Transcriptome profiling of grapefruit flavedo following exposure to low temperature and conditioning treatments uncovers principal molecular components involved in chilling tolerance and susceptibility

PILAR MAUL1, GREGORY T. MCCOLLUM1, MICK POPP2, CHARLES L. GUY3 & RON PORAT4*

1United States Department of Agriculture, US Horticultural Research Laboratory, 2001 S. Rock Road, Fort Pierce, FL 34945, USA, 2Interdisciplinary Center for Biotechnology Research, PO Box 100156, Gainesville, FL 32610, USA, 3Department of Environmental Horticulture, University of Florida, Gainesville, FL 32611, USA and 4Department of Postharvest Science, Agricultural Research Organization, The Volcani Center, PO Box 6, Bet Dagan 50250, Israel

ABSTRACT

A pre-storage conditioning (CD) treatment of 16 °C for 7 d enhanced chilling tolerance of grapefruit and reduced the development of chilling injuries during storage at 5 °C. To gain a better understanding of the molecular mechanisms involved in the responses of citrus fruit to low temperatures, we performed genome-wide transcriptional profiling analysis of RNA isolated from grapefruit flavedo using the newly developed Affymetrix Citrus GeneChip microarray. Utilizing very restrictive cut-off criteria, including pair-wise ANOVA comparisons significantly different at \( P < 0.05 \) and induction or repression of transcript levels by at least fourfold, we found that out of 30 171 probe sets on the microarray, 1345 probe sets were significantly affected by chilling in both control and CD-treated fruits, 509 probe sets were affected by chilling specifically in the CD-treated fruits, and 417 probe sets were specifically expressed in chilling-sensitive control fruits. Overall, exposure to chilling led to expression arrest of general cellular metabolic activity, including concretive down-regulation of cell wall, pathogen defence, photosynthesis, respiration, and protein, nucleic acid and secondary metabolism. On the other hand, chilling enhanced adaptation processes that involve changes in the expression of transcripts related to membranes, lipid, sterol and carbohydrate metabolism, stress stimuli, hormone biosynthesis, and modifications in DNA binding and transcription factors.

Key-words: chilling; conditioning; post-harvest.

INTRODUCTION

Chilling referring to low non-freezing temperatures is one of the major environmental factors influencing the growth, development, survival and geographical distribution of plants (Levitt 1972). Whereas most plant species from temperate regions can acclimatize to cold, and can survive exposure to low or freezing temperatures during the winter, plants of tropical and subtropical origin are severely injured or killed when exposed to chilling temperatures between 0 and 13 °C (Lyons 1973; Lynch 1990; Wang 1990). Exposure of chilling-sensitive plants to low temperatures leads to various physiological alterations, such as increased electrolyte leakage through the cell membranes and decreased photosynthetic capacity and respiration rates. Symptoms of chilling injury (CI) include cessation of growth, wilting, chlorosis, necrosis and, eventually, plant death (Lyons 1973; Graham & Patterson 1982; Allen & Ort 2001). In addition to its adverse effects on plant growth and development, chilling sensitivity also imposes major limitations on the post-harvest storage and handling of fruits and vegetables, because it necessitates storage at relatively high temperatures, which enhance deterioration and spoilage (Paul 1990; Kader 2002).

As for other subtropical crops, exposure of citrus fruits to low storage temperatures causes the development of CI manifested as dark sunken lesions (pitting) of collapsed tissues throughout the peel surface (Fig. 1). Among citrus fruits, grapefruits, limes, pummelos and lemons are especially sensitive to chilling and, therefore, are commercially stored at relatively high temperatures of 11–13 °C (Chalutz, Waks & Schiffmann-Nadel 1985; Kader & Arpaia 2002). Although citrus fruits are chilling sensitive, several commercial treatments can enhance chilling tolerance and can reduce the development of CI following cold storage. The best characterized and probably most effective treatment to enhance chilling tolerance comprises pre-storage temperature conditioning (CD) at 16 °C for 7 d, just before the transfer to cold storage (Hatton & Cubbedge 1982, 1983; McDonald, McCollum & Nordby 1991; Porat et al. 2000, 2003) (Fig. 1).

In contrast to our knowledge of plant responses to other abiotic stresses, such as freezing, drought, salinity and heat, only little is known regarding the molecular basis of chilling tolerance, or of the signal transduction networks.
there have been very few and limited studies of the changes in the transcriptome in chilling-sensitive species, during acclimation to chilling. Suppression subtractive hybridization techniques have been used to show that a high-temperature heat-CD treatment at 37 °C for 3 d significantly increased chilling tolerance in ‘Fortune’ mandarins correspondingly induced the expression of particular transcription factors and stress-related genes (Sanchez-Ballesta et al. 2003). Using PCR-cDNA subtraction libraries and conducting a grapefruit flavedo expressed sequence tags (EST) sequencing project, we have recently identified additional chilling-responsive and heat- and CD-induced genes whose expression is related to the acquisition of chilling tolerance (Sapitnitskaya et al. 2006). A recent study with sunflower seedlings using nylon microarrays containing 8000 unigenes has demonstrated that exposure to chilling temperatures (7 and 15 °C) results in extensive down-regulation of transcripts involved in cellular metabolism. Accordingly, it has been suggested that chilling-sensitive plants may respond to low temperatures differently from chilling-tolerant species (Hewezi et al. 2006).

In the present study, we conducted a genome-wide transcriptional profiling analysis with the newly developed Affymetrix Citrus GeneChip microarray to identify COR genes and to detect likely molecular mechanisms involved in the acquisition of chilling tolerance and susceptibility in grapefruit. The Affymetrix Citrus GeneChip consists of 30 171 probe sets which represent citrus transcripts that have been designed on the basis of bioinformatics analysis generated from the HarvEST Citrus database. This database includes 166 284 DNA sequences collected from 72 different citrus cDNA libraries, including cDNA libraries prepared by us from the flavedo tissue (the outer coloured layer of the peel) of control and CD-treated grapefruit. Our analysis revealed genome-wide changes in the transcriptome of grapefruit following exposure to chilling, indicating the existence of both transcriptional governed arrest in general metabolic activity as well as transcriptional governed metabolic adaptation processes to low temperatures. These new findings shed light onto the molecular and biochemical mechanisms that determine chilling tolerance and susceptibility in a commercially important, chilling-sensitive crop.

MATERIALS AND METHODS

Plant material, chilling and CD treatments

Grapefruits (Citrus paradisi Macf., cv. ‘Marsh’) were harvested in September from a commercial grove in La Belle, FL. Mature fruits were harvested, washed and air-dried at room temperature. Afterwards, half of the fruits were transferred directly to storage at 5 °C (chilling), and the remainder was first kept at 16 °C for 7 d (CD) and afterwards was transferred to 5 °C (CD + chilling). The fruits were stored at 5 °C for up to 8 weeks. Each treatment included four boxes, each containing 30 fruits (total of 120 fruits per treatment). For CI evaluations, the fruits were
sorted into four categories according to their CI severity: none (score 0, no pitting), slight (score 1, a few scattered pits), moderate (score 2, pitting covering up to 30% of the fruit surface) and severe (score 3, extensive pitting covering >30% of the fruit surface). The CI index was determined for each treatment by multiplying the number of fruits in each category by their score, and then by dividing this sum by the total number of fruits assessed. In addition, the percentage of fruit displaying CI symptoms (scores 1–3) was determined.

Transcript profiling and data analysis

Transcript profiling followed the minimum information about a microarray experiment (MIAME) protocol recommendations (http://www.mged.org/Workgroups/MIAME/miame.html). RNA was isolated from the flavedo of fruits given four different treatments: (1) time zero (immediately after harvest), (2) after CD (7 d at 16 °C), (3) after 2 weeks at 5 °C and (4) after CD + 2 weeks at 5 °C. Total RNA was extracted using a modified guanidinium thiocyanate/acid phenol method (Strommer, Gregerson & Vayda 1993), and was further purified and concentrated with the RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. For each treatment, we made three separate RNA extractions, each from flavedos collected from five different fruits. The RNA samples were further prepared for hybridization according to the protocols outlined in the GeneChip Expression Analysis Technical Manual, and were hybridized to the Affymetrix Citrus GeneChip microarray (Affymetrix, Santa Clara, CA, USA). The experiment was conducted three times, to yield three replicate measurements from separate RNA extractions at each time point. Hybridizations were performed at the University of Florida Shands Cancer Center (UFSCC)/Interdisciplinary Center for Biotechnology Research (ICBR) Microarray Core Facility at the University of Florida, Gainesville, FL. Data analysis was performed using the Affymetrix Microarray Suite 5.0 (MAS5.0) statistical algorithms (Affymetrix). The probability of differences among treatments was determined with the MAS5.0 statistical programme. One-way analysis of variance (ANOVA) and Tukey’s honestly significant difference (HSD) pairwise comparisons tests were applied to the resulting signal values to identify probe sets that exhibited significant changes in signal levels at P ≤ 0.05. The Affymetrix data files were imported into the GeneSpring 7.0 software (Silicon Genetics, Redwood City, CA, USA). Normalization was performed in two steps: (1) ‘per chip normalization’, in which each measurement in an array was divided by the 50th percentile of all measurements in that array; and (2) ‘per gene normalization’, in which each measurement for a particular gene was divided by the 50th percentile of all measurements for that gene in all the arrays. Hierarchical cluster analysis was used to organize the genes with similar expression patterns into groups.

Principal component analysis (PCA)

PCA was performed with the S-Plus 2000 software package, standard edition, release 3 (Insightful, Berlin, Germany) on log10-transformed relative responses, log10 (R). The denominator of the quotient R was the average response of non-treated control samples at time zero (R = Nt × avgN). Log-transformed relative responses were averaged at each time point. Transcripts with more than one missing time point or low overall variance were excluded. Overall, PCA was conducted as described previously for profiling of both Arabidopsis transcripts and metabolites (Kaplan et al. 2004, 2007).

RT-PCR analysis

Semi-quantitative RT-PCR analysis was performed to confirm the microarray results. Total RNA was pretreated with RNase-free DNase I (Amersham Biosciences, Piscataway, NJ, USA), and RT-PCR analysis was performed with the TITAN one-tube RT-PCR kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s instructions. A 50 ng aliquot of total RNA was used as a template in RT-PCR with each pair of specific primers. The number of PCR cycles for each primer set was determined empirically, to ensure that the PCR products obtained remained in the linear phase of amplification. Amplification reactions with 18S rRNA primers were run for 18 cycles to ensure the presence of equal amounts of RNA in all treatments. The reactions with all other primer sets continued for 25–35 cycles. The temperature profiles used for PCR were 94 °C for 30 s, and up to 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1.5 min. Gene-specific primers were designed using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) (Rozen & Skaletsky 2000), with cDNA sequences derived from EST libraries present in the Affymetrix Citrus GeneChip. All primers were tested for specificity by PCR followed by electrophoresis. Generation of a single band was followed by cloning and DNA sequencing, to confirm the identity of the PCR products involved. Images were captured and band density was quantified by image densitometry with the Kodak 1D Scientific Imaging System software (Eastman Kodak Co., Rochester, NY, USA). RT-PCR reactions for each transcript were performed in triplicate, and mean relative expression values and SEs were determined.

Databases and programmes

The National Center for Biological Information (NCBI) nucleotide Basic Local Alignment and Search Tools (BLAST) (Altschul et al. 1997), the Gene Ontology (GO) functional categorization tool and AraCyc metabolic pathway databases were used. The GO and AraCyc databases were downloaded from The Arabidopsis Information Resource (TAIR) home page (http://www.arabidopsis.org/).
RESULTS

Effects of CD and chilling on the transcriptome of grapefruit

Control ‘Marsh’ grapefruits developed CI after 3–4 weeks of cold storage at 5 °C. However, keeping the fruit for 7 d at a moderate temperature of 16 °C (CD treatment) prior to continuous storage at low temperatures enhanced chilling tolerance and reduced the development and severity of CI following 8 weeks of storage at 5 °C by 79% (Fig. 1).

The effects of CD and chilling on the transcriptome of grapefruit were determined using RNA isolated from the flavedo of fruits given four different treatments: (1) time zero (immediately after harvest), (2) after CD (7 d at 16 °C), (3) after 2 weeks at 5 °C and (4) after CD + 2 weeks at 5 °C. We chose to determine transcript abundance after the particular time point of 2 weeks at 5 °C because, although this period was long enough to affect the fruit significantly, it was much too short for the development of visible CI symptoms to appear. Overall, about 75% of the total probe sets on the microarray yielded validated signals with RNA from the flavedo. Differential signal intensities for a very high percentage of the probe sets (40% – 9127 probe sets) were significantly (P ≤ 0.001) affected by either the CD or the chilling treatment.

PCA was used to validate the repeatability of the microarray data across replications. PCA was also used to identify variances in global gene expression phenotypes (Scholz et al. 2005; Kaplan et al. 2007). The PCA analysis revealed the occurrence of marked differences in gene expression patterns among all four tested treatments (Fig. 2). Furthermore, the transcriptome profiles of all three separate RNA samples of the time zero, CD and chilling treatments were tightly clustered together (Fig. 2).

Based on one-way ANOVA pairwise comparisons (P ≤ 0.05), 6151 probe sets were identified whose expression differed between the CD and time-zero treatments, and 7561 and 7478 probe sets whose expression differed between the chilling and time-zero treatments, and between the CD + chilling and time-zero treatments, respectively. Because we observed such marked statistical differences among treatments, we designated another criterion for the selection of CD- and chilling-responsive genes: induction or repression of transcript levels by a factor of at least 4 (log2 ≥ 2 or log2 ≤ −2). This led to the identification of 911 probe sets (296 up-regulated and 614 down-regulated) whose expression changed by at least fourfold after the CD treatment, and 1762 (505 up-regulated and 1260 down-regulated) and 1854 probe sets (549 up-regulated and 1305 down-regulated) whose expression changed by a factor of at least 4 between the chilling and the time-zero treatments, and between the CD + chilling and the time-zero treatments, respectively (Table 1).

In order to define which probe sets were related to chilling tolerance and which to chilling susceptibility, we compared transcript responses that were significantly affected by chilling in control untreated chilling-sensitive fruits with those induced during chilling in the CD-treated chilling-tolerant fruits and found that 1345 probe sets were affected by chilling in both control and CD-treated fruits. This group of transcripts was designated as belonging to a ‘chilling-response regulon’. The 509 probe sets that were unique to the CD-treated fruits were assigned to a ‘chilling-tolerance regulon’. The 417 probe sets that were unique to the chilling-sensitive control fruits, this group was designated as the ‘chilling-stress regulon’ (Table 2). The spatial and unique expression patterns of the chilling-response, chilling-tolerance and chilling-stress regulons are depicted in the hierarchical clustering analysis (Fig. 3). It can be seen that in the common chilling-response regulon, many genes that were highly expressed at time zero (red colour) were markedly repressed in both chilling and (CD + chilling)-treated fruits, whereas another cluster of genes with very low expression levels at time zero (green colour at the bottom of the diagram) was markedly induced by the chilling treatment (Fig. 3). In contrast to these findings, transcripts of the CD-treated fruit in the chilling-tolerance regulon were specifically induced or repressed during chilling, whereas in the chilling-stress regulon, only transcripts of control untreated fruit were significantly affected by chilling (Fig. 3).

RT-PCR analysis

To confirm the validity of the microarray gene expression data, and the accuracy and reliability of the defined chilling-responsive regulons, we chose four genes from each regulon...
(two up-regulated and two down-regulated), and conducted semi-quantitative RT-PCR analysis with gene-specific primers (Table 3). The results show that the observed RT-PCR gene expression data were extremely similar to those achieved with the citrus GeneChip (Fig. 4). Furthermore, multiple linear regression analysis between the transcript abundance data observed by the microarray and obtained in the RT-PCR studies revealed a very high correlation with $r^2$ values between 0.92 and 0.99 for all 12 genes tested (Table 3). Overall, the observed RT-PCR data strongly supported the microarray gene expression data and confirmed the differences in gene expression patterns among the defined chilling-responsive regulons.

**Functional categorization of chilling-responsive genes**

After defining the chilling-responsive regulons, we performed nucleotide blast searches in order to identify the putative functions of the various differentially expressed genes. It was found that, out of 1345, 509 and 417 genes in the chilling-response, chilling-tolerance and chilling-stress regulons, respectively, 843, 342 and 241 genes, respectively, had known or homology-based predicted functions. Thus, approximately 60% of the transcripts in the various chilling-responsive regulons encoded proteins with known functions, and all the other transcripts either represented unrecognized ESTs or encoded unknown, unnamed, expressed or putative proteins.

Functional categorization of the various transcripts of each regulon provided new insights into the possible molecular mechanisms involved in citrus fruit responses to chilling temperatures (Table 4). It was found that exposure to chilling resulted in massive overall down-regulation of transcripts related to cell wall metabolism, defence against pathogens; photosynthesis; respiration; the phenylpropanoid, flavonoid, and terpenoid biosynthesis pathways; water channels; and senescence-related transcripts (Table 4). For instance, in the common chilling-response regulon, exposure to chilling resulted in the down-regulation of 60 transcripts encoding cell wall proteins and in the up-regulation of only six of them, including two transcripts – CN188649 and CB29078 – that actually encoded pectin methyl esterase inhibitors. In the case of pathogen defence proteins, we found that exposure to chilling resulted in down-regulation of 24 probe sets and slight up-regulation of only two of them (Table 4). Following exposure of the fruit to chilling, we observed a general repression of transcripts involved in secondary metabolism (repression of 113 as compared to induction of 21 probe sets), including transcripts involved in phenylpropanoid, flavonoid and terpenoid biosynthesis pathways. Nevertheless, it is worth noting that following exposure to chilling, we did not detect any major changes in the levels of transcripts involved in the carotenoid biosynthesis pathway (down-regulation of just a single probe set).

In contrast with the extensive down-regulation of transcripts involved in various aspects of cellular metabolism,
exposure to chilling also governed cellular adaptation processes, including both up- and down-regulation of transcripts related to amino acids, carbohydrate, DNA binding and transcription factors, hormones, lipid metabolism, membrane proteins, transporters and stress proteins (Table 4). In the chilling-response regulon, we found that exposure to chilling resulted in extensive down-regulation of transcripts encoding protein kinases (41 probe sets down and only 9 up), but up-regulation of those encoding protein phosphatases (eight probe sets up and only one down) (Table 4). These results suggest that exposure to chilling may have activated dephosphorylation processes.

In an effort to obtain additional biological information regarding the molecular and biochemical responses that occur in citrus fruits in response to chilling, we further performed bulk nucleotide blast searches to identify Arabidopsis homologs of the citrus chilling-response genes. This enabled us to use both the GO annotations and the AraCyc metabolic pathway resources available at the TAIR site (http://www.arabidopsis.org/). By applying a cut-off criterion of E-value \( \leq 10^{-4} \), we identified Arabidopsis homologs for about 45–50% of the cold-responsive citrus genes. By comparing the gene profiles of the citrus chilling-response regulon with about 4000 randomly chosen flavedo-expressed genes, we performed additional functional categorization analysis by means of the GO database according to the heading Biological Processes (Fig. 5). We found that the chilling-response regulon included over-representation of transcripts related to ‘response to biotic or abiotic stimulus’ and ‘response to stress’ proteins, but under-representation of transcripts related to ‘protein metabolism’, ‘cell organization and biogenesis’ and ‘DNA or RNA metabolism’ (Fig. 5).

**Hormone biosynthesis**

Upon examining the effects of chilling on transcripts involved in hormone biosynthesis or catabolism, we found that chilling enhanced the expression of an ACC synthase transcript (CV704207), which is the key limiting enzyme in the ethylene biosynthesis pathway (Yang & Hoffman 1984), and remarkably increased the expression of three
transcripts (CB292529, DN797172 and CF830826) encoding 9-cis-epoxycarotenoid dioxygenase 2, a key enzyme for ABA biosynthesis (Thompson et al. 2000) (Supplementary Table S1). Exposure to chilling caused the down-regulation of the expression of an ABA-glucosyltransferase transcript involved in ABA catabolism (Nambara & Marion-Poll 2005) (Supplementary Table S1). Chilling also resulted in the down-regulation of a transcript encoding ent-kaurenoic acid oxidase (BQ624758), a key enzyme in gibberellic acid (GA) biosynthesis (Helliwell et al. 1999), and of lipoxygenase, allene oxide cyclase and 12-oxo-phytodienoate reductase transcripts involved in the biosynthesis of JA (Creelman & Mullet 1997) (Supplementary Table S1). In sum, exposure to chilling increased the expression of ethylene and ABA biosynthesis genes, but repressed GA and JA biosynthesis genes. In addition to the common fruit response to chilling (chilling-response regulon), exposure of the CD-treated fruit (chilling-tolerance regulon) to chilling further suppressed the expression of ethylene and ABA biosynthesis genes, but repressed GA and JA biosynthesis genes. In addition to the common fruit response to chilling (chilling-response regulon), exposure of the CD-treated fruit (chilling-tolerance regulon) to chilling further suppressed the expression of SAM synthase, ACC oxidase and ent-kaurenoic acid oxidase transcripts, resulting in specific down-regulation of ethylene biosynthesis and further suppression of GA biosynthesis (Supplementary Table S1). The effects of chilling on the expression of all ACC synthase and ACC oxidase probe sets (the key enzymes involved in ethylene biosynthesis) present on the Affymetrix Citrus GeneChip is shown in Fig. 6. It can be seen that exposure to chilling induced the expression of a particular ACC synthase gene (CV704207) in both control and CD-treated fruits. However, whereas control chilling-sensitive fruits retained very high expression levels of two ACC oxidase transcripts (CX305211 and CB322167), chilling markedly suppressed ACC oxidase transcript levels in the CD-treated chilling-tolerant fruits. Furthermore, the suppression of ACC oxidase gene expression was probably directly related to the pre-storage CD treatment, because CD by itself was sufficient to reduce ACC oxidase transcript levels (Fig. 6).

**Transcription factors**

Global transcript profiling analysis revealed that exposure to chilling markedly affected the expression patterns of many transcription factor genes (Supplementary Table S2). Overall, exposure to chilling induced the expression of 17, 10 and 3, and decreased the expression of 33, 11 and 13 probe sets encoding transcription factors in the chilling-response, chilling-tolerance and chilling-stress regulons, respectively. The expression patterns of four genes from each regulon (two up-regulated and two down-regulated) were confirmed by RT-PCR, and multiple linear regression analysis was performed.

---

**Table 3.** Genes and primers used for RT-PCR analysis and comparison between the Citrus GeneChip microarray and RT-PCR gene expression data

<table>
<thead>
<tr>
<th>Affymetrix ID No.</th>
<th>GenBank accession no.</th>
<th>Annotation</th>
<th>Forward primer 5′→3′</th>
<th>Reverse primer 5′→3′</th>
<th>Correlation between microarray and RT-PCR (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cit.17235</td>
<td>CF83082</td>
<td>9-Cis-epoxycarotenoid dioxygenase</td>
<td>gatggttatatctgtcatt</td>
<td>ctgggagtcacaaagtt</td>
<td>0.94</td>
</tr>
<tr>
<td>Cit.36677</td>
<td>DN619429</td>
<td>β-Amylase</td>
<td>tacatacacaagagcaag</td>
<td>ctatacataaatcatac</td>
<td>0.98</td>
</tr>
<tr>
<td>Cit.2093</td>
<td>CX071708</td>
<td>Expansin</td>
<td>ctggctacagacggctt</td>
<td>ctatacataaatcatac</td>
<td>0.94</td>
</tr>
<tr>
<td>Cit.2945</td>
<td>CV887291</td>
<td>Endo-β-1,4-glucanase</td>
<td>atgtgacatagttggct</td>
<td>ctatacataaatcatac</td>
<td>0.94</td>
</tr>
<tr>
<td>Cit.15009</td>
<td>CX304634</td>
<td>Sucrose-phosphate synthase</td>
<td>gagacgccagagatgcag</td>
<td>ctatacataaatcatac</td>
<td>0.97</td>
</tr>
<tr>
<td>Cit.15760</td>
<td>CX640929</td>
<td>UTP-glucose glucosyltransferase</td>
<td>agatagagagggtagtgag</td>
<td>ctatacataaatcatac</td>
<td>0.99</td>
</tr>
<tr>
<td>Cit.30473</td>
<td>CX044246</td>
<td>Caffeic acid 3-O-methyltransferase</td>
<td>ctgataagccatcggctc</td>
<td>ctatacataaatcatac</td>
<td>0.92</td>
</tr>
<tr>
<td>Cit.30535</td>
<td>AF321533</td>
<td>ACC oxidase</td>
<td>gaaattagcagagcatgct</td>
<td>ctatacataaatcatac</td>
<td>0.98</td>
</tr>
<tr>
<td>Cit.26262</td>
<td>CN184508</td>
<td>Ripening-related protein</td>
<td>gaagcaattaccagctcag</td>
<td>gatctattgacctgttgg</td>
<td>0.99</td>
</tr>
<tr>
<td>Cit.289</td>
<td>CF835645</td>
<td>Esterase</td>
<td>tgttaaattaatggtagttg</td>
<td>acctattgacctgttgg</td>
<td>0.99</td>
</tr>
<tr>
<td>Cit.20545</td>
<td>CF417087</td>
<td>Lipid-transfer protein</td>
<td>gtcaggtgaagggtagttg</td>
<td>acctattgacctgttgg</td>
<td>0.98</td>
</tr>
<tr>
<td>Cit.15492</td>
<td>DN906153</td>
<td>Peptide transporter</td>
<td>gtcaggtgaagggtagttg</td>
<td>acctattgacctgttgg</td>
<td>0.97</td>
</tr>
</tbody>
</table>

The expression patterns of four genes from each regulon (two up-regulated and two down-regulated) were confirmed by RT-PCR, and multiple linear regression analysis was performed.
Transcriptome profiling of grapefruit following chilling

9-Cis-epoxycarotenoid dioxygenase

Signal intensity

UTP-glucose glucosyltransferase

Esterase

Signal intensity

β-Amylase

Sucrose phosphate synthase

Ripening protein

Signal intensity

β-1,4-Glucanase

ACC oxidase

Lipid-transfer protein

Signal intensity

Expansin

Caffeicacid 3-O-methyltransferase

Peptide transporter

Signal intensity

Time zero Chilling CD + chilling CD Chilling CD + chilling CD Chilling CD + chilling CD Chilling CD + chilling

Chilling-response regucn

Chilling-tolerance regucn

Chilling-stress regucn

Up-regulated transcripts

Down-regulated transcripts

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd, Plant, Cell and Environment, 31, 752–768
chilling-tolerance and chilling-stress regulons, respectively (Supplementary Table S2). In the common chilling-response regulon, exposure to chilling resulted in mixed regulation (both up- and down-regulation) of various classes of transcription factors, such as AP2 domain, EREBP, bHLH, bZIP, MADS, no apical meristem and MYB (Supplementary Table S2). On the other hand, chilling specifically induced the expression of SCARECROW (CB291008), a repressor of GA signalling (Silverstone, Ciampaglio & Sun 1998), and of a particular PWWP domain transcription factor gene. In contrast, chilling specifically repressed the expression of auxin response factors, and of homeodomain, phantastica, TINY and WRKY transcription factors (Supplementary Table S2). An example of the marked effects of chilling (with or without the pre-storage CD treatment) on the suppression of various transcription factor genes was demonstrated by means of clustering analysis (Fig. 7a). Moreover, the clustering analysis data suggest that the expression of different types of transcription factors may be coordinately regulated during chilling (Fig. 7a). In the chilling-tolerance regulon, chilling specifically induced the expression of two heat shock factors (HSFs), gigantea, a MADS box and another SCARECROW probe set (Supplementary Table S2). As seen in the clustering analysis, the induction of the HSF transcripts and also of an additional MYB transcription factor gene was mainly attributed to the CD treatment, and their transcript levels somewhat decreased during subsequent exposure to chilling (Fig. 7b). Within the chilling-tolerance regulon, we further detected specific and unique down-regulation of the pathogenesis-related transcriptional activator pti5, and of another EREBP gene. Finally, in the chilling-stress regulon, we identified specific up-regulation of a dehydration-induced MYB transcript and of an NAC-domain transcription factor; the latter is known to be involved in regulation of senescence and stress responses (Hegedus et al. 2003; Guo & Gan 2006) (Supplementary Table S2).

**Lipid metabolism**

Chilling grapefruits resulted in changes in the abundance of transcripts related to lipid and sterol metabolism. The chilling-response regulon includes up-regulation of various probe sets involved in fatty acids and phospholipid biosynthesis, and also of several lipases involved in phospholipid degradation (Supplementary Table S3). In addition, grapefruit responded to chilling with a marked increase of cycloartenol synthase transcripts (CX545154), the key enzyme and committed step towards sterol biosynthesis (Benveniste 2004). Furthermore, chilling increased, by approximately 16-fold, the abundance of steroleosin (CX675291), an enzyme involved in sterol signalling.

### Table 4. Functional categorization of chilling-responsive genes in grapefruit

<table>
<thead>
<tr>
<th>Functional categorization</th>
<th>Chilling-response regulon</th>
<th>Chilling-tolerance regulon</th>
<th>Chilling-stress regulon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Up</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Amino acid</td>
<td>12</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>20</td>
<td>45</td>
<td>15</td>
</tr>
<tr>
<td>Carotenoid</td>
<td>–</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>–</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>Cell wall/defence</td>
<td>6</td>
<td>60</td>
<td>3</td>
</tr>
<tr>
<td>DNA binding</td>
<td>3</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>1</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Hormone</td>
<td>6</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Ion transport</td>
<td>6</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Lipid</td>
<td>12</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Membrane proteins</td>
<td>2</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>Metabolite transport</td>
<td>7</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Pathogen defence</td>
<td>2</td>
<td>24</td>
<td>–</td>
</tr>
<tr>
<td>Phenylpropanoid</td>
<td>2</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Photosynthesis</td>
<td>–</td>
<td>12</td>
<td>–</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>5</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Protein kinase</td>
<td>9</td>
<td>41</td>
<td>3</td>
</tr>
<tr>
<td>Protein phosphatase</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Respiration</td>
<td>1</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td>RNA</td>
<td>7</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Senescence</td>
<td>1</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Stress proteins</td>
<td>7</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>1</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Transcription factors</td>
<td>17</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>Water channels</td>
<td>–</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Secondary metabolism</td>
<td>21</td>
<td>113</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>203</td>
<td>640</td>
<td>102</td>
</tr>
</tbody>
</table>

The data represent the number of genes with known function from each regulon belonging to different functional groups.

© 2008 The Authors

Nevertheless, exposure to chilling resulted in down-regulation of 24 probe sets involved in fatty acid and phospholipid metabolism, and decreased amounts of transcripts involved in later stages of sterol biosynthesis (Supplementary Table S3).

In the chilling-tolerance regulon, we observed unique increases in two probe sets encoding fatty acid desaturases, which are known to play a major role in protecting plants from CI (Miquel et al. 1993). In addition, the chilling-tolerance regulon included fourfold induction of a transcript encoding ceramide glucosyltransferase (CF417498), a key enzyme that catalyses the initial step in glycosphingolipid biosynthesis (Ichikawa et al. 1996) (Supplementary Table S3). The marked and specific increases of the fatty acid desaturase and ceramide glucosyltransferase transcripts in the (CD + chilling)-treated fruit is further illustrated in the clustering analysis (Fig. 8).

In the chilling-stress regulon, we observed only specific decreases of transcripts involved in lipid and sterol metabolism (Supplementary Table S3). Especially worth noticing is the specific down-regulation of two transcripts involved in wax biosynthesis (acyl coenzyme A reductase and the fatty acid condensing enzyme CUT1, CX046518 and CF838037, respectively) and of three probe sets encoding lipid-transfer proteins thought to be involved in wax deposition (Kunst & Samuels 2003) (Supplementary Table S3). These changes in transcript abundance suggest that wax biosynthesis and deposition processes may be repressed in control chilling-sensitive fruits.

**Carbohydrate metabolism**

A principal factor in carbohydrate metabolism under stress conditions is regulation of the balance between biosynthesis and breakdown of starch. The enzyme β-amylase degrades starch to maltose, whose further conversion leads to increased glucose, fructose and sucrose levels (Kaplan, Dong & Guy 2006). Overall, the effects of CD and chilling on the expression of all β-amylase probe sets present on the Affymetrix Citrus GeneChip are shown in Fig. 9. It can be seen that the expression of 7 out of 12 different β-amylase probe sets was significantly and markedly induced by chilling: the mean signal intensity value of all the various citrus β-amylase probe sets increased by 4.2-fold in control fruits and by 5.5-fold in CD-treated chilling-tolerant fruits (Fig. 9). Thus, exposure to chilling clearly favoured starch degradation, which would result in increased accumulation of soluble sugars. In addition to the effects of chilling on β-amylase transcript levels, we found that CD and CD + chilling increased the expression levels of a specific sucrose-phosphate synthase (SPS1) transcript (CX304634) by 26- and 29-fold, respectively (data not shown). Because SPS1 catalyses sucrose synthesis, it is...
possible that increased accumulation of sucrose in the flavedo may contribute to chilling tolerance.

**Stress stimulus**

Proline, trehalose, mannitol and gamma-aminobutyric acid (GABA) are stress metabolites with compatible solute-like properties that accumulate at low temperatures and protect membranes and proteins from freezing damage (Yancey et al. 1982; Carpenter & Crowe 1988; Bouche & Fromm 2004). By using the AraCyc metabolic pathway database, we found that exposure of citrus fruit to chilling (with or without the CD treatment) resulted in a significant 16-fold increase of D1-pyrroline-5-carboxylate synthase transcript (CN190655), encoding a key enzyme that catalyses the first two steps in the biosynthesis pathway of proline from L-glutamate (Hu, Delauney & Verma 1992). In addition, chilling also caused a fourfold increase in the transcript level of trehalose-6-phosphate synthase (CX299159), which catalyses the first step in the biosynthesis pathway of trehalose (Eastmond et al. 2002).

Chilling also resulted in a decrease in the abundance of mannitol dehydrogenase transcripts, CV884329 and CK934767, by factors of 15- and 19-fold, respectively, suggesting that mannitol degradation is suppressed by chilling. Regarding GABA accumulation, we found that exposure to chilling reduced the abundance of a succinate semialdehyde dehydrogenase transcript (CX672087) by a factor of about 10-fold. This enzyme irreversibly oxidizes succinate semi-aldehyde to succinate and, thus, provides the main enzyme responsible for GABA catabolism (Bouche et al. 2003).

**Vitamins and antioxidant defence**

The antioxidant capacity of the cells depends on the presence and activity of the reactive oxygen-scavenging enzymes catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD), and on the accumulation of compounds with antioxidant properties, such as vitamins C and E (Mittler 2002). By using AraCyc metabolic pathways, we found that chilling significantly increased the abundance of one of two different citrus CAT transcripts (CX671191) by 23- and 16-fold in control and CD-treated fruits, respectively. We did not detect any significant effect of chilling on either APX or SOD transcript levels.

Regarding vitamins C and E biosynthesis pathways, we found that in control chilling-sensitive fruits (chilling-stress regulon), but not in CD-treated chilling-tolerant fruits, exposure to chilling significantly decreased the abundance of transcripts encoding mannose-1-phosphate guanylytransferse and guanosine diphosphate-mannose 3,5-epimerase, two enzymes involved in the biosynthesis of ascorbate (vitamin C) (Wolucka et al. 2001), and of 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase, encoding an enzyme catalyzing the first committed step in α-tocopherol (vitamin E) biosynthesis (Eckardt 2003). Therefore, maintenance of vitamins C and E contents may be necessary in order to reduce antioxidant damage under chilling temperatures.

**DISCUSSION**

Although chilling is one of the most important environmental factors affecting plant growth and post-harvest storage of horticultural crops, surprisingly, little is known regarding the molecular mechanisms involved in conferring chilling tolerance or susceptibility in chilling-sensitive crops. In the present study, we performed genome-wide transcriptional profiling analysis in order to identify and characterize the molecular components involved in the acquisition of chilling tolerance or susceptibility in citrus fruits. The Affymetrix Citrus GeneChip used in this study was commercially released in 2006. In our experiments, we detected signal from 75% of all the probe sets on the microarray, which indicates that we achieved a very high hybridization level of trehalose-6-phosphate synthase (CX299159), which catalyses the first step in the biosynthesis pathway of trehalose (Eastmond et al. 2002).

Chilling also resulted in a decrease in the abundance of mannitol dehydrogenase transcripts, CV884329 and CK934767, by factors of 15- and 19-fold, respectively, suggesting that mannitol degradation is suppressed by chilling. Regarding GABA accumulation, we found that exposure to chilling reduced the abundance of a succinate semi-aldehyde dehydrogenase transcript (CX672087) by a factor of about 10-fold. This enzyme irreversibly oxidizes succinate semi-aldehyde to succinate and, thus, provides the main enzyme responsible for GABA catabolism (Bouche et al. 2003).

### Figure 6.

Effects of conditioning (CD) (7 d at 16 °C) and chilling (2 weeks at 5 °C) treatments on the expression levels of all ACC synthase (a) and ACC oxidase (b) probe sets present on the Affymetrix Citrus GeneChip. Data are means of three measurements. The GenBank accession numbers of the ACC synthase and ACC oxidase probe sets Cit.4491, Cit.18037, Cit.14093, Cit.5687, Cit.21723, Cit.30535, Cit.1236 and Cit.1235 are CX643923, AJ012696, CN189566, CV704207, CX305211, CB322167, CF834731 and CX642130, respectively.
Figure 7. Hierarchical cluster analysis. (a) A cluster of the chilling-response regulon including transcription factors whose expression was down-regulated by chilling (2 weeks at 5 °C). (b) A cluster of the chilling-tolerance regulon including transcription factors whose expression was particularly up-regulated by the conditioning (CD) treatment (7 d at 16 °C). HSF, heat shock factor.

Figure 8. Hierarchical cluster analysis of the chilling-tolerance regulon including transcripts encoding fatty acid desaturases and ceramide glucosyltransferase whose expression was up-regulated by the [conditioning (CD) + chilling] treatment (7 d at 16 °C + 2 weeks at 5 °C).
the general down-regulation of cellular metabolism transcripts, we found that chilling also activated various adaptation processes, with effects that included significant changes (both up- and down-regulation) of transcripts encoding membrane proteins, lipid and sterol metabolism, carbohydrate metabolism, stress stimulus, hormone biosynthesis, and DNA binding and transcription factors (Table 4, Supplementary Tables S1–S3). Overall, a model illustrating the principal responses of citrus fruits to chilling is presented in Fig. 10. It can be seen that whereas, on the one hand, chilling initiated a coordinated arrest in expression related to general metabolic activity, on the other hand, it activated various adaptation processes. These adaptation processes included changes in hormone biosynthesis genes, such as induction of transcripts related to biosynthesis of ethylene and ABA, and depression of transcripts related to GA and JA biosynthesis (Supplementary Table S1); induction of transcription factors such as SCARECROW, a repressor of GA signalling; and suppression of other transcription factors, such as auxin response factors (suppression of growth) and WRKY transcription factors (suppression of pathogen defence responses) (Supplementary Table S2); changes in transcripts encoding lipid and sterol metabolism enzymes, including marked induction of cycloartenol synthase, which is the key enzyme that catalyses the committed step towards sterol biosynthesis (Benveniste 2004) (Supplementary Table S3); induction of β-amylase transcripts involved in starch breakdown (Fig. 9); induction of gene expression involved in the accumulation of various stress metabolites, including proline, trehalose, mannitol and GABA; and increase in CAT transcript levels (Fig. 10).

In general, we found that exposure to a low temperature of 5 °C for 2 weeks had tremendous effects on the transcriptome of grapefruit where the expression of approximately 7500 genes was significantly \((P \leq 0.05)\) affected by chilling. Utilizing very restrictive cut-off criteria for selection of CD- and chilling-responsive genes, we still identified more than 1700 probe sets that were affected by exposure to chilling (Table 1). These criteria were (1) probability of difference among treatments at \(P \leq 0.001\), (2) pairwise one-way ANOVA comparisons significantly different at \(P \leq 0.05\) and (3) induction or repression of transcript levels by at least fourfold. Among the chilling-responsive genes, a large group of 1345 probe sets \((371 \text{ up-regulated and } 974 \text{ down-regulated})\) were commonly affected by chilling in both control chilling-sensitive fruits and CD-treated chilling-tolerant fruits (Table 2). This group of transcripts was termed the ‘chilling-response regulon’, and it comprised transcripts involved in the natural and basic response of citrus fruit to low temperatures. Within the chilling-response regulon, we identified massive down-regulation of transcripts related to cell wall and defences against pathogens, photosynthesis, respiration, protein metabolism, DNA and RNA metabolism, secondary metabolism, water channels and senescence (Fig. 5, Table 4). Similar extensive down-regulation of cellular metabolism transcripts and proteins following exposure to chilling has recently been reported for other chilling-sensitive crops, such as sunflower and rice seedlings (Hewezī et al. 2006; Yan et al. 2006). Nevertheless, besides efficiency. Moreover, both PCA and semi-quantitative RT-PCR analysis of selected genes confirmed the reliability and accuracy of the microarray data we obtained (Figs. 2 & 4, Table 3).

In general, we found that exposure to a low temperature of 5 °C for 2 weeks had tremendous effects on the transcriptome of grapefruit where the expression of approximately 7500 genes was significantly \((P \leq 0.05)\) affected by chilling. Utilizing very restrictive cut-off criteria for selection of CD- and chilling-responsive genes, we still identified more than 1700 probe sets that were affected by exposure to chilling (Table 1). These criteria were (1) probability of difference among treatments at \(P \leq 0.001\), (2) pairwise one-way ANOVA comparisons significantly different at \(P \leq 0.05\) and (3) induction or repression of transcript levels by at least fourfold. Among the chilling-responsive genes, a large group of 1345 probe sets \((371 \text{ up-regulated and } 974 \text{ down-regulated})\) were commonly affected by chilling in both control chilling-sensitive fruits and CD-treated chilling-tolerant fruits (Table 2). This group of transcripts was termed the ‘chilling-response regulon’, and it comprised transcripts involved in the natural and basic response of citrus fruit to low temperatures. Within the chilling-response regulon, we identified massive down-regulation of transcripts related to cell wall and defences against pathogens, photosynthesis, respiration, protein metabolism, DNA and RNA metabolism, secondary metabolism, water channels and senescence (Fig. 5, Table 4). Similar extensive down-regulation of cellular metabolism transcripts and proteins following exposure to chilling has recently been reported for other chilling-sensitive crops, such as sunflower and rice seedlings (Hewezī et al. 2006; Yan et al. 2006). Nevertheless, besides
Some of the changes in the transcriptome of citrus, a chilling-sensitive crop, to chilling (5 °C) resembled those observed in the transcriptome of Arabidopsis, a chilling-tolerant plant, following cold acclimation at 4 °C. For example, changes in lipid composition, increases in ABA biosynthesis transcripts, induction of β-amylase gene expression and induction of genes involved in the accumulation of compatible solutes, such as sucrose, glucose, proline, trehalose, mannitol and GABA (Fowler & Thomashow 2002; Hannah, Heyer & Hincha 2005; Vogel et al. 2005; Kaplan et al. 2007). Nevertheless, in the present study, we identified various low-temperature adaptation responses that appeared to be unique to citrus fruits and that have not been reported before, for example, the marked induction of ACC synthase and decrease in GA and JA biosynthetic transcripts (Fig. 6, Supplementary Table S1); induction and repression of different transcription factor genes from those of the well-characterized C-repeat binding factor (CBF) cold-responsive pathway in Arabidopsis (Supplementary Table S2) (Vogel et al. 2005); and induction of transcripts involved in sterol biosynthesis and metabolism (Supplementary Table S3). Another outstanding difference between the low-temperature responses of chilling-sensitive citrus fruits and chilling-tolerant Arabidopsis plants is that in citrus, exposure to chilling resulted in a massive down-regulation of gene expression (Tables 1, 2 & 4), whereas in Arabidopsis, low temperature rather favoured up-regulation of gene expression (Fowler & Thomashow 2002; Hannah et al. 2005; Vogel et al. 2005). Regardless of these observed similarities and differences in transcript profiling of chilling-sensitive citrus fruits as compared to chilling-tolerant Arabidopsis rosettes in response to chilling, it should be noted that both represent different types of tissues at different physiological and growth stages and, therefore, most likely adopt different protection strategies to cope with exposure to low temperatures. For example, young Arabidopsis rosettes respond to chilling by governing growth retardation and coordination of photosynthetic activity (Hannah et al. 2005), while grapefruit flavedo is rather a mature non-photosynthetic tissue with already completed its growth phase.

In the present study, we identified a group of 509 probe sets (178 up-regulated and 331 down-regulated) that were significantly affected by chilling specifically in the CD-treated, chilling-tolerant fruits (Fig. 3, Table 2). This group of transcripts was termed the ‘chilling-tolerance regulon’, and we considered that it comprised transcripts that were most likely involved in conferring chilling tolerance. Within this regulon, we identified some marked and unique acclimation processes. Firstly, we found that whereas chilling induced ACC synthase gene expression in control fruits, it strongly suppressed ACC oxidase transcript levels in CD-treated fruits (Fig. 6). These observations indicate that induction of ethylene biosynthesis may play a crucial role in causing chilling susceptibility. Furthermore, in the chilling-tolerance regulon, we identified a further decrease in ent-kaurene oxidase transcript levels and a parallel increase of an additional transcript of SCARECROW, suggesting further suppression of GA biosynthesis and signal transduction as compared to untreated fruits (Supplementary Tables S1–S2). Secondly, following CD, we identified marked increases in two HSF transcripts (Fig. 7b); because it is known that heat treatments can enhance chilling tolerance, these HSFs may play an important role in acclimation response (Sabehat, Lurie & Weiss 1998; Li et al. 2003). Thirdly, in the chilling-tolerance regulon, we observed specific increases of two fatty acid desaturase transcripts (Fig. 8). These findings are in full agreement with previous studies that demonstrated the importance of fatty acid desaturases in increasing membrane fluidity and chilling tolerance (Murata et al. 1992; Miquel et al. 1993). In addition, we found that the CD treatment also induced the expression of ceramide glucosyltransferase, a key enzyme that catalyses the initial step in glycosphingolipid biosynthesis (Ichikawa et al. 1996) (Fig. 8), indicating that glycosphingolipids may play an important role in regulating membrane properties and chilling tolerance. Fourthly, in addition to the common induction of starch breakdown, we found a 26-fold increase of SPS1 transcript levels after CD, which suggests that enhanced accumulation of sucrose may improve chilling tolerance. Fifthly, in the chilling tolerance regulon, we observed further down-regulation of transcripts involved in secondary metabolism, especially those involved in phenylpropanoid biosynthesis (Table 4). Overall, the main molecular mechanisms related to the acquisition of chilling tolerance are summarized in Fig. 11a.

The last group of 417 probe sets (134 up-regulated and 283 down-regulated) were significantly affected by chilling specifically in control chilling-sensitive fruits (Fig. 3, Table 2). This group of transcripts was termed the ‘chilling-stress regulon’, and we considered that it comprised transcripts related to chilling susceptibility. Within this regulon, it is worth noticing that we identified only down-regulation of lipid and sterol metabolism transcripts, suggesting that adaptations in lipid metabolism are crucial for low-temperature fitness (Supplementary Table S3). Furthermore, in the chilling-stress regulon, we identified marked down-regulation of transcripts for enzymes involved in wax biosynthesis and deposition. This finding is very reasonable, because it is known that the content and composition of the epicuticular wax affect chilling tolerance and that post-harvest application of wax coatings reduces CI (McDonald, Nordby & McCollum 1993; Wild 1993). In the chilling-stress regulon, we also identified specific increases in transcript levels of two stress-related transcription factors: a dehydration-induced MYB and an NAC-domain protein (Supplementary Table S2). This indicates that the control untreated fruits activated stress-related gene expression. The specific induction of the NAC-domain transcription factor in the pitted area of chilling-damaged orange fruits has recently been reported by others (Gao et al. 2007). Finally, in the chilling-stress regulon, we detected down-regulation of transcripts involved in the biosynthesis of ascorbate and α-tocopherol. These compounds are antioxidants involved in scavenging reactive oxygen species and in...
Figure 11. Models describing the molecular mechanisms involved in the acquisition of chilling tolerance (a) and susceptibility (b) in grapefruit. (a) Adaptation processes defined in the chilling-tolerance regulon including repression of ethylene and gibberellic acid (GA) biosynthesis transcripts, up- and down-regulation of particular transcription factors, modifications in lipid metabolism, induction of sucrose accumulation and repression of secondary metabolism. (b) The molecular processes defined in the chilling-stress regulon including down-regulation of transcripts related to lipid and steroid metabolism and of wax biosynthesis and deposition, up-regulation of specific stress-related transcription factors and down-regulation of antioxidant biosynthesis transcripts. EREB, ethylene-responsive element binding; FAD, fatty acid desaturase; HSF, heat shock factor; PR, pathogenese-related; TF, transcription factor.

preventing lipid peroxidation; they are required for chilling and stress tolerance in general (Guo et al. 2005; Munne-Bosch 2005). Overall, the molecular mechanisms associated with the chilling-stress regulon and chilling susceptibility are summarized in Fig. 11b.

ACKNOWLEDGMENTS

This chapter is a contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, no. 491/07. This research was supported by Research Grant No. IS-3499-03R from the United States–Israel Binational Agricultural Research and Development Fund (BARD).

REFERENCES


Wolucka B.A., Persiau G., Van Doorsselaere J., Davey M.W.,


Received 19 October 2007; received in revised form 10 January 2008; accepted for publication 11 January 2008

SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Table S1. Effects of conditioning (CD) and chilling treatments on the expression of hormone biosynthetic genes in grapefruit. Data include transcripts differentially expressed at \( P \leq 0.05 \) and induced or repressed by a factor of at least 4.

Table S2. Effects of conditioning (CD) and chilling treatments on the expression of transcription factor genes in grapefruit. Data include transcripts differentially expressed at \( P \leq 0.05 \) and induced or repressed by a factor of at least 4.

Table S3. Effects of conditioning (CD) and chilling treatments on the expression of lipid biosynthetic genes in grapefruit. Data include transcripts differentially expressed at \( P \leq 0.05 \) and induced or repressed by a factor of at least 4.

This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-3040.2008.01793.x

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.