Journal of Experimental Botany, Vol. 63, No. 8, pp. 3307–3319, 2012
doi:10.1093/jxb/ers070 Advance Access publication 9 March, 2012
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Gene expression in *Citrus sinensis* fruit tissues harvested from huanglongbing-infected trees: comparison with girdled fruit

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Received 1 November 2011; Revised 8 February 2012; Accepted 13 February 2012

Abstract

Distribution of viable *Candidatus* Liberibacter asiaticus (*Ca*Las) in sweet orange fruit and leaves ('Hamlin' and 'Valencia') and transcriptomic changes associated with huanglongbing (HLB) infection in fruit tissues are reported. Viable *Ca*Las was present in most fruit tissues tested in HLB trees, with the highest titre detected in vascular tissue near the calyx abscission zone. Transcriptomic changes associated with HLB infection were analysed in flavedo (FF), vascular tissue (VT), and juice vesicles (JV) from symptomatic (SY), asymptomatic (AS), and healthy (H) fruit. In SY 'Hamlin', HLB altered the expression of more genes in FF and VT than in JV, whereas in SY 'Valencia', the number of genes whose expression was changed by HLB was similar in these tissues. The expression of more genes was altered in SY 'Valencia' JV than in SY 'Hamlin' JV. More genes were also affected in AS 'Valencia' FF and VT than in AS 'Valencia' JV. Most genes whose expression was changed by HLB were classified as transporters or involved in carbohydrate metabolism. Physiological characteristics of HLB-infected and girdled fruit were compared to differentiate between HLB-specific and carbohydrate metabolism-related symptoms. SY and girdled fruit were smaller than H and ungirdled fruit, respectively, with poor juice quality. However, girdling did not cause misshapen fruit or differential peel coloration. Quantitative PCR analysis indicated that many selected genes changed their expression significantly in SY flavedo but not in girdled flavedo. Mechanisms regulating development of HLB symptoms may lie in the host disease response rather than being a direct consequence of carbohydrate starvation.

Key words: *Candidatus* Liberibacter asiaticus, carbohydrate restriction, greening, microarray, symptom development.

Introduction

Huanglongbing (HLB or 'greening') is a destructive citrus disease caused by a fastidious, phloem-restricted bacterium, *Candidatus* Liberibacter spp. HLB is present in many major citrus-producing countries worldwide and affects all known citrus species. A few citrus species display differential susceptibility (Folimonova *et al.*, 2009), but their value for citrus improvement breeding has not been fully realized. Of the three species of HLB bacteria known (daGraça, 1991; Planet *et al.*, 1995; Teixeira *et al.*, 2005), only the Asian form (*Candidatus* Liberibacter asiaticus; *Ca*Las) has been confirmed in Florida (Manjunath *et al.*, 2008). *Ca*Las is transmitted via the Asian citrus psyllid and by grafting infected scions. Concern over transmission via seed was due to identification of *Ca*Las genomic material on and in seed removed from symptomatic fruit; however, seed as a viable pathway for HLB transmission remains uncertain (Albrecht and Bowman, 2009; Kim *et al.*, 2009; Hartung *et al.*, 2010; Hilf, 2011).

Symptoms of HLB have been well documented (Bové, 2006). Leaf symptoms include vein yellowing and blotchy mottle; leaf symptoms have been mistaken for minor nutrient deficiencies. Leaf size is reduced, and symptomatic leaves prematurely abscise at the laminar or petiole abscission zones. Twig dieback, tree decline, and tree mortality occur

several months to years after infection. Global gene expression changes in leaves assessed by microarray analysis indicated a large number of physiological processes impacted by HLB. Major changes in starch metabolism and assimilate transport were implicated (Albrecht and Bowman, 2008; Kim *et al.*, 2009). Genes encoding P-proteins markedly accumulated. Such proteins function to maintain sieve plate turgor pressure after injury (Knoblauch and van Bel, 1998, Knoblauch *et al.*, 2001) and may participate in the formation of host-derived callose that blocks photosynthate transport in HLB-infected vascular tissue. Vascular blockage leads to massive starch accumulation in symptomatic HLB leaf tissue (Etxeberria *et al.*, 2009). Phloem plugging in plasmodesmata connecting companion cells and sieve elements and pores of sieve tubes, excessive storage of fixed carbon in the form of starch in leaves, and destruction of the photosynthetic apparatus (Albrecht and Bowman, 2008; Sagaram and Burns, 2009; Koh *et al.*, 2011) in leaf tissues could limit assimilate transport and impact symptom development.

In contrast to leaf studies, little is known about global gene expression changes associated with HLB in fruit tissues. Fruit symptoms are readily distinguishable but non-specific (Bové, 2006). Symptomatic fruit are located on symptomatic branches, canopy sectors, or whole tree canopies. Such fruit are lopsided and small. Colour development is poor and may only 'break' on the stem end, leaving the majority of the fruit surface green. HLB-impacted fruit have an altered carbohydrate and phytohormone balance (Rosales and Burns, 2011). The fruit abscission zone located at the pedicel–fruit interface can be orange in colour, and columella vascular bundles are brown. Symptomatic fruit abscise prematurely. Seed abortion is common. If seed from symptomatic fruit are filled or partially filled, they are dark in colour. Early reports of juice quality impacts indicated that juice from HLB fruit was bitter and had numerous off-flavours (McClean and Schwartz, 1970; daGraça, 1991; Stokstad, 2006). Juice of symptomatic mature fruit was lower in juice percentage, °Brix, and °Brix/% acid ratio, and higher in acidity compared with asymptomatic and healthy fruit (Dagulo *et al.*, 2010; Plotto *et al.*, 2010). Such a juice profile is similar to that of immature fruit. Bitter flavonone compounds limonin, nomalin, some terpenes, and linalool were significantly higher in juice from HLB-infected fruit. The appearance and juice quality of asymptomatic fruit were similar to those of healthy fruit.

*Ca*Las was unequally distributed in the phloem of infected plants, with the highest titre found in the pedicel (Tatineni *et al.*, 2008). Although bacterial aggregates do not plug sieve elements (Kim *et al.*, 2009), the presence of a high titre in the pedicel suggests that the fruit vasculature could be blocked by callose. As a result, carbohydrate and nutrient supply to this sink tissue could be restricted and contribute to symptom development. In this work, fruit characteristics, physiological changes, and gene expression were compared in healthy, asymptomatic, and symptomatic citrus fruit tissues in two commercially important citrus cultivars to determine if measured changes could be associated with HLB infection, and symptom and biomarker development. HLB-associated changes in fruit tissues with girdling were also compared to determine if fruit symptoms were specific to HLB or generally related to restricted carbohydrate movement.

Materials and methods

Plant materials

Microarray analysis: Healthy (H), symptomatic (SY), and asymptomatic (AS) fruit samples were collected from 18-year-old 'Hamlin' and 'Valencia' sweet orange trees grafted on 'Swingle' citrumelo growing in the field in Lake Placid, FL, USA. Trees were randomly selected in a 30 acre block. Quantitative real-time PCR (qRT-PCR) analysis was performed in fruit and leaf tissues to determine the presence or absence of *Ca*Las as described below and by Li *et al.* (2006). H was collected from qRT-PCR-negative trees. SY and AS were selected from symptomatic trees (qRT-PCR-positive trees). Selection of SY and AS in the field was by visual observation. SY were smaller in size, poorly coloured, and misshapen, while AS were visually similar to H in size, colour, and shape. AS were frequently located on asymptomatic branches or canopy sectors of qRT-PCR-positive trees. Four replicate qRT-PCR-positive and -negative trees were selected and 25 fruit from each were harvested. 'Hamlin' and 'Valencia' fruit were harvested on 13 December 2007 and 1 April 2009, respectively. Fruit tissues removed from the 25 fruit sample/tree were pooled into a single replicate sample. Juice vesicle tissue (JV), vascular tissue (VT), and fruit flavedo (FF) were removed from each fruit and pooled. Fruit were sliced at the equator and JV removed with a knife. To collect VT, ~1.5 cm of vascular tissue immediately distal to the calyx abscission zone was removed. Once excised, contaminating albedo was removed with a razor blade. FF was removed from the fruit equatorial region using a kitchen-type potato peeler. Contaminating albedo was trimmed away from FF and discarded.

Girdling experiments

Bark was removed on PCR-negative 'Hamlin' trees grafted on 'Swingle' rootstock in groves located in Lake Alfred, FL near the end of stage II fruit growth (Pozo and Burns, 2009) in July 2010. Twigs with a single subtending fruit were selected for girdling. Bark to be removed was located ~10 cm proximal to the fruit. An 8 mm wide section of bark fully (fully girdled or FG) or half encircling (half-way girdled or HG) the circumference of the twig was removed. Leaves between the girdle and fruit were removed. Ungirdled (UG) fruiting twigs with a single subtending fruit served as controls. Four biological replicates composed of two trees each (four fruit per replicate) were harvested on 8 December 2010.

Titre determination

Fruit and leaf tissues were collected from 25 randomly selected qRT-PCR-positive symptomatic or asymptomatic, or PCR-negative healthy branches or trees. VT, JV, and FF were collected as described above. Fruit abscission zones (FAZs) were removed using a 4 mm diameter cork borer. The borer was slipped over the pedicel and pushed through the calyx and fruit peel. The FAZ location was visually determined, and trimmed to 6 mm in length by 4 mm in width using a razor blade. For the fruit pedicel (FPD), ~1 cm of tissue proximal to the FAZ was collected. Leaf midribs (LMs) were collected using a scalpel to remove the leaf blade (LB) and petiole. LB was collected and the petiole was discarded. A leaf with a branch attached was used for laminar abscission zone (LAZ) and petiole abscission zone (PAZ) collection. Approximately 4 mm thick LAZs and PAZs were excised using a razor blade. Filled and collapsed seeds were collected as described below. In all cases, tissues were frozen in liquid nitrogen after excision and stored at –80 °C until needed.

Measurement of fruit characteristics

To determine fruit characteristics at harvest, fruit diameter and weight, pedicel diameter, seed morphology, and juice quality (% juice; °Brix/% acid) were analysed. Juice was extracted from fruit by hand and analysed as described (Dagulo *et al.*, 2010). Filled seeds, collapsed seeds, and aborted seeds were removed. Seeds were classified visually by size and colour. Filled seeds were covered with a thick outer seed coat. Amber-coloured seeds that were partially filled were defined as collapsed. Aborted seeds were devoid of endosperm and embryo, dark in colour, flat in shape, and small in size. Starch, sucrose (Rosales and Burns, 2011), and chlorophyll *a*/*b* measurements were performed as described (Mackinney, 1941). Peel colour measurement and extraction and quantification of total carotenoids were performed following the method of Alferez *et al.* (2006).

Genomic DNA and RNA extraction, qRT-PCR analysis, and gene selection for girdling versus HLB comparisons

Total genomic DNA and RNA were extracted and the reverse transcription procedure for total RNA was performed according to Liao and Burns (2010). A qRT-PCR-based approach that targeted the bacterial 16S RNA (Li *et al.*, 2006) was utilized for *Ca*Las detection and quantification. To quantify viable bacteria in tissues, total genomic DNA (0.1 μg) and RNA (0.02 μg) were used as templates in 25 μl reactions. The constitutively expressed cytochrome oxidase (COX) gene was used as the internal calibrator in multiplex reactions. Primer and probe sequences were designed according to Li *et al.* (2006). TaqManUniversal Master Mix II (Applied Biosystems, Foster City, CA, USA) was used for the reaction, and qRT-PCRs were performed using a 7500 Fast Real-Time PCR system (Applied Biosystems) as described by Liao and Burns (2010). The *Ca*Las concentration was quantified (cells per μg of total DNA) according to Tatineni *et al.* (2008). Bacterial 16S rRNA expression was estimated relative to the sample with the lowest expression.

As the greatest shift of gene expression occurred in SY FF (see the Results), a comparison was performed using this gene set to differentiate expression of unique genes in HLB-impacted tissues from that in girdled tissues. Fifteen genes were selected according to the following criteria. First, only the genes shared in SY FF of 'Hamlin' and 'Valencia' with gene expression changes of ⩾8.0 were considered. There were 40 candidate genes in total (Supplementary Table S1 available at *JXB* online). Secondly, gene sequences had to be identical to the functional homologues demonstrated by published research. Out of 40 genes, 27 candidate genes fit this criterion. Thirdly, selected genes had to respond to pathogen infection and/or girdling as indicated by published research. Out of 27 genes, 15 genes fit this criterion. These 15 genes included six genes involved in disease or defence response [*CsSULF* (Howarth *et al.*, 2003), *CsSUR2* (Krinke *et al.*, 2009), *CsNCED* (Fan *et al.*, 2009), *CsLHCB* (Mur *et al.*, 2010), *CsELIP* (Wierstra and Kloppstech, 2000; Dietrich *et al.*, 2010), and *CsATC* (Ray *et al.*, 2003)]. Six genes played a role in regulating starch metabolism, a pathway shown to be affected by girdling (Li *et al.*, 2003): *CsSB1*, *CsSB2*, *CsSD1*, *CsSD2*, *CsSD3*, and *CsSD4*. Finally, three genes were reported to be involved in disease responses (Fischer and Bennett, 1991; Chen *et al.*, 2003; Balaji *et al.*, 2008) and changed by girdling (Murayama *et al.*, 2006): *CsPG*, *CsACO*, and *CsACS1*.

FF gene expression was determined using qRT-PCR. Specific primer sets (Supplementary Table S2 at *JXB* online) used were designed according to the partial cDNA sequences cloned from *Citrus sinensis* 'Hamlin' or 'Valencia'. qRT-PCRs were performed using SYBR®Green PCR Master Mix (Applied Biosystems), and gene expression was analysed according to Liao and Burns (2010). Sequences of partial genes and the qRT-PCR amplicons were highly similar to the specific genes in the NCBI database. Fruit tissue gene expression was estimated relative to the expression level of each gene in H samples.

Microarray analysis

Global gene expression in FF, VT, and JV tissue from SY, AS, and H was analysed using an Affymetrix subgenomic array containing 30,279 expressed sequence tags (ESTs) from various *Citrus* species (Affymetrix, Santa Clara, CA, USA). cDNA generation, array analysis, and statistical tests were performed as a service at the Interdisciplinary Center for Biotechnology Research Microarray Core facility at the University of Florida (Gainesville, FL, USA). ESTs with significant expression changes (*P*-value <0.001; false discovery rate ⩽0.01 with ⩾2-fold changes in expression) were selected for further analysis. EST identities were confirmed using BLAST at the NCBI. Functional assignment of identified genes was accomplished using the Plant Metabolic Network (http://www.plantcyc.org:1555/ARA/class-tree?object=Pathways) service, KEGG (http://www.genome.jp/kegg/pathway.html), and Pathway Studio 7 package (Ariadne, Rockville, MD, USA). The R statistical package was used for statistical analysis of data presented in Tables 2 and 5. A subgenomic array file containing original data was uploaded to the Gene Expression Omnibus website (GEO). Detailed results of the microarray experiments are listed in Supplementary Table S3 at *JXB* online.

Results

Candidatus *Liberibacter asiaticus* (CaLas) titre

To determine viable bacteria in fruit and leaf tissues, qRT-PCR analysis was performed that targeted 16S rDNA or 16S rRNA of *Ca*Las. The *Ca*Las concentration was proportional to the relative expression of 16S rRNA in all tissues tested (data not shown), indicating that relative expression of 16S rRNA was sufficient to estimate the population of viable bacteria in fruit and leaf tissues. *Ca*Las was unevenly distributed in SY and AS fruit and leaf tissues harvested from SY 'Valencia' orange trees (Table 1; Tatineni *et al.*, 2008). *Ca*Las was not detected in apparently H trees. SY tissues had a higher *Ca*Las titre than AS when it was detected. *Ca*Las was found in all SY tissues examined except

Table 1. Detection of CaLas 16S rRNA from fruit and leaf tissues of 'Valencia' using qRT-PCR

	Fold expression ratio of *Ca*Las 16S rRNA, mean ±SD		
	H	**AS**	**SY**
Leaf tissues			
Leaf midribs (LM)	ND (0)	9.1a (2)	234.2 a (12)
Laminar abscission zone (LAZ)	ND (0)	0.04 c (1)	2.24 b (6)
Petiole abscission zone (PAZ)	ND (0)	0.1 b (6)	99.4 a (16)
Fruit tissues			
Fruit pedicel (FPD)	ND (0)	100 a (8)	1181.4 b (25)
Fruit abscission zone (FAZ)	ND (0)	160 a (10)	3157.2 a (25)
Vascular tissue (VT)	ND (0)	194.0 a (7)	2032.1 ab (25)
Juice vesicle (JV)	ND (0)	ND (0)	0.6 d (4)
Fruit flavedo (FF)	ND (0)	13.6 b (3)	29.2 cd (6)
Filled seeds	ND (0)	ND (0)	ND (0)
Collapsed seeds	ND (0)	ND (0)	107.0 c (8)

ND, not detected. Numbers in parentheses indicate the number of trees within a 25 tree total that were PCR positive.
Values within a column for each organ followed by the same letters were not significantly different as determined by Duncan's multiple range test; $P < 0.01$.

filled seeds. In AS tissues, PCR verified the presence of *Ca*Las in LM, FPD, FAZ, VT, and FF. A higher bacterial titre was found in VT, FPD, and FAZ compared with other SY or AS tissues. In some cases, PCR failed to detect the presence of *Ca*Las in SY or AS midrib tissue, even though visual inspection indicated that trees were infected. To overcome this problem, leaf midribs and FPD tissues in each 'Hamlin' and 'Valencia' orange replicate tree were routinely checked for the presence of *Ca*Las for experiments described below. In cases where PCR failed to detect *Ca*Las in SY leaf midribs, the organism was always found in FPD.

Comparison between HLB-impacted and girdled fruit

HLB impacted fruit characteristics, seed number, flavedo carbohydrate content and pigmentation, and juice quality of 'Hamlin' and 'Valencia' oranges. Fruit diameter and weight, pedicel diameter, and flavedo carbohydrate content were significantly reduced in SY (Table 2). Seed contained within H, AS, and SY fruit differed in number and type. In both cultivars, decreasing numbers of filled seeds were found in AS, and SY fruit. The number of amber-coloured collapsed seed was significantly greater in SY than in H or AS fruit. Aborted seed number was not significantly different but was numerically higher in SY fruit. Flavedo pigmentation was also altered. SY fruit were greener, as indicated by a significantly lower chlorophyll *a*/*b* ratio. The chlorophyll content was higher and the total carotenoid content significantly lower in SY as compared with AS and H, except in 'Hamlin', where the chlorophyll *b* content in AS fruit was numerically lower than in SY. Significant changes in pigmentation were measured in 'Hamlin' between H and AS fruit, whereas these changes were observed between AS and SY in 'Valencia'. Juice quality was impacted by HLB. The percentage juice in SY 'Hamlin' fruit did not change in HLB-impacted fruit, but was lower in 'Valencia' SY. In 'Hamlin', SY juice was lower in °Brix than in H or AS, but the percentage acid was unchanged; consequently, the °Brix/% acid ratio was lower in SY juice when compared with H. In contrast, the percentage acid was significantly higher in SY 'Valencia' juice, but the effect on the °Brix/% acid ratio was similar to that of 'Hamlin'.

Visual appearance and characteristics of HLB-impacted fruit and girdled fruit were compared. SY were small, poorly coloured, misshapen, and contained aborted seeds (Bové, 2006; Table 2; Fig. 1A). Fruit from FG stems were similar in size to SY fruit, but FG were greener and not misshapen (Table 2; Fig. 1A, B). Visual differences were not apparent between H and AS or UG and HG fruit. SY and FG fruit were lower in seed number, starch, and sugar, and had changes in juice quality similar to H or UG fruit, respectively. Carotenoid content was reduced in SY when compared with H, but increased in FG flavedo when compared with UG.

Table 2. Fruit characteristics, seed number, flavedo carbohydrate content and pigmentation, and juice quality in apparently healthy, HLB-infected, and girdled fruit tissues

	Hamlin (HLB)			Valencia (HLB)			Hamlin (girdled)		
	H	AS	SY	H	AS	SY	UG	HG	FG
Fruit characteristics									
Fruit diameter (mm)	71.5 a	68.8 a	53.2 b	73.7 a	76.5 a	58.4 b	64.4 a	64.8 a	50.5 c
Fruit weight (g)	194.3 a	196.6 a	109.9 b	208.5 a	214.5 a	122.3 b	134.1 a	137.1 a	67 c
Pedicel diameter (mm)	3.7 a	3.6 a	3 b	3.7 a	3.7 a	3.1 b	3.2 a	3.2 a	3 a
Seed number/fruit									
Filled seeds	11 a	4.2 b	0.3 c	9 a	3.8 b	0.7 c	7.9 a	6.5 a	0 b
Collapsed seeds	0 b	0 b	1.6 a	0 b	0 b	1.8 a	0 b	0 b	2.6 a
Aborted seeds	1.1 a	0.7 a	2.5 a	1.3 a	0.8 a	2 a	2.9 a	2.7 a	2.6 a
Flavedo carbohydrate content									
Starch (mg g^{-1} DW)	22.8 a	19.1 a	3.8 b	32.4 a	30.4 a	4.4 b	19.9 a	21.3 a	3.2 b
Sugar (mg g^{-1} DW)	246 a	212.4 a	39.1 b	70.6 a	68.4 a	17.9 b	205.6 a	196.4 a	34.5 b
Flavedo pigmentation									
a/*b* ratio	0.18 a	0.17 a	–0.2 b	0.18 a	0.19 a	–0.12 b	0.06 a	0.03 a	–0.34 b
Chlorophyll *a* (μg g^{-1} FW)	7.6 c	17.2 b	36.9 a	7.9 b	7.9 b	21.5 a	7 b	14.9 b	151.5 a
Chlorophyll *b* (μg g^{-1} FW)	11.4 b	17.4 ab	35.8 a	5.2 b	4.9 b	15.7 a	9.5 b	15.8 b	122.9 a
Total chlorophyll (μg g^{-1} FW)	19 c	34.6 b	72.7 a	13.1 b	12.8 b	37.2 a	16.5 b	30.7 b	274.4 a
Total carotenoid (μg g^{-1} FW)	26.1 a	12.9 b	15.4 b	53.1 a	58.9 a	32.1 b	14.7 b	15.1 b	23.5 a
Juice quality									
% Juice	52.1 a	49.9 a	48.8 a	53.2 a	52.9 a	46.1 b	44.7 a	47.2 a	40.5 a
°Brix	11.3 a	11.5 a	9.1 b	11.6 a	11.2 a	9.3 b	12 a	11.6 a	8.6 b
% acid	0.75 a	0.8 a	0.78 a	0.85 b	0.85 b	0.91 a	0.72 b	0.75 b	1.02 a
°Brix/% acid	15.1 a	14.3 ab	11.7 b	13.5 a	13.1 a	10.2 b	16.9 a	15.6 a	8.6 b

H, healthy; AS, asymptomatic; SY, symptomatic; UG, ungirdled; HG, fruiting twig girdled half way around its circumference; FG, fruiting twig fully girdled around its circumference.
% Juice is the percentage ratio of total juice weight and fruit weight.
Values within a row and cultivar followed by the same letters were not significantly different as determined by Duncan's multiple range test; $P < 0.01$.

Number and functional identification of HLB-responsive ESTs in fruit tissues

Notable changes in transcript levels were measured in HLB-impacted fruit tissues. When SY and H were compared in 'Hamlin', the number of genes changing expression in FF and VT was greater when compared with JV (Fig. 2A). In contrast, the number of genes changing expression in 'Valencia' was similar in the three tissues. The number of genes changing in 'Valencia' JV in response to HLB was greater than in 'Hamlin'. Since 'Valencia' AS tissues were collected, additional comparisons were made in this cultivar. More FF and VT genes changed expression when comparing H with AS, whereas more JV genes changed expression when comparing AS with SY (Fig. 2B). Very few JV ESTs changed when comparing AS with H.

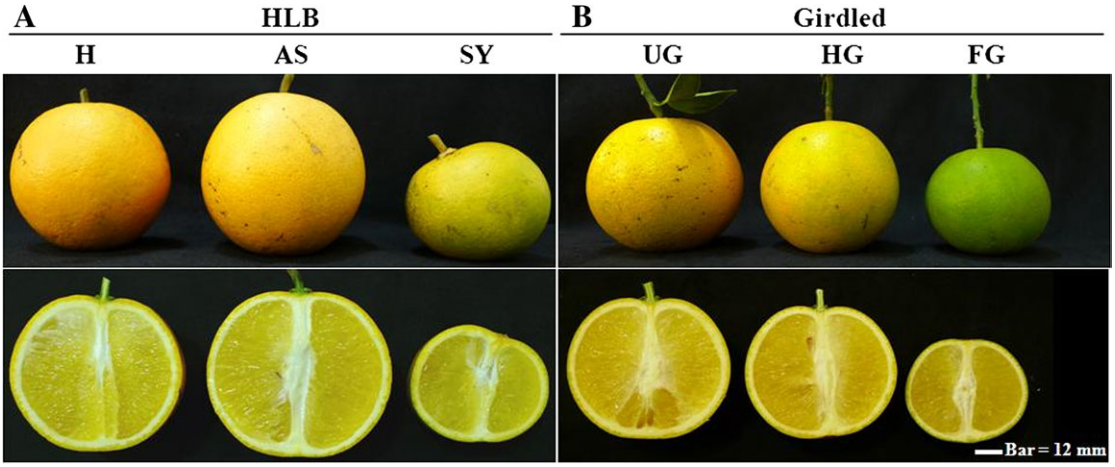

Fig. 1. HLB-infected (A) and girdled (B) fruit from 'Hamlin' trees. Healthy, H; asymptomatic, AS; symptomatic, SY; ungirdled, UG; fruiting stem girdled half way around its circumference, HG; fruiting stem fully girdled around its circumference, FG.

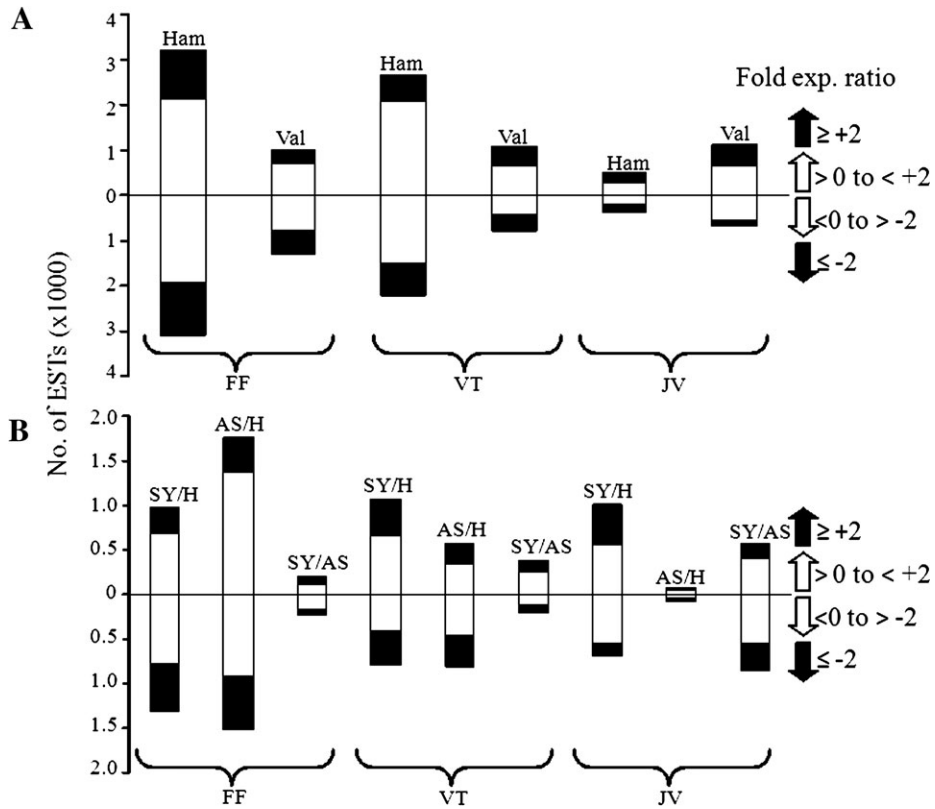

Fig. 2. EST expression in HLB-affected fruit tissues. Changes in EST expression with false discovery rate ⩽0.01 and *P*-value $<1\times10^{-3}$ are presented. Bars above and below the zero line represent induced and repressed ESTs, respectively. Black and white bars indicate the number of transcripts that changed ⩾2-fold or between 0 and <2-fold, respectively. (A) EST number comparison of symptomatic (SY) flavedo (FF), vascular tissue (VT), and juice vesicle (JV) tissues compared with healthy (H) controls in 'Hamlin' (Ham) and 'Valencia' (Val); and (B) EST number comparison between SY, asymptomatic (AS) and H FF, VT, and JV tissues in Val.

Predicted functional analysis was performed to assign ESTs to transcripts that were significantly induced or repressed, and to characterize metabolic shifts due to HLB. Functionally identified genes were grouped into functional categories. In 'Hamlin' and 'Valencia', 25 major categories (Fig. 3) were identified that represented >180 unique metabolic pathways and protein groups (Supplementary Table S3 at *JXB* online). Functional groups with the highest number of genes changing expression in response to HLB included those encoding proteins associated with various transporters, carbohydrate metabolism, genetic information processes, phytohormone metabolism, defence responses, photosynthesis/light signalling, and stress response/senescence. Over 160 genes had open reading frames with unidentified function and grouped as unidentified proteins.

Shared genes impacted by HLB in FF, VT, and JV

To determine common genes changing expression in HLB-impacted tissues, shared genes in VT and FF whose expression changed ⩾6-fold in at least one of the tissues of either cultivar were compiled (Table 3). In FF and VT, genes that regulate transport systems for sugar and zinc were decreased, while cyclic nucleotide and sulphate transporter gene expression was increased. Expression of genes involved in starch biosynthesis was down-regulated, whereas invertase inhibitor, trehalose biosynthesis, and inositol metabolism gene expression increased. Genes for UDP-glucose metabolism were affected. Differential regulation of genes for phytohormone-related pathways including auxin, gibberellins, abscisic acid, jasmonate, and ethylene metabolism was observed. Wounding- and senescence-related genes increased expression. Up-regulation of four genes for photosystem I (PSI)PSI/II subunits occurred in HLB-impacted FF and VT. Genes responsible for electron transfer and light signalling were affected. Genes for pattern establishment, cell wall metabolism, and cutin/wax transportation were significantly shifted, as were genes for fatty acid reduction (fatty acyl-CoA reductase) and lipid biosynthesis and metabolism. Asparagine synthetase expression was up-regulated. Two genes associated with reactive oxygen species (ROS) scavenging and antioxidant networks during oxidative stress were up-regulated. Genes involved in pigment metabolism were also differentially expressed, including increased expression of two genes for light-harvesting complexes (LHCB6 and LHCA1) and decreased expression of two genes that regulate flavonoid biosynthesis. Finally, genes encoding NAD(P)H dehydrogenase that are responsible for electron transport in mitochondria were down-regulated.

To determine common gene changes in HLB-impacted JV, a table of shared genes was compiled whose expression changed ⩾2-fold (Table 4). Included in these were genes whose encoded proteins were associated with transport, carbohydrate and phytohormone metabolism, disease resistance, photosynthesis and chlorophyll development, cell development and cell wall metabolism, lipid metabolism, ROS, flavonoid and terpene biosynthesis, and conversion of aldehydes, alcohols, and esters. A comparison of genes listed in Tables 3 and 4 revealed that JV, VT, and FF had 12 genes in common (Table 4). If the stringency of VT and

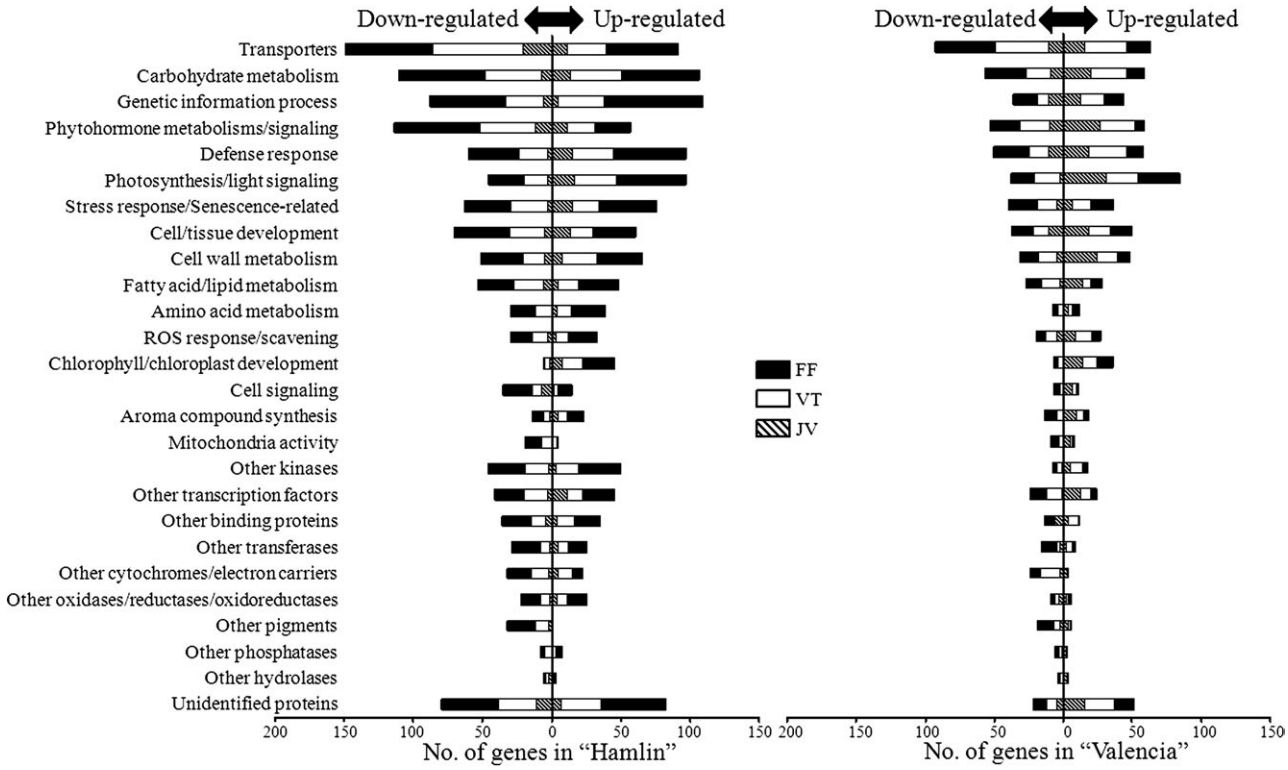

Fig. 3. Number of HLB-affected genes of symptomatic (SY) fruit tissues in *Citrus sinensis* cv. 'Hamlin' (left panel) and 'Valencia' (right panel) sorted by functional category. Flavedo, FF (black bars); vascular tissue, VT (white bars); juice vesicle tissue, JV (striped bars).

Table 3. The 47 shared genes significantly changed in symptomatic VT and FF by HLB infection

Putative function (citrus gene)	Fold change				Affymetrix ID
	Hamlin		Valencia		
	VT	FF	VT	FF	
Transporters/transportation systems					
Glucose-6-P transporter	–6.2	–14	–2	–2	Cit.9620.1.S1_s_at
Carbohydrate/sugar:H symporter	–10	–2	–2	–3.5	Cit.37918.1.S1_at
Sugar transporter	–8.4	–2.2	–2.1	–4.8	Cit.29937.1.S1_s_at
Zinc transporter	–6.3	–2	–2	–3.6	Cit.11460.1.S1_at
Cyclic nucleotide gated channel	7.2	8.6	4.8	2	Cit.20819.1.S1_at
Sulphate transporter 3;5 (*CsSULF*)	11.4	39.8	2	3.6	Cit.20694.1.S1_s_at
Carbohydrate metabolism					
Cell wall/vacuolar inhibitor of fructosidase	7	7	2	2	Cit.17506.1.S1_at
Glucose-1-P adenylyltransferase (*CsSB1*)	–2	–8	–2.7	–2.5	Cit.13437.1.S1_s_at
1,4-α-Glucan branching enzyme	–2.4	–6	–2	–2	Cit.3957.1.S1_s_at
UDP-D-glucose/UDP-D-galactose 4-epimerase	4	6	2.6	4.8	Cit.30402.1.S1_at
UDP-glucose 6-dehydrogenase	–3.9	–12	–2	–2	Cit.6084.1.S1_s_at
Trehalose phosphatase/synthase 6	2	6.3	2	2	Cit.17244.1.S1_s_at
Myo-inositol oxygenase	7.3	10.7	2.1	2	Cit.10133.1.S1_s_at
Phytohormone metabolism/signalling					
Cytochrome P450 monooxygenase 83B1 (*CsSUR2*)	4.1	17	2.4	3.9	Cit.25089.1.S1_at
Auxin/aluminium-responsive protein	7.5	12.2	2.9	2	Cit.13706.1.S1_s_at
Gibberellin-regulated family protein	–2.4	–23	–2.4	–2	Cit.9890.1.S1_s_at
Nine-*cis*-epoxycarotenoid dioxygenase (*CsNCED*)	–2	–8.9	–2	–5.5	Cit.8156.1.S1_at
12-Oxophytodienoate reductase	–2.2	–15	–2	–2	Cit.10684.1.S1_at
ACC oxidase (*CsACO*)	2	–8	3.2	–3.5	Cit.21723.1.S1_s_at
Disease-responsive/senescence-related					
Wound-responsive family protein	5.2	8.7	2.6	2.2	Cit.31091.1.S1_at
Senescence 1; dark inducible1	2	7.9	2	3.6	Cit.21724.1.S1_at
Photosynthesis/photorespiration/light signalling					
Photosystem II subunit Q1	3.8	7.7	2.2	3.7	Cit.9960.1.S1_s_at
Photosystem I subunit E-2	3.9	6.2	2.6	2.2	Cit.1842.1.S1_s_at
Photosystem I subunit D-2	3.4	7.5	3.3	2.8	Cit.8947.1.S1_s_at
Photosystem I subunit D-1	2.8	6	4.4	2.3	Cit.8928.1.S1_s_at
Proton gradient regulation 5	–4	–14	–6.4	–7.1	Cit.2850.1.S1_s_at
Late elongated hypocotyls; DNA binding/transcription factor	–3.3	–6.4	–3.3	–4.3	Cit.29406.1.S1_s_at
Cell/tissue development and cell wall metabolism					
Vein patterning 1	–6.2	–4.4	–2	–4.6	Cit.3107.1.S1_s_at
Maternal effect embryo arrest 14	3.1	7.6	2.1	2.7	Cit.18537.1.S1_at
Polygalacturonate 4-α-galacturonosyltransferase	–4	–6	–2	–2	Cit.28150.1.S1_at
Pectinesterase inhibitor	7	7	2	2	Cit.17506.1.S1_at
Cutin/wax transporter	–15	–3.8	–11	–10	Cit.25840.1.S1_s_at
Fatty acid/lipid and amino acid metabolism					
Fatty acyl-CoA reductase; alcohol-forming	–2	–6	–2.3	–2.8	Cit.4931.1.S1_s_at
Monogalactosyldiacylglycerol synthase 2	–6.8	–2	–2.6	–2.4	Cit.21988.1.S1_s_at
Lipase	5.5	17.5	4.5	3.5	Cit.16331.1.S1_s_at
Sterol methyltransferase 1	–2.2	–6	–2.2	–3.6	Cit.10660.1.S1_at
Asparagine synthetase	2.4	19	2.2	3.2	Cit.10060.1.S1_s_at
ROS scavenging and antioxidants					
Thioredoxin	5.7	7.4	4.2	2.5	Cit.20400.1.S1_s_at
Protein disulphide isomerase (*CsATC*)	8.6	31.5	4.7	3.9	Cit.31451.1.S1_s_at
Chlorophyll development					
Light-harvesting complex LHCB6 (*CsLHCB*)	2.5	9.7	3.1	3.1	Cit.37563.1.S1_s_at
Light-harvesting complex LHCA1	2.3	6.7	3.9	2.8	Cit.10648.1.S1_s_at
Oxidoreductase/protochlorophyllide reductase	2.7	7.5	3.8	3.2	Cit.9939.1.S1_s_at
Early light-inducible protein (*CsELIP*)	–6.4	–8	–5.5	–5.2	Cit.19769.1.S1_x_at
Flavonoid biosynthesis					
4-Coumarate-CoA ligase	–3.5	–2.8	–2	–6.1	Cit.25648.1.S1_s_at

Table 3. *Continued*

Putative function (citrus gene)	Fold change				Affymetrix ID
	Hamlin		Valencia		
	VT	FF	VT	FF	
p-Coumarate 3-hydroxylase	–2.3	–3.6	–2.9	–13	Cit.10353.1.S1_at
Mitochondrial activity					
NAD(P)H dehydrogenase A	–6.5	–3.8	–2.4	–3.6	Cit.9327.1.S1_at
NAD(P)H dehydrogenase B	–6.1	–3.8	–2.4	–3.5	Cit.18517.1.S1_s_at

Only those genes whose expression changed ⩾6.0 in at least one of the tissues of either cultivar are presented.
Up-regulated (positive) and down-regulated (negative) gene expression patterns compared with the healthy control are shown.

FF comparisons was reduced to 2-fold, only four additional genes would be shared between the three tissues (data not shown). These were the genes for fructosidase, auxin/aluminium-responsive protein, CCR-like senescence-associated protein, and PSII subunit P.

Comparison of expression between HLB-impacted and girdled flavedo

qRT-PCR was performed to differentiate expression of genes in HLB-impacted tissues from that in girdled tissues. Of 15 genes selected, significant expression changes in 10 genes were observed in SY but not in FG flavedo when compared with the healthy or ungirdled controls, respectively (Table 5). Those genes included a transporter (*CsSULF*), those involved in carbohydrate metabolism (*CsSB1*, *CsSB2*, and *CsSD1*), phytohormone metabolism (*CsSUR2*, *CsNCED*, and *CsACS1*), chlorophyll degradation (*CsELIP*), cell wall metabolism (*CsPG*), and ROS scavenging (*CsATC*). Expression of a gene for ethylene biosynthesis, *CsACO*, was down-regulated in SY flavedo but it was up-regulated in FG flavedo when compared with control. Shared genes whose expression changed in a similar way included α-amylase 3 (*CsSD2*), β-amylase 9 (*CsSD3*), β-amylase 8 (*CsSD4*), and LHCB6 (*CsLHCB*).

Discussion

In this study, qRT-PCR targeting a *Ca*Las 16S rRNA template (Kim and Wang, 2009) was used to estimate the quantity of HLB bacteria and differentiate viable from non-viable bacterial cells in HLB-affected tissues of citrus. Living bacteria existed in most HLB-infected fruit tissues examined, but was significantly higher in tissues surrounding the fruit abscission zone. Such tissues are rich in vascular tissue where *Ca*Las is restricted (Bové, 2006; Shokrollah *et al.*, 2010). As distances from the fruit abscission zone increased, *Ca*Las titre diminished. *Ca*Las was poorly detected in fruit tissues with limited fruit vascular connection and direct phloem loading activity such as juice vesicle tissue and filled seed (Koch and Avigne, 1990). Practical *Ca*Las detection may be more successful if fruit tissues closest to the fruit/pedicel interface are utilized for qRT-PCRs.

HLB impacts on fruit characteristics, seed number and condition, flavedo carbohydrate content and pigmentation, peel colour, fruit size, and effects on juice quality are known (Bové, 2006; Baldwin *et al.*, 2009; Bassanezi *et al.*, 2009; Etxeberria *et al.*, 2009; Dagulo *et al.*, 2010). Pigment changes and transition in seed condition measured in AS required destructive sampling methods, making these characteristics poor choices for practical early HLB detection. With the remaining fruit characteristics, significant changes were only measured in SY. Reduced fruit size, misshapen fruit, and colour change remain important non-destructive diagnostic characteristics for identifying HLB-impacted fruit.

Physiological aberrations that underlie HLB symptom development remain uncertain. Phloem plugging leads to starch accumulation in leaves and destruction of the photosynthetic apparatus (Albrecht and Bowman, 2008; Etxeberria *et el.*, 2009; Kim *et al.*, 2009; Sagaram and Burns, 2009). Reduction in flavedo starch and sucrose content in symptomatic HLB fruit (Rosales and Burns, 2011) occurs due to depletion of reserves and restriction of transported carbohydrate from source leaves. Reduction and restriction of carbohydrate movement into citrus fruit has negative consequences for fruit growth (Gomez-Cardenas *et al.*, 2000). There was a close correlation between pedicel diameter and fruit size, with fruit growth rate depending on the capacity of vascular tissue to deliver phloem-transported materials to the fruit (Bustan *et al.*, 1995). When vascular tissue transport is blocked at the pedicel, the response is to rebalance supply and demand in affected fruit by mobilizing its starch reserves. Once depleted, fruit growth is arrested.

Restricting carbohydrate movement into fruit by fully girdling fruiting twigs (Goren *et al.*, 2004) caused fruit to display similar symptoms to HLB, with notable exceptions. First, total carotenoid content was lower in SY but higher in FG when compared with H and UG, respectively. Rivas *et al.* (2011) reported increased synthesis and accumulation of the carotenoid pool in leaves of girdled branches, pointing to a girdling affect. Secondly, girdled fruit were not misshapen, even if the fruiting stem was girdled only halfway around its circumference. Flavedo isolated from

Table 4. The 48 shared genes significantly changed in symptomatic 'Hamlin' and 'Valencia' JV by HLB infection

Putative function (citrus gene)	Fold change		Affymetrix ID
	Ham JV	Val JV	
Transporters/transportation systems			
Glucose-6-P transporter[a]	–3.3	–3.3	Cit.9620.1.S1_s_at
Carbohydrate/sugar:H symporter[a]	–2	–2	Cit.37918.1.S1_at
Sugar transporter[a]	–3.5	–2.1	Cit.29937.1.S1_s_at
Vacuolar-type ATPase subunit	2.3	2	Cit.28343.1.S1_at
Cyclic nucleotide gated channel[a]	2.6	2.8	Cit.20819.1.S1_at
Sulphate transporter 3;5 (*CsSULF*)[a]	13.3	2	Cit.16104.1.S1_at
Carbohydrate metabolism			
β-Fructofuranosidase/fructosidase	–2	–2	Cit.26169.1.S1_s_at
β-Fructofuranosidase/vacuolar invertase	–2.7	–2	Cit.10661.1.S1_at
Sucrose-phosphate-synthase	–2.6	–2	Cit.15009.1.S1_at
Myo-inositol oxygenase[a]	4.2	3.3	Cit.10133.1.S1_s_at
UTP-glucose-1-phosphate uridylyltransferase	2	2	Cit.16956.1.S1_x_at
Phytohormone metabolism/signalling			
Auxin/aluminium-responsive protein	9.2	2	Cit.13706.1.S1_s_at
UDP-glycosyltransferase	2.2	2	Cit.21798.1.S1_at
Gibberellin-regulated family protein[a]	–2.2	–2	Cit.9890.1.S1_s_at
Ethylene-responsive transcription factor	2.8	2	Cit.2675.1.S1_s_at
Disease-responsive/senescence-related proteins			
Serine-type endopeptidase inhibitor	3.1	2.3	Cit.21195.1.S1_at
Bon-associated protein/hypersensitive responsive protein	4.4	2.6	Cit.17388.1.S1_at
Isoflavonereductase	–2.3	–2.5	Cit.9371.1.S1_at
Heat shock protein 21	3.2	2.1	Cit.30648.1.S1_s_at
Senescence 1; dark inducible1[a]	4.7	2.1	Cit.21724.1.S1_at
CCR-like senescence-associated protein	2.1	2.6	Cit.10672.1.S1_s_at
Photosynthesis light and dark reaction			
Photosystem II subunit Q	2.3	2	Cit.6148.1.S1_at
Photosystem II subunit X	2	2.1	Cit.5461.1.S1_at
Photosystem II subunit P	2	2	Cit.9791.1.S1_x_at
Photosystem I subunit K	2	2.1	Cit.21356.1.S1_s_at
Ribulose-bisphosphate carboxylase	4.4	3	Cit.1816.1.S1_s_at
Ribulose-bisphosphate carboxylase small chain 3B	2	2.7	Cit.37846.1.S1_s_at
Phosphoenolpyruvate carboxykinase 1	2.8	2	Cit.19543.1.S1_at
Cell/tissue development and cell wall metabolism			
Arabinogalactan protein 16	2	2	Cit.17450.1.S1_at
NAC domain-containing protein 71	2.2	2.7	Cit.12214.1.S1_s_at
3-Ketoacyl-CoA synthase	2.1	2.1	Cit.26284.1.S1_at
Late embryogenesis abundant group 1 domain-containing protein	2	2.1	Cit.9395.1.S1_s_at
Invertase/pectin methylesterase inhibitor	4.2	7.4	Cit.5370.1.S1_s_at
Beta-Galactosidase	2.9	2	Cit.11079.1.S1_at
Beta-Xylosidase	3.1	2	Cit.28480.1.S1_s_at
Fatty acid/lipid and amino acid metabolism			
Phosphatidylethanolamine (PE)-binding protein	2.4	2	Cit.445.1.S1_s_at
Asparagine synthetase[a]	3.3	2	Cit.10060.1.S1_s_at
ROS scavenging and antioxidants			
Superoxide dismutase	–3	–3.5	Cit.6189.1.S1_s_at
Peroxidase 21	2	2	Cit.3966.1.S1_s_at
Chlorophyll development			
Light-harvesting complex LHCB6 (*CsLHCB*)[a]	2	2.4	Cit.37563.1.S1_s_at
Light-harvesting complex LHCB4	2	2.7	Cit.9248.1.S1_s_at
Light-harvesting complex LHCA1[a]	2.3	2.1	Cit.10648.1.S1_s_at
Chlorophyll *a*/*b*-binding protein 3	3.1	3	Cit.29329.1.S1_x_at
Oxidoreductase/protochlorophyllide reductase[a]	2.2	2	Cit.9939.1.S1_s_at
Flavonoid and terpene biosynthesis; alcohol and easter metabolism)			
Naringenin-chalcone synthase	2.1	11.9	Cit.21179.1.S1_at
Terpene synthase	2.1	4.9	Cit.1435.1.S1_at

Table 4. *Continued*

Putative function (citrus gene)	Fold change		Affymetrix ID
	Ham JV	Val JV	
Alcohol dehydrogenase	2.3	3.6	Cit.3120.1.S1_s_at
Carboxyesterase	2.0	2.1	Cit.13050.1.S1_at

[a] Genes shared with FF and VT as shown in Table 3. In other words, those are the genes changed in all symptomatic tissues.
Only those genes whose expression changed ⩾2.0 fold are presented.
Up-regulated (positive) and down-regulated (negative) gene expression patterns compared with the healthy control are shown.

Table 5. Comparison of expression of 15 genes in HLB-impacted and girdled flavedo in 'Hamlin' using qRT-PCR analysis

Putative function (citrus gene)	Fold change					
	H	AS	SY	UG	HG	FG
Transporters						
Sulphate transporter 3;5 (*CsSULF*)[a]	1 b	242 a	195 a	7.8 b	22 b	9.4 b
Carbohydrate metabolism						
Glucose-1-P adenylyltransferase (*CsSB1*)[b]	1 a	0.13 b	0.07 b	1.3 a	1.3 a	1 a
Granule-bound starch synthase (*CsSB2*)[b]	1 b	0.5 bc	0.1 c	1.3 ab	1.5 a	1 b
α-Amylase (*CsSD1*)[b]	1 b	4.4 ab	6.9 a	0.3 c	0.3 c	0.4 c
α-Amylase 3 (*CsSD2*)[b]	1 b	3.5 ab	8.5 a	1.5 b	1.5 b	4.8 a
β-Amylase 9 (*CsSD3*)[b]	1 b	7.0 a	7.1 a	2.7 b	3.1 b	11 a
β-Amylase 8 (*CsSD4*)[b]	1 c	43 b	77 a	7.9 c	12 c	101 a
Phytohormone metabolism						
Cytochrome P450 monooxygenase 83B1 (*CsSUR2*)[a]	1 b	31 ab	87 a	12 ab	5.8 b	47 ab
Nine-*cis*-epoxycarotenoid dioxygenase (*CsNCED*)[a]	1 b	7.3 a	7.8 a	0.7 b	1 b	2.5 b
ACC synthase 1 (*CsACS1*)[a,b]	1 a	0.15 b	0.07 b	0.7 a	1.6 a	2.8 a
ACC oxidase (*CsACO*)[a,b]	1 b	0.6 b	0.16 c	1.1 b	0.5 b	4.4 a
Light reaction						
Light-harvesting complex LHCB6 (*CsLHCB*)[a]	1 b	15 ab	24 a	2.4 b	6.2 ab	34 a
Chlorophyll degradation						
Early light-inducible protein (*CsELIP*)[a]	1 a	0.2 b	0.03 c	0.8 a	0.8	0.9 a
Cell wall metabolism						
Polygalacturonase (*CsPG*)[a,b]	1 b	1.3 ab	2.6 a	0.6 b	0.6 b	1 b
ROS scavenging						
Protein disulphide isomerase (*CsATC*)[a]	1 b	86 a	74 a	0.4 b	0.4 b	7.8 b

[a] Gene expression reported to respond to pathogen infection (see the Materials and Methods for references).
[b] Gene expression reported to be affected by girdling (see the Materials and Methods for references).
The relative expression of each gene was compared. Means within each gene followed by different letters are significantly different from healthy (H) control as determined by Duncan's multiple range test; $P < 0.01$

misshapen locations of SY was higher in abscisic acid and auxin content, and hypodermal cell area was greater when compared with normal-sized areas of the same fruit (Rosales and Burns, 2011). Since seeds are a rich source of auxins (Crane, 1964), varied but low SY filled seed populations contribute to asymmetric distribution of auxins and fruit growth.

Global transcriptome expression profiles were examined to determine the impact of HLB on fruit metabolism and symptom development. Microarray analysis identified many categories of metabolism that were affected by HLB, but no category appeared to be specific to the disease (García-Marcos *et al.*, 2009; Sarowar *et al.*, 2011). Based purely on number, SY 'Hamlin' responded to infection with more genes changing expression than SY 'Valencia'. A general field observation is that the response of 'Hamlin' to HLB infection is greater than that of 'Valencia'. However, since the duration of infection could not be determined in trees selected for this work, we cannot rule out that temporal or other interacting factors contributed to this difference. Nevertheless, comparisons between tissues within a cultivar can be made as a point-in-time evaluation. In this regard, FF and VT of 'Hamlin' appeared more responsive to HLB than JV, but similar numbers were measured in FF, VT, and JV from 'Valencia'.

A goal of many HLB research programmes is to identify disease biomarkers that could be used for early disease detection. FF from AS 'Valencia' had the highest number of genes changing expression when compared with H. This was not the case with JV, supporting published reports indicating that AS juice impacts are less when compared with

its H juice counterpart (Baldwin *et al.*, 2009; Dagulo *et al.*, 2010). Although gene groups associated with HLB infection represented a broad array of metabolic changes associated with host–pathogen interactions, specific transcripts may be good indicators of HLB. For example, the *CsSULF* homologue of the low-affinity sulphate transporter *SULTR3:5* was significantly overexpressed in SY and AS 'Hamlin' and 'Valencia' fruit and leaf (data not shown) tissues but not in FG. Carbohydrate starvation *per se* does not appear to impact *CsSULF* expression. However, root system dysfunction due to phloem plugging limits sulphur uptake and vascular transport, and disrupts sulphur pools throughout the plant. Sulphur deprivation increased expression of *SULTR3:5* (Kataoka *et al.*, 2004), suggesting that *CsSULF* overexpression was a direct consequence of sulphur deficiency resulting from root system decline rather than local vascular restriction or HLB specifically. Identification of specific transcript biomarkers will require evaluation of gene expression and the complexity of the whole plant physiological response to HLB.

Some carbohydrates are different or more abundant in HLB leaves (Hawkins *et al.*, 2010). Although reduced carbohydrate accumulation in HLB fruit could be a consequence of dysfunctional sieve elements in the phloem, disruption of cellular carbohydrate metabolic regulation and transport could be another contributing factor to carbohydrate imbalance. Numerous genes involved in carbohydrate transport and metabolism changed expression in 'Hamlin' and 'Valencia'. In fact, several genes involved in starch metabolism that changed expression after girdling (Li *et al.*, 2003) were altered in HLB and girdled FF, supporting the hypothesis that in addition to disrupted carbohydrate transport in phloem, HLB and fruit stem girdling also induce major changes in the cellular metabolism of carbohydrates. Such changes can disrupt fruit growth and lead to small fruit size typical of HLB-affected fruit and fruit from girdled stems. Since girdling did not result in misshapen fruit or irregular peel colour typical of HLB-affected fruit and several representative genes for specific pathways were affected by HLB but not girdling, the mechanisms regulating the development of these symptoms may lie in the host disease response rather than being a direct consequence of carbohydrate starvation.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. The 40 shared genes significantly changed in symptomatic FF by HLB infection. Only those genes whose expression changed ⩾8.0 in 'Hamlin' are presented.

Table S2. Quantitative RT-PCR primer sets used to determine differential expression between symptomatic and girdled flavedo.

Table S3. Functional identification and functional category of changed genes in fruit tissues in response to HLB. Only ESTs with significant expression changes (false discovery rate ⩽0.01, and *P*-value $<1\times10^{-3}$) with ⩾2-fold changes in expression are presented.

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