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Effects of harvest time on chilling tolerance and the transcriptome of 'Wonderful' pomegranate fruit

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ABSTRACT

We observed that harvest time markedly affected chilling tolerance of 'Wonderful' pomegranate fruit; early-harvested fruit were extremely chilling sensitive, whereas late-harvested ones were relatively chilling tolerant. Damage to inner membranes is the most obvious phenotypic damage observed in pomegranate fruit exposed to chilling. To elucidate the molecular mechanisms that govern chilling tolerance of pomegranate fruit, we conducted RNA-Seq analysis of inner membrane tissues from early- and late-harvested fruit on harvest day and after a 2-week exposure to a cold quarantine treatment at 1 °C. Pair-wise comparisons revealed that 6853 transcripts were significantly ($p \leq 0.01$) induced or repressed by a factor of at least 4 after exposure to chilling in early-harvested fruit, as compared with 8000 transcripts in late-harvested ones. In early-harvested, chilling-sensitive fruit most (63%) differentially expressed transcripts were down-regulated by cold storage, whereas in late-harvested fruit most (62%) differentially expressed transcripts were up-regulated, indicating activation of adaptation processes. The results demonstrate that transcripts related to several regulatory, metabolic, and stress-adaptation pathways were specifically induced in late-harvested fruit while suppressed in early-harvested, chilling-sensitive fruit. These regulatory mechanisms included activation of jasmonic acid and ethylene biosynthesis and signal transduction pathways, induction of various stress-related transcription factors, including AP2/ ERFs, MYBs, WRKYs, bHLH, homeobox, and HSFs. The observed changes in transcripts related to metabolic pathways involved primary and secondary carbohydrate metabolism, including activation of starch degradation and of galactinol and raffinose biosynthesis genes. Finally, we observed up-regulation of transcripts corresponding to stress-tolerance, most notably heat shock proteins.

1. Introduction

Global export of pomegranates (*Punica granatum* L.) to fly-free zones necessitates implementation of cold quarantine treatments. For example, the cold-quarantine treatment against the Mediterranean fruit fly (*Ceratitus capitate*) approved by the US Animal and Plant Health Inspection Service (APHIS) involves exposure of the fruit to an internal temperature below 1.1 °C for at least 14 d (USDA, 1976; Powell, 2003). However, pomegranate fruit are chilling-sensitive; and when exposed to low temperatures they might develop chilling injuries (CI), manifested primarily as internal browning of the white spongy tissue surrounding the arils and the inner membranes, and as pitting on the outer peel surface (Elyatem and Kader, 1984; Opara et al., 2015; Pareek et al., 2015). The optimal safe temperatures recommended for postharvest storage of 'Wonderful' pomegranates are 5 °C for storage periods of up to 2 months, and 7.2 °C for longer storage periods (Elyatem and Kader, 1984; Crisosto et al., 1996).

In a previous study, we found that harvest time had a dramatic effect on the CI sensitivity of 'Wonderful' pomegranates: early-harvested fruit were extremely sensitive to chilling, mid-season ones moderately sensitive, and late-harvested fruit were relatively chilling tolerant and showed no CI symptoms even after 4 weeks of cold storage at 1 °C followed by 1 week under shelf-life conditions at 20 °C (Kashash et al., 2016). In order to investigate the molecular mechanisms involved in acquisition of chilling tolerance in 'Wonderful' pomegranate fruit, we conducted RNA-Seq analysis of inner membrane tissues from early- and late-harvested fruit, on harvest day and after 2 weeks of cold storage at 1 °C. We thereby identified differentially expressed transcripts that were up- or down-regulated in early- and late-harvested fruit, respectively.

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2. Materials and methods

2.1. Plant material and storage conditions

Pomegranate fruit, cv. 'Wonderful', were harvested from the Lachish experimental farm, in the southern lowland area of Israel, at two different periods during the 2016 season; early-harvested fruit on September 21 and late-harvested ones about a month later on October 25. After harvest, the fruit were brought to the Department of Postharvest Science at the Volcani Center, and were placed in cold storage at 1 °C for 2 weeks. Five boxes were taken from each harvest, each containing 9–11 fruit.

2.2. Chilling injury evaluations

Chilling injury was evaluated according to the severity of chilling symptoms, manifested as internal browning of the white spongy tissue and inner membranes, as described by Kashash et al. (2016); it was conducted after 2 weeks of cold storage at 1 °C followed by 1 week under shelf-life conditions at 20 °C. The fruit were sorted and scored according to their CI severity: 0 = none; 1 = slight, i.e., a few scattered brown spots; 2 = moderate browning on up to 30% of the white inner tissues; and 3 = severe, i.e., extensive browning covering > 30% of the white inner tissues. The CI index was calculated by multiplying the number of fruit in each category by their score, and dividing by the total number of fruit assessed. The CI percentage was based on the total number of fruit with CI symptoms regardless of their severity.

2.3. Color measurements

For color measurements, 10 fruit per treatment were randomly chosen and their peel color was determined by measuring their hue angle with a Chromometer, Model CR-400 (Minolta, Tokyo, Japan); a hue angle of ~90° represents yellow, ~60° orange, and ~30° red. Data are means of 10 measurements, one per each fruit.

2.4. Juice soluble solids and titratable acidity

Total soluble solids (TSS) content in the juice was determined with a PAL-1 digital refractometer (Atago, Tokyo, Japan), and acidity percentages were measured by titration to pH 8.3 with 0.1 M NaOH by means of a Model CH-9101 automatic titrator (Metrohm, Herisau, Switzerland). Each measurement comprised five replications, each using juice collected from three different fruit, i.e., a total of 15 fruit per measurement.

2.5. RNA isolation, cDNA library construction and RNA-Seq

Inner membranes of 'Wonderful' pomegranate fruit were collected at four different time-points: (i) early harvest, time zero (designated as Early-T0); (ii) early harvest + 2 weeks at 1 °C (Early-chilling); (iii) late harvest at time zero (Late-T0); and (iv) late harvest + 2 weeks at 1 °C (Late-chilling). The collected tissues were frozen in liquid nitrogen and stored at -80 °C pending RNA extraction. Overall, we collected three samples for each time point, each comprising inner membrane tissues collected from three different fruit. Total RNA was extracted according to the CTAB protocol (Chang et al., 1993). RNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and RNA purity and integrity were further verified with a Model 2100 Total RNA BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Library preparation and sequencing were performed at the Crown Institute for Genomics, in the Weizmann Institute of Science, Rehovot, Israel. Twelve single-end RNA-Seq libraries with a length of 125 nucleotides were prepared with a Hiseq 2000 instrument (Illumina Inc., San Diego, CA, USA) and separated on two different lanes.

2.6. Transcriptome analysis: mapping and assembly

For transcriptome analysis the raw reads were filtered and cleaned; the SortMeRNA tool was used to filter out rRNA (Kopylova et al., 2012) and then the Trimmomatic tool was used to remove adaptors (Bolger et al., 2014). Then, the FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html, version 0.0.13.2) was used to trim read-end nucleotides with quality scores < 30. Clean reads were aligned to the *Punica granatum* (pomegranate) genome extracted from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/assembly/GCA_002201585.1/) by using Tophat2 software (v 2.1) (Kim et al., 2013). Gene abundance was estimated with the Cuffflinks software (v 2.2) (Trapnell et al., 2010) combined with gene annotations from the NCBI.

2.7. Differential expression analysis

Differential expression was analyzed with the DESeq2 R package (Love et al., 2014). Genes that differed from the control by a factor greater than four, with an adjusted *p*-value of no more than 0.001, were considered differentially expressed (Benjamini and Hochberg, 1995). Heatmap visualization was performed with the R Bioconductor software (Gentleman et al., 2004). Functional categorization and incorporation into metabolic pathways were conducted with the MapMan software (Thimm et al., 2004), and Venn diagrams were generated with the online tool at bioinformatics.psb.ugent.be/webtools/Venn/.

3. Results

3.1. Effects of harvest time on chilling tolerance

We examined the chilling tolerances of early- and late-harvested 'Wonderful' pomegranate fruit. The ripening indices of the early- and late-harvested fruit used in this study are presented in Table 1, which shows that juice TSS levels were significantly higher in late-harvested fruit, whereas acidity levels decreased and were somewhat lower in late-harvested fruit. Furthermore, the hue angle of the peels was ~44° for early-harvested fruit and ~22° for late-harvested fruit, i.e., the peels of late-harvested fruit were darker and redder than those of early-harvested ones (Table 1).

It was found that early-harvested fruit were extremely chilling sensitive; they exhibited severe chilling damage, manifested as internal browning, after 2 weeks of cold storage at 1 °C followed by 1 week under shelf-life conditions at 20 °C (Fig. 1). In contrast, late-harvested fruit were relatively chilling tolerant and did not show CI symptoms under similar storage conditions (Fig. 1). Overall, the CI index scores of early- and late-harvested fruit were 1.93 and 0.05, respectively (Fig. 1). It should be noted that CI symptoms were not visually apparent immediately after 2 weeks of cold storage at 1 °C but rather appeared only after transference to shelf-life conditions at 20 °C.

Table 1
Ripening indices of early- and late-harvested 'Wonderful' pomegranate fruit on the day of harvest. TSS and acidity data represent means ± SE of 5 replications, and Hue angle data represent means ± SE of 10 replications. Means followed by different letters are statistically different according to Student's *t*-test at $p \leq 0.05$.

	TSS (%)	Acidity (%)	Hue angle (H°)
Early-harvest	15.72 ± 0.15 b	1.51 ± 0.06 a	43.98 ± 1.39 a
Late-harvest	16.47 ± 0.09 a	1.23 ± 0.15 a	21.78 ± 1.79 b