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Full Length Article

Expression Profiling of Huanglongbing Disease in Kinnow (*Citrus reticulata*) and Succari (*Citrus sinensis*) Leaves

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Abstract

Huanglongbing (HLB), also called as citrus greening disease, is the most destructive and prevalent disease of citrus. In this study, for HLB diagnosis in the leaf samples of Succari sweet orange [*Citrus sinensis* (L.) Osbeck] from field, conventional polymerase chain reaction (PCR) technique was employed. Asian citrus psyllid was collected from HLB positive sweet orange field trees and released on healthy plants of succari and kinnow for infestation in the growth room under controlled conditions. *Candidatus* Liberibacter asiaticus was detected in the inoculated citrus by Taqman based real time PCR using primers set HLBas fpr. SYBR green based real time qPCR was performed to differentiate expression of seven genes in HLB infected and healthy leaf samples of Succari sweet orange and Kinnow mandarin. Conventional PCR results were obtained with discrete bands having amplicon size of 1160bp, 800bp and 700bp for OI1/OI2c, OI2/23S1 and A2/J5, respectively. Succari proved to be good indicator plant for HLB symptoms expression. Gene expression data analysis results represented Kinnow mandarin is less susceptible to HLB as compared to Succari sweet orange. © 2017 Friends Science Publishers

Keywords: *Candidatus* Liberibacter asiaticus; Citrus; Citrus psyllid; Gene expression; HLB management

Introduction

Huanglongbing (HLB) is the most serious infectious disease of citrus and major threat to citrus industry (Hall *et al*., 2012; Volk and Suszkiw, 2013). In regions, where HLB is endemic, citrus trees produce unmarketable fruit as it abscise prematurely and mostly die within 5 to 8 years (Baldwin *et al*., 2010). Pakistan ranks at the 13th position in the world with 2150 thousand tones production of citrus from an area of 198 thousand hectare (FAOSTAT, 2013). From 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 in the revenue of Pakistan. Bahrain, Dubai, Indonesia, Kuwait, Malaysia, Netherlands, Oman, Qatar, Russia, Saudi Arabia, Singapore, and UK are the major market places of Pakistan's kinnow. Kinnow was brought to Indo Pak from Riverside (California), 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 (Johnson, 2006).

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. Up to 2014, approximately 33% revenue of the total production of the state has been wasted due to HLB and more than 8000 persons associated with citrus industry lost their jobs (Chin *et al.*, 2014). *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: *Candidatus* Liberibacter asiaticus (*Ca.* Las), *Candidatus* Liberibacter africanus (*Ca.* Laf) (Koizumi, 1995) and *Candidatus* Liberibacter americanus (*Ca.* Lam) (Teixeira *et al*., 2005). The complete genomes of all three bacterium have been sequenced: 1.23 Mbp 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: *Diaphorina citri* Kuwayama (Hemiptera: Sternorryncha: Lividae) and *Trioza erytreae* del Guercio(Hemiptera: Sternorrynca: Triozidae) 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

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not appear in the host plant immediately after pathogen infection (Chin *et al.*, 2014). HLB symptoms development under greenhouse environment from grafting may take 3 to 12 months (Lopes *et al*., 2009). For successful management of HLB, a powerful observation of disease epidemic with rapid and accurate detection of pathogen is very important but, the detection of the pathogen is very difficult because of its low titer and uneven distribution in its citrus hosts (Bove, 2006; Li *et al*., 2007). 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; Martinelli *et al.*, 2015). 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). Chohan *et al.* (2007) first time confirmed the presence of HLB bacterium in psyllid and plant host at molecular level in Peshawar, Pakistan. In 2011, HLB was first time confirmed by conventional PCR in the leaves of *Citrus sinensis* cv. Succari at Faisalabad district of Punjab province, Pakistan (Yaqub *et al*., 2017). Molecular detection of the pathogen in HLB diseased plants for comparisons of gene expression in response to HLB infection were not studied yet in Pakistan. Therefore, it is the dire need of this study to use molecular technology for HLB bacterium detection, *Candidatus* Liberbacter asiaticus transmission by psyllid vector to citrus host plants and compare gene expression changes in response to HLB infection in kinnow mandarin and Succari sweet orange with healthy controls.

Materials and Methods

Experimental Details and Treatments

Nursery establishment: To study the gene expression changes in citrus in response of HLB, seeds of kinnow and sweet orange cultivar succari were sown in controlled conditions of greenhouse of Institute of Horticultural Sciences (IHS), University of Agriculture Faisalabad (UAF) Pakistan. Plants were kept free of any other graft transmissible disease except huanglongbing.

HLB Pathogen Transmission by Asian Citrus Psyllid

For expression profiling of huanglongbing disease in citrus, succari (*Citrus sinensis*) and kinnow (*Citrus reticulata* Blanco) plants were inoculated by Asian Citrus Psyllid (ACP) for HLB bacterium transmission according to a modified protocol from Nava *et al*. (2007). A sweet orange orchard with a huge population of ACP nymphs and adults with numerous symptoms of HLB on leaves was selected for ACP collection.

Symptomatic leaves for HLB diagnosis were collected from that sweet orange field and subjected to molecular studies for the detection of HLB bacterium *Candidatus* Liberibacter. 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 amplicon size of 1160bp and 703bp for OI1/OI2c and A2/J5 respectively (Fig. 1). After the detection of HLB pathogen, ACP was collected from those trees and released on succari and kinnow plants for bacterium transmission. One year old, five plants of each genotype were subjected for inoculation by ACP. ACP was released on those plants fortnightly up to one year.

Real-time PCR for *Candidatus* Liberibacter Asiaticus Detection

HLB bacterium detection in artificially ACP-transmitted HLB diseased plants samples were 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). 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 min followed by 40 cycles of denaturation at 95°C for 10 sec and annealing at 58°C for 20 sec.

RNA Extraction

RNA extraction from whole leaf with three biological replicates was carried out at (CABB), University of Agriculture Faisalabad (UAF), Pakistan 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 addition of 300 µL phenol to the above mix. Tubes were incubated at 65ºC for 5 min followed by centrifugation at 13000 rpm for 10 min. To supernatant, added equal volume of phenol chloroform (1:1) and centrifuged for 10 min. 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 complimentary DNA using NEB kit for cDNA synthesis according to the manufacturer's instructions. Complementary DNA was then stored at -80ºC for downstream processes.

Expression Profiling of HLB in Citrus

Expression of seven genes including: sulfate transferase (CsSULF), glucose-1-phosphate adenyl transferase (CsSB1), starch synthase (CsSB2), alpha amylase (CsSD1), alpha amylase 3 (CsSD2), beta amylase 9 (CsSD3) and cytochrome P450 mono oxygenase 83B1 (CsSUR) was studied by real time qPCR as per Liao and Burns (2012) studies. A 2x SYBR green ready to use, SensiMix™ SYBR & Flourescein master mix (BIOLINE, USA) was used. Sequence from actin gene (Table 1) was used as reference gene for gene expression analysis (Staiger *et al*., 2000). Thermocycle conditions for SYBR green based PCR reactions were: one cycle of initial denaturation at 95ºC for 10 min followed by 39 cycles of denaturation, annealing and extension at 95ºC for 15 sec, 60ºC for 15 sec and 72ºC for 15, 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 gene of interest and HKG= average Ct values of 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 of both varieties. As far as the expression of HLB symptoms in the infested plants of succari and kinnow are concerned, typical symptoms of HLB started to appear after nine months of ACP release in leaves of both citrus varieties. More prominent symptoms of vein yellowing and blotchy mottling were found on succari as compared to kinnow, revealing sweet orange a good indicator plant of HLB symptoms. Twisted new flushes arose on both varieties.

Real-time PCR for *Candidatus* Liberibacter Asiaticus Detection

All of the inoculated kinnow and succari plants were found to carry HLB bacterium upon qPCR analysis. We obtained mean Ct value of 25.06 for kinnow mandarin and 20.0 for sweet orange plants for16S rDNA of *Candidatus* Liberibacter asiaticus. Low mean Ct values for sweet orange indicate its susceptibility and higher number of Las.

Expression Profiling of Huanglongbing Disease in Citrus

Gene expression data analysis results for relative quantity and fold change for all of the seven genes in both genotypes of citrus in response of HLB infection indicated up regulation of starch synthase (*CsSB2*) and alpha amylase 3 (*CsSD2*) in kinnow. Sulfate transferase (*CsSULF*) gene was down regulated in kinnow while beta amylase 9 (*CsSD3*) was expressed equally in healthy and HLB infected kinnow.Glucose-1-phosphate adenyl transferase (*CsSB1*), alpha amylase (*CsSD1*) and cytochrome P450 mono oxygenase 83B1(*CsSUR2*) were not amplified in kinnow.In case of succari sweet orange, sulphate transferase (*CsSULF*), beta amylase 9 (*CsSD3*) and cytochrome P450 mono oxygenase 83B1(*CsSUR2*)were up regulated while glucose-1-phosphate adenyl transferase (*CsSB1*) and granule bound starch synthase (*CsSB2*) were down regulated. Alpha amylase (*CsSD1*) did not amplify in Succari like Kinnow (Fig. 2). Gene expression values with fold change compared to healthy controls are presented in Table 2. The negative values are representing down regulation whereas, positive values indicate up regulation of the respective gene in each genotype of citrus.

Discussion

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

Up regulation of *CsSULF* transporter gene in succari suggests sulfur deficiency, indicating week root system (Kataoka *et al*., 2004; Liao and Burns, 2012). In kinnow, *CsSULF* gene was down regulated indicating strong root system for plant survival. 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 down regulation in HLB infected plants relate to the starch synthesis. No

Table 1: List of primers used for expression profiling of HLB in citrus by qPCR

Citrus gene	Gene function	Orientation	Primer sequences (5′ → 3′)
CsSULF	Sulfate transferase	forward	ACAGGAATTGCCGCATTTAGAG
		reverse	AAACCGAATCCTTCCCAATGAC
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

Table 2: Comparison of expression of 7 genes in HLB infected Kinnow and sweet orange using qRT PCR analysis

Citrus gene	Fold change	
	Kinnow	Succari
Sulfate transferase *(CsSULF)*	-24	97
Glucose-1-phosphate adenyl transferase *(CsSB1)*	Not amplified	-7
Starch synthase *(CsSB2)*	25	-2
Alpha amylase *(CsSD1)*	not amplified	not amplified
Alpha amylase 3 *(CsSD2)*	9	1
Beta amylase 9 (*CsSD3)*	1	2
Cytochrome P450 mono oxygenase 83B1 *(CsSUR2)*	not amplified	4

amplification of this gene in kinnow mandarin suggests no starch accumulation in the said variety. CsSB1 was down regulated in HLB infected sweet orange cv. Succari. Liao and Burns (2012) and Martinelli *et al*. (2015) also reported such results.

Starch synthase (CsSB2) is responsible for starch accumulation in HLB infected leaves (Kim *et al*., 2009). In present study, this gene was upregulated in Kinnow. The upregulation of the same gene was described in response to HLB infection by Martinelli *et al*. (2015). CsSB2 was down regulated in sweet orange. Majority of citrus genotypes vary in susceptibility for HLB pathogen. A study on response of lemon towards *Ca.* L. asiaticus infection resulted in 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 both genotypes of citrus 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 while it was equally expressed in healthy and HLB infected sweet orange. Similar results of CsSD2 for sweet orange were described by Liao and Burns (2012). 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 upregulation of CsSD3 in Succari. Phytohormone metabolism related gene cytochrome P450 mono oxygenase83B1(CsSUR2) was upregulated in Succari sweet orange pointing towards synthesis and breakdown of

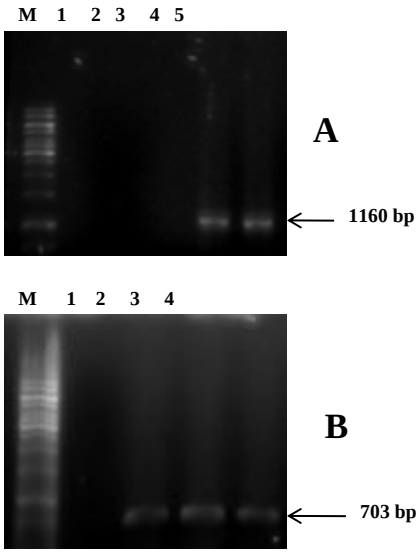

Fig. 1: Gel electrophoresis of PCR product from field samples of sweet orange. A. DNA amplified with primer pair OI1/OI2c. M=1Kb DNA ladder (Fermentas), Lane 1= No template control, lane 2 and 3= DNA amplified from healthy succari sweet orange for *Candidatus* Liberibacter asiaticus and lane 4-5= DNA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

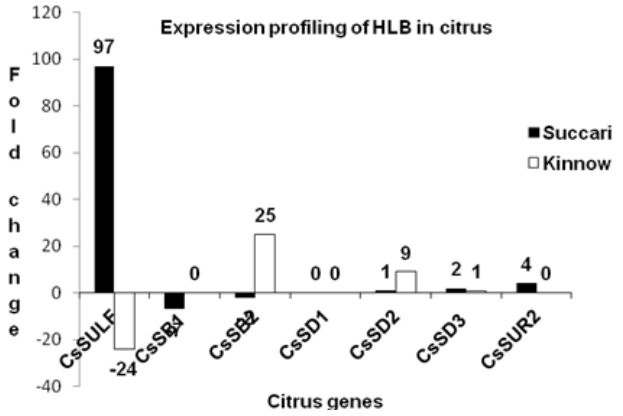

Fig. 2: Gene expression with fold change in HLB affected Kinnow and Succari leaves. Bars below zero with negative values are representing down regulation whereas, bars above zero with positive values indicate up regulation of the respective gene in a given genotype of citrus

hormones responsible for leaf formation and shedding, fruit development and ripening while this gene was not expressed in kinnow. Albrect and Bowman (2008) described genes for cytochrome P450 family that were upregulated upto 6 fold in 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 Kinnow mandarin also.

Conclusion

From 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. More accumulation of starch occurs in HLB infected leaves of succari as a result of down regulation of CsSB1 and CsSB2 and no expression or amplification of starch degrading gene CsSD1. No amplification of CsSB1 gene in kinnow results in no accumulation of starch in the phloem cells. Starch accumulation in succari causes phloem plugging and results in early decline of succari than kinnow. Further studies are needed to find more genes responsible for HLB symptom expression and tolerance in commercially important varieties of citrus.

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