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Changes in the Transcriptome of 'Mor' Mandarin Flesh during Storage: Emphasis on Molecular Regulation of Fruit Flavor Deterioration

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ABSTRACT: Mandarin sensory acceptability deteriorates rapidly after harvest. To gain a better understanding of the molecular processes that occur in 'Mor' mandarin flesh and contribute to flavor deterioration during storage, we performed genome-wide transcriptional profiling analysis using the Affymetrix Citrus GeneChip. Out of 30,171 probe sets representing citrus transcripts on the microarray, expression of 1,139 probe sets was significantly ($q \leq 0.01$) altered by factors of at least 4 after 6 weeks of cold storage at 5 °C, of which 745 (65%) were downregulated and 394 (35%) upregulated. Overall, storage led to expression arrest of general cellular and metabolic activity, but enhanced lipid and amino acid catabolism, most probably to form substrates for the tricarboxylic acid (TCA) cycle and energy production. In addition, storage enhanced gene expression of pyruvate decarboxylase (PDC), a key enzyme in anaerobic respiration and the ethanol fermentation pathway. Taken together, we propose that induction of amino acids and fatty acids degradation leads to accumulation of volatiles involved in formation of "fruity" (overripe) and "musty" aromas, respectively, whereas induction of ethanol fermentation metabolism leads to formation of "alcohol" flavor, all of which generate off-flavors after harvest.

KEYWORDS: aroma, citrus, flavor, mandarin, off-flavor, postharvest, storage, transcriptome

INTRODUCTION

During the past decade, mandarins gained in economic importance in world citrus markets, with production forecast of over 20 million metric tons in 2010.[1] However, despite their attractive appearance and convenience for consumption, mandarins are much more perishable than other citrus varieties, and suffer from much shorter storage lives of just a few weeks after harvest.[2,3] One of the major problems in maintaining mandarin fruit quality is rapid deterioration in flavor and sensory acceptability, mainly because of accumulation of off-flavor volatiles.[2,4]

Mandarin flavor is determined by a combination of basic taste, aroma, and mouth-feel sensations that are perceived simultaneously during eating.[5,6] The taste of mandarin fruit is principally governed by the levels of sugars and acids in the juice sacs, and the ratio between them; the latter relationship is termed the fruit ripening ratio or total soluble solids to titratable acidity (TSS:TA) ratio.[7,8] Mandarin aroma derives from a mixture of dozens of volatiles, including alcohols, aldehydes, ketones, terpenes/hydrocarbons, esters and others, which contribute a variety of floral, fruity, minty, woody, mushroom, etc. aromas.[6,9,10] The overall mouth-feel sensation is influenced by the texture of the segment membranes surrounding the juice sacs, gumminess, and presence of particular macromolecules such as pectin, which may create a certain mouth-feel.[11]

In a previous study, with the aid of a trained taste panel, we showed that prolonged storage negatively affected the flavor of 'Mor' mandarins. Extending postharvest storage from 3 to 6 weeks resulted in a gradual deterioration in sensory acceptability from between "good" and "excellent" at harvest to "good" after 3 weeks and only "fair" after 6 weeks.[12] The observed deterioration in fruit flavor during storage was attributed to a slight decrease in sourness, a gradual decrease in typical mandarin flavor, and enhanced accumulation of off-flavors. Biochemical analysis revealed that sugar levels in the juice sacs remained more or less constant during storage, whereas acidity levels decreased from 1.5% at harvest to 1.3% and just 1.0% after 3 and 6 weeks respectively.[12] By using head space (HS) gas chromatography mass spectrometry (GC−MS) we detected slight decreases in levels of some aroma volatiles and significant (at least 3-fold, with $q \leq 0.05$) increases in levels of 12 others during prolonged storage. The volatiles that increased after harvest belonged to three major biochemical pathways: ethanol fermentation metabolism (acetaldehyde and ethanol); amino acid catabolism, mainly via leucine and isoleucine degradation pathways (ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, and 3-methylbutanol); and fatty acid catabolism (ethyl acetate, ethyl propanoate, ethyl hexanoic acid, (*E*)-2-hexenal, (*E*)-2-octenal, and ethyl dodecanoate).[12] Worth notice is that five out of the 12 volatiles whose levels increased during storage were ethanol esters, which suggests they might be esterification products of amino and fatty acid catabolism derivatives and ethanol. Overall, we consider that the observed increases in ethanol fermentation, amino acid- and fatty acid-derived volatiles are probably responsible for detected alcohol, "fruity" (overripe), and "musty" off-flavors, respectively.[6,12]

In the present study, we conducted genome-wide transcriptional profiling analysis of 'Mor' mandarin flesh (juice sacs and segment membranes) during postharvest storage by using the Affymetrix Citrus Genome Array (Affymetrix, Santa Clara, CA,

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USA), in order to characterize changes in the transcriptome after harvest and to identify genes that might be involved in the observed changes in fruit flavor. The Affymetrix Citrus Genome Array consists of 30,171 probe sets that represent citrus transcripts. To the best of our knowledge, although there have been previous studies involving transcriptome analysis of citrus flesh during ripening and development[13,14] there have been none that addressed the processes occurring in the flesh during postharvest storage. Other transcriptome analysis studies focused on gene expression patterns in flavedo tissue (the colored outer layer of the peel) but not on those in the flesh.[15,16]

Our analysis revealed genome-wide changes in the transcriptome of mandarin flesh during storage, indicating the existence of both transcriptionally governed arrest of general cellular activities —including concretive downregulation of cell-wall, signaling, and transport processes—and transcriptionally governed metabolic adaptations in lipid, amino acid, and fermentation metabolism. These new findings shed light on the molecular and biochemical mechanisms that operate in mandarin flesh during storage, especially with regard to energy production and flavor deterioration.

MATERIALS AND METHODS

Plant Material and Storage. Mature 'Mor' mandarins (*Citrus reticulata* Blanco) were harvested from a commercial citrus plantation at Nir Zvi, Israel. On the day of harvest, fruits were washed, sorted, and treated with commercial Zivdar wax emulsion (Safepack Products, Kfar Saba, Israel), the wax most commonly used for coating citrus fruits in Israel. For postharvest evaluations, fruits were stored for 3 or 6 weeks at 5 °C and ~85% relative humidity (RH), followed by 5 days of shelf life conditions at 20 °C.

Transcript Profiling Analysis. Total RNA was isolated from the flesh of 'Mor' mandarins by phenol/chloroform extraction and precipitation with LiCl, according to standard procedures.[17] The treatments were as follows: 1, time zero; 2, short storage (3 weeks at 5 °C + 5 days at 20 °C); and 3, prolonged storage (6 weeks at 5 °C + 5 days at 20 °C). For each treatment, we performed three separate RNA extractions, each involving peeled segments from three different fruits. The RNA samples were further prepared for hybridization according to the protocols outlined in the GeneChip Expression Analysis Technical Manual, and were then hybridized to the Affymetrix Citrus Genome Array (Affymetrix, Santa Clara, CA, USA), which includes 30,171 probe sets, representing different citrus transcripts. Hybridizations were performed at the Genome Technology Center of the Hebrew University of Jerusalem, Israel. Data analysis, including background subtraction, normalization, summarization, differential expression determination, and false discovery rate (FDR) correction, was performed with the Partek Genomics Suite (Partek GS) statistical and data visualization program (Partek Inc., St. Louis, MO). One-way analysis of variance (ANOVA) and false discovery rate (FDR) step-up values of $q \leq 0.01$ and changes by factors of at least 4.0 were used to identify differentially expressed probe sets.

In order to assign the differentially expressed mandarin genes into functional categories and metabolic pathways, we identified corresponding homologous genes of *Arabidopsis thaliana* by using either the NCBI nucleotide BLAST search tool[18] or the Affymetrix Microarray Platform Translator (MPT) (http://www.plexdb.org/modules/MPT/mpt_Input.php). By applying a cutoff criterion of *E*-value $\leq 10^{-5}$, we identified *Arabidopsis* homologues for about 85−90% of the differentially expressed mandarin genes, and imported them into the MapMan software (http://gabi.rzpd.de/projects/MapMan/) in order to perform functional categorization and to identify corresponding metabolic pathways.[19]

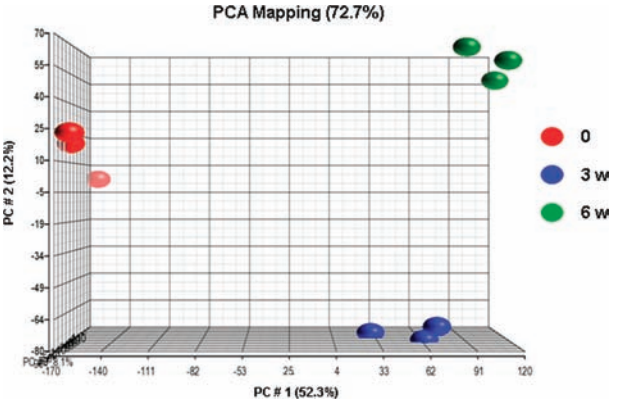

Figure 1. Principal component analysis (PCA) of 'Mor' mandarin flesh transcript profiles. Red = time zero; blue = after 3 weeks of storage at 5 °C; green = after 6 weeks of storage at 5 °C. The three axes account for 72.7% of the total variance among treatments.

Statistical Analysis. Statistical analysis, principal component analysis (PCA), and generation of heat maps were performed with the Partek Genomics Suite. Clustering analysis was performed with Expander, a gene expression analysis and visualization software suite (http://acgt.cs.tau.ac.il/expander/). For the PCA and for hierarchical-clustering and heat maps, means of three independent measurements were used. The PCA was applied to the various treatments by using the default parameters of Partek; the dispersion matrix was calculated by the correlation method and the eigenvectors were normalized.

RESULTS

Changes in the Transcriptome of Mandarin Flesh during Storage. In order to gain a better understanding of the molecular mechanisms that occur in mandarin flesh during postharvest storage, we collected RNA from 'Mor' mandarin flesh immediately after harvest (time zero) and after 3 and 6 weeks of cold storage at 5 °C, and performed a genome-wide transcriptional profiling analysis by means of the Affymetrix Citrus GeneChip.

To confirm repeatability across replications of the observed microarray data, we applied PCA to the various RNA samples. This revealed the occurrence of marked differences in gene expression patterns among the three treatments, and showed that the transcriptome profiles of the three separate RNA samples of each treatment were tightly clustered together (Figure 1). Furthermore, examination of the X axis, which accounted for 52.3% of the total variance among samples, showed that the biggest differences among treatments were those between the time-zero samples and the stored-fruit samples (Figure 1).

To identify transcripts that exhibited significant changes in their abundance after short or prolonged storage, we performed pairwise comparisons, and selected transcripts that had ANOVA and FDR step-up values of $q \leq 0.01$ and that were induced or repressed by a factor of at least 4 after 3 or 6 weeks of storage, as compared with their initial level at time zero. Overall, out of 30,171 probe sets representing citrus transcripts on the microarray, we identified 717 probe sets (278 upregulated and 439 downregulated) whose expression changed after 3 weeks of storage, and 1,139 probe sets (394 upregulated and 745 downregulated) whose expression changed after 6 weeks of storage (Table 1). It can be seen that most of the transcripts that were differentially expressed during storage were downregulated,

Table 1. Effects of Postharvest Storage on the Transcriptome of 'Mor' Mandarin Flesh[a]

	probe sets differentially expressed at $q \leq 0.01$ and induced or repressed by a factor of at least 4		
pair-wise comparison	upregulated	downregulated	total
3 weeks/time 0	278 (39%)	439 (61%)	717
6 weeks/time 0	394 (35%)	745 (65%)	1,139

[a] Data include numbers of probe sets on the citrus Affymetrix Citrus Genome Array that were differentially expressed during storage as compared with time zero at $q \leq 0.01$ and induced or repressed by a factor of at least 4.

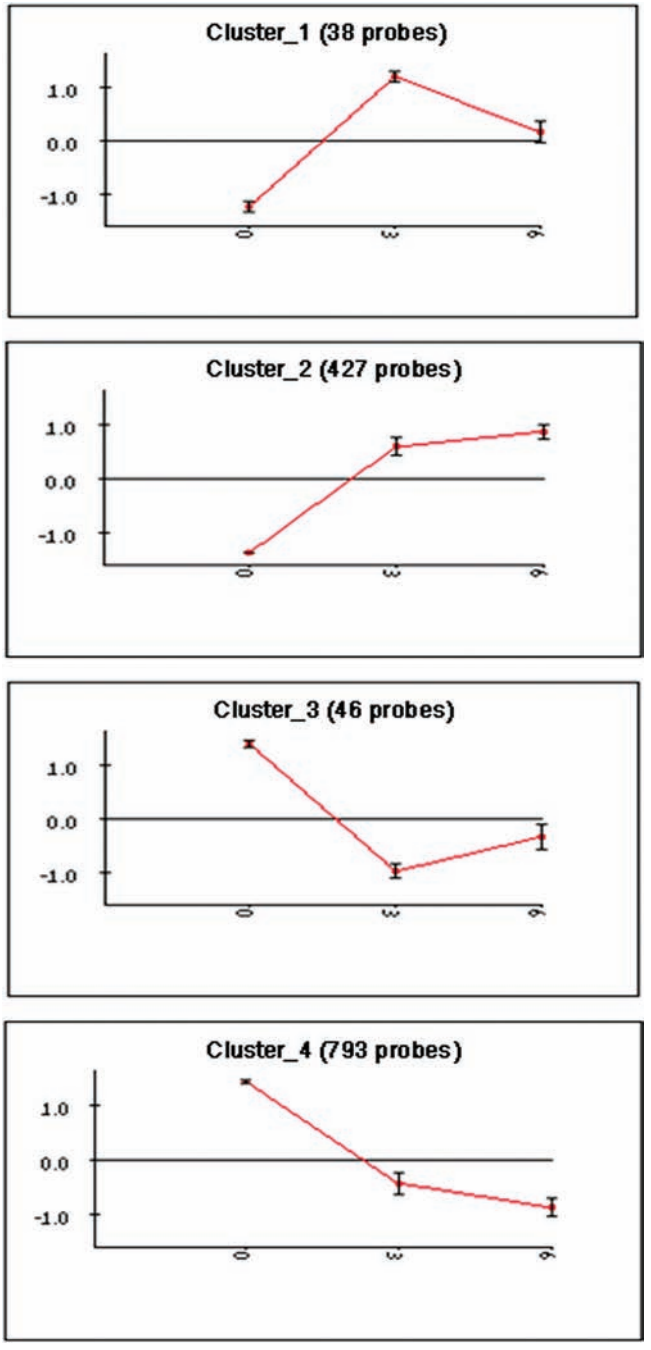

Figure 2. Clustering analysis of storage-regulated differentially expressed transcripts of 'Mor' mandarin flesh. Clustering analysis was performed with Expander, a gene expression analysis and visualization software suite (http://acgt.cs.tau.ac.il/expander/).

either after 3 weeks (61%) or after 6 weeks (65%) of storage (Table 1).

To discriminate between different patterns of gene expression during storage, we used the Expander gene expression analysis and visualization software, and identified four main different gene clusters (Figure 2). The first cluster (cluster 1) included 38 probe sets that were upregulated after 3 weeks but downregulated after 6 weeks (up → down regulon). The second cluster (cluster 2) included 427 probe sets that were continuously upregulated throughout storage (up regulon). The third cluster (cluster 3) included 46 probe sets that were downregulated after 3 weeks but upregulated after 6 weeks (down → up regulon). The fourth and largest cluster included 739 probe sets that were continuously downregulated throughout storage (down regulon) (Figure 2).

Functional Categorization of Differentially-Expressed Genes. For functional categorization, we identified *Arabidopsis thaliana* homologues for 85−90% of the citrus genes in the various clusters, and imported them into the MapMan software.[19] Overall, it can be seen that storage led to massive downregulation of genes involved in general cellular and metabolic activity (Table 2). For example, we observed downregulation of 32, 26, 10, 46, and 48 transcripts belonging to cell wall, lipid metabolism, nucleotide metabolism, signaling, and transport categories, respectively, as compared with only very minor upregulation of 1, 5, 1, 7, and 7 genes, respectively, in each of these categories (Table 2). In contrast to the extensive downregulation of transcript levels during storage, we observed concretive upregulation of transcripts involved in photosynthesis, glycolysis, and fermentation, which are processes required for respiration and energy production (Table 2).

Effects of Storage on Metabolism and Energy Production. After harvest and detachment from the tree, the fruit becomes perishable and depends on its own carbohydrate reserves for respiration and energy production. The common metabolic resources for respiration and energy production are proteins, carbohydrates, and lipids, which may be utilized to form acetyl-CoA, the direct precursor of the tricarboxylic acid (TCA) cycle and the subsequent oxidative phosphorylation chain[20] (Figure 3). Alternatively, energy may be produced via activation of anaerobic respiration following conversion of pyruvate to acetaldehyde and ethanol.[21,22] In the present study, we evaluated changes in transcript levels of genes involved in catabolism of sugars, amino acids and fatty acids, and ethanol-fermentation metabolism.

Carbohydrate Metabolism. During storage, we observed an overall cellular effort to produce and maintain cellular pools of sucrose and free sugars. This effort was manifested via three different metabolic pathways. First, during fruit storage we detected upregulation of transcripts involved in photosynthesis and sugar production. For example, although the fruits were stored in the dark and already had lost their photosynthesis capability, we detected significant upregulation of eight and 11

Table 2. Functional Categorization of Postharvest Storage-Induced Genes in 'Mor' Mandarin Flesh[a]

functional categorization	up → down	up	down → up	down
photosynthesis	8	11	0	4
major CHO metabolism	0	2	0	3
minor CHO metabolism	1	2	0	8
glycolysis	0	3	0	0
fermentation	0	2	0	0
gluconeogenesis/glyoxylate cycle	0	2	0	3
OPP	0	0	0	1
TCA/transformation	0	0	0	0
electron transport/ATP synthesis	0	0	0	3
cell wall	**0**	**1**	**1**	**32**
lipid metabolism	**0**	**5**	**0**	**26**
N-metabolism	0	1	0	1
amino acid metabolism	0	2	0	5
S-assimilation	0	0	0	0
metal handling	0	1	0	3
secondary metabolism	0	8	1	16
hormone metabolism	0	9	3	24
cofactor and vitamin	0	1	0	1
tetrapyrrole synthesis	0	1	0	1
stress	8	19	1	20
redox regulation	1	4	0	8
polyamine metabolism	0	0	0	2
nucleotide metabolism	**0**	**1**	**0**	**10**
misc	0	26	4	50
RNA and transcription	2	30	1	43
DNA	0	11	0	6
protein	2	35	0	48
signaling	**0**	**7**	**1**	**46**
cell	0	9	0	17
"Micro RNA"	0	0	0	0
development	1	9	0	18
transport	**2**	**7**	**2**	**48**
not assigned	3	70	9	127

[a] Functional categorization was performed according to MapMan (http://gabi.rzpd.de/projects/MapMan/). The functional groups of "cell wall", "lipid metabolism", "nucleotide metabolism ", "signaling" and "transport" that were massively downregulated during storage are shown in boldface.

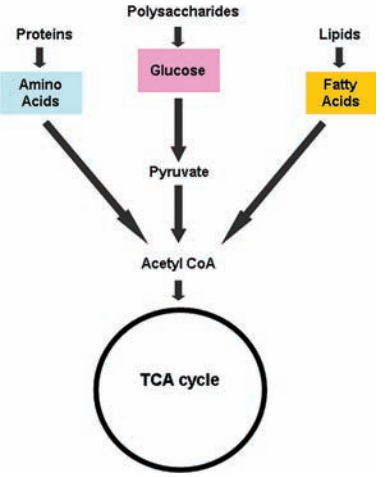

Figure 3. Schematic diagram illustrating various metabolic routes leading to production of acetyl-CoA, the direct substrate for the TCA cycle and energy production.

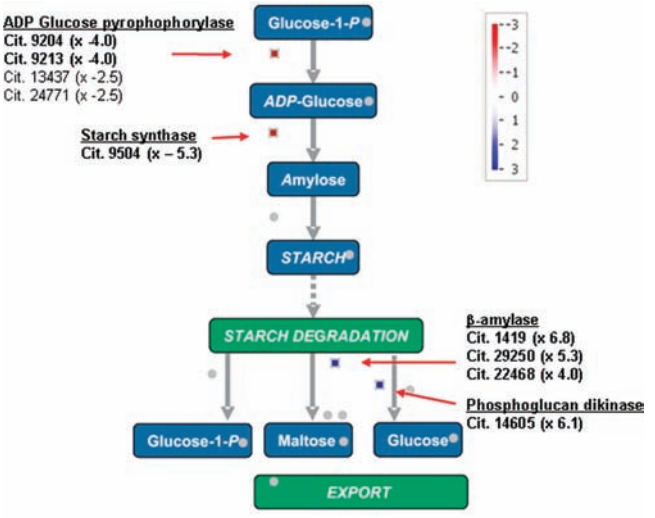

Figure 4. Expression patterns of storage-regulated differentially expressed genes of 'Mor' mandarin flesh involved in starch synthesis and breakdown. The image was taken from the MapMan software. Blue color represents upregulated genes, and red color represents downregulated genes. The change ratio of each transcript after 6 weeks of storage, as compared with its initial level at time zero, is indicated in parentheses. Transcripts differentially expressed at $q \leq 0.01$ and induced or repressed by a factor of at least 4 are printed in bold.

transcripts involved in, photosystems I and II light harvesting complexes in both the up → down and the up regulons, respectively (Table 2). Thus, although obviously unsuccessful, cells of mandarin flesh tried to activate photosynthesis processes, most likely in order to synthesize and accumulate free sugars. Second, during fruit storage, we detected significant downregulation of transcripts involved in starch synthesis and upregulation of transcripts involved in starch breakdown, which favored accumulation of free sugars (Figure 4). More specifically, we detected significant decreases in transcript levels involved in starch biosynthesis, including two ADP glucose pyrophosphorylase probe sets (Cit. 9204 and 9213) by a factor of 4.0, and of a starch synthase probe set (Cit. 9504) by a factor of 5.3. In contrast, we detected parallel increases in transcript levels involved in starch degradation, including three β-amylase probe sets (Cit. 1419, Cit. 29250 and Cit.22468) by factors of 6.8, 5.3 and 4.0, respectively, and of a phosphoglucan dikinase probe set (Cit. 14605) by a factor of 6.1 (Figure 4). Third and last, during fruit storage we observed downregulation of transcripts involved in secondary metabolism of sugars. Especially worth notice was the significant downregulation of five raffinose synthase transcripts (Cit. 21765, Cit. 35345, Cit. 2027, Cit. 1013 and Cit. 3070) by factors of 9.6, 23.5, 7.8, 23.4 and 20.3, respectively (data not shown). Raffinose synthase synthesizes raffinose from sucrose and galactinol, and its inhibition during storage helps to maintain the cellular reserves of sucrose. Taken together, the processes we observed during fruit storage indicate an overall aim by the cells to accumulate and maintain sugars levels, which was manifested in activation of carbohydrate synthesis (upregulation

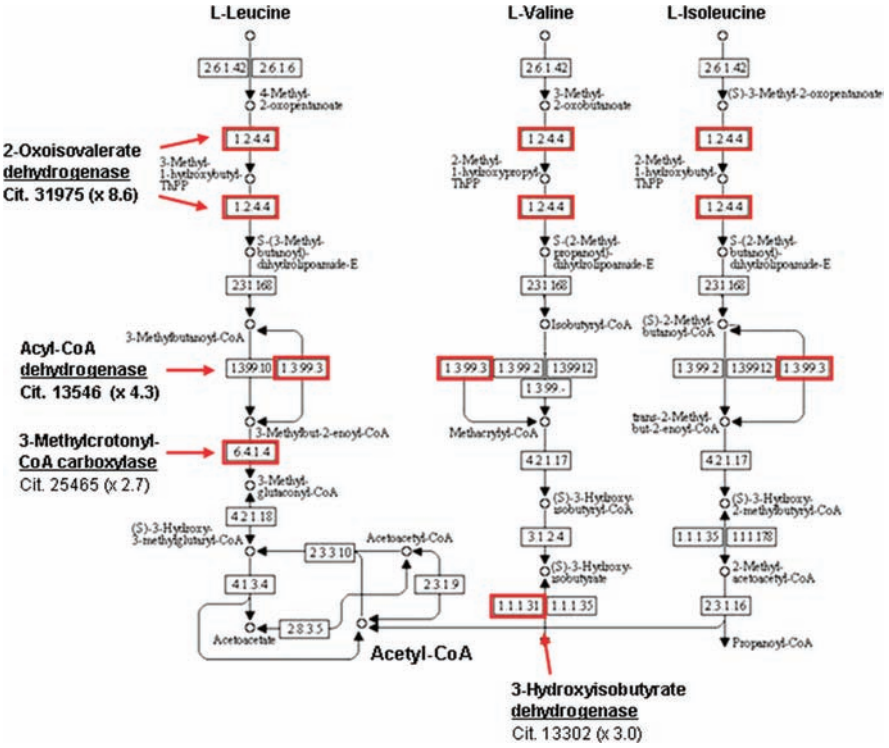

Figure 5. Schematic diagram of branched-chain amino acids catabolism pathway. The diagram was taken from the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway. Transcripts differentially expressed during storage of 'Mor' mandarin are marked in red. The change ratio of each transcript after 6 weeks of storage, as compared with its initial level at time zero, is indicated in parentheses. Transcripts differentially expressed at $q \leq 0.01$ and induced or repressed by a factor of at least 4 are printed in bold.

of photosynthetic genes); suppression of starch synthesis and induction of starch breakdown; and suppression of secondary metabolism of sucrose (downregulation of raffinose synthase transcripts).

Amino Acid Catabolism. During fruit storage, we observed significant upregulation of transcripts involved in catabolism of branched-chain amino acids. For instance, after 6 weeks of storage we observed significant increases in transcript levels of 2-oxoisovalerate dehydrogenase (Cit. 31975), acyl-CoA dehydrogenase (Cit. 13546), 3-methylcrotonyl-CoA carboxylase (Cit. 25465) and 3-hydroxyisobutyrate dehydrogenase (Cit. 13302), by factors of 8.6, 4.3, 2.7 and 3.0, respectively (Figure 5); all of these are specifically involved in degradation of branched-chain amino acids, which results in formation of acetyl-CoA, the direct precursor of the TCA cycle (Figure 3). None of the transcripts in the branched-chain amino acids catabolism pathway was suppressed during storage.

Fatty Acid Catabolism. During fruit storage we observed significant upregulation of transcripts involved in catabolism of lipids and fatty acids. For example, after 6 weeks of storage we observed significant increases in transcript levels of triacylglycerol lipase (Cit. 5724) and lipase1 (Cit. 12050)—both involved in cleavage of free fatty acids from triglycerides—by factors of 8.4 and 5.6, respectively (Figure 6). Furthermore, during storage we observed remarkable upregulation of an acyl CoA synthetase probe set (Cit. 31542) responsible for formation of fatty acyl CoA's by a factor of 10.2, and of various transcripts involved in β-oxidation of fatty acids that leads to formation of acetyl-CoA. For example, after 6 weeks of storage we observed increases in transcript levels of acyl-CoA oxidase (Cit. 15402), acetyl CoA acyltransferase (Cit. 25712) and acyl-CoA dehydrogenase (Cit. 13546) by factors of 3.1, 2.1 and 4.3, respectively (Figure 6).

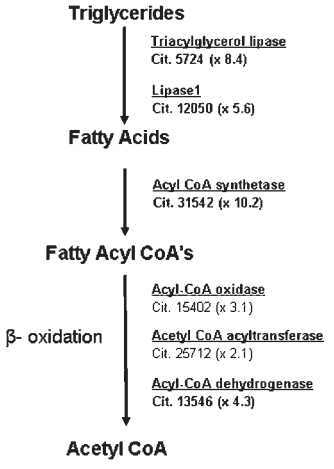

Figure 6. Schematic diagram of triglycerides and fatty acids catabolism pathway. Transcripts differentially expressed during storage of 'Mor' mandarins are indicated on the right. The change ratio of each transcript after 6 weeks of storage, as compared with its initial level at time zero, is indicated in parentheses. Transcripts differentially expressed at $q \leq 0.01$ and induced or repressed by a factor of at least 4 are printed in bold.

Ethanol Fermentation Metabolism. Pyruvate, which is the end product of the glycolysis pathway, either can be utilized to form acetyl-CoA, the direct substrate for aerobic respiration via the TCA cycle, or may be utilized in energy production by anaerobic respiration via the ethanol fermentation pathway (Figure 7A). During fruit storage, we observed significant upregulation of various pyruvate decarboxylase (PDC) transcripts (Cit. 30531, Cit. 12778, Cit. 23700

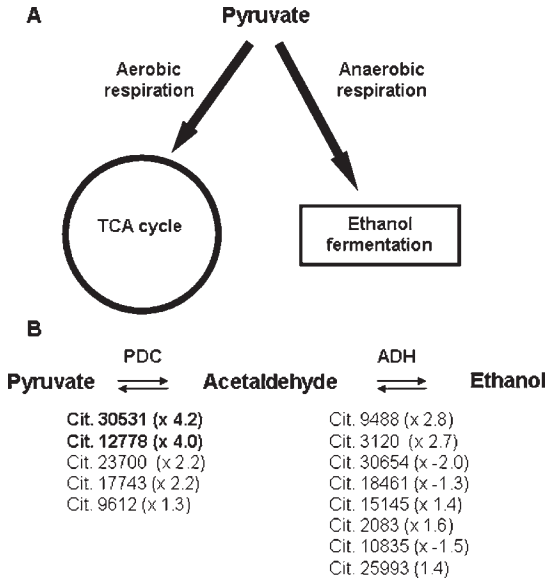

Figure 7. Schematic diagram of anaerobic respiration and ethanol fermentation pathway. A, Schematic diagrams of the aerobic and anaerobic respiration pathways. B, Schematic diagram of ethanol fermentation metabolism pathway. The change ratios of all *PDC* and *ADH* transcripts after 6 weeks of storage, as compared with their initial level at time zero, are indicated in parentheses. Transcripts differentially expressed at $q \leq 0.01$ and induced or repressed by a factor of at least 4 are printed in bold.

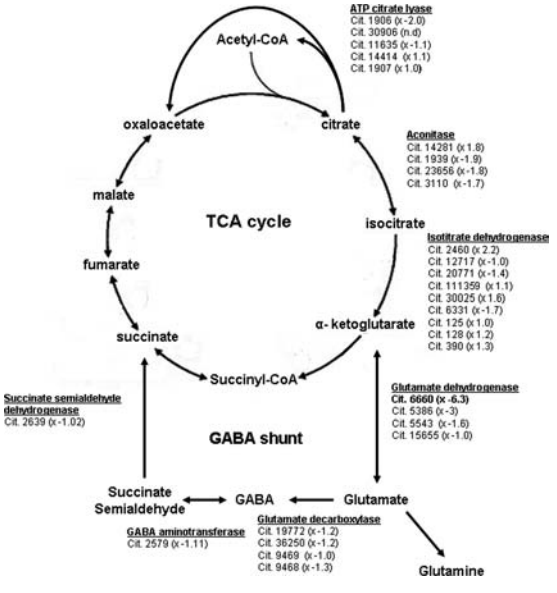

Figure 8. Schematic diagram of citric acid catabolism pathways. The change ratios of all GABA shunt and citric acid catabolism transcripts after 6 weeks of storage, as compared with their initial level at time zero, are indicated in parentheses. Transcripts differentially expressed at $q \leq 0.01$ and induced or repressed by a factor of at least 4 are printed in bold.

and Cit. 17743) by factors of 4.2, 4, 2.2 and 2.2, respectively; and of two alcohol dehydrogenase (ADH) transcripts (Cit. 9488 and Cit. 3120) by factors of 2.8 and 2.7, respectively (Figure 7B). Overall, upregulation of PDC transcripts and expression of some ADH transcripts most likely leads to activation of anaerobic respiration and of ethanol fermentation metabolism during storage.

Citric Acid Catabolism. During fruit storage there is a gradual decrease in citric acid levels, which leads to loss of sourness, and this phenomenon has been attributed to either activation of the GABA shunt pathway, ATP citrate lyase, or of other enzymes in the TCA cycle, such as aconitase and isocitrate dehydrogenase (Figure 8).[13,23−26] In the present study, we did not observe any increases in transcript levels that are assumed to be involved in citric acid catabolism. For example, we have not observed any induction of transcripts in the GABA shunt pathway, which utilizes citric acid to form glutamine for amino acid synthesis. On the contrary, after 6 weeks of storage we observed a significant decrease, by a factor of −6.3, of a glutamate dehydrogenase transcript (Cit. 6660), which is responsible for routing α-ketoglutarate from the TCA cycle toward the GABA shunt pathway and production of glutamate (Figure 8). Moreover, during fruit storage we observed a slight downregulation of an ATP citrate lyase transcript (Cit. 1906) by a factor of −2.0 (Figure 8). Finally, we have not observed any noteworthy changes in either aconitase or isocitrate dehydrogenase transcript levels (Figure 8).

DISCUSSION

The main goals of the present study were (1) to characterize the global changes that occur in the transcriptome of mandarin flesh during storage, and (2) to evaluate the molecular mechanisms that might be involved in regulation of fruit flavor deterioration after harvest. With regard to characterization of the global changes in the transcriptome of mandarin flesh, we found that storage resulted in massive downregulation of genes involved in general cellular and metabolic activity, including concretive downregulation of transcripts belonging to cell wall, lipid metabolism, nucleotide metabolism, signaling, and transport categories, which led to overall transcriptionally governed arrest of general metabolic activity (Tables 1 and 2). A similar trend of transcriptionally governed arrest of general metabolic activity was observed also in grapefruit peel tissue (the flavedo) after cold storage.[16]

With regard to evaluation of the molecular mechanisms that might be involved in regulation of fruit flavor deterioration, we have not observed any differentially expressed transcripts belonging to particular aroma volatile biosynthesis pathways, such as those of terpenes, aldehydes, etc. Nevertheless, during mandarin storage we identified upregulation of transcript levels involved in amino acid and fatty acid catabolism pathways; therefore, we suggest that increased availability of amino acid and fatty acid substrates may be responsible for the enhanced accumulation of amino acid- and fatty acid-derived volatiles during storage (Figures 5 and 6). Furthermore, during storage we observed upregulation of transcripts involved in ethanol fermentation metabolism, which may be responsible for the observed accumulation of ethanol and acetaldehyde during storage (Figure 7).

In many postharvest evaluation studies, it was reported that sugar levels in the juice sacs remained more or less constant or, perhaps, even slightly increased during storage.[6,12,27−29] Indeed, in the present study of 'Mor' mandarins, we observed, at the transcriptional level, an overall effort in the cells to maintain reserve pools of sucrose and free sugars in the flesh; this effort was manifested in induction of photosynthesis genes, suppression of starch synthesis and induction of its breakdown, and suppression of secondary metabolism of primary sugars, e.g., by downregulation of raffinose synthase transcripts (Figure 4). The exact reason

for the maintenance of high sugar levels in citrus juice sacs after harvest in not known with certainty, but it is reasonable to assume that high sugar levels render the fruits more attractive to animals and thus might impart an evolutionary advantage in seed dispersal.

As indicated above, sugars probably are not the main source of carbohydrates for respiration and energy production during mandarin storage, and therefore we suggest that activation of catabolism of both branched-chain amino acids and fatty acids appears to provide the preferred substrates for production of acetyl-CoA, the immediate precursor of the TCA cycle and subsequent energy production (Figure 3). In particular, we identified in mandarin flesh specific upregulation of transcripts suggested to be involved in catabolism of branched-chain amino acids, such as leucine, isoleucine and valine, that are able to form acetyl-CoA, and therefore also are termed ketogenic amino acids,[30] but we did not detect any induction of catabolism of other types of amino acids (Figure 5, Table 2). Accordingly, the amino acid-derived volatiles that accumulated in 'Mor' mandarins during storage, such as ethyl 2-methylbutanoate, ethyl 2-methylpropanoate and 3-methyl butanol, were indeed derived from the branched-chain amino acids leucine and isoleucine.[12,31] These amino acid-derived aroma volatiles were described as having a "fruity" odor[32] but, on the other hand, accumulation of high concentrations of these fruity volatiles might elicit a perception of undesired "overripe" attributes.[33,34]

In addition to amino acid degradation, catabolism of fatty acids also leads to formation of acetyl-CoA, the precursor of the TCA cycle (Figure 3). In this study, during fruit storage we observed significant upregulation of transcripts encoding: lipases involved in cleavage of free fatty acids; acyl CoA synthetase, responsible for formation of fatty acyl CoA's; and transcripts involved in β-oxidation leading to formation of acetyl-CoA (Figure 6). These observations are consistent with our previously reported findings that various fatty acid-derived volatiles, such as ethyl acetate, ethyl propanoate, ethyl hexanoic acid, (*E*)-2-hexenal, (*E*)-2-octenal, and ethyl dodecanoate, accumulated in 'Mor' mandarins juice sacs during storage, and we suppose that the increase in these fatty acid-derived volatiles may be the result of increased availability of free fatty acids that provide substrates for their biosynthesis.[12,35,36] Many of the fatty-acid derived volatiles have an "oily" odor[32] and thus may contribute to a "musty" off-flavor sensation.[29]

During mandarin storage, in addition to amino acid and fatty acid catabolism, we also observed upregulation of *PDC* transcripts, resulting in enhanced ethanol fermentation metabolism (Figure 7). This finding provides, for the first time, molecular support for the observed well-known increases in contents of acetaldehyde and ethanol volatiles during postharvest storage of mandarins.[4,6,12,29,37] The induction of *PDC* transcript levels was probably a result of the build-up of anaerobic conditions in the internal atmospheres of wax-coated fruits.[27,38,39]

In light of these findings, we offer a general model of the molecular changes that occur in the transcriptome of mandarin flesh during storage and that are responsible for production of off-flavor volatiles and impaired sensory acceptability. According to this model, we propose that the juice sac cells tend to maintain their internal sugar pools and, therefore, activate degradation of branched-chain amino acids and fatty acids in order to produce acetyl-CoA as a substrate for the TCA cycle and energy production. As a result of the increased availability of branched-chain amino acids (mainly leucine and isoleucine) and free fatty acids, there is enhanced production and accumulation of various amino acid-derived and fatty acid-derived volatiles that are responsible for sensations of "fruity" ("overripe") and "oily" ("musty") off-flavors, respectively (Figure 9). In addition, build up of anaerobic conditions in the internal atmosphere of wax-coated fruits enhances *PDC* gene expression and anaerobic respiration, thereby resulting in enhanced accumulation of ethanol responsible for causing "alcohol" off-flavor (Figure 9). In addition to our previous study with 'Mor' mandarins,[12] recent GC−MS volatile analysis studies conducted in Israel with 'Or' mandarins[40] and in California with 'W. Murcott' and 'Owari' mandarins[29] yielded similar findings regarding enhanced accumulation of amino acid-derived, fatty acid-derived, and ethanol fermentation volatiles during postharvest storage, thus supporting the general validity of the proposed model (Figure 9).

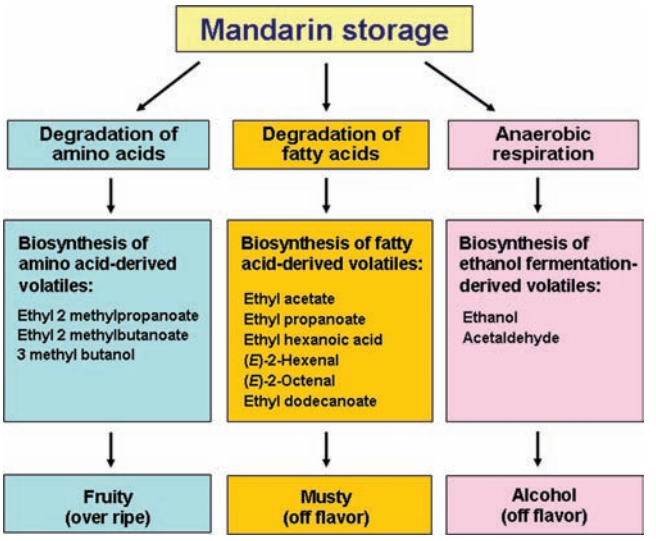

Figure 9. A model describing the molecular changes that occur in the transcriptome of 'Mor' mandarin flesh during storage, and that are responsible for production of off-flavor volatiles and impaired sensory acceptability.

Finally, besides the accumulation of off-flavor volatiles, there is also a gradual decrease in acidity levels during storage, which results in a decrease in sourness and flavor acceptability.[6,12,27,29] Nevertheless, our transcriptome analysis study did not reveal any clue to the possible regulation of citric acid catabolism at the molecular level: we did not observe upregulation of either GABA-shunt pathway transcripts, *ATP citrate lyase* transcripts, or *aconitase* and *isocitrate dehydrogenase* transcripts, all of which were proposed to be involved in citric acid catabolism.[13,23−25] Thus, elucidation of the molecular regulation of citric acid catabolism during mandarin storage still awaits further investigation.

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