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Development of ChillPeach genomic tools and identification of cold-responsive genes in peach fruit

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Abstract The ChillPeach database was developed to facilitate identification of genes controlling chilling injury (CI), a global-scale post-harvest physiological disorder in peach. It contained 7,862 high-quality ESTs (comprising 4,468 unigenes) obtained from mesocarp tissues of two full-sib progeny contrasting for CI, about 48 and 13% of which are unique to *Prunus* and *Arabidopsis*, respectively. All ESTs are in the Gateway® vector to facilitate functional assessment of the genes. The data set contained several putative SNPs and 184 unigenes with high quality SSRs, of which 42% were novel to *Prunus*. Microarray slides containing 4,261 ChillPeach unigenes were printed and used in a pilot experiment to identify differentially expressed genes in cold-treated compared to control mesocarp tissues, and in vegetative compared to mesocarp tissues. Quantitative RT-PCR (qRT-PCR) confirmed microarray results for all 13 genes tested. The microarray and qRT-PCR analyses indicated that ChillPeach is rich in putative fruit-specific and novel cold-induced genes. A website (http://bioinfo.ibmcp.upv.es/genomics/ChillPeachDB) was created holding detailed information on the ChillPeach database.

Keywords ChillPeach · Chilling injury · Mesocarp · Peach · Microarray · qRT-PCR

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Introduction

World production of peaches and nectarines is about 11 million tons (Lurie and Crisosto 2005). The major producing countries are China, Italy, and the United States in the northern hemisphere, and Chile, South Africa and Australia in the southern hemisphere. Fresh-market peaches are produced in the northern hemisphere from April through September, and in the south, from November to March. Americans eat approximately 4.41 lbs of peaches per capita per year. This consumption level is low when compared to other fresh fruit such as apple (35.28 lbs) and banana (19.85 lbs) (Crisosto 2006). A major reason for low consumption of fresh peach and nectarine is a physiological disorder called chilling injury (CI) or internal breakdown. CI development is the consequence of storing fruit in cold temperatures to extend fruit market life.

Marketing and shipment of peach fruit, either within a large country such as the United States or China, or export to other countries can require a lengthy storage period. Also, the global industry can take advantage of the on and off seasons between the two hemispheres to exchange fruit for marketing. This requires long periods (at least 3–5 weeks of market life—shipment, handling at production and receiving) between the time fruit is harvested to

when it finally reaches consumers. CI is encountered in all these cases, and it is responsible for consumer dissatisfaction and reduced consumption.

CI symptoms include lack of juiciness (mealiness or woolliness), flesh browning, black pit cavity, flesh translucency (gel breakdown), red pigment accumulation (bleeding), loss of flavor, development of off flavors, and failure to ripen (Lurie and Crisosto 2005). Because most of these symptoms develop during fruit ripening after cold storage, the CI problem is usually not noticed until fruit reaches consumers (Bruhn et al. 1991; Crisosto 2006), thereby reducing consumer satisfaction.

Physiology of CI has received considerable attention. Several postharvest treatments have been proposed to mitigate the problem of CI in peach and nectarine fruit. These include warming interruptions during cold storage, treatment with plant growth regulators, controlled atmosphere, and intermittent warming and controlled delayed cooling (for review see Lurie and Crisosto 2005). Preharvest factors influencing CI were also reviewed by Lurie and Crisosto (2005) including fertilization, irrigation regimes, canopy position of fruit, and crop load and size. Manipulation of these factors has provided only short-term solution to CI problem. A long-term solution is highly desirable and relies on understanding the genetic basis of its inheritance. Elucidation of the inheritance mechanism will enable the breeding of new CI-resistant cultivars, and provide a better understanding of the problem within existing cultivars.

Progress is currently being made in unraveling the genetics of CI. A peach linkage map (Pop-DG) developed by crossing a processing peach cultivar 'Dr. Davis' with a fresh market cultivar 'Georgia Belle' is proving very useful for identifying genes and molecular markers associated with CI control (Peace et al. 2005a; Ogundiwin et al. 2007). One major quantitative trait locus (QTL) and a few minor QTLs have been localized for mealiness, browning and bleeding using the Pop-DG map (Peace et al. 2005a, 2006; Ogundiwin et al. 2007). A gene encoding a cell wall modifying enzyme, endopolygalacturonase (endoPG) co-localized with the major QTL affecting both mealiness and bleeding (Peace et al. 2005a, b). Another gene in the anthocyanin biosynthesis pathway, leucoanthocyanidin dioxygenase (PpLDOX), mapped to the same genomic region where the major browning QTL was identified (Ogundiwin et al. 2008).

Genomic information is accumulating for fruit crops in the Rosaceae family such as apple (Newcomb et al. 2006), apricot (Grimplet et al. 2005), peach (Horn et al. 2005; ESTree Consortium 2005), and strawberry (Folta et al. 2005), and there are current whole genome sequencing efforts for apple and peach. Extensive Rosaceae expressed sequence tag (EST) data is accessible from the Genome Database for Rosaceae (GDR: www.bioinfo.wsu.edu/gdr) for functional genomic study of fruit quality. The first publicly available peach microarray was μPeach 1.0, constructed by the Italian Consortium for Genomics in *Prunus* (ESTree Consortium 2005). This microarray contains 4,806 unigenes expressed in peach fruit, 70% of which were expressed during fruit ripening (ESTree Consortium 2005). Given the significance of CI to the stone fruit industry, a dedicated genomics toolkit is indispensable to expedite CI-resistance gene discovery. Such toolkit should be enriched with sequences of genes that are directly involved with CI development to improve the precision of candidate gene mapping. We report here the development of a specialized database (ChillPeach) to target genes expressed during CI development, and the construction of a cDNA microarray containing probes for them. Fruit tissue obtained from two full-sib progeny individuals of Pop-DG with contrasting susceptibility to CI and subjected to various cold storage durations and ripening regimes were used to develop the ChillPeach EST collection. The features of this EST collection, the results of a preliminary test of the microarray developed from it, and validation by real-time quantitative RT-PCR (qRT-PCR) of a set of cold-responsive genes identified in the microarray analysis are also reported as proof of the applicability of the ChillPeach dataset to CI functional genomics study.

Materials and methods

Plant material and CI measurements

Fruit tissue from the mesocarp of two progeny trees of the Pop-DG mapping population were used for developing EST libraries. These two genotypes represent extremes of susceptibility (one resistant, one susceptible) to mealiness and internal browning in Pop-DG. The CI phenotype of the two genotypes was confirmed over 3 years of observation prior to this study. For both, fruit tissue was obtained at several stages of cold storage and ripening as follows: M = mature at 12–14 lb fruit firmness, R = mature with 2–4 days ripening at 20°C to 2–3 lb firmness, TR = tree-ripe—picked at 2–3 lb firmness, CS-1 = M + 1 week cold storage at 5°C, CSR-1 = M + CS-1 + R, CS-2 = M + 2 weeks cold storage at 5°C, CSR-2 = M + CS-2 + R. For CI induction, fruit were forced-air cooled at 0–2°C within 6 h of harvest and then stored at 5°C with 90% relative humidity. At 1 and 2 weeks after cold storage, observations were made on the mesocarp for mealiness and browning after fruit were cut in halves through the suture plane. Mesocarp tissue was collected from three fruit for each treatment listed above, bulked and immediately frozen in liquid nitrogen before storing at −80°C until it was used for RNA isolation. For

microarray experiments, tissues from leaves and roots of the two full-sib progeny were also used.

Synthesis of cDNA and library construction

Two libraries were constructed: one with full-length non-normalized ESTs (PP1), and the other with full-length normalized ESTs (PPN). Total RNA was isolated from 4 g of pooled mesocarp tissue of three fruit in each treatment using the method described by Meisel et al. (2005). For both libraries, 10 μg RNA of M, TR and R, and 30 μg RNA of CS1, CSR-1, CS-2 and CSR-2 were pooled and used for cDNA synthesis. The PPN library was slightly different from PP1 because the former contained additional 60 μg RNA of mealy fruit from the CSR-2 treatment. Purification of poly(A)$^+$ RNA from pools was conducted with Oligotex® mRNA Purification System (Qiagen) following manufacturer instructions. The Clontech's SMART PCR cDNA Synthesis kit was used to construct the libraries, and primers SMART IV oligonucleotide and CDS III/3′ (SMART^{TM} cDNA Library Construction Kit Clontech) containing *Sfi*I restriction site were used.

Normalization of the PPN library was carried out using the properties of the enzyme DSN (Duplex-specific nuclease) (Evrogen) as described in Zhulidov et al. (2004) but with modification (M.C. Marques and M.A. Perez-Amador, personal communication) in the primers as indicated below. Briefly, in the DSN normalization method, double stranded cDNA is denatured and subsequently allowed to re-hybridize. Following re-association, the double stranded DNA fraction (formed by most abundant transcripts) is degraded by DSN and the equalized single stranded fraction is amplified by PCR. The primers used for the first and second rounds of amplification of normalized cDNA were M1-5′ (5′-AAGCAGTGGTATCAACGCAGAGT-3′) and M1-3′ (5′-ATTCTAGAGGCCGAGGCGG-3′), and M2-5′ (5′-AAGCAGTGGTATCAACGCAG-3′) and M2-3′ (5′-ATTCTAGAGGCCGAGGCG-3′), respectively.

In both libraries, cDNA was size-selected by agarose gel electrophoresis, and only fragments that were 0.5 to 4 kb long were used. cDNAs were digested with *Sfi*I and cloned into Gateway vector pENTR1A (Invitrogen) modified to contain a *Sfi*I restriction sites in the polylinker (kindly provided by M.C. Marques and M.A. Amador, unpublished).

Ligated products were used to transform One Shot MAX Efficiency DH5α-T1 Competent Cells (Invitrogen) following manufacturer instructions. The selection of transformed colonies was carried out on LB agar plates supplemented with kanamycin. Randomly-selected clones were grown overnight in standard selective bacterial growth media, and plasmids were isolated by alkaline lysis using Perfect-Prep kit (Eppendorf). Sequencing reactions were carried out on plasmids from the 5′-end of the cDNA inserts, using an ABI 3100 capillary automatic sequencer (Applied Biosystems) with fluorescent dye terminator technology.

Sequence analysis and functional annotation

ESTs processing, assembly and annotation was performed by using EST2uni (http://bioinf.comav.upv.es/est2uni), an open parallel software package for automated EST pre-processing, assembly and unigene annotation (Forment et al. 2008). EST2uni analysis pipeline featured the following standard EST analysis tools: Raw sequences and base confidence scores were obtained from raw chromatogram files using the program PHRED (Ewing and Green 1998; Ewing et al. 1998). Low-quality and cloning vector regions were removed from the sequences with LUCY (Chou and Holmes 2001), and ESTs that were left with <100 non-vector good-quality bases after trimming were discarded from further analyses. Repetitive elements and low-complexity regions were masked with RepeatMasker (http://www.repeatmasker.org) and SeqClean (http://compbio.dfci.harvard.edu/tgi/software), respectively. For repeat masking in this study, the eucotyledons-specific repeats database was used. Vector sequence contaminants were also removed with SeqClean, using NCBI's UniVec database (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html). Assembly of reads in contigs and singletons to estimate the redundancy of the ESTs, determine the consensus sequences of the redundant ones, and obtain the unigene set, was made with TGICL (Pertea et al. 2003), using the following default parameters: 30 bases minimum overlap length, 94% minimum percent identity for overlaps, and 30 bases maximum length of unmatched overhangs. Poly(A/T) tails and open reading frames (ORFs) were predicted using ESTScan (Iseli et al. 1999). ESTScan was also used to obtain reverse complimentary sequences of the unigenes when necessary.

Di-, tri-, and tetra-nucleotide simple sequence repeats (SSR) were detected with Sputnik (http://espressosoftware.com/pages/sputnik.jsp). Putative single nucleotide polymorphisms (SNPs) were found by EST2uni using a locally developed algorithm. As ESTs have frequent sequencing errors, only positions with a quality score above 39 were considered, and sequence discrepancies between ESTs in the same contig were marked as putative SNPs only if the polymorphism was confirmed by more than one EST in the contig. Because cDNA libraries were constructed using oligo-dT primer for the reverse transcriptase reaction and ESTs were obtained by 5′-end sequencing, unigenes were aligned with the *Arabidopsis* complete proteins database to determine if they included the translation initiation codon and predict the presence of full-length cDNA clones for each unigene.

For the functional annotation of ChillPeach unigenes, BLASTx was carried out in EST2uni against: (1) the UniRef90 non-redundant protein clusters database

(http://www.ebi.ac.uk/uniref; Suzek et al. 2007; downloaded October 2006: UniProtKB release 8.9 of October 2006), (2) the predicted full set of *Arabidopsis thaliana* proteins provided by TAIR (http://www.arabidopsis.org; downloaded September 2006: TAIR6 of November 2005), and (3) the predicted full set of *Oryza sativa* proteins provided by TIGR (http://www.tigr.org; downloaded October 2006: TIGR rice pseudomolecule release 4.0 of January 2006). BLASTn searches were also made in EST2uni against the following sets of public peach DNA sequences: (1) the *Prunus persica* sequences at GenBank (downloaded from NCBI October 2006), (2) the peach EST collection at ESTreeDB (http://www.itb.cnr.it/estree; downloaded December 2006: Release 5 of September 2006), (3) the *Prunus* EST collection at GDR (http://www.bioinfo.wsu.edu/gdr/genus/prunus; downloaded December 2006: EST *Prunus* Unigene version 3 of June 2006), (4) the TIGR transcript assembly database for all public plant ESTs, or PlantTA (Childs et al. 2007; http://plantta.tigr.org ; downloaded December 2006), and (5) the TIGR transcript assembly database for all public *Prunus persica* ESTs (downloaded December 2006: Release 2 from June 2006). All these analyses were performed using BLAST default parameters and an arbitrary non-stringent threshold of 10^{-5} E-value.

Unigenes were annotated with the description of the most similar UniRef90 cluster of proteins. When no significantly similar UniRef90 cluster was found, unigenes were annotated with the first informative (i.e., not containing words such as "unknown", "anonymous", or "hypothetical") description of the BLAST hits, if any, against the rest of databases analyzed, in the following order: TIGR Plant transcripts, TIGR Peach transcripts, *Arabidopsis* proteins, *Oryza sativa* proteins, GenBank *Prunus persica* DNA sequences, GDR *Prunus* ESTs, and ESTreeDB. Unigenes were annotated as highly similar to the first BLAST hit when the E-value was lower than 10^{-15}. BLASTX hits with an E-value higher than 10^{-10} were not considered for annotation. Gene Ontology (GO) annotation of the *Arabidopsis* most similar protein was used for GO annotation of the peach unigenes.

A bi-directional BLAST comparison was also performed with *Arabidopsis* and rice sequence databases to obtain a set of putative orthologs. In these analyses, two sequences were considered orthologs when each one was the first hit in a BLAST search with the other.

Microarray slide printing

A microarray was constructed to represent all the unigenes in the ChillPeach database. To reduce spot cross-hybridization within gene families in the microarray experiments (Everstz et al. 2001), a set of unigene clusters or "superunigenes", grouping different unigenes with extensive sequence overlap (more than 300 bp with more than 90% identity, and covering more than 50% of the length of one of the unigenes), was obtained from the cDNA clone collection using BLAST. One cDNA clone that was the best representative for each superunigene was selected to be printed on the glass slides. The two criteria used in choosing representative cDNA clone were: (1) EST sequence length greater than 300 bp and covering at least 90% of the unigene consensus sequence, and (2) GC content not greater than 80% in a 70 base-long sliding window. Where more than one clone in a superunigene satisfied all the criteria, the one with longest 5′ sequence was selected to ensure that full-length clones were used for slide printing. Where no clone in a superunigene satisfied all the criteria, the criteria were progressively relaxed until a representative clone was selected.

DNA probes for microarrays were obtained by PCR from each of the selected cDNA clones in the cDNA plasmid collection. PCR reactions were carried out in a final volume of 100 µl using 4 ng plasmid DNA, 400 nM of each primer, and 200 µM dNTPs. Primers used were pENTR-forward (5′-GGCTTTAAAGGAACCAATTCAG-3′) and pENTR-reverse (5′-GCAATGCTTTCTTATAATGCCAAC-3′). Amplifications were performed in a 96-well format and PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30°s and 72°C for 2 min 30 s, and a final 7 min extension at 72°C. The reaction products were analyzed on agarose gels and PCR products were purified using ExcelaPure 96-Well UF Plate (EdgeBioSystems). Finally, only representative clones with a single product in the PCR reaction were printed in the microarray.

Before printing, purified PCR products corresponding to 4,261 selected cDNA clones were transferred to 384-well plates at a final concentration of 100–200 ng/µl in 5 µl PRONTO™ (Universal spotting buffer, Corning) and reorganized using a robotic liquid dispenser (MultiPROBRE® Iiex Robotic Liquid Handling System, Packard BioScience Company). Lucidea Universal ScoreCard (Amersham) spike controls were diluted in 100 ng/µl PRONTO™ and spotted on the array for quality evaluation. Each of the calibration and negative controls from the Lucidea kit were spotted several times across the whole area of the array. Every selected ChillPeach clone was spotted once. All samples were spotted on Corning UltraGAPS glass slides, using a MicroGrid II spotting device from Biorobotics. After printing, slides were cross-linked at 150 mJ and stored.

Microarray hybridization, scanning and data analyses

For microarray experiments, equal amounts of RNA samples from CS-1, CSR-1, CS-2, and CSR-2 were pooled to

form the cold-treated sample (CT), and equal amounts of RNA samples from M, R, and TR were pooled to form the non-cold treated sample (NC). RNA was also isolated from leaves and roots of the two full-sib progeny, pooled and treated as vegetative sample (Vg). A pool of equal amounts of RNA from all mesocarp tissue and vegetative samples was made and treated as a pool reference (PR). To obtain differential gene expression values, three technical replicates from each sample pool—CT, NC, and Vg—were hybridized and CT versus NC and Vg versus NC obtained after each of the log2 ratios of fluorescence values were normalized against those of the PR.

RNA samples for microarray hybridization were amplified using the method of Van Gelder et al. (1990). Briefly, 1 μg of total RNA of each sample (CT, NC, and Vg) and PR was amplified and aminoallyl-labelled using MessageAmp® II aRNA kit (Ambion, http://www.ambion.com) and 5-(3-aminoallyl)-2′-deoxyuridine-5′-triphosphate (aa-dUTP, Ambion), following manufacturer's instructions. Approximately 40–50 μg of amplified RNA (aRNA) was obtained. For each sample and the PR, 7.5 μg of aminoallyl-labelled aRNA was re-suspended in 0.1 M Na_2CO_3 (pH 9.0) and labelled with either Cy3 or Cy5 Mono NHS Ester (CyTM Dye Postlabelling Reactive Dye Pack, Amersham). The samples were purified with MegaclearTM (Ambion) following manufacturer instructions. Incorporation of Cy3 and Cy5 was measured using 1 μl of the probe in a Nanodrop spectrophotometer (Nanodrop Technologies Inc.; http://www.nanodrop.com/).

Microarray hybridization of samples and PR to the ChillPeach microarray slides was performed manually using Telechem Hybridization Chambers (Corning), following manufacturer instructions. Briefly, slides were re-hydrated and UV-cross-linked, and then pre-hybridized 45 min at 42°C in 5× SSC, 0.1%SDS, 0.1 mg/ml BSA, 10 mM EDTA pH 8, washed twice for 30 s in milliQ water (Millipore) and in isopropanol for 30 s. Arrays were drained by centrifugation at 528*g* for 2 min. For each hybridization, 100 pmol of each Cy5-labelled sample was mixed with 100 pmol of Cy3-labelled PR. Fluorescence values were normalized against those of the reference PR by dividing the log2 values obtained at 635/532 nm. Three technical replicates were made, one of them dye-swapped. Mixture was dried in a speed-vac, and re-suspended in 34 μl water, 4 μl EDTA 0.5 M pH 8 and 2 μl polyA (10 μg/μl). This mix was heated for 3 min at 95°C and 40 μl of 2× hybridization buffer (50 μl formamide 50 μl, 25 μl 20× SSC, 2 μl 10%SDS) was added. Hybridization was done overnight at 42°C.

After hybridization, slides were washed in 2× SSC, 0.1% SDS for 5 min at 42°C, 0.1× SSC, 0.1% SDS for 10 min at room temperature, 0.1× SSC for 5 min at room temperature four times, and 0.01× SSC for 5 min at room temperature four times. Arrays were drained by centrifugation at 528*g* for 2 min. Slides were scanned with a GenePix 4000B scanner (Axon Instruments) at 10 μm resolution, 100% laser power, and different PMT values to adjust the ratio to 1.0. Microarray images were analyzed and globally normalized using GenePix 4.1 (Axon Instruments) software. Only spots with background-subtracted intensity greater than two-fold the mean background intensity in at least one channel were selected for analysis. Data files were imported into Acuity 4.0 (Axon Instruments) and normalized by using the Lowess normalization method. Finally, only spots with valid values in at least five of the six analyzed hybridizations were considered for further analyses. Mean and standard deviation of values were calculated from each sample as log2 values, and later normalized to the median of the expression in NC sample.

To detect differentially expressed genes in cold-treated fruits or in vegetative tissues compared to non-cold treated fruits, data were analyzed with the SAM package (Significance Analysis of Microarray, Tusher et al. 2001). Statistical significance was assessed using two class (unpaired) SAM analysis, with a false discovery rate of 5%, q-value $\leq$0.05, and two-fold change cut-off. ChillPeach genes up-regulated in cold-treated tissues were compared with ColdArrayDB (http://cold.stanford.edu/cgi-bin/data.cgi)—a database that contains global expression profiles of *Arabidopsis* genes in response to cold. The search conditions used were: chip type—23 k, experiment—cold treatment, growth medium—plate and soil, expression level >50, and fold change >2.

Real-time qRT-PCR analysis

Thirteen genes were selected from the microarray results for qRT-PCR analysis. Total RNA was isolated and purified from treatments M, CS-1, and CSR-1 of each of the two full sib progeny as described above in two biological replicates.

One microgram of total RNA was used to synthesize first-strand cDNA using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). cDNA samples were diluted in a total volume of 100 ml. Two microliter of cDNA was used for qRT-PCR using SYBR Green PCR master mix (Applied Biosystems) following manufacturer's recommendations and an ABI Prism 7000 sequence detection system (Applied Biosystems). Each biological replicate was assayed in triplicate. Gene-specific oligonucleotide primers were designed using Primer Express® version 2.0 software (Applied Biosystems). Primer information is shown in Supplemental Table 1. Expression levels for target genes were calculated relative to *Initiation Factor elF-4-Gamma* using the DDthreshold cycle (Ct) method (Applied Biosystems). Pairwise comparisons were made on the $2^{-\Delta\Delta C_T}$ values between the two full sib progeny and among the three treatments (M, CS-1 and CSR-1) using Student's t-test.

Results

ChillPeach EST assembly

Two dedicated cDNA libraries PP1 (non-normalized) and PPN (normalized) were generated from mesocarp of two full-sib peach progeny that contrast for susceptibility to mealiness and browning (major symptoms of CI). All clones are in the Gateway® vector. Sequence information of 8,432 clones randomly selected from both (768 ESTs from PP1 and 7,664 from PPN) is available in the ChillPeach database (Table 1). The nucleotide sequence data have also been submitted to GenBank and assigned the accession numbers FC860626–FC868487. After vector and low quality sequence trimming, 7,868 high quality ESTs were obtained with average sequence length of 640 bases, 7,058 (90%) of which were longer than 500 bases. Following sequence assembly of this EST dataset based on 30 bases minimum overlap length, 94% minimum percent identity for overlaps, and 30 bases maximum length of into 1,480 contigs (4,468 unigenes total), indicating a redundancy of 43% for both unmatched overhangs, 2,988 singletons were identified and the remaining ESTs clustered libraries. The number of ESTs per contig ranged from 2 to 34, while most contigs (83%) contained four or less ESTs.

Using EST2uni, a structured MySQL database was created and populated with information about all the data including cDNA libraries, isolated clones, raw sequences, and results of all bioinformatics analyses performed. The website to access this ChillPeach database is http://bioinfo.ibmcp.upv.es/genomics/ChillPeachDB. The website provides a powerful data mining tool with an advanced querying interface and high integration among all kinds of data.

ChillPeach novelty

When compared with other public databases the ChillPeach EST database showed some uniqueness both at nucleotide (sequence novelty obtained from BLASTn searches) and protein (functional novelty obtained from BLASTx searches) levels (Table 2). BLASTn and BLASTx matches of the ChillPeach unigenes to the NCBI (National Center for Biotechnology Information) *Prunus* EST collection showed that 2,331 (52%) ChillPeach unigenes did not share significant nucleotide sequence similarity, and 28% were functionally unique. Of the remaining 2,137 with similar sequences in the NCBI *Prunus* EST collection, at least 31% had either full length or were longer than their NCBI counterparts at the 5′ UTR. Similar searches of *Prunus* ESTs in the TIGR PlantTA (http://www.plantta.tigr.org), ESTree (http://www.itb.cnr.it/estree), and GDR (http://www.bioinfo.wsu.edu/gdr) databases revealed that 52–72% of ChillPeach unigene sequences were new to these databases. Functional uniqueness to these databases ranged between 26 and 55%. Out of the total 4,468 ChillPeach unigenes, 563 sequences (13%) have no *Arabidopsis* ortholog and 179 (4%) have no ortholog in any plant database based on results of BLASTx search of all plant transcripts in the PlantTA of TIGR. Analysis of those

Table 2 Novelty of the ChillPeach database compared to available relevant databases

Database[a]	Novelty[b]	
	Functional	Sequence[c]
NCBI	1,270	2,331
TAIR	563	ne
Rice	737	ne
PlantTA_all-plants	179	ne
PlantTA_peach	1,288	2,463
ESTree	2,473	3,238
GDR	1,163	2,357
All peach databases	1,103 (25%)	2,148 (48%)

[a] All databases were downloaded for local analysis in September–December, 2006. NCBI = NCBI public peach (*Prunus persica*) mRNA sequences including ESTs—71,093 sequences; TAIR = complete set of *Arabidopsis* proteins—30,690 sequences; Rice = complete set of *Oryza sativa* proteins—62,827 sequences; PlantTA_all-plants = public plant transcripts assembled at TIGR—3,572,230 sequences; PlantTA_peach = *Prunus persica* transcripts assembled from TIGR—28,441 sequences; ESTree = collection of *Prunus persica* EST sequences—20,924 sequences; GDR = 83,751 *Prunus* (not only peach) sequences from Genome Database for Rosaceae

[b] Novelty = number of unigenes in the ChillPeach database that do not produce significant match at the protein (functional novelty) and nucleotide(sequence novelty) level with sequences in the queried database; E-value $>1e^{-10}$

[c] ne = not evaluated

Table 1 Features of the ChillPeach cDNA libraries

Library	Clones	High-quality ESTs	Mean EST length (bp)	Singletons	Contigs	Unigenes	Redundancy (%)[a]	Library-specific unigenes	Novelty (%)[b]
PP1	768	720	644.7	212	306	518	28	229	44
PPN	7,664	7,142	639.4	2,776	1,463	4,239	41	3,950	93
Total	8,432	7,868		2,988	1,480	4,468	40		

[a] Redundancy = (1 − (Unigenes/ESTs)) × 100

[b] Novelty = (Library-specific unigenes/Unigenes) × 100

sequences corresponding to *Arabidopsis* orthologs indicated that more than 45% of them were likely to be full length.

SSRs and SNPs

Sequence information of the ChillPeach unigene set revealed the existence of 184 perfect di-, tri-, and tetranucleotide microsatellites or SSRs representing 4% of all ChillPeach unigenes (Table 3). Out of these 184 EST SSRs, 77 (42%) are novel to *Prunus*. The number of observed dinucleotide SSRs was more than twice that of trinucleotides and ten times more than tetranucleotides. All but three ChillPeach dinucleotides were AG repeats, representing 97% of the dinucleotide repeats and 58% of all SSRs. Only two AC repeats and one AT repeat were observed, and no CG repeat was present in the dataset. Among the trinucleotide repeats, AAG repeats were the most abundant (34%) followed by ATC repeats (19%). Analysis of the positions of the SSRs relative to putative initiation (Met) and stop codons in the ChillPeach dataset showed that 109 (59%) fell in the putative 5'UTR region, and 14 (7.6%) were found in the 3'UTR region, while 56 (30%) were located within the coding sequence and the positions of the remaining five repeats could not be determined.

Sequence analysis of the ESTs within each of the 396 contigs containing at least four ESTs showed occurrence of 70 (18%) single nucleotide polymorphisms (SNPs). These putative SNPs were confirmed by at least two EST pairs within the respective contigs. When more than one SNP was found in a unigene, their distribution in the ESTs was found consistent with the existence of different alleles.

Table 3 Summary of microsatellites in ChillPeach database

Repeat composition	No. of unigenes	% of ChillPeach SSRs
Dinucleotide repeats		
AG/GA/CT/TC	106	57.6
AC/CA/GT/TG	2	1.1
AT/TA	1	0.5
Trinucleotide repeats		
AAC/ACA/CAA/GTT/TGT/TTG	6	3.3
AAG/AGA/GAA/CTT/TCT/TTC	22	12.0
ACC/CAC/CCA/GGT/GTG/TGG	4	2.2
ACG/CGA/GAC/CGT/GTC/TCG	2	1.1
ACT/CTA/TAC/AGT/TAG/GTA	1	0.5
AGC/CAG/GCA/TGC/CTG/GCT	8	4.4
AGG/GGA/GAG/TCC/CTC/CCT	9	4.9
ATC/CAT/TCA/GAT/ATG/TGA	12	6.5
Tetranucleotide repeats		
AAAG	3	1.6
ACAG	3	1.6
AAAC	2	1.1
AAGG	1	0.5
AATG	1	0.5
ACTC	1	0.5
Total	184	

Functional annotation

Of the 4,468 ChillPeach unigenes, only 163 could not be annotated with an informative description other than "expressed protein", "hypothetical protein", etc., based on their similarity to sequences in external databases (see "Material and methods"). However, a total of 2,851 ChillPeach unigenes (64%) that shared significant similarity with *Arabidopsis* proteins fell into various Gene Ontology (GO) functional classes other than 'unknown', as determined by the GO term assigned to the most similar *Arabidopsis* protein. Another 1,054 unigenes were similar to *Arabidopsis* proteins annotated as "biological process unknown", "molecular function unknown" and/or "cellular component unknown". Only 13% of the unigenes (563 sequences) did not have a match in *Arabidopsis* and could not be annotated with GO functional terms. Of the annotated unigenes, 2,165 (76%) were annotated in the Molecular Function ontology, 1,699 (60%) in the Biological Process ontology, and 1,926 (68%) in the Cellular Component ontology (Fig. 1).

Of the 2,148 ChillPeach unigenes with *Prunus* sequence novelty, 1,855 were assigned to the Molecular Function ontology, 844 to the Biological Processes ontology, and 1,386 to the Cellular Component ontology. Of the 1,103 ChillPeach unigenes with *Prunus* functional novelty, 868 were annotated in the Molecular Function ontology, 367 in the Biological Process ontology, and 668 in the Cellular Component ontology. Unigenes containing SSRs were also classified, and of the 184 total, 131 fell into the Molecular Function ontology, 68 in the Biological Process ontology, and 109 in the Cellular Component ontology. Novel unigenes and those with SSRs shared similar distributions within the ontology classes with those conducted for the entire ChillPeach unigene set.

Microarray analysis

We demonstrated the usefulness of ChillPeach unigenes in microarray studies by conducting a targeted microarray study comparing cold-treated peach fruit mesocarp tissue to non-cold treated, and by comparing vegetative tissue to mesocarp tissue.

Cold-responsive ChillPeach ESTs

In our study of differential gene expressions in cold-treated peach fruit, 399 genes were identified as differentially

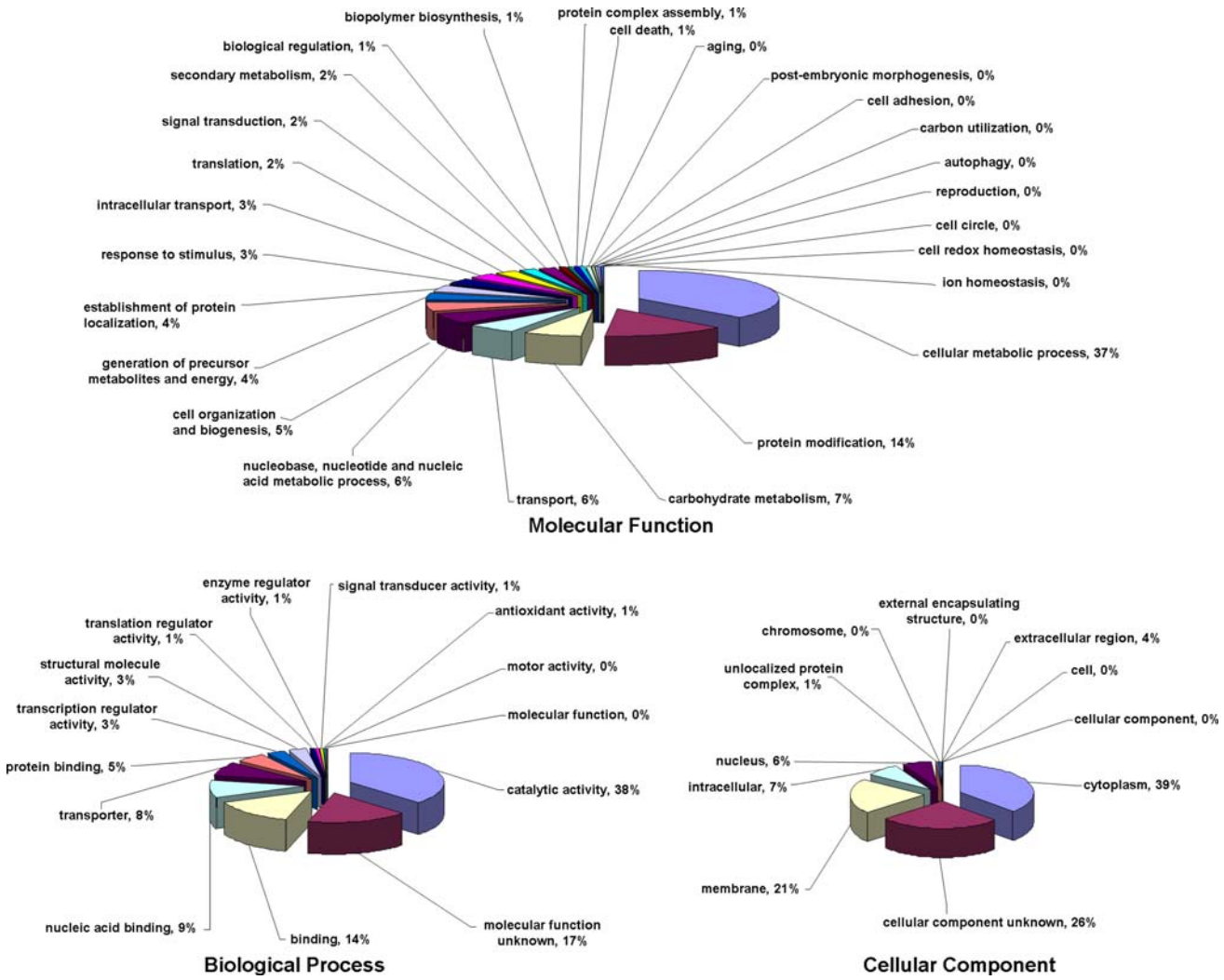

Fig. 1 Functional distribution of ChillPeach unigenes based on GO functional categorization

expressed (287 up-regulated and 112 down-regulated) in cold-treated mesocarp tissue sample using two criteria: a False Discovery Rate (FDR) and q-values <0.05. Of the 287 up-regulated genes, 48 (16.7%) were novel *Prunus* genes, 40 (14%) had no *Arabidopsis* orthologs, and 14 were novel to both *Prunus* and *Arabidopsis*. In contrast, only 8 of the 112 down-regulated genes (7%) were novel in *Prunus* and 7 genes (6%) had no *Arabidopsis* orthologs.

Of the 287 up-regulated cold-responsive genes, there were 74 with ≥2.0-fold increase, and 27 of these had ≥2.8-fold increase (Table 4). The gene α-L-arabinofuranosidase topped the list of genes with significant expression in cold-treated mesocarp tissue samples with 10.3-fold increase, closely followed by a dehydrin 2 gene (8.5-fold increase), a chitinase gene (6.5-fold increase), a little protein 1 gene (5.9-fold increase) and a protein kinase gene (4-fold increase). Of the 74 genes with ≥2.0-fold increase 28 were novel to *Prunus*. For genes down-regulated in cold-treated mesocarp tissue, there were nine genes with <0.5-fold expression, two of which were novel to *Prunus* (Table 4). Five of these were heat shock proteins. Others were CBF1, pectinesterase PPE8B precursor, granule-bound starch synthase 1 (chloroplast precursor), and T17B22.3 protein genes. Of the 74 genes with >2.0 fold change, 59 had *Arabidopsis* orthologs. When these 59 ChillPeach genes were compared to the expression profile of the ColdArrayDB, 23 of these were also found to be differentially expressed in cold-treated *Arabidopsis* vegetative tissue (Table 5); 11 genes were up-regulated and 12 were down-regulated. Of the remaining 36 genes, 30 were not responsive to cold in *Arabidopsis* vegetative tissue (fold-change <2), and six were absent in the expression profile of the ColdArrayDB (Table 6).

Fruit versus vegetative tissue microarray

A total of 950 genes (434 up-regulated and 516 down-regulated) were identified as significantly differentially expressed (FDR and q-values <0.05) in vegetative tissue

Table 4 List of ChillPeach genes with ≥2.0 fold increase or decrease in expression in cold-treated compared non-treated mesocarp tissue samples

Unigene functional annotation	ChillPeach ID	Fold change[a]	Novelty[b]
Up-regulated			
Alpha-L-arabinofuranosidase/beta-D-xylosidase	CL801Contig1	10.25	Yes
Dehydrin 2	CL85Contig1	8.52	No
Chitinase Ib	CL698Contig1	6.43	No
Little protein 1	PPN043F06-T7_c_s	5.86	No
Protein kinase; NAF	PPN080C05-T7_c_s	4.50	Yes
Putative RING zinc finger ankyrin protein	CL1011Contig1	4.31	No
At2g20670/F23N11.1	CL481Contig1	4.28	No
At4g33690	PP1004F11-T7_c_s	4.18	No
No annotation available	PPN048E11-T7_c_s	4.17	No
1.00E-172	PPN049D06-T7_c_s	3.81	No
Beta-galactosidase precursor (EC 3.2.1.23)	CL1325Contig1	3.72	No
Sulfate transporter 3.1	PPN065F08-T7_c_s	3.46	No
41 kD chloroplast nucleoid DNA binding protein	PPN018D10-T7_c_s	3.43	No
0.00E + 00	PPN046H04-T7_c_s	3.34	No
AJ824832 S3 *Prunus persica* cDNA clone S38F24, mRNA sequence	PP1000E03-T7_c_s	3.27	No
F19K23.17 protein	PP1005B10-T7_c_s	3.26	No
Arabidopsis thaliana genomic DNA, chromosome 3, P1 clone: MJK13	PPN069D06-T7_c_s	3.23	No
Anthocyanidin 3-O-glucosyltransferase	PPN007E12-T7_c_s	3.21	No
No annotation available	PPN048D07-T7_c_s	3.16	No
Glutamate dehydrogenase 2	PP1004F04-T7_c_s	3.09	No
D-3-phosphoglycerate dehydrogenase, chloroplast precursor	PP1003B08-T7_c_s	3.06	No
Zeaxanthin epoxidase, chloroplast precursor	CL377Contig1	3.06	No
Polygalacturonase-inhibiting protein	CL146Contig1	3.02	No
Thaumatin-like protein 1 precursor	PPN003H07-T7_c_s	2.97	No
Alanine–glyoxylate aminotransferase 2 homolog 2, mitochondrial precursor	CL1351Contig1	2.94	Yes
Hly-III related proteins	PPN065E08-T7_c_s	2.93	Yes
F9L1.4 protein	CL351Contig2	2.85	Yes
Dbj|BAA78736.1	CL569Contig1	2.77	No
Cinnamoyl-CoA reductase-like protein	CL362Contig1	2.68	No
Putative aspartate aminotransferase	CL149Contig2	2.66	No
Putative inosine-uridine nucleoside N-ribohydrolase	CL1188Contig1	2.57	Yes
MADS box transcription factor	PPN004D05-T7_c_s	2.56	Yes

Table 4 continued

Unigene functional annotation	ChillPeach ID	Fold change[a]	Novelty[b]
Prunus dulcis transcript; similar to ABA-inducible protein [*Fagus sylvatica*]	CL964Contig1	2.56	No
No annotation available	PPN049B03-T7_c_s	2.55	No
F19P19.4 protein	PP1004A08-T7_c_s	2.54	No
Alcohol acyl-transferase	PPN009E09-T7_c_s	2.54	Yes
Tonoplast intrinsic protein	PP1003C07-T7_c_s	2.54	No
Arabidopsis thaliana genomic DNA, chromosome 3, P1 clone: MLN21	PPN069H05-T7_c_s	2.53	Yes
Indole-3-acetic acid-induced protein ARG2	CL704Contig1	2.48	No
Gb|AAD31066.1	PPN017G08-T7_c_s	2.40	No
C-terminal domain phosphatase-like 2	PPN070H08-T7_c_s	2.40	Yes
Putative seed imbibition protein	CL1468Contig1	2.34	No
F21O3.6 protein	PPN071E03-T7_c_s	2.34	Yes
AJ826928 S3 *Prunus persica* cDNA clone S39D20, mRNA sequence	CL201Contig1	2.33	No
Emb|CAB89401.1	PPN024C05-T7_c_s	2.32	Yes
Gossypium raimondii transcript; similar to Protein phosphatase 2C [*Medicago truncatula*]	PPN069G12-T7_c_s	2.30	Yes
RING-H2 finger protein RHB1a	PPN077H09-T7_c_s	2.30	Yes
YUP8H12R.13 protein	PPN026F04-T7_c_s	2.28	Yes
Asparagine synthetase	CL283Contig1	2.28	No
Sesquiterpene cyclase	PPN001B06-T7_c_s	2.28	Yes
Amino acid transporter	CL1123Contig1	2.24	No
No annotation available	PPN041G05-T7_c_s	2.22	Yes
F20N2.11	CL638Contig1	2.20	Yes
OSJNBb0039L24.13 protein	CL1095Contig1	2.19	No
Glutaredoxin, eukaryotic and viruses	PPN048A04-T7_c_s	2.19	No
Ribonuclease 2 precursor	PPN014H05-T7_c_s	2.17	No
MYB transcription factor MYB93	PPN003G06-T7_c_s	2.15	No
Sucrose responsive element binding protein	CL757Contig1	2.13	No
Putative aspartate aminotransferase	CL149Contig1	2.13	No
T20H2.5 protein	CL1185Contig1	2.12	Yes
Protein phosphatase 2C-like	PPN054D04-T7_c_s	2.11	Yes
Protease-associated PA; Proteinase inhibitor I9, subtilisin propeptide	PPN078F09-T7_c_s	2.11	No
No annotation available	PPN002C06-T7_c_s	2.10	Yes

Table 4 continued

Unigene functional annotation	ChillPeach ID	Fold change[a]	Novelty[b]
Prunus persica transcript; similar to BZIP protein BZ2 [*Arabidopsis thaliana* (Mouse-ear cress)]	PPN019A01-T7_c_s	2.07	Yes
YUP8H12.27 protein	PPN017G10-T7_c_s	2.07	Yes
Putative ripening-related protein	CL86Contig2	2.07	No
Serine protease-like protein	PPN007C09-T7_c_s	2.07	No
AT3g24470/MXP5_4	PPN030H04-T7_c_s	2.06	No
Putative tyrosine aminotransferase	PPN018G03-T7_c_s	2.05	Yes
DbjlBAA18864.1	CL852Contig1	2.04	Yes
UPI00004C154C; phytanoyl-CoA dioxygenase domain containing 1	CL483Contig1	2.03	Yes
Protein kinase	CL550Contig1	2.01	Yes
No annotation available	PPN048B04-T7_c_s	2.01	Yes
Chalcone synthase 2	CL792Contig1	2.01	No
Down-regulated			
Granule-bound starch synthase 1, chloroplast precursor	PPN017F12-T7_c_s	0.49	No
T17B22.3 protein	PPN039G04-T7_c_s	0.49	No
Heat shock cognate 70 kDa protein 2	CL823Contig1	0.44	No
Pectinesterase PPE8B precursor	PPN002G04-T7_c_s	0.44	No
Heat shock 70 kDa protein	PPN068G11-T7_c_s	0.42	Yes
18.1 kDa class I heat shock protein	PPN027F03-T7_c_s	0.41	No
CBF1	CL242Contig1	0.40	No
Low molecular weight heat-shock protein	PPN038E08-T7_c_s	0.38	No
Heat shock protein 83	CL1174Contig1	0.37	Yes

[a] Significant fold increase or decrease of expression in cold-treated mesocarp tissue compared to non-treated samples, $q \leq 0.001$

[b] Unigenes that are novel (sequence-based) to *Prunus*

sample compared with non-cold treated mesocarp tissue. Of the 434 genes that were preferentially expressed in vegetative tissue, 69 (14.9%) were novel *Prunus* genes, 66 (15.2%) had no *Arabidopsis* orthologs, and 21 (5%) were novel to both. Of the 515 down-regulated genes, 66 (12.8%) were novel *Prunus* genes, 52 (10%) had no *Arabidopsis* orthologs, and 11 were new to both. Among those with higher values in vegetative tissue, 16 had $\geq 2^{10}$-fold increase in expression compared to mesocarp tissue (Table 7). Sixty-three genes had 2^5- to 2^{10}-fold, and 260 genes had 4- to 32-fold increase in expression, respectively. Most of these genes were easily identifiable markers of photosynthetic tissues.

A total of 515 genes were significantly over-expressed in mesocarp tissue as compared to the vegetative tissue. Of these, expression of 45 genes was 2^5 to 2^{10} fold higher in mesocarp than in vegetative tissues and therefore could be good candidates for mesocarp-specific genes. Eleven of the genes with $>2^7$ fold increase in mesocarp are shown in Table 7. Of the differentially expressed genes, 322 showed expression ratios of 2^2 to 2^5 fold higher in mesocarp than in vegetative tissues.

qRT-PCR analysis

The results of single gene qRT-PCR analysis of all selected 13 genes corroborated the results of microarray analysis (Fig. 2, Table 8). The 13 genes comprised of six genes that were cold-induced in both peach and *Arabidopsis* (ColdArrayDB) (Table 5), five genes that were up-regulated in peach but not reported in *Arabidopsis* (Table 6), and two genes that were up-regulated in peach but down regulated in *Arabidopsis* (Table 5). Both microarray and qRT-PCR analyses showed that all the 13 genes were up-regulated in cold treated mesocarp tissues of both progeny (Fig. 2). The most notable difference in the qRT-PCR analysis was observed for *Thau* (Thaumatin-like precursor) and *Chit* (Chitinase) genes where their relative expression in some of the treatments was about 10 times the relative expression of other genes (Fig. 2). Expression of *Thau* and *Chit* increased by 1,107-fold and 185-fold, respectively, in CS-1 compared to M of the resistant progeny; and by 167-fold and 60-fold, respectively, in the susceptible progeny (Table 8). In addition, *ST1* (sulfate transporter) also had 167-fold change in the resistant progeny. Cold-induced genes with greater fold change in the resistant progeny compared to the susceptible progeny were *Thau*, *Chit*, *ST1*, *Ara* (α-L-arabinofuranosidase), *B-Gal* (beta-galactosidase precursor), *AGAT* (Alanine-glyoxylate aminotransferase 2), *ARG2* (Indole-3-acetic acid-induced protein ARG2), *CCR* (cinnamoyl-CoA reductase) *PGDH* (D-3-phosphoglycerate dehydrogenase chloroplast precursor) and *ZEP* (zeaxanthin epoxidase). Two genes had higher expression in the susceptible progeny

Table 5 Cold-responsive genes in common between peach mesocarp tissue (ChillPeachDB) and *Arabidopsis* vegetative tissue (ColdArrayDB)

Unigene functional annotation	Peach (ChillPeachDB)[a]		Arabidopsis (ColdArrayDB)		Sequence similarity (E-value)
	ID	Fold change	ID	Fold change[b]	
Chitinase Ib	CL698Contig1	6.43	At3g12500	2.43	1.0^{e-80}
Sulfate transporter 3.1	PPN065F08-T7_c_s	3.46	At3g51895	2.07	1.00^{e-100}
D-3-phosphoglycerate dehydrogenase, chloroplast precursor	PP1003B08-T7_c_s	3.06	At1g17745	2.22	7.0^{e-75}
Polygalacturonase-inhibiting protein	CL146Contig1	3.02	At5g06860	8.74	1.0^{e-122}
Thaumatin-like protein 1 precursor	PPN003H07-T7_c_s	2.97	At1g20030	5.84	3.0^{e-54}
Indole-3-acetic acid-induced protein ARG2	CL704Contig1	2.48	At4g02380	4.22	2.0^{e-14}
F21O3.6 protein	PPN071E03-T7_c_s	2.34	At2g39650	2.15	7.00^{e-68}
YUP8H12R.13 protein	PPN026F04-T7_c_s	2.28	At1g79270	3.63	8.0^{e-94}
F20N2.11	CL638Contig1	2.20	At1g55690	2.03	1.0^{e-109}
Putative tyrosine aminotransferase	PPN018G03-T7_c_s	2.05	At5g53970	4.45	1.0^{e-65}
Chalcone synthase 2	CL792Contig1	2.01	At5g13930	3.91	9.0^{e-46}
Alpha-L-arabinofuranosidase/beta-D-xylosidase	CL801Contig1	10.25	At5g49360	−18.15	1.00^{e-163}
Protein kinase; NAF	PPN080C05-T7_c_s	4.50	At3g17510	−2.17	1.00^{e-104}
Beta-galactosidase precursor (EC 3.2.1.23)	CL1325Contig1	3.72	At3g13750	−7.49	1.0^{e-102}
F19K23.17 protein	PP1005B10-T7_c_s	3.26	At1g62250	−2.47	1.0^{e-31}
Arabidopsis thaliana genomic DNA, chromosome 3, P1 clone: MJK13	PPN069D06-T7_c_s	3.23	At4g27450	−18.83	8.00^{e-91}
Hly-III related proteins	PPN065E08-T7_c_s	2.93	At5g20270	−2.76	4.00^{e-68}
Alcohol acyl-transferase	PPN009E09-T7_c_s	2.54	At5g48930	−2.07	5.0^{e-73}
Tonoplast intrinsic protein	PP1003C07-T7_c_s	2.54	At2g36830	−3.41	7.0^{e-71}
EmbICAB89401.1	PPN024C05-T7_c_s	2.32	At5g65380	−4.43	4.00^{e-54}
Asparagine synthetase	CL283Contig1	2.28	At3g47340	−2.53	0.00
OSJNBb0039L24.13 protein	CL1095Contig1	2.19	At3g61260	−2.17	9.0^{e-36}
Putative ripening-related protein	CL86Contig2	2.07	At5g02230	−5.84	5.00^{e-68}

[a] Only ChillPeach genes with ≥2.0 fold change were compared with *Arabidopsis* genes in the ColdArrayDB

[b] Maximum fold change in both biological samples at any time point

than the resistant progeny: *Ank* (ring zink finger ankyrin) and *Anth* (anthocyanidin-3-O-glucosyltransferase). The fold change of *PGIP* (polygalacturonase inhibiting protein) was similar for both CI-resistant and susceptible progeny.

The qRT-PCR analysis also showed that the expression of all genes dropped drastically during ripening (CSR-1), although the expression of some genes such as *Chit* and *Thau* (in both progeny), *Ara* and *B-Gal* (in susceptible progeny only), and *PGIP*, *ST1* and *ZEP* (in resistant progeny only) was still significantly higher in CSR-1 than in normal fruit (M) (Table 8).

Discussion

ChillPeach database (http://bioinfo.ibmcp.upv.es/genomics/ChillPeachDB) has been created containing a set of functional genomic tools to facilitate speedy detection of genetic

Table 6 Cold-induced genes in peach mesocarp tissue (ChillPeachDB) that are not cold-regulated in *Arabidopsis* or are absent in ColdArrayDB

Unigene functional annotation	Peach (ChillPeachDB)[a]		Arabidopsis (ColdArrayDB)		Sequence similarity (E-value)
	ID	Fold change	ID	Fold change[b]	
Putative RING zinc finger ankyrin protein	CL1011Contig1	4.31	At2g28840	NS	1.00^{e-102}
At2g20670/F23N11.1	CL481Contig1	4.28	At4g32480	NP	8.00^{e-46}
At4g33690	PP1004F11-T7_c_s	4.18	At4g33690	NS	1.00^{e-36}
Anthocyanidin 3-O-glucosyltransferase	PPN007E12-T7_c_s	3.21	At3g50740	NS	2.00^{e-32}
Glutamate dehydrogenase 2	PP1004F04-T7_c_s	3.09	At5g07440	NS	2.00^{e-90}
Zeaxanthin epoxidase, chloroplast precursor	CL377Contig1	3.06	At5g67030	NS	0.00^{e+00}
Alanine–glyoxylate aminotransferase 2 homolog 2, mitochondrial precursor	CL1351Contig1	2.94	At2g38400	NS	1.00^{e-134}
F9L1.4 protein	PPN014H10	2.85	At1g15110	NS	1.00^{e-101}
Dbj|BAA78736.1	CL569Contig1	2.77	At5g67140	NP	1.00^{e-52}
Cinnamoyl-CoA reductase-like protein	CL362Contig1	2.68	At4g30470	NS	9.00^{e-83}
Putative aspartate aminotransferase	CL149Contig2	2.66	At1g80360	NS	1.00^{e-94}
Putative inosine-uridine nucleoside N-ribohydrolase	CL1188Contig1	2.57	At2g36310	NS	1.00^{e-45}
Prunus dulcis transcript; similar to ABA-inducible protein [*Fagus sylvatica* (Beechnut)]	CL964Contig1	2.56	At5g38760	NP	8.00^{e-11}
MADS box transcription factor	PPN004D05-T7_c_s	2.56	At2g22540	NS	2.00^{e-20}
F19P19.4 protein	PP1004A08-T7_c_s	2.54	At2g33360	NS	3.00^{e-28}
Arabidopsis thaliana genomic DNA, chromosome 3, P1 clone: MLN21	PPN069H05-T7_c_s	2.53	At3g14360	NS	2.00^{e-80}
C-terminal domain phosphatase-like 2	PPN070H08-T7_c_s	2.40	At5g01270	NS	4.00^{e-25}
Gb|AAD31066.1	PPN017G08-T7_c_s	2.40	At3g26140	NS	4.00^{e-91}
Putative seed imbibition protein	CL1468Contig1	2.34	At3g57520	NS	1.00^{e-34}
RING-H2 finger protein RHB1a	PPN077H09-T7_c_s	2.30	At4g00335	NS	5.00^{e-41}
Sesquiterpene cyclase	PPN001B06-T7_c_s	2.28	At5g23960	NP	3.00^{e-46}
Amino acid transporter	CL1123Contig1	2.24	At5g09220	NS	2.00^{e-46}
Glutaredoxin, eukaryotic and viruses	PPN048A04-T7_c_s	2.19	At5g63030	NS	4.00^{e-40}
Ribonuclease 2 precursor	PPN014H05-T7_c_s	2.17	At2g39780	NS	1.00^{e-85}
MYB transcription factor MYB93	PPN003G06-T7_c_s	2.15	At5g47390	NS	3.00^{e-75}
Putative aspartate aminotransferase	CL757Contig1	2.13	At5g67300	NP	2.00^{e-58}
Putative aspartate aminotransferase	CL149Contig1	2.13	At1g80360	NS	1.00^{e-129}
T20H2.5 protein	CL1185Contig1	2.12	At3g49070	NS	9.00^{e-60}
Protease-associated PA; Proteinase inhibitor I9, subtilisin propeptide	PPN078F09-T7_c_s	2.11	At5g67360	NS	5.00^{e-59}
Protein phosphatase 2C-like	PPN054D04-T7_c_s	2.11	At1g03590	NS	1.00^{e-108}
YUP8H12.27 protein	PPN017G10-T7_c_s	2.07	At1g05120	NS	1.00^{e-73}

Table 6 continued

Unigene functional annotation	Peach (ChillPeachDB)[a]		Arabidopsis (ColdArrayDB)		Sequence similarity (E-value)
	ID	Fold change	ID	Fold change[b]	
Serine protease-like protein	PPN007C09-T7_c_s	2.07	At4g10520	NP	3.00^{e-44}
AT3g24470/MXP5_4	PPN030H04-T7_c_s	2.06	At4g13345	NS	1.00^{e-92}
DbjlBAA18864.1	CL852Contig1	2.04	At3g19900	NS	9.00^{e-65}
UPI00004C154C; phytanoyl-CoA dioxygenase domain containing 1	CL483Contig1	2.03	At2g01490	NS	1.00^{e-122}
Protein kinase	CL550Contig1	2.01	At2g01820	NS	4.00^{e-96}

[a] Only ChillPeach genes with ≥2.0 fold change were compared with *Arabidopsis* genes in the ColdArrayDB

[b] Fold change at any time point, NS = not significant (fold change <2), ND = not present in ColdArrayDB

Table 7 List of ChillPeach genes with $\geq 2^{10}$ fold increase and $\geq 2^{-7}$ fold decrease in expression in vegetative tissue compared to mesocarp tissue samples

Unigene functional annotation	ChillPeach ID	Fold change[a]
Up-regulated		
Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor	CL530Contig1	18.75
Cytochrome b6-f complex iron-sulfur subunit, chloroplast precursor	CL106Contig1	15.90
Phosphoglycerate kinase	PPN035C04-T7_c_s	14.27
Ferredoxin–NADP reductase, leaf-type isozyme, chloroplast precursor	PPN050C04-T7_c_s	11.92
Glutathione transferase	PP1006G04-T7_c_s	11.57
MADS box transcription factor	PPN004D05-T7_c_s	11.22
F6A14.17 protein	PPN041E05-T7_c_s	11.06
F21O3.6 protein	PPN071E03-T7_c_s	10.91
At1g06690/F4H5_17	PPN071G04-T7_c_s	10.81
Protein At1g73650	CL1216Contig1	10.74
Prunus dulcis transcript; similar to ABA-inducible protein [*Fagus sylvatica* (Beechnut)]	CL964Contig1	10.53
At4g33690	PP1004F11-T7_c_s	10.40
Gossypium hirsutum transcript; similar to *Arabidopsis thaliana* genomic DNA, chromosome 3	PPN040H01-T7_c_s	10.19
Chitinase Ib	CL698Contig1	10.06
Serine/threonine kinase	PPN013H01-T7_c_s	10.03
Alcohol acyl-transferase	PPN009E09-T7_c_s	10.02
Down-regulated		
Flavin-containing monooxygenase, putative	PPN034D04-T7_c_s	−9.47
Ethylene receptor	PPN054G06-T7_c_s	−8.12
Snakin-1	PP1000F05-T7_c_s	−8.09
Leucine-rich repeat protein; contains similarity to elicitor-inducible receptor EIR	PPN026G08-T7_c_s	−7.83
YSL transporter 1	PP1009F11-T7_c_s	−7.77
UPI000034F553; ATP binding/kinase/protein kinase/protein serine/threonine kinase/protein-tyr…	CL170Contig1	−7.57
Mitogen-activated protein kinase	PPN057B03-T7_c_s	−7.39
Heme oxygenase 1	CL900Contig1	−7.29
Heat shock protein 83	CL1174Contig1	−7.20
PDR-like ABC-transporter	PPN032F06-T7_c_s	−7.06
Putative pod-specific dehydrogenase SAC25	CL1233Contig1	−7.05

[a] Significant fold increase or decrease of expression (in exponents of 2) in vegetative tissue compared to mesocarp tissue samples, $q \leq 0.001$

factors responsible for CI in peach and nectarine and, possibly, other stone fruits. The ChillPeach EST libraries were developed using two peach genotypes that contrast for CI but have a common genetic background as they were progeny of a single cross (Pop-DG). 'Dr. Davis' and 'Georgia Belle', parents of the two genotypes, are canning

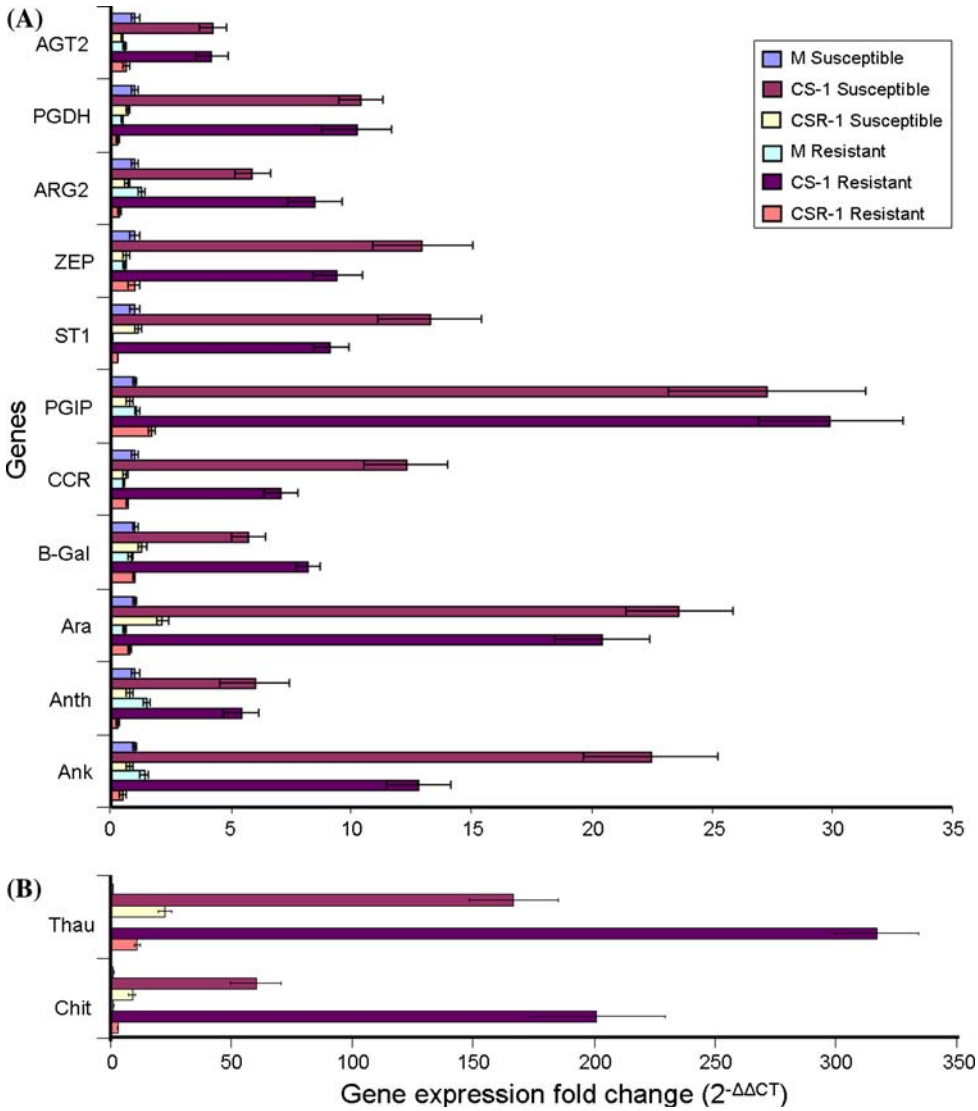

Fig. 2 Genes chosen for data validation by real time qRT-PCR. Shown are relative levels of differential gene expression among treatments. Genes in group B were charted on a different scale because their expression in some of the treatments was about 10 times the expression of genes in group A. The data represented the mean of two biological and three technical replicates. Gene expression levels were normalized against peach *Initiation Factor elF-4-Gamma*. The level of each analyzed gene transcript in mature (M) susceptible sample was set to one and the level of the remaining sample was calculated relative to this reference. Error bars show the standard error of the mean for each treatment

and fresh market peach cultivars, respectively. 'Dr. Davis' is resistant to CI while 'Georgia Belle' is susceptible. The two progeny were observed for 3 consecutive years for expression of CI and one was consistently resistant and the other consistently susceptible.

A peach genetic linkage map was earlier constructed using the Pop-DG segregating population from which the two genotypes used in this study were chosen (Peace et al. 2005a, 2006; Ogundiwin et al. 2007). Quantitative genetic analyses of CI symptoms have also been conducted and several QTLs have been identified using the Pop-DG map (Peace et al 2005a, 2006; Ogundiwin et al. 2007). This linkage map is syntenic with the *Prunus* reference T × E linkage map (Aranzana et al. 2003; Dirlewanger et al. 2004). This direct correspondence between the two maps allows studies in Pop-DG to benefit from the bin mapping strategy developed from the T × E population (Howad et al. 2005). The genomic positions of putative CI genes that are identified through microarray analyses involving ChillPeach unigenes can therefore be determined using the Pop-DG map directly or indirectly through T × E bin mapping, and correspondence with positions of CI QTLs will be a strong indication of the involvement of the respective genes in CI.

About half of the EST sequences in the ChillPeach database have not been reported for *Prunus* in available databases including NCBI, TAIR, PlantTA, ESTree, and GDR. This high novelty was probably due to the normalization of the PPN library. Usually, 10–20 abundant genes (several thousand mRNA copies per cell) account for at least 20% of the cellular mRNA mass, several hundred genes of medium abundance (several hundred mRNA copies per cell) comprise 40–60% of the mRNA mass, and several thousand rare genes (<10 mRNA copies per cell) may account for 20–40% of the mRNA mass (Carninci et al. 2000). Hence, straightforward random sequencing of

Table 8 Gene expression fold change between mesocarp tissues of normal (M) and cold-treated (CS-1), and between M and cold treated plus ripening (CSR-1) using real time qRT-PCR

Gene code	ChillPeachDB ID	Fold change[a]			
		CS-1/M[b]		CSR-1/M[b]	
		Susceptible	Resistant	Susceptible	Resistant
AGAT	CL1351Contig1	4.27***	7.43***	0.47***	1.17^{NS}
Ank	CL1011Contig1	22.47***	9.30***	0.76**	0.36***
Anth	PPN007E12	5.60***	3.50***	0.80*	0.19***
Ara	CL801Contig1	23.64***	36.26***	2.14***	1.44^{NS}
ARG2	CL704Contig1	5.89***	6.65***	0.67^{NS}	0.25^{NS}
B-Gal	CL1325Contig1	5.71***	9.96***	1.29**	1.16^{NS}
CCR	CL362Contig1	12.28***	13.40***	0.62***	1.30^{NS}
Chit	CL698Contig1	60.09***	185.41***	9.07***	2.94***
PGDH	PP1003B08	10.40***	22.20***	0.70***	0.60^{NS}
PGIP	CL146Contig1	27.30***	27.60***	0.75**	1.58***
ST1	PPN065F08	13.27***	166.60***	1.10^{NS}	5.12***
Thau	PPN003H07	166.74***	1107.05***	22.60***	38.48***
ZEP	CL377Contig1	12.96***	16.70***	0.61^{NS}	1.69**

[a] Fold change was calculated using $2^{-\Delta\Delta CT}$ of two biological and three technical replicates; *, **, and *** = significant at $P \leq 0.05$, 0.01, and 0.001, respectively, NS = not significant

[b] Student t-test assuming equal variances among samples

clones from standard cDNA libraries is inefficient for discovering rare transcripts, owing to the repeated occurrence of intermediately and highly abundant cDNA (Zhulidov et al., 2004). This was the case with the PP1 library. In the PPN library, however, the prevalence of clones representing abundant transcripts was decreased by normalization before sequencing. This significantly increased the efficiency of random sequencing and the discovery of novel *Prunus* genes.

Therefore the large contribution this limited EST dataset is making to already available databases reflects the paucity of tree fruit functional genomic information and justifies its development. The database should aid the discovery of genes controlling CI that would otherwise be missed if analyses were to rely solely on previously available *Prunus* ESTs. This database should also be of interest to other tree fruit functional genomics researchers who may be involved in other aspects of fruit physiology. All ChillPeach ESTs are cloned in a pENTR1A GATEway® vector (Invitrogen), and this should facilitate rapid sub-cloning in a range of expression and gene silencing vectors to make assays of gene function much easier.

It is common to observe SSRs in cDNA sequence datasets, and the ChillPeach EST database was not different in this regard. A total of 184 perfect di, tri- and tetranucleotide repeats were observed among the 4,468 unigenes, representing 4% frequency. This frequency is rather low compared to 17% SSR frequency in apple non-redundant EST sequences reported by Newcomb et al. (2006). The inbreeding nature of peach compared to outbreeder apple, and the fact that the apple EST dataset was obtained from extensive tissue types of seven cultivars as opposed to only mesocarp tissue of two full-sib peach genotypes, could explain the low SSR frequency reported here. Despite the total SSR frequency disparity there was good correspondence in many structural components of the SSRs between the two datasets. The dinucleotide repeat group in the ChillPeach SSR dataset contained almost exclusively (97%) AG repeats. AC and AT repeats had 1.8 and 0.9% frequency, respectively, and no occurrence of CG repeat was observed. AG repeats was also reported to dominate the dinucleotide repeats in apple with 88% frequency, AC and AT repeats had 4 and 7.6% frequency, and CG repeats was almost non-existent with 0.05% frequency (Newcomb et al. 2006). Among the trinucleotide repeats, AAG repeats were the most frequent for both ChillPeach and apple. A greater number of the SSRs were found in the 5′ UTR compared to the 3′ UTR, as reported for apple (Newcomb et al. 2006). SSRs are valuable molecular markers and those derived from ESTs such as ChillPeach ESTs may prove to be a good addition to *Prunus* mapping efforts, giving researchers a plethora of functional markers to choose from. A large proportion (42%) of the ChillPeach EST SSRs is new to *Prunus* and approximately 30% of the SSRs were located within coding regions.

Another class of molecular marker obtainable from EST datasets is SNP. Only 70 SNPs, confirmed by more than two ESTs per contig, were observed from the redundant ChillPeach ESTs. This number appeared lower than reported for

apple where 18,408 SNPs were detected from 126,209 redundant EST sequences (Newcomb et al. 2006). However, only 396 contigs of our 4,468 ChillPeach unigenes (8.9%) had four or more ESTs and were those analyzed for SNP detection. This lower redundancy in ChillPeach dataset (due in part to lower number of ESTs, but mainly a direct consequence of the normalization of the PPN library from which most ESTs were sequenced), could partly explain the apparent lower SNP frequency compared to the much larger apple dataset. In addition, as explained in the case of SSR above, higher heterozygosity of an outbreeder apple, and the multiple tissue and cultivars utilized for data collection may be other contributing factors.

To demonstrate the utility of ChillPeach and to generate a cDNA microarray for gene expression analyses of CI in *Prunus*, a preliminary microarray experiment was conducted. The microarray experiments successfully discriminated differentially expressed genes between cold-treated and normal mesocarp tissues, and between vegetative and mesocarp tissues. Some of these genes may be involved with CI because mesocarp tissues with CI were represented in the cold-treated samples. Of the 4,261 genes on the ChillPeach microarray slides, at least 516 were significantly up-regulated in mesocarp tissue.

Arabidopsis orthologs of cold-induced ChillPeach genes varied in their response to cold treatment when compared to their ChillPeach counterparts. Some *Arabidopsis* ChillPeach orthologs were similarly up-regulated in both species while some had opposite response (up-regulated in peach but down-regulated in *Arabidopsis*). Several *Arabidopsis* orthologs of cold-induced ChillPeach genes were non-responsive to cold treatment in *Arabidopsis* vegetative tissue, and a few were not found in the *Arabidopsis* expression profile. The genes with similar expression between the two species supported the utility of ChillPeach for microarray studies while those that were non-responsive to cold or absent in the *Arabidopsis* expression profile may be fruit-specific genes because vegetative tissues were used in the *Arabidopsis* experiments as opposed to the fruit tissue in ChillPeach. This is particularly significant for the functional genomics analyses of CI for which the database is designed. CI occurs as a post-harvest physiological disorder in fruit; therefore, it is essential for the success of CI genetic analyses that the platform to be used in global gene expression should be transcriptionally active in the fruit as in the case of ChillPeach ESTs. About 14% of the differentially expressed genes in cold-treated tissue were new *Prunus* genes and 38% of the genes with $\geq$2.0-fold increases in expression were novel to *Prunus*. This underscores the suitability of ChillPeach dataset for tackling CI problems.

Among the highly expressed genes in cold-treated mesocarp tissue were common stress-induced genes in plants including dehydrin 2, chitinase, RING zinc finger ankyrin protein, ABA-inducible protein, BZIP protein BZ2, chalcone synthase, protein kinase, and others. Other classes of genes such as ripening-related, and sugar and amino acid transport genes were also up-regulated. Whether or not this list includes genes involved in CI control will become clearer as this microarray platform is used specifically for this purpose and efforts to answer this question are underway. The down-regulated genes included heat shock proteins (HSPs). Cold temperatures are known to induce expression of HSPs. The reason for the down-regulation of these genes in our microarray experiment is not clear. The cold-treated fruit used for the analysis included those that were sampled immediately after cold treatment and those that were ripened at room temperature for about 48 h after cold storage. The exposure to room temperature could possibly explain in part why HSPs were down-regulated in this study.

Real-time qRT-PCR analysis confirmed the qualitative and quantitative results of microarray analysis for all 13 genes tested. All genes showed induced expression in cold-treated mesocarp tissue in both analyses. When gene expression results of microarray and qRT-PCR analyses are compared at the quantitative level, all 13 genes had greater fold change in qRT-PCR analysis of CS-1 versus M. However, all but two genes in both progenny had lower fold change in qRT-PCR analysis of CSR-1 versus M. It should be noted that microarray experiment compared a pool of normal mesocarp tissues (no cold treatment) to a pool of mesocarp tissues that included CS-1 and CSR-1 treatments. The expression values observed in the microarray analysis seemed to be a balance between the qRT-PCR values for CS-1 and CSR-1. Therefore, there appears to be a general agreement in the quantitative fold change of the genes considered in both analyses.

The validation of microarray results is a further proof of the usefulness of the ChillPeach platform for transcriptome studies in peach. Furthermore, the qRT-PCR analysis revealed some differences in the expression of tested genes between CI-resistant and susceptible full-sib progeny and between tissues subjected to cold treatment (CS-1) and those that were allowed to ripen after cold treatment (CSR-1). The high transcript accumulation of all the genes in cold-treated fruit was drastically depleted within 2–4 days of subsequent ripening. However, the transcript level of many genes in the ripened cold-treated mesocarp was still significantly higher than the transcript level in normal tissue. The depletion of transcripts during ripening could indicate the involvement of these genes in the ripening/softening process. It is possible that the transcripts have been converted to their respective proteins important in the ripening/softening and other associated processes.

CI symptoms (mealiness, browning, bleeding and loss of flavor) are observed during ripening/softening after cold treatment of most CI susceptible peach cultivars. The observed pattern of transcript accumulation of the qRT-PCR-tested genes could indicate their involvement in CI

development. Detailed transcriptome analysis focusing on each of the CI symptoms will be necessary to associate these genes with any specific symptom.

Of the 13 genes validated by qRT-PCR, 10 had significantly higher expression in cold-treated CI-resistant progeny compared to its cold-treated susceptible counterpart. Two genes had opposite reaction (i.e. higher expression in susceptible progeny), and the expression of one gene was similar to both progeny. The 12 genes with differential expression in the two full-sib progeny may be associated with genotype differences including resistance/susceptibility to CI. Further studies are needed to confirm the involvement of these genes in CI development. Such studies should consider separate pools of several resistant and susceptible full sib progeny for each of the CI-symptoms.

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