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Transcriptome and metabolome analysis of *Citrus* fruit to elucidate puffing disorder

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ABSTRACT

A systems-level analysis reveals details of molecular mechanisms underlying puffing disorder in *Citrus* fruit. Flavedo, albedo and juice sac tissues of normal fruits and fruits displaying symptoms of puffing disorder were studied using metabolomics at three developmental stages. Microarrays were used to compare normal and puffed fruits for each of the three tissues. A protein–protein interaction network inferred from previous work on *Arabidopsis* identified hub proteins whose transcripts show significant changes in expression. Glycolysis, the backbone of primary metabolism, appeared to be severely affected by the disorder, based on both transcriptomic and metabolomic results. Significantly less citric acid was observed consistently in puffed fruits. Gene set enrichment analysis suggested that glycolysis and carbohydrate metabolism were significantly altered in puffed samples in both albedo and flavedo. Expression of invertases and genes for sucrose export, amylose–starch and starch–maltose conversion was higher in puffed fruits. These changes may significantly alter source–sink communications. Genes associated with gibberellin and cytokinin signaling were downregulated in symptomatic albedo tissues, suggesting that these hormones play key roles in the disorder. Findings may be applied toward the development of early diagnostic methods based on host response genes and metabolites (i.e. citric acid), and toward therapeutics based on hormones.

1. Introduction

Citrus is a source of complex carotenoids with anti-oxidant properties important to human health. The citrus fruit consists of two distinct regions: the pericarp (peel) and endocarp (juice sacs). The pericarp consists of two tissue layers: the outer flavedo and the albedo. Flavedo contains cellulose and other components such as essential oils, paraffin waxes, steroids, triterpenoids, fatty acids, pigments, the bitter compound limonene, and enzymes. It is rich in oil glands and composed of several cell layers that become progressively thicker toward the inside. It is covered with wax and contains few stomata. In ripe fruit, flavedo cells contain chromoplasts with carotenoids (xanthophyll) inside, synthesized from chlorophyll, that are responsible for the fruit's color change during ripening. The albedo, or mesocarp, is the fleshy middle layer of the pericarp, between the exocarp and the endocarp. It is generally colorless and spongy, and changes its character and thickness throughout fruit development. Its properties determine ease of peeling. Albedo tissue is very physiologically active and is a place where disorder and disease symptoms commonly appear [1,2]. Many physiological disorders occur preharvest and are believed to result from either mineral deficiency (creasing, boron and, copper deficiency) or weather (water spots, zebra skin, sunburn, wind scar, and freezing injury) [3].

Several candidate genes with altered expression during peel development were reported in a broad survey of gene expression during rind development in "Washington Navel" orange using a customized microarray platform [4]. Normal development of albedo tissue is of particular interest because consumers avoid

Abbreviations: ABA, abscisic acid; APG6, albino pale green 6; BA, brassinosteroid; CDC2, cell division control 2; CHO, carbohydrate; FDR, false discovery rate; FPS2, farnesyl diphosphate synthase 2; GABA, γ-aminobutyric acid; GA, gibberelic acid; GAs, gibberelins; GASA, gibberellin-responsive protein 4; GC–MS, gas chromatography–mass spectrometry; GSEA, gene set enrichment analysis; HB, homeobox; HSP, heat shock proteins; IAA, indole-3-acetic acid; NADH, nicotinamide adenine dinucleotide (reduced form); PEP, phosphoenolpyruvate; PPDK, pyruvate orthophosphate dikinase; PPI, protein–protein interactions; SA, salicylic acid; TCA, tricarboxylic acid; TF, transcription factor.

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fruits that are visually abnormal. Processed and fresh citrus production is seriously threatened by diseases and physiological disorders, threatening the livelihood of individual growers and depriving consumers of locally produced fresh fruit. Peel disorders diminish fruit quality and thus the profits of growers and producers. One of the best-known physiological disorders is "puffing" disorder, characterized by albedo breakdown and separation between peel and pulp. This leads to disintegration of the albedo tissue, causing the formation of air spaces and albedo with weaker mechanical resistance during peeling in mature fruits. Symptoms increase if the peel continues growing after the pulp has completed its development. The cause of this fruit disorder is currently unknown, although it seems to be associated with water exchange regulation through peel tissues or mineral nutrition [5]. Before the present study, no investigations were performed to elucidate molecular causes of the disorder.

This study examined the transcriptome and metabolome of citrus fruits to understand the disorder at the molecular level. Transcriptomics, the quantification of transcripts in a cell, includes the use of next-generation techniques [6–9] and microarrays [10–12]. Metabolomics may be a good complement to transcriptomics and proteomics [13–16]. The metabolome is more directly related to phenotype than transcripts or proteins because metabolic fluxes are governed not only by gene expression, but also by post-transcriptional and post-translational events. Gas chromatography–mass spectrometry (GC–MS) is typically used for non-targeted metabolite profiling of volatile, thermally stable polar and non-polar metabolites [17].

A microarray analysis was performed on three different fruit tissues (flavedo, albedo, and juice sacs) to compare normal and puffed samples, followed by metabolomic profiling of peel and juice sacs. The two primary objectives were: (1) to identify genes and metabolites with variability corresponding to symptoms of puffing disorder; and (2) to identify pathways that describe network regulation responsible for the disorder. Fruit peel tissues can serve as a biological sensor of abiotic and biotic stresses. They contain key biomarkers for early diagnosis and possible therapies for diseases and disorders. The identification of genes, metabolites and regulatory interactions from this study provides knowledge essential for the development of early diagnostic methods and novel therapeutic strategies based on small molecule applications.

2. Materials and methods

2.1. Plant material and experimental design

Symptomatic and healthy control "Navel" orange fruits were harvested at three different developmental stages (immature, intermediate, and mature) from an orchard located in San Diego County, California, USA. Symptoms of puffing become apparent at the mature "Navel" orange stage. Thus immature and intermediate fruits that were harvested from trees that showed the typical puffing symptoms in mature fruit during the previous season were considered to be symptomatic. The study included four immature, four intermediate, and four mature symptomatic fruits, each comprising one biological replicate. Two healthy fruits comprised two biological replicates of control samples. Mature, healthy fruits of five different citrus cultivars (mandarin [*Citrus reticulata*], "Valencia" and "Navel" orange [*Citrus sinensis*], lemon [*Citrus* × *limon*], grapefruit [*Citrus* × *paradisi*]) were purchased from a food market located in Davis, CA, USA. Three fruits comprised three biological replicates from each of the five different healthy citrus cultivars. At the time of collection of each fruit sample, a 1 cm-thick equatorial disk and four sections (N, S, E, and W) were cut per fruit. Flavedo, albedo, and juice sac from each section was dissected, ground in liquid nitrogen and stored at −80 °C until analyzed. Metabolomic analysis was conducted at different developmental stages (immature, intermediate, and mature) for each of the three tissues, and for both symptomatic and control fruits.

2.2. Metabolomic analysis

Analysis of metabolites was conducted at the Metabolomics Core facility at the UC Davis Genome Center. "Navel" orange samples were collected at three developmental stages (immature, intermediate, and mature), for each of the three tissues, for both symptomatic and control samples. Metabolites from 20 to 50 mg frozen, ground samples were extracted with 1 mL pre-chilled solvent mixture (dH_2O:MetOH:$CHCl_3$ 1:2.5:1), vortexed for about 10 s and shaken for 6 min at 4 °C. Samples were then centrifuged for 2 min at 14,000 rpm and supernatant was removed. 500 μL aliquots of supernatant were concentrated to complete dryness in a speed vacuum concentrator. Dry samples were stored at −20 °C until analyzed. Reconstituted metabolite samples were analyzed with a Pegasus III TOF GC-mass spectrometer that can profile 400 compounds. Relative concentrations were determined by peak area (mm^2). All peak detections were manually checked for false positive and false negative assignments. These mass spectra were then compared to known and commercially available mass spectral libraries. Statistical analysis was performed using pairwise comparison to determine significant differences. Target GC–MS metabolite analysis was performed for sucrose, fructose, glucose, citric, and malic acid.

2.3. RNA extraction

For transcript analysis, total RNA was extracted from 2 g of pooled (N + S + E + W) sections of each fruit and tissue (albedo, flavedo, or juice sac). Total RNA was extracted using a two-day modified hot borate procedure [18], followed by RNA purification using Qiagen RNeasy cleanup kit (Qiagen; Valencia, CA). RNA concentration and purity were assessed by UV spectrophotometry. RNA was stored at −80 °C until analyzed.

2.4. Microarray analysis

RNA labeling was performed according to instructions in the GeneChip One Cycle Target Labeling Kit (Affymetrix, Inc., Santa Clara, CA, USA). Citrus microarrays were purchased from Affymetrix. Each microarray contains over 30,171 probe sets corresponding to 33,879 citrus gene transcripts. Fragmentation, hybridization, scanning, and image data processing were performed according to Affymetrix protocols at the Microarray Core Facility in the Medical Microbiology and Immunology Department, University of California, Davis (USA).

2.5. Statistical analysis of microarray data

The raw probe intensities obtained after scanning individual citrus chips were processed using the RMA method to normalize and scale the data [19]. To determine which genes were differentially expressed among different groups, one-way ANOVA was used to obtain *p*-values for each gene. All *p*-values were BH-adjusted for multiple hypotheses [20]. Genes with adjusted *p*-values < 0.05 were considered differentially expressed (up or downregulated). R package LMGene [21] was used to perform a one-way ANOVA and adjust for multiple hypotheses. R package limma was used to make pairwise comparisons between groups and identify citrus fruit- and tissue-specific genes.