Stress responses in citrus peel: Comparative analysis of host responses to Huanglongbing disease and puffing disorder

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ABSTRACT

A comparison between transcriptomic responses to puffing disorder and Huanglongbing disease was conducted to decipher differences and similarities in gene and pathway regulation induced by abiotic (puffing) and biotic stresses (Huanglongbing) in citrus peel tissues. We functionally analyzed two previously published datasets: the first obtained for the study of puffing disorder using an Affymetrix citrus microarray and the second consisting of a deep sequencing analysis of symptomatic responses to Huanglongbing disease. Transcriptomic data were mined using bioinformatic tools to highlight genes and pathways playing a key role in modulating responses to different types of stress in citrus fruit. Puffing disorder was linked to altered expression of genes involved in abiotic stress, vesicle transport, and protein targeting while Huanglongbing disease induced biotic stress responses and transport pathways. Sucrose and starch metabolism were the most significantly regulated pathways in both the two stresses. Huanglongbing disease significantly affected secondary metabolism (phenylpropanoid, flavonoid, and terpenoid pathways) while puffing disorder was more linked to primary metabolism (fatty acid, pentose phosphate, and glycerolipid pathways). Key genes were analyzed by qRT-PCR to define possible host biomarkers specific to each stress or which could act as general indicators of stress. Ethylene-related genes in the fruit peel were more affected by Huanglongbing than puffing. Gibberellin signaling genes (GASA1 and GASA5) were repressed under both stresses. Huanglongbing upregulated key genes involved in biotroph responses such as methylsalicylate and WRKY70. A protein–protein network revealed that heat shock proteins were major, transcriptionally regulated hubs under stress conditions as shown by the repression of HSP82.

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1. Introduction

The genus Citrus is affected by diseases and disorders that cause serious challenge to production, reducing fresh fruit quality, yield, and thus, grower profits. In case of diseases HLB, symptoms occurrence is too late to introduce an intervention to manage the disease because the pathogen has already established the secondary spread of the disease. A similar situation also exists with physiological disorders like puffing where the physiological manifestation of the disorder precedes the observable phenotype. It is essential that a therapeutic intervention to be effective would need to be deployed before the overt symptoms/observable phenotype occurs at an asymptomatic stage. Global climate change is expanding the range of abiotic stresses and the spectrum of disease causing pathogens (Marcais et al., 2004). It has been shown that higher levels of carbon dioxide have been linked to increases in fungal pathogen load in tall grass prairie (Mitchell et al., 2003). It is imperative that diagnostic methods/tools are developed to monitor plant health so abiotic or biotic stresses can be diagnosed while intervention is still possible to prevent losses due to yield reduction and poor fruit. Plants subjected to stress manifest an “induced stress response” (ISR) that could be used to improve diagnostics (Agudelo-Romero et al., 2008).

Transcriptomics has proved difficult to identify plant genes specifically induced by each pathogen or physiological stress. The molecular analysis in plant stress response is important not only to clarify mechanisms of disease establishment and progression but
also to identify host biomarkers that could improve the speed and efficiency of diagnosis. The technologies that allow a massive parallel analysis of mRNA and proteins provides a deep insight into gene regulatory networks that mediate host responses to stresses. Microarray and RNA-seq are both technologies that allow such massive parallel analysis of the transcriptome. However, it is a challenge to establish a causal-link to a set of genes that play a key role in the stress response and may be used for rapid asymptomatic diagnosis from among the hundreds or thousands of significantly regulated genes. It is very important to identify those genes that may be used to distinguish different types of stresses at asymptomatic stage. A possible strategy may be the analysis of a subset of genes, the simultaneous regulation of which may be specific for a disease, in a complementary approach to traditional tools targeting the pathogen, such as molecular and serological assays.

Puffing is a citrus physiological disorder characterized by a split between peel and pulp and the production of aerial spaces through dissolution of the albedo: the white, fleshy, spongy middle layer of the pericarp or peel (Kuraoka, 1962). It is typical of mandarins, particularly the easy-peel varieties, but may occur in other citrus fruits when fruit are left on the tree until overripe. Symptoms occur in fully ripe fruit and can render the fruit unmarketable (Garcialuis et al., 1985). No pathogen has been found in affected fruit, but the disorder has been linked to water exchange regulation through the peel or high nitrogen fertilization (Kawase and Hirose, 1981). Albedo is a very metabolically active tissue, particularly during rind development and maturation, when modifications occur in cellular structure, shape, and pectin composition (Kuraoka, 1962). Gene regulation underlying rind development in citrus peel has been investigated previously using a customized microarray platform with selected candidate genes (Goudeau et al., 2008).

Huanglongbing disease (HLB), also called “citrus greening”, is a worldwide destructive citrus disease that is caused by several Candidatus Liberibacter species (Bove, 2006). Candidatus Liberibacter asiaticus (CaLs), currently present in North and South America (Teixeira et al., 2005), belongs to the alpha subdivision of the proteobacteria (Jagoueix et al., 1994). The bacterium is phloem-limited and transmitted by the Asian citrus psyllid (Diaphorina citri). Symptoms are mainly due to the host response itself: no genes that produce toxic compounds have been found in the genome sequence, the bacteria is a biotroph endophyte, and typical symptoms, such as asymmetric yellowing, starch accumulation, and callose production are directly linked to altered source–sink relationships due to altered expression of key genes involved in carbohydrate metabolism. Microarray analysis of artificially infected mature leaf tissues has been conducted to examine gene regulation in response to CaLas attacks under controlled conditions (Albrecht and Bowman, 2008; Kim et al., 2008; Fan et al., 2011, 2012). Recently, next-generation sequencing (NGS) technologies have been applied to characterize the fruit from afflicted trees (Martinelli et al., 2012) and to examine how the pathogen transcriptionally regulates source–sink relationships (Martinelli et al., 2013).

This paper compares two previously published transcriptomic datasets to determine similarities and differences in how the gene regulatory networks in citrus fruit peel respond to a biological (Huanglongbing) and a physiological (puffing) stress. The two research works were conducted in fruit peel tissues at symptomatic stage. The identification of gene patterns affected only in one of the two stresses or commonly-regulated in both conditions may help to improve, respectively, the detection of these stresses or identify general induced stress response. The analysis was conducted at three levels: genes, pathways and inferred protein networks through the integration of the two large scale already published transcriptomic datasets.

The differences in gene expression, metabolic pathways, and inferred protein networks provide clues to potential biomarkers of stress onset, which may assist growers in specifically (1) manage different stresses or (2) identify general stress responses.

2. Material and methods

2.1. Plant material and experimental design

We functionally analyzed and integrated two previously published transcriptomic datasets: a study of peel responses to puffing disorder using Affymetrix citrus arrays (Ibanez et al., 2014) and an analysis of transcriptomic changes in response to Huanglongbing disease (Martinelli et al., 2012). We briefly summarize below the materials and methods used to generate the two datasets.

The puffing disorder study sampled symptomatic and healthy ‘Navel’ orange fruits harvested at mature stage (Ibanez et al., 2014). The four fruits harvested of each type and developmental stage were considered a biological replicate. For analysis of responses to HLB, we compared the data generated from mature ‘Valencia’ sweet oranges harvested from symptomatic and apparently healthy trees in the same orchard (Martinelli et al., 2012). For these samples, five to ten fruits were combined to make a biological replicate.

2.2. RNA extraction

Total RNA was extracted from pooled peel tissue sections at the four opposite sides of the fruit samples from both the two types of symptomatic fruits using the hot borate method (Wan and Wilkins, 1994), followed by RNA purification using an RNA/DNA cleanup kit (Qiagen; Valencia, CA) (Ibanez et al., 2014). RNA concentration and purity were assessed by UV spectrophotometry. The RNA was stored at −80 °C in RNase-free water.

2.3. Microarray analysis for the study of puffing

Raw data obtained by the previous published study (Ibanez et al., 2014) were statistically re-analyzed using R package LMGene (Roke, 2004) to perform one-way ANOVA and to assign a p-value for each gene. All p-values were adjusted for multiple hypotheses using R package nulltest (Benjimini and Hochberg, 2006) and genes with adjusted p-values <0.05 were considered differentially expressed. One-way ANOVA was used to adjust the data for multiple hypotheses (Ibanez et al., 2014). R package limma (Pollard and van der Laan, 2004) was employed to perform pairwise comparison between different groups. The list of genes differentially regulated in response to puffing disorder in peel was the sum of the significantly puffing-regulated genes for the flavedo (outer) and albedo (inner) fruit peel tissues.

2.4. Next-generation sequencing and data processing of HLB-infected fruit

Details of sample preparation for the next-generation sequencing, read trimming, assembly and annotation was previously published (Martinelli et al., 2012). The five biological replicates of the symptomatic or asymptomatic fruits were equally combined to produce one cDNA library for each condition. HLB-regulated genes were those that presented a p-value <0.01 and a Log2 Fold Change >1.5 or <−1.5). Only the pairwise comparison between symptomatic and apparently healthy fruits (SY_vs_AH) was functionally analyzed. Apparently healthy trees were asymptomatic but HLB-positive.
2.5. Functional analysis and data mining

The differentially regulated genes belonged to different pathways (Table S1). For the bioinformatic and protein network analysis, an Arabidopsis hortolog was assigned to each differentially regulated Citrus Affymetrix identifier (puffing) or citrus NCBI unigene (HLD). Functional classifications were obtained using MapMan software (Thimm et al., 2004) using the most updated Arabidopsis mapping file downloaded from MapMan website. The list of differentially regulated genes obtained by the two studies was compared. Upregulated genes in only one of the two studies were assigned to a standard positive value that was different between puffing disorder and HLD (these genes were, respectively, light and dark red). Similarly downregulated genes in only one of the two studies were assigned to two different negative values. Commonly regulated genes in the two studies were assigned to 0 (white in figure). PathExpress software was used to interpret pathway regulation under the two different stresses (Goffard and Weiller, 2007). The same list of upregulated and downregulated genes previously used for Mapman were employed for pathway enrichment analysis. Puffing- and HLD-differentially regulated pathways were those having a p-value <0.05 (no p-value adjustment).

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For the protein–protein network analysis, a protein–protein interaction (PPI) network was inferred for Citrus based on PPIs in Arabidopsis (Geisler-Lee et al., 2007). Proteins encoded by differentially regulated genes in response to the two different stresses were highlighted. Networks were visualized using Graphviz software. PCA analysis was performed with SAS software (SAS Institute, NC, USA) with default settings using the same list of genes differentially regulated used for PathExpress and Mapman analysis. This analysis was limited to those genes that were present in both transcriptome datasets, determined by the corresponding Affymetrix identifiers for each unigene analyzed by RNA-seq. The key pathways analyzed were hormones (59 genes), secondary metabolism (56), and stress responses and volatiles (254).

2.6. Real time quantitative TaqMan PCR

HLD symptomatic and puffing symptomatic fruits were analyzed by qRT-PCR. Four biological replicates were used for each analyzed gene. Each biological replicates consists of a mix of the four pieces at opposite sides of the fruit. For each target gene, PCR primers and a TaqMan probe were designed using Primer Express software and default parameters (Applied Biosystems, Foster City, CA). A list of primers used for each analyzed gene was reported (Table S2). The Quantitect Reverse Transcription Kit (Qiagen) was employed to combine DNase and cDNA synthesis. Real time amplification was performed with TaqMan Universal PCR Master Mix, Taqman probes (Applied Biosystems), and primers (Operon Technologies) in a total volume of 12 μL using 50 ng cDNA. Amplifications were performed on a StepOne Real Time PCR system (Applied Biosystems) as previously described (Martinelli et al., 2013). A standard curve was determined to calculate the amplification efficiency. Values for each gene ranged between 96 and 102%.

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Ct values were obtained using adjusted thresholds for each gene. Citrus sinensis elongation factor 1 alpha (EF-1α) (accession number: AY498567) was employed as a reference gene. This gene was already used as housekeeping gene of qRT-PCR analysis in the two published papers validated by this study (Martinelli et al., 2012; Ibanez et al., 2014).

\[ \Delta Ct \] was calculated by subtracting the average EF-1α Ct from the average Ct of each specific gene.

3. Results

3.1. Gene set enrichment analysis and PCA

A gene set enrichment analysis was initially performed to determine which metabolic pathways were affected by the two stresses. The most significant changes were in sucrose and starch metabolism in response to both stresses (Table 1). In puffing disorder, significant changes also occurred in the biosynthesis of unsaturated fatty acids, the pentose phosphate pathway, glycerolipids, and phenylalanine metabolism. HLD disease perturbed different secondary pathways, such as synthesis of phenylpropanoids, anthocyanins, and carotenoids.

PCA plots were made of key pathways involved in plant stress biology: hormones, secondary metabolism, stress responses, and volatiles (Fig. S1). All four plots clearly separated the HLB pool sample from the four biological replicates of puffing disorder. Puff and HLB samples were clearly separated by PC1 that explains 59.9% of the sample variability for stress response pathway, 60% for genes involved in volatiles, 64% (hormones) and 54.7% (secondary metabolism). PC2 was able to explain up to 28.4–31.8% of variability between the four considered samples.

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3.2. Metabolic Overview

To gain insight into distinct genes of significantly regulated pathways, functional categorization of the transcriptomic data was performed using the Mapman knowledgebase. There were clear differences in the transcriptomes of citrus peel subjected to the two stresses (Fig. 1). Expression of several genes involved in secondary metabolism was similarly (and strongly) affected by both stresses: terpenes, phenylpropanoids, and phenolics. Huanglongbing disease strongly induced genes for the light reactions of photosynthesis, amino acid biosynthesis, and catabolism.

Puffing primarily affected the TCA cycle, glycolysis, and mitochondrial electron transport. Genes involved in cell wall modification and restricting were also differentially regulated by the two stresses. Genes encoding reversibly glycosylated proteins and involved in hemicellulose synthesis were only affected by puffing disorder. HLB mostly affect expression of genes encoding pectate lyases and polygalacturonases. Few genes involved in cell wall metabolism were commonly regulated by the two stresses, such as a fucosyltransferase, a beta-xylosidase 2, a pectinesterase and a pectinacetylxylosesterase.

Table 1

<table>
<thead>
<tr>
<th>Pathways (p-values &lt; 0.05)</th>
<th>Puffing</th>
<th>Huanglongbing disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch and sucrose metabolism</td>
<td>0.003</td>
<td>0.007</td>
</tr>
<tr>
<td>Biosynthesis of unsaturated fatty acids</td>
<td>0.015</td>
<td>n.s.</td>
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<tr>
<td>Pentose phosphate pathway</td>
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<td>n.s.</td>
</tr>
<tr>
<td>Glycerolipid metabolism</td>
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<td>0.027</td>
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<tr>
<td>Phenylalanine metabolism</td>
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<tr>
<td>Phenylpropanoid biosynthesis</td>
<td>n.s.</td>
<td>0.002</td>
</tr>
<tr>
<td>α-Linolenic acid metabolism</td>
<td>n.s.</td>
<td>0.027</td>
</tr>
<tr>
<td>Anthocyanin biosynthesis</td>
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</tr>
<tr>
<td>Selenoamino acid metabolism</td>
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<td>0.039</td>
</tr>
<tr>
<td>Carotenoid biosynthesis – general</td>
<td>n.s.</td>
<td>0.046</td>
</tr>
</tbody>
</table>
3.3. Cell functions and primary metabolism

Interesting differences were observed between the two stresses in cell functions (Fig. 2). Although it is not correct to compare the total number of differentially regulated genes due to the fact that different methodology (and consequently thresholds) were used, it is possible to compare within the same stress the number of up- or down-regulated genes. HLB mostly upregulated biotic stress while puffing showed a higher number of repressed genes involved in both biotic and abiotic stress (Table S3). Particularly, HLB upregulated genes encoding acidic endochitinase (CHIB1), chitinase class IV, non race-specific disease resistance 1 (NDR1) and several pathogenesis-related proteins (NBS-LRR class, leucine-rich repeat family proteins). Puffing enhanced expression of genes involved in drought responses, such as dehydration-responsive proteins-related, rare-cold-inducible 2B, drought-responsive family proteins. Primary metabolic genes involved in photosynthesis reactions and encoding for photosystem II subunit R, oxygen evolving enhancer 3, photosystem II reaction center family protein, and redox chain reactions were upregulated during HLB (Fig. 3A).

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Key genes involved in raffinose metabolism (Fig. 3B) were transcriptionally affected under both stresses but particularly by puffing disorder. However, CaLas infection enhanced transcription of genes encoding enzymes involved in galactinol, stachyose, and glucose synthesis. Invertase family genes were upregulated under both stresses (Fig. 4). The first two steps of starch biosynthesis were significantly repressed under both stresses as shown by the down-regulation of glucose-1-phosphate-adenyltransferase and starch synthase. Genes involved in starch degradation to produce glc-1-phosphate were similarly regulated.

3.4. Signaling, protein degradation and synthesis

Receptor-like kinases are signal transduction agents for plant molecular responses to biotic attacks. HLB disease induced transcriptomic changes in this gene category, but puffing disorder did not (Table S4). Different classes of leucine-rich repeat (LRR) family proteins were somewhat affected. Some genes encoding for LRR protein XI were enhanced while others were inhibited. Genes encoding for receptor kinase DUF26 were more abundant as were cysteine-rich kinases RLK6 and RLK10.

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In response to CaLas attack, the expression of genes encoding ubiquitin E3 ring and F-box family proteins, proteasome maturation factor, and ubiquitin protein ligase were significantly affected.

Fig. 1. Metabolic overview of transcriptomic responses to Puffing and Huanglongbing disease in Citrus fruit peel tissues.
Several categories of E3 ring proteins were induced, such as copine-related, U-box domain-containing protein, jumonji, beta-catenin repeat family protein, and zinc ion binding proteins. Conversely, the beta-catenin repeat family protein, zinc finger C3HC4 proteins, zinc ion binding were inhibited. Functions as protein synthesis and amino acid activation were more involved in response to Puffing disorder than in HLB disease (Fig. S2).

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3.5. Transcription factors

As expected transcription factors were drastically affected by both stresses (Table S4). WRKY70 was strongly induced by CaLas infection while puffing repressed WRKY23, WRKY57, WRKY32, and WRKY75. MYB factors and the AP2-EREBP family were also altered. Different members of MYB were specifically regulated by puffing or HLB. Conversely, AS1 and MYB44 were significantly inhibited by CaLas infection while MYB3, MYB4, MYB59 and MYB91 were repressed in puffing. The AP2-EREBP proteins CBF1 and CBF2 were more abundant in HLB disease while RAP2.4 and ERF were enhanced in puffing. Puffing disorder repressed expression of homeobox (HB), MADS, Aux/IAA, trihelix, SET-domain and histone-related. Conversely, the disorder enhanced other functional categories, such as CH2 Zinc finger, bromodomain, GRAS, and nucleosome assembly. The key transcription factors ATAF1 (NAC) and TGA1 (bZIP) were upregulated by HLB.

3.6. Hormone-related pathways

Hormones are primary regulators of stress response pathways, so changes in hormone synthesis were key elements of the similarities and differences in gene regulatory networks between the two stresses (Table S5). Several genes involved in cytokinin and gibberelin metabolism and signal transduction were downregulated in both stresses. The gibberelin GAS1 and two gibberelin responsive proteins were repressed with HLB infection while ent-kaurene synthase and ent-kaurenoic acid hydroxylase 1 were less abundant in puffing disorder. Some genes were differentially regulated with both stresses, such as histidine kinase 1 (cytokinin pathway), GA2, GAS4, and gibberelin responsive proteins. Ethylene-related genes were also involved in the response to both stresses as shown by the high number of commonly regulated genes, such as 1-aminocyclopropane carboxylate oxidase, ethylene response factor
(ERF) 1 and ERT2, and ethylene responsive protein. As expected in fruit peel tissues infected by CaLas, the jasmonate and salicylic acid defense response pathways were induced while puffing disorder did not affect the expression of these genes. Some key auxin synthesis genes were induced by HLB (ILL2, GH3.1, GH3.10) while puffing differentially affected auxin-related pathways.

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3.7. Secondary metabolism

Terpenoid biosynthesis was one of the most affected pathways in response to CaLas infection (Table S5). Several transcripts encoding for terpene synthases were repressed while lanosterol synthase 1 and β-amanin were induced. Puffing disorder repressed genes encoding for farnesyl diphasate synthases 1 and 2, geranylgeranyl pyrophosphate (GGP) synthase, and GGP-reductase, all involved in the isoprenoid pathway (Table S5). Lanosterol and a terpene synthase (At3g14520) were also differentially regulated in response to the disorder. The gene regulation of terpenoid and carotenoid pathways have been linked to the modified volatile profile observed in HLB-symptomatic fruits.

Phenylpropanoids, lignins, and lignans were differently regulated by the two stresses and the expression of only a few genes was affected by both stresses (Fig. 5A). The production of important pheno- nolic volatiles may be affected by HLB and puffing as shown by the higher abundance of genes involved in cinnamic acid, p-coumaric and coniferyl aldehydes, and alcohol synthesis (Fig. 5B).

3.8. Energy metabolism

While repression of phosphofructokinase was observed in puffing disorder, this gene was induced in HLB disease. In puffing disorder, an induction was observed for genes involved in pyruvate and phosphoenolpyruvate (PEP) metabolism. Transcripts involved in fermentation processes were more abundant in the disorder, including one encoding l-lactate dehydrogenase (Fig. 1). At the same time, puffing was associated with general repression of genes involved in the TCA cycle and regulation of mitochondrial redox reactions. In puffing disorder, an upregulation of fermentation reaction catalyzed by lactate dehydrogenase was observed.

3.9. Protein–protein interaction network analysis

We inferred citrus protein–protein interactions (PPIs) basing on an Arabidopsis protein interaction network database (Geisler-Lee et al., 2007), obtaining 24,505 predicted pairwise interactions between NCBI citrus unigenes. This “citrus inferred interactome file” was used to determine key regulated genes encoding highly interactive proteins (hubs) and proteins connecting hubs associated with puffing disorder and/or HLB disease (Fig. 6). The PPI network distinguishes proteins encoded by genes uniquely regulated by HLB or puffing from those commonly regulated and associated to both stresses (Table 2). Proteins were categorized by pathway to understand which protein–protein networks were peculiar to each stress condition. Each node was characterized by three features: the number of PPI interactions (indicated by size), the pathway (color), and its presence/absence under puffing disorder and HLB disease.

Fig. 4. Functional categorization of differentially regulated genes involved in sucrose and starch metabolism.
Fig. 5. Pathways involved in secondary metabolism. (A) Categories of genes involved in the biosynthesis of different secondary metabolites. (B) Genes involved in secondary metabolism volatiles.

Most interactive proteins were specifically regulated in HLB or puffing disorder. However, heat shock proteins (HSP), such as HSP82, HSP60, HSP70, and Heat shock factor A4 were encoded by genes that were differentially expressed in both stresses. Several transcription factors were also involved in both stress responses: DUF861, splicing factor PWI domain-containing protein, and TOC1 (timing of CAB1.1). Transcription factors were more involved in the response to puffing disorder than HLB disease. Major hubs were RNA polymerase II accessory factor, small nuclear ribonucleoprotein F, KOW domain-containing transcription factor, ubiquitin family protein, and small nuclear ribonucleoprotein D. Conversely, there were also transcription factors involved in the PPI networks specific to HLB disease, such as DNA polymerase processivity factor, CSL zinc finger domain-containing protein, and BEL1-like homeodomain 1. Protein synthesis and modification was a peculiar characteristic of the network of both stresses, as shown by the high number of interactive proteins. Based on the number of interactions, we identified several hubs belonging to different pathways that play a major role in the response to puffing. These hubs included important proteins, such as HSP 70-7, RNA pol II accessory factor, methylallyltransferase/geranyltransferase, ribosomal protein S5 family protein, and small nuclear ribonucleoprotein F. Most of these proteins perform RNA processing. Major hubs in the PPI networks of HLB disease were protein synthesis enzymes, such as elongation factor Tu, C-terminal domain-containing protein, and elongation factor 1B-γ. Proteins linked to lipid metabolism, such as LAG1 (Longevity assurance gene 1), galactolipase/hydrolase/phospholipase, and quinone reductase family protein were mostly limited to the PPI network of HLB response. Interestingly, proteins of signaling pathways were more
associated with HLB responses, such as 1-phosphatidylinositol-4-phosphate 5-kinase/ zinc ion binding, Rho-GTPase-activating protein, and Rac-like GTP-binding protein. Many proteins involved in the response to HLB disease have unknown functions. These proteins could be involved in the plant–pathogen interactions and might include pathogen-specific proteins.

3.10. qRT-PCR analysis

For qRT-PCR analysis, 19 genes with key roles in important pathways (sugar and starch metabolism, transcription factors, hormones, stress responses, or major hubs in the PPI network) were selected (Table 3). The aim was to compare their pattern of expression in disordered or diseased peel tissues to the respective healthy control.

Invertase and sucrose transporter genes were clearly induced in HLB-symptomatic peel tissues. Invertase was also significantly upregulated by puffing disorder. These results confirm the general trend observed in both transcriptomic datasets. ADP glucose pyrophosphorylase was significantly inhibited by both stresses while starch synthase was enhanced only in response to HLB.

The repression of key genes in gibberellin (GASA1 and GASA5) and cytokinin transduction signal (ENT-kaurenoic acid hydrolase) in both disordered and diseased peel tissues confirmed the general trend of this class of genes observed in large scale transcriptomic analysis. Ethylene-related pathways were significantly linked to HLB symptoms. Auxin-related genes were clearly more abundant with HLB disease. As expected, methyltransferase, a key transcript in response to biotrophs, was clearly upregulated in response to

CaLas infection while it was unregulated in disordered fruits. Two ethylene responsive genes and ACC synthase were clearly induced by CaLas. Among transcription factors, WRKY7 and NAC-1 were more abundant in HLB-symptomatic samples but unregulated in puffing disorder. The expression pattern of highly interactive proteins, such as HSP82 and GTP binding protein confirmed the trend seen during transcriptomic analysis.

Principal component analysis (PCA) was used to reduce the great dimensionality of the gene expression data (Fig. 7). The analysis was performed to normalize possible bias caused by the amount
Table 2
Important key interactive proteins (“hubs”) encoded by differentially expressed genes in fruits affected by Puffing and HLB disease. Arabidopsis gene identifier, of protein–protein interactions, pathway and predictive annotation were indicated.

<table>
<thead>
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<th>AGI</th>
<th>N PPI</th>
<th>Pathway</th>
<th>Annotation</th>
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<td>At5g23290</td>
<td>76</td>
<td>RNA.regulation</td>
<td>c-Myc binding protein, Heat shock protein 70</td>
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HLB disease

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<td>Microtubule-end-binding protein</td>
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<td>At1g59520</td>
<td>51</td>
<td>Metal handling.binding,</td>
<td>Serine O-acetyltransferase</td>
</tr>
<tr>
<td>At1g13580</td>
<td>58</td>
<td>Lipid metabolism.'exotics'</td>
<td>Longevity assurance homolog 3</td>
</tr>
<tr>
<td>At1g06400</td>
<td>50</td>
<td>Protein.synthesis.elongation</td>
<td>Elongation factor 1-gamma</td>
</tr>
</tbody>
</table>

Commonly regulated between the two stresses

<table>
<thead>
<tr>
<th>AGI</th>
<th>N PPI</th>
<th>Pathway</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>At5g24090</td>
<td>29</td>
<td>Stress.biotic</td>
<td>Acidic endochinase precursor</td>
</tr>
<tr>
<td>At4g18880</td>
<td>16</td>
<td>Stress.abiotic.heat</td>
<td>Heat shock transcription factor A4</td>
</tr>
<tr>
<td>At3g29000</td>
<td>30</td>
<td>Amino acid metabolism.synthesis.</td>
<td>Homocysteine S-methyltransferase</td>
</tr>
<tr>
<td>A2g33210</td>
<td>107</td>
<td>Stress.abiotic.heat</td>
<td>Chaperonin CPN60-like 1</td>
</tr>
<tr>
<td>At2g29990</td>
<td>25</td>
<td>Mitochondrial electron transport/ATP synthesis.</td>
<td>NADH dehydrogenase</td>
</tr>
<tr>
<td>At2g07690</td>
<td>18</td>
<td>DNA.synthesis/chromatin structure</td>
<td>McM family protein</td>
</tr>
<tr>
<td>At1g19030</td>
<td>57</td>
<td>Stress.abiotic.heat</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>At1g10070</td>
<td>49</td>
<td>Amino acid metabolism.synthesis.</td>
<td>Branched-chain-amino-acid transaminase</td>
</tr>
<tr>
<td>At1g02500</td>
<td>21</td>
<td>Amino acid metabolism.synthesis.</td>
<td>S-Adenosylmethionine synthetase 1</td>
</tr>
</tbody>
</table>

Table 3
Gene expression data by qRT-PCR. Letters indicate significant differences for each of the two pairwise comparisons using ANOVA (p value = 0.05) and post-hoc test.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Puffing</th>
<th>Huanglongbing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy</td>
<td>Diseased</td>
</tr>
<tr>
<td>ACC synthase</td>
<td>0.25 ± 0.21</td>
<td>0.94 ± 0.53</td>
</tr>
<tr>
<td>ERF1</td>
<td>0.39 ± 0.15</td>
<td>0.19 ± 0.15</td>
</tr>
<tr>
<td>ERT2</td>
<td>0.26 ± 0.12</td>
<td>0.20 ± 0.21</td>
</tr>
<tr>
<td>Ethylene signaling</td>
<td>0.35 ± 0.15</td>
<td>0.19 ± 0.15</td>
</tr>
<tr>
<td>GASAS5</td>
<td>1.04 ± 0.23 a</td>
<td>0.38 ± 0.19 b</td>
</tr>
<tr>
<td>GH3.3</td>
<td>0.02 ± 0.007 b</td>
<td>0.21 ± 0.11 a</td>
</tr>
<tr>
<td>Heat shock protein 82</td>
<td>1.24 ± 0.24 a</td>
<td>0.62 ± 0.22 b</td>
</tr>
<tr>
<td>GTP binding</td>
<td>0.46 ± 0.23</td>
<td>0.97 ± 0.45</td>
</tr>
<tr>
<td>Glicogen starch initiat.</td>
<td>0.29 ± 0.25</td>
<td>0.71 ± 0.53</td>
</tr>
<tr>
<td>GH3.4</td>
<td>0.02 ± 0.007 b</td>
<td>0.21 ± 0.11 a</td>
</tr>
<tr>
<td>Invertase</td>
<td>0.19 ± 0.06 b</td>
<td>0.60 ± 0.06 a</td>
</tr>
<tr>
<td>NAC-1</td>
<td>206.44 ± 45.33</td>
<td>152.34 ± 101.94</td>
</tr>
<tr>
<td>Sucrose transporter</td>
<td>0.48 ± 0.05</td>
<td>1.42 ± 0.74</td>
</tr>
<tr>
<td>ADPase</td>
<td>208.21 ± 81.27 a</td>
<td>41.59 ± 33.27 b</td>
</tr>
<tr>
<td>WRKY70</td>
<td>4.81 ± 2.82</td>
<td>3.62 ± 2.27</td>
</tr>
<tr>
<td>Lipid transfer protein</td>
<td>0.30 ± 0.05</td>
<td>0.37 ± 0.30</td>
</tr>
<tr>
<td>GASAT1</td>
<td>146.26 ± 16.58 a</td>
<td>79.84 ± 12.31 b</td>
</tr>
<tr>
<td>ENT Kae. acid hydr.</td>
<td>0.36 ± 0.09 a</td>
<td>0.09 ± 0.07 b</td>
</tr>
<tr>
<td>Methylsaliclydate</td>
<td>0.001 ± 0.001</td>
<td>0.004 ± 0.003</td>
</tr>
</tbody>
</table>

of collected material for each class or other factors that may skew results. The relative expression of the genes analyzed by qRT-PCR was used to construct the PCA plot. Principal component analysis was then applied to the ratio matrix of this gene list to examine the contribution of each target gene to the separation of the sample classes. Principal component 1 accounts for approximately 68% of the variability while principal component 2 was explain another additional 26%. PCA showed that overall pattern of expression of the analyzed genes was able to clearly separate uninfected and HLB-infected trees while puffing and healthy fruits showed to be very similar.

4. Discussion

Analysis of host responses to stresses in specific tissues may deliver important biomarkers to complement direct pathogen
detection and improve diagnosis in case of diseases or disorders with similar and confused symptoms (Baratto et al., 2005). While this analysis would not substitute for direct identification of a specific pathogen, a warning that a condition of plant stress exists might allow some remedial action to be taken before irreversible damage occurs.

4.1. Sucrose and starch metabolism are activated in response to both stresses

Gene set enrichment analysis showed that starch and sucrose metabolism was the most significantly regulated pathways under both stresses. This led us to hypothesize that molecular regulation of this pathway might indicate a general induced stress condition. Stressed fruit showed altered expression of invertase, sucrose synthase, fructokinase, sucrose–phosphate–synthase, ADP-glucose transferase, and starch synthase in response to both stresses. HLB-infected fruits remain more green and therefore photosynthesizing, producing extra sugar that might require transport or synthesis into starch. Photosynthesis is not significantly affected in puffing disorder since disordered fruits have same color of normal ones.

Invertase was clearly upregulated by both puffing disorder and HLB. The invertase gene family helps regulate glucose breakdown for cell energy production and sucrose partitioning, which controls the relative sink strength of plant tissues (Rötsch, 1999). It has been hypothesized that invertase may play a role as an extra-cellular indicator for pathogen infection (Jang and Sheen, 1994) because activating defense mechanisms requires the induction of sink metabolism for energy production. Thus, pathogens can reduce photosynthetic capacity and induce sink metabolism (Cernadas et al., 2008).

Modulation of invertase and other genes involved in sucrose metabolism when plants are under stress would affect source–sink communications and transport of assimilates. The progression of HLB is marked by ongoing disruption of source–sink relationships (Kim et al., 2008; Martinelli et al., 2013). Other phloem-limited plant pathogens like phytoplasmas and spiroplasmas are also associated with dysfunctions in sugar metabolism (Renaudin, 2006). Upregulation of glucose-6-phosphate transporter2 in leaves (a source tissue) during HLB disease or citrus bacterial canker increased the uptake of glucose-6-phosphate, an essential substrate for starch synthesis, into plastids and caused starch accumulation (Albrecht and Bowman, 2008; Cernadas et al., 2008). Starch accumulation in leaves then reduced photosynthesis and transport of assimilates to the rest of the plant. Conversely, starch biosynthesis was reduced in HLB-infected fruits, a sink tissue. At the same time, genes involved in photosynthetic light reactions were induced (Martinelli et al., 2012), a possible measure to compensate for reduced ability to import carbon.

4.2. Specific responses to different stresses in peel tissues

PCA analysis was used to compare gene expression changes due to puffing and HLB in key pathways, such as hormones, secondary metabolism, stress responses, and volatiles. The two stresses induced specific changes in different pathways.

During abiotic stresses like puffing disorder, the extra energy produced might fuel defenses against oxidative stress. Reactive oxygen species (ROS) increase under environmental stress or biotic attacks, causing destruction of cell components. Soluble sugars are intermediates in ROS-producing metabolic pathways but they also contribute to ROS scavenging by activating NADPH–producing metabolic pathways, such as the oxidative pentose–phosphate (OPP) pathway (Rolland et al., 2002). It is possible that invertase genes are induced by ROS to provide the glucose from which ROS-scavenging compounds are ultimately synthesized.

Puffing disorder induced genes involved in drought responses while HLB disease caused transcriptional upregulation of biotic stress pathways (cell wall modifications and pathogenesis-related proteins). These differences are consistent with the different symptoms induced by each stress. Puffing disorder is characterized by degradation of the albedo, giving rise to aerial spaces, a cracked and vulnerable albedo, and oversized fruits. The downregulation of genes involved in protein and amino acid synthesis may be a response to this degradation of the albedo in severely disordered fruits. Nucleic acid repair and transcription were also stimulated by puffing disorder. HLB disease increased protein degradation via ubiquitin-dependent proteins and transport-related functions. A key cell function that can clearly characterize the response to HLB disease in the fruits is the sugar signaling. This is maybe considered another proof that the alteration of sugar signaling and nutrient transport are clearly implied in the fruit symptoms of this disease. The reduced cell cycling and division induced by HLB could cause the small, severely malformed fruit typical of the late stage of the disease. Indeed, HLB–symptomatic fruits typically remain partially immature and poorly colored, consistent with the observed enhanced photosynthetic light reactions.

HLB infection greatly altered secondary metabolism, particularly synthesis of phenylpropanoids, anthocyanins, and carotenoids. Upregulated genes coding for synthesis of phenolic alcohols (caffeyl and coumaryl) have previously been associated with plant defense responses to other biotic and abiotic stresses (Moura et al., 2010). HLB increased expression of genes for biosynthesis of mono- and di-terpenes. This evidence is intriguing and confirms previous findings of volatile emissions of fruits in response to biotic stresses, such as CTV and HLB (Sankaran et al., 2010). Ongoing studies of the volatile profiles emitted by fruit peel seek to detect biomarkers for early diagnosis of HLB disease (Dandekar et al., 2010). Advanced methods based on volatile analysis of asymptomatic detection of plant disease directly in the field have been developed (Martinelli et al., 2015). The clear differences in the pathways that synthesize volatile compounds is particularly intriguing because it opens the possibility of diagnosing different stresses using volatile sensors, such as e-nose or the differential Mobility Spectrometer (DMS).

It is interesting that some of the genes analyzed in this study were upregulated in asymptomatic fruits, such as ERT2, GTP-Binding, Lipid Tranfer Protein, Invertase.

4.3. Hormonal crosstalk regulation in response to Citrus peel stresses

Classic hormones and their crosstalk play crucial roles in resistance to adverse environmental conditions (Pieterse et al., 2009).

Genes controlling the synthesis of jasmonates, salicylic acid, and ethylene were enhanced in HLB disease while auxin-related genes responded to puffing disorder, a result of the different nature of the two stresses. CAs is considered a biotroph, although the specific mechanisms of its pathogenicity are poorly understood. It is unclear why jasmonate and ethylene-related pathways were upregulated in infected fruits, since these pathways are typically associated with response to necrotrophic pathogens. Gibberellin and cytokinins were mostly downregulated under both stresses. Their differential regulation may drastically affect fruit metabolism. Gibberellins (GAs) are essential endogenous regulators of plant growth and development. Gibberellin treatments can decrease the occurrence of several physiological disorders in citrus (Chun et al., 2003). Citrus creasing, a disorder characterized by cracks in the albedo, is also partially mitigated by gibberellic acid applications (Greenberg et al., 2010). Thus, regulation of gibberellins affects the occurrence of metabolic physiological disorder in fruit. Interestingly, GAS1 and GAS5 involved in gibberellin synthesis and
response were repressed during HLB disease. These genes were downregulated also in immature fruits of HLB symptomatic trees (Martinielli et al., 2012). At this developmental stage no symptoms are evident and fruits appear as those of apparently healthy trees. Gibberellins may be linked to key significant changes in glycolysis observed in disordered fruits, as they can induce changes in glycolysis and affect sugar metabolism, particularly pyruvate and phosphoenolpyruvate (PEP) metabolism (Konishi et al., 2004). Gibberellin applications should be investigated as a possible treatment to mitigate the symptoms of puffing disorder in citrus fruit.

Cytokinin concentration and composition control the source or sink strength of tissues by regulating carbon fixation, assimilation, partitioning of primary metabolites, and cell cycle activity (Hedden et al., 2006). Cytokinins stimulate chloroplast development and chlorophyll biosynthesis (Hudson et al., 2013) and increase the rate of photosynthesis (Sakamoto et al., 2006). Several cytokinin-related genes were repressed under both HLB and puffing disorder; they may regulate the common induced responses that altered source–sink communications, and thus, fruit development. However, no data are present on the effect of downregulation of cytokinins in peel fruit tissues and the effect of cytokinin applications to reduce symptoms in disease or disorders has not been tested.

### 4.4. Gene regulatory and protein–protein interaction networks

Several downregulated genes in puffing disorder were encoding transcription factors belonging to NAC, Aux/IAA families. The NAC subfamilies help control stress responses, senescence, organ formation and development, and hormone signaling (Olsen et al., 2005). They are suggested to have a role in the crosstalk between different signaling pathways since they are co-regulated by specific sets of stimuli, including phytohormones, dehydration, or wounding (Olsen et al., 2005).

HLB induced WRKY genes (Martinielli et al., 2012, 2013). WRKY transcription factors have many regulatory roles in response to biotic and abiotic stresses (Qiu and Yu, 2009). WRKY70 upregulation is linked to HLB while this gene was not affected by puffing disorder. It is worthy to notice that WRKY70 was upregulated in immature fruits of HLB symptomatic trees (Martinielli et al., 2012). Two closely related WRKY transcription factors (WRKY25 and WRKY33) are associated with salt stress responses in Arabidopsis (Jiang and Deyholos, 2006). Overexpression of the tobacco WRKY57 gene conferred tolerance to drought stress in soybean, indicating that this transcription factor plays a role in plant tolerance to abiotic stress (Zhang et al., 2008). It has been suggested that ROS could be sensed by different redox-response transcription factors, including members of the WRKY transcription factor family (Li et al., 2009).

Some key heat shock proteins (HSPs) were regulated in response to both HLB and puffing disorder and represent major hubs in the protein networks. These highly conserved proteins are also induced in cells subjected to elevated temperatures, other environmental stresses (Almoguera et al., 1995), and pathogen infection (Maimbo et al., 2007). HSP70 and heat shock factor A4 were induced by both HLB and puffing disorder. In plant cells, HSP70 and HSP82 are involved in signal transduction leading to plant defenses. HSP70 interacts with an SA-induced protein kinase in Nicotiana to alter the hypersensitive response and basic resistance (Maimbo et al., 2007). The protein–protein network analysis showed that HSP82 was the most interactive protein in the network and expression analysis revealed that the gene is differentially expressed in both puffing disorder and HLB disease. This leads to the hypothesis that a major cause of symptoms under both stresses might be protein misfolding. Other heat shock proteins were specific to only one of the two stresses. Indeed, it is possible that an increase in ROS combined with downregulation of HSPs might combine to induce the stress symptoms typical of HLB. ROS species were likely to be generated by the upregulation of genes involved in light reactions, with consequent induction of protein misfolding and degradation. Altered expression of transcription factors and other proteins involved in RNA synthesis was typical of puffing disorder. Hub proteins, such as small nuclear ribonucleoprotein F and ELF7 are potential targets for modulating the response to puffing using information from public databases of transcriptomic data (Zimmermann et al., 2005).

### 5. Conclusions

We compared two different gene expression datasets obtained from citrus fruit peel affected by puffing disorder or HLB disease to discover common and specific genes, pathways, and cell functions regulated by the stresses. Sugar and starch metabolism were the most significantly regulated pathways in the two stresses and might be considered an early general indicator of induced stress response. Several differentially regulated genes involved in hormones, such as gibberellins and ethylene may trigger the fruit metabolic changes that lead to symptom occurrence. While puffing disorder affected pathways involved in protein synthesis and activation, HLB disease altered functions, such as cell cycle and organization, metal handling, transport, and protein degradation and modification. Oxidative stress and downregulation of heat shock proteins may play a key role in the symptom appearance of both stresses. Further studies will focus also on the regulation of key proteins that allow communication between otherwise disjoint regions. Future studies of the therapeutic use of hormone-based application in fruits are highly desirable.

### References


