

## ORIGINAL ARTICLE

# Identification of Genes Expressed Differentially in Grapefruit Infected with *Candidatus Liberibacter asiaticus* in the Late Stage of Disease

Hosein Gholampour<sup>1,2</sup>, Maryam Ghayeb Zamharir<sup>1</sup>, Jaber Karimi<sup>2</sup>, Naser Farrokhi<sup>3</sup>, Ali Alizadeh<sup>1</sup> and Peyman Taheri<sup>2</sup>

<sup>1</sup> Prokaryote Laboratory, Plant Disease Department, Iranian Research Institute of Plant Protection, Tehran, Iran

<sup>2</sup> Agricultural Biotechnology Group, Agriculture Department, Shahed University, Tehran, Iran

<sup>3</sup> Agriculture Department, Shahrood University of Technology, Shahrood, Semnan Province, Iran

## Keywords

*Candidatus* Leiberibacter asiaticus, cDNA-AFLP, grapefruit, Huanglongbing, Iran

## Correspondence

M. Ghayeb Zamharir, Prokaryote Laboratory, Plant Disease Department, Iranian Research Institute of Plant Protection, Tehran, Iran.  
E-mail: zamharir2005@yahoo.com

Received: January 6, 2014; accepted: March 31, 2014.

doi: 10.1111/jph.12273

## Abstract

Citrus greening or Huanglongbing (HLB), a destructive disease of citrus worldwide, was reported from south of Iran in 2007. The molecular basis of compatibility and disease development in this system is poorly understood. We have carried out a cDNA-AFLP analysis to analyse gene expression of grapefruit infected by *Candidatus* Leiberibacter asiaticus in the late infection stage. We have applied a cDNA-AFLP approach on grafted infected grapefruit trees at the representing symptoms stage in susceptible host. Selective amplifications with 10 primer combinations allowed the visualization of approximately 24 transcript-derived fragments (TDFs) in the leaves of graft-inoculated trees, which were differentially expressed. We sequenced 14 fragments, which were identified as grapefruit transcripts after homology searching, whereas 12 were not homologous to sequences in NCBI databases. Many grapefruit genes spanning almost all functional categories were upregulated during infection, especially genes involved in ATP synthesise, cytochrome P450 synthesise, isoflavone 2'-hydroxylase, zeaxanthin epoxidase, cellulose synthase, DNA repair protein, aconitate hydratase 2 and citrus tristeza virus resistance gene. This study provides the first global catalogue of grapefruit genes expressed during inoculation, together with their functional annotations. This will help to elucidate the molecular basis of the resistance process and identify genes and chemicals that could help to inhibit the pathogen.

## Introduction

*Candidatus* Leiberibacter asiaticus is an obligate biotrophic plant pathogen that causes citrus greening disease, also called Huanglongbing or yellow dragon disease, a devastating disease resulting in significant economic losses of citrus (Zhao 1981; Capoor 1963). *Ca.* Leiberibacter asiaticus (Las) is a phloem-inhabiting prokaryotes transmitted by the Asian citrus psyllid (*Diaphorina citri*) in Asia and America (Chen et al. 1973; Bove 2006). The HLB liberibacters were among the first bacteria to receive 'Candidatus' designations

according to the rules established for uncultured organisms (Murray and Schleifer 1994). Phylogenetically, the closest relatives of the liberibacters are members of the alpha-2 subgroup of the Proteobacteria (Jagoueix et al. 1994; Woese et al. 1984).

The distribution of (Las) in the host tissue is still widely unknown, but seasonal changes of tissue specificity could play a role. The HLB bacterium has been reported to inhabit living phloem cells, a different environment from foliar and intercellular spaces, and avoids the extracellular surface receptors encountered by many pathogens (Kim et al. 2009). At the tissue

level, Las infection can cause localized pockets of necrotic phloem scattered throughout the vascular system of mature leaves that block the translocation stream. Other anatomical aberrations were observed and are believed to be reactions to the blockage (i.e. massive accumulation of starch in the plastids, disordered cambial activity with excessive formation of phloem, soon to become necrotic). Leaf mottle symptoms associated with HLB are probably the consequence of these alterations (Bove 2006; Schneider 1968).

Some molecular aspects of the infection process are known, but details of the pathology of Las infections are still unresolved (Slisz et al. 2012).

Bacterial infection of plants, in general, causes extensive changes of gene expression involved in plant defence, environmental stress response metabolism, protein metabolism, transport, energy and others (Scheideler et al. 2002; Schenk et al. 2000; Tao et al. 2003). Transcriptome analysis has been used as a straight forward approach to identify the genes whose expression is altered in citrus leaves in response to the HLB inoculation [Kim et al. 2009; Albrecht and Bowman 2008, 2011]. These studies led to the identification of several hundred or thousand genes that are up- or downregulated by the HLB bacterial infection. The majority of these genes can be grouped into metabolism, transport and response to stimulus. The microarray analysis indicated that HLB infection significantly affected expression of genes which are associated with sugar metabolism, plant defence, phytohormone and cell wall metabolism (Ute and Kim 2008; Kim et al. 2009).

Other studies of citrus response to the HLB bacterial infection show that genes involved in carbohydrate and nitrogen metabolic processes, transport, defence, signaling and hormone response were overrepresented in the HLB response. Transport plays a key role in the citrus response to the HLB bacterial infection. Moreover, analysis of a phloem protein subnetwork indicates a role for this protein and zinc transporters or zinc-binding proteins in the citrus HLB defence response (Zheng and Zhao 2013).

Plants have evolved multiple defence mechanisms in response to pathogen attack, and the pathogens have evolved multiple counter measures to host defences (Hammond-Kosack and Jones 1996; Gachomo et al. 2003). At the molecular level, plant defence responses depend on the combination of a specific set of dominant R genes in the plant and a corresponding set of dominant avirulence (Avr) genes in the pathogen. Expression of the Avr genes triggers plant defence responses governed by the product of the R

gene (Bogdanove 2002). Plant defence responses in incompatible responses include the hypersensitive response resulting in localized cell death, structural alterations and production of plant defence molecules such as antimicrobial proteins (Grant and Mansfield 1999).

Despite the many visual and physiological observations on HLB-affected citrus plants worldwide, the molecular determinants for the HLB disease have yet to be established. Understanding the basis of susceptibility would greatly assist in the development of new control strategies and the identification of pathogen and host factors required for disease progression. In this study, we applied a cDNA-amplified fragment length polymorphism (AFLP) approach to identify genes that may be expressed differentially in grapefruit trees infected with Las. Understanding the basis of susceptibility or tolerance to the pathogen will assist greatly in the development of new control strategies and the identification of pathogen and host factors that are required for disease progression.

## Materials and Methods

### Plant material and inoculation

Ten healthy greenhouse-grown 1-year-old grapefruit (*Citrus paradisi*) plants were used in this experiment. The healthy grapefruits were native type Jiroft which have a health certificate from the Plant Protection Organization (PPO) of Iran. The spacemen of Las-infected orange were grafted to healthy grapefruit. Purity of the inoculum has been tested using OI1/OI2c (Jagoueix et al. 1996a; Fujikawa and Iwanami 2012) and A2/J5 (Hocquellet et al. 1999b) primer sets for confirming single infection. All plant material used for inoculation was confirmed to be free of *Citrus tristeza virus* by RT-PCR analysis. The grafted plants were covered for 1 month with plastic bags to increase humidity and were arranged randomly on the greenhouse bench. They were kept under natural light conditions at 25–28°C. The greening-infected branches in grapefruit were sampled 5 months after inoculation when Las was detectable in grapefruit by PCR (Hocquellet et al. 1999a,b).

### Detection of Las infection by PCR

Total DNA was extracted from leaf samples (vascular tissues of leaf veins and petioles) using the method originally described by Daire et al. (1997) with some modifications. Briefly, 1 g of tissue was homogenized

at room temperature in disposable plastic bags with a ball-bearing device in 7 ml of CTAB buffer (3% CTAB, 1 M Tris-HCl pH 8.2, mM EDTA and 1.4 M NaCl) with the extemporaneous addition of 0.2% 2-mercaptoethanol; 1 ml was transferred to an Eppendorf tube and incubated in a water bath at 65°C for 20 min. After extraction with 1 ml of chloroform, nucleic acids were precipitated from the aqueous phase with an equal volume of isopropanol, collected by centrifugation, washed with 70% ethanol, dried, dissolved in 150 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6) and stored at -20°C until use. DNA amplification of a region of the 16S rRNA phytoplasma gene was performed in 25 µl total reaction volume in an Applied Biosystem thermal cycler. PCR primers was A2 (5'-TATAAAGGTTGACCTTTTCGAGTTT-3') and J5 (5'-ACAAAAGCAGAAATAGCACGAACAA-3') (Jagoueix et al. 1996b).

#### RNA isolation and cDNA synthesis

Samples were collected 6–7 months after inoculation when pathogen could be detected by PCR assay. Sampling was performed from leaves above grafting region. Total RNAs of infected and non-infected were extracted from all replicates using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis and cDNA-AFLP analysis were performed in 4 replicates. 2 µg of total RNA was used to synthesize from cDNA using RevertAid H Minus First Strand cDNA Synthesis kit (Fermentase, Germany) according to the manufacturer's instructions. Second strand cDNA synthesis was performed by adding the first strand mix to 150 µl total volume of 15 µl 10× cDNAII buffer, 35 U DNA Polymerase I (Fermentase, Germany), 3 U RNase H (Fermentase, Germany), 1.00 µl dNTPs (25 mM) and incubating for 2 h at 16°C. The resulting double-stranded cDNA was purified according to Powell and Gannon (2002). The concentration of cDNAs was determined using spectrophotometer (Bio-Rad) and their quality was determined by electrophoresis on 1.2% agarose gel.

#### cDNA-AFLP

An aliquot (500 ng) of double-stranded cDNA was used for AFLP analysis as described by Bachem et al. (1996) with the following modifications. The template for cDNA-AFLP was digested by two pair restriction enzyme, *EcoRI/MseI* (Fermentase, Germany). AFLP reactions were performed according to Bachem et al. (1998). Selective amplification products were

separated on a 10% polyacrylamide gel (Bassam et al. 1991). The gels were dried onto 3MM Whatman paper (Whatman, Maidstone, UK).

#### Cloning, sequencing and bioinformatic characterization

The bands of interest were excised from the gels. DNA fragments were extracted from denaturing gels using a razor blade. Each gel slice was incubated in 10 µl of distilled water for 10 min at 96°C. 10 µl of eluent was then PCR-amplified under the same conditions as for the selective PCR. PCR product was separated on a 10% polyacrylamide gel to confirm whether polymorphism segment was chosen correctly (Wu 2006). In order to select differentially expressed TDFs, the profiles of infected and uninfected samples were compared across replicates. The TDFs that were differentially abundant between the two types of samples, infected and non-infected plants, were selected only when the same tendency was also observed in all replicates.

The recovered products reamplified by primer pair E-0/M-0 and P-0/M-0 to provide sufficient DNA for cloning and then sequenced using the fluorescent automated sequencing facility at the Fazabiotech Company (Iran).

The sequences were compared with sequences in NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) and TAIR (<http://www.arabidopsis.org/Blast/index.jsp>) non-redundant databases using Blastn, Blastx, and tBlastx sequence alignment programs (Altschul et al. 1990).

#### Semiquantitative analysis

Semiquantitative was carried out on RNA derived from both infected and non-infected Grapefruit samples as previously described by Rey et al. (2000). Primer pairs were designed using OLIGOTECH software (version 7) (Table 1). Reaction conditions (20 µl

**Table 1** Primer and their sequences is designed for semiquantitative analysis

Primer	Sequences
552R	5'-CGGGGCGTCTAACTCATAGA-3'
552F	5'-GGGGAAAGACAACCTTGGA-3'
CGF	5'-CGGGGCGTCTAACTCATAGA-3'
CGR	5'-GGGGAAAGACAACCTTGGA-3'
18s R	5'-GCGGTAATTCAGCTCCAA-3'
18sF	5'-TAATTTCTCAAAGTAACAGCG-3'

volumes) were optimized by changing the primer concentration and annealing temperature to minimize primer-dimer formation and to increase PCR efficiency. Each run included standard dilutions and negative reaction controls. The mRNA expression level of each gene of interest and of the ribosomal protein 18S rRNA, chosen as a housekeeping gene, was determined in parallel for each sample in three times for healthy and infected samples. Relative band intensities were calculated through dividing the intensity of each interested band by that of the 18S rRNA band and standardized with respect to the length of DNA fragments.

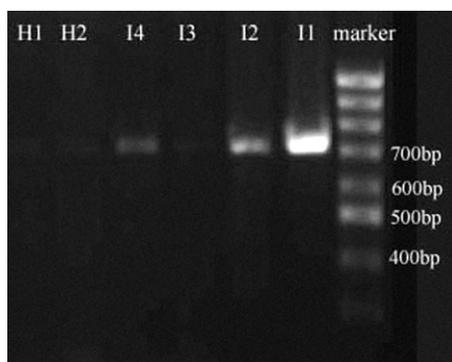
## Results

Five months after grafting, Las was detectable in inoculated grapefruit (Fig. 1). Nucleotide blast analysis with BlastX revealed that the bacterium was a variant 'Ca. L. asiaticus' (Fig. 1).

### cDNA-AFLP analysis

A total of 40–100 different transcript-derived fragments (TDFs) were detected on each given gel, ranging from 50–800 bp (Fig. 2). As a result of cDNA-AFLP analysis, a total of 26 TDFs were identified as differentially expressed.

Based on pairwise comparisons of 26 TDFs, 14 of 26 sequenced TDFs showed similarity to gene sequences in databases but 12 TDFs did not show homology to any nucleotide or amino acid sequence. All of the 26 sequences of TDFs were submitted to the NCBI database (Table 2). Because the genome of grapefruit has not been sequenced, these results are expected.



**Fig. 1** Agarose gel electrophoresis of PCR product of grapefruit infected by *Candidatus* *Liberibacter asiaticus* (I1–I4) and healthy plants (H1–H2). Marker is 100 bp DNA ladder.

### Functional categories of grapefruit transcripts modulated by Las infection

Each transcript was functionally annotated through careful analysis of the scientific literature and the Gene Ontology Database. Of 26 TDFs, 14 TDFs (54%) were assigned to one of categories including stress response/defence, cell metabolism, protein synthesis/destination, photosynthesis and energy and unknown function. The annotation of the molecular function of each individual protein is given in Table 2.

### Verification of representative genes by semiquantitative PCR

To validate cDNA-AFLP expression profiles, the expression level of 2 TDFs were studied by semiquantitative RT-PCR for verifying the expression patterns identified in the cDNA-AFLP study. These TDFs were chosen as they represented almost all the different functional categories identified (Fig. 3).

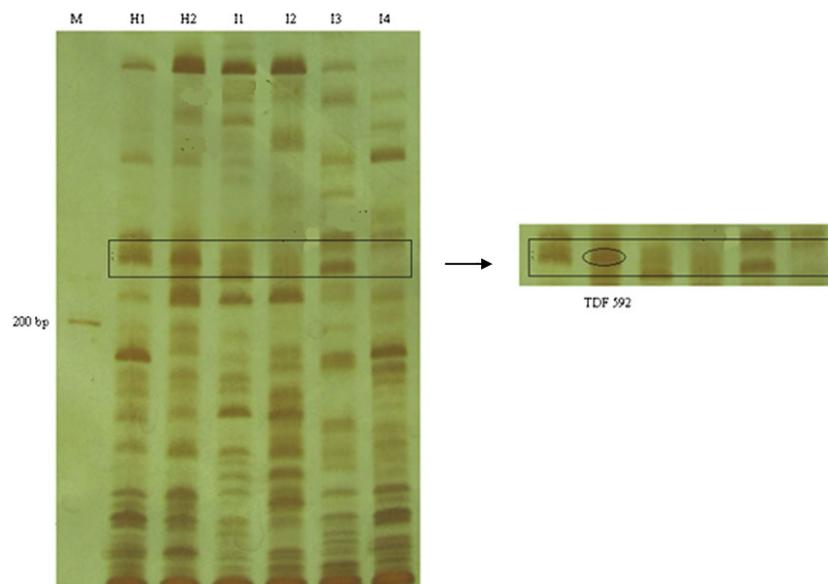
TDF 552 was more abundant in infected plants, but TDF 575 (*Aconitate Hydratase 2 (aco2)* gene) was abundant in infected plants (Table 2 and Fig. 3). 18S rRNA gene of greening was used as reference gene for data normalization (Carlos 2004). Semiquantitative analysis showed that the expression of the selected genes was in good agreement with profiles detected by cDNA-AFLP (Fig. 3).

## Discussion

A comparative transcriptomic analysis was carried out by cDNA-AFLP between healthy grapefruit and plants infected by Las. We carried out a cDNA-AFLP analysis on leaf samples of healthy control and infected plants at a late development stage of infection. This stage was chosen because Las is well established and its concentration at this stage are abundant enough to allow plant/pathogen interaction to be established, also plant cells are still active and can maintain pathogen survival (Zamharir et al. 2011).

As far as we are aware, this is the first gene expression analysis of the compatible interaction between 'Ca. *Liberibacter asiaticus*' and grapefruit trees. We observed transcriptional changes that affected the expression of several genes related to physiological functions that would affect most leaves in infected tissues.

The cDNA-AFLP method for global transcriptional analysis is an open architecture technology that is appropriate for gene expression studies in non-model species. This is because prior sequence data are not

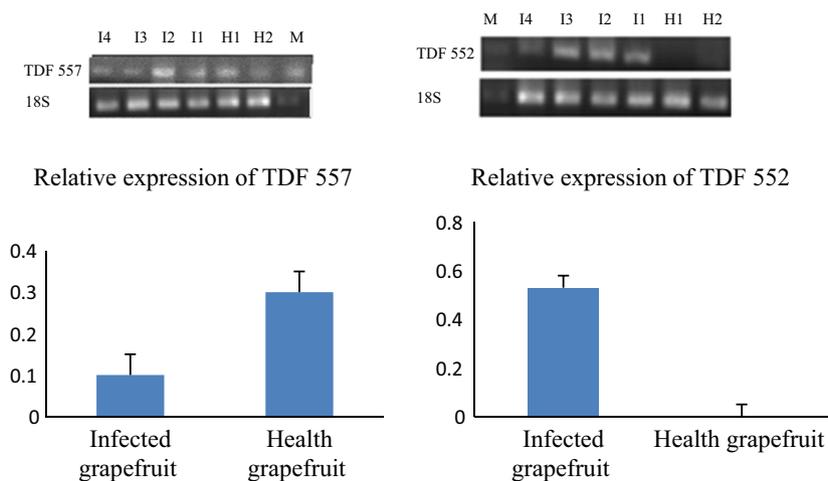


**Fig. 2** Representative results of polyacrylamide gel of cDNA-AFLPs generated by the primer combinations E11/MCG. Wells H1-H2, I1-I4 and M present non-infected, infected and 100 bp DNA size marker, respectively. TDF 592 represents a differentially expressed transcript-derived fragments (DE-TDFs) that were identified as *Vitis vinifera* cellulose synthase-like protein H1-like.

**Table 2** Homologies of TDFs to sequences in the databases

TDF	Length (bp)	I/R	Annotation (plant, accession number)	E-value
<b>Stress response/defence</b>				
552	512	I	<i>Poncirus trifoliata</i> citrus tristeza virus resistance gene locus (AF506028.1)	2e-77
572	516	I	IXM_002266629.2  <i>Vitis vinifera</i> DNA repair protein (LOC100243413), mRNA	6e-47
597	288	I	DNA repair (Rad51) family protein	3e-04
<b>Cell metabolism</b>				
575	384	R	mRNA for <i>Aconitate Hydratase 2 (aco2 gene)</i>	2e-17
595	451	R	<i>Citrus sinensis ZEP1o gene for zeaxanthin epoxidase (AB548572.1)</i>	3e-22
592	309	I	<i>Vitis vinifera</i> cellulose synthase-like protein H1-like (LOC100248315), mRNA (XM_003633251.1)	8e-26
<b>Protein synthesis/destination</b>				
594	369	I	rRNA <i>Arabidopsis thaliana</i>	1e-05
5113	381	I	Picobiliphyte sp. MS584-22 18S ribosomal RNA gene, partial sequence (JN934892.1)	9e-07
571	405	R	Phormium tenax 18S ribosomal RNA gene, partial sequence	1e-164
553	369	I	<i>Citrus aurantium</i> 26S ribosomal RNA gene, complete sequence (AY177420.1)	5e-148
<b>Photosynthesis and Energy</b>				
534	351	I	<i>Pyrus communis</i> monooxygenase-like mRNA (AY436775.1)	2e-04
5134	352	I	<i>Ricinus communis</i> cytochrome P450, putative, mRNA (XM_002524896.1)	3e-08
5133	309	I	<i>ATP synthase CF1 alpha subunit [Citrus sinensis]</i> , (YP_740460.1)	7e-07
611	300	I	<i>Citrus sinensis</i> chloroplast (DQ864733.1)	4e-06
613	250	I	<i>Citrus sinensis</i> chloroplast (DQ864733.1)	4e-06
<b>Unknown function</b>				
5134	327	I	TDF13 <i>Brassica napus</i> leaves cDNA-AFLP transcript-derived fragment	2e-12
593	311	I	gb GT222018.2  gh1574 <i>Candidatus Phytoplasma aurantifolia</i> cDNA-AFLP <i>Citrus aurantiifolia</i>	0.29
555	307	I	<i>Populus trichocarpa</i> predicted protein, mRNA (XM_002315037.1)	3e-18
596	380	I	— <sup>a</sup>	
573	404	I	— <sup>a</sup>	
5112	562	I	— <sup>a</sup>	
591	400	I	— <sup>a</sup>	
551	290	I	— <sup>a</sup>	
554	280	R	— <sup>a</sup>	
556	400	R	— <sup>a</sup>	
557	230	R	— <sup>a</sup>	
612	250	I	— <sup>a</sup>	

<sup>a</sup>According to similarity analyses of TDF sequences using BLAST.



**Fig. 3** Semiquantitative analysis of 2 DA-TDFs. Y axis represent the relative expression compared with housekeeping gene.

required for the visual identification of differentially expressed transcripts, in contrast to other approaches.

#### Infection with Las causes widespread gene induction in grapefruit

Of the identified TDFs, 54% were upregulated in response to infection, whereas only 46% were downregulated in response to infection which could reflect the exploitation of cellular resources and the induction of defence responses by the Las.

#### Stress responses and defence

Several genes that were modulated in grapefruit trees by infection with '*Ca. Liberibacter asiaticus*' were related to defence and response to stress. The expression of a tristiza resistance homologue gene (*ctz*) (TDF 552) was induced. *Ctv* is a single dominant gene in NBS-LRR resistance gene group that is present in *Poncirus trifoliata* and that causes resistance to *Citrus tristeza virus* (Mariângela et al. 2007). Plant resistance (R) genes encode immune receptors that recognize pathogens directly or indirectly and activate defence responses (Fujikawa and Iwanami 2012). The expression levels of R genes have to be regulated tightly due to costs to the fitness of plants that are associated with maintaining R-protein mediated resistance (Jones and Dangl 2006). The results of citrus database (CitEST database) show that the *Ctv* present in EST libraries of different species of citrus infected by *Xylella fastidiosa* and it is causing resistance to the pathogen (Simone and Helaine 2007). Quantitative study of this TDF indicated that its expression induced during infection of grapefruit by Las (Fig. 3). It is possible that TDF 552 involves in molecular process of resistance grapefruit

to pathogen and act as tolerance factor in citrus infected by greening pathogen.

Tow TDFs in this study were similar to DNA repair protein. The DNA repair proteins are involved in transcription regulation of the genes that acting in response to pathogen infection (Junqi et al. 2011). Studies show approximately half of the PR genes were upregulated by '*Ca. Liberibacter asiaticus*' infection (Kim et al. 2009). Thus, our work supports the idea that up- and downregulation of PR genes indicates that the host has reacted to invasion and infection by the HLB pathogen in a pattern that has been reported by others.

#### Cell Metabolisms

Several genes modulated by grapefruit-Las interaction were related to defence and response to stress. The expression of aconitate hydratase gene (Aco) in grapefruit plants inoculated by greening pathogen was downregulated (Javier et al. 2010). It has been shown that NO and ROS produced in defence response to *Tobacco mosaic virus* will have an effect on the activity of mitochondrial aconitate hydratase gene (Aco). Disabling aconitate hydratase gene damage to mitochondrial energy metabolism pathway and causing an accumulation of citrate in the mitochondria. Citrate levels may act as a defensive strategy because it stimulates the production of citrate and the resistance of plants such as potato, soybean, Arabidopsis and tobacco to tobacco mosaic virus and other viruses related to its accumulation (Duroy et al. 2000). Other study in *Olea europaea* subsp. *europaea* infected with *Pseudomonas savastanoi* pv. *savastanoi* was shown aconitate hydratase was upregulated in response to the increasing of bacteria population. Therefore, changes

in this protein is a marker for overpopulation of bacteria in the tissues of infected plants (Campos et al. 2009) (Table 2), and it can be concluded that reducing the activity of this enzyme in mitochondria and citrate level in grapefruit, prevents bacteria proliferation and increasing population of citrus greening bacteria and progression of disease.

We also identified a zeaxanthin epoxidase transcript levels decreased in response to pathogen (Corinne et al. 1998). Zeaxanthin cycle is essential for protecting the photosynthetic apparatus from light oxidation (Corinne et al. 1998). Studies show that reducing the activity or inactivation of the zeaxanthin epoxidase enzyme cause accumulation of zeaxanthin pigment in the tissues (Beatrycze et al. 2009). Carotenoids are compounds necessary for photosynthesis (Crispin and Barry 2006). Thus, the role of this enzyme in infected grapefruit by citrus greening pathogen is a photosynthetic response to protect the grapefruit photosynthesis system against *Ca. Liberibacter asiaticus* attacks.

Another transcript expression increased in grapefruit was the gene associated with cellulose synthesis. Cellulose is the most abundant molecule in biology; it is involved in microfibre forming in cell walls of plants and in plant resistance and flexibility (Hee et al. 2011). Cellulose is the major component of the cell wall structure, and plays a pivotal role in the invasion and penetration of bacteria and fungi to plant (Adrienne et al. 2007).

#### Protein synthesis and destination

We identified several TDFs that were related to protein metabolism. Among these were genes that encoded ribosomal proteins. The expression of three ribosomal proteins was induced, whereas another ribosomal protein was repressed. This suggests that the infection results in a general induction of protein turnover, which could reflect an adaptive response in the plants to remove misfolded proteins that have accumulated as a result of stress (Polesani et al. 2008).

#### Conclusion

This study report expression analysis of genes involved in the interaction of grapefruit trees with '*Ca. Liberibacter asiaticus*'. The cDNA-AFLP technique allowed several novel genes to be identified from grapefruit trees, because a significant proportion of the TDFs are not currently represented in citrus databases. Our data showed that infection resulted in

the upregulation of grapefruit trees transcripts in all major functional categories. These results will serve as a basis to address new questions and design new experiments to elucidate the biology of plant-*Ca. Liberibacter asiaticus* interactions and the associated reprogramming of the host metabolism. They might also pave the way to identify genes that can be targeted to elevate plant resistance or inhibit the growth and reproduction of the pathogen. However, further research is required to elucidate the roles of these genes in the susceptibility/resistance of grapefruit trees to '*Ca. Liberibacter asiaticus*', and to determine how strategies might be developed to incorporate these genes into molecular breeding programs.

#### References

- Adrienne R, David A, Daigo T. (2007) Cytoskeleton and cell wall function in penetration resistance. *Curr Opin Plant Biol* 10:342–348.
- Albrecht U, Bowman KD. (2008) Gene expression in *Citrus sinensis* (L.) Osbeck following infection with the bacterial pathogen *Candidatus Liberibacter asiaticus* causing Huanglongbing in Florida. *Plant Sci* 175:291–306.
- Albrecht U, Bowman KD. (2011) Transcriptional response of susceptible and tolerant citrus to infection with *Candidatus Liberibacter asiaticus*. *Plant Sci* 185–186:118–130.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410.
- Bachem CWB, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M, Visser RGF. (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *Plant J* 9:745–753.
- Bachem CWB, Oomen RJFJ, Visser RGF. (1998) Transcript imaging with cDNA-AFLP: a step-by-step protocol. *Plant Mol Biol Report* 16:57–173.
- Bassam B, Caetanoanollles G, Gresshoff P. (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* 196:80–83.
- Beatrycze N, Wojciech S, Kazimierz S. (2009) New transgenic line of *Arabidopsis thaliana* with partly disabled zeaxanthine poxidase activity displays changed carotenoid composition, xanthophyll cycle activity and non-photochemical quenching kinetics. *J Plant Physiol* 166:1045–1056.
- Bogdanove AJ. (2002) Protein-protein interactions in pathogen recognition by plants. *Plant Mol Biol* 50:981–989.

- Bove JM. (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *J Plant Pathol* 88:7–37.
- Campos A, Costa G, Coelho V, Fevereiro P. (2009) Identification of bacterial protein markers and enolase as a plant response protein in the infection of *Olea europaea* subsp. *europaea* by *Pseudomonas savastanoi* pv. *savastanoi*. *Eur J Plant Pathol* 125:603–616.
- Capoor SP. (1963) Decline of citrus trees in India. *Bull Natl Inst Sci, India* 24:48–64.
- Carlos EF. (2004). Transcriptional profiling on trees affected by citrus blight and identification of an etiological contrast potentially associated with the disease. University of Florida.
- Chen M, Miyakawa T, Matsui C. (1973) Citrus likubin pathogens in the salivary glands of *Diaphorina citri*. *Phytopathology* 63:194–195.
- Corinne A, Charlotte B, Anne F, Bruno S, Christian M, Thierry S, Annie M. (1998) Expression studies of the zeaxanthin epoxidase gene in *Nicotiana glauca*. *Plant Physiol* 118:1021–1028.
- Crispin A, Barry J. (2006) Carotenoid accumulation and function in seeds and non-green tissues. *Plant, Cell Environ* 29:435–445.
- Daire X, Clair D, Reinert W, BoudonPadieu E. (1997) Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. *Eur J Plant Pathol* 103:507–514.
- Duroy A, David W, Jo D, Robert N, Daniel F. (2000) Nitric oxide modulates the activity of tobacco aconitase. *Plant Physiol* 122:573–582.
- Fujikawa T, Iwanami T. (2012) Sensitive and robust detection of citrus greening (huanglongbing) bacterium "*Candidatus* Liberibacter asiaticus" by DNA amplification with new 16S rDNAspecific primers. *Mol Cell Probes* 26:194–197.
- Gachomo EW, Shonukam OO, Kotchoni SO. (2003) The molecular initiation and subsequent acquisition of disease resistance in plants. *Am J Biotechnol* 2:26–32.
- Grant M, Mansfield J. (1999) Early events in host-pathogen interactions. *Curr Opin Plant Biol* 2:312–319.
- Hammond-Kosack KE, Jones JDG. (1996) Resistance gene-dependent plant defense responses. *Plant Cell* 8:1773–1791.
- Hee JK, Norimoto M, David DF, Barbara AT. (2011) Functional analysis of *Gossypium hirsutum* cellulose synthase catalytic subunit 4 promoter in transgenic *Arabidopsis* and cotton tissues. *Plant Sci* 180:323–332.
- Hocquellet A, Bové J, Garnier M. (1999a) Isolation of DNA from the uncultured "*Candidatus* Liberibacter" species associated with citrus huanglongbing by RAPD. *Curr Microbiol* 38:176–182.
- Hocquellet A, Toorawa P, Bové JM, Garnier M. (1999b) Detection and identification of the two *Candidatus* Liberibacter species associated with citrus huanglongbing by PCR amplification of ribosomal protein genes of the b-operon. *Mol Cell Probes* 13:373–379.
- Jagoueix S, Bové JM, Garnier M. (1994) The phloem-limited bacterium of greening disease of citrus is a member of the a subdivision of the Proteobacteria. *Int J Syst Bacteriol* 44:379–386.
- Jagoueix S, Bové JM, Garnier M. (1996a) PCR Detection of the two Liberibacter species associated with greening disease of citrus. *Mol Cell Probes* 10:43–50.
- Jagoueix S, Bové JM, Garnier M. (1996b) PCR detection of the two '*Candidatus*' Liberibacter species associated with greening disease of citrus. *Mol Cell Probes* 10:43–50.
- Javier T, Guillermo S, Manuel T, Manuel C. (2010) The aconitate hydratase family from citrus. *BMC Plant Biol* 10:222.
- Jones JDG, Dangl JL. (2006) The plant immune system. *Nature* 444:323–329.
- Junqi S, Wendy E, Shui W, Shunping Y, Ek HT, Xinnian D. (2011) DNA repair proteins are directly involved in regulation of gene expression during plant immune response. *Cell Host Microbe* 9:115–124.
- Kim JS, Sagaram US, Burns JK, Li JL, Wang N. (2009) Response of sweet orange (*Citrus sinensis*) to '*Candidatus* Liberibacter asiaticus' infection: microscopy and microarray analyses. *Phytopathology* 99:50–57.
- Mariângela C, Irving J, Maria L, Marco A, Sílvia O, Juliana F, Alessandra A, Raquel L, Marcelo S, Marcos A. (2007) Differential expression of genes identified from *Poncirus trifoliata* tissue inoculated with CTV through EST analysis and in silico hybridization. *Genet Mol Biol* 303(suppl):972–979.
- Murray RGE, Schleifer KH. (1994) Taxonomic notes: a proposal for recording the properties of putative taxa of prokaryotes. *Int J Syst Bacteriol* 44:174.
- Polesani M, Desario F, Ferrarini A, Zamboni A, Pezzotti M, Kortekamp A, Polverari A. (2008). cDNA-AFLP analysis of plant and pathogen genes expressed in grapevine infected with *Plasmopara viticola*. *Bmc Genomics* 9:142.
- Powell R, Gannon F. (2002) Purification of DNA by Phenol Extraction and Ethanol Precipitation. Oxford, Oxford University Press.
- Rey JM, Pujo P, Callier P, Cavailles V, Freiss G, Maudelonde T, Brouillet JP. (2000) Semiquantitative reverse transcription-polymerase chainreaction to evaluate the expression patterns of genes involved in the oestrogen pathway. *J Mol Endocrinol* 24:433–440.
- Scheideler M, Schlaich NL, Fellenberg K, Beissbarth T, Hauser NC, Vingron M, Slusarenko A, Hoheisel JD. (2002) Monitoring the switch from housekeeping to pathogen defense metabolism in *Arabidopsis thaliana* using cDNA arrays. *J Biol Chem* 277:10555–10561.
- Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM. (2000) Coordi-

- nated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proc Natl Acad Sci USA* 97:11655–11660.
- Schneider H. (1968) Anatomy of greening-diseased sweet orange shoots. *Phytopathology* 58:1155–1160.
- Simone G, Helaine C. (2007). Putative resistance genes in the CitEST database. *Genet Mol Biol* 30:(3 suppl):931–942.
- Slisz AM, Breksa AP, Mishchuk DO, McCollum G, Slupsky CM. (2012) Metabolomic analysis of citrus infection by ‘*Candidatus Liberibacter*’ reveals insight into pathogenicity. *J Proteome Res* 11:4223–4230.
- Tao Y, Xie Z, Chen W, Glazebrook J, Chang HS, Han B, Zhu T, Zou G, Katagiri F. (2003) Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* 15:317–330.
- Ute A, Kim DB. (2008) Gene expression in *Citrus sinensis* (L.) Osbeck following infection with the bacterial pathogen *Candidatus Liberibacter asiaticus* causing Huang-longbing in Florida. *Plant Sci* 175:291–306.
- Woese CR, Stackebrandt E, Weisburg WG. (1984) The phylogeny of purple bacteria: the alpha subdivision. *Syst Appl Microbiol* 5:315–326.
- Wu M. (2006) Development of a simple and powerful method, cDNA AFLPSSPAG, for cloning of differentially expressed genes. *Afr J Biotech* 5:2423–2427.
- Zamharir MG, Mardi M, Alavi SM, Hasanzadeh N, Khayyam Nekouei M, Zamanizadeh HR, Alizadeh A, Salekdeh GH. (2011) Identification of genes differentially expressed during interaction of Mexican lime tree infected with “*Candidatus Phytoplasma aurantifolia*”. *BMC Microbiol* 11:1.
- Zhao XY. (1981) Citrus yellow shoot disease (huang-longbing) in China- a review. *Proc Intern Soc Citricul* 1:466–469.
- Zheng ZL, Zhao Y. (2013) Transcriptome comparison and gene coexpression network analysis provide a systems view of citrus response to ‘*Candidatus Liberibacter asiaticus*’ infection. *BMC Genomics* 14:27.