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Transcriptional response of susceptible and tolerant citrus to infection with *Candidatus* Liberibacter asiaticus

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ABSTRACT

Candidatus Liberibacter asiaticus (Las), a non-culturable phloem-limited bacterium, is the suspected causal agent of huanglongbing (HLB) in Florida. HLB is one of the most devastating diseases of citrus and no resistant cultivars have been identified to date, though tolerance has been observed in the genus *Poncirus* and some of its hybrids. This study compares transcriptional changes in tolerant US-897 (*Citrus reticulata* Blanco × *Poncirus trifoliata* L. Raf.) and susceptible 'Cleopatra' mandarin (*C. reticulata*) seedlings in response to infection with *Las* using the Affymetrix GeneChip citrus array, with the main objective of identifying genes associated with tolerance to HLB. Microarray analysis identified 326 genes which were significantly upregulated by at least 4-fold in the susceptible genotype, compared with only 17 genes in US-897. Exclusively upregulated in US-897 was a gene for a 2-oxoglutarate (2OG) and Fe(II)-dependant oxygenase, an important enzyme involved in the biosynthesis of plant secondary metabolites. More than eight hundred genes were expressed at much higher levels in US-897 independent of infection with *Las*. Among these, genes for a constitutive disease resistance protein (CDR1) were notable. The possible involvement of these and other detected genes in tolerance to HLB and their possible use for biotechnology are discussed.

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1. Introduction

Citrus huanglongbing (HLB), also known as "citrus greening" is possibly the most destructive disease of citrus. It is distributed throughout most citrus producing countries worldwide [1], where it generates substantial economic losses in heavily affected areas. The suspected causal agent of HLB is a fastidious, phloem-limited bacterium of the genus *Candidatus* Liberibacter [2,3] which has not yet been obtained in pure culture. Three different bacterial species are associated with HLB in citrus: *Candidatus* Liberibacter asiaticus, found in all HLB-affected countries except Africa [1], *Candidatus* Liberibacter africanus, presently restricted to Africa [4], and *Candidatus* Liberibacter americanus, limited to Brazil until recently [5], but now also reported in China [6]. Transmission of the pathogens occurs through the insect vectors *Diaphorina citri* Kuwayama, the Asian citrus psyllid, or *Trioza erytrea* Del Guercio, the African citrus psyllid. Other modes of transmission in citrus are by dodder (*Cuscuta* sp.) and through grafting with diseased budwood [7].

Typical leaf symptoms observed in HLB-affected citrus plants are an asymmetric blotchy mottling of older leaves [8] and a range of chlorotic patterns, often resembling zinc-deficiency symptoms, followed by twig-dieback, reduced fruit production, and tree decline at advanced stages of the disease. Blockage of the translocation stream due to the plugging of sieve elements along with phloem necrosis appears to be a major factor of the disease process [9,10].

Infection of plants with pathogens usually results in a series of defense responses such as the hypersensitive reaction, the production of reactive oxygen species, cell wall fortifications, the synthesis of pathogenesis-related proteins, and the production of phytoalexins [11]. Analyzing modifications of host transcriptional activity during this response can produce valuable information on the mechanisms of the host–pathogen interaction and may result in the development of new plant protection strategies [12]. Microarray technology is a tool to simultaneously analyze transcriptional changes of thousands of genes and has been used in numerous studies of plant bacterial, fungal and other diseases [13–16]. The transcriptional response of citrus to *Ca. L. asiaticus* using a citrus microarray was described by Albrecht and Bowman [17] and Kim et al. [18] for sweet orange (*Citrus sinensis* L.) and revealed important information about the mechanisms of HLB disease development. Other array-based gene expression studies conducted in citrus include the response of *Citrus* and *Poncirus* species to infection with *Phytophthora parasitica* [19] and citrus tristeza virus [20].

HLB affects all known citrus species and citrus relatives with little known resistance [8,21–23] and no known cure exists at present. Current management strategies are the removal of infected trees, elimination of the insect vector through use of insecticides, and nutritional applications. However, several studies reported the

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absence of well-defined disease symptoms in trifoliate orange (*Poncirus trifoliata* L. Raf.) and some of its hybrids in the presence of HLB, indicating tolerance of this genotype to the disease [8,22,24,25].

P. trifoliata and some of its hybrids are known for their resistance to citrus tristeza virus (CTV), a phloem-limited pathogen causing phloem-necrosis in susceptible rootstocks [26]. Although resistance appears to be associated with the failure of the virus to move through the tissue [27], the exact mechanism of resistance remains unknown. Extracts from trifoliate orange have also been widely used in folk medicine as a remedy for gastritis, dysentery, inflammation, and other ailments and several antibacterial compounds have been identified from fruit and seed [28,29]. In addition, compounds derived from this genotype were shown to be effective in the treatment of cancer through inhibition of cancer cell proliferation and induction of apoptosis [30,31]. It is possible that similar compounds restricting Liberibacter proliferation or movement are present within the phloem resulting in tolerance of *Poncirus* and its hybrids to HLB.

A recent study conducted in our laboratory demonstrated tolerance of US-897, a hybrid of trifoliate orange and the susceptible 'Cleopatra' mandarin (*Citrus reticulata* Blanco), to *Ca. L. asiaticus*, the presumed causal agent of HLB in Florida [25]. Trees of this genotype do not display noticeable disease symptoms in greenhouse- or field location despite infection with the pathogen, and rate and speed of infection are significantly reduced compared with the susceptible mandarin genotype. It was suggested that tolerance of US-897 may be expressed through suppression of bacterial multiplication in the infected tissue.

Using the Affymetrix GeneChip citrus genome microarray, this study compares gene expression of tolerant US-897 and susceptible 'Cleopatra' mandarin in response to infection with *Ca. L. asiaticus* (*Las*) with the main objective of discovering genes possibly involved in tolerance to HLB. We hypothesized to find genes in US-897 that are involved in plant defense, either through constitutively higher expression compared with the susceptible genotype or through induced expression in response to bacterial invasion. Such genes may be suitable as potential targets for biotechnology approaches, providing one strategy to possibly control this devastating disease of citrus.

2. Materials and methods

2.1. Plant material and inoculation

For the first experiment, 21 greenhouse-grown 15 month-old US-897 (*C. reticulata* × *P. trifoliata*) seedlings and 21 greenhouse-grown 15 month-old 'Cleopatra' mandarin (*C. reticulata*) seedlings were used. Fifteen plants of each genotype were inoculated in December 2008 by grafting two bark- or bud-pieces and two leaf pieces from infected greenhouse-grown 'Valencia' (*C. sinensis*) plants, PCR-positive for *Ca. L. asiaticus* and symptomatic for HLB, onto each plant. Six plants of each genotype were inoculated with disease-free tissue pieces obtained from healthy greenhouse-grown 'Valencia' plants to be used as non-infected controls. Inoculations were performed pair-wise to ensure that plants from both genotypes received tissue pieces from the same source. For the second experiment, 40 greenhouse-grown nine month-old seedlings of both genotypes were inoculated in November 2009 as described above. Twelve plants each were inoculated with disease-free tissue pieces and 28 plants were inoculated with infected tissue.

Plants were arranged randomly on the greenhouse bench and kept under natural light conditions at a temperature of 21–28 °C. Plants were irrigated as needed and fertilized every three weeks using a water-soluble fertilizer mix, 20N-10P-20K (Peters Professional, The Scotts Company, Marysville, OH). Micronutrients (Micro Key Palm and Ornamental Formulation, Brandt Consolidated, Springfield, IL) and additional iron (Sequestrene 138 Fe, Becker Underwood, Ames, IA) were applied in February 2009 to plants from experiment 2. Plants were pruned immediately after graft-inoculation to promote new leaf growth and HLB disease development. Plants from experiment 1 were additionally pruned 24 weeks after inoculation (wai).

2.2. PCR detection of Ca. L. asiaticus

Four to six fully expanded leaves were collected from each greenhouse plant in eight-week (experiment 1) or six-week intervals (experiment 2). Leaves were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Leaf petioles were used for PCR detection of the bacterium.

Petioles were ground in liquid nitrogen with a mortar and pestle. One-hundred milligrams of ground tissue was used for DNA extraction. DNA was extracted using the Plant DNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, yielding 20–30 ng DNA per extraction. Real-time PCR assays were performed using primers HLBas (5′-TCGAGCGCGTATGCAATACG-3′) and HLBr (5′-GCGTTATCCCGTAGAAAAAGGTAG-3′) and probe HLBp (5′-AGACGGGTGAGTAACGCG-3′) developed by Li et al. [32]. Amplifications were performed using an AB7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the QuantiTect Probe PCR Kit (Qiagen) according to the manufacturer's instructions. All reactions were carried out in a 20 μL reaction volume using 5 μL DNA. Additional PCR assays were performed on a subset of samples using primers COXf and COXr in combination with probe COXp [32] to confirm uniformity of DNA preparations and loadings.

2.3. RNA extraction, labeling and hybridization

RNA from selected plants was extracted from leaf samples collected at 32 wai (experiment 1) and at 30 wai (experiment 2). Plants were selected for RNA analysis based on uniformity of leaf symptom development and C_t-values after PCR detection of *Las*. Six plants were selected from each genotype and treatment.

One gram of leaf tissue was ground in liquid nitrogen with a mortar and pestle and resuspended in 10 mL guanidinium isothiocyanate buffer [33]. Total RNA was extracted according to Strommer et al. [34] with slight modifications. Phenol/chloroform/isoamylalcohol (25:24:1) extraction was followed by two extractions with chloroform/isoamylalcohol and precipitation of RNA with isopropanol at −20 °C overnight. RNA was pelleted by centrifugation at 10,000 × *g* and 4 °C for 1 h, resuspended in 5 mL water and precipitated overnight at 0 °C with an equal volume of 8 M LiCl. After centrifugation at 10,000 × *g* and 4 °C for 1 h, RNA was washed twice with 70% ethanol, air-dried and dissolved in 500 μL of water. RNA was further purified using the RNeasy® MinElute Cleanup kit (Qiagen) according to the manufacturer's instructions. Total RNA was DNase-treated using the TURBO DNA-*free*-Kit™ (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Samples collected from experiment 1 were used for microarray analysis and qRT-PCR validation experiments. Prior to analysis, equal amounts of RNA from two samples were combined, resulting in three non-infected and three infected biological replicates for both genotypes. Samples from experiment 2 were used for selected qRT-PCR experiments only employing six biological replicates per genotype and treatment.

Microarray experiments were performed using the Affymetrix GeneChip® Citrus Genome Array (Affymetrix, Santa Clara, CA) which contains 33,879 citrus transcripts based on EST sequences from several species of *Citrus*

and *Poncirus* and some of their hybrids. A description of the array is available at the manufacture's website (http://www.affymetrix.com/products/arrays/specific/citrus.affx). Twelve arrays, representing three non-infected and three infected biological replicates of both genotypes, were used in the study. Biotinylated cRNA were prepared according to the Affymetrix GeneChip 3′ IVT Express protocol from 250 μg of total RNA (Affymetrix P/N 702646 Rev. 1). Following fragmentation, 12.5 μg of cRNA were hybridized for 16 h at 45 °C on the citrus array. GeneChips were washed and stained in a GeneChip Fluidic Station 450 (Affymetrix) and scanned using a GeneChip 7G Scanner (Affymetrix). Labeling, hybridization, and scanning were performed at the ICBR Microarray Core facility, University of Florida, Gainesville, USA. The microarray data have been submitted to the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo); accession number GSE30502.

2.4. Microarray data analysis

Data analysis was performed using Partek Genomic Suite 6.5 (Partek Inc., MO, USA, www.partek.com). Robust Multi-array Analysis (RMA) normalization [35] of probe intensity values was executed using default settings. Two-way analysis of variance (ANOVA) was performed using the model $Y_{ijk} = \mu + \text{genotype}_i + \text{treatment}_j + \text{genotype} \times \text{treatment}_{ij} + \varepsilon_{ijk}$, where μ is the common effect for the whole experiment, ε_{ijk} represents the random error present in the kth observation on the ith genotype jth treatment. Contrasts were performed based on differences between genotypes and infected and control state. Gene lists were created for genes with p-values ≤ 0.05, adjusted by the false-discovery rate (FDR) correction [36], and expression level changes of at least 4-fold. Probe set target sequences were aligned to the *Arabidopsis thaliana* genome using WU-BLAST2 on www.arabidopsis.org (The Arabidopsis Information Resource (TAIR)) and locus identifiers were used to obtain the GO annotation and functional characterization based on top hits with an E value $<10^{-5}$.

2.5. Real-time qRT-PCR analysis

To validate results obtained with microarray analysis, quantitative reverse transcription real-time PCR (qRT-PCR) was performed with 18 selected genes using the same RNA preparations that were used for the microarray (Supplement Table S1). Ten of these genes were further employed for gene expression experiments using RNA samples obtained from experiment 2. Genes with different expression profiles in response to infection with *Las* and with higher expression levels in US-897 compared with 'Cleopatra' were selected. Selected genes were regarded as biologically interesting based on their predicted function retrieved from TAIR. Amplifications were performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Fifty nanograms of DNase-treated RNA were used in a total volume of 20 μL. Gene-specific primers (Supplement Table S1) with calculated melting temperatures of 60 °C ± 0.5 °C were designed using Primer3 [37]. Melting curve analysis was performed to ensure amplification of a single product and the absence of primer-dimers. For relative quantification of gene expression the $2^{-\Delta\Delta C_t}$ method was applied according to Livak and Schmittgen [38], using C_t-values of 18S rRNA for normalization. Fold changes based on qRT-PCR analysis were compared with fold changes based on signal intensities obtained from the microarray.

3. Results

3.1. Ca. L. asiaticus detection and disease expression

At 32 wai 'Cleopatra' seedlings selected from experiment 1 were PCR-positive for *Las* with C_t-values ranging from 22.0 to

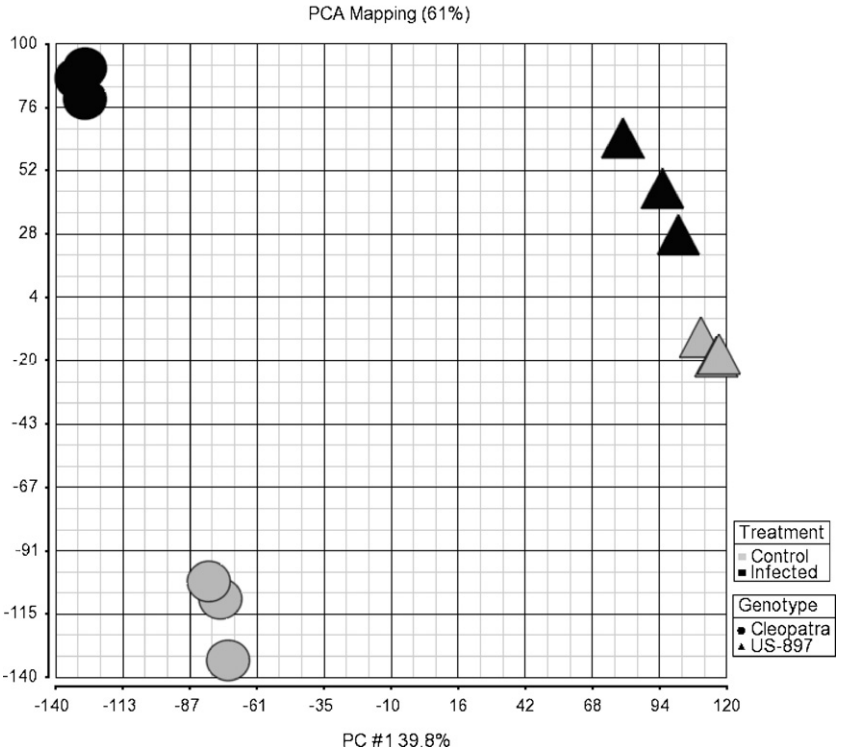

Fig. 1. Principal component analysis (PCA) of gene expression in susceptible 'Cleopatra'- and tolerant US-897 seedlings non-infected (control) and infected with *Ca. L. asiaticus* after microarray analysis using the Affymetrix citrus GeneChip.

24.5 (C_t-average = 22.6) and displayed prominent disease symptoms such as leaf chlorosis, blotchy mottle and reduced growth. US-897 seedlings from this experiment did not display any distinct leaf symptoms or growth abnormalities throughout the study, despite PCR detection of the bacterium based on C_t-values ranging from 26.5 to 28.9 (C_t-average = 27.3). Similar to experiment 1, growth of 'Cleopatra' seedlings from experiment 2 was reduced and leaves displayed severe chlorosis. C_t-values of selected plants were between 20.4 and 21.0 (C_t-average = 20.8) at 30 wai for 'Cleopatra' and between 22.3 and 26.1 (C_t-average = 23.9) for US-897. Sixteen weeks after inoculation and shortly after the micronutrient/iron treatment most US-897 plants from experiment 2 developed nutrient burn-like leaf symptoms. These symptoms were observed in non-infected and infected US-897 seedlings, but not in any of the 'Cleopatra' seedlings. Affected leaves were not included in the sample collection.

3.2. Microarray analysis

ANOVA identified 16,362 genes as differentially expressed ($p < 0.05$) across the study and 9397 genes which varied in response to infection with *Las*. The interaction of genotype and treatment revealed 3412 differentially expressed genes. Principal component analysis showed a distinct clustering of genotypes and treatment with 39.8% of variance explained by PC1 and 21.1% of variance explained by PC2 (Fig. 1).

3.3. Genes up-regulated in 'Cleopatra' in response to infection

Infection with *Las* significantly induced 326 genes in leaves of 'Cleopatra' seedlings by 4- or more-fold. Of these, 61% were identified with orthologs to *Arabidopsis*. More than 8% of the annotated genes were associated with the cell wall and 6.5% were assigned each to the chloroplast and to the extracellular space (Table 1). For the endomembrane category 11.2% of genes were identified. The majority of genes were related to other or unknown cellular components.

Within the group of genes associated with cell organization and biogenesis (3.8%) several genes coding for members of the expansin family and for an endo-1,4-β-glucanase, key regulators of cell wall extension during cell growth, were induced to levels exceeding 6-fold. Of the genes involved in protein metabolism, those for ribosomal proteins, leucin-rich repeat protein kinases, a HXXXD-type acyl transferase, and disease resistance-responsive (dirigent) family proteins, the latter known to be involved in defense response and lignan biosynthesis, were most prominent. More than 15% of annotated genes were classified as responding to abiotic or biotic stimulus and to stress. Among this group were genes for an MRP subfamily/ABC transporter, a cytosolic copper/zinc superoxide dismutase (CSD1), an S-adenosylmethionine synthetase, a thaumatin-like protein (PR5), a chloroplast lipoxygenase, a constitutive disease resistance protein (CDR1), and a chlorophyllase, most of which are generally associated with plant defense responses to pathogens. Up-regulation of these proteins ranged from 10- to nearly 40-fold. Transcripts for an oxidoreductase, associated with the detoxification of reactive carbonyls in the chloroplasts, were 6-fold more abundant in infected 'Cleopatra'. Also induced, though to lower levels, were other defense-related genes, such as those for phenylalanine ammonia-lyase, chitinase A and several peroxidases. Less than 1% of genes coding for transcription factors were induced upon infection. Most dominant within this group was the gene for a Myb-like HTH transcriptional regulator family protein which was induced by nearly 200-fold. More than 7% of annotated genes were coding for proteins associated with transport processes. Most highly induced within this group were genes for a glucose-6-phosphate/phosphate transporter, a zinc transporter 5 precursor (ZIP5), a lipid transfer protein, and several MRP-like ABC transporters. Several other genes coding for members of the zinc transporter family were induced by 4 to 5-fold. Prominent genes coding for proteins involved in other biological processes were those for a pathogenesis related protein (PR1) and a pyridoxal phosphate-dependant transferase. Within the large group of genes associated with other metabolic processes (25.3%) were several coding for proteins involved in carbohydrate metabolism, such as genes for galactose mutarotase-like proteins, ADP-glucose pyrophosphorylase, glycosyl hydrolase family proteins, and galactinol synthase 4. Transcripts for a protein with sucrose synthase activity and a neutral invertase, both associated with the degradation of sucrose, were 4-fold more abundant upon infection. Also strongly induced were several genes coding for enzymes involved in oxidation–reduction processes, such as 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenases, which are involved in the biosynthesis of plant secondary metabolites. Within the group of genes coding for proteins with unknown biological function (9.2%), transcripts for phloem protein 2-B15 were 503-fold more abundant in infected seedlings compared with non-infected seedlings. A different isoform of this protein was induced by more than12-fold in response to infection. Transcripts for a Kunitz family trypsin and protease inhibitor protein were 32-fold more abundant in infected plants. Other genes highly induced in infected 'Cleopatra' seedlings were without known ortholog to *Arabidopsis*. A complete list of

Table 1
Biological process and cellular component of genes up- or down-regulated in leaves of 'Cleopatra mandarin' (Cleo)- and US-897 seedlings in response to infection with *Ca. L. asiaticus*.

	Annotations (%)		
	Up_{Cleo}	Up_{US-897}	$Down_{Cleo}$
GO biological process			
Cell organization and biogenesis	3.8	4.3	0.8
Developmental processes	3.1	0	5.7
Electron transport or energy pathways	0.3	0	0
Other biological processes	7.7	26.1	11.5
Other cellular processes	23.5	21.7	23.8
Other metabolic processes	25.3	26.1	16.4
Protein metabolism	3.1	0	2.5
Response to abiotic or biotic stimulus	7.1	8.7	12.3
Response to stress	8.4	4.3	13.1
Signal transduction	0.5	0	4.9
Transcription	0.8	0	0.8
Transport	7.4	8.7	4.9
Unknown biological processes	9.2	0	3.3
Total annotations	392	23	122
GO cellular component			
Cell wall	8.5	36.4	0
Chloroplast	6.5	0	15.3
Cytosol	2.7	0	1.4
ER	1.2	0	1.4
Extracellular	6.5	9.1	1.4
Mitochondria	2.3	0	2.8
Nucleus	3.5	0	6.9
Other cellular components	15.0	9.1	2.8
Other cytoplasmic components	11.2	9.1	18.1
Other intracellular components	11.5	9.1	16.7
Other membranes	11.2	9.1	12.5
Plasma membrane	4.6	0	0
Plastid	2.7	0	8.3
Ribosome	1.5	0	0
Unknown cellular components	11.2	18.2	12.5
Total annotations	260	11	72

All genes differentially expressed by at least 4-fold in response to infection with *Ca. L. asiaticus* and with *p*-values ≤0.05, adjusted by the false-discovery rate (FDR) correction, were included. Probe sets were aligned to the *Arabidopsis* genome using WU-BLAST 2 on www.arabidopsis.org (The Arabidopsis Information Resource (TAIR)) and locus identifiers were used to obtain the GO annotation and functional categorization. Note that individual gene products may be assigned more than one biological process or cellular location.

genes can be found in the Supplementary material (Supplement Table S2).

3.4. Genes up-regulated in US-897 in response to infection

Only 17 genes were induced by four- or more-fold in US-897 seedlings in response to infection with *Las* (Table 2). Of these, 14 genes were also up-regulated in infected 'Cleopatra' seedlings, though mostly to higher levels compared with infected US-897 seedlings. *Arabidopsis* orthologs were identified for 10 of the genes, of which several (36%) were coding for proteins associated with the cell wall (Table 1). One of these, coding for a GA-responsive GAST1 protein homolog (GASA5) thought to be involved in unidimensional cell growth, was up-regulated in US-897 but not in 'Cleopatra'. Only one gene, coding for a peroxidase superfamily protein and only moderately induced upon infection in US-897 and in 'Cleopatra' leaves, was associated with response to stress. The two genes most highly induced in US-897 in response to infection were those for a glucose-6-phosphate/phosphate transporter, which mediates the import of glucose-6-phosphate, an essential substrate for starch biosynthesis, in the plastids. Genes for this transporter were induced by 12–19-fold in US-897, though increases in 'Cleopatra' were up to 50-fold for this enzyme. The gene most highly (>10-fold) induced in the category for other cellular/metabolic processes was that for a 2-oxoglutarate and Fe(II)-dependent oxygenase associated with hydrogen peroxide-mediated programmed cell death and secondary metabolic processes. This gene was not significantly induced in 'Cleopatra' seedlings upon infection. Transcripts for a different 2-oxoglutarate and Fe(II)-dependent oxygenase, involved in oxidation-reduction processes, were most abundant in infected 'Cleopatra' leaves. Of the unknown genes, two for an alpha/beta-hydrolase were up-regulated by 5- to 8-fold in US-897, one of which was not significantly induced in 'Cleopatra'. The most noteworthy gene for which no *Arabidopsis* ortholog was found was induced to similarly high levels in both genotypes as observed for the genes coding for the glucose-6-phosphate/phosphate transporter.

3.5. Genes down-regulated in response to infection

Infection with *Las* significantly reduced transcript levels for 68 genes in 'Cleopatra' seedlings by four- or more-fold, whilst no genes were significantly repressed by more than 4-fold in infected US-897 seedlings. Of the 68 genes identified in 'Cleopatra', 68% were annotated with orthologs to *Arabidopsis*. A large percentage of annotated genes were associated with chloroplasts (15.3%) or plastids (8.3%). Nearly 7% were assigned to the nuclear compartment. The majority of genes were within the group of other or unknown cellular components. Within the group of genes associated with developmental processes, a gene for a cytochrome P450 monooxygenase, an important regulator of leaf formation and organ growth, was repressed by nearly 7-fold. Transcripts for a gibberellin 20-oxidase, also involved in leaf development and cell growth, were 4-fold less abundant in response to infection. Several genes for proteins involved in protein folding were moderately down-regulated. Notable within the group of genes responding to stress or to abiotic and biotic stimuli were those for a wound-responsive family protein and a chloroplastic superoxide dismutase CSD2, which is associated with the detoxification of superoxide radicals in the chloroplast. Two genes for a mitochondrial substrate carrier protein were repressed by 8- to 10-fold. Most strongly repressed (−22.4%) in response to infection with *Las* was a gene for a protein of the thioredoxin superfamily, whose members are major components of the plant cell redox regulatory system. A gene for isopentenyltransferase 3 involved in the biosynthesis of cytokinins was repressed by more than 12-fold. Transcripts for two genes coding for a galactinol synthase 2, associated with carbohydrate metabolic processes, were 6–8-fold less abundant in infected 'Cleopatra' leaves. Also involved in carbohydrate metabolism, specifically in the starch catabolic process, were two genes for a chloroplast beta-amylase which were repressed by up to 5-fold. Most strongly repressed within the group of genes with unknown function was a gene for an exostosin family protein. A complete list of genes can be found in the Supplementary material (Supplement Table S3).

3.6. Genes with higher expression levels in US-897

More than 800 transcripts were found to be at least four times more abundant in US-897 seedlings compared with 'Cleopatra' seedlings independent of infection with *Las*. Of the 559 transcripts that were ≥10-fold more abundant in non-infected or infected US-897 seedlings in comparison to 'Cleopatra' (Supplement Table S4) only 19% were identified to have orthologs to *Arabidopsis*. Annotated transcripts with ≥32-fold higher abundance in US-897 are depicted in Table 3. A considerable proportion of the annotated genes was categorized as responding to stress (13%) and to abiotic and biotic stimulus (11%). The majority of genes were involved in other metabolic processes (23%) and other cellular processes (20%). More than 34% of transcripts were classified within the group of other intracellular or other cytoplasmic components. The proportion of genes associated with the chloroplasts or the plastids was 14% and 7%, respectively. More than 9% were associated with the endomembrane system.

Within the category of genes involved in cell organization and biogenesis a gene for a gibberellic acid (GA)-responsive GAST1 protein homolog (GASA5) was notable because, in addition to being constitutively more expressed in US-897, expression levels were significantly induced in this genotype in response to infection. Genes for a progesterone-5beta-reductase-like protein (VEP1), essential for xylem and phloem pattern formation, were prominent within the developmental category. The largest percentage (31%) of annotated genes with ≥32-fold higher expression levels in US-897 were associated with responses to stress and to abiotic and biotic stimuli. Most noteworthy within this group were the genes for a constitutive disease resistance 1 (CDR1) protein, a pheide a oxygenase (PAO), a copper/zinc superoxide dismutase 1 (CDS1), and a vitamin C defective 2 protein (VTC2), all of which are associated with defense responses to bacteria. Within the category of other biological processes, a gene for a plastidic glucose transporter (GLT1), which is important for the export of starch degradation products, was identified. Among the genes involved in other cellular or metabolic processes were those for cellulose synthase-like proteins and an UDP-glucosyltransferase 71B1. Several genes without identifiable ortholog to *Arabidopsis* exhibited >500-fold higher expression levels in US-897 seedlings compared with 'Cleopatra' seedlings independent of infection with *Las*.

Twenty-two genes with four- or more-fold higher expression levels in US-897 compared with 'Cleopatra' were also found to be significantly induced in 'Cleopatra' seedlings in response to infection (Table 4). However, expression levels observed in infected 'Cleopatra' leaves never exceeded the levels observed in non-infected or infected US-897 leaves. Most notable within this group were genes for a constitutive disease resistance (CDR1) protein, associated with salicylic acid-mediated defense response to bacteria, an oxidoreductase and a gene without *Arabidopsis* ortholog which were induced by more than 4-fold in 'Cleopatra' in response to infection. Also typically associated with defense responses to pathogens and with responses to other abiotic stresses were the genes for glyceraldehyde-3-phosphate dehydrogenase, an osmotin-like protein, a phytoalexin deficient 4 protein (PAD4), a plant defensin (PDF2.2), and a peroxidase superfamily protein. Genes for several UDP-glycosyltransferases involved in plant

Table 2
Genes induced in US-897 leaves in response to infection with *Ca. L. asiaticus*. All genes induced ≥4-fold with *p*-values ≤0.05, adjusted by the false-discovery rate (FDR) correction, are depicted. Fold changes for 'Cleopatra' are shown for comparison.

Probe set ID[a]	US-897		'Cleopatra'		AGI number[c]	Gene product[d]
	p_{FDR}	Fold change[b]	p_{FDR}	Fold change[b]		
Cell organization and biogenesis						
Cit.20082.1.S1_x_at	1.2E−04	4.4	ns		AT1G75750.1	GA-responsive GAST1 protein homolog (GASA5)
Response to stress						
Cit.23268.1.S1_s_at	1.7E−04	4.0	8.6E−04	2.9	AT5G05340.1	Peroxidase superfamily protein
Transport						
Cit.9625.1.S1_s_at	4.3E−05	19.2	1.4E−05	31.0	AT1G61800.1	Glucose-6-phosphate/phosphate transporter 2
Cit.22602.1.S1_at	1.1E−04	11.6	3.5E−06	49.7	AT1G61800.1	Glucose-6-phosphate/phosphate transporter 2
Cit.950.1.S1_s_at	3.8E−05	4.0	1.4E−06	8.6	AT3G18280.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
Other biological/cellular/metabolic processes						
Cit.940.1.S1_s_at	2.7E−06	10.2	ns		AT3G13610.1	2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Cit.35640.1.S1_s_at	7.8E−05	4.7	1.6E−03	2.7	AT4G08950.1	EXORDIUM (EXO)
Cit.18521.1.S1_at	5.6E−05	4.6	1.1E−06	13.6	AT3G19000.1	2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Unknown biological process						
Cit.6544.1.S1_at	2.6E−06	7.5	ns		AT3G15650.2	Alpha/beta-hydrolases superfamily protein
Cit.7635.1.S1_at	5.8E−05	5.2	1.2E−05	7.8	AT3G15650.2	Alpha/beta-hydrolases superfamily protein
No hit						
Cit.9620.1.S1_s_at	2.7E−05	23.6	1.3E−05	32.8		
Cit.31379.1.S1_at	3.1E−09	7.0	1.5E−10	17.0		
Cit.214.1.S1_s_at	2.5E−06	5.5	8.3E−10	115.5		
Cit.12214.1.S1_s_at	6.8E−08	5.1	5.4E−09	9.6		
Cit.12427.1.S1_at	2.6E−06	5.1	7.7E−08	13.1		
Cit.12214.1.S1_at	4.6E−06	4.5	2.4E−07	9.2		
Cit.10244.1.S1_s_at	6.0E−04	4.2	1.3E−06	28.6		

Probe sets were aligned to the *Arabidopsis* genome using WU-BLAST 2 on www.arabidopsis.org (The Arabidopsis Information Resource (TAIR)) and locus identifiers were used to obtain the GO annotation and functional categorization. Underlined probe set IDs indicate genes used for qRT-PCR confirmation experiments. ns, no significant change.

[a] Affymetrix citrus GeneChip® Probe set ID.

[b] Relative gene transcript level in leaves of infected plants compared with leaves of non-infected plants.

[c] *Arabidopsis* genome initiative number.

[d] Gene product predicted by TAIR based on top hits with an *E* value <10^{-5}.

secondary metabolism and playing a potential role in stress or defense responses were also noteworthy.

3.7. Quantitative RT-PCR validation

Quantitative RT-PCR analysis performed with 18 genes using the RNA samples from experiment 1 revealed expression patterns similar to the results obtained by microarray analysis for the majority of genes. Both methods produced very similar expression patterns for the genes coding for progesterone-5beta-reductase-like protein (VEP1), ADP-glucose pyrophosphorylase, thioredoxin superfamily protein, constitutive disease resistance 1 protein (CDR1; Cit.23704.1.S1_at), cytosolic copper/zinc superoxide dismutase (CSD1), zinc transporter 5 precursor (ZIP5), cytochrome P450 monooxygenase (CYP78A5), UDP-glycosyltransferase 73B3 (UGT73B3), Kunitz family trypsin and protease inhibitor protein, myb-like HTH transcriptional regulator family protein, phloem protein 2-B15 (PP2-B15), chloroplastic copper/zinc superoxide dismutase (CSD2), 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein, and glucose-6-phosphate/phosphate transporter 2, although the extent of differential expression often differed between the two methods (Fig. 2). Similar expression patterns were also observed for constitutive disease resistance 1 protein (CDR1; Cit.28117.1.S1_s_at) and pathogenesis-related plant defensin (PDF2.2) although expression levels were higher in infected 'Cleopatra' samples compared with the microarray data. For two of the selected genes, vitamin C defective 2 (VTC2) and oxidoreductase zinc-binding dehydrogenase family protein, results from qRT-PCR analysis did not agree with those obtained from the microarray. Both genes showed higher expression levels in US-897 according to the array data, but not according to PCR analysis.

Results from qRT-PCR analysis performed with selected genes using RNA samples from experiment 2 are summarized in Table 5. Genes coding for progesterone-5beta-reductase-like protein (VEP1), thioredoxin superfamily protein, pathogenesis-related plant defensin (PDF2.2), phloem protein 2-B15 (PP2-B15), and glucose-6-phosphate/phosphate transporter 2 showed expression patterns similar to those obtained after qRT-PCR analysis of samples from experiment 1. Unlike observations from experiment 1, transcripts for cytosolic copper/zinc superoxide dismutase (CSD1) and 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein were not induced in US-897 in response to infection with *Las*, although they were more abundant in this genotype compared with 'Cleopatra'. Transcripts for constitutive disease resistance 1 protein (CDR1; Cit.23704.1.S1_at), while much more abundant in US-897, were not induced in infected 'Cleopatra' seedlings. Expression levels for constitutive disease resistance 1 protein (CDR1; Cit.28117.1.S1_s_at) and UDP-glycosyltransferase 73B3 (UGT73B3) did not differ much between genotypes and treatments, contrary to the results from the first experiment.

4. Discussion

Huanglongbing, the perhaps most destructive disease of citrus, affects all known citrus species and relatives and no resistant genotypes have been identified as yet. However, results from several studies suggested tolerance of trifoliate orange and trifoliate hybrids, since no well-described disease symptoms were detectable in plants inoculated with the HLB pathogen [8,22,24].

Table 3
Genes with higher expression levels in US-897 leaves compared with 'Cleopatra' (Cleo) leaves. Only annotated genes with expression levels of ≥32-fold in non-infected (Ctrl) or *Ca. L. asiaticus*-infected (Inf) US-897 compared with 'Cleopatra' and *p*-values ≤0.05, adjusted by the false-discovery rate (FDR) correction, are depicted.

Probe set ID[a]	AGI number[b]	Gene product[c]	Array signal (log_2)			
			$Cleo_{Ctrl}$	$Cleo_{Inf}$	US-897_{Ctrl}	US-897_{Inf}
Cell organization and biogenesis						
Cit.24341.1.S1_s_at	AT2G28110.1	Member of glycosyltransferase family 47	2.5	2.5	8.4	8.4
Cit.20082.1.S1_x_at	AT1G75750.1	GA-responsive GAST1 protein homolog (GASA5)	3.7	4.3	7.3	9.4
Developmental processes						
Cit.1299.1.S1_x_at	AT4G24220.1	Progesterone-5beta-reductase-like protein (VEP1, vein patterning 1)	3.7	3.5	10.7	10.7
Cit.1302.1.S1_x_at	AT4G24220.1	Progesterone-5beta-reductase-like protein (VEP1, vein patterning 1)	2.6	2.6	8.9	8.7
Cit.29061.1.S1_at	AT3G63088.1	ROTUNDIFOLIA like 14 (RTFL14)	2.8	2.6	8.5	8.2
Electron transport and energy pathway						
Cit.12285.1.S1_x_at	AT1G53310.1	Phosphoenolpyruvate carboxylase 1	2.3	2.0	8.6	8.5
Protein metabolism						
Cit.11907.1.S1_at	AT3G27925.1	DegP protease 1	4.1	3.8	8.9	9.0
Cit.9769.1.S1_x_at	AT1G01300.1	Eukaryotic aspartyl protease family protein	2.4	2.2	7.3	8.2
Cit.550.1.S1_at	AT4G13780.1	Methionyl-tRNA synthetase	2.5	2.4	7.6	7.5
Response to stress/response to abiotic and biotic stimulus						
Cit.8006.1.S1_s_at	AT4G15910.1	Drought induced 21 (DI21)	5.4	3.9	13.0	12.6
Cit.23704.1.S1_at	AT5G33340.1	Constitutive disease resistance 1 (CDR1)	3.3	4.1	11.2	11.5
Cit.8866.1.S1_x_at	AT4G26850.1	Vitamin C defective 2 (VTC2)	4.8	4.3	11.1	11.2
Cit.8905.1.S1_at	AT1G23740.1	Oxidoreductase, zinc-binding dehydrogenase family protein	3.2	3.5	10.8	10.8
Cit.182.1.S1_s_at	AT1G08830.1	Copper/zinc superoxide dismutase 1 (CDS1)	2.7	2.5	9.9	9.9
Cit.11617.1.S1_at	AT3G44880.1	Pheide a oxygenase (PAO)	1.9	1.7	9.5	9.4
Cit.9500.1.S1_at	AT2G29500.1	HSP20-like chaperones superfamily protein	2.5	2.3	8.6	8.8
Cit.13876.1.S1_at	AT5G18100.1	Putative peroxisomal CuZnSOD (copper/zinc superoxide dismutase 3)	1.9	2.1	8.6	8.7
Cit.11308.1.S1_at	AT1G75280.1	Isoflavone reductase, putative	2.0	2.3	7.0	7.7
Transport						
Cit.20504.1.S1_x_at	AT1G01620.1	Plasmamembrane intrinsic protein 1C	3.1	2.8	11.3	11.5
Cit.2927.1.S1_s_at	AT5G23810.1	Nonfunctional amino acid transporter AAP7	6.4	5.7	11.2	10.9
Cit.8420.1.S1_at	AT1G66240.1	Homolog of anti-oxidant 1 (ATX1)	2.2	2.3	10.8	10.9
Cit.2928.1.S1_s_at	AT5G09220.1	Member of AAAP family, amino acid permease 2	3.7	3.1	8.8	8.3
Other biological processes						
Cit.12711.1.S1_at	AT5G16150.1	Putative plastidic glucose transporter (GLT1)	2.5	2.4	8.1	8.0
Other cellular						
Cit.1801.1.S1_at	AT2G02400.1	NAD(P)-binding Rossmann-fold superfamily protein	5.6	5.1	10.1	10.6
Cit.20662.1.S1_s_at	AT2G32530.1	Similar to cellulose synthase (cellulose synthase like B3)	3.6	3.4	9.9	9.6
Cit.15437.1.S1_at	AT2G32530.1	Similar to cellulose synthase (cellulose synthase like B3)	1.8	1.6	7.8	7.4
Other metabolic processes						
Cit.27468.1.S1_s_at	AT2G45550.1	Member of CYP76C (cytochrome P450)	4.0	4.1	9.5	9.1
Cit.12475.1.S1_at	AT3G21750.1	UDP-glucosyltransferase 71B1	3.6	4.1	8.6	8.6
Unknown biological process						
Cit.1723.1.S1_at	AT2G47400.1	CP-12 domain-containing protein 1 (CP12-1)	5.8	5.1	11.0	11.0
Cit.10279.1.S1_at	AT3G57520.1	Seed imbibition 2 (SIP2)	5.1	5.1	10.3	10.3
Cit.16427.1.S1_at	AT1G78955.1	Camelliol C synthase 1 (CAMS1)	4.8	4.2	9.8	9.5
Cit.24387.1.S1_at	AT3G04650.1	FAD/NAD(P)-binding oxidoreductase family protein	5.7	4.3	9.7	9.4
Cit.5623.1.S1_at	AT5G04700.1	Ankyrin repeat family protein	4.3	4.7	9.5	9.3
Cit.9384.1.S1_x_at	AT5G61820.1	MAC9.6	4.2	4.2	9.1	9.2
Cit.3051.1.S1_at	AT1G79390.1	Unknown protein	3.5	3.6	8.9	8.7
Cit.16765.1.S1_at	AT4G35150.1	O-methyltransferase family protein	2.5	2.6	8.1	8.5
Cit.29042.1.S1_at	AT1G74640.1	Alpha/beta-hydrolases superfamily protein	2.8	2.5	8.4	8.1
Cit.28378.1.S1_at	AT1G31780.1	Unknown protein	2.8	2.4	7.8	7.8
Cit.15777.1.S1_at	AT4G16660.1	Heat shock protein 70 (Hsp 70) family protein	2.2	2.1	7.4	7.3

Probe sets were aligned to the *Arabidopsis* genome using WU-BLAST 2 on www.arabidopsis.org (The Arabidopsis Information Resource (TAIR)) and locus identifiers were used to obtain the GO annotation and functional categorization. Underlined probe set IDs indicate genes used for qRT-PCR confirmation experiments.

[a] Affymetrix citrus GeneChip® Probe set ID.

[b] *Arabidopsis* genome initiative number.

[c] Gene product predicted by TAIR based on top hits with an *E* value $<10^{-5}$.

A recent detailed study conducted in our laboratory demonstrated tolerance of the trifoliate hybrid US-897 to *Ca*. L. asiaticus (Las), the pathogen associated with HLB in Florida [25]. The main objective of this study was to identify genes associated with tolerance to HLB through comparison of US-897 with its susceptible parent, 'Cleopatra' mandarin.

Microarray analysis detected 16,362 genes as differentially expressed across the two genotypes and 9397 genes which responded significantly to infection with *Las*. Genes with a differential expression of at least 4-fold and a *p*-value of ≤0.05 after FDR correction were extracted. Based on these criteria, 326 genes were significantly induced in leaves of 'Cleopatra' seedlings compared with only 17 genes in US-897. Many genes induced in the susceptible genotype, which expressed obvious disease symptoms at the time of sample collection, were involved in cell organization and biogenesis processes, such as the genes coding for expansins, key regulators of cell wall extension during cell growth [39]. The high abundance of transcripts for these genes is likely associated with the production of replacement phloem due to phloem necrosis caused by Liberibacter infection [9,10]. In addition, several pathogenesis-related (PR) genes, such as thaumatin, chitinase, phenylalanine ammonia-lyase, and peroxidase, which are generally involved in plant defense against pathogens and adaptation to other stresses [40], were induced in 'Cleopatra' in response to infection. This shows that even a susceptible host does not remain passive, but initiates defense responses to counteract the damaging effects of the pathogen. This phenomenon is termed basal resistance [41]. Whether a response ultimately results in

Table 4
Genes with higher expression levels in US-897 leaves compared with 'Cleopatra' (Cleo) leaves and significantly induced in 'Cleopatra' in response to infection with *Ca. L. asiaticus*. All genes with ≥4-fold higher expression levels in US-897 and *p*-values ≤0.05, adjusted by the false-discovery rate (FDR) correction, are depicted.

Probe set ID[a]	AGI number[b]	Gene product[c]	Array signal (log_2)			
			$Cleo_{Ctrl}$	$Cleo_{Inf}$	US-897_{Ctrl}	US-897_{Inf}
Electron transport or energy pathways						
Cit.6867.1.S1_at	AT3G07800.1	Thymidine kinase	4.7	6.0	7.6	8.0
Response to stress/response to abiotic and biotic stimulus						
Cit.8902.1.S1_s_at	AT1G23740.1	Oxidoreductase, zinc-binding dehydrogenase family protein	6.4	9.0	11.2	11.2
Cit.28117.1.S1_s_at	AT5G33340.1	Constitutive disease resistance (CDR1)	6.1	9.4	10.3	10.8
Cit.8105.1.S1_s_at	AT1G13440.1	Glyceraldehyde-3-phosphate dehydrogenase C2 (GAPC2)	8.1	9.7	10.4	10.7
Cit.2831.1.S1_s_at	AT2G02100.1	Predicted pathogenesis-related protein of the plant defensin family (PDF2.2)	6.6	8.5	9.7	9.9
Cit.28883.1.S1_at	AT4G34131.1	UDP-glucosyl transferase 73B3 (UGT73B3)	4.4	5.6	9.2	9.0
Cit.28883.1.S1_x_at	AT4G34135.1	UDP-glucosyl transferase 73B3 (UGT73B3)	4.3	5.8	9.1	9.0
Cit.34096.1.S1_s_at	AT3G01190.1	Peroxidase superfamily protein	4.7	6.3	7.9	7.8
Cit.2116.1.S1_s_at	AT4G11650.1	Osmotin-like protein (OSM34)	3.8	5.3	6.6	6.5
Cit.21700.1.S1_at	AT3G52430.1	Phytoalexin deficient 4 (PAD4)	2.5	3.6	5.6	5.5
Transport						
Cit.14342.1.S1_at	AT3G62150.1	P-glycoprotein 21 (PGP21)	2.9	4.2	5.4	5.3
Other metabolic processes						
Cit.4771.1.S1_s_at	AT4G24340.1	Phosphorylase superfamily protein	6.6	7.7	9.9	10.2
Cit.11435.1.S1_s_at	AT3G19000.1	2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	6.3	9.5	9.4	8.9
Cit.5439.1.S1_at	AT5G49690.1	UDP-glycosyltransferase superfamily protein	5.6	7.3	8.5	8.9
Cit.2994.1.S1_s_at	AT3G26210.1	Putative cytochrome P450	2.5	4.1	6.7	7.5
Unknown biological process						
Cit.17307.1.S1_at	AT4G14305.1	Peroxisomal membrane 22 kDa (Mpv17/PMP22) family protein	3.7	4.7	6.0	6.2
No hit						
Cit.2048.1.S1_s_at			4.3	6.8	7.5	9.0
Cit.5444.1.S1_at			2.5	3.6	6.9	6.9
Cit.5150.1.S1_at			3.0	4.2	6.2	6.9
Cit.15992.1.S1_at			2.5	4.4	5.5	7.4
Cit.9824.1.S1_at			3.4	5.3	5.6	6.8

Probe sets were aligned to the *Arabidopsis* genome using WU-BLAST 2 on www.arabidopsis.org (The Arabidopsis Information Resource (TAIR)) and locus identifiers were used to obtain the GO annotation and functional categorization. Underlined probe set IDs indicate genes used for qRT-PCR confirmation experiments. Ctrl, non-infected control plants; Inf, plants infected with *Ca. L. asiaticus*.

[a] Affymetrix citrus GeneChip® Probe set ID.

[b] *Arabidopsis* genome initiative number.

[c] Gene product predicted by TAIR based on top hits with an *E* value $<10^{-5}$.

Table 5
Comparison of gene expression in non-infected (Ctrl) and *Ca. L. asiaticus*-infected (Inf) 'Cleopatra' (Cleo)- and US-897 seedlings from experiment 1 (Exp 1) and experiment 2 (Exp 2) based on qRT-PCR analysis.

Probe set ID[a]	Gene product[b]		Relative gene expression			
			$Cleo_{Ctrl}$	$Cleo_{Inf}$	US-897_{Ctrl}	US-897_{Inf}
Cit.1299.1.S1_x_at	Progesterone-5β-reductase-like protein (VEP1, vein patterning 1)	Exp 1	1.0	0.6	7.2	7.9
		Exp 2	1.0	0.7	11.5	12.3
Cit.18719.1.S1_at	Thioredoxin superfamily protein	Exp 1	27.3	1.0	26.8	31.9
		Exp 2	17.8	1.0	262.6	113.2
Cit.23704.1.S1_at	Protein with aspartic protease activity (CDR1, constitutive disease resistance 1)	Exp 1	1.0	28.9	50,591.9	65,052.7
		Exp 2	1.0	2.3	8233.7	6802.3
Cit.28102.1.S1_s_at	Cytosolic copper/zinc superoxide dismutase CSD1	Exp 1	1.0	60.3	3.7	24.3
		Exp 2	1.0	8.4	85.2	33.4
Cit.28117.1.S1_s_at	Protein with aspartic protease activity (CDR1, constitutive disease resistance 1)	Exp 1	1.0	26.8	9.8	27.4
		Exp 2	1.0	2.2	3.8	3.3
Cit.2831.1.S1_s_at	Predicted pathogenesis-related protein of the plant defensin family (PDF2.2)	Exp 1	1.0	8.7	10.3	7.9
		Exp 2	1.0	21.3	13.6	32.0
Cit.28883.1.S1_x_at	UDP-glycosyltransferase 73B3 (UGT73B3)	Exp 1	1.0	4.6	18.2	14.7
		Exp 2	1.0	2.2	1.3	2.5
Cit.35955.1.S1_at	Phloem protein 2-B15 (PP2-B15)	Exp 1	1.0	55,070.1	46.0	363.7
		Exp 2	1.0	352.9	78.6	207.9
Cit.940.1.S1_s_at	2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Exp 1	1.0	2.7	3.2	155.9
		Exp 2	1.0	2.7	312,624.8	376,288.3
Cit.9625.1.S1_s_at	Glucose-6-phosphate/phosphate transporter 2	Exp 1	1.0	281.9	1.0	43.2
		Exp 2	1.0	22.5	2.2	12.9

Gene expression was normalized to expression of 18S rRNA and is presented as mean fold differences relative to non-infected or infected 'Cleopatra' samples. Six biological replicates were used per genotype and treatment.

[a] Affymetrix citrus GeneChip® Probe set ID.

[b] Gene product predicted by TAIR based on top hits with an *E* value $<10^{-5}$.

resistance or susceptibility depends on the speed and magnitude of the defense mechanisms employed [42] and the ability of the pathogen to suppress them. Evidently, responses exhibited by 'Cleopatra' seedlings were not sufficient to hinder multiplication and spread of *Las* throughout the plant, resulting in disease expression and plant decline. It is unclear to what extent the disease symptoms are caused directly by the bacterium or by the plant defense responses directed against it.

Also associated with defense to bacteria and stress is the gene for a cytosolic copper/zinc superoxide dismutase (CSD1), which was highly up-regulated in infected 'Cleopatra' seedlings. Superoxide dismutases are among the most potent antioxidants in nature and are important constituents of the cellular defense against oxidative stress [43]. Although the gene also appeared to be induced in infected US-897 seedlings, expression changes did not pass significance criteria. However, a different gene for CSD1 was found to be

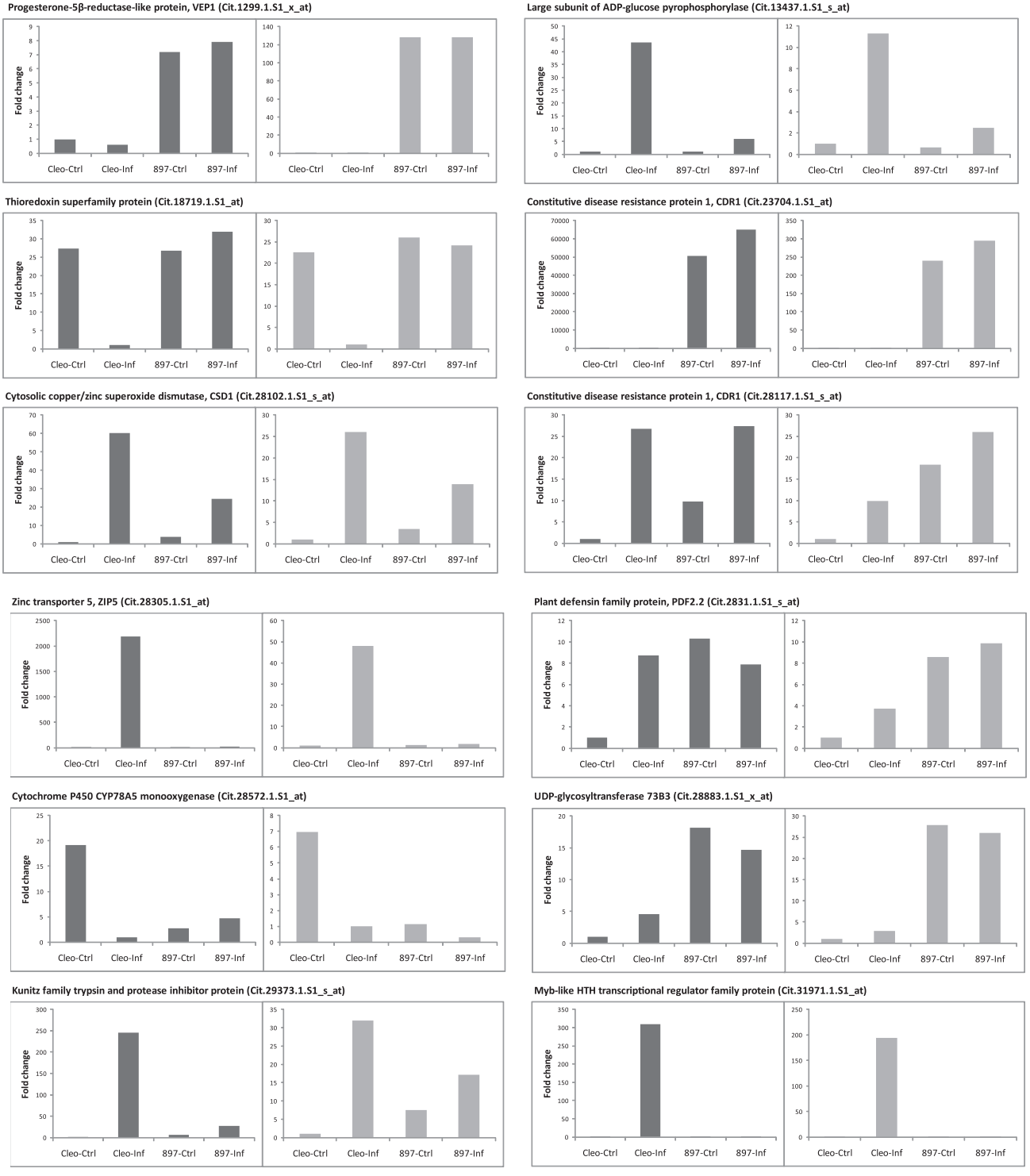

Fig. 2. Quantitative RT-PCR analysis (black bar graphs) in comparison with microarray analysis (grey bar graphs) of selected genes expressed in non-infected (Ctrl) and *Ca. L. asiaticus*-infected (Inf) 'Cleopatra' (Cleo)- and US-897 (897) seedlings. Gene expression was normalized to expression of 18S rRNA and is presented as mean fold differences relative to non-infected or infected 'Cleopatra' samples.

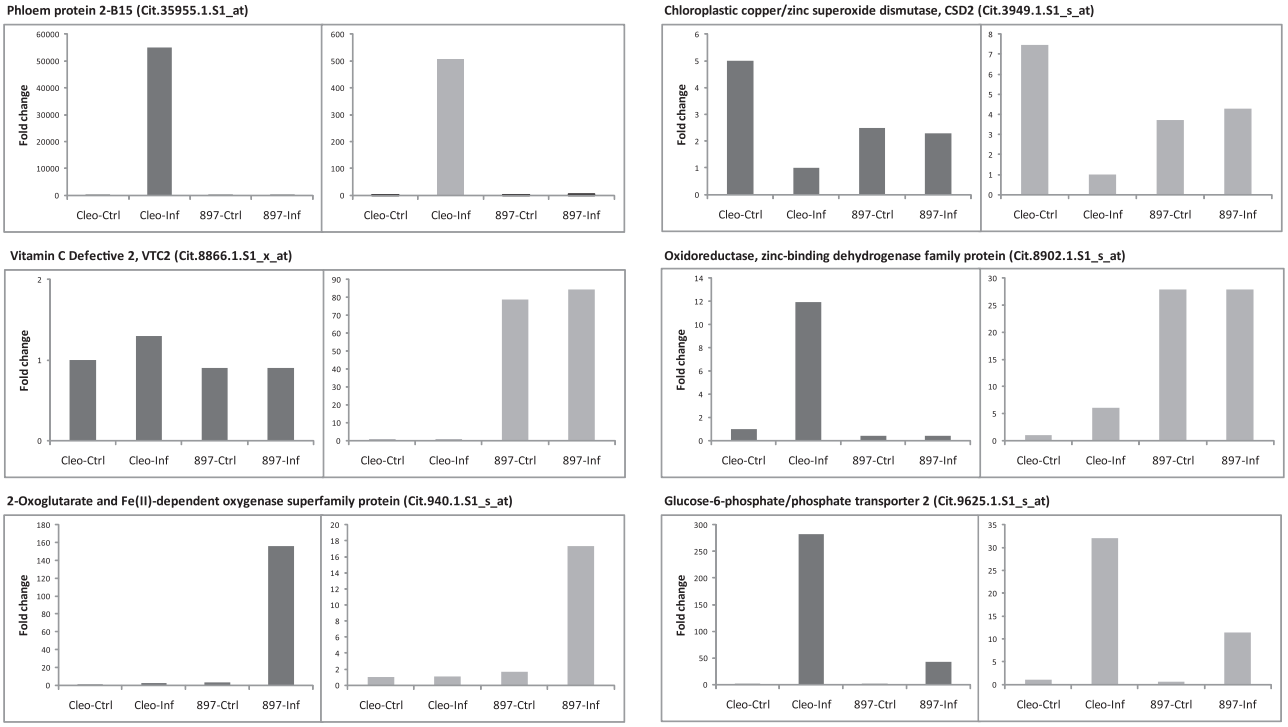

Fig. 2. (*Continued*).

expressed at much higher levels in US-897 independent of infection with *Las*, suggesting its possible involvement in the protection against *Las*.

Transcripts for a constitutive disease resistance protein (CDR1) were strongly induced in infected 'Cleopatra' seedlings and more abundant in US-897 seedlings independent of infection. CDR1 was shown to encode an apoplastic aspartic protease capable of activating salicylic-acid-mediated resistance mechanisms through the release of a mobile peptide signal [44]. Over-expressions of CDR1 resulted in over-expression of other defense-related genes and enhanced resistance to bacterial and fungal pathogens in *Arabidopsis* and rice [44,45]. Another gene coding for a different form of CDR1 exhibited expression levels in US-897 exceeding those observed in 'Cleopatra' by more than 200-fold. The larger abundance of transcripts for CDR1 in US-897 seedlings compared with 'Cleopatra' suggests the possible involvement of these genes in tolerance to HLB.

Among the genes associated with transcription, a Myb-like transcriptional regulator family protein was induced by nearly 200-fold in infected 'Cleopatra' seedlings but not in infected US-897 seedlings. MYB proteins are part of the large family of transcription factors which are key factors in the regulatory networks controlling development, metabolism and response to biotic and abiotic stresses [46]. Several of its members were shown to be essential for early signaling in rhizobacteria-induced systemic resistance [47] and for salicylic acid-mediated control of the hypersensitive response to bacterial pathogens [48]. Induction of a Myb transcription factor in grapevine appeared to be associated with resistance to infection with Bois Noir phytoplasma, which, like Liberibacter is a phloem-limited pathogen [16]. Expression of a Myb-like transcriptional regulator in citrus infected with *Las* does not seem to be associated with tolerance to HLB, but appears to be characteristic for the symptomatic stage of the disease. This is in agreement with the results from a transcriptional analysis of infected sweet orange plants previously conducted in this laboratory [17]. It is unclear whether expression of this gene at an earlier stage of infection would enable the plants to counteract the detrimental effects of HLB.

Citrus plants affected by HLB typically develop nutritional deficiency-like symptoms, particularly those usually associated with zinc deficiency [49]. Several genes for a zinc transporter 5 precursor (ZIP5) were up-regulated in 'Cleopatra' leaves infected with *Las* and displaying nutritional deficiency-like chlorosis. The up-regulation of genes for these transporters was shown to be associated with increased zinc uptake in transgenic barley and in the zinc hyperaccumulating plant *Thlaspi caerulescens* [50,51]. Induced expression of genes for ZIP5 in citrus affected by HLB is likely an attempt of the host to increase the uptake of zinc in response to disease-induced deficiency.

Most strongly induced in 'Cleopatra' leaves in response to infection with *Las* was the gene for a phloem protein 2-B15. This gene was not induced in US-897 seedlings. Phloem protein 2 (PP2), a lectin-like protein, besides being involved in the differentiation of vascular tissue [52], is associated with the plugging of sieve plates in response to wounding and the defense against pathogens and insects [53]. Accumulation of PP2 at the sieve plates in conjunction with phloem necrosis and blockage of the translocation stream was demonstrated by Kim et al. [18] and Achor et al. [10] for HLB-affected citrus and appears to be a major factor of disease symptom development. Interestingly, the deposition of PP2 in combination with callose at the sieve plates was associated with the recovery of apple trees from apple proliferation disease caused by the phloem-limited pathogen *Candidatus* Phytoplasma mali [54]. Like the Myb transcriptional regulator protein, PP2 is characteristic for the late, symptomatic stage of HLB disease and appears to be a last attempt of the citrus host to restrict further spread of the pathogen within the sieve tubes [17]. Though not significantly induced in US-897 seedlings in response to infection, transcript levels for PP2 were generally higher in this genotype compared with the non-infected 'Cleopatra' seedlings based on the results from qRT-PCR analysis, suggesting the possible involvement of this protein in tolerance to HLB. Earlier induction of this protein in susceptible genotypes

through genetic engineering may be one strategy to limit the spread of Liberibacter in citrus.

In addition to phloem necrosis, a characteristic symptom of HLB-affected citrus is the accumulation of starch in the chloroplasts of infected leaves [9,10,55]. A gene for the large subunit of ADP-glucose pyrophosphorylase (AGPase), they key enzyme for starch biosynthesis in plants [56], was strongly induced in 'Cleopatra' leaves in response to infection. This gene was also induced in US-897 leaves as determined by qRT-PCR and microarray, though increases were below 4-fold according to the latter. In addition, several genes for a glucose-6-phosphate/phosphate transporter (GPT) were found to be induced to very high levels in both, susceptible 'Cleopatra' seedlings and tolerant and symptomless US-897 seedlings. Thus, the up-regulation of GPT genes appears to be one of the earliest and most pronounced transcriptional events in response to infection with *Las*. Results from a previous study on sweet orange plants confirm this notion [17]. GPT mediates the import of glucose-6-phosphate, essential substrate for starch synthesis, in the plastids [57]. A recent study found that volatiles emitted by pathogens affect primary carbohydrate metabolism and induce higher levels of starch in leaves of a variety of plants [58]. Starch over-accumulation was accompanied by the up-regulation of glucose-6-phosphate transporters and other enzymes associated with starch biosynthesis. Acquisition of nutrients from a host organism is essential for the survival and reproduction of pathogens and microbial strategies such as the targeting of sugar efflux transporters have evolved to gain access to the supply of glucose available in the host tissue [59]. The strong up-regulation of GPT genes suggests that similar mechanisms may be employed by Liberibacter to ensure its survival in the citrus host.

The gene most repressed in 'Cleopatra' leaves infected with *Las*, was that for a thioredoxin family protein. Besides playing an important role in redox-related responses to abiotic and biotic stresses [60], thioredoxins are involved in the mediation of post-translational activation of AGPase [56]. Thus, down-regulation of these proteins appears to be linked to the starch accumulation observed in HLB-affected citrus. This is in accordance with the observations of Ezquer et al. [58] who observed that microbial volatile-induced starch accumulation was accompanied by the down-regulation of thioredoxins and enzymes involved in starch breakdown. The involvement of thioredoxins in the regulation of plant disease resistance was demonstrated by Rivas et al. [61]. The interaction of these enzymes with other enzymes involved in ROS scavenging processes, such as superoxide dismutases [62], is illustrated by the similar expression profile obtained for superoxide dismutase CSD2. No genes were observed to be down-regulated by more than 4-fold in US-897 seedlings in response to infection with *Las*.

Among the few genes exclusively induced to high levels in US-897 seedlings upon infection with *Las* was a gene for a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase. 2OG and iron-dependant dioxygenases are involved in the biosynthesis of plant secondary metabolites such as flavonoids, hydroxyproline-rich proteins and gibberellins, though their exact biochemical function has not always been confirmed [63]. A recent study identified Fe(II)-and 2OG-dependant dioxygenase as the pivotal enzyme in the biosynthesis of the coumarin scopoletin in *Arabidopsis* [64]. Coumarins are widespread compounds in rutaceous plants, which include the genera *Citrus* and *Poncirus* [65,66]. Based on their induction following various stress events and their antimicrobial activities, coumarins are thought to be an important component of plant defense. Several other genes coding for 2OG and Fe(II)-dependent oxygenases were also observed to be induced in 'Cleopatra' upon infection and/or were expressed at much higher levels in US-897 independent of infection. These results suggest an important role for these enzymes in the defense response to *Las* and tolerance to HLB.

More than eight hundred genes showed transcription levels which were at least 4-fold higher in US-897 seedlings compared with 'Cleopatra' seedlings, independent of infection with *Las*. Genes for a progesterone-5β-reductase-like protein (VEP1) were amongst the nearly three hundred genes with more than 32-fold higher expression levels in the tolerant genotype. VEP1 (vein patterning 1), a cell death domain-containing cytosolic protein, was shown to be an important, wound-inducible regulator of vascular strand development in *Arabidopsis* [67]. Interestingly, the protein shows high similarity to *Digitalis lanata* progesterone-5β-reductase, a key enzyme involved in the biosynthesis of cardiac glycosides, which, besides being widely used as drugs for the treatment of cardiac failure, play a role in plant defense against herbivores [68,69]. The inducible expression of progesterone-5β-reductases in response to different stresses such as heat, ethylene and H_2O_2 was demonstrated by Pérez-Bermúdez et al. [69]. It is unclear whether VEP1 contributes to tolerance to HLB in US-897, but its involvement with vascular differentiation and cardenolide metabolism make it an interesting candidate for further studies.

Transcripts for a plastidic glucose transporter (GLT1) were more than 40-fold more abundant in US-897 seedlings independent of infection. In *Arabidopsis*, this transporter was shown to be involved in the export of photoassimilates from chloroplasts and plays an essential role in the transport of starch degradation products, plant growth and development [70]. The high expression levels for this gene in US-897 may be part of the reason this genotype, like other trifoliates and trifoliate hybrids, does not display the distinct disease symptoms associated with the starch accumulation usually observed in HLB-susceptible plants. Over-expression of this gene in combination with an HLB-inducible promoter may be one strategy to remediate the negative effect of HLB in citrus.

Prominent among the many genes with considerably higher expression levels in US-897 were several genes for a variety of different UDP-glycosyl transferases (UGT), some of which were moderately induced in 'Cleopatra' in response to infection. UGTs are members of the large family of glycosyltransferases which catalyze the transfer of UDP-activated sugars to low molecular weight substrates, including all major classes of secondary metabolites. Glycosylation allows the stabilization and storage of potent toxic metabolites in high concentrations within the cell, allowing the plant to release a battery of highly aggressive chemicals in response to attack by pathogens or herbivores [71]. Langlois-Meurinne et al. [72] demonstrated the importance of the glycosyltransferase genes UGT73B3 and UGT73B5 in resistance to *Pseudomonas syringae* pv *tomato* in *Arabidopsis*. Though not induced in response to *Las*, these genes were more highly expressed in US-897 compared with 'Cleopatra'. Down-regulation of a pathogen-responsive UGT reduced accumulation of the coumarin scopoletin and weakened virus resistance in tobacco, signifying a crucial role for this enzyme in plant defense [73]. It is likely that UGTs and other glycosyltransferases also play a role in the resistance or tolerance of trifoliate orange and its hybrids to CTV and HLB, thus making them potential candidates for genetic engineering technologies.

Within the family of genes for pathogenesis-related proteins, genes for an osmotin-like protein (OSM34) of the PR-5 family and for a plant defensin (PDF2.2) of the PR-12 family were expressed at higher levels in US-897 compared with 'Cleopatra' independent of infection. The gene for PDF2.2 was also significantly induced in infected 'Cleopatra' seedlings based on the results from microarray- and qRT-PCR analysis. Plant defensins are small, cysteine-rich peptides, which are generally involved in the defense against fungi, but have also been shown to exhibit activity against bacteria and insects [74]. The overexpression of different defensins resulted in

enhanced resistance of plants to a wide range of fungal species [75]. It remains to be seen if this group of antimicrobial peptides will also induce resistance to bacterial pathogens such as Liberibacter.

Quantitative RT-PCR experiments were performed with selected genes to validate the results of microarray analysis. Most of the genes showed very similar expression profiles in microarray and PCR analysis, although the extent of differential expression often varied. Discrepancies between both methods were observed for the genes for a vitamin C defective 2 (VTC2) protein and an oxidoreductase, zinc-binding dehydrogenase family protein. Microarray and qRT-PCR data often result in disagreement due to the fundamental differences between the two techniques, and filtering of the microarray data for measures of quality was shown to be the most critical factor [76]. Since genes in this study were filtered based on significant ($p \leq 0.05$) expression level differences of at least 4-fold after FDR correction, it is assumed that the observed discrepancies were the result of cross-hybridization of the array probes with other genes.

Additional qRT-PCR experiments were conducted on leaf samples from a second experiment involving the same genotypes and treatments. Though most of the selected genes showed similar expression profiles compared with experiment 1, several differences were observed, such as for one form of constitutive disease resistance CDR1 protein and for UDP-glycosyltransferase 73B3, which did not appear to be expressed at much higher levels in US-897 compared with 'Cleopatra', contrary to the observations from the first experiment. The reason for this is unclear, but may be associated with developmental differences of the plants in the two experiments. The genes for 2OG and Fe(II)-dependant oxygenase and for cytosolic superoxide dismutase CSD1 were not induced in infected US-897 plants, but much higher expressed in both non-infected and infected plants of this genotype compared with 'Cleopatra'. Stress, possibly caused by a nutritional treatment during the course of the experiment, is one possible explanation for these findings.

In conclusion, a large number of genes were found to be induced in susceptible 'Cleopatra' seedlings in response to infection with *Las*. Many of these were defense-related genes, demonstrating that even susceptible hosts initiate defense responses, however insufficient, to restrict multiplication and spread of the pathogen throughout the plant. In contrast, only few genes were induced in the tolerant genotype US-897. Yet, a large number of genes possibly involved in pathogen defense were expressed at much higher levels in this genotype independent of infection. It appears that tolerance of US-897 to *Las* is associated with the constitutively higher expression of defense-related or other genes rather than with an induced expression in response to bacterial infection. Expressing these genes using biotechnology approaches may be one strategy to counteract the detrimental effects of HLB in citrus. Further experiments using trifoliate orange and other trifoliate hybrids are in progress to clarify the role of the genes identified in this study in the defense against Liberibacter.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2011.09.008.

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