Comparative Transcriptional and Anatomical Analyses of Tolerant Rough Lemon and Susceptible Sweet Orange in Response to ‘*Candidatus Liberibacter asiaticus*’ Infection

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Although there are no known sources of genetic resistance, some *Citrus* spp. are reportedly tolerant to huanglongbing (HLB), presumably caused by ‘*Candidatus Liberibacter asiaticus*’. Time-course transcriptional analysis of tolerant rough lemon (*Citrus jambhiri*) and susceptible sweet orange (*C. sinensis*) in response to ‘*Ca. L. asiaticus*’ infection showed more genes differentially expressed in HLB-affected rough lemon than sweet orange at early stages but substantially fewer at late time points, possibly a critical factor underlying differences in sensitivity to ‘*Ca. L. asiaticus*’. Pathway analysis revealed that stress responses were distinctively modulated in rough lemon and sweet orange. Although microscopic changes (e.g., callose deposition in sieve elements and phloem cell collapse) were found in both infected species, remarkably, phloem transport activity in midribs of source leaves in rough lemon was much less affected by HLB than in sweet orange. The difference in phloem cell transport activities is also implicated in the differential sensitivity to HLB between the two species. The results potentially lead to identification of key genes and the genetic mechanism in rough lemon to restrain disease development and maintain (or recover) phloem transport activity. These potential candidate genes may be used for improving citrus tolerance (or even resistance) to HLB by genetic engineering.

Citrus huanglongbing (HLB) or citrus greening is a highly destructive disease in many citrus production areas worldwide. It was first found in China in the early 20th century (Zhao 1981) and recently discovered in Brazil and Florida (United States) (Bove 2006), and threatens the future viability of the citrus industries. This disease is presumably caused by ‘*Candidatus Liberibacter* spp.’, a phloem-inhabiting α -proteobacteria, and is transmitted by two kinds of phloem-feeding citrus psyllids, *Diaphorina citri* and *Trioza erytreae*. HLB results in a range of symptoms that vary with citrus varieties and species,

including yellow shoots, blotchy mottled leaves, lopsided fruit, and severe tree decline. All known citrus species and cultivars can be affected by HLB. However, host response to infection and development of symptoms in greenhouse conditions or in the field are significantly different among the various citrus species (Folimonova et al. 2009; Koizumi et al. 1993), and some citrus species and relatives were considered tolerant to this disease. Generally, sweet orange and grapefruit are very susceptible; some lemon and lime display tolerance, evidenced by less severe symptoms and much slower or no decline in both greenhouse and field plantings; and *Poncirus trifoliata* appears to be most tolerant to HLB; however, the work supporting the concept was not reproducible (Folimonova et al. 2009).

Disease symptom development is considered to be the consequence of molecular, cellular, and physiological changes and defense responses in a plant host interacting with a pathogen (O’Donnell et al. 2003). In incompatible plant–microbe interactions, defense responses include hypersensitive reaction resulting in localized cell death, cell wall modifications, and synthesis of plant defense molecules (Hammond-Kosack and Jones 1996). In contrast, suppression of host responses such as basal defenses is crucial for pathogenesis in compatible microbe–plant interactions (Nomura et al. 2005). Understanding host response following ‘*Candidatus Liberibacter asiaticus*’ inoculation and subsequent progression of HLB could aid the development of new strategies to control the disease. Two research groups have reported on the global gene expression changes of sweet orange in response to ‘*Ca. L. asiaticus*’ infection, showing that genes involved in a number of processes such as cell defense, photosynthesis, and carbohydrate metabolism were differentially expressed (Albrecht and Bowman 2008; Kim et al. 2009). Albrecht and Bowman (2008) conducted experiments covering the early and late time points of HLB infection but they applied a pooling strategy to the RNA samples, which might result in loss of important gene expression information and increase of false positives (Affymetrix 2004). Kim and associates (2009) only investigated the transcriptional profile at a late stage of the disease development. A recent study reported the transcriptional changes in tolerant US-897 (*Citrus reticulata* Blanco $\times$ *P. trifoliata* L. Raf.) and susceptible ‘Cleopatra’ mandarin in response to the infection of ‘*Ca. L. asiaticus*’ only at an advanced stage of HLB disease development, when symptoms were fully expressed (Albrecht and Bowman 2011b). Because US-897 is an intergeneric hybrid

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of *Citrus* spp. and *Poncirus* spp., comparing host responses throughout a time course of ‘*Ca. L. asiaticus*’ infection between two more closely related *Citrus* varieties should provide a more complete understanding of the disease mechanisms in cultivated *Citrus* spp. and may lead to a strategy to manage the disease by increasing such tolerance in highly susceptible cultivars through adjusted horticultural practices or genetic modifications.

Rough lemon is considered tolerant to HLB, compared with sweet orange and other highly susceptible varieties. Once infected and symptomatic, rough lemon can be rejuvenated by continual growth of new shoots with few or no foliar symptoms and repeat in many growing seasons, whereas sweet orange exhibits continuous quick growth retardation and eventual die back. US-897 is tolerant to the infection of ‘*Ca. L. asiaticus*’ based on the observation that it exhibits no distinct disease symptoms associated with HLB, except for few foliar symptoms in a small number of plants at later stages of the disease (Albrecht and Bowman 2011a). The underlying mechanisms of recovery in ‘*Ca. L. asiaticus*’-infected rough lemon may be quite different from the tolerant response of US-897 (Albrecht and Bowman 2011b).

The objective of this study was to compare host responses to ‘*Ca. L. asiaticus*’ between highly susceptible sweet orange (*C. sinensis* L. Osbeck) and tolerant rough lemon (*C. jambhiri* Lush.) throughout the time course from inoculation to full dis-

ease expression. Microarray analysis was carried out at 5, 17, and 27 weeks after inoculation (WAI) and at the time immediately before inoculation using the Affymetrix Citrus GeneChip. Phloem ultrastructure and phloem loading of rough lemon and sweet orange in response to ‘*Ca. L. asiaticus*’ infection were also compared. These results will enhance understanding of HLB susceptibility and tolerance mechanisms in citrus, and lead to the development of new tools to improve citrus tolerance, possibly even to develop resistance, to this disease.

RESULTS

‘*Ca. L. asiaticus*’ detection and HLB symptom development.

‘*Ca. L. asiaticus*’ detection was conducted by quantitative real-time polymerase chain reaction (PCR). Because ‘*Ca. L. asiaticus*’ is unevenly distributed in citrus (Teixeira et al. 2008), it is difficult to judge which plant contains more ‘*Ca. L. asiaticus*’. Therefore, we used the quantitative real-time PCR results (cycle threshold [C_T] values) as qualitative data to confirm whether rough lemon and sweet orange plants were infected with ‘*Ca. L. asiaticus*’ (i.e., whether ‘*Ca. L. asiaticus*’ positive or not). In the three graft-inoculated rough lemon plants selected for microarray analysis, ‘*Ca. L. asiaticus*’ was detected by quantitative real-time PCR around 20 WAI. In the three selected graft-inoculated sweet orange plants, one was confirmed to be ‘*Ca. L. asiaticus*’ positive around 20 WAI and

Fig. 1. Typical leaf symptoms of huanglongbing (HLB)-affected rough lemon and sweet orange around 27 weeks after inoculation (WAI). **A**, Branch from mock-inoculated rough lemon; **B**, branch from HLB-affected rough lemon showing disease-related symptoms on mature leaves and no symptoms on young leaves; **C**, branch from mock-inoculated sweet orange; **D**, branch from HLB-diseased sweet orange showing disease-related symptoms.

the other two at 27 WAI. No HLB-specific symptoms were observed before 17 WAI for rough lemon and sweet orange; typical symptoms such as blotchy leaf mottle and yellowing emerged around 20 WAI and developed toward 27 WAI. Afterward, with severe and obvious blotchy mottle symptoms on mature and old leaves of sweet orange plants, their growth was significantly inhibited, rarely putting out new shoots toward the end of the experiment; however, rough lemon plants, without obvious inhibition of plant growth, kept flushing new shoots with few or no symptoms, although blotchy mottle was commonly found on mature and old leaves (Fig. 1). No '*Ca. L. asiaticus*' was detected in mock-inoculated rough lemon or sweet orange throughout the experiment.

Overview of microarray analysis.

The genome-wide Affymetrix citrus microarray, containing 33,879 citrus transcripts, was used to compare the global gene expression profiles of rough lemon and sweet orange representing tolerant and susceptible citrus, respectively, in response to '*Ca. L. asiaticus*' infection. Time-course experiments, including three time points (5, 17, and 27 WAI) for each species, were conducted. Log₂-fold change of gene expression ('*Ca. L. asiaticus*' inoculated versus mock inoculated) of each probe was obtained. The numbers of differentially expressed probes were displayed under two significance levels ($P \leq 0.05$ or false discovery rate [FDR] ≤ 0.05), respectively, in combination with a cutoff value of twofold change (i.e., log₂ ratio ≥ 1 or log₂ ratio ≤ -1) (Fig. 2). There were 683, 411, and 1,055 probe sets in rough lemon and 181, 1,037, and 3,476 probe sets in sweet orange differentially expressed ($P \leq 0.05$) at 5, 17, and 27 WAI, respectively. Under more stringent criteria (i.e., FDR ≤ 0.05), fewer differentially expressed probe sets were found (Fig. 2B). No significant changes of gene expression were displayed at 5 WAI for either rough lemon or sweet orange data sets (FDR $\leq$

0.05). Only 1 probe set (Cit.39005.1.S1_at) was downregulated (FDR ≤ 0.05) at 17 WAI in sweet orange but 207 probe sets showed changed expression levels at 17 WAI in rough lemon. Overall, upregulation predominated downregulation along with time for both species, except at 5 WAI in rough lemon ($P \leq 0.05$) (Fig. 2A). At 27 WAI, many more genes were differentially expressed in sweet orange than in rough lemon (Fig. 2).

Comparison of gene expression between rough lemon and sweet orange plants immediately before grafting inoculation was also conducted. In total, 351 genes were differentially expressed (fold change ≥ 2 , $P \leq 0.05$) in rough lemon compared with sweet orange independent of '*Ca. L. asiaticus*' infection (Supplementary Table S1). Among them, only 30 genes had change of expression $\geq$ fourfold. The transcriptional levels of 13 genes were more than fourfold higher in rough lemon compared with sweet orange. However, none of these genes was found to be modulated by '*Ca. L. asiaticus*' infection in either rough lemon or sweet orange (Supplementary Table S2). Among the 17 genes with higher basal expression in sweet orange compared with rough lemon, the most noteworthy was a gene coding for GAST1 protein homolog 1 (GASA1). It was more than 15-fold abundant in sweet orange than in rough lemon, and was induced by more than 11-fold in response to '*Ca. L. asiaticus*' infection in sweet orange (27 WAI) but not changed in '*Ca. L. asiaticus*'-inoculated rough lemon. Four genes with unknown functions (Cit.842.1.S1_at, Cit.8198.1.S1_x_at, Cit.17803.1.S1_at, and Cit.20194.1.S1_at) shared a similar trend of expression with GASA1. They may be potential HLB-susceptible genes.

Significantly HLB-modulated pathways at each time point.

To provide a comparative overview of the responses to '*Ca. L. asiaticus*' in rough lemon and sweet orange across time points, a condensed heat-map-like display of changed pathways was generated by PageMan (Usadel et al. 2006). Here, the expression data of probe sets with $P \leq 0.05$ at each time point were used. These data files can be found in Supplementary Tables S3 to S8. The pathways showing qualitatively different or similar regulations in the two species across time points were revealed by visual inspection (Fig. 3). Pathways related to photosynthesis and response to heat stress were downregulated in both infected rough lemon and sweet orange compared with their own controls, although these changes were not shown at the same time points in the two species. It could be inferred that photosynthesis in HLB-affected citrus is inhibited to some extent, which agrees with the suggestion of Albrecht and Bowman (2008). However, many other processes were distinctively and differentially modulated by '*Ca. L. asiaticus*' infection in rough lemon and sweet orange. Notably, genes involved in cell wall-related pathways (e.g., cell wall pectin esterases) were mostly downregulated at the early stage (5 WAI) of infection in rough lemon and upregulated at the late stage (27 WAI); in contrast, a generally opposite trend was observed in the '*Ca. L. asiaticus*'-sweet orange interaction (Fig. 3). Different transcriptional changes were also observed in other gene functional categories (e.g., lipid metabolism, β -1,3-glucan hydrolases, and GDSL-motif lipases). These differences were highlighted at 27 WAI, when typical HLB symptoms were already expressed in diseased plants. Interestingly, significant upregulation of both pathogenesis-related (PR) proteins and WRKY domain transcription factors were only observed at 27 WAI in sweet orange, indicating a strong stress response. Moreover, hormone metabolisms were differentially modulated in rough lemon and sweet orange (Fig. 3). It is noteworthy that, at the early time point of infection, genes related to abscisic acid metabolism showed coordinated upregulation in diseased rough lemon, whereas genes involved in the ethylene pathway

Fig. 2. Number of differentially expressed genes at each time point in the '*Candidatus Liberibacter asiaticus*'-inoculated rough lemon and sweet orange versus their own mock-inoculated controls (fold change ≥ 2) at the significant levels of **A**, $P \leq 0.05$ or **B**, false discovery rate (FDR) ≤ 0.05 . WAI = weeks after inoculation.

Fig. 3. Comparative PageMan display of modulated pathways in huanglongbing (HLB)-affected rough lemon and sweet orange. The log₂-fold change of gene expression ('*Candidatus* Liberibacter asiaticus'-inoculated plants versus mock-inoculated controls) was input into PageMan and subjected to a Wilcoxon test. Results were shown as a false-color heat-map-like display. Pathways significantly upregulated are colored in red, while those colored in green are significantly downregulated. Pathways without significant changes are white. Names of pathways of interest are indicated on the right panel. RL5WAI, RL17WAI, and RL27WAI indicate 5, 17, and 27 weeks after inoculation (WAI) of rough lemon; SO5WAI, SO17WAI, and SO27WAI indicate 5, 17, and 27 WAI of sweet orange. PS, photosynthesis; CHO, carbohydrate.

were mostly upregulated in infected sweet orange. These results suggest that rough lemon and sweet orange likely possess distinctive mechanisms in response to '*Ca. L. asiaticus*' infection. Other significantly changed functional gene categories, such as

carbohydrate metabolism, signaling, protein, and transport, were also observed. A complete list of HLB-modulated pathways for both rough lemon and sweet orange data sets was provided in Supplementary Table S9.

Fig. 4. Display of huanglongbing (HLB)-modulated stress responses at 27 weeks after inoculation (WAI) of **A**, rough lemon and **B**, sweet orange using data sets. The $\log_2$ -fold change of gene expression ('*Candidatus Liberibacter asiaticus*'-inoculated plants versus mock-inoculated controls) was analyzed using MapMan. Small squares colored in red and green represent genes up- and downregulated, respectively. A false-color scale was used and all the values were given on a $\log_2$ scale. Color saturates at a fourfold change (i.e., $\log_2$ ratio = 2 or -2). Red boxes and arrows indicate the upregulated processes and genes while green ones indicate the downregulated processes and genes.

Differential stress responses of rough lemon and sweet orange to '*Ca. L. asiaticus*' infection.

Stress-response-related pathways, such as cell wall, hormone metabolism, and transcription factor families, displayed differential responses in HLB-affected rough lemon and sweet orange plants (Fig. 3). The differentially expressed genes associated with stress responses were mapped into the Biotic Stress Pathway using MapMan software (Thimm et al. 2004).

Cell wall metabolism was one of the most dramatically altered pathways in both HLB-affected rough lemon and sweet orange (Fig. 4; Supplementary Fig. S1). In rough lemon, 66, 23, and 74 cell wall-related genes were differentially expressed at 5, 17, and 27 WAI, respectively. Most cell wall-related genes were downregulated at the early time point of '*Ca. L. asiaticus*' infection (5 WAI), whereas expression levels of a large number of cell wall genes were elevated toward 27 WAI. It is

very interesting that an opposite trend was observed in sweet orange (i.e., coordinated upregulation at the beginning of '*Ca. L. asiaticus*' infection but downregulation at the late stage [27 WAI]). The expression data were presented in Supplementary Table S10. These modulated genes were categorized into a wide range of cell wall processes such as cell wall modification, cell wall proteins, cell wall pectin esterases, precursor synthesis, cellulose synthesis, and cell wall degradation. It is indicated that cell wall components and structures are likely undergoing dramatic changes in HLB-affected citrus. Expression levels of many cell wall genes were significantly altered as early as 5 WAI in rough lemon but not until 17 WAI in sweet orange, suggesting that earlier cell wall responses to '*Ca. L. asiaticus*' bacterium occurred in the former.

The most remarkable change of transcriptional profiling occurred at 27 WAI for both rough lemon and sweet orange,

Table 1. Huanglongbing (HLB)-modulated cell wall-associated genes at 27 weeks after inoculation (WAI) of both rough lemon and sweet orange data sets

Gene product	27 WAI ^a		AGI ^b
	Rough lemon	Sweet orange	
Cell-wall precursor synthesis			
UDP-glucose 6-dehydrogenase	1.09	-1.83	AT5G15490
dTDP-glucose 4,6-dehydratase	0.83	-2.64	AT3G62830
UDP-L-rhamnose synthase/UDP-glucose 4,6-dehydratase	0.79	-2.39	AT1G78570
Cell-wall cellulose synthesis			
Cellulose synthase-like C5	1.7	-3.95	AT4G31590
Cellulose synthase-like A9	1.2	-2.49	AT2G21770
Cellulose synthase-like D3	0.71	-1.63	AT3G03050
Cellulose synthase-like D5	1.13	-1.43	AT1G02730
Cellulose synthase-like B4	-0.79	0.72	AT2G32540
Cellulose synthase-like B2	-0.63	0.84	AT2G32620
Cellulose synthase-like G2	1.12	2.19	AT4G24000
Cell expansin protein COB (COBRA)	1.03	-1.41	AT5G60920
Cell-wall hemicellulose synthesis			
Exostosin family protein	-0.46	-0.71	AT5G16890
Cell-wall proteins			
Fasciclin-like arabinogalactan 1	1.95	-2.49	AT5G55730
Fasciclin-like arabinogalactan 9	1.98	-1.98	AT2G14890
Fasciclin-like arabinogalactan 10	2.55	-1.3	AT3G60900
Arabinogalactan 19	2.64	0.91	AT1G68725
Extensin family protein	1.72	-3.44	AT4G29240
Extensin 3	1.92	-1.33	AT1G21310
Proline-rich extensin-like family protein	-0.54	-0.54	AT2G43150
Reversibly glycosylated polypeptide 1	0.81	-1.79	AT3G02230
Reversibly glycosylated polypeptide, putative	0.52	-0.91	AT5G16510
Reversibly glycosylated polypeptide 3	0.83	-0.87	AT3G08900
Cell-wall degradation			
Glycosyl hydrolase family 3 protein	-0.56	0.53	AT3G47040
Cellulase 5	1.68	0.97	AT1G22880
Glycoside hydrolase family 28 protein	1.75	-3.37	AT3G61490
Polygalacturonase, putative	2.65	-3.01	AT1G70370
Glycoside hydrolase family 28 protein	0.94	-1.51	AT3G62110
Pectate lyase family protein	1.32	-1.17	AT3G24670
Polygalacturonase	3.56	-0.43	AT1G23760
Cell-wall modification			
Xyloglucan endotransglucosylase 9	1.41	-3.98	AT4G03210
Xyloglucan endotransglucosylase 33	2.13	-2.84	AT1G10550
Expansin A4	1.03	-2.32	AT2G39700
Xyloglucan:xyloglucosyl transferase	0.56	-1.44	AT5G57560
Xyloglucan endotransglucosylase 6	0.69	-1.3	AT4G25810
Xyloglucan endotransglucosylase 27	0.54	-0.78	AT2G01850
Xyloglucan endotransglucosylase related 2	-0.46	1.2	AT1G14720
Cell-wall pectin esterases			
Enzyme inhibitor/pectinesterase	1.3	-2.38	AT1G11580
PME3 pectinesterase	2.15	-2.27	AT3G14310
PME1 pectinesterase	1.51	-1.74	AT1G53840
Pectinacetyltransferase, putative	1.44	-2.16	AT5G26670

^a Log₂-fold change of gene expression ('*Candidatus Liberibacter asiaticus*' versus mock-inoculated control) at 27 WAI of rough lemon and sweet orange.

^b *Arabidopsis* Genome Initiative (AGI) numbers of *Arabidopsis* orthologs of HLB-modulated citrus genes. The differentially expressed probe sets from the Affymetrix Citrus GeneChip were annotated based on the closest hits in the *Arabidopsis* genome. Note that different probes can be matched to the same gene. In this case, the expression data of these probes were averaged.

when '*Ca. L. asiaticus*' bacterium was detected and HLB symptoms appeared (Fig. 4). The results showed that 40 differentially expressed cell wall-associated genes were observed at 27 WAI in both data sets (Table 1). Of these, most genes increased their expression in affected rough lemon but were downregulated in diseased sweet orange. For instance, a group of genes encoding for arabinogalactan proteins (AGP) and xyloglucan endotransglycosylases (XET) were significantly induced or suppressed by more than twofold in HLB-affected rough lemon and sweet orange, respectively, compared with their own mock-inoculated controls.

The transcriptional change of an important large enzyme family, β -1,3-glucanase, had a similar trend with the cell wall-associated genes in both data sets (Fig. 4). This is expected because β -1,3-glucanase is closely related to cell wall metabolism. Few changes of β -1,3-glucanase transcripts were observed at 5 and 17 WAI; dramatic changes appeared at 27 WAI, at which time both '*Ca. L. asiaticus*'-inoculated rough lemon and sweet orange expressed leaf yellowing and blotchy mottle symptoms. In all, 15 β -1,3-glucanase genes showed upregulation at 27 WAI in diseased rough lemon; in contrast, the transcripts of 10 β -1,3-glucanase decreased by two- to 10-fold in infected sweet orange compared with the mock-inoculated control (Fig. 4).

PR proteins play an important role in plant defense against pathogens. However, no significant induction of PR transcripts was observed at early stages (5 and 17 WAI) in either rough lemon- or sweet orange-'*Ca. L. asiaticus*' interactions. By contrast, a disease resistance family protein and a trypsin and protease inhibitor family protein or Kunitz family protein were repressed by nearly four- and eightfold, respectively, at 5 WAI of infected rough lemon compared with the mock-inoculated control. At 27 WAI, more PR genes were modulated in both infected rough lemon and sweet orange (Fig. 4). Among the 17 modulated PR genes in rough lemon, only three disease resistance-responsive genes and a trypsin and protease inhibitor family gene or Kunitz family gene were significantly upregulated (fold change ≥ 2). At 27 WAI in sweet orange, 14 of the 32 differentially expressed PR genes were induced by two- to eightfold (Fig. 4). These genes mostly belong to the coiled-coil nucleotide-binding site leucine-rich repeat (NBS-LRR) class and Toll interleukin-1 receptor NBS-LRR class of disease resistance gene families. Nevertheless, several PR genes were downregulated (e.g., an acidic endochitinase gene and a disease resistance gene were repressed by 6.1- and 3.6-fold, respectively). Intriguingly, a resistance (R)-gene-mediated disease resistance pathway was turned on at 27 WAI in sweet orange (Fig. 4). Upstream of the defense genes, transcripts of genes homologous to *Arabidopsis* WRKY19 transcription factor, AtRLP33, EDS1, and a transmembrane receptor were induced by more than twofold (Fig. 4).

In addition, a set of MYB-related transcription factor genes displayed significant upregulation at 27 WAI of infected sweet orange (Fig. 4B). For instance, citrus orthologs of *Arabidopsis* LH1-, RVE1-, and CCA1-like transcription factor genes were induced by two- to fourfold. However, they were downregulated in infected rough lemon at both 17 and 27 WAI (Fig. 4A). MYB transcription factors play important roles in many physiological processes, such as light signaling pathway (Rawat et al. 2009) and stress response (Nagaoka and Takano 2003; Raffaele et al. 2008; Vaillau et al. 2002). Specifically, LH1-, RVE1-, and CCA1-like transcription factors play critical roles in regulating circadian rhythms (Rawat et al. 2009; Schaffer et al. 1998; Wang and Tobin 1998). Altered expression of these genes can cause abnormal circadian rhythms which, in turn, result in disorders of clock-regulated pathways, such as photosynthesis, transport of sugars, and starch metabolism

(Harmer et al. 2000). Interestingly, repression of photosynthesis pathway genes and disorders of carbohydrate metabolism (genes and metabolites) have been observed in HLB-affected sweet orange (Albrecht and Bowman 2008; Fan et al. 2010, 2011). It is suggested that the transcriptional changes of these MYB-related transcription factors may correlate to the interference of photosynthesis and carbohydrate metabolism in '*Ca. L. asiaticus*'-infected plants.

Validation of microarray data by quantitative reverse-transcription PCR.

To validate the microarray data using quantitative reverse-transcription (RT) PCR (qPCR), 18 genes distributed among different biological processes were selected, especially pathways significantly modulated in HLB disease progression such as carbohydrate metabolism, cell wall metabolism, or stress response. In total, 54 data points from different time points from both rough lemon and sweet orange data sets were included. The detailed information and expression data of the selected genes were presented in Supplementary Table S11. The $\log_2$ ratio values from qPCR were plotted against $\log_2$ ratio values from the microarray analysis. A high correlation ($R^2 = 0.8$) was obtained (Supplementary Fig. S2). Additionally, 50 of the 54 data points showed the same tendency of transcriptional modulation between the two methods. These results confirmed the reliability and accuracy of the microarray data in this study.

Changes of phloem ultrastructure and phloem loading activity in HLB-affected rough lemon and sweet orange.

Phloem plugging is considered to be a major factor inhibiting the transport of photosynthate in HLB-affected citrus plants and leads to starch accumulation in leaves and eventual tree decline (Achor et al. 2010; Etxeberria et al. 2009; Kim et al. 2009; Koh et al. 2012). Callose and phloem protein 2 are the two types of reported plugging materials deposited in the sieve elements of HLB-affected sweet orange leaves (Achor et al. 2010). However, thus far, callose deposition has not been demonstrated in the phloem of HLB-affected rough lemon.

A callose synthase (CALS) gene homologous to *Arabidopsis* CALS1 (AT1G05570) was upregulated by 1.8- and 5.4-fold ('*Ca. L. asiaticus*' inoculated versus mock inoculated) at 27 WAI in rough lemon and sweet orange, respectively. It is suggestive that callose may also accumulate in the phloem tissue of infected rough lemon, though perhaps at a lesser rate. Observed under transmission electron microscopy (TEM), obvious accumulation of some smooth materials (presumed callose) was observed in the sieve elements of diseased rough lemon leaves with symptoms, compared with the basic level of callose formation in the mock-inoculated healthy controls (Fig. 5A and C). Moreover, collapse of phloem cells and starch accumulation can also be seen in the midribs of rough lemon symptomatic leaves, as well as in the same type of tissue from sweet orange (Fig. 5). In the HLB-affected but symptomless leaves, callose accumulation was sometimes found in a few sieve elements (by observing multiple sections) of both rough lemon and sweet orange samples but no phloem cell collapse was observed (Fig. 5B and E). These results indicate that the phloem ultrastructure of leaf midribs in '*Ca. L. asiaticus*'-infected rough lemon was affected as much as in infected sweet orange, and the transportation of photosynthate in phloem may be interrupted.

Detached leaves treated with a phloem tracer, 5,6-carboxy-fluorescein diacetate (5,6-CFDA), were used to confirm the functionality of phloem loading and transport of in '*Ca. L. asiaticus*'-infected rough lemon and sweet orange. The green fluorescence of 5,6-CFDA spread all over the midribs and minor

veins of healthy leaves from mock-inoculated rough lemon and sweet orange plants (Fig. 6); by contrast, a large amount of 5,6-CF was deposited at the treated sites of HLB-affected symptomatic leaves, indicating an inhibition of phloem loading in both diseased rough lemon and sweet orange leaves. Surprisingly, the phloem transportation in the midribs of HLB-affected leaves revealed an obvious difference between diseased rough lemon and sweet orange. The fluorescence was clearly detected in the midribs of diseased rough lemon leaves, including leaves with and without symptoms (Fig. 6A). However, it was barely detected in the midribs of symptomatic sweet orange leaves; also, the fluorescence signal was much weaker in the midribs of leaves without symptoms than that in healthy controls (Fig. 6B). It was indicated that phloem loading and transport is severely interrupted in HLB-affected sweet orange but is substantially less affected in diseased rough lemon. The sustained transport activity in rough lemon phloem may be a significant explanation for the differences in disease progression and host growth between rough lemon and sweet orange.

DISCUSSION

Citrus species have shown significant differences in disease symptom development, growth rate, and survival period when infected with '*Ca. L. asiaticus*' but little is known at the genomic levels about these phenomena. It is critical to investigate and understand the mechanisms from all possible perspectives underlying the differences between susceptible and tolerant types, to facilitate the development of tolerant or even

resistant varieties, or to adjust horticultural management to promote such tolerance. Prior to an ultimate solution, such adjustment to prolong the productive life of HLB-affected citrus trees would be useful to the industry; currently, many citrus growers in Florida have approached HLB management by enhanced fertilization applications, which have been successful to some degree, though the mechanism through which this occurs is currently unknown. Comparisons were made of the transcriptional changes of tolerant rough lemon and susceptible sweet orange after inoculation with '*Ca. L. asiaticus*' and during the subsequent disease development. It was shown that differentially expressed genes tended to be turned on or off earlier in infected rough lemon than in infected sweet orange, although a much more robust change in gene expression at 27 WAI was indicated in sweet orange than in rough lemon (Fig. 2). The contrast in gene expression responses between the two varieties likely indicates that timing of changes in expression patterns may be a critical factor for their differences in susceptibility (sensitivity) to the disease. Together with the results of anatomical analysis, the mechanisms of rough lemon tolerance and sweet orange susceptibility are discussed below.

Possible tolerance mechanisms of rough lemon to HLB.

Although callose-plugged phloem sieve elements and inhibition of phloem loading were observed in both HLB-diseased rough lemon and sweet orange leaves, phloem transport was less affected in the former than in the latter (Fig. 5). Less inhibition of phloem transport activities in symptomatic mature leaves of infected rough lemon may be due to formation of

Fig. 5. Transmission electron micrographs of cross sections of leaf midribs from rough lemon and sweet orange (displaying the phloem area). **A, B, and C,** Midribs from healthy leaves of mock-inoculated plants, leaves without symptoms, and leaves with symptoms from huanglongbing (HLB)-affected plants of rough lemon, respectively. **D, E, and F,** Midribs from healthy leaves of mock-inoculated plants, leaves without symptoms, and leaves with symptoms from HLB-affected plants of sweet orange, respectively. **G,** Enlarged view of representative sieve element in midribs of mock-inoculated rough lemon or sweet orange; **H and I,** enlarged view of plugged sieve elements in midribs from HLB-affected rough lemon and sweet orange, respectively. Ca, callose (presumed); SE, sieve element; St, starch; *, phloem cell collapse. Bars = 2 μ m (A–C, E, F, and H) and 500 nm (D, G, and I).

new functional phloem tissues, implicated by the upregulation of cell wall biosynthesis genes (Fig. 4). Interestingly, the root systems of diseased rough lemon plants in the greenhouse looked similar to those of the mock-inoculated plants 1 year after inoculation, whereas the root systems of diseased sweet orange plants had degraded substantially (J. Fan, C. Chen, R. H. Brlansky, F. G. Gmitter, Jr. *unpublished data*). It is likely that photosynthate was still transported in the phloem of affected rough lemon leaves to sink organs such as roots.

β -1,3-Glucanase can degrade callose (β -1,3-glucan) and is suggested to be important in regulating callose deposition during pathogenesis (Beffa et al. 1996). A group of β -1,3-glucanase genes was upregulated similarly at the late stage of HLB-affected rough lemon (Fig. 4) which, taken together with lesser upregulation of a CALS gene homologous to *Arabidopsis CALS1* (AT1G05570), might result in decreasing the callose deposition to some extent in the phloem of diseased rough lemon plants. As a result, the consequence of phloem plugging can be less severe which, in turn, reduces the inhibition of phloem transport (Fig. 6). These factors might contribute to the recovery of infected rough lemon plants. By contrast, the considerable repression of β -1,3-glucanase genes observed in diseased sweet orange may lead to greater callose accumulation and, thus, severe interference with phloem transport. However, further research needs to be carried out to confirm the quanti-

tative relation between the callose deposition in sieve elements and the inhibition of phloem transport. Meanwhile, the other type of phloem plugging material, PP2 protein, should be qualitatively and quantitatively compared between diseased rough lemon and sweet orange, because our results indicated that *pp2* gene expression was not significantly changed in HLB-affected rough lemon, though it was highly induced at 27 WAI in affected sweet orange. This result is similar to the findings of Albrecht and Bowman (2011b) that transcript levels for PP2 were not induced in tolerant US-897 in response to '*Ca. L. asiaticus*' infection but were dramatically elevated in susceptible Cleopatra mandarin.

AGP are a major class of structural proteins in the plant cell wall and play an important role in plant growth and development (Park et al. 2003). It is also suggested that AGP participates in stress-tolerance mechanisms, such as salt, water stress, and heat tolerance (Yamada et al. 2010). A fasciclin-like AGP (FLA) gene in *Arabidopsis*, *AtFLA11*, is likely involved in the formation of the secondary cell wall (Ito et al. 2005). XET is a key enzyme regulating cell wall expansion (Darley et al. 2001) and involved in many aspects of cell wall biosynthesis (e.g., the formation of secondary walls of xylem or phloem cells) (Bourquin et al. 2002). Secondary walls are essential for withstanding high turgor pressure in phloem sieve tubes. A set of genes encoding for cell wall protein AGP and XET were significantly induced at a late stage of HLB-affected rough lemon (Table 1), possibly leading to the change of secondary cell wall and the enhancement of tolerance to stresses such as high turgor pressure. Repression of these genes at a late stage of infected sweet orange may decrease the host ability to adapt to the '*Ca. L. asiaticus*'-altered cellular environment.

Altogether, maintaining or recovering of phloem transport activity and possible enhancement of stress tolerance may contribute to rough lemon tolerance to HLB. It likely represents a mechanism different from the HLB tolerance of US-897 that is suggested to be related to the high basal expression of defense-associated genes (Albrecht and Bowman 2011b), presumably derived from its *Poncirus* parent. It should be noted that trees of *Poncirus*, a sexually compatible genus related to *Citrus*, are deciduous and undergo winter dormancy, are relatively slower-growing, and exhibit several distinctive morphological differences in comparison with *Citrus* spp. Comparing two *Citrus* types much more closely related but with distinctive differences in HLB tolerance, such as rough lemon and sweet orange, highlights other additional host HLB-tolerance mechanisms that may be exploited for disease management.

Delayed defense response may contribute to HLB disease development of sweet orange.

Global change of gene expression can be observed in both compatible and incompatible plant-pathogen interactions (Espinoza et al. 2007; Gibly et al. 2004). A critical difference between the two interactions is that timely recognition of the invading pathogen and rapid and intense activation of plant defense response occur in the latter. For instance, *PR* genes are rapidly induced during an incompatible interaction in bacterial speck disease-resistant tomato plants (Jia and Martin 1999). At 27 WAI of '*Ca. L. asiaticus*' infection, an *R*-gene-mediated defense pathway, including a WRKY transcription factor, a mitogen-activated protein kinase RLP33, and many other defense-related genes, was switched on in infected sweet orange (Fig. 4). It suggests that sweet orange is able to activate its defense mechanism at the late stage of HLB infection, similar to the results reported by our previous work (Fan et al. 2011) and the work of Kim and associates (2009) and Albrecht and Bowman (2011b). In addition, our results showed that inoculation of rough lemon and sweet orange with '*Ca. L. asiaticus*' did not

Fig. 6. Transportation of carboxyfluorescein in source leaves of **A**, rough lemon and **B**, sweet orange affected by huanglongbing. Lane 1, healthy leaves (from mock-inoculated plants) treated with water; lane 2, healthy leaves (from mock-inoculated plants) treated with 5,6-carboxyfluorescein diacetate (5,6-CFDA); lane 3, leaves without symptoms from '*Candidatus Liberibacter asiaticus*'-positive plants confirmed by quantitative polymerase chain reaction [qPCR] treated with 5,6-CFDA; lane 4, leaves showing blotchy mottle symptoms (from '*Ca. L. asiaticus*'-positive plants confirmed by qPCR) treated with 5,6-CFDA; lane 5, leaves showing blotchy mottle symptoms (from '*Ca. L. asiaticus*'-positive plants) treated with water. Pictures in the upper panels of A and B were taken under a Dark Reader Transilluminator DR-88M (Clare Chemical Research Inc.), and those in the lower panels were taken under a Stemi SV11 Fluorescent Microscope (Carl Zeiss). The green fluorescence in the leaves represents carboxyfluorescein.

result in any considerable induction of defense genes at early stages (5 and 17 WAI), inferring that both rough lemon- and sweet orange-*'Ca. L. asiaticus'* interactions are compatible.

Activation of defense responses can lead to induction of programmed cell death and callose deposition which, in turn, prevents or slows the spread of pathogens. Nevertheless, our data demonstrated that the defense response of sweet orange was delayed until the late stage (27 WAI) of *'Ca. L. asiaticus'* infection, at which time the bacterium already reached high titer and likely spread throughout much of the plant's phloem system. Consequently, severe impairments of phloem ultrastructure and function such as phloem plugging (including callose and PP2 protein) and phloem necrosis occurred, as previously reported in HLB-diseased sweet orange (Achor et al. 2010; Etxeberria et al. 2009; Folimonova et al. 2009; Kim et al. 2009; Schneider 1967, 1968). In addition, loading and transport of photosynthate in the phloem of infected sweet orange were greatly inhibited (Fig. 6), which may lead to carbohydrate starvation of sink organs (e.g., roots) and quick tree decline (Etxeberria et al. 2009; Koh et al. 2012).

Conclusion.

Comparative transcriptional profiling results indicate that differential stress responses occur in rough lemon and sweet orange infected with *'Ca. L. asiaticus'*. Phloem transport activity in the midribs of leaves is considerably impaired in diseased sweet orange but much less significantly affected in infected rough lemon. The results highlight some underlying differences between tolerance and susceptibility mechanisms of divergent types of citrus to HLB disease. These results also provide potential candidate genes, such as those coding for cell wall proteins, β -1,3-glucanases, and GASA1, that might be manipulated for improving citrus tolerance or resistance to HLB by genetic engineering approaches.

MATERIALS AND METHODS

Plant material.

Two-year-old seedlings of rough lemon (*C. jambhiri* Lush.) and 'Madam Vinous' sweet orange (*C. sinensis* L. Osbeck) were graft-inoculated with bud wood from *'Ca. L. asiaticus'*-infected 'Carrizo' citrange (*C. sinensis* L. Osbeck $\times$ *P. trifoliata* L. Raf.) trees kept under greenhouse conditions to provide an inoculum source for various HLB-related experiments. For controls, the plants were grafted with bud wood from healthy Carrizo trees. All these plants were kept in a United States Department of Agriculture Animal and Plant Health Inspection Service and Center for Disease Control-approved and secured greenhouse at the University of Florida, Citrus Research and Education Center, Lake Alfred. Quantitative real-time PCR was performed to confirm the presence of *'Ca. L. asiaticus'* in the inoculum source and inoculated plants as described (Li et al. 2006). Fully expanded leaves were sampled separately from three *'Ca. L. asiaticus'*-inoculated plants and three mock-inoculated plants (used as controls) of each species at 5, 17, and 27 WAI. Note that fully expanded leaves with HLB-specific symptoms were collected from infected plants at 27 WAI, representing an advanced stage of HLB disease development. For comparison of basal gene expression between rough lemon and sweet orange, fully expanded leaves were sampled separately from three healthy rough lemon and three healthy sweet orange plants before graft inoculation. Leaves were immediately frozen in liquid nitrogen and stored at -80°C until use.

RNA extraction and microarray experiments.

Total RNA from each sample was extracted using the RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.) and contaminated

DNA was eliminated with the DNA-free Kit (Ambion, Austin, TX, U.S.A.), according to the manufacturer's instructions; elimination of DNA was confirmed by qPCR and gel electrophoresis. The concentration of RNA was measured in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.). RNA quality was evaluated using an Agilent Bioanalyzer Model 2100 (Agilent Technologies, Palo Alto, CA, U.S.A.).

Microarray analysis using the Affymetrix microarray platform was performed at the Interdisciplinary Center for Biotechnology Research Microarray Core, University of Florida, Gainesville. According to the manufacturer's instructions, microarray experiments were carried out using the GeneChip 3' IVT Express kit and the GeneChip Citrus Genome array (containing up to 33,879 citrus transcripts) (Affymetrix, Santa Clara, CA, U.S.A.). Forty-two arrays, representing three *'Ca. L. asiaticus'*-inoculated and three mock-inoculated (used as controls) individual biological replicates at 5, 17, and 27 WAI, and three individual biological replicates of baseline sample for each citrus species, were used in this study. Briefly, cDNA was synthesized from 250 ng of total RNA and template for in vitro transcription, during which a biotin-modified nucleotide was incorporated. The biotin-labeled amplified RNA was then purified and fragmented. Samples were hybridized with rotation at 4°C for 16 h to the Affymetrix GeneChip Citrus Genome Arrays. The arrays were washed and stained with the reagents supplied in GeneChip hybridization wash and stain kit (Affymetrix) on an Affymetrix Fluidics Station 450 and were scanned with a GeneChip 7G Scanner (Affymetrix).

Data analysis.

The array data were analyzed using BioConductor, 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 2004). FDR was generated by the Benjamini and Yekutieli approach (Benjamini and Yekutieli 2001). The microarray data are presented in accordance with the Minimum Information About a Microarray Experiment guidelines.

Due to the unavailability of citrus genome annotation at the time the data were analyzed, probe sets represented on the Affymetrix Citrus GeneChip were annotated by searching against the *Arabidopsis thaliana* genome via the HarVEST database. The corresponding *Arabidopsis* Genome Initiative numbers of *Arabidopsis* orthologs were used instead of probe ID numbers on the Affymetrix Citrus GeneChip for the following analyses. Differentially expressed probe sets ($P \leq 0.05$) were used to identify significantly changed pathways at each time point. Pathway analysis was performed using PageMan (Usadel et al. 2006), which is a built-in application in the free-downloadable software MapMan. A Wilcoxon test was applied and a statistics-based overview of changed pathways from global gene expression alterations was provided. The same data sets were also imported into MapMan to map the differentially expressed genes into specific pathways. It should be noted that multiple probe sets from citrus genome microarray may be annotated to the same gene from *Arabidopsis* genome. In this case, average expression data of these probe sets were used, since no duplicate gene can be imported into MapMan.

qPCR.

The same RNA samples used for microarray experiments were also used for qPCR. RT was performed with the QuantiTect Reverse Transcription Kit (Qiagen) according to the manu-

facturer'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 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.

qPCR was carried out in an Applied Biosystems 7500 Fast system (Applied Biosystems, Inc., Foster City, CA, U.S.A.) using a QuantiTect SYBR Green PCR Kit (Qiagen). A reaction mixture (20 µl) consisted of 2× QuantiTect SYBR Green PCR Master mix, 0.3 µM each primer, and 1 µl of the finished RT reaction. qPCR was started from one cycle at 95°C for 15 min, followed by 40 cycles at 94°C for 15 s, 58°C 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. In total, six replicates were used for each sample (three biological replicates × two technical replicates).

Glyceraldehyde 3-phosphate dehydrogenase was used as a reference gene to provide relative quantification for the target genes upon '*Ca. L. asiaticus*' infection (Mafra et al. 2012). Primer sequences were listed in Supplementary Table S12. PCR efficiencies of the reference and target genes were determined by generating standard curves based on serial dilutions of sweet orange genomic DNA, which was extracted from tender leaves using the cetyltrimethylammonium bromide method (Doyle and Doyle 1990). The amplification efficiency was automatically calculated using the 7500 Fast system SDS software. The relative quantification of gene expression level was determined by the comparative C_T method $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen 2001), where $\Delta\Delta C_T = (C_{T, \text{target}} - C_{T, \text{reference}})_{\text{HLB}} - (C_{T, \text{target}} - C_{T, \text{reference}})_{\text{Control}}$. Standard deviation and *P* value were calculated using a simple *t* test in SAS 9.1 (SAS Institute, Cary, NC, U.S.A.) as described by Yuan and associates (2006).

TEM and phloem loading assessment.

Fully expanded leaves were collected from the same set of plants used in microarray experiments. One year after inoculation, multiple leaves with and without symptoms were sampled from at least three '*Ca. L. asiaticus*'-infected plants; healthy leaves were sampled from mock-inoculated plants as controls. For TEM, leaf midribs were cut in pieces approximately 2 to 3 mm² and processed as described by Etteberria and associates (2009). Micrographs were taken with an AMT digital camera (Advanced Microscopy Techniques Corp., Danver, MA, U.S.A.) connected to a Morgagni 268 transmission electron microscope (FEI Company, Hillsboro, OR, U.S.A.).

Phloem-loading experiments were carried out with detached leaf samples based on the method of Roberts and associates (1997), with modifications. Briefly, approximately 1 cm² of the abaxial surface at two sites of each leaf was gently abraded with fine sandpaper, and 10 µl of 5,6-CFDA at 300 µg/ml was applied to each abraded area. Leaves treated with equal amounts of distilled water were used as controls for assessing tissue autofluorescence. After 24 h, the leaves were observed for fluorescence under a Dark Reader Transilluminator DR-88M (Clare Chemical Research Inc., Dolores, CO, U.S.A.), and photos were taken with a Nikon D80 digital camera (Nikon Corporation, Tokyo). Higher magnification pictures were made under a fluorescence stereomicroscope Stemi SV11 (Carl Zeiss, Oberkochen, Germany).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BioConductor database: www.bioconductor.org
 HarvEST database:
harvest-web.org/hweb/bin/chipsearch.wc?wsize=1259x608
 MapMan database: www.gabipd.org/projects/MapMan