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Research Article

Comparison of gene expression changes in susceptible, tolerant and resistant hosts in response to infection with *Citrus tristeza virus* and huanglongbing.

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Abstract

The pathogens *Candidatus* Liberibacter asiaticus (Las) and *Citrus tristeza virus* (CTV) are both phloem limited and have significant economic impact on citrus production wherever they are found. Studies of host resistance have indicated that *Poncirus trifoliata* has tolerance or resistance to both pathogens, suggesting that there may be some common factors in the 2 kinds of resistance. We have conducted studies of host gene expression changes that occur in response to infection to gain further insight. Controlled inoculation by grafting infected budwood was used to infect potted greenhouse plants of Cleopatra mandarin (*Citrus reticulata*), US-897 (*C. reticulata* x *P. trifoliata*), and US-942 (*C. reticulata* x *P. trifoliata*) with CTV and with Las, the pathogen associated with the disease huanglongbing (HLB). Stem and leaf tissue was collected at 10, 20, and 30 weeks after inoculation, DNA and RNA were extracted and subjected to qPCR and RT-qPCR analysis. Few differences in gene expression were observed between mock-inoculated and CTV-inoculated plants. Differences between mock-inoculated and Las-inoculated plants were most pronounced in susceptible Cleopatra plants and at the later stages of infection. Notable was the higher expression of a gene for miraculin-like protein 2 and other defense-related genes in US-897 and US-942 plants independent of infection. It is hypothesized that tolerance or resistance of US-897 and US-942 is associated with a higher constitutive expression of defense-related or other genes associated with the *P. trifoliata* parentage, rather than with induced expression in response to bacterial infection.

Keywords: citrus, *Poncirus trifoliata*, huanglongbing, *Citrus tristeza virus*, rootstock, gene expression, US-897, US-942

Introduction

Citrus tristeza virus (CTV) and *Candidatus* Liberibacter asiaticus (Las) are both pathogens that can have devastating effects on infected citrus trees and are similar in their phloem localization. It is hypothesized that comparisons of host responses to the 2 pathogens will help us understand the infection process and provide clues about effective ways to improve host plant resistance or tolerance. This study compared 3 citrus rootstock cultivars for response to Las and CTV infection. We measured the time to detection of infection by real-time PCR (qPCR) and reverse transcription-qPCR (RT-qPCR), the amount of pathogen present as measured by qPCR and RT-qPCR, the development of host plant symptoms, the effect on plant growth, and the level of expression for 18 genes by RT-qPCR, at 3 time points following inoculation.

Materials and methods

Plant Material

One-year-old seedlings of the genotypes Cleopatra mandarin (*Citrus reticulata*), US-897 (*C. reticulata* 'Cleopatra' x *Poncirus trifoliata* 'Flying Dragon') and US-942 (*C. reticulata* 'Sunki' x *P. trifoliata* 'Flying Dragon') were used. These genotypes have varying degrees of tolerance/resistance to CTV and Las, with Cleopatra being the most susceptible and US-942 the most tolerant.

Inoculation

Five plants per genotype were mock-inoculated with buds from healthy sweet orange plants. Twelve plants per genotype were inoculated with buds from Las-infected and HLB-symptomatic (blotchy mottle) sweet orange plants. Eight plants per genotype were inoculated with buds from CTV (strain T68-1)-infected symptomless sweet orange plants. Three buds were used to inoculate

each plant. Inoculations were performed in October 2012. Plants were cut back immediately after inoculation and managed to allow no more than 2 new shoots to develop.

Sample collection

Shoots were severed and the basal 2 cm were collected and stored at -20 °C for Las/CTV detection. Apical young portions of each branch were removed, leaving only mature tissue (10 to 15 cm of each branch, including stem and leaves) to be used for gene expression analysis. Tissue (2 to 6 g per sample) was immediately frozen in liquid nitrogen and stored at -80 °C. Samples were collected at 10 weeks after inoculation (wai), 20 wai, and 30 wai. Weight of shoots was determined at 30 wai.

Las/CTV detection

Stem pieces were ground in liquid nitrogen with a mortar and pestle, and 100 mg of the resulting ground tissue was used for DNA or RNA extraction. DNA was extracted using the Plant DNeasy Mini Kit (Qiagen, Hilden, Germany) and qPCR assays were performed using primers HLBas and HLBr and probe HLBp (Li et al. 2006). RNA was extracted using the Plant RNeasy Mini Kit (Qiagen) and RT-qPCR assays were performed using primers UTR1 and UTR2 and probe 181T (Bertolini et al. 2008). Amplifications were carried out in an AB7500 real-time PCR system (Applied Biosystems, Foster City, CA) using the QuantiTect Probe PCR Kit (Qiagen) for Las, and the QuantiTect Probe RT-PCR Kit (Qiagen) for CTV.

Gene expression analysis

Three plants with similar disease progression were selected from each genotype and time point, resulting in a total of 81 samples. Stems and leaves were ground in liquid nitrogen with a mortar and pestle. Total RNA was extracted from 300 mg of ground tissue using the Plant RNeasy Mini Kit (Qiagen). Contaminating DNA was removed using the TURBO DNA-free Kit (Ambion, Austin, TX). Amplifications were performed in an AB7500 real-time PCR system using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). Gene-specific primers with calculated melting temperatures of 60 °C ± 0.5 °C were designed using Primer 3 (Rosen and Skaletsky 2000). Relative quantification of gene expression was performed applying the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), using Ct-values of 18S rRNA (Yan et al. 2012) and GAPC2 (Mafra et al. 2012) for normalization.

Results

Las/CTV detection

The percentage of Las- and CTV-inoculated plants with Ct-values ≤ 33 after real-time PCR analysis was lowest for US-942 seedlings and highest for Cleopatra seedlings at 20 wai. At 30 wai most or all plants were infected with Las or CTV, respectively (Fig. 1). Mean Ct-values were highest (corresponding to lowest pathogen genome numbers) for infected US-942 seedlings. Lowest Ct-values (corresponding to highest pathogen genome numbers) were found for Las-infected Cleopatra seedlings and T68-infected Cleopatra and US-897 seedlings, respectively.

Disease symptoms

No disease symptoms were detected in Las-inoculated plants at 10 wai. At 20 wai 58% of Las-inoculated Cleopatra plants showed reduced growth, chlorosis of leaves, and reduction of leaf size (Fig. 1). The same symptoms were observed for 75% of Las-inoculated Cleopatra plants at 30 wai. No disease symptoms were detected in mock-inoculated plants, in CTV-inoculated Cleopatra plants or in Las- and CTV-inoculated US-897 and US-942 plants throughout the experiment.

Shoot masses determined at 30 wai were significantly (ANOVA, $P<0.05$) different in Cleopatra and US-897 seedlings in response to infection with Las (Table 1).

Gene expression

Genes were selected based on our previous results for Las (Albrecht and Bowman 2012) and based on published results for CTV (Cristofani-Yali et al. 2007; Gandía et al. 2007; Liu et al. 2012). Expression results of 14 genes are summarized in Table 2. Four additional genes (CTV.22, caffeic acid O-methyltransferase, aquaporin, and a putative DnaJ-like protein) did not show any differences in gene expression between treatments and genotypes and are not presented. Gene expression differed between time points independent of the treatment, which is likely the result of differences in the developmental stage of the plant tissue at the time of collection, and other factors such as daylight, temperature, and water status. Fold changes in gene expression are therefore presented relative to non-infected Cleopatra controls within each time point.

Only in Cleopatra seedlings infected with Las was differential expression (> 4-fold) of genes observed at 10 wai. These included the genes coding for miraculin-like protein 2 (MLP), Glucose-6-phosphate/phosphate transporter (GPT), lipoxygenase (LOX), and RNA-dependent RNA polymerase I (RDR1) (Table 2). This response was observed only after inoculation with Las but not with CTV. Transcripts for Myb-like HTH transcriptional regulator family protein (MYB), Grape RAP4-4-like ethylene transcription factor (RAP4), a constitutive disease resistance protein 1 (CDR1), MLP, chitinase, GPT, cytosolic copper/zinc superoxide dismutase (CSD1), and oxidoreductase were more than 4-fold induced in Cleopatra in response to Las infection at 20 or 30 wai. Except for CSD1, none of these genes were induced in response to CTV. In US-897 seedlings, transcripts for the genes coding for MYB, RAP4, MLP, chitinase, GPT, CSD1, and 2-Oxoglutarate (2OG) and Fe (II)-dependent oxygenase (2OG-FeII) were more than 4-fold induced at 20 or 30 wai in response to Las. Except for chitinase and CSD1, these genes were also induced in response to CTV at one or both of these time points.

Induced expression of genes in US-942 seedlings was observed only in response to Las for RAP4, CDR1 (Cit.28117) and chitinase at 30 wai. Transcripts for CDR1 (Cit.23704), GPT, GLT1, and RDR1 were less abundant in Las-infected US-942 seedlings than in controls at some of the time points. Reduced abundance of transcripts for GPT was also observed in response to CTV at 30 wai. Most notable was the considerably higher abundance of transcripts for MLP in US-897 and US-942 seedlings compared with Cleopatra even in the non-infected state. Transcripts for plant defensin 2 (PDF2) were also more abundant in these 2 genotypes at 10 wai. Expression of genes for CDR1 (Cit.23704), GLT1, and 2OG-FeII was not detected in Cleopatra seedlings throughout the study. Expression of CSD1 was not observed for US-942.

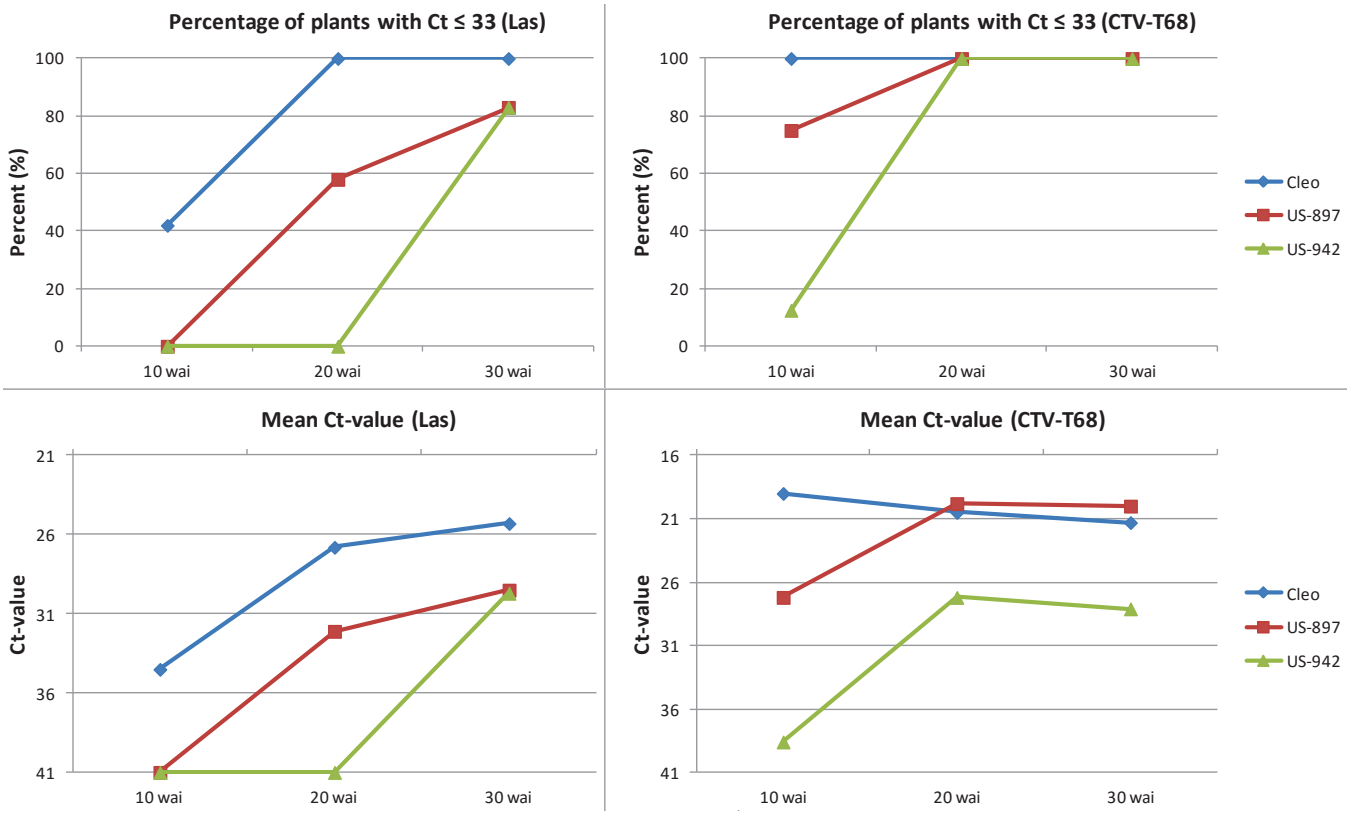

Fig. 1. Percentage of plants with Ct-values ≤ 33 after PCR detection of Las- and CTV-inoculated plants, and mean Ct-values.

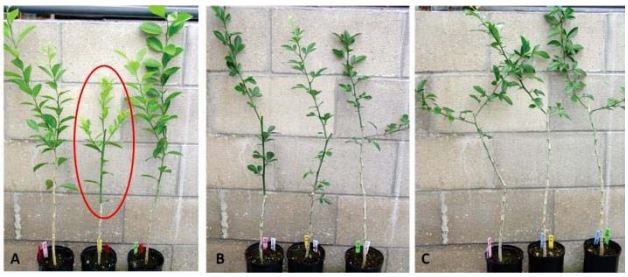

Fig. 2. Cleopatra- (A), US-897- (B), and US-942- (C) seedlings 20 weeks after inoculation. In each picture, CTV-infected, Las-infected, and non-infected seedlings are presented on the left, middle, and right, respectively. Disease symptoms (stunting and chlorosis) were only observed in Las-infected Cleopatra seedlings (red oval).

Table 1
Shoot masses in mock-inoculated (ctrl), CTV-inoculated and Las-inoculated Cleopatra, US-897, and US-942 seedlings 30 weeks after inoculation.

	Average shoot mass (g)					
	Cleopatra	Tukey HSD	US-897	Tukey HSD	US-942	Tukey HSD
Ctrl	25.6	b	16.8	a	24.1	a
CTV	22.2	ab	16.9	a	24.1	a
Las	14.5	a	12.2	a	20.8	a
P	0.0174		0.0336		> 0.05	

Means in the same column followed by the same letter are not significantly different (P = 0.05) according to Tukey's HSD test.

Table 2
Fold changes in gene expression of mock-inoculated (ctrl), CTV-inoculated and Las-inoculated Cleopatra, US-897, and US-942 seedlings 10, 20, and 30 weeks after inoculation.

	Cleopatra			US-897			US-942		
	Ctrl	**CTV**	**Las**	**Ctrl**	**CTV**	**Las**	**Ctrl**	**CTV**	**Las**
Myb-like HTH transcriptional regulator family protein(Cit.31971.1.S1_at)									
10 wai	1	0.2	1.6	0.1	0	0.3	0	0.1	0
20 wai	1	0.9	54.8	0.5	9.8	9.3	0.4	0.1	0.3
30 wai	1	1.2	69	0.4	1	7.6	0.6	0.6	1.2
Grape RAP4-4-like ethylene transcription factor(Cit.3534.1.S1_s_at)									
10 wai	1	0.3	4.7	1.9	1.9	0.7	0.1	0.2	0.1
20 wai	1	1.3	24.6	0.8	1.7	4.7	0.3	0.2	0.1
30 wai	1	0.3	0.7	0.1	0.6	1.4	0	0	5.7
Constitutive disease resistance protein CDR1 (Cit.28117.1.S1_s_at)									
10 wai	1	0.5	1.4	0.7	0.9	0.8	0.4	0.4	0.4
20 wai	1	0.5	3.3	2.1	3.5	3.6	2.2	1.3	3.1
30 wai	1	0.7	4.2	1.1	3.1	3.4	1.2	2	5.2
Constitutive disease resistance protein CDR1 (Cit.23704.1.S1_at)									
10 wai	1	0.5	0.2	486	430	716	460	242	76.9
20 wai	1	0.4	6.3	4587.5	5525	7637.5	4187.5	2618.8	298
30 wai	1	0.2	0.5	905	2565	1181.7	416.7	915	1047.3
Miraculin-like protein 2 (AF283533.1)									
10 wai	1	1.8	107	163.7	93.8	227.9	119.3	102	49.2
20 wai	1	0.2	87.9	21.4	36.5	79.9	25.6	29.8	56.3
30 wai	1	0.8	19	3.5	11.9	24.5	6.4	4.5	12.5
Acidic class II chitinase (orange1.1g026315m)									
10 wai	1	0.6	2.1	1.6	1.4	1.4	1.4	1.1	2.1
20 wai	1	0.7	29.4	6.6	6.7	23.2	2.8	1.7	5.2
30 wai	1	1.2	10.4	1	2.3	7.7	0.6	1	12
Glucose-6-phosphate/phosphate transporter GPT (Cit.9625.1.S1_s_at)									
10 wai	1	0.1	9.1	3.2	6.3	0.5	1.2	12.8	6.9
20 wai	1	0.2	162.9	1.8	7.8	3.8	36.2	10.2	5.2
30 wai	1	7.2	11.4	0.6	1.9	9.1	19.3	0.8	2
Plastidic glucose transporter GLT1 (Cit.12711.1.S1_at)									
10 wai	1	7.2	1.2	333072	332129	291279	397451	352812	150857
20 wai	1	1.3	46.6	649698.6	733056.8	1052044.6	1081541.9	666129.7	160751.4
30 wai	1	2.7	3.2	46062.1	80535	61163.6	59770.7	96783.6	75894.3
Cytosolic copper/zinc superoxide dismutase CSD1 (Cit.28102.1.S1_s_at)									
10 wai	1	0.9	2.9	0.9	0.8	1.5	0	0	0
20 wai	1	4.8	18.3	0.3	0.5	3	0	0	0
30 wai	1	1	28	1	1	5	0	0	0
Oxidoreductase(Cit.8902.1.S1_s_at)									
10 wai	1	0.9	1.2	0.1	0.1	0.1	0.1	0.1	0.1
20 wai	1	1.3	18.8	0.1	0.1	0.1	0	0.1	0.1
30 wai	1	0.5	2.6	0	0	0.1	0.1	0.1	0
2-Oxoglutarate (2OG) and Fe (II)-dependent oxygenase (Cit.940.1.S1_s_at)									
10 wai	1	0.3	0.3	1871	3738	2604	1026	867	239
20 wai	1	1.2	0.6	840	5322.7	12214.7	6426.7	5393.3	5486.7
30 wai	1	1.1	9.1	25374.2	73013.6	52750	6406.1	1624.2	8563.6
Lipoxygenase (Cit.8206.1.S1_s_at)									
10 wai	1	1	6.1	0.8	0.8	0.9	0.6	0.5	0.4
20 wai	1	0.9	3.7	0.5	1	2	1.1	1.1	0.7
30 wai	1	1	1.2	0.4	0.6	0.5	1	1	1.5
Plant defensin PDF2 (Cit.2831.S1_s_at)									
10 wai	1	1.1	0.6	5.7	4.5	6.3	4.7	3.4	4
20 wai	1	1.4	0.9	2.2	2.5	3.1	1.5	1.9	2
30 wai	1	0.8	0.6	1.1	1.1	1.1	1	0.8	1.1
RNA-dependent RNA polymerase I RDR1 (orange1.1g002586m)									
10 wai	1	1	5.9	8.6	5.1	8.2	5.5	3.8	1.1
20 wai	1	0.5	0.7	0.5	0.7	0.4	0.3	0.6	0.6
30 wai	1	0.7	0.6	0.6	0.9	0.6	0.8	0.8	0.9

Gene target sequence identifiers (Affymetrix, GenBank, or Phytozome) are in parentheses.

Discussion

qPCR and RT-qPCR analyses showed that the speed of transmission of Las and CTV was highest in the susceptible genotype (Cleopatra) and lowest in the most tolerant genotype (US-942). Correspondingly, the most pathogen genomes (lowest Ct-values) were found in Cleopatra seedlings, which also exhibited severe chlorosis and significant growth reductions in response to Las. Infection with CTV did not result in any leaf abnormalities in this genotype. Las- and CTV-inoculated US-897 and US-942 plants did not experience significant growth reductions and remained free of foliar disease symptoms throughout the study. RT-qPCR analysis showed that gene expression changes were most prominent in Cleopatra and in response to infection with Las, thus corresponding to disease severity and the degree of susceptibility. Gene expression changes were also observed in US-897 plants in response to Las and CTV, but at later time points compared with Cleopatra. Gene expression changes in US-942 plants were detected only at 30 wai and were limited to a few genes. Several genes, such as those for constitutive disease resistance protein 1, 2-oxoglutarate (2OG), Fe (II)-dependent oxygenase, and miraculin-like protein 2 were more abundantly or exclusively expressed in US-897 and US-942 plants.

Most of the genes analyzed in this study have been implicated in the defense response to biotic and abiotic stresses in other plants (Albrecht and Bowman 2012). For example, Myb-like HTH transcriptional regulator family proteins are involved in salicylic acid-mediated control of the hypersensitive response to bacterial pathogens and are associated with resistance to infection with Bois Noir phytoplasma in grapevine. Over-expression of the constitutive disease resistance proteins CDR1 resulted in over-expression of other defense-related genes and enhanced resistance to bacterial and fungal pathogens in arabidopsis and rice. Miraculin-like proteins have been shown to have bioinsecticidal activity against lepidopteran pests, and to be induced in response to pathogens other than Las or CTV in citrus and other plant pathogen systems. Chitinases have long been implicated in the resistance response to fungal- and other pathogens and different abiotic stresses. Other genes, such as those coding for glucose-6-phosphate/phosphate transporter GPT and plastidic glucose transporter GLT1, play important roles in carbohydrate metabolism. The effect of Las and of other phloem-limited pathogens on carbohydrate metabolism and in particular, the accumulation of starch, has been demonstrated in many studies. Surprisingly, expression of an RNA-dependent RNA polymerase I, which is associated with salicylic acid-mediated antiviral defense, was only affected by Las but not by CTV.

In conclusion, tolerance or resistance of US-897 and US-942 does not appear to be related to an earlier and more pronounced expression of defense-related genes. The transcriptional responses to Las, and to a smaller extent to CTV, observed in this study appear to be part of a basal resistance response, which is apparently not sufficient to suppress the pathogenic effect of Las in susceptible genotypes. It is hypothesized that tolerance or resistance of US-897 and US-942 to Las is associated with a higher constitutive expression of defense-related or other genes associated with the *P. trifoliata* parentage, rather than with induced expression in response to bacterial infection. The gene region associated with resistance to CTV, unlike Las, has been characterized in *P. trifoliata* (Gmitter et al. 1996), facilitating marker-assisted selection of resistant genotypes.

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References

Albrecht U, Bowman KD. 2012. Transcriptional response of susceptible and tolerant citrus to infection with *Candidatus* Liberibacter asiaticus. Plant Science. 185-186:118-130.

Bertolini E, Moreno A, Capote N, Olmos A, de Luis A, Vidal E, Pérez-Panadés J, Cambra M. 2008. Quantitative detection of *Citrus Tristeza virus* in plant tissues and single aphids by real-time PCR. Eur J Plant Pathol. 120:177-188.

Cristofani-Yali M, Berger IJ, Targon MPN, Takita MA, Dorta SdeO, Freitas-Astúa J, de Souza AA, Boscariol-Camargo RL, Reis MS, Machado MM. 2007. Differential expression of genes identified from *Poncirus trifoliata* tissue inoculated with CTV through EST analysis and *in silico* hybridization. Gen Mol Biol. 30(3):972-979.

Gandía M, Conesa A, Ancillo G, Gadea J, Forment J, Pallás V, Flores R, Duran-Vila N, Moreno P, Guerri J. 2007. Transcriptional response of *Citrus aurantifolia* to infection by *Citrus tristeza virus*. Virology. 367:298-306.

Gmitter FG, Xiao SY, Huang S, Hu XL, Garnsey SM, Deng Z. 1996. A localized linkage map of the *Citrus tristeza virus* resistance gene region. Theor Appl Genet. 92:688-695.

Li W, Hartung JS, Levy L. 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. J Microbiol Methods. 66:104-115.

Liu Y, Wang G, Wang Z, Yang F, Wu G, Hong N. 2012. Identification of differentially expressed genes in response to infection of a mild *Citrus tristeza virus* isolate in *Citrus aurantifolia* by suppression

subtractive hybridization. Sc Horticulturae. 124:144-149.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. Methods. 25:402-408.

Mafra V, Kubo KS, Alves-Ferreira M, Ribeiro-Alves M, Stuart RM, Boava LP, Rodrigues CM, Machado MM. 2012. Reference genes for accurate transcript normalization in Citrus genotypes under different experimental conditions. PLoS ONE. 7(2):e31263. doi:10.1371/journal.pone.0031263

Rosen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, editors. Bioinformatics methods and protocols: Methods in molecular biology. Totowa (NJ):Humana Press. p. 365-386.

Yan J, Feirong F, Long G, Qin L, Deng Z. 2012. Selection of reference genes for quantitative real-time RT-PCR analysis in citrus. Mol Bio Rep. 39:1831-1838.