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Comparative transcript profiling of a peach and its nectarine mutant at harvest reveals differences in gene expression related to storability

Anurag Dagar · Clara Pons Puig · Cristina Marti Ibanez · Fiorenza Ziliotto · Claudio Bonghi · Carlos H. Crisosto · Haya Friedman · Susan Lurie · Antonio Granell

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Abstract Gene expression at harvest was compared for two stone fruit cultivars, a peach and its near-isogenic nectarine mutant, using two microarray platforms, μPEACH1.0 and ChillPeach. Together, both platforms covered over 6,000 genes out of which 417 were differentially expressed between the fruits of the two cultivars at a p value of 0.05. A total of 47 genes in nectarine and 60 genes in peach were at least twofold higher relative to each other. Nectarine had much better storage characteristics than peach and could be stored for over 5 weeks at 5 °C without storage disorders. In an attempt to determine whether gene expression at harvest could give an indication of storage potential, the expression analysis of the two cultivars was compared to that of two genotypes with different sensitivities to chilling injury. Principal component analysis of gene expression results across four fruit types differing in chilling sensitivity resulted in 41 genes whose expression levels separated the fruits according to sensitivity to storage disorders, suggesting that the genes have a role in cold response adaptation.

Keywords Rosaceae · Microarray · Peach · Nectarine · Chilling injury

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Anurag Dagar and Clara Pons Puig contributed equally to this publication.

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A. Dagar (✉) · H. Friedman · S. Lurie
Department of Postharvest Science of Fresh Produce, Agricultural Research Organization—The Volcani Center, Bet Dagan, Israel
e-mail: anurag@agri.gov.il

A. Dagar
Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel

C. Pons Puig · C. Marti Ibanez · A. Granell
IBMCP CSIC-Universidad Polite´cnica, E-48022 Valencia, Spain

F. Ziliotto · C. Bonghi
Department of Environmental Agronomy and Crop Science, University of Padova, Agripolis-35020 Legnaro (Padova), Italy

C. H. Crisosto
Plant Sciences Department, University of California, Davis 1 Shields Ave., Davis, CA 95616, USA

Introduction

Nectarines arose as peach mutants, and their inheritance pattern is consistent with the glabrous skin characteristic controlled by a single recessive gene (Blake 1932). Most aspects of nectarine trees, leaves, and flowers are indistinguishable from those of peach; however, peach researchers have noted differences between peaches and nectarines that extend beyond those of lack of pubescence alone. These differences include fruit size, shape, firmness, external color, aroma, flavor, and disease resistance (Wen et al. 1995a, b). The nectarine character is controlled by a recessive gene, *g*, which determines the glabrous character of the fruit skin, and has no close linkage with other phenotypic markers (Dirlewanger et al. 1998). The hypothesis is that nectarines are phenotypes for a minor, nonlethal mutation, which includes the gene causing pubescence of the fruit skin but includes closely linked genes

that have other roles in the mesocarp of the fruit. Alternatively, it is possible that the nectarine phenotype may arise as an alteration in a single regulatory gene that controls the expression of other genes. This latter interpretation is consistent with genetic evidence and molecular characterization of the two known classes of glabrous mutations in *Arabidopsis thaliana* (L.) Heynh., glabrous (g11) and transparent testa glabrous (ttg), both of which are transcription factors (Maes et al. 2008; Walker et al. 1999). The organoleptic characteristics of organic acid and sugar content have been mapped to diverse linkage maps in peach and are not found associated with the glabrous G locus (Dirlewanger et al. 1999). In addition to differences in taste between peach and nectarine, the latter has been found to store better (Crisosto et al. 1999; Lurie and Crisosto 2005). This property is a valuable one for the global stone fruit industry, where shipment of fresh fruit from one continent to another is common. However, the reasons for the better storage of nectarines are not known.

One method for examining the molecular basis underlying the differences between peach and nectarine fruit is to utilize microarrays. For fruit crops, microarray platforms so far developed are mainly home-brewed, permitting robust, reproducible results to be obtained and to investigate the expression of thousands of genes at once. Two microarray platforms have been developed for peach, μPEACH1.0 and ChillPeach. The μPEACH1.0 microarray, developed by an Italian consortium, (ESTree), represents about 4,800 oligonucleotide probes corresponding to a set of unigenes expressed during the last stages of fruit development (ESTree Consortium 2005). The ChillPeach, a cDNA microarray, comprising 4,261 unigenes, was obtained from harvested, ripened, and stored fruit of two full-sibling peach progeny contrasting for chilling injury sensitivity (Ogundiwin et al. 2008). Using the two microarrays, both developed from peach fruit, increased the number of genes being investigated, since the overlap between the two platforms is only about 29 % (Granell, unpublished).

A nectarine mutant "Yuval" arose in 2002 within an "Oded" peach population from a commercial orchard in central Israel. Because the peach cultivar was of high quality, the mutation of interest was propagated. Nectarine was found to be more resistant to chilling injury (flesh browning and woolly texture) than the "Oded" peach after prolonged storage (Dagar et al. 2011). The objective of this study was to compare the molecular attributes at harvest of both fruits at the level of gene expression using transcriptomic analyses of this near-isogenic material. Thus, the comparison studied here brought us closer to elucidating the molecular basis for the pleiotropic effects of the peach-to-nectarine mutation, including the differential response to cold storage. Since it was of interest to see if gene expression at harvest could reflect the storage potential of the two fruits, we also compared the gene expression at harvest of these two fruits to that of genotypes from the peach population with different susceptibilities (sensitive and tolerant) to chilling injury, which were used to make the ChillPeach microarray. This gave us a greater spread of storage potential, since the four groups of cultivars had storage potential ranging from 2 to 7 weeks (Ogundiwin et al. 2008; Dagar et al. 2011).

Materials and methods

Plant material and treatments

The experiments were carried out with a peach [*Prunus persica* (L.) Batsch "Oded"] and its nectarine mutant [*P. persica* (L.) Batsch, var. nectarine "Yuval"] in 2008. The peach and nectarine fruits were harvested from two adjacent commercial orchards in Israel. The fruits were stored immediately at 5 °C, removed from cold storage after 3, 5, and 7 weeks, and subsequently held at 20 °C for 3 days for ripening after each removal. At the time of observation, 20 fruits from each cultivar were examined. At harvest, five representative fruits were cubed, weighed, flash frozen in liquid nitrogen, and stored at −80 °C for further analyses.

Fruit measurements

Fruit sections about 1 cm in from the peel were examined under transmitted light microscopy and photographed using a Nikon Eclipse 50i microscope and a Nikon Digital Sight DS-L1 camera system. The cell size was measured using ImageJ, a Java-based image-processing program. Ethylene production by the peach and nectarine fruits at harvest was determined by a gas chromatograph (GC; Varian 3300, Walnut Creek, CA, USA) with a flame ionization detector and an alumina column. Each fruit was individually sealed in a jar (600 mL) for 1 h at 20 °C; a 5-mL gas sample was taken with a syringe and loaded on the GC for analysis. Firmness, soluble solids content (SSC), and titratable acidity (TA) were measured following the protocol as described earlier (Zhou et al. 2000). Fruit quality including expressible juice and the flesh disorders of bleeding, browning, and wooliness after removal from storage and 3 days of shelf life at 20 °C were measured as described in Dagar et al. (2011).

Transcriptome analysis

ChillPeach microarray

ChillPeach was developed from pooling expressed sequence tags (ESTs) of peach from harvest through storage for 3 weeks. From 7,862 ESTs, 4,261 cDNA unigenes were used to construct the microarray (Ogundiwin et al. 2008). For the ChillPeach microarray analysis, total RNA from frozen mesocarp (4 g) tissue of "Oded" peach, "Yuval" nectarine, and tissues of

sensitive and tolerant peach at harvest as described by Ogundiwin et al. (2008) was made using the method described by Meisel et al. (2005). The concentration and purity of the extracted RNA was assayed by ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA) and its integrity was checked on agarose gels. RNA (1 μg) for microarray hybridization was amplified using the method of Van Gelder et al. (1990).

Transcriptome analyses and hybridization procedure of "Oded" peach, "Yuval" nectarine, and sensitive and tolerant peach fruit at harvest to the ChillPeach microarray slides were performed after a direct comparison design. Probe synthesis and labeling, hybridization procedure, and data analyses were carried out as described by Ogundiwin et al. (2008). To obtain differential gene expression values, three replicates of "Oded," four replicates of "Yuval" nectarine, and three replicates of the two peach genotypes used to make the ChillPeach microarray were hybridized against the pool reference as described by Ogundiwin et al. (2008), one of which was a dye swap. To detect differentially expressed genes in treatments, data were analyzed with the Significance Analysis of Microarray (SAM) package (Tusher et al. 2001). Statistical significance was assessed using two-class (unpaired) SAM analysis for the treatments with a false discovery rate of 5 % and q value ≤0.05. The mean of the values of the differentially expressed genes was calculated for each sample as $\log_2$ values. Functional enrichment was performed using a homemade Perl script according to Tavazoie et al. (1999). Further, for each gene, the expression ratio ($\log_2$ scale) between "Yuval" nectarine and "Oded" peach was carried out. Genes were annotated by hand following the Gene Ontology (GO) categories and other databases such as NCBI (http://www.ncbi.nlm.nih.gov), Google scholar (http://scholar.google.com), EBI (http://www.ebi.ac.uk), Swiss-Prot (http://www.expasy.org/sprot), Prosite (http://www.expasy.org/prosite), BRENDA (http://www.brenda-enzymes.org), TAIR (http://www.arabidopsis.org), the Gene Index Project (http://compbio.dfci.harvard.edu/tgi/plant.html), KEEG (http://www.genome.ad.jp/kegg), PlantCyc (http://www.plantcyc.org), and Plant Transcription Factor Database 2.0 (plntfdb.bio.uni-potsdam.de/v2.0/; Riano-Pachon et al. 2007). In order to compare "Oded" peach and "Yuval" nectarine with the chilling-tolerant and chilling-sensitive peaches, one-way analysis of variance ($p<0.05$) and principal component analysis (PCA) were performed utilizing replicates of all four fruit types.

μPEACH1.0 microarray

μPEACH1.0 is an oligo-based microarray constructed from ESTs expressed during different stages of ripening of climacteric peach fruit. It contains 4,806 specific 70-mer oligos (Trainotti et al. 2006). Total RNA was isolated as described above. Fifty micrograms of total RNA was treated with 10 U of RQ1 RNase-free DNAse (Promega) and 1 U of RNAguard (RNase INHIBITOR) (Amersham) for 30 min and then purified by phenol–chloroform according to the manufacturer's instructions. The concentration and purity of the extracted RNA was assayed as above, and its integrity was checked on agarose gels. Transcriptome analyses of "Oded" peach and "Yuval" nectarine fruit were performed following a direct comparison design. Probe synthesis and labeling, hybridization procedure, and data analyses were carried out as described by Ziliotto et al. (2008) and Falara et al. (2011). Each comparison was repeated at least twice, one of which was a dye swap. Metric quality for the arrays was evaluated by the MIDAS software, included in the TM4 package (http://www.tm4.org) developed at TIGR (http://www.tigr.org) by using intensity box plots, MA plots, RI plots, and Z score histograms. To detect differentially expressed genes, data were analyzed with the SAM package (Tusher et al. 2001). Statistical significance was assessed using two-class (unpaired) SAM analysis for the comparisons with a false discovery rate of 5 %. After normalization, the ratios were transformed to their $\log_2$ values. The $\log_2$ transformation converted the expression values to an intuitive linear scale that represented twofold differences. The genes that were differentially expressed between peach and nectarine at harvest were identified. A threshold for the hybridization signal ratio, expressed as $\log_2$, was set to be higher than 1 and lower than −1 for selecting upregulated and downregulated genes, respectively. Genes were annotated as described by Bonghi et al. (2011).

Real-time quantitative reverse transcriptase-PCR analysis

The transcript abundance of selected genes that were differentially expressed between peach and its near-isogenic nectarine mutant with over twofold change and were common between the μPEACH1.0 and ChillPeach microarray results, as well as a few genes that came from the PCA across the four cultivars, were validated with quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses. The expression levels for the genes were calculated relative to the *Initiation Factor eIF-4-Gamma* (*eIF-G*) gene as described by Ogundiwin et al. (2008), and the expression level of each analyzed gene transcript in the "Oded" peach was set to one and the level of the "Yuval" nectarine was calculated relative to this reference. Total RNA (1 μg) was used to synthesize full-length cDNA using the Versco™ cDNA kit (Applied Biosystems, USA). Primers and cDNA concentrations used for the reactions were predetermined as described to enable linear and high-efficiency response (http://www.abgene.com/downloads/article-SYBRoptimise.pdf). The reaction mixture contained forward and reverse primers and Power SYBR Green PCR Master Mix (Applied Biosystems, USA) in a 10-μL total sample volume. Reactions were analyzed on a Rotor-Gene 3000 PCR machine (Corbett Life Research, Australia) using 35 cycles of 95 °C for 10 s, 60 °C for 15 s,

72 °C for 20 s, and 80 °C for 10 s. Data obtained were analyzed with the Rotor-Gene 6 software. Primer sequences and amplicon lengths are given in Additional Table S1. The qBase Quantification Software (http://medgen.ugent.be/qbase/) was used and data are expressed according to the delta-delta-Ct method. Each biological sample was examined in duplicate with two to three technical replicates. Gene-specific oligonucleotide primers were designed using Primer Express® version 2.0 software (Applied Biosystems, USA).

Results and discussion

Ripeness and storage parameters

There was no significant difference in cell size or ethylene production between the peach and the nectarine fruits at harvest (Table 1). Average cell size measured in the fruit of both cultivars beginning 3 weeks before harvest was 66 μm and increasing to 80 and 81 μm at harvest in the peach and nectarine fruits, respectively. The literature discussing the cell size has been reported in Dagar et al. (2011). In addition, the significantly smaller fruit and higher SSC and TA in nectarines have also been reported and discussed in Dagar et al. (2011). Wu et al. (2003) and Cantin et al. (2009) also reported a significant positive correlation between TA and SSC in peaches and nectarines. It might be possible that this result is because of the colocalization of quantitative trait loci involved in SSC and acid contents in peach (Dirlewanger et al. 1999). The similar ethylene production and cell size of these near-isogenic fruits indicated that, at harvest, both were at the same physiological stage.

In contrast, the peach cultivar and the derived nectarine showed dramatic differences in the way they withstood extended periods of cold storage. These results were reported and discussed in Dagar et al. (2011). In cultivars sensitive to chilling injury, disorders appeared after 2 or 3 weeks of 5 °C storage (Zhou et al. 2001). In this regard, both cultivars were relatively resistant to chilling injury; however, nectarines were more resistant than peaches. These results are in agreement with the findings of Crisosto et al. (1999) who reported that nectarines have better storage and shipping characteristics than peaches.

In summary, peach and near-isogenic nectarine, which were at a similar physiological stage at harvest, showed differences in the way they responded to cold storage. We anticipate that those differences may already be in part reflected at the transcript level of the fruits at harvest.

Microarray analysis

Two microarray platforms, ChillPeach and μPEACH1.0, were used to compare nectarine and its peach progenitor. The overlap of the two platforms is 29 % (Granell, unpublished). A total of 2,584 ChillPeach probes (~61 %) met the threshold for hybridization quality. A total of 222 ChillPeach unigenes were significantly different at a p value <0.05 (Additional Table S2). Of these, 69 genes were more highly expressed in nectarine than peach and 153 were more expressed in peach than nectarine.

In the μPEACH1.0 microarray, a total of 2,358 genes met the threshold of hybridization quality for the two fruits. There were 195 genes showing statistical significance at p <0.05 between peach and nectarine (Additional Table S3). Of these, 57 genes had higher expression in nectarine than in peach and 138 were higher in peach.

A comparison of the functional categorization of the differentially expressed genes in the two platforms is shown in Table 2. It was found that, in the ChillPeach and μPEACH1.0 microarray platforms, nectarine was lower than peach in abundance of the majority of transcripts related to cell wall, lipid metabolism, RNA transcription regulation, signal transduction pathway, trafficking machinery and membrane dynamics, and transport. Exceptions to this trend were a few categories including secondary metabolism (in ChillPeach microarray platform), antioxidant system (in μPEACH1.0 microarray platform), RNA posttranscriptional regulation (in μPEACH1.0 microarray platform), and RNA translation and protein assembly (in μPEACH1.0 microarray platform), with more genes higher in nectarine compared to peach.

Few studies in peaches and nectarines have utilized the global gene expression and transcriptome analysis approaches to elucidate chilling injury, and all of them were focused either during cold storage or during ripening (shelf life) after cold storage. Pegoraro et al. (2010) reported that, during ripening of two different peach cultivars, previously

Table 1 Representative ripeness parameters at harvest of the "Oded" peaches and "Yuval" nectarines

Cultivar	Weight (g)	Ethylene (μL kg^{-1} h^{-1})	Cell size (μm)	SSC (%)	TA (%)	Firmness (newtons)
Oded	140A	0.67A	80A	12.2A	0.52A	49.7A
Yuval	106B	0.62A	81A	13.7B	0.63B	51.3B

Average values followed by a different capital letter are statistically different between the "Oded" peach and "Yuval" nectarine according to two-sample t test at a significance level of $p \leq 0.05$ conducted with MINITAB 14 (Minitab, Inc.)

Table 2 Functional categorization of differentially expressed genes of ChillPeach and μPEACH1.0 microarray platforms as well as common genes between the two microarray platforms in the "Yuval" nectarine compared to its peach progenitor "Oded" at harvest ("Yuval"/"Oded")

Functional categorization	"ChillPeach"		"μPEACH1.0"		Common genes		Total
	Up	Down	Up	Down	Up	Down	
Amino acid metabolism	1	3	–	4	–	1	9
Antioxidant system	3	4	4	1	1	2	15
Cell wall-related	4	10	3	13	–	4	34
Chromatin status and regulation	–	1	2	–	–	–	3
Circadian clock	–	–	1	–	–	–	1
Cofactor and vitamin metabolism	1	4	1	3	–	–	9
Cytoskeleton organization and biogenesis	1	3	1	1	–	2	8
Energy production	1	2	3	2	–	1	9
Glycolysis/pentose phosphate pathway	1	1	–	–	–	1	3
Homeostasis	–	2	2	1	–	1	6
Lipid metabolism	2	6	1	8	1	–	18
Nucleotide metabolism	–	4	–	2	–	2	8
Other carbohydrate metabolism	2	2	1	6	1	–	12
Other nucleic acid metabolic process	1	2	3	1	–	–	7
Posttranslational protein modification	2	2	1	–	–	–	5
Programmed cell death	–	1	–	–	–	–	1
Protein degradation	4	8	–	5	–	–	17
Pyruvate metabolism	–	2	2	1	–	–	5
RNA posttranscriptional regulation	8	6	–	–	–	–	14
RNA transcription regulation	2	9	1	7	–	–	19
RNA translation and protein assembly	4	2	1	3	–	–	10
Secondary metabolism	9	6	6	17	–	3	41
Signal transduction pathway	3	8	3	10	–	6	30
Structure maintenance proteins	2	2	2	–	–	–	6
Sulfur metabolism	–	1	–	1	–	–	2
Trafficking machinery and membrane dynamics	3	11	–	2	–	–	16
Transport	1	8	2	9	–	2	22
Tricarboxylic acid cycle	1	–	–	–	–	–	1
Unknown function	12	27	13	16	1	–	69
Total	68[a]	137[a]	53[a]	113[a]	4	25	400

Functional categorization and annotation was performed according to GO categories and information published about genes in databases

[a]Because of genes with no corresponding homologues (including *Arabidopsis*) in gene databases, the total number of functional categorized upregulated and downregulated genes in ChillPeach and μPEACH1.0 microarray was 205 and 166 instead of 222 and 195, respectively

stored under cold storage (30 days at 4 °C followed by a 5-day shelf life) and controlled atmosphere, the differential expression of genes involved in transport and cell wall metabolism analyzed by quantitative PCR were lower in the peach resistant to woolliness compared to the susceptible peach. The authors suggested that prevention of chilling injury in peaches and nectarines is not completely dependent on the increase in abundance, but may also be tied to the decrease in abundance of these groups of genes.

The genes from the two platforms were further filtered to those which were more than twofold different between the two fruits (Table 3). There were a total of 49 (22 and 27 with higher and lower expressions, respectively, in nectarine compared to peach) genes in ChillPeach and 58 genes (25 and 33 with higher and lower expressions, respectively, in nectarine compared to peach) in μPEACH1.0. The pattern of lower expression of genes in nectarine than peach was maintained in this case as well. There were nine genes that were common between the two platforms with over twofold different expression, and most of these genes (seven) were lower in nectarine and higher in peach (Fig. 1). The expression of selected common genes between the two platforms was validated by qRT-PCR (Additional Table S1 and Additional Fig. 1)

In summary, the overlap between the two microarray platforms is small enough so that, together, the two platforms give a broader view than either one alone. In addition, the fact that, in both platforms, the shared genes have similar expression patterns further supports our approach of using these two independent microarrays to investigate differences in gene expression between the two cultivars.

Table 3 List of genes with ≥2.0-fold increase or decrease in expression in "Yuval" nectarine compared to "Oded" peach mesocarp tissue samples at harvest

Unigene functional annotation	AT ID	Source ChillPeach/ μPEACH1.0	ID	Fold change[a]
Upregulated in nectarine				
Antioxidant system				
Metallothionein-like protein	AT5G02380	μPEACH1.0	Contig490	2.20[b]
Catalase	AT4G35090	ChillPeach	CL884Contig1	2.21
Thioredoxin family	AT5G61440	μPEACH1.0	Contig3385	2.24
Glutathione S-transferase GST 22	AT2G30860	ChillPeach	PPN040C03-T7_c_s	2.36
Glutathione S-transferase	AT5G17220	ChillPeach	CL372Contig1	3.35
Dehydrogenase/reductase (SDR)	AT4G13250	ChillPeach	CL466Contig1	4.33
Carbohydrate metabolism				
Glyoxalase I, putative (lactoylglutathione lyase)	AT1G11840	μPEACH1.0	Contig126	3.08
Cell wall-related				
Endo-1,3;1,4-β-D-glucanase	AT3G23600	ChillPeach	CL480Contig1	2.09
Glycosyltransferase	AT3G15350	ChillPeach	PP1004E08-T7_c_s	2.09
Endo-1,4-β-D-glucanase	AT1G64390	μPEACH1.0	Contig2196	2.17
β-Galactosidase precursor	AT3G13750	ChillPeach	CL1325Contig1	4.31
Chromatin status and regulation				
Coiled-coil protein	AT4G30200	μPEACH1.0	Contig2128	2.61
Cell cycle checkpoint RAD17	AT5G66130	ChillPeach	PPN040G09-T7_c_s	2.17
Energy production				
Photosystem I reaction center subunit	AT5G64040	μPEACH1.0	Contig5456	2.01
Signal peptidase subunit	AT1G15820	μPEACH1.0	Contig4905	2.16
Lipid and secondary metabolism				
UDP-sulfoquinovose:DAG	AT5G01220	μPEACH1.0	Contig2505	2.01
Sulfolipid synthase	AT5G01220	ChillPeach	CL484Contig1	2.31[c]
T3B23.2/T3B23.2 protein	AT2G28305	μPEACH1.0	Contig2139	2.05
Haloacid dehalogenase-like hydrolase family	AT5G59480	μPEACH1.0	Contig2160	2.10
FAD-linked oxidoreductase	AT2G34790	μPEACH1.0	Contig1907	2.37
Putative ripening-related P-450	AT2G45550	ChillPeach	CL130Contig1	2.39
Chalcone synthase 2	AT5G13930	ChillPeach	CL792Contig1	2.78
S-adenosylmethionine methyltransferase	AT1G16650	ChillPeach	PP1001H05-T7_c_s	2.88
Protein degradation				
Mitochondrial processing peptidase	AT3G16480	ChillPeach	CL408Contig1	2.63
RNA regulation				
RNA recognition motif	AT4G33690	ChillPeach	PP1004F11-T7_c_s	2.11
Eukaryotic TF	AT1G58110	ChillPeach	CL882Contig1	3.03
Hypothetical protein	AT1G64390	μPEACH1.0	Contig2200	3.59
Signal transduction pathway				
COG2200: FOG: EAL domain	AT4G33270	μPEACH1.0	Contig2830	2.01
Gigantea protein	AT1G22770	ChillPeach	CL943Contig1	2.45
Diacylglycerol kinase 1	AT5G07920	ChillPeach	PPN004A11-T7_c_s	2.64
Protein kinase	AT5G48380	ChillPeach	PPN042D03-T7_c_s	3.41
Stress response				
Early response to dehydration	AT4G22120	ChillPeach	PPN031G09-T7_c_s	2.10
Dehydrin	AT1G20440	μPEACH1.0	Contig1528	3.14
Dehydration-responsive RD22	AT5G25610	μPEACH1.0	Contig973	2.80
Structure maintenance proteins				
DnaJ-like protein	AT1G56300	ChillPeach	CL1421Contig1	2.34
Transport				

Table 3 (continued)

Unigene functional annotation	AT ID	Source ChillPeach/ μPEACH1.0	ID	Fold change[a]
Nodulin-related protein	AT4G34950	μPEACH1.0	Contig4838	3.06
Unknown function				
Hypothetical protein	AT5G20700	μPEACH1.0	Contig728	2.03
Little protein 1	Not available	ChillPeach	PPN043F06-T7_c_s	2.10
ERD4 protein	AT1G30360	μPEACH1.0	Contig251	2.11
Unknown protein	AT5G18130	μPEACH1.0	Contig1296	2.11
CIP7	Not available	μPEACH1.0	Contig1512	2.15
Hypothetical protein	AT3G27090	μPEACH1.0	Contig2017	2.22
Hypothetical protein	Not available	μPEACH1.0	Contig208	2.26
F3I6.25 protein	AT1G24310	ChillPeach	PPN011B09-T7_c_s	2.30
Auxin-repressed protein	AT1G28330	μPEACH1.0	Contig734	2.67
Unknown protein	AT2G15890	μPEACH1.0	Contig2103	3.16
Unknown protein	AT3G52070	μPEACH1.0	Contig1419	3.38
Downregulated in nectarine				
Acid metabolism				
Glutamate dehydrogenase 2	AT5G07440	ChillPeach	PP1004F04-T7_c_s	0.48[c]
Asparagine synthase	AT4G27450	μPEACH1.0	Contig1501	0.49
Carbohydrate metabolism				
NAD-dependent sorbitol dehydrogenase	AT5G51970	μPEACH1.0	Contig636	0.39
Trehalose-6-phosphate phosphatase	AT5G51460	μPEACH1.0	Contig3551	0.40
Cell wall-related				
Ripening-related protein-like (invertase)	AT5G51520	μPEACH1.0	Contig938	0.25
Xyloglucan endotransglucosylase	AT1G10550	ChillPeach	CL907Contig1	0.27
Pectinesterase	AT1G76160	μPEACH1.0	Contig1944	0.37
Endopolygalacturonase	AT3G59850	μPEACH1.0	Contig420	0.39
Extensin-like protein	Not available	μPEACH1.0	Contig2430	0.44
Pectinesterase PPE8B precursor	AT3G43270	ChillPeach	PPN002G04-T7_c_s	0.45
Lipopolysaccharide biosynthesis protein	AT4G12730	μPEACH1.0	Contig2512	0.49
Chromatin regulation				
hnRNP	AT3G20890	ChillPeach	CL1071Contig1	0.38
Cytoskeleton organization				
CIG1	AT5G38710	μPEACH1.0	Contig760	0.27
At4g14960 tubulin	AT5G19780	ChillPeach	PPN021H05-T7_c_s	0.38
Tubulin alpha-5 chain	AT5G19770	μPEACH1.0	Contig1500	0.46
Energy production				
ATPase-like protein	AT3G10420	μPEACH1.0	Contig5213	0.43
Chloroplast precursor	AT3G22840	ChillPeach	CL460Contig1	0.47[c]
Lipid and secondary metabolism				
S-adenosylmethionine decarboxylase	AT3G25570	ChillPeach	PPN035E11-T7_c_s	0.29[c]
S-adenosylmethionine decarboxylase	AT3G02470	μPEACH1.0	Contig1003	0.38
1-aminocyclopropane-1-carboxylate	AT1G05010 oxidase	μPEACH1.0	Contig64	0.41
Ceramide glucosyltransferase	AT2G19880	ChillPeach	CL1355Contig1	0.42
Enoyl-CoA hydratase	AT5G65940	μPEACH1.0	Contig60	0.44
Omega-6 fatty acid desaturase	AT3G12120	μPEACH1.0	Contig835	0.45
Expressed protein	AT2G19880	μPEACH1.0	Contig1094	0.49
Gibberellin 2-oxidase	AT1G02400	μPEACH1.0	Contig544	0.46
Cytochrome P450, putative	AT3G14660	μPEACH1.0	Contig4560	0.47
NADPH-cytochrome P450 reductase	AT4G30210	μPEACH1.0	Contig2300	0.47
Flavonoid 1-2 rhamnosyltransferase	AT5G65550	μPEACH1.0	Contig205	0.48
Nitrate metabolism				

Table 3 (continued)

Unigene functional annotation	AT ID	Source ChillPeach/ μPEACH1.0	ID	Fold change[a]
Nitrate reductase	AT1G77760	μPEACH1.0	Contig494	0.37
Nucleotide metabolism				
Ripening-related protein	AT5G02230	ChillPeach	CL86Contig2	0.33[c]
MutT domain protein-like	AT5G47650	μPEACH1.0	Contig950	0.41
Protein degradation				
Subtilisin-like serine protease	AT2G05920	ChillPeach	PPN065B02-T7_c_s	0.38
Ubiquitin-specific protease	AT4G24560	ChillPeach	PP1001G11-T7_c_s	0.48
RNA regulation				
Arabidopsis thaliana genome chromosome 3, P1 clone: MOE17	AT3G20890	ChillPeach	CL1071Contig1	0.49
BZIP protein BZ2	Not available	ChillPeach	PPN019A01-T7_c_s	0.28
Signal transduction pathway				
Phi-1	AT4G08950	μPEACH1.0	Contig4454	0.46
Hypothetical protein	Not available	μPEACH1.0	Contig3709	0.30
CBL-interacting protein kinase 6	AT4G30960	μPEACH1.0	Contig2777	0.40[b]
Abscisic acid-inducible protein kinase	AT4G40010	μPEACH1.0	Contig1514	0.40
Serine–threonine protein kinase	AT1G78290	ChillPeach	PPN010B11-T7_c_s	0.49[c]
SOS2-like protein kinase	AT4G30960	μPEACH1.0	Contig2449	0.44
Bet v I allergen family	AT2G26040	μPEACH1.0	Contig1109	0.46
Putative serine–threonine kinase	Not available	ChillPeach	CL1059Contig1	0.46
Leucine-rich repeat protein kinase	Not available	μPEACH1.0	Contig134	0.47
Serine–threonine protein kinase	AT1G78290	ChillPeach	PPN010B11-T7_c_s	0.48
Stress response				
Generic methyltransferase	AT4G00750	ChillPeach	CL420Contig1	0.45
Phototropic response NPH family	AT1G50280	ChillPeach	CL412Contig1	0.48
Hypoxia-responsive protein	AT5G27760	ChillPeach	PP1000D03-T7_c_s	0.49[c]
Transport				
Sodium dicarboxylate cotransporter-like	AT5G47560	μPEACH1.0	Contig1515	0.29
ChaC-like family protein	AT5G26220	ChillPeach	PPN027H10-T7_c_s	0.34
Putative peptide transporter	AT3G01350	ChillPeach	PPN029A02-T7_c_s	0.42
PIN1-like auxin transport protein	AT1G73590	μPEACH1.0	Contig3721	0.42
Nitrate transporter NRT1-2	AT1G18880	ChillPeach	PPN024D02-T7_c_s	0.45
Unknown function				
ChaC-like family protein-like	AT5G26220	ChillPeach	PPN027H10-T7_c_s	0.32
T76725	AT1G10740	μPEACH1.0	Contig3616	0.38
AM290178 *Prunus persica* fruit	Not available	ChillPeach	PPN044E02-T7_c_s	0.44
At5g03345 Pm52 protein	AT5G03345	ChillPeach	PPN036E10-T7_c_s	0.45
Gb|AAD25142.1	AT2G17240	ChillPeach	PPN019A03-T7_c_s	0.47
Gb|AAD50054.1	AT1G50280	ChillPeach	CL412Contig1	0.48
Chain A, agglutinin	Not available	μPEACH1.0	Contig1764	0.49

AT Arabidopsis thaliana

[a] Significant fold increase or decrease of expression in "Yuval" nectarine compared to "Oded" peach mesocarp, $p<0.05$

[b] μPEACH1.0 common genes from gene expression comparison of ChillPeach and μPEACH1.0 microarray results of "Yuval" nectarine and "Oded" peach ("Yuval"/"Oded") fruits

[c] ChillPeach common genes from gene expression comparison of ChillPeach and μPEACH1.0 microarray results of "Yuval" nectarine and "Oded" peach ("Yuval"/"Oded") fruits

Among the genes common in both microarrays over twofold higher in nectarine was *sulfolipid synthase*, which is involved in lipid metabolism (validated by qRT-PCR; Additional Fig. 1). Other genes twofold higher in nectarine compared to peach included *glutathione S-transferase GST22* (validated by qRT-PCR; Additional Fig. 1); *catalase*

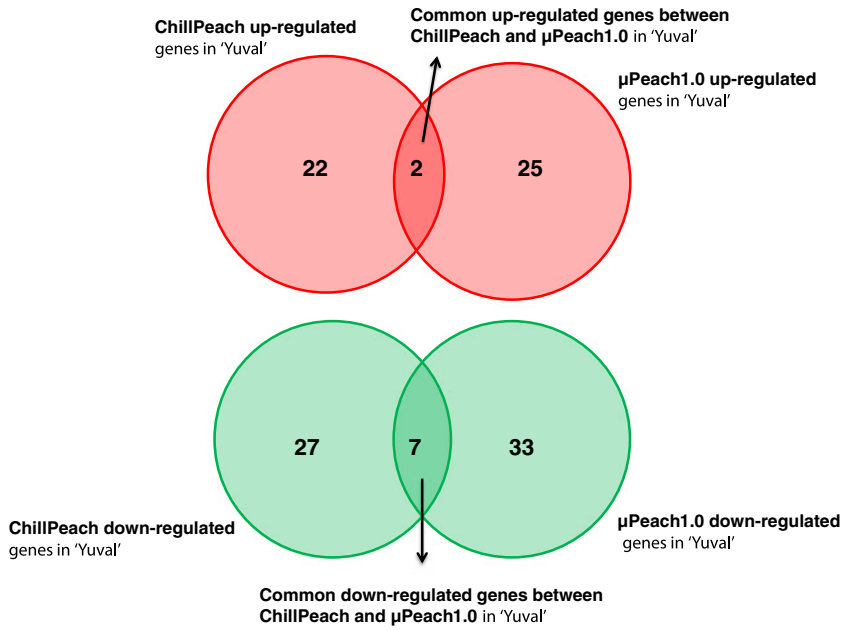

Fig. 1 Venn diagram comparing the significantly and differentially expressed transcripts in "Yuval" compared to "Oded" with fold changes ≥2.0. The expression patterns of the common nine (two upregulated and seven downregulated in "Yuval") statistically significant transcripts between the ChillPeach and µPEACH1.0 microarray platforms are reported

and *thioredoxin*, related to antioxidant system; *endo-1,4-β-D-glucanase* and *β-galactosidase*, precursors involved in cell wall degradation; mitochondrial processing peptidase involved in protein degradation; and *chalcone synthase 2*, related to secondary metabolism. In contrast, among the common genes higher in peach compared to the nectarine mutant were *glutamate dehydrogenase 2*, related to amino acid metabolism; *S-adenosylmethionine decarboxylase*, related to secondary metabolism; *calcineurin B-like (CBL)-interacting protein kinase 6* and *serine–threonine protein kinase*, involved in signal transduction pathway; and a gene encoding hypoxia-responsive protein in the stress responses (the selected genes were validated by qRT-PCR as listed in Additional Table S1 and shown in Additional Fig. 1). Other genes higher in peach compared to nectarine included: *trehalose-6-phosphate phosphatase*, involved in carbohydrate metabolism; *xyloglucan endo-transglucosylase*, *endopolygalacturonase*, and *pectinesterase*, precursors involved in cell wall degradation; *ceramide glucosyltransferase* and *omega-6 fatty acid desaturase*, involved in lipid metabolism; *gibberellin 2-oxidase* and *flavonoid 1-2 rhamnosyltransferase*, related to secondary metabolism; and a number of stress response genes. Overall, 47 genes were ≥2.0-fold higher in nectarine and 60 in peach (Table 3). Hence, in the present study, we found that, at harvest, nectarine had more genes with lower expression compared to peach. This suggests that, perhaps, either a positive regulator responsible for the expression of these genes is mutated in peach to yield a nectarine phenotype or some of the transcripts expressed in nectarine are not represented since both microarray platforms were constructed using ESTs from peaches.

Most of the studies on transcriptome analysis were performed on fruit at the beginning or during the development of chilling injury. Gonzalez-Aguero et al. (2008) utilized a nylon macroarray from a ripe peach fruit cDNA library to study gene expression changes of "O'Henry" peach fruit. The authors compared healthy fruit (juicy; at 7 days ripening after harvest) with chilling-injured fruit (woolly, nonjuicy; at 7 days ripening after 2 weeks cold storage at 4 °C) and found 106 genes including cell wall metabolism- and endomembrane trafficking-related genes to be differentially expressed between juicy and woolly fruit. In addition, they also found lower expression of *cobra*, *endopolygalacturonase*, *cinnamoyl-Co-A-reductase*, and *rab11* genes in woolly fruit compared to juicy fruit.

A more comprehensive study of changes in "O'Henry" peach fruit was performed with comparative EST transcript profiling (Vizoso et al. 2009). They identified genes differentially expressed during ripening, in response to cold storage, or combined effects of cold storage and ripening. They ordered 1,402 normalized unigenes into 13 clusters according to the gene expression patterns. Among the 114 genes in the cluster that increased in expression in fruits in cold storage, there were six genes that had been identified by Ogundiwin et al. (2008) in their study of the ESTs used to make the ChillPeach microarray. Ogundiwin et al. (2008) utilized the ChillPeach microarray to compare cold-stored fruit tissue to fruit at harvest. There were 287 genes significantly higher and 74 lower in cold-stored fruit compared to nonripe fruit at harvest. Of the 287 upregulated genes, 74 were ≥2-fold higher, and of the 74 downregulated genes, nine were <0.5-fold expression. Twelve of these genes are also present as differentially expressed in "Oded" peach and "Yuval" nectarine at harvest, and most were higher in nectarine than peach. These

Table 4 Genes that separate fruit according to storage potential as seen in Fig. 2

Unigene annotation	AT ID	ChillPeach ID	Functional annotation
Upregulated in cold-tolerant fruit			
Metallothionein-like protein	Not available	CL294Contig1	Antioxidant
Glutathione S-transferase GST22	AT2G30860	PPN040C03-T7_c_s	Antioxidant system
Endo-1,4-β-mannosidase	AT5G66460	PP1003C05-T7_c_s	Cell wall-related
F17A17.37 protein	AT3G08030	CL18Contig2	Cell wall-related
Ribosomal protein S3Ae	AT3G04840	CL646Contig1	RNA translation and protein assembly
Chalcone synthase 2	AT5G13930	CL792Contig1	Secondary metabolism
Putative WD-repeat protein	AT4G02730	CL1412Contig1	Signal transduction pathway
Heat shock cognate 70 kDa protein 2	AT3G12580	CL823Contig1	Structure maintenance proteins
Tonoplast intrinsic protein	AT2G36830	PP1003C07-T7_c_s	Transport and trafficking
T19F11.6 protein	AT3G11660	PPN014C03-T7_c_s	Trafficking machinery and membrane dynamics
Unknown protein	Not available	PPN068F02-T7_c_s	Unknown function
Downregulated in cold-tolerant fruit			
Glutamate dehydrogenase 2	AT5G07440	PP1004F04-T7_c_s	Amino acid metabolism
Indole-3-acetic acid-induced protein ARG2	AT4G02380	CL704Contig1	Antioxidant system
Glutathione reductase	AT3G24170	PPN073D11-T7_c_s	Antioxidant system
Trehalose-6-phosphate phosphatase	AT5G65140	CL877Contig1	Other carbohydrate metabolism
Arabidopsis thaliana genome chromosome 5, P1 clone:MNJ7	AT5G47530	CL1286Contig1	Cell wall-related
Ubiquinone COQ4 homolog	AT2G03690	CL652Contig1	Cofactor and vitamin metabolism
Protein At2g43840	AT2G43840	PPN049C06-T7_c_s	Cofactor and vitamin metabolism
Putative postsynaptic protein CRIPT	AT1G61780	CL319Contig1	Cytoskeleton organization and biogenesis
Ceramide glucosyltransferase	AT2G19880	CL1355Contig1	Lipid metabolism
Allantoinase	AT4G04955	PPN048D05-T7_c_s	Nucleotide metabolism
Os04g0623400 protein	AT5G26940	CL261Contig1	RNA posttranscriptional regulation
Pod-specific dehydrogenase SAC25	AT5G02540	CL1233Contig1	Secondary metabolism
Pod-specific dehydrogenase SAC25	AT5G02540	CL495Contig1	Secondary metabolism
NADPH-cytochrome P450 oxydoreductase isoform 2	AT4G30210	CL1418Contig1	Secondary metabolism
Cytochrome P450 monooxygenase CYP72A59	AT3G14660	CL1206Contig1	Secondary metabolism
CBL-interacting protein kinase	AT4G30960	CL340Contig1	Signal transduction pathway
Serine–threonine kinase	AT5G58380	PPN013H01-T7_c_s	Signal transduction pathway
Serine–threonine kinase [*Persea americana*]	Not available	CL1059Contig1	Signal transduction pathway
Rust resistance gene ABC1041	AT2G28930	CL827Contig1	Signal transduction pathway
DnaJ protein	AT2G21510	PPN009H11-T7_c_s	Structure maintenance proteins
Multidrug resistance-associated protein 10	AT3G62700	PPN023B08-T7_c_s	Transport
Nitrate transporter (NTL1); 53025–56402	AT1G69850	CL1251Contig1	Transport
COP1-interacting protein 7	AT4G27430	CL1460Contig1	Trafficking machinery and membrane dynamics
Arabidopsis thaliana genomic chromosome 5, P1 clone:MSH12	AT5G13660	CL245Contig1	Unknown function
At5g03345	AT5G03345	PPN036E10-T7_c_s	Unknown function
RING finger-like protein	AT5G19430	PPN038H07-T7_c_s	Unknown function
AM290178 *Prunus persica* fruit skin mature fruit	Not available	PPN044E02-T7_c_s	Unknown function
Ubiquitin-conjugating enzyme E2-like protein	AT2G33770	CL752Contig1	Protein degradation
Gb|AAC18972.1	AT5G67370	PPN049A10-T7_c_s	Unknown function
Arabidopsis thaliana, mRNA sequence	AT2G27830	PPN055E08-T7_c_s	Unknown function

included *β-galactosidase precusor*, *chalcone synthase 2*, and genes encoding little protein 1 (Table 3), heat shock 70 protein, and tonoplast intrinsic protein (Table 4), while lower in nectarine were genes encoding BZIP protein and a putative ripening protein and *glutamate dehydrogenase 2* gene (Table 3).

A number of studies have been published utilizing the μPEACH1.0 microarray. These studies have examined peach fruit development (Bonghi et al. 2011), fruit ripening (Trainotti et al. 2006; Ziliotto et al. 2008), and the influence of various hormones on fruit development and ripening (Trainotti et al. 2007; Ziosi et al. 2008; Rasori et al. 2010; Bonghi et al. 2011). The microarray has also been used to investigate transcript profiling of apricot during development and ripening (Manganaris et al. 2011). One study examined two cultivars of peaches ripened after storage of 4 or 6 weeks (Falara et al. 2011). They found a number of cell wall-modifying proteins (β-D-xylosidase and expansin) and stress proteins (HSPs, dehydrin, and PR-4B) whose expression increased in ripening without storage and after storage in the chilling injury-resistant cultivar. This corresponds with the findings in this study, which found a *dehydrin* gene (Table 3), genes encoding an HSP (Table 4), and cell wall enzymes (Table 3) more highly expressed in nectarine than in peach.

It was of interest to see if differences in gene expression shown at harvest could be used to predict storage behavior, so a correlation study between gene expression and this trait was conducted. To widen the comparison of storage behavior, we utilized also the results of gene expression at harvest of the fruits that had been used to prepare the ChillPeach microarray. A quantitative comparison was possible as the "Yuval" nectarine, "Oded" peach, and mature tolerant and sensitive peach samples (Ogundiwin et al. 2008) were hybridized against the same reference pool. These samples were pools of different sibling genotypes coming from a breeding population and were either sensitive (S; showing chilling injury after 1 week of cold storage plus shelf life) or tolerant (T; developing chilling injury after 2–3 weeks of cold storage plus shelf life). A PCA was obtained with the expression values for the 222 differentially expressed genes in the ChillPeach microarray (Additional Table S2) for the mature sensitive (M-S), mature tolerant (M-T), "Oded" peach (O), and "Yuval" nectarine (Y) representing the four types of mature samples with increasing tolerance to cold stress: M-S<M-T<O<Y. The analysis revealed that, in the PCA space, samples were arranged so that, according to the depicted diagonal of PCA1 and PCA2, they could be projected into it according to their tolerance (Fig. 2). We next selected genes contributing to the loading plots on the ends of this diagonal in the score plot PCA1/2. In Fig. 2, the pattern of expression of 41 genes (listed in Table 4) selected could be seen with empty circles repressed (DR; downregulated) with tolerance and solid circles induced (UR; upregulated) with tolerance. This indicated that, out of the 222 genes that could contribute to the differences in the physiology and postharvest behavior, 41 of them were also able to explain the way four different genotypes are distributed according to their tolerance to cold stress in storage. Selected genes of these 41 were validated by qRT-PCR as listed in Additional Table S1 and shown in Additional Fig. 1.

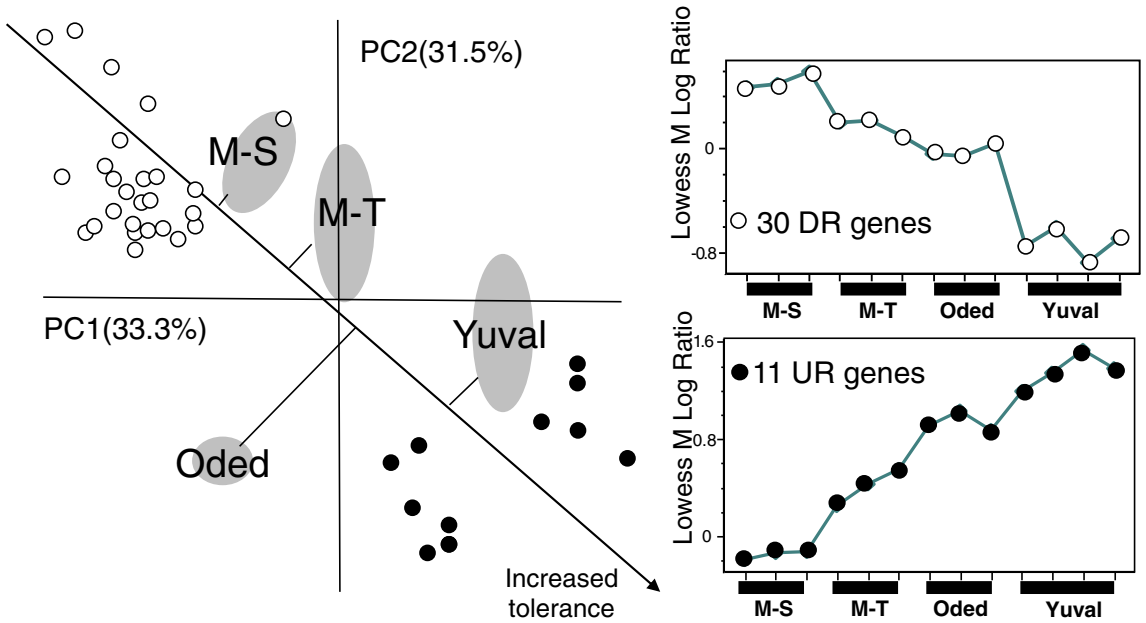

Fig. 2 A PCA showing the score plots obtained using the 222 genes that showed statistical significance between "Yuval" and "Oded" but including also the results from the M-S and M-T. The position of the samples from the score plots is indicated. The genes contributing to loading plots at the ends of the diagonal in the score plot of PCA1 and PCA2 are shown with *solid circles* for genes higher in tolerant fruit and *open circles* for genes higher in sensitive fruit. The *insets* show the expression patterns of these genes; 30 genes repressed (*DR*; downregulated) with tolerance and 11 genes induced (*UR*; upregulated) with tolerance. The *x*-axis of the insets indicates the expression of the replicates of the four fruits, with highest expression of the 30 repressed with tolerance genes in the M-S and highest expression of the induced with tolerance genes in "Yuval"

Out of these 41 genes, 11 had higher expression in the chilling-tolerant fruit than in the chilling-sensitive fruit and 30 had lower expression in the chilling-tolerant fruit (Table 4). In examining the functional categories of these genes, it was found that the antioxidant system, structure and maintenance proteins (chaperones), and transport and trafficking categories had about the same number of genes with higher and lower expressions in the chilling-tolerant fruit compared to the chilling-sensitive fruit. Other major categories with lower expression in the chilling-tolerant fruit compared to the chilling-sensitive fruit were cofactor and vitamin metabolism, signal transduction pathway, and secondary metabolism. It is interesting to note that many of these genes are not more than twofold different in expression in nectarine compared to peach and, therefore, are absent from Table 3. Three of the genes higher in the cold-tolerant fruit were over twofold higher expression in nectarine, while nine genes were less than twofold higher expression in nectarine. The remaining 30 downregulated genes in the chilling-tolerant fruit were significantly different, but not by twofold.

In the PCA results of genes separating the cold-tolerant fruit from the cold-sensitive fruit, there were three times fewer genes with higher expression in the cold-tolerant fruit than in the cold-sensitive fruit (Table 4). Despite that general pattern, more antioxidant and other protective genes were expressed over twofold higher in nectarine than in peach (Table 3). Among the antioxidant genes in the cold-tolerant side was *glutathione S-transferase GST22* (validated by qRT-PCR; Additional Fig. 1). The glutathione S-transferase protein belongs to the tau subfamily and is involved in cellular detoxification due to its ability to conjugate endobiotic and xenobiotic compounds to glutathione (Nilo et al. 2010; Lo Piero et al. 2009). The higher expression levels of glutathione S-transferase in nectarine are in agreement with previous findings of Nilo et al. (2010), where the authors found that increased accumulation of a glutathione S-transferase protein in the cold-stored peach fruit is associated with the fruits' increased capacity to withstand low-temperature stress.

Signal transduction pathways include entire physiological processes from signal reception to cellular response. There were fewer genes in this category with higher expression in nectarine than in peach and also in the PCA separating chilling-tolerant fruits from chilling-sensitive fruits (Table 4).

The roles of various kinases as important signal transducers during low-temperature stress have been demonstrated, although their signaling pathways are not fully understood. Surprisingly, we found more genes with higher expression in the chilling-sensitive fruits compared to the chilling-resistant fruits. One of the kinases was a CBL-interacting protein kinase, which is activated by a CBL in the presence of Ca^{2+}. However, in *Arabidopsis*, CBL-1 has been reported as a positive regulator of salt and drought responses but a negative regulator of cold response (Cheong et al. 2003). Therefore, the higher expression of CBL-interacting kinase gene (validated by qRT-PCR; Additional Fig. 1) in chilling-sensitive peach might act as a negative regulator of low-temperature stress and thus reduce its resistance to the stress.

Chilling injury is a multigene process, and the discovery of genes that have emerged from our study may be a step in understanding this complex process. Differences that are found at harvest might indicate how well different cultivars of peaches and nectarines can be stored, and this can lead to the identification of candidate genes which will be of use to breeding chilling-resistant cultivars.

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