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Genes involved in the regulation of starch metabolism and defense response determine resistance in citrus against huanglongbing

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Abstract

Different diseases and pests affect the genus citrus, resulting in reduced quality and taste of fresh fruit, low production and ultimately less profit for the growers. Huanglongbing (HLB) is the major problem for citrus-producing countries including Pakistan all over the world. Asian citrus psyllid (ACP) was collected from HLB positive sweet orange cv. succari (*Citrus sinensis* (L.) Osbeck) field trees and released on healthy plants of succari in the growth room under controlled conditions for the infestation to obtain bud/grafts for inoculation in experimental mandarin plants. SYBR green-based real-time qPCR was performed to differentiate the expression of carbohydrate metabolism and defense response-related selected genes in HLB infected and healthy leaf samples of kinnow, citrus sunki, parsons special and sun chu sha. Gene expression data analysis results represented that glucose-1-phosphate adenyl transferase, starch synthase and cytochrome P450 monooxygenase 83B1 genes may play a role in the tolerance against HLB.

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Introduction

Different diseases and pests affect the genus citrus, resulting in reduced quality and taste of fresh fruit, low production and ultimately less profit for the growers (Martinelli *et al*., 2015). Huanglongbing (HLB) is the major problem for citrus-producing countries including Pakistan all over the world. In regions, where HLB is endemic, citrus trees produce unmarketable fruit as it abscises prematurely and mostly trees die within 5 to 8 years (Baldwin *et al*., 2010). Pakistan ranks at the 14th position in the world with 1907.4 thousand tones production of citrus from an area of 198 thousand hectares (FAOSTAT, 2017). From a 95.6% share of total citrus produced by Punjab, about 80% citrus includes kinnow mandarin (Tahir, 2014). Citrus is one of the most important fruit crops contributing to the revenue of Pakistan. Bahrain, Dubai, Indonesia, Kuwait, Malaysia, Netherlands, Oman, Qatar, Russia, Saudi Arabia, Singapore, and the UK are the major market places of Pakistan's kinnow. Kinnow was brought to Indo Pak from Riverside (California), the USA in 1940. Sargodha and its neighboring areas including Faisalabad, Toba Tek Singh, Jhang and Sahiwal are the main districts that produce good quality kinnow (Aslam *et al*., 2017a). Per hectare yield of citrus in Pakistan is lower as compared to the majority of countries of the world due to many reasons, HLB is one of them. In Florida, USA, HLB was reported in 2005.

Candidatus Liberibacter, a Gram-negative, non-culturable and phloem limited bacterium is the causal organism of HLB (Li *et al*., 2009). There are three types of this bacterium and complete genomes of all three bacteria have been sequenced: 1.23Mbp for *Ca*. L. asiaticus (Duan *et al*., 2009), 1.195201 Mbp for *Ca*.L. americanus (Wulff *et al*., 2014) and 1.192232 Mbp for *Ca*. L. africanus (Lin *et al*., 2015). The natural vector of the pathogen is citrus psyllid. There are two species of psyllid vector: the Asian Citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Sternorryncha: Lividae) and the African Citrus psyllid, *Trioza erytreae* Del Guercio, reported for HLB transmission (Aubert, 1987). The *Diaphorina citri* Kuwayama is a natural vector of both *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter americanus, while *Trioza erytreae* is the vector of *Candidatus* Liberibacter africanus (Bove, 2006; Lin *et al*., 2015).

Lopsided, small sized and uneven colored fruits produce on HLB diseased plants. Early flowering in HLB diseased plants has also been observed (Albrecht and Bowman, 2008; Martinelli *et al*., 2012). HLB symptoms do not appear in the host plant immediately after pathogen infection (Chin *et al*., 2014). HLB symptoms development under greenhouse environment from grafting may take 4 to 12 months (Yaqub *et al*., 2019). It is very important to understand the citrus host response to pathogen for the development of HLB management strategies. Various studies have been conducted to identify genes and proteins in response of HLB pathogen in leaves, juice vesicles and fruit peel (Kim *et al*., 2009). Studies on gene expression changes revealed a number of different processes like, photosynthesis, carbohydrate metabolism, cell defense and transport. For gene expression/transcriptome profiling of HLB in citrus, along with microarray and qRTPCR, a high throughput sequencing technique known as RNA-Seq is also being used (Martinelli *et al*., 2015). Molecular detection of the pathogen in HLB diseased plants for comparisons of gene expression in response to HLB infection has been studied in kinnow and succari (Aslam *et al*., 2017a & b). Different strategies are being used for the management of HLB, but still, there is no report of the complete cure of the disease. The present study was an effort to study the expression of some selected genes in response to HLB infection in a commercially important mandarin group of citrus to determine resistance against HLB.

Materials and methods

Nursery establishment

To study the gene expression changes in citrus in the response of HLB, seeds of exotic and indigenous mandarins were sown under controlled conditions of the greenhouse of the Institute of Horticultural Sciences (IHS), University of Agriculture Faisalabad

(UAF) Pakistan (Table1). From eight sown genotypes, four were survived including kinnow, citrus sunki, parson's special and sun chu sha. Plants were kept free of any other graft transmissible disease except huanglongbing.

Inoculation of graft source by Asian citrus psyllid

For expression profiling of huanglongbing disease in citrus, healthy succari (*Citrus sinensis*) plants were inoculated by Asian Citrus Psyllid (ACP) captured from HLB positive field trees for HLB bacterium transmission in the growth room under controlled conditions (Fig. 1 A&B). ACP was released on those plants fortnightly up to one year (Yaqub *et al*., 2019). Positivity for *Candidatus* Liberibacter asiaticus of the field sweet orange plants was confirmed by conventional PCR using primer pairs OI1/OI2c and A2/J5 as described by Jagoueix *et al*. (1996) and Hocquellet *et al*. (1999) respectively. Discrete bands were obtained with an amplicon size of 1160bp and 703bp for OI1/OI2c and A2/J5 respectively (Fig. 2). Three plants for each genotype of citrus mandarin with the same size and health were inoculated and three plants for each genotype were isolated as healthy control with no inoculation. Molecular studies for detection and expression profiling of HLB in citrus sunki, kinnow, parson's special and sun chu sha were carried out after one year of inoculation.

Real-time PCR for Candidatus Liberibacter asiaticus detection:

HLB bacterium detection in artificially ACP-transmitted HLB diseased plant samples was done through real-time quantitative PCR at the University of California Riverside (UCR), USA. The DNA was extracted from about 0.5 g midribs and petioles of leaves by CTAB (cetyltrimethylammonium bromide) method modified from protocol 3 of Ruangwong and Akarapisan (2006) (Yaqub *et al*., 2017). Quantitative PCR was conducted using 16S rDNA based primer-probe set HLBasfpr, specific to Las (5´→3´ sequences: forward GTCGAGCGCGTATGCAATAC, reverse TGCGTTATCCCGTAGAAAAAGGTAG and probe AGACGGGTGAGTAACGCG). A primer-probe set based on plant cytochrome oxidase (COX) gene was used as a positive internal control to assess the quality of the DNA extracts (5´→3´ sequences: forward GTATGCCACGTCGCATTCCAGA, reverse GCCAAAACTGCTAAGGGCATTC and probe ATCCAGATGCTTACGCTGG) as described by Li *et al*. (2006). Cycling conditions of PCR consisted of an initial denaturation step at 95 °C for 3 minutes followed by 40 cycles of denaturation at 95 °C for 10 seconds and annealing at 58 °C for 20 seconds.

RNA extraction

Total RNA extraction from the whole leaf with three biological replicates was carried out according to Sambrook and Russell, 2001. About 0.2 g leaf sample was ground in liquid nitrogen. 300 µL RNA extraction buffer (5% SDS, 200 mM Tris HCl, 50 mM sodium acetate, 10 mM EDTA) was added followed by the addition of 300 µL phenol to the above mix. Tubes were incubated at 65 °C for 5 minutes followed by centrifugation at 13000 rpm for 10 minutes. To supernatant, added an equal volume of phenol-chloroform (1:1) and centrifuged for 10 minutes. To the supernatant, added absolute ethanol and 3M sodium acetate. RNA pellets were dissolved in d_3H_2O.

Synthesis of cDNA

Total RNA extracted was converted to complementary DNA using NEB kit for cDNA synthesis according to the manufacturer's instructions. Complementary DNA was then stored at -80 °C for downstream processes.

Expression profiling of HLB in mandarins

Although primer pairs for fifteen genes were optimized for gene expression experiment by qPCR, among those genes, starch synthesis, carbohydrate metabolism and cell defense were selected. Expression of six genes including: glucose-1-phosphate adenyl transferase (CsSB1), starch synthase (CsSB2), alpha-amylase (CsSD1), alpha-amylase 3 (CsSD2), beta-amylase 9 (CsSD3) and cytochrome P450 monooxygenase 83B1 (CsSUR) was studied by real-time qPCR (Liao and Burns 2012; Aslam *et al*., 2017a). A 2x SYBR green ready to use, SensiMix™ SYBR & Flourescein master mix

(BIOLINE, USA) was used. Sequence from actin gene (Table 2) was used as a reference gene for gene expression analysis (Staigers *et al.*, 2000). Thermocycle conditions for SYBR green-based PCR reactions were: one cycle of initial denaturation at 95 °C for 10 minutes followed by 39 cycles of denaturation, annealing and extension at 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 15 seconds respectively.

Statistical analysis

Relative expression of the said genes was calculated using the software, CFX manager version 3.0.1224.1015(Bio-Rad). Calculations for the relative quantity, accurate normalization and fold change of gene expression were done according to Pfaffl, (2001) and Vandesompele *et al.* (2002) using formula: for relative quantity, ΔCt = GOI- HKG Where:

GOI= average Ct values of the gene of interest and HKG= average Ct values of the housekeeping gene. For normalization, Δ ΔCt = ΔCt experimental samples - ΔCt controls Fold change = $2^{(-\Delta\,\Delta Ct)}$

Results

HLB pathogen transmission by Asian citrus psyllid

After six weeks of the ACP release, we were able to see the colonies of nymphs on the new flushes.

As far as the expression of HLB symptoms in the infested plants of succari is concerned, typical symptoms of HLB started to appear after nine months of ACP release in leaves revealing sweet orange a good indicator plant of HLB symptoms (Fig. 1C).

Table 1. Citrus germplasm comprising mandarin group sown for the detection and expression profiling of huanglongbing.

Sr. No.	Group	Cultivar	*Binomial*	Accession No.
1	Mandarin	Citrus Sunki	*Citrus sunki*	PI 539678
2	Mandarin	Cleopatra	*Citrus reshni*	PI539492
3	Mandarin	Kinnow	*Citrus reticulata* Blanco	Pak
4	Mandarin	Kinokuni	*Citrus kinokuni*	PI 539270
5	Mandarin	Parson's Special	*Citrus reticulata*	PI539497
6	Mandarin	Sun Chu Sha	*Citrus reticulata*	PI 539544
7	Mandarin	Nules	*Citrus clementina* hort.ex Tanaka	Pak
8	Mandarin	Scarlet Emperor	*Citrus reticulata*	PI 539505

Real-time PCR for Candidatus Liberibacter asiaticus detection

All of the inoculated succari plants and mandarin varieties were found to carry HLB bacterium upon qPCR analysis for16S rDNA of *Candidatus* Liberibacter asiaticus. Mean Ct values of infested mandarins and succari indicate a higher number of *Ca*. Las (Fig. 3).

Table 2. List of primers used for expression profiling of HLB in citrus mandarins by qPCR.

Citrus gene	Gene function	Orientation	Primer sequences (5′ → 3′)
CsSB1	Glucose-1-phosphate adenyl transferase	forward	CCTCCTTCTAAGATGCTTGATGCT
		reverse	GCACCTTCTGATATGCAAGATCG
CsSB2	Starch synthase	forward	CAGTAGATGTGGATGCAGTGTCC
		reverse	GCCGTCAATTCCAGGTTCAC
CsSD1	Alpha amylase	forward	GGTATCCTCCAAGCTGCTGTG
		reverse	ACTTTATCCGATGGGAATGGC
CsSD2	Alpha amylase3	forward	AAGGAATAAAATCCACTGCCGTAG
		reverse	CTTGGAGGTTCATAATGACCTGGT
CsSD3	Beta amylase 9	forward	AAGAATTTTGCGAGAGCTTTAAGTCT
		reverse	CCAACTCCAGGGATTTTGCTAC
CsSUR2	Cytochrome P450 mono oxygenase 83B1	forward	GCGGCG ACTATGGTTTGG
		reverse	CCT TTTTCATCACTCTAGGATGCA
Actin	ATPase	forward	TCACAGCACTTGCTCCAAGCA
		reverse	TGCTGGAAGGTGCTGAGGGA

Expression profiling of huanglongbing disease in citrus mandarins

For expression profiling of HLB in citrus, primer pairs for 6 genes were selected based on their functions. Five genes involved in the regulation of starch metabolism were; glucose-1-phosphate adenyl transferase *(CsSB1)*, granule bound starch synthase *(CsSB2)*, alpha-amylase *(CsSD1)*, alpha-amylase 3 *(CsSD2)* and beta-amylase 9 (*CsSD3)*. Cytochrome P450 monooxygenase 83B1 *(CsSUR2)* involved in defense response is a phytohormone related gene.

Relative quantities of the target genes were determined to healthy control of each genotype.

Table 3. Differential expression of 6 genes with fold changes in 4 genotypes of HLB infected mandarin group of citrus using qRT PCR analysis.

Sr. No.	Cultivar	Fold change					
		CsSB1	*CsSB2*	*CsSD1*	*CsSD2*	*CsSD3*	*CsSUR*
1	Citrus Sunki	=	-22	-3		Not amplified	Not amplified
2	Kinnow	Not amplified	25		9	=	Not amplified
3	Parson's Special	-454	2		5	-2	Not amplified
4	Sun Chu Sha	-2	-36		Not amplified	3	Not amplified

For normalization, the actin gene was used as reference or housekeeping. Upregulated and down-regulated gene expression values with fold change compared to healthy controls are presented in Table 3. The negative values are representing downregulation whereas, positive values indicate the up-regulation of the respective gene in a given genotype of citrus.

Discussion

For expression profiling of huanglongbing disease, HLB bacterium was transmitted by ACP in succari. The optimum range of temperatures for the growth of ACP was maintained between 25-28 ^{0}C according to Liu and Tsai (2000). In the present study, taqman based qPCR targeting 16S rRNA gene for Las detection (Li *et al*., 2006) in HLB infected genotypes of mandarin including citrus sunki, kinnow, parson's special and sun chu sha and succari sweet orange was performed. Bacterial titer based on cycle threshold (Ct) values were found significantly higher in succari with mean Ct value of 20 as compared to kinnow with mean Ct value 25.06.

Glucose-1-Phosphate adenyl transferase (*CsSB1*) is also known as ADP glucose pyrophosphorylase. This enzyme takes part in starch and sucrose metabolism. qPCR results for CsSB1 expression associated with up-regulation as well as downregulation in HLB infected plants relate to the starch synthesis. No amplification of this gene in kinnow suggests no starch accumulation in the said variety. CsSB1 was down-regulated in HLB infected Parson's special and Sun chu sha. This result is in agreement with the results of Liao & and Burns (2012) and Martinelli *et al.* (2015).

Starch synthase (CsSB2) is responsible for starch accumulation in HLB infected leaves (Kim *et al*., 2009). In the present study, this gene was upregulated in kinnow and parson's special. The upregulation of the same gene was described in response to HLB infection by Martinelli *et al.* (2015). CsSB2 was down-regulated in citrus sunki and sun chu sha.

The majority of citrus genotypes vary in susceptibility for HLB pathogen, the response of lemon towards *Ca.* L. asiaticus infection results in an increased quantity of starch synthase (Nwugo *et al*., 2013). Alpha-amylase (CsSD1) act on starch at any place and break it down into maltose and glucose etc. qRT PCR results for CsSD1 gene expression revealed no amplification in three genotypes of citrus mandarin except citrus sunki indicating agreement with the results of HLB infected and girdled fruit tissues from Liao and Burns (2012). Alpha amylase3 (CsSD2) was up-regulated in kinnow and parson's special while it was not expressed in healthy and HLB infected citrus sunki and sun chu sha as described for sweet orange by Liao

and Burns (2012).

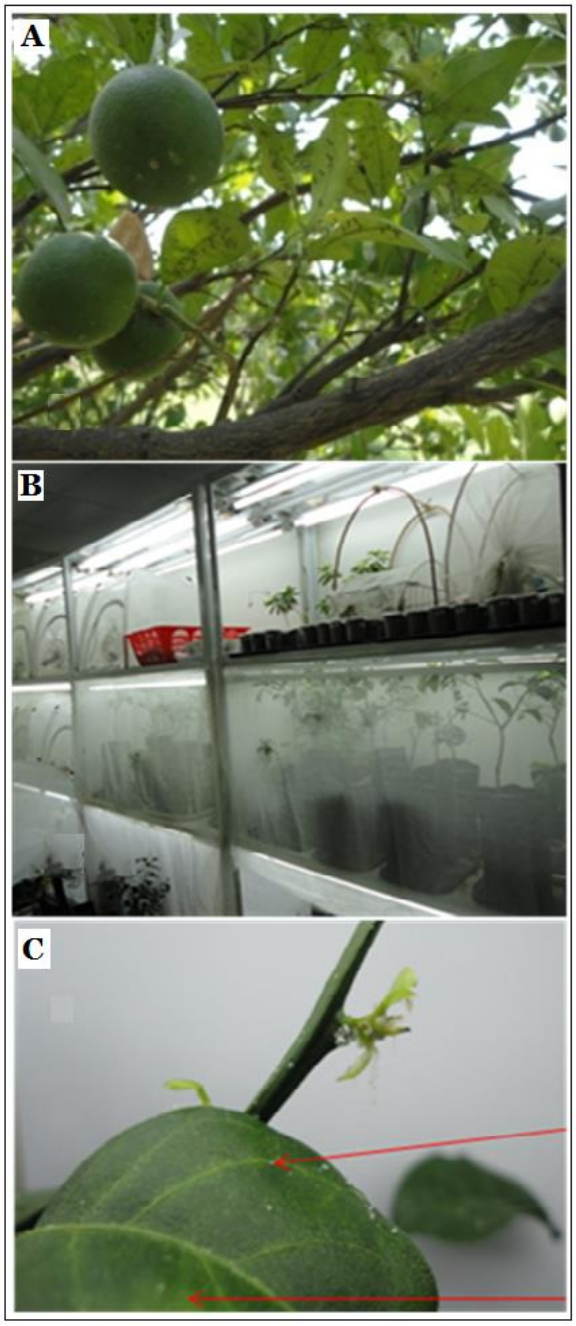

Fig. 1. Huanglongbing positive source plants for gene expression studies in mandarin group of citrus: A, Sweet orange cv. succari field tree with huge population of Asian citrus psyllid. ACP were captured from these trees and released on greenhouse raised healthy sweet orange for candidates liberibacter asiaticus transmission to obtain source of graft for gene expression studies; B, growth room having sweet orange plants for release and infestation by ACP; C, Graft source plant of sweet orange having leaf with prominent vein yellowing symptom of HLB after infestation by ACP in growth room.

During fruit ripening, beta-amylase (CsSD3) degrades starch into maltose, causing sweetness in ripe fruit (Grennan, 2006). qPCR results for gene expression changes in HLB infected leaf samples revealed the upregulation of CsSD3 in sun chu sha. Phytohormone metabolism-related gene cytochrome P450 monooxygenase 83B1 (CsSUR2) was not amplified in any of the four mandarins pointing towards synthesis and breakdown of hormones responsible for leaf formation and shedding, and fruit development.

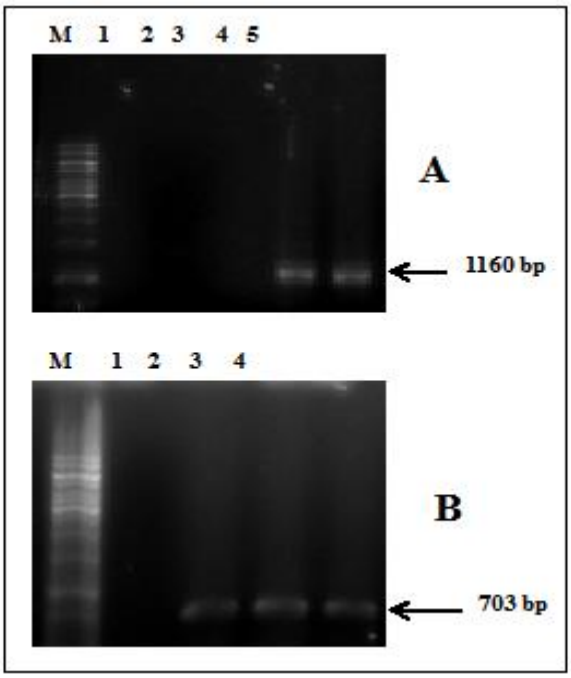

Fig. 2. Gel electrophoresis of PCR product from field samples of sweet orange. A. DNA amplified with primer pair OI1/OI2c. M=1Kb DNA ladder (Fermentas), Lane 1= No template control, lane 2 and 3= DNA amplified from healthy succari sweet orange for *Candidatus* Liberibacter asiaticus and lane 4-5= DNA amplified for *Candidatus* Liberibacter asiaticus from field sweet orange; B. DNA amplified with primer pair A2/J5. Lane 1= No template control, lane 2 - 4= DNA amplified from field sweet orange for *Candidatus* Liberibacter asiaticus, M = 1Kb DNA ladder (Fermentas).

Albrect and Bowman (2008) described genes for cytochrome P450 family that were upregulated up to 6 fold in sweet orange. No amplification of CsSUR2 gene indicates resistance in all of the four mandarin varieties against HLB. As it has been observed that leaf size goes very small and in an upright position in

the response of HLB but kinnow mandarin does not die as early as sweet orange. Up till now, gene expression studies have been done mostly on *Citrus sinensis* cultivars only (Albrecht and Bowman, 2012; Liao and Burns, 2012; Nwugo *et al.*, 2013; Du *et al.*, 2015) but in this study, we have investigated the response of mandarins towards HLB.

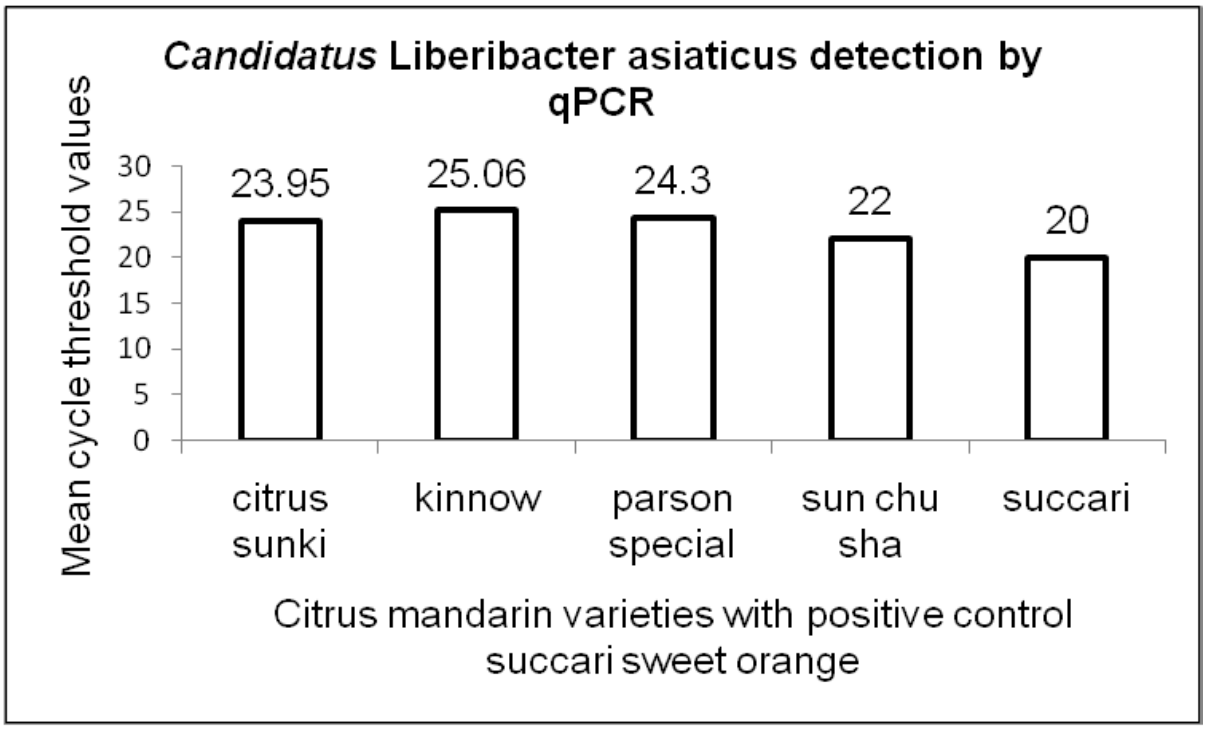

Fig. 3. Mean cycle threshold values in citrus mandarin group and succari DNA for the detection of *Candidatus* Liberibacter asiaticus.

Conclusion

From the expression analysis of the studied genes, it is concluded that carbohydrate metabolism plays a major role in the identification of tolerance in different genotypes of citrus against HLB. Glucose-1-Phosphate adenyl transferase (*CsSB1*), Starch synthase (CsSB2), and cytochrome P450 monooxygenase 83B1 (CsSUR2) genes are suspected to play a role in tolerance against HLB. Further studies are needed to find more genes responsible for HLB symptom expression and tolerance in commercially important varieties of citrus. After identification of tolerant or susceptible gene through expression profiling, the tolerant gene could be incorporated or susceptible gene could be silenced in a susceptible genotype of citrus to manage HLB.

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