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Comparative iTRAQ proteome and transcriptome analyses of sweet orange infected by ''*Candidatus* Liberibacter asiaticus''

Jing Fan[a,b], Chunxian Chen[b], Qibin Yu[b], Ronald H . Brlansky[b], Zheng-Guo Li[a,*] and Frederick G. Gmitter Jr[b,*]

[a]Chongqing University, College of Bioengineering, Department of Bioengineering, Key Laboratory of Biorheological Science and Technology, Ministry of Education, Chongqing 400030, China

[b]University of Florida, Institute of Food and Agricultural Sciences, Department of Horticultural Sciences, Citrus Research and Education Center, Lake Alfred, FL 33850, USA

Correspondence
*Corresponding authors,
e-mail: zhengguoli@cqu.edu.cn;
fgmitter@ufl.edu

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Citrus Huanglongbing (HLB) has been threatening citrus production worldwide. In this study, a comparative proteomic approach was applied to understand the pathogenic process of HLB in affected sweet orange leaves. Using the isobaric tags for relative and absolute quantification (iTRAQ) technique, we identified 686 unique proteins in the mature leaves of both mock-inoculated and diseased 'Madam Vinous' sweet orange plants. Of the identified proteins, 20 and 10 were differentially expressed in leaves with and without symptoms of HLB (fold change > 2.5), respectively, compared with mock-inoculated controls. Most significantly, upregulated proteins were involved in stress/defense response, such as four miraculin-like proteins, chitinase, Cu/Zn superoxide dismutase and lipoxygenase. Microarray analysis also showed that stress-related genes were significantly upregulated at the transcriptional level. For example, remarkable upregulations of miraculin-like proteins and Cu/Zn superoxide dismutase transcripts were observed. Moreover, the transcriptional patterns of miraculin-like protein 1 and Cu/Zn superoxide dismutase were examined at different stages of HLB disease development. Combined with the transcriptomic data, the proteomic data can provide an enhanced understanding of citrus stress/defense responses to HLB.

Introduction

Citrus Huanglongbing (HLB, or greening) is one of the most severe diseases of citrus. It is distributed in most Asian, African and American countries and has caused substantial economic losses (Bove 2006, Halbert 2005). The presumed causal agent of HLB, ''*Candidatus* Liberibacter spp.'', is a gram-negative, phloem-inhabiting α-proteobacteria, and includes three known species, ''*Candidatus* Liberibacter asiaticus'' (*C*Las), ''*Candidatus* Liberibacter africanus'' and ''*Candidatus* Liberibacter americanus''. The disease is transmitted by two kinds of phloem-feeding citrus psyllids, *Diaphorina citri* and *Trioza erytreae*. All known citrus species and citrus relatives can be infected. HLB symptoms include leaf blotchy mottle, yellow shoots, small and misshapen fruits frequently with color inversion, aborted seeds and poor juice quality; starch accumulation and phloem damage are often observed anatomically (Kim et al. 2009). Control of HLB depends on exclusion

Abbreviations – *C*Las, ''*Candidatus* Liberibacter asiaticus''; CSD, citrus sudden death; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; HLB, Huanglongbing; HPLC, high-pressure liquid chromatography; iTRAQ, isobaric tags for relative and absolute quantification; MS, mass spectrometry; PR, pathogenesis-related; SCX, strong cation exchange; WAI, weeks after inoculation.

of the pathogen from a citrus-producing area, use of disease-free propagating material, inoculum reduction through removal of infected trees as soon as they are detected, and control of psyllid vectors using insecticides or biological agents. An extended latency period between infection with *C*Las and symptom expression results in the undetectable spread of the presumed causal organism over time, greatly complicating control strategies based on inoculum reduction.

Plant disease symptom development is considered to be the consequence of a number of molecular, cellular and physiological changes, and may also be associated with host defense responses (O'Donnell et al. 2003). Some information has already been produced to describe the molecular basis of host response to *C*Las infection. Two studies have reported on changes in the global gene expression profiles using the Affymetrix GeneChip® Citrus Genome Microarray following infection of sweet orange and development of symptoms (Albrecht and Bowman 2008, Kim et al. 2009). Differentially expressed genes have been found to be involved in a number of processes such as cellular defense, photosynthesis and carbohydrate metabolism. However, the host response to *C*Las infection at the proteome level has yet to be described.

Changes at the transcriptional level (mRNA) do not necessarily manifest in the same magnitude at the protein level because of posttranscriptional, translational and/or posttranslational regulations (Gygi et al. 1999, Washburn et al. 2003). For instance, mRNA produced in abundance may be degraded rapidly or translated inefficiently, leading to a small amount of protein; furthermore, many transcripts can give rise to more than one protein through alternative splicing. Finally, protein degradation rates also can play a critical role in protein content (Belle et al. 2006). Proteomic science is rapidly advancing beyond a simple cataloging of proteins. It is being widely used to study functional and regulatory aspects of proteins, e.g. comparative proteomics, protein–protein interactions and protein modifications (Chen and Harmon 2006). However, low sensitivity in protein analysis is a technical bottleneck, because amplification of proteins cannot be carried out as is performed routinely with DNA or RNA. The widely used two-dimensional gel electrophoresis-based approach is likely to identify primarily the most abundant and soluble proteins (Chen and Harmon 2006). An alternative and more powerful approach, isobaric tags for relative and absolute quantification (iTRAQ), has been developed. It uses isotope labeling coupled with multidimensional liquid chromatography and tandem mass spectrometry (MS), thereby enabling sensitive assessment and quantification of protein levels (Chen and Harmon 2006, Gan et al. 2007, Pierce et al. 2008). In this study, an eight-channel iTRAQ technique was used to characterize the proteome change in mature leaves from *C*Las-inoculated sweet orange plants (*Citrus sinensis*) compared with that from mock-inoculated controls. To increase the probability of detecting differentially expressed proteins, leaf samples were collected at 7 months after inoculation representing advanced stage of HLB infection process, as it has been found that more transcriptional changes were detected at late stage than early stage (Albrecht and Bowman 2008). In addition, transcriptional changes in response to HLB infection were also investigated using the Affymetrix GeneChip Citrus Genome Array, and transcriptional and proteomic changes were compared.

Materials and methods

Plant material

Two-year-old seedlings of 'Madam Vinous' sweet orange (*C. sinensis*) were inoculated by grafting with bud sticks from HLB-diseased, polymerase chain reaction (PCR)-positive sweet orange plants. For mock-inoculated controls, the same types of plants were grafted with bud sticks from HLB-free, PCR-negative sweet orange. All these plants were kept under controlled conditions, side by side in a US Department of Agriculture–Animal and Plant Health Inspection Service (APHIS)/Centers for Disease Control and Prevention (CDC)-approved and secured greenhouse at University of Florida, Citrus Research and Education Center, Lake Alfred, FL. The presence of *C*Las, the bacterium associated with HLB, was confirmed by quantitative real-time PCR as described (Li et al. 2006). Typical HLB symptoms, yellowing, blotchy mottle and/or variegated chlorosis of leaves (Fig. 1), first appeared 4 months after inoculation. Due to the variation of HLB symptom development among individual plants over time, two

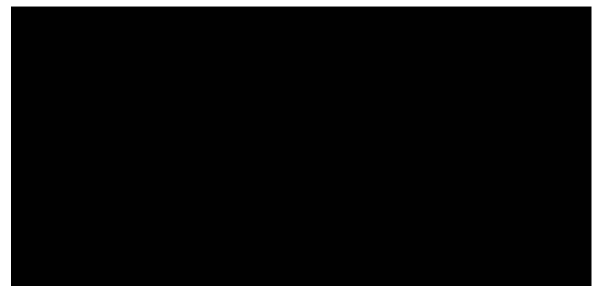

Fig. 1. Mature leaves sampled from mock-inoculated and HLB-diseased 'Madam Vinous' sweet orange plants. The left leaf was from mock-inoculated plant; the middle one having no HLB symptoms and the right one showing HLB typical blotchy mottle symptoms sampled from an infected plant (PCR positive for *C*Las).

comparable HLB-diseased plants were selected for proteomic experiments. At 7 months after inoculation, mature leaves with and without symptoms were sampled from the two infected plants, and healthy mature leaves from two mock-inoculated plants. For microarray experiments, mature leaves were sampled from three individual HLB-diseased plants regardless of symptom development, and healthy leaves from three mock-inoculated plants as control. For quantitative reverse transcription (RT)-PCR, leaves were sampled at 5, 17 and 27 weeks after inoculation (WAI) from three individual healthy or HLB-diseased plants, the latter confirmed by quantitative real-time PCR.

Protein extraction

Protein extraction was performed based on the study by Omar et al. (2007). Briefly, approximately 0.2 g leaf tissue was ground in liquid nitrogen. Seven hundred microliters of extraction buffer [50 m*M* Tris–HCl (pH 7.5), 150 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.1% Triton-X-100, 5 m*M* dithiothreitol (DTT)] plus 7× protease inhibitor solution made from Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN) were added into each sample. The extract was mixed completely, shaken for 30 min at 4°C and centrifuged at 18 404 g for 10 min at 4°C. The supernatant was transferred to a new tube and quantified using Protein Assay Kit I from Bio-Rad Laboratories (Hercules, CA) in a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Protein processing and iTRAQ labeling

As described by Zhu et al. (2009) but with modifications, 100 μg protein of each sample was precipitated in 80% cold acetone at −20°C overnight, centrifuged at 30 425 g for 20 min at 4°C and washed once with 80% cold acetone. After protein precipitation, the pellet of each sample was dissolved in 0.1% sodium dodecyl sulfate (SDS), 500 m*M* triethylammonium bicarbonate, pH 8.5. These samples were reduced, alkylated and trypsin-digested prior to labeling with the eight-channel iTRAQ Reagents Multiplex Kit according to manufacturer's instructions (Applied Biosystems, Foster City, CA). Two control samples were labeled with iTRAQ tags 113 and 114, two HLB-diseased symptomless leaf samples were labeled with tags 115 and 116 and two leaf samples with symptoms were labeled with tags 117 and 118.

Strong cation-exchange fractionation, liquid chromatography and mass spectrometry

After labeling, the samples were mixed and dried. They were then resuspended in 3% acetonitrile, 0.1% acetic acid, 0.01% trifluoroacetic acid, followed by loading on a MacroSpin Vydac C18 reverse-phase minicolumn. The washing and elution were conducted following instructions from the manufacturer (The Nestgroup Inc., Southborough, MA). The eluates were dried down and fractionated using a strong cation-exchange (SCX) column. Peptides were eluted at a flow rate of 200 μl min^{-1} with a linear gradient of 0–20% solvent B (25% v/v acetonitrile, 500 m*M* ammonium formate) over 90 min, followed by ramping up to 100% solvent B in 5 min and holding for 10 min. The absorbance at 280 nm was monitored, and a total of 25 fractions were collected and pooled into 10 fractions. As described previously (Zhu et al. 2009), each SCX fraction was lyophilized and redissolved in solvent A (3% acetonitrile v/v, 0.1% acetic acid v/v) plus 0.01% trifluoroacetic acid. The peptides were loaded onto a C18 capillary trap cartridge (LC Packings, San Francisco, CA) and then separated on a 15-cm nanoflow C18 column (PepMap 75 μm inner diameter, 3 μm, 100 Å) (LC Packings) at a flow rate of 200 nl min^{-1}. The high-pressure liquid chromatography (HPLC) instrument and the quadrupole time-of-flight (QSTAR XL) MS system were the same as previously described (Chen 2006). Peptides were eluted from the HPLC column by a linear gradient from 3% solvent B (96.9% acetonitrile v/v, 0.1% acetic acid v/v) to 40% solvent B for 2 h followed by ramping up to 90% solvent B in 10 min. Peptides were sprayed into the orifice of the mass spectrometer, which was operated in an information-dependent data acquisition mode where an MS scan followed by three MS/MS scans of three highest abundance peptide ions were acquired in each cycle (Chen 2006).

Data analysis

The MS/MS data were analyzed by a thorough search considering biological modifications against the NCBI subset green plants fasta database (downloaded on September 11, 2009) using the Paragon™ Algorithm (Shilov et al. 2007) of ProteinPilot v3.0 software suite (Applied Biosystems). According to Zhu et al. (2009), fixed modification of methylmethane thiosulfate-labeled cysteine, fixed iTRAQ modification of free amine in the N-terminus and lysine and variable iTRAQ modifications of tyrosine were considered. Parameters such as trypsin digestion, precursor mass accuracy and fragment ion mass accuracy are built-in settings of the software. The raw peptide identification results from the Paragon Algorithm were further processed by the ProGroup™ Algorithm. The ProGroup Algorithm uses the peptide identification results to determine the minimal set of confident proteins.

Unused ProtScore and total ProtScore are reported for protein identification. The Unused ProtScore prevents reuse of the same peptide evidence to support the detection of more than one protein. Thus, it is the real indicator of protein confidence. The software calculates a percentage confidence which reflects the probability that the hit is a false positive, so that at the 99% confidence level, there is a false-positive identification rate of 1%. The low confidence peptides do not identify a protein by themselves, but may support the presence of a protein identified using other peptides (Shilov et al. 2007). Performing the search against a concatenated database containing both forward and reversed sequences allowed estimation of the false discovery level. For protein relative quantification, only MS/MS spectra unique to a particular protein and where the sum of the signal-to-noise ratio for all the peak pairs was greater than 9 were used for protein relative quantification (software default settings; Applied Biosystems). Unused score $\geq$1.3 (corresponding to a confidence limit of 95%) was required for all the identified proteins.

RNA extraction and microarray experiments

Total RNA from each sample was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA), and contaminating DNA was eliminated using the DNA-*free* Kit (Ambion, Austin, TX), according to the manufacturer's instructions. The concentration of RNA was measured in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was evaluated by an Agilent Bioanalyzer Model 2100 (Agilent Technologies, Palo Alto, CA).

RNA samples were sent to the Interdisciplinary Center for Biotechnology Research Microarray Core, University of Florida, Gainesville, FL. According to the manufacturer's instructions, microarray experiments were carried out using the GeneChip 3′ IVT Express Kit and the GeneChip® Citrus Genome Array (Affymetrix, Santa Clara, CA).

The array data were analyzed using BioConductor (http://www.bioconductor.org), which is primarily based on the R programming language (Gentleman et al. 2004). Data normalization was performed by the Robust Multichip Analysis approach in an R package, Affy (Bolstad et al. 2003). Limma linear models were used to assess differential expression, and then an empirical Bayes method was applied to moderate the standard errors (Smyth et al. 2005). Due to the unavailability of citrus genome annotation, probe sets represented on the Affymetrix Citrus GeneChip were annotated by searching against *Arabidopsis thaliana* genome in the database of HarvEST (http://harvest-web.org/hweb/bin/chipsearch.wc?wsize =1259x608). The corresponding *Arabidopsis* orthologs were used in the following analysis. To identify the significantly affected pathways, the differentially expressed genes ($P \leq 0.05$) were analyzed by PageMan (Usadel et al. 2006). A Wilcoxon test was applied and Benjamini and Hochberg approach corrected P values were generated (Benjamini and Hochberg 1995). This approach to data analysis provides a statistics-based overview of changed pathways from global gene expression alterations.

Quantitative reverse transcription-PCR

RT was performed with the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instruction. Briefly, 1 μg of total RNA was incubated with 7× gDNA wipeout buffer at 42°C for 5 min. The reaction mixture was then added into the RT master mix in a final volume of 20 μl, incubated at 42°C for 30 min and inactivated at 95°C for 3 min.

Quantitative PCR (qPCR) was carried out in the Applied Biosystems 7500 Fast system (Applied Biosystems) using a QuantiTect SYBR Green PCR Kit (Qiagen). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene to provide relative quantification for the target genes miraculin-like protein 1 and Cu/Zn superoxide dismutase. Primer sequences were as follows: GAPDH-F 5′-GGAAGGTCAAGATCGGAATCAA-3′ and GAPDH-R 5′-CGTCCCTCTGCAAGATGACTCT-3′ for the reference gene; MLP1-F 5′-GGCCACAAAACCTCAGTTGGGC-3′ and MLP1-R 5′-TGTCCGCGCTAATTCCACCGC-3′ for miraculin-like protein 1 gene; SOD-F 5′-GCCTCTCTGG TTTGCCGCCT-3′ and SOD-R 5′-AAACCGTCAGCTCC AGCAGGT-3′ for Cu/Zn superoxide dismutase gene. A reaction mixture (20 μl) consisted of 2× QuantiTect SYBR Green PCRMaster mix, 0.3 (for *GAPDH* and *MLP1*) or 1 μ*M* (for *SOD*) of each primer and 1 μl of the finished RT reaction. qPCR conditions comprised one cycle at 95°C for 15 min, followed by 40 cycles at 94°C for 15 s, 58°C (for *GAPDH* and *MLP1*) or 54°C (for *SOD*) for 30 s and 72°C for 30 s. Melting curve analysis and electrophoresis were performed to verify the specificity and identity of the qPCR products. A total of six replicates were used for each sample (three biological replicates × two technical replicates).

PCR efficiencies of the reference and target genes were determined by generating standard curves based on serial dilutions of sweet orange genomic DNA (extracted from young, tender leaves of sweet orange using cetyltrimethylammonium bromide (CTAB) method as described; Doyle and Doyle 1990). The amplification efficiency was automatically

calculated using the 7500 FAST SYSTEM SDS software. The relative quantification of target gene transcripts was determined by the comparative C_T method $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001), where $\Delta\Delta C_T = (C_{T,Target} - C_{T,GAPDH})_{HLB} - (C_{T,Target} - C_{T,GAPDH})_{Control}$. *P* values were calculated using a simple *t*-test in SAS 9.1 (SAS Institute, Cary, NC) as described by Yuan et al. (2006).

Results

Protein identification and quantification

Total protein was extracted from mature leaves of mock-inoculated and *C*Las-inoculated sweet oranges (with and without symptoms, Fig. 1), as described (Omar et al. 2007). The protein yields ranged from 5.88 to 8.94 mg g^{-1} fresh weight, without significant differences among the three types of leaves. Using iTRAQ, a total of 686 unique proteins were identified (unused ProtScore > 1.3) from control and HLB-diseased mature leaves (Table S1). Of these, 263 proteins were quantified with iTRAQ ratios.

To determine differentially expressed proteins between HLB-diseased mature leaves and mock-inoculated controls, the correlation coefficient between two biological replicates was evaluated first. The two mock-inoculated controls (i.e. tags 113 and 114) were used as denominators, respectively. Only the proteins that were quantified with iTRAQ ratios were used to calculate correlation coefficient. The ratios (HLB-diseased samples without or with symptoms vs mock-inoculated controls) were then log-transformed and plotted against each other. As shown in Fig. 2, all the correlation coefficients between two biological replicates were approximately 0.8 or more, thereby indicating the biological reproducibility of HLB-regulated protein expression. Further, the biological variations between two replicates were estimated to set an optimal cut-off value of fold change for determining significantly altered differential protein expression levels. Fold changes were generated between two biological replicates of control samples, and HLB-diseased samples

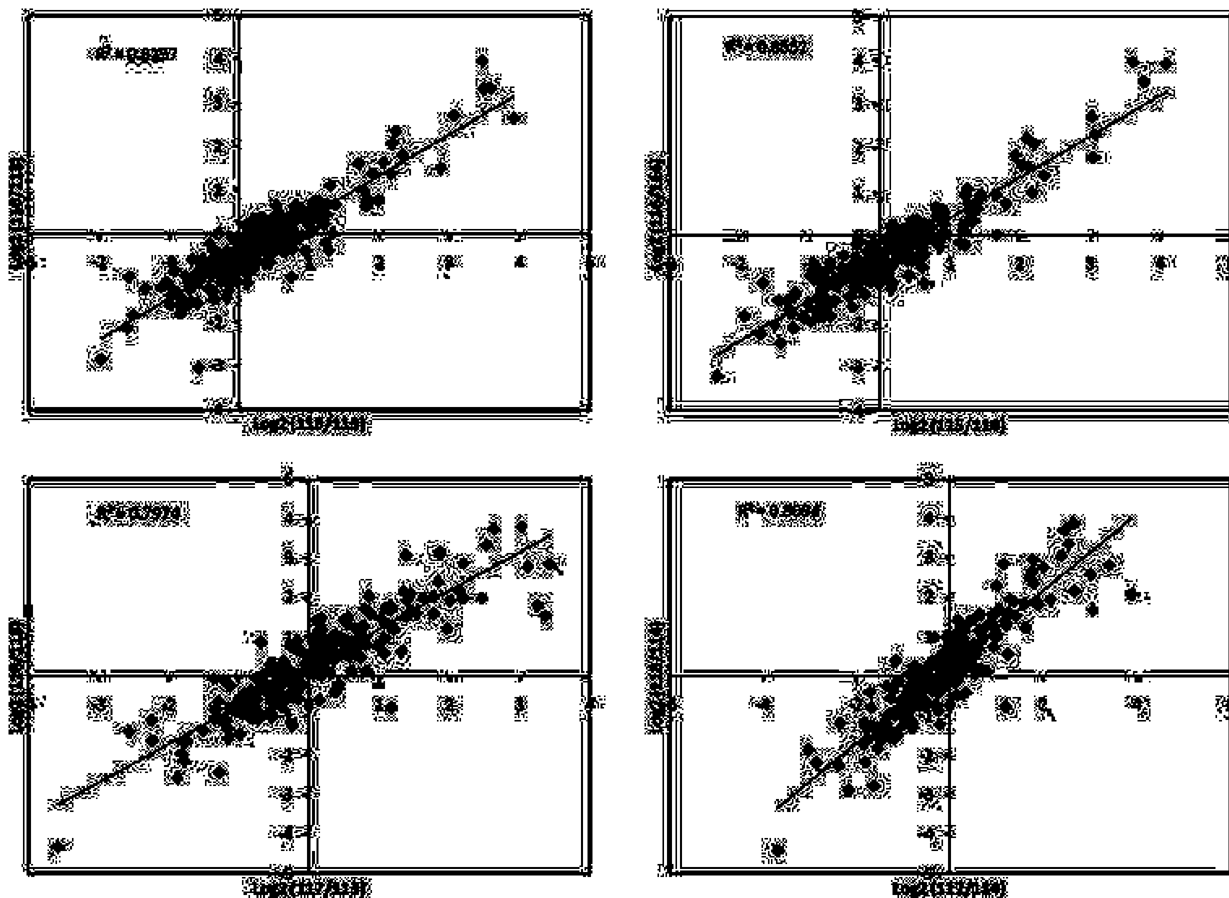

Fig. 2. Correlation coefficients of biological replicates. The ratios of quantified proteins were log-transformed and plotted between two biological replicates.

Table 1. Evaluation of biological variance of proteome data. 95% proteins had fold changes less than 2.5 within biological replicates.

Percentage of proteins	Fold change (114/113)	Fold change (116/115)	Fold change (118/117)
99	<2.38	<3.29	<3.40
95	<1.75	<2.50	<2.37
50	<1.18	<1.44	<1.29

with and without symptoms, respectively. The variations within two biological replicates were presented in Table 1. It was found that 95% proteins had fold changes less than 2.5 within biological replicates. Therefore, a 2.5-fold change was used as an optimal threshold to achieve 95% confidence ($P < 0.05$) for measuring differentially expressed proteins in HLB-diseased samples.

Differentially expressed proteins in HLB-infected sweet orange

Only proteins which were identified by three or more peptides and had >2.5-fold change in at least three of four comparisons (i.e. tag 115/113, 116/113, 115/114 and 116/114 for HLB-diseased symptomless samples vs mock-inoculated controls; tag 117/113, 118/113, 117/114 and 118/114 for HLB-diseased samples with symptoms vs controls) were considered to be differentially expressed proteins (Table S2). The quantities of 10 and 20 proteins in the HLB-infected leaves without and with symptoms, respectively, were significantly changed compared with the mock-inoculated controls (Table 2). The changes of all 10 proteins in symptomless leaves were also observed with similar trends in leaves with symptoms, although the fold changes were mostly different. Of the 20 proteins that were found in different quantities in HLB-diseased sweet orange, a total of 13 and 7 were up- and downregulated, respectively. For example, chitinase, Cu/Zn superoxide dismutase, lipoxygenase and four miraculin-like proteins were highly induced by 3.6–13.7-fold in both types of HLB-diseased leaves. In addition, several proteins such as beta-1,3-glucanase and ATPase alpha subunit were only significantly induced in leaves with HLB symptoms. However, all the downregulated proteins were annotated as unknown function (Table 2). Using the same criteria as above, the proteomes of leaves with and without symptoms were compared and no significant change of protein amount was observed (data not shown).

Comparative analysis of proteome and transcriptome data

For comparison, transcriptome analysis was performed using leaves of HLB-diseased sweet orange compared with healthy leaves from mock-inoculated plants. The transcriptome data have been deposited at the Gene Expression Omnibus database (http://www.ncbi.nih.gov/geo) under accession number GSE29633. Of the 33 879 probe sets on the Affymetrix citrus microarray, 24 604 present calls were detected in this study. In total, the expression of 3476 probe sets was significantly changed ($P \le 0.05$, fold change ≥ 2), with 1953 upregulated and 1523 downregulated. The 20 transcripts most induced in HLB-affected leaves were shown in Table 3. The transcripts Cit.7043.1.S1_at encoding miraculin-like protein 2 and Cit.28102.1.S1_s_at encoding Cu/Zn superoxide dismutase were remarkably induced by 219- and 83-fold, respectively, which agreed with the accumulation of their protein products (Table 2). Similar trends of deregulation were observed between other differentially expressed proteins and transcripts. For instance, both the protein and transcriptional levels of lipoxygenase, beta-1,3-glucanase and cysteine protease Cp were elevated in HLB-diseased sweet orange compared with mock-inoculated control (Table 2). However, the fold changes between the two levels were not matchable, and the correlation coefficient of the overall proteome and transcriptome data was very low (data not shown). This is expected because mRNA levels are not always consistent with protein levels due to the posttranscriptional, translational and/or posttranslational regulations (Gygi et al. 1999, Washburn et al. 2003).

Furthermore, PageMan software was used for pathway analysis of the differentially expressed transcripts with $P \le 0.05$. Significantly changed pathways (Benjamini and Hochberg approach corrected P value ≤ 0.05; Benjamini and Hochberg 1995) were displayed in Table 4. Stress response and transcription regulation pathways, such as pathogenesis-related (PR) proteins and WRKY domain transcription factor family, were significantly upregulated. Interestingly, the HLB-induced proteins, such as chitinase, Cu/Zn superoxide dismutase, lipoxygenase and miraculin-like protein, have been reported to be involved in response to abiotic or biotic stimulus (see section Discussion). These results indicate that similar pathways were affected both at the protein and transcriptional levels in HLB-diseased sweet orange.

Validation of differentially expressed proteins by qPCR

To further validate the proteome data, qPCR was performed to investigate the transcriptional patterns of two differentially expressed proteins, miraculin-like protein 1 and Cu/Zn superoxide dismutase, at multiple stages of HLB development in diseased sweet orange compared

Table 2. Differentially expressed proteins in mature leaves without (Asym) and with (Sym) symptoms from HLB-diseased sweet orange plants compared with mock-inoculated controls (Con). Only average ratios were shown here. Please see Table S1 for the ratios of all the comparisons among replicates. N.S., not significantly changed protein expression. The numbers in bold and italic in the column of 'Microarray ratio' represent values with $P \leq 0.05$. [a]Since an annotated citrus genome sequence is not available, data were analyzed by a thorough search against the NCBI subset green plants to identify proteins from this study. [b]Affymetrix Citrus GeneChip Probe ID of the closest hit of the differentially expressed protein.

<table>
<tr><th></th><th></th><th></th><th colspan="2">iTRAQ ratio</th><th>Microarray ratio</th><th></th><th></th></tr>
<tr><th>GenBank ID[a]</th><th>Protein</th><th>Species</th><th>Asym/Con</th><th>Sym/Con</th><th>HLB/Con</th><th>Probe ID[b]</th><th>E value</th></tr>
<tr><td>Upregulated</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>gi|1220144</td><td>Chitinase</td><td>Citrus sinensis</td><td>13.65</td><td>10.14</td><td>6.29</td><td>Cit.8276.1.S1_x_at</td><td>1.00E-145</td></tr>
<tr><td>gi|220029665</td><td>Putative miraculin-like protein 2</td><td>Citrus unshiu</td><td>3.62</td><td>8.92</td><td>0.54</td><td>Cit.730.1.S1_x_at</td><td>1.00E-125</td></tr>
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<tr><td>gi|2274917</td><td>Cu/Zn superoxide dismutase</td><td>C. sinensis</td><td>7.42</td><td>8.00</td><td>83.11</td><td>Cit.28102.1.S1_s_at</td><td>3.00E-69</td></tr>
<tr><td>gi|87299375</td><td>Miraculin-like protein 1</td><td>C. jambhiri</td><td>3.98</td><td>7.41</td><td>1.32</td><td>Cit.29373.1.S1_s_at</td><td>1.00E-100</td></tr>
<tr><td>gi|18461098</td><td>Lipoxygenase</td><td>C. jambhiri</td><td>4.47</td><td>6.16</td><td>8.36</td><td>Cit.9904.1.S1_s_at</td><td>0</td></tr>
<tr><td>gi|119367468</td><td>Putative miraculin-like protein 2</td><td>Citrus cv. Shiranuhi</td><td>4.05</td><td>6.08</td><td>17.77</td><td>Cit.7966.1.S1_x_at</td><td>1.00E-110</td></tr>
<tr><td>gi|116006688</td><td>Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit</td><td>Guatteria zamorae</td><td>8.28</td><td>5.87</td><td>0.77</td><td>Cit.25047.1.S1_at</td><td>9.00E-71</td></tr>
<tr><td>gi|4586021</td><td>Cytoplasmic aconitate hydratase</td><td>Arabidopsis thaliana</td><td>N.S.</td><td>4.37</td><td>0.81</td><td>Cit.3110.1.S1_s_at</td><td>0</td></tr>
<tr><td>gi|2274915</td><td>Beta-1,3-glucanase</td><td>C. sinensis</td><td>N.S.</td><td>4.22</td><td>4.86</td><td>Cit.9703.1.S1_at</td><td>0</td></tr>
<tr><td>gi|255640167</td><td>Unknown</td><td>Glycine max</td><td>4.70</td><td>3.77</td><td>0.86</td><td>Cit.9901.1.S1_s_at</td><td>1.00E-117</td></tr>
<tr><td>gi|151547430</td><td>Cysteine protease Cp</td><td>C. sinensis</td><td>N.S.</td><td>3.19</td><td>1.56</td><td>Cit.8566.1.S1_s_at</td><td>0</td></tr>
<tr><td>gi|89112876</td><td>ATPase alpha subunit</td><td>Lamium sp. Qiu 95019</td><td>N.S.</td><td>2.77</td><td>1.16</td><td>Cit.17976.1.S1_at</td><td>1.00E-157</td></tr>
<tr><td>Downregulated</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>gi|225464995</td><td>Hypothetical protein isoform 1</td><td>Vitis vinifera</td><td>N.S.</td><td>0.33</td><td>0.57</td><td>Cit.1478.1.S1_at</td><td>0</td></tr>
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<tr><td>gi|255626027</td><td>Unknown</td><td>G. max</td><td>N.S.</td><td>0.26</td><td>1.32</td><td>Cit.18004.1.S1_s_at</td><td>2.00E-64</td></tr>
<tr><td>gi|225429074</td><td>Hypothetical protein</td><td>V. vinifera</td><td>N.S.</td><td>0.24</td><td>1.16</td><td>Cit.3018.1.S1_at</td><td>1.00E-178</td></tr>
<tr><td>gi|255630681</td><td>Unknown</td><td>G. max</td><td>0.31</td><td>0.22</td><td>1.62</td><td>Cit.18106.1.S1_at</td><td>4.00E-55</td></tr>
<tr><td>gi|225458424</td><td>Hypothetical protein</td><td>V. vinifera</td><td>N.S.</td><td>0.20</td><td>0.98</td><td>Cit.9106.1.S1_s_at</td><td>1.00E-162</td></tr>
</table>

Table 3. The 20 most strongly induced transcripts in HLB-diseased sweet orange compared with mock-inoculated plants

Probe ID	Log2 ratio (HLB/control)	*P* value	Closest BLASTX hit in GenBank	E value	Description
Cit.7043.1.S1_at	7.772	0.01588	AAG38518	5.00E-37	Miraculin-like protein 2
Cit.60.1.S1_at	6.598	0.00333	AAF61436	2.00E-28	Lipid-transfer protein precursor
Cit.28102.1.S1_s_at	6.377	0.00266	CAA03881	7.00E-67	Cu/Zn superoxide dismutase
Cit.6827.1.S1_x_at	6.238	0.00417	AAD50034	2.00E-79	Very similar to SRG1
Cit.7918.1.S1_at	6.092	0.00215	AAG60148	7.00E-83	Copper amine oxidase, putative
Cit.15697.1.S1_s_at	5.821	0.00073	NP_917058	2.00E-54	Putative leucine rich repeat containing protein kinase
Cit.31369.1.S1_at	5.778	0.00254	AAD52097	3.00E-75	Receptor-like kinase CHRK1
Cit.35955.1.S1_at	5.354	0.038	AAM64609	5.00E-49	Phloem-specific lectin PP2-like protein
Cit.13019.1.S1_at	5.123	0.00651	AAD50032	9.00E-65	SRG1
Cit.11563.1.S1_at	4.995	0.03795	A54523	7.00E-06	Histidine-rich protein
Cit.11460.1.S1_at	4.933	0.00311	AAK37761	4.00E-83	Zinc transporter protein ZIP1
Cit.17464.1.S1_at	4.903	0.00898	NP_188746	2.00E-69	ABC transporter family protein
Cit.28009.1.S1_at	4.885	0.00409	NP_173510	1.00E-55	Phosphate transporter family protein
Cit.12040.1.S1_s_at	4.826	0.00252	AAM20235	5.00E-59	Putative metal-binding isoprenylated protein
Cit.5973.1.S1_s_at	4.808	0.03038	No hit	–	–
Cit.13036.1.S1_at	4.73	0.00368	BAB09229	6.00E-17	Unnamed protein product
Cit.18726.1.S1_at	4.689	0.00521	AAS76741	1.00E-18	Copper amine oxidase, putative
Cit.13018.1.S1_s_at	4.535	0.00655	AAD50032	1.00E-98	SRG1
Cit.9300.1.S1_s_at	4.524	0.00069	BAA74434	3.00E-69	Very similar to SRG1
Cit.16636.1.S1_at	4.518	0.00867	BAA07324	2.00E-52	Ethylene-responsive element-binding protein

with mock-inoculated control. Citrus *GAPDH* gene was used as the reference gene. The absence of non-specific PCR products and primer dimers was confirmed by melting curve analysis and electrophoresis (data not shown). Before applying the comparative C_T method (Livak and Schmittgen 2001), we verified that the PCR efficiencies of the target and reference genes were approximately equal to 1(data not shown).

Interestingly, the transcript level of miraculin-like protein 1 was significantly upregulated ($P < 0.05$) by 4.48- and 3.77-fold in the HLB-diseased plants compared with the mock-inoculated controls at 5 and 17 WAI, respectively, at which time points no obvious HLB symptoms were observed. By contrast, no significant change of miraculin-like protein 1 transcript level was found at 27 WAI (Table 5), when the HLB-infected plants already displayed severe symptoms such as blotchy mottle and yellowing of the leaves. Remarkably, over 177-fold induction of Cu/Zn superoxide dismutase transcripts was detected at late stage (27 WAI) of HLB-diseased sweet orange; no significant change occurred at early stages including 5 and 17 WAI (Table 5). It is indicated that the transcriptional levels of miraculin-like protein 1 and Cu/Zn superoxide dismutase are upregulated at different stages of HLB infection, although their protein products both accumulate at advanced stage of disease development (7 months after inoculation, Table 2).

Discussion

Citrus HLB is a destructive citrus disease associated with the fastidious, phloem-limited bacterium, *C*. Liberibacter spp. Understanding host response to HLB could help to unravel its pathogenic processes and further develop novel control strategies to manage the disease. Although the changes of host gene expression to HLB infection have been reported at the transcriptional level (Albrecht and Bowman 2008, Kim et al. 2009), this study is the first attempt to investigate it at the protein level. As it has been shown that more differentially expressed transcripts were observed at late stage than early stage of HLB disease development (Albrecht and Bowman 2008), in this work, we chose to collect leaf samples at 7 months after inoculation representing advanced stage, so as to detect more proteomic changes. In total, 686 unique proteins were identified from mature leaves of mock-inoculated and HLB-diseased sweet orange. As a limited number of biological replicates were used in this experiment, we carefully evaluated the biological variations and estimated an optimal cut-off fold change in identification of differentially expressed proteins. On the basis of the good correlation coefficients of biological replicates and fold change cut-off value of 2.5, 10 proteins were found to be differentially expressed in HLB-diseased symptomless leaves. Again, similar changes of these 10 proteins were found in leaves with HLB symptoms. Seven out of the 10 shared proteins were highly induced

Table 4. Significantly changed pathways in HLB-diseased sweet orange based on transcriptome analysis. All the probe sets with $P \leq 0.05$ from the transcriptome data were analyzed by PageMan (Thimm et al. 2004) to determine significantly changed pathways in HLB-diseased sweet orange mature leaves, compared with mock-inoculated controls. Upregulated pathways are highlighted in grey. [a]Bins are a set of hierarchical functional categories as reported by Thimm et al. (2004). [b]Benjamini and Hochberg approach corrected P value (Benjamini and Hochberg 1995).

Bin[a]	Pathway	*P* value[b]
1.1.1	PS_lightreaction_photosystem II	0.012539
10	Cell wall	1.23*E* − 16
10.1	Cell wall_precursor synthesis	0.003701
10.5	Cell wall_cell wall proteins	1.99*E*-06
10.5.1	Cell wall_cell wall proteins_AGPs	5.72*E*-04
10.6	Cell wall_degradation	0.042916
10.6.3	Cell wall_degradation_pectate lyases and polygalacturonases	0.004526
10.7	Cell wall_modification	0.002788
10.8	Cell wall_pectin*esterases	0.04803
10.8.1	Cell wall_pectin*esterases_PME	0.048875
11	Lipid metabolism	2.62*E*-04
11.1	Lipid metabolism_FA synthesis and FA elongation	0.003372
11.8	Lipid metabolism_'exotics' (steroids, squalene, etc.)	0.017649
20	[illegible]	0.012539
20.1	[illegible]	2.62*E*-04
20.1.7	[illegible]	0.012539
26	Misc	0.003982
26.3	Misc_gluco-, galacto- and mannosidases	0.026255
26.4	Misc_beta-1,3 glucan hydrolases	0.01778
26.19	Misc_plastocyanin-like	0.025739
26.21	Misc_protease inhibitor/seed storage/lipid-transfer protein (LTP) family protein	0.03692
26.28	Misc_GDSL-motif lipase	0.002788
27	[illegible]	7.04*E*-08
27.3	[illegible]	1.73*E*-05
27.3.26	[illegible]	0.015875
27.3.32	[illegible]	4.97*E*-05
29.2.1.2	Protein_synthesis_ribosomal protein_eukaryotic	0.014187
29.5.1	protein_degradation_subtilases	0.003701
30.2.3	Signaling_receptor kinases_leucine rich repeat III	7.95*E*-06
30.2.12	[illegible]	0.021865
31	Cell	0.003701
31.1	Cell_organization	0.017882
34.19	Transport_major intrinsic proteins	0.042916
35	[illegible]	0.04469

and also involved in stress/defense responses, i.e. four miraculin-like proteins, chitinase, Cu/Zn superoxide dismutase and lipoxygenase (Table 2). Transcripts of miraculin-like protein, Cu/Zn superoxide dismutase and lipoxygenase also remarkably increased (Table 4).

Table 5. Transcriptional patterns of miraculin-like protein 1 and Cu/Zn superoxide dismutase at different time points after inoculation in HLB-diseased sweet orange plants, compared with mock-inoculated controls.

Gene	Week after inoculation	Fold change (HLB/control)	*P* value
Miraculin-like protein 1	5	4.48	0.0333
	17	3.77	0.0002
	27	1.28	0.3589
Cu/Zn superoxide dismutase	5	1.33	0.8209
	17	0.66	0.7463
	27	177.91	<0.0001

Miraculin, which can modify a sour taste into a sweet taste, is a plant glycoprotein first extracted from the miracle berry (*Richadella dulcifera*) (Brouwer et al. 1968). Recently, two distinct miraculin-like proteins, RlemMLP1 and RlemMLP2, were characterized in rough lemon (*Citrus jambhiri*) (Tsukuda et al. 2006). It was shown that they have protease inhibitor activities and are likely to be involved in defense response against pathogens. Their transcripts were not detected in leaves but increased to very high levels after wounding and inoculation with conidia of *Alternaria alternata* (Tsukuda et al. 2006). During the development of citrus sudden death (CSD) disease, a miraculin-like protein was suppressed in the bark of diseased-susceptible plants but not in the tolerant plants (Cantu et al. 2008). The authors suggested that the defense reactions were inhibited in susceptible citrus plants, highlighted by the suppression of miraculin-like protein and chitinases. Plant chitinases belong to PR protein families and are associated with disease resistance and systemic acquired resistance (Kasprzewska 2003, Ryals et al. 1996). It has been reported in citrus that the expression of chitinases can be induced by ultraviolet irritation, wounding and various treatments that elicit fruit pathogen resistance (Porat et al. 1999, 2001). Cu/Zn superoxide dismutase is a critical component of the active oxygen-scavenging system of plants and contributes to cellular defense against oxidative stress (Gupta et al. 1993). Modification of its expression in transgenic plants can improve plant stress tolerance (Gupta et al. 1993). Plant lipoxygenases have been implicated in responses to abiotic stresses, synthesis of abscisic acid or jasmonic acid and microbial attack (Brash 1999). Lipoxygenase transcript level and its enzymatic activity were induced by wounding and microbe attack in rough lemon leaves (Gomi et al. 2002). It might lead to the production of antimicrobial compounds, such as hydroperoxides and hydroxyl derivatives of linoleic and linolenic acids, or jasmonic acid, a signal compound involved in plant defense systems (Gardner 1991, Siedow 1991).

Both proteome and transcriptome data suggest that stress/defense response is activated in sweet orange affected by HLB, at least at the late stage of this disease development when severe symptoms were already expressed in diseased plants. Two differentially expressed stress-responsive proteins, miraculin-like protein 1 and Cu/Zn superoxide dismutase, were further investigated by qPCR at different stages of the HLB infection process. The results indicate that the transcriptional level of miraculin-like protein 1 tends to be elevated at early stages of HLB disease development, while the Cu/Zn superoxide dismutase transcripts accumulate at advanced stages.

In conclusion, global analysis of protein profiles using iTRAQ technology has succeeded in identifying 20 and 10 differentially expressed proteins, respectively, in leaves with and without symptoms from HLB-diseased sweet orange. Interestingly, all the differentially expressed proteins identified in symptomless leaves were also found to have similar changes in leaves with symptoms. These proteins may be good candidates for biomarkers to identify HLB-diseased plants prior to the expression of typical symptoms. However, similar changes of protein expression may be caused by other abiotic stresses such as wounding and pathogen infections other than *C*Las. Thus, it may be inappropriate to use them as biomarkers individually, but it may be possible to develop an HLB-specific protein profile with the combined changes of these proteins.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Total proteins identified from control and HLB-diseased mature leaves (unused ProtScore >1.3).

Table S2. Differentially expressed proteins in HLB-affected sweet orange mature leaves compared with healthy controls.

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