.72-fold) of GPT2 (A0A067EH66) in protein level. These results suggest that starch biosynthesis-associated enzymes may be up-regulated during the early disease development of
HLB, which agrees with several previous works[14, 22, 37]. Here, CsGBSS1 with high induced change fold was chosen to prepare its antibodies, and the western analysis confirmed that this protein was indeed up-regulated in response to CLas infection (Fig. 4C). This is the first time to validate the result via western blotting analysis, particularly in citrus petiole.

The photosystem was significantly suppressed by CLas-infection

CLas disturbs the metabolic system of plants and results in yellowing and blotchy on leaves[38], according with the striking results that all photosynthesis-related proteins identified here showed down-regulation, except that the expression of chlorophyllase(A0A067DGV8) was significantly increased by 1.67 times(Additional file 5: Table S3). This manifests that pathogens can also indirectly degrade chlorophyll by inducing plants to produce chlorophyll enzymes. It was the first time that we used proteomics to found the chlorophyllase was induced upregulation in citrus petiole where were directly parasitized by CLas, and eliminated interference from other tissues that are not directly in contact with the pathogen. Combined with Western blot and proteomics data, it is assumed that photosynthesis was inhibited not only by destroying the PSI reaction center subunit II(A8C172), but also directly promoting the synthesis of chlorophyll hydrolase(A0A067DGV8) in the early stage of HLB infection. These results suggest that CLas-infection had significant negative effects on citrus photosystem by regulating the CsPSI and chlorophyllase in early infection stage.

Rubisco is an enzyme involved in the first major step of carbon fixation and catalyzes the carboxylation of ribulose-1,5-bisphosphate[39], and it in general is used as a housekeeping protein in study. Agree with previous studies, Rubisco has been shown to be depressed in response to CLas-infection[11, 22]. The possible explanations of the reduction of Rubisco are that the accumulation of starch and sucrose metabolism suppresses its production, or the deficiencies of nutrients causes its degradation[40]. 3 oxygen-evolving enhancer (OEE) proteins and a PSII 10 kDa polypeptide, which are involved in PSII oxygen evolving complex assembly, were observed a more than 1.5-fold decrease (Table 2 and Additional file 5: Table S3). OEEs are involved in splitting water into proton and oxygen molecules, and are required for PSII assembly/stability[41, 42]. OEEs have been shown to stabilize the manganese cluster [43], although a previous study showed that the manganese content was not significantly reduced in CLas-infected plants[22]. Citrus plants with Mn-deficiency also display symptoms, including reticular light/dark green and chlorosis of leaves, which are similar with CLas-infected plants. Reduced Mn is available form for plants, it has been demonstrated that pathogens can oxidize Mn to oxidized form, which is unavailable for plants[40]. Decreased OEEs may not stabilize the Mn in available form, resulting in Mn-deficiency symptoms without decrease of its content. The DEPs in PSI were mainly involved in forming complexes with ferredoxin (Fd) and ferredoxin-oxidoreductase (FNR), and assisting the docking of the Fd to PSI and interaction with FNR. Fd/FNR oxidation-reduction system has effect on the production of NADPH, which further affects the processes of carbon fixation[44]. We showed here that the level of several PSI-related proteins including CsPSI was reduced in CLas-infected plants (Table 2 and Fig. 5C).
This seems to contradict the up-regulation of carbohydrate metabolism discussed above, thus how CLas mediates photosystem and carbohydrate metabolism needs further study.

The up-regulation of starch synthesis-related proteins in plant tissues during CLas infection has been previously reported[12, 22, 26, 37] and we earlier showed that CLas-induced up-regulation of starch and sucrose metabolism-related proteins occurred in the petiole where pathogens reside in. The accumulation of photosynthates could inhibit the photosynthesis by suppressing photosynthetic activity via a negative feedback mechanism[45]. Our findings indicated that CLas could proactively promote starch synthesis to depress photosystem indirectly and directly in the early infection stage, suggesting that the activity of the PS might be one of early targets during HLB disease development. Increasing studies have demonstrated that chloroplast and photosynthesis play important roles in plant defense responses, including production of reactive oxygen species (ROS), reactive nitrogen oxide intermediates (NOI), hormones SA and JA[46–50].

**Effects of CLas-infection on plant defense responses**

Significantly, a lot of hormone-related proteins were differentially expressed in plants in response to CLas. SA plays important roles in plant defense and in activating systemic acquired resistance (SAR)[51–53]. Bacterial PAMPs, such as flg22, can induce SA accumulation. Previous study has been reported that CLas can also encode a 452-amino acid flagellin protein containing a conserved 22 amino acid domain (flg22) [54]. This would be one reason for the increase of SA production in CLas-infection plants (Fig. 6D). In addition, a protein (A0A067FR53) involved in the EDS1-dependent intrinsic and indispensable resistance signaling pathway was elevated 1.59-fold (Additional file 5: Table S3). EDS1 is able to encode a lipase that modulates SA-dependent disease resistance[55]. The SA-mediated CsPR1 and CsPR5 proteins showed a similar regulatory pattern with SA-related proteins (Table 3). And these PR proteins have been reported to be induced in response to pathogen attack and are associated with disease resistance and SAR[56, 57]. Interestingly, JA signaling pathway was also activated by CLas-infection. In addition to CsLOX2.1, many DEPs related to the JA pathway, including 4 glutathione S-transferases and a 4-coumarate—CoA ligase (Additional file 3: Table S1), were induced while some of them were reduced. The SA/JA content in CLas-infected petioles was indeed relatively higher than that in mock petioles (Fig. 6C,D). These results indicate that the SA and JA pathways were induced in this study, which is different from a previous study[37]. Generally, JA pathway is required for resistance of plants against necrotrophic pathogens, while SA signaling pathway is activated against biotrophic pathogens, and JA can antagonize SA signaling[58]. CLas is a biotrophic pathogen, the activation of JA may antagonize the content of SA level to some extent, contributing to HLB disease development. A recent study has reported that CLas is able to encode a SA hydroxylase to degrade SA, contributing to disease development[59]. At the meantime, a patent on citrus plant resistant to HLB has been approved (Dutt, Manjul (Winter Haven, FL, US), Grosser, Jude (Winter Haven, FL, US)2017. Citrus plants resistant to huanglongbing,United States, University of Florida Research Foundation, Inc. (Gainesville, FL, US) 20170073700. [http://www.freepatentsonline.com/y2017/0073700.html](http://www.freepatentsonline.com/y2017/0073700.html). The present invention relates to transgenic
citrus trees resistant to HLB through overexpression of AtNPR1, and the results demonstrated that
overexpressing the AtNPR1 can result in effective HLB resistance in citrus. NPR1 is a key regulator in the
signal transduction pathway that leads to SAR, and transduction of the SA signal requires the function of
NPR1[60]. It would be an important research to clarify how CLas mediate SA and JA crosstalk during its
infection. In fact, CLas affected multiple hormone-mediated immune responses. A auxin (A0A067ETE6)-
induced protein was up-regulated, suggesting that the auxin-regulated pathways were activated by CLas
infection. Moreover, several DEPs involved in the signal transduction of abscisic acid (A0A067FB99) and
brassinosteroid (A0A067FLJ4) were also induced.

Furthermore, Secondary metabolites play significant roles in disease resistance, including
phenylpropanoid-containing substances based on different biosynthesis pathways[61].
Phenylpropanoids are found to serve as essential components of a number of structural polymers and to
defend against pathogens[62]. It has been demonstrated that lignification and reinforcement of cell walls
are important processes of plants in response to pathogen infection[61, 63]. Generally, the activity of
several enzymes involved in lignin biosynthesis increases under pathogen infection, following the
accumulation of lignin. Lignification of cell walls limits the spread of enzymes and toxins of fungi to the
host, and also limits the absorption of water and nutrients from the host. Many lignin biosynthesis-related
proteins were down-regulated in our study (Fig. 6A and Additional file 5: Table S3). In particular, a number
of peroxidases were down-regulated in response to CLas-infection, implicating that phenylpropanoid
biosynthesis pathway might play significant roles on preventing CLas early infection and CLas might
inhibit the formation of lignin by depressing the peroxidases to contribute to disease infection. Therefore,
genes of many elicitors that can promote phenylpropanoid biosynthesis are potential targets for breeding
CLas-tolerant cultivar.

**Conclusions**

In this study, a comparative proteome analysis of Citrus petiole protein profiles using TMT-labeled
technology was carried out. A total of 777 DEPs were identified in response to CLas, which is significantly
more than that identified in previous studies. We mainly showed 3 physiological and molecular processes
that many DEPs involved. The data suggested that CLas infection could promote the carbohydrate
metabolism (DEPs were generally significantly up-regulated), depress the photosystem (DEPs were
significantly down-regulated) and activate/inhibit defense responses (some of DEPs were significantly
up-regulated). Western blot analysis was carried out here to validate the reliability of proteome data with
4 representative DEPs of CsGBSS1, CsPSI, CsSAMT and CsLOX2.1. The data expand the CLas-mediated
protein catalog in citrus plants. Identifying the early plant responses in response to CLas infection in
petioles contributes to facilitating the researches on citrus resistance against CLas.

**Methods**

**Plants materials**
Two-year-old seedlings of sweet orange (Citrus sinensis cv. Newhall) were obtained from the Institute of Citrus Research located in Ganzhou, Jiangxi Province, China. They were grafted with bud sticks from qPCR-positive sweet orange plants. For mock-inoculated controls, the same types of plants were grafted with bud sticks from qPCR-negative sweet orange plants. All plants were grown under controlled conditions (natural photoperiod at 25 to 28°C) in an insect-proof greenhouse. Starting at 2 months after grafting, each plant was detected biweekly using qPCR for CLas as described[64]. The CLas in leaf petioles was first observed at 3–4 months post grafting, and typical HLB symptoms appeared at 4–5 months post inoculation. One month after firstly observing CLas by qPCR was considered as the early infection stage. Three biological replicate samples of PCR-positive and PCR-negative plants were collected to extract total proteins for proteomic analysis. About 10–15 leaf petioles containing similar CLas quantity from 1–2 trees were sampled as a biological replicate.

**Plant protein extraction, digestion and TMT-labeling**

The petiole sample was ground in a mortar in the presence of liquid nitrogen. The cell powder was transferred to 50-ml centrifuge tubes, 10 ml of lysis buffer (8 M urea, 2% SDS, 1× Protease Inhibitor Cocktail (Roche Ltd. Basel, Switzerland)) was added to each sample, followed by sonication on ice and centrifugation at 13,000 g for 15 min at 4°C. The supernatant was transferred to a fresh tube. For each sample, proteins were precipitated with ice-cold acetone at −20°C overnight, the precipitations were cleaned with 50% ethanol and 50% acetone three times. 200 μg proteins was diluted by buffer (100 mM Tris, pH 8.0, 8 M urea) to 100 μl, then the solution was added 11 μl DTT (1 M) and incubated at 37 °C for 1 h. The treated samples were added into 10 kDa ultrafiltration tube (Millipore, MA, USA) and centrifuged at 12,000 g for 10 min. Then 100 μl 55 mM iodoacetamide (IAA) was added to ultrafiltration tube and incubated for 20 min protected from light at room temperature. After that, 50 mM triethylammonium bicarbonate (TEAB) was used as exchange buffer. Then proteins were tryptic digested with sequence-grade modified trypsin (Promega, WI, USA) overnight at 37 °C in a 1:50 trypsin-to-protein mass ratio, and the resultant peptide mixture was labeled using chemicals from the TMT reagent kit (Pierce Biotechnology, IL, USA). Proteins were labeled with the TMT as follows: TMT Sample1 was labeled with 131/129 (CLas/Mock), TMT Sample2 was labeled with 130/127, and TMT Sample3 was labeled with 128/131. Samples were then dried in vacuo.

**High pH reverse phase separation**

The peptide mixtures were redissovled in the buffer A (20 mM ammonium formate in water, pH 10.0, adjusted with ammonium hydroxide), and then fractionated by high pH separation using Ultimate 3000 system (ThermoFisher scientific, MA, USA) connected to a reverse phase column (XBridge C18 column, 4.6 mm × 250 mm, 5 μm, (Waters Corporation, MA, USA). High pH separation was performed using a linear gradient. Starting from 5% to 45% buffer B (20 mM ammonium formate in 80% ACN, pH 10.0, adjusted with ammonium hydroxide) in 40 min. The column was re-equilibrated at initial conditions for
10 min. The column flow rate was maintained at 1ml/min and column temperature was maintained at 30°C. 12 fractions were collected, each fraction was dried in a vacuum concentrator for the next step.

**Low pH NANO-HPLC-MS/MS analysis**

The fractions were resuspended with 30 μl solvent buffer C (water with 0.1% formic acid) respectively, separated by nanoLC and analyzed by on-line electrospray tandem mass spectrometry. The experiments were carried out on an Easy-nLC 1000 system (Thermo Fisher Scientific, MA, USA) connected to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, MA, USA) equipped with an online nano-electrospray ion source. 10 μl peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm × 2cm), with a flow of 10 μl/min for 3 min and subsequently separated on the analytical column (Acclaim PepMap C18, 75 μm × 15 cm) with a linear gradient, from 3% to 32% buffer D (ACN with 0.1% formic acid) in 120 min. The column was re-equilibrated at initial conditions for 10 min. The column flow rate was maintained at 300nL/min. The electrospray voltage of 2 kV versus the inlet of the mass spectrometer was used. The fusion mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350–1550) were acquired with a mass resolution of 120K, followed by sequential high energy collisional dissociation (HCD) MS/MS scans with a resolution of 30 K. The isolation window was set as 1.6 Da. The AGC target was set as 400000. MS/MS fixed first mass was set at 110. In all cases, one microscan was recorded using dynamic exclusion of 45 seconds.

**Database searches and bioinformatics analysis**

Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by Mascot Distiller version 2.6.1. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1). Mascot was set up to search the UniProt_ *Citrus sinensis* database assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 5.0 PPM. Carbamidomethyl of cysteine and TMT6plex of lysine and the n-terminus were specified in Mascot as fixed modifications. Deamidated of asparagine and glutamine, oxidation of methionine and acetyl of the n-terminus were specified in Mascot as variable modifications.

Scaffold (version Scaffold_4.6.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Scaffold Q+ (version Scaffold_4.6.2, Proteome Software Inc., Portland, OR) was used to quantitate TMT Label Based Quantitation peptide and protein identifications. Normalization was performed iteratively (across samples and spectra) on intensities, as described in Statistical Analysis of Relative Labeled Mass Spectrometry Data from Complex Samples Using ANOVA[65]. Medians were used for averaging. Spectra
data were log-transformed, pruned of those matched to multiple proteins, and weighted by an adaptive intensity weighting algorithm. Differentially expressed proteins were determined by applying Mann-Whitney Test with unadjusted significance level $p < 0.05$ corrected by Benjamini-Hochberg.

Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). Proteins were classified by GO annotation based on three categories: biological processes, cellular components and molecular functions. The Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) database was used to annotate protein pathways.

**Quantitative real-time PCR**

Total RNA from citrus petioles was extracted using a plant RNA kit (Qiagen, Valencia, CA). Reverse transcription was carried out with the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer’s instruction. Total DNA from citrus petioles was extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Amplification was performed in an ABI7500 Real-Time PCR system with SYBR Fast qPCR Mix (Takara, Dalian, China). Citrus GADPH gene was used as internal references to normalize the amount of RNA in different reactions. The relative mRNA quantities were calculated from the threshold cycle using the $\Delta \Delta Ct$ method[66]. Each experiment was repeated three times. The details of the primers used in this experiment, please see (Additional file 7: Table S4).

**Determination of the endogenous levels of salicylic acid and jasmonic acid**

For the SA and JA content assay, CLas-inoculated and control citrus samples were collected in three replicates. Petiole tissues were collected, weighed and frozen in liquid nitrogen. For each sample, 0.1 g of the frozen tissue was extracted and quantitated for free SA and JA, as described previously[25, 67]. In brief, the tissue was ground into powder and homogenized in 1 mL of methanol-H$_2$O-acetic acid (80:19:1). After extraction overnight at 4°C and centrifugation, the supernatant was re-extracted with the solution described previously. After the addition of 1 mL chloroform and further centrifugation, the organic phase containing the free SA was dried in a speed vacuum with heat (∼40°C). The residue was resuspended in 0.5 mL of methanol, filtered and analyzed by Ultra Performance Liquid Chromatography (UPLC). UPLC was performed on an ACQUITY UPLC@BEHC18 column (50 mm x 2.1 mm, 1.7 μm) run at 40°C with a flow rate of 0.4 mL min$^{-1}$. The analytes were eluted from the column with a mixed solvent of water with 0.1% acetic acid (solvent A) and methanol with 0.1% acetic acid (solvent B) using a linear gradient mode. The ratio of A and B was 90:10 from 0 s to 3 min, and this ratio changed linearly from 90:10 to 10:90 between 3 and 4 min. The ratio of 90:10 was finally maintained from 4 to 7 min. The authenticity of the SA/JA from citrus petiole extract was verified based on the retention times and spectral properties, which matched perfectly to those of commercial SA/JA standards.
Cloning, expression and purification of proteins

The coding sequence of targeted protein fragment was cloned into pET28a/pET32a (Novagen) and transformed into *E. coli* strain BL21(DE3) for expression. The refolding of the recombinant proteins expressed in inclusion bodies was carried out as previously described[68]. The recombinant proteins were purified via 6×His tag, and then detected by SDS-PAGE with the molecular mass marker (RTD6105, Beijing Real-Times Biotechnology Co. Ltd., China. The concentration of the purified protein was measured using the BCA™ Protein Assay Kit (TransGen Biotech), and the protein was then frozen at −80°C in small aliquots until use.

Preparation of polyclonal antibody and Western blot analysis

The aim band was excised and used as antigens for antibody production. Antibodies were produced in rabbit by HuaAn Biotechnology Company. Citrus petioles were ground in liquid nitrogen and then added to 2 ml of protein extraction buffer (50 mM Tris, pH 8.0, 10 mM NaCl, 1% SDS, 1 mM EDTA, 0.5% (v/v) 2-mercaptoethanol, 1 mM PMSF, 0.1 mM DTT and 0.1% (v/v) Triton X–100). The mixtures were centrifuged at 4°C for 30 min at 12,000g, and the supernatant was transferred into a 15-ml centrifuge tube. The protein concentration was determined using the BCA protein assay kit (TransGen Biotech). Total proteins (20 μg) from each sample were separated by SDS-PAGE and transferred onto membranes (PVDF), and the blots were analyzed using respective antibodies.

Declarations

Abbreviations

CLas: Candidatus Liberibacter asiaticus; DEPs: differentially expressed proteins; FDR: False Discovery Rate; FNR: ferredoxin-oxidoreductase; GO: Gene ontology; GPT2: Glucose–6-phosphate/phosphate translocator 2; HLB: Huanglongbing; JA: jasmonic acid; NOI: reactive nitrogen oxide intermediates; OEE: oxygen-evolving enhancer; PP2: Phloem protein 2; PSI: photosystem I; ROS: reactive oxygen species; SA: salicylic acid; SAR: systemic acquired resistance.

Authors’ contributions

- BL designed the experiments, analysed all data, and wrote the draft manuscript, YZ and SCW participated in the main experiments and collected the data. SCW also took part in data analysis and assisted in the preparation of the manuscript. DWQ supervised the overall study and revised the whole manuscript. All authors have read and approved the final version of the manuscript.
- Acknowledgements
- We are grateful to Dr. Tian Fang for reading this manuscript and giving some comments.
Funding

This work was supported by the Agricultural Science and Technology Innovation Program of Chinese Academy of Agricultural sciences (CAAS-XTCX2016013) to Dewen Qiu, the National Key R & D Program of China (2018YFD0201500) to Shuangchao Wang, and China Scholarship Council (201903250116) to Bo Li. The funders had no roles in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials

All the supporting data are included as additional files.

- Ethics approval and consent to participate
- Not applicable.
- Consent for publication
- Not applicable.
- Competing interests
- The authors declare that they have no competing interests.

References

1. Graça JVD, Korsten L: *Citrus Huanglongbing: Review, Present status and Future Strategies. Diseases of Fruits & Vegetables* 2004:229–245.

2. Shen W, Halbert SE, Dickstein E, Manjunath KL, Shimwela MM, Bruggen AHCV: *OCCURRENCE AND IN-GROVE DISTRIBUTION OF CITRUS HUANGLONGBING IN NORTH CENTRAL FLORIDA*. *Journal of Plant Pathology* 2013, 95(2):361–371.

3. Do CTD, Luc DJ, Eveillard S, Cristina ME, Wc DJJ, Takao YP, Aparecido LS, Beozzo BR, Juliano AA, Saillard C: *Citrus huanglongbing in São Paulo State, Brazil: PCR detection of the ‘Candidatus’ Liberibacter species associated with the disease*. *Molecular & Cellular Probes* 2005, 19(3):173–179.

4. Gottwald TR: *Current epidemiological understanding of citrus huanglongbing*. *Annu Rev Phytopathol* 2010, 48(48):119–139.

5. Tsai JH, Wang JJ, Liu YH: *SEASONAL ABUNDANCE OF THE ASIAN CITRUS PSYLLID, DIAPHORINA CITRI (HOMOPTERA: PSYLLIDAE) IN SOUTHERN FLORIDA*. *Fla Entomol* 2002, 85(3):446–451.

6. Lopes SA, Bertolini E, Frare GF, Martins EC, Wulff NA, Teixeira DC, Fernandes NG, Cambra M: *Graft Transmission Efficiencies and Multiplication of ‘Candidatus Liberibacter americanus’ and ‘Ca. Liberibacter asiaticus’ in Citrus Plants*. *Phytopathology* 2009, 99(3):301–306.
7. Sagaram US, DeAngelis KM, Trivedi P, Andersen GL, Lu SE, Wang N: Bacterial Diversity Analysis of Huanglongbing Pathogen-Infected Citrus, Using PhyloChip Arrays and 16S rRNA Gene Clone Library Sequencing. Appl Environ Microb 2009.

8. BOVE JM: Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. Journal of Plant Pathology 2006, 88(1):7–37.

9. Gottwald TR: Current Epidemiological Understanding of Citrus Huanglongbing*. Annu Rev Phytopathol 2010, 48(48):119–139.

10. Kim J, Sagaram US, Burns JK, Li J, Wang N: Response of Sweet Orange (Citrus sinensis) to ‘Candidatus Liberibacter asiaticus’ Infection: Microscopy and Microarray Analyses. Phytopathology 2009, 99(1):50–57.

11. Fan J, Chen C, Yu Q, Brlansky RH, Gmitter FG: Comparative iTRAQ proteome and transcriptome analyses of sweet orange infected by. Physiol Plantarum 2011, 143(3):235–245.

12. Zhong Y, Cheng C, Jiang N, Jiang B, Zhang Y, Wu B, Hu M, Zeng J, Yan H, Yi G: Comparative Transcriptome and iTRAQ Proteome Analyses of Citrus Root Responses to Candidatus Liberibacter asiaticus Infection. Plos One 2015, 10(6):e126973.

13. Slisz AM, Breksa AP, Mishchuk DO, McCollum G, Slupsky CM: Metabolomic analysis of citrus infection by ‘Candidatus Liberibacter’ reveals insight into pathogenicity. J. Proteome Res. 2012, 11(8):4223–4230.

14. Albrecht U, Bowman KD: Gene expression in Citrus sinensis (L.) Osbeck following infection with the bacterial pathogen Candidatus Liberibacter asiaticus causing Huanglongbing in Florida. Plant Sci 2008, 175(3):291–306.

15. Etxeberria E, Gonzalez P, Achor D, Albrigo G: Anatomical distribution of abnormally high levels of starch in HLB-affected Valencia orange trees. Physiological & Molecular Plant Pathology 2010, 74(1):76–83.

16. Folimonova SY, Achor DS: Early Events of Citrus Greening (Huanglongbing) Disease Development at the Ultrastructural Level. Phytopathology 2010, 100(9):949–958.

17. Achor DS, Etxeberria E, Wang N, Folimonova SY, Albrigo LG: Sequence of Anatomical Symptom Observations in Citrus Affected with Huanglongbing Disease. Plant Pathology Journal 2010, 9(2):56–64.

18. Huber DM HS: Managing nutrition to control plant disease. Landbauforschung Volkenrode 2007, 313–322(57).

19. Mafra V, Martins PK, Francisco CS, Ribeiro-Alves M, Machado MA: Candidatus Liberibacter americanus induces significant reprogramming of the transcriptome of the susceptible citrus genotype. Bmc Genomics 2013, 14(1):247.
20. Valente A, Diann A, GF, Gene A, Nian W, A. CR: Transcriptional and Microscopic Analyses of Citrus Stem and Root Responses to Candidatus Liberibacter asiaticus Infection. Plos One 2013, 8(9):e73742.

21. Federico M, L. US, Ute A, L. RR, L. PM, Monica B, Vincent B, Joseph F, Elizabeth L, Weixiang Z: Transcriptome Profiling of Citrus Fruit Response to Huanglongbing Disease. Plos One 2012, 7(6):e38039.

22. Nwugo CC, Lin H, Duan Y, Civerolo EL: The effect of 'Candidatus Liberibacter asiaticus' infection on the proteomic profiles and nutritional status of pre-symptomatic and symptomatic grapefruit (Citrus paradisi) plants. Bmc Plant Biology 2013, 13(1):59.

23. Dinant S, Clark AM, Zhu Y, Vilaine F, Thompson GA: Diversity of the Superfamily of Phloem Lectins (Phloem Protein 2) in Angiosperms. Plant Physiol 2003, 131(1):114–128.

24. Beneteau J, Renard D, Marché L, Douville E, Lavenant L, Rahbé Y, Dupont D, Dinant FOVA: Binding Properties of the N-Acetylglucosamine and High-Mannose N-Glycan PP2-A1 Phloem Lectin in Arabidopsis. Plant Physiol 2010, 153(3):1345–1361.

25. Zhang C, Shi H, Chen L, Wang X, Lü B, Zhang S, Liang Y, Liu R, Qian J, Sun W: Harpin-induced expression and transgenic overexpression of the phloem protein geneAtPP2-A1 in Arabidopsis repress phloem feeding of the green peach aphid Myzus persicae. Bmc Plant Biology 2011, 11(1):11.

26. Martinelli F, Reagan RL, Dolan D, Fileccia V, Dandekar AM: Proteomic analysis highlights the role of detoxification pathways in increased tolerance to Huanglongbing disease. Bmc Plant Biology 2016, 16(1):167.

27. Wang Y, Lijuan Z, Xiaoyue Y, Ed S, Feng L, Yongping D: Transcriptome Profiling of Huanglongbing (HLB) Tolerant and Susceptible Citrus Plants Reveals the Role of Basal Resistance in HLB Tolerance. Frontiers in Plant Science 2016, 7(74).

28. Fan J, Chen C, Yu Q, Brlansky RH, Li ZG, Gmitter FG: Comparative iTRAQ proteome and transcriptome analyses of sweet orange infected by "Candidatus Liberibacter asiaticus". Physiol Plant 2011, 143(3):235–245.

29. Zhan Y, Wu Q, Chen Y, Tang M, Sun C, Sun J, Yu C: Comparative proteomic analysis of okra (Abelmoschus esculentus L.) seedlings under salt stress. 2019, 20(1).

30. Yao L, Yu Q, Huang M, Hung W, Grosser J, Chen S, Wang Y, Gmitter FG: Proteomic and metabolomic analyses provide insight into the off-flavour of fruits from citrus trees infected with 'Liberibacter asiaticus'. Hortic Res 2019, 6:31.

31. Nwugo CC, Doud MS, Duan YP, Hong L: Proteomics analysis reveals novel host molecular mechanisms associated with thermotherapy of 'Ca. Liberibacter asiaticus'-infected citrus plants. Bmc Plant Biology 2016, 16(1):253.
32. Yun Z, Chun-zhen C, Nong-hui J, Bo J, Yong-yan Z, Bo W, Min-lun H, Ji-wu Z, Hua-xue Y, Gan-jun Y: *Comparative Transcriptome and iTRAQ Proteome Analyses of Citrus Root Responses to Candidatus Liberibacter asiaticus Infection*. Plos One 2015, 10(6):e126973.

33. Yang QS, Wu JH, Li CY, Wei YR, Sheng O, Hu CH, Kuang RB, Huang YH, Peng XX, Mccardle JA: *Quantitative Proteomic Analysis Reveals that Antioxidation Mechanisms Contribute to Cold Tolerance in Plantain (Musa paradisiaca L.; ABB Group) Seedlings*. Molecular & Cellular Proteomics 2012, 11(s1):1853.

34. Liao HL, Burns JK: *Gene expression in Citrus sinensis fruit tissues harvested from huanglongbing-infected trees: comparison with girdled fruit*. J Exp Bot 2012, 63(8):3307–3319.

35. Smith, M. A: *The Biosynthesis of Starch Granules*. Biomacromolecules 2001, 2(2):335–341.

36. Kunz HH, Häusler RE, Fettke J, Herbst K, Niewiadomski P, Gierth M, Bell K, Steup M, Flügge UI, Schneider A: *The role of plastidial glucose–6-phosphate/phosphate translocators in vegetative tissues of Arabidopsis thaliana mutants impaired in starch biosynthesis*. Plant Biol (Stuttg) 2010, 12 Suppl 1:115–128.

37. Xu M, Ya L, Zheng Z, Zehan D, Yang T, Xiaoling D, Ji-Hong L: *Transcriptional Analyses of Mandarins Seriously Infected by 'Candidatus Liberibacter asiaticus'*. Plos One 2015, 10(7):e133652.

38. Freitas DS, Carlos EF, Gil MC, Vieira LG, Alcantara GB: *NMR-Based Metabolomic Analysis of Huanglongbing-Asymptomatic and -Symptomatic Citrus Trees*. J. Agric. Food Chem. 2015, 63(34):7582–7588.

39. Carmo-Silva AE, Salvucci ME: *The Regulatory Properties of Rubisco Activase Differ among Species and Affect Photosynthetic Induction during Light Transitions*. Plant Physiol 2013, 161(4):1645–1655.

40. Huber D, Haneklaus S: *Managing nutrition to control plant disease*. Landbauforsch Volk 2007, 57(57):313–322.

41. Yi, X.: *The PsbQ Protein Is Required in Arabidopsis for Photosystem II Assembly/Stability and Photoautotrophy under Low Light Conditions*. J Biol Chem 2006, 281(36):26260.

42. Murakami R, Ifuku K, Takabayashi A, Shikanai T, Endo T, Sato F: *Characterization of an Arabidopsis thaliana mutant with impaired psbO, one of two genes encoding extrinsic 33-kDa proteins in photosystem II*. Febs Lett 2002, 523(1–3):142.

43. Yi X, McChargue M, Laborde S, Frankel LK, Bricker TM: *The Manganese-stabilizing Protein Is Required for Photosystem II Assembly/Stability and Photoautotrophy in Higher Plants*. J Biol Chem 2005, 280(16):16170–16174.
44. Pushkar Y, Yano J, Sauer K, Boussac A, Yachandra VK: Structural changes in the Mn4Ca cluster and the mechanism of photosynthetic water splitting. Proc Natl Acad Sci U S A 2008, 105(6):1879–1884.

45. Iwai M, Takizawa K, Tokutsu R, Okamuro A, Takahashi Y, Minagawa J: Isolation of the elusive supercomplex that drives cyclic electron flow in photosynthesis. Nature 2010, 464(7292):1210–1213.

46. Fan J, Chen C, Bransky RH, Jr FGG, Li ZG: Changes in carbohydrate metabolism in Citrus sinensis infected with 'Candidatus Liberibacter asiaticus'. Plant Pathol 2010, 59(6):1037–1043.

47. Coll NS, Epple P, Dangl JL: Programmed cell death in the plant immune system. Cell Death & Differentiation 2011, 18(8):1247–1256.

48. Grant MR, Jones JDG: Hormone (Dis)harmony Moulds Plant Health and Disease. Science 2009, 324(5928):750–752.

49. Kangasjarvi S, Neukermans J, Li S, Aro EM, Noctor G: Photosynthesis, photorespiration, and light signalling in defence responses. J Exp Bot 2012, 63(4):1619–1636.

50. Nomura H, Komori T, Uemura S, Kanda Y, Shimotani K, Nakai K, Furuichi T, Takebayashi K, Sugimoto T, Sano S: Chloroplast-mediated activation of plant immune signalling in Arabidopsis. Nature Communications 2012, 3(926):926.

51. Padmanabhan MS, Dinesh-Kumar SP: All Hands on Deck—The Role of Chloroplasts, Endoplasmic Reticulum, and the Nucleus in Driving Plant Innate Immunity. Molecular plant-microbe interactions: MPMI 2010, 23(11):1368.

52. An C, Mou Z: Salicylic Acid and its Function in Plant Immunity. Journal of Integrative Plant Biology 2011, 53(6):412–428.

53. Aritua V, Achor D, Gmitter FG, Albrigo G, Wang N: Transcriptional and Microscopic Analyses of Citrus Stem and Root Responses to Candidatus Liberibacter asiaticus Infection. Plos One 2013, 8(9):e73742.

54. Liu G, Ji Y, Bhuiyan NH, Pilot G, Selvaraj G, Zou J, Wei Y: Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in Arabidopsis. Plant Cell 2010, 22(11):3845–3863.

55. Zou H, Gowda S, Zhou L, Hajeri S, Chen G, Duan Y: The destructive citrus pathogen, ‘Candidatus Liberibacter asiaticus’ encodes a functional flagellin characteristic of a pathogen-associated molecular pattern. PLoS ONE 2012, 7(9):e46447.

56. Feys BJ, Moisan LJ, Newman MA, Parker JE: Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. Embo J 2014, 20(19):5400–5411.

57. Van Loon LC, Rep M, Pieterse CMJ: Significance of Inducible Defense-related Proteins in Infected Plants. Annu.rev.phytopathol 2006, 44(1):135–162.
58. Zhang Y, Liang Y, Qiu D, Yuan J, Yang X: *Comparison of cerato-platanin family protein BcSpl1 produced in Pichia pastoris and Escherichia coli*. *Protein Expression & Purification* 2017, 136:20–26.

59. Mohamed El Oirdi TAER: *Botrytis cinerea Manipulates the Antagonistic Effects between Immune Pathways to Promote Disease Development in Tomato*. *Plant Cell* 2011, 23(6):2405–2421.

60. Li J, Pang Z, Trivedi P, Zhou X, Wang N: ‘*Candidatus Liberibacter asiaticus*’ Encodes a Functional Salicylic Acid (SA) Hydroxylase That Degrades SA to Suppress Plant Defenses. *Molecular plant-microbe interactions: MPMI* 2017, 30(8):620.

61. Kinkema M, Dong WFX: *Nuclear Localization of NPR1 Is Required for Activation of PR Gene Expression*. *Plant Cell* 2000, 12(12):2339–2350.

62. Xu L, Zhu L, Tu L, Liu L, Yuan D, Jin L, Long L, Zhang X: *Lignin metabolism has a central role in the resistance of cotton to the wilt fungus Verticillium dahliae as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry*. *J Exp Bot* 2011, 62(15):5607–5621.

63. Ferrer JL, Austin MB, Jr. Stewart C, Noel JP: *Structure and function of enzymes involved in the biosynthesis of phenylpropanoids*. *Plant Physiol Biochem* 2008, 46(3):356–370.

64. Naoumkina MA, Zhao Q, Gallego-Giraldo L, Dai X, Zhao PX, Dixon RA: *Genome-wide analysis of phenylpropanoid defence pathways*. *Molecular Plant Pathology* 2010, 11(6):829–846.

65. Li W, Hartung JS, Levy L: *Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associate with citrus huanglongbing*. *J Microbiol Meth* 2006, 66(1):104–115.

66. Oberg AL, Mahoney DW, Eckel-Passow JE, Malone CJ, Wolfinger RD, Hill EG, Cooper LT, Onuma OK, Spiro C, Therneau TM: *Statistical Analysis of Relative Labeled Mass Spectrometry Data from Complex Samples Using ANOVA*. *J Proteome Res* 2008, 7(1):225–233.

67. Schmittgen TD, Livak KJ: *Analyzing real-time PCR data by the comparative CT method*. *Nature Protocols* 2008, 3(6):1101–1108.

68. Matsuura H, Aoi A, Satou C, Nakaya M, Masuta C, Nabeta K: *Simultaneous UPLC MS/MS analysis of endogenous jasmonic acid, salicylic acid, and their related compounds*. *Plant Growth Regul* 2009, 57(3):293.

69. Zhang Y, Liang Y, Qiu D, Yuan J, Yang X: *Comparison of cerato-platanin family protein BcSpl1 produced in Pichia pastoris and Escherichia coli*. *Protein Expr. Purif.* 2017, 136:20–26.

**Tables**

Due to technical limitations, tables are only available as a download in the supplemental files section.
Figures

Figure 1

Phenotype symptoms of Citrus leaves at 4 months post CLas-inoculation. (A) Leaves from CLas/mock-inoculated sweet orange plants. The left leaves were from CLas-inoculated plant showing HLB blotchy mottle symptoms, and the right leaves were from mock-inoculated control plant. (B) The presence of CLas was confirmed by qPCR.

Figure 2

QC validation of MS data and the volcano plot for the identified proteins. (A) Length distribution of all identified peptides. (B) Distribution of identified proteins in each replicate was shown by a Venn Diagram. (C) All identified proteins were shown by a Volcano Plot. Differentially expressed proteins were shown as red (up-regulation) and green (down-regulation) spots.
Figure 3

Categorization of differentially expressed proteins (DEPs) in response to CLas-infection. GO analysis (A) and KEGG pathway (B) analysis of all DEPs in response to CLas infection. GO analysis, containing 3 main categories of biological processes, molecular functions and cellular components, was carried out to understand the functions of the DEPs. P values of all GO terms and KEGG pathways is lower than 0.05. Number on the right of column in (A) means the p value.
Figure 4

Differentially expressed proteins involved in starch and sucrose metabolism. (A) The sketch map of KEGG pathway on starch and sucrose metabolism. Red frames correspond to up-regulation. (B) Expression and purification of a 20-kD fragment of the CsGBSS1. The fragment was inserted into plasmid pET32a containing a 17-kD Trx-tag, the recombinant plasmid was then transformed into E. coli BL21(DE3) for expression. M, protein molecular weight markers. Lane 1 and 2 show proteins expressed by E. coli induced without/with IPTG after transformation with the recombinant plasmid, respectively. Lane 3 corresponds to the purified protein and shows a 37-kD single band. The proteins were stained with Coomassie Brilliant Blue. (C) Western blotting analysis of CsGBSS1 in CLas-infected and control citrus petioles. CsGBSS1 has a predicted molecular mass of 67 kD. 20 μg extracted proteins were loaded into each lane, and three replicates were carried out. Coomassie brilliant blue (CBB) served as a loading control.
Figure 5

Differentially expressed proteins involved in photosystem. (A) The sketch map of KEGG pathway on photosystem. Green frames correspond to down-regulation. (B) Expression and purification of CsPSI. The CsPSI was inserted into plasmid pET28a, the recombinant plasmid was then transformed into E. coli BL21(DE3) for expression. M, protein molecular weight markers. Lane 1 and 2 show proteins expressed by E. coli induced without/with IPTG after transformation with the recombinant plasmid, respectively. Lane 3 corresponds to the purified protein and shows a 28-kD single band. The proteins were stained with Coomassie Brilliant Blue. (C) Western blotting analysis of the indicated protein in CLas-infected and control citrus petioles. CsPSI has a predicted molecular mass of 22 kD. 20 μg extracted proteins were loaded into each lane, and three replicates were carried out.
Figure 6

Differentially expressed proteins involved in citrus defense responses. (A) The sketch map of KEGG pathway on phenylpropanoid biosynthesis. Red and green frames correspond to up-regulation and down-regulation, respective. (B) Expression and purification of a 15-kD fragment of the CsSAMT and CsLOX2.1, respectively. The fragment was inserted into plasmid pET28a (CsSAMT) or pET32a (CsLOX2.1), the recombinant plasmid was then transformed into E. coli BL21(DE3) for expression. M, protein molecular weight markers. Lane 1/4 and 2/5 show proteins expressed by E. coli induced without/with IPTG after transformation with the recombinant plasmid, respectively. Lane 3/6 corresponds to the purified CsSAMT and CsLOX2.1, showing a 18-kD and 35-kD single band, respectively. The proteins were stained with Coomassie Brilliant Blue. (C) Western blotting analysis of the indicated proteins in CLas-infected and control citrus petioles. CsSAMT and CsLOX2.1 have predicted molecular mass of 39 kD and 101 kD, respectively. 20 μg extracted proteins were loaded into each lane, and three replicates were carried out. (D) The induction of SA and JA levels in citrus petiole responses to CLas infection. Double asterisks indicate a statistically significant difference (p < 0.01 by Student’s t-test).
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile6FIGURES3.tif
- Additionalfile3TableS1.xlsx
- Additionalfile7TableS4.docx
- Additionalfile4TableS2.xlsx
- Additionalfile2FIGURES2.tif
- table3.docx
- table2.docx
- table1.docx
- Additionalfile1FIGURES1.tif
- Additionalfile5TableS3.xlsx