Identification and characterization of two putative genes encoding acetyl-coenzyme A carboxylase subunits that are possibly associated with internal browning during cold storage of 'Hass' avocados (*Persea americana* Mill.)

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A B S T R A C T

Cold storage is widely used to extend the postharvest life of 'Hass' avocados (*Persea americana* Mill.). However, prolonged low-temperature storage results in the development of chilling-induced physiological disorders. Additionally, the response of avocados to cold storage depends on their stage of maturity, as late-harvest avocados are more susceptible to developing physiological disorders than those that are harvested early in the season. To understand the molecular mechanisms that cause physiological disorders in cold-stored 'Hass' avocados, we sought to identify and characterize the lipid metabolism-related genes. In this work, we focused our analysis on the genes that encode a multi-subunit acetyl-CoA carboxylase enzyme (MS-ACCase), which is one of the key enzymes in fatty acid biosynthesis. Two avocado MS-ACCase subunits were identified by *in silico* analysis, a biotin carboxylase (BC) and a biotin carboxyl carrier protein (BCCP). Transcriptional qPCR analyses of both identified gene subunits were performed in avocados that had been harvested both early and late in the season after 40 d of storage at 0 and 5 °C and subsequent ripening at 20 °C. For both harvest dates, *PamACCase-BC* and *PamACCase-BCCP* transcripts increased in ripe fruit after cold storage at 5 °C; this increase was significant for *PamACCase-BCCP*. A similar expression pattern was observed in ripe avocados from early-harvested fruit after cold storage at 0 °C; however, significant down-regulation of *PamACCase-BC* and *PamACCase-BCCP* expression was observed in ripe late-harvested avocados, suggesting that these fruit have senesced. A control study involving late-harvested avocados demonstrated that the expression of these genes did not change throughout ripening. Thus we suggest that the MS-ACCase *BCCP* subunit might be transcriptionally regulated during physiological disorder development in cold-stored 'Hass' avocados.

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1. Introduction

Low temperature storage between 4 and 7 °C is the most commonly used method to extend the postharvest life of avocado fruit (Bower and Cutting, 1988; Woolf et al., 2004). A major limitation of 'Hass' avocado storage is the development of physiological disorders at low temperatures, some of which are related to chilling injury (CI) (Woolf et al., 2003). These disorders may be present during cold storage or after transferring the fruit to room temperature (Florissen et al., 1996; Covarrubias et al., 2007) and depend on storage temperature and duration (Bower and Cutting, 1988). CI symptoms during extended cold storage are manifested in several ways, such as internal browning, vascular browning and increased pathogen susceptibility (García-Rojas et al., 2012).

Avocados harvested at the end of the season (late harvest) have a higher incidence of cold storage-induced disorders (Dixon, 2003), indicating that maturity at harvest and ripeness are important factors in determining avocado fruit susceptibility to physiological disorder development (García-Rojas et al., 2012).

CI-induced internal browning has been linked to peroxidase and polyphenol oxidase activities (Wongsheree et al., 2009). However, other metabolic processes may be altered in cold-injured fruit (Nilo et al., 2010). For example, CI is accompanied by alterations of the cell membrane conformation and structure (Badea and Basu, 2009), particularly because of lipid degradation (Campos et al., 2003). These metabolic processes trigger changes at the transcriptional and post-transcriptional levels and differential gene expression (Badea and Basu, 2009). In previous experiments using suppression subtractive hybridization (SSH), we identified genes encoding key lipid metabolism enzymes that were differentially expressed in late-harvested avocados that were cold-stored for 40 d at 5 °C (García-Rojas et al., 2012). From the SSH library, a gene encoding acetyl CoA carboxylase (ACCase) was identified.

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There is evidence that plant ACCase is highly regulated and is a major control point for *de novo* fatty acid biosynthesis (Schmid and Ohlrogge, 2002). This enzyme catalyzes the adenosine triphosphate (ATP)-dependent carboxylation of acetyl-CoA to malonyl-CoA in a primary reaction that occurs during *de novo* fatty acid biosynthesis (Li et al., 2010). In plants, plastid ACCase provides malonyl-CoA for 16- 18-carbon fatty acid biosynthesis, while cytosolic ACCase produces the malonyl-CoA used for elongation of these fatty acids to 20 carbons or more as well as for flavonoid, stilbenoid, malonic acid, and other metabolite biosynthesis (Ke et al., 2000; Li et al., 2010). Two structurally distinct multi-subunit (MS) and multi-functional (MF) ACCase forms have been identified in plants (Schulte et al., 1997). Most plants have both forms; the MS is expressed in plastids, and the MF is expressed in the cytosol. One exception to this observation is the grass family, which expresses the MF form in both locations (Sasaki and Nagano, 2004). The MS-ACCase is composed of independent polypeptides, biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and carboxyltransferase (CT) complex subunits α and β (αCT and βCT) (Shintani et al., 1997), whereas the MF is a large polypeptide with four subunit domains (Shorrosh et al., 1995). The identification of MS-ACCase has been difficult because this enzyme is extremely labile and difficult to isolate (Sasaki and Nagano, 2004). Therefore, an alternative method to protein purification involves the isolation and characterization of the genes that encode each of the plastid ACCase subunits.

Because ACCase might provide malonyl-COA constitutively for the production of fatty acids for membrane synthesis and maintenance (Roesler et al., 1994), we sought to isolate and characterize the plastid subunit genes that encode for MS-ACCase and to investigate the relationship between these genes and cold storage-induced physiological disorders in early- and late-season 'Hass' avocados.

2. Materials and methods

2.1. Fruit material

Avocado fruit (*Persea americana* Mill. cv. Hass) were harvested both early and late from a commercial orchard in Quillota (32°52′ S, 71°15′ W), Chile. Dry matter was used as a harvest index. The avocados were collected with 24.1 and 28.5% of dry matter average in October (early harvest) and February (late harvest), respectively. Medium-sized fruit were selected and transported to the INIA postharvest laboratory. For postharvest storage experiments, the fruit were stored at 5 and 0 °C for 40 d with subsequent storage at 20 °C until the fruit ripened. Postharvest storage at 0 °C was performed to induce avocado chilling injury. Additionally, a control assay was conducted with late-harvest avocados that were harvested and immediately stored at 20 °C until they were ripe. In addition, leaves (L), buds (B), flowers (F), developing fruit (DF) and ripe fruit (RF) were stored at −80 °C until further molecular analyses.

2.2. Fruit parameters

The avocados were evaluated for dry matter content; physiological disorders, including external damage and internal browning; color changes; and flesh firmness. Dry matter determination was performed by oven drying. For the drying process, half of each vertically sliced fruit was peeled, the seed coat was removed and the flesh was chopped and weighed. Each sample was dried for 24 h at 103 °C until a constant weight was reached.

External damage was assessed after cold storage at 0 and 5 °C, and internal browning and flesh firmness were assessed after cold storage and again after ripening at 20 °C. External damage was manifested as skin surface blackening, skin spotting or irregular dark patches on the skin. A hedonic scale was used to classify the internal browning (flesh graying) from 1 to 5 (1: no occurrence, 2: slight, 3: moderate, 4: moderately severe and 5: severe). Flesh firmness was measured using a penetrometer (Effegi, Milan, Italy) equipped with a 4 mm plunger tip. Skin color was assessed visually using a hedonic scale with scores from 1 to 5. For molecular analyses, the flesh of six fruit from each condition were frozen in liquid nitrogen, homogenized and stored at −80 °C until use. For conditions that presented physiological disorders, those fruits that showed the highest internal browning intensity within each condition were selected for molecular analyses.

2.3. RNA isolation and cDNA synthesis

The total RNA from the avocado samples was isolated using a modified hot borate method (Gudenschwager et al., 2012). *In vitro*-synthesized RNA from the *DAP* gene (dihydrodipicolinate reductase) was obtained from ATCC (number 87486) and was added as a spike mRNA to normalize the expression data (González-Agüero et al., 2008). The first cDNA strand was obtained by reverse transcription with MMLV-RT reverse transcriptase using 2 μg of total RNA as a template (Promega, Madison, WI, USA) and oligo dT primers (Invitrogen, Breda, The Netherlands).

2.4. Isolation and in silico analysis of ACCase cDNA sequences

An *in silico* search with the BLASTN program (Altschul et al., 1990) was performed to identify the avocado expressed sequence tags (EST) encoding for MS-ACCase proteins using *Arabidopsis* BC (NM_122927), BCCP (NM_121557), αCT (NM_129360) and βCT (NC_000932) as queries. To obtain full-length cDNA of the avocado-identified gene subunits, *PamACCase-BC* and *PamACCase-BCCP*, RACE-PCR assays were performed according to the GeneRacer kit instructions (Invitrogen, Breda, Netherlands). Primer sequences for 3′ and 5′ RACE amplifications were based on the ESTs found and were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, USA). The primer sequences were as follows: *PamACCase-BC* (1f) 5′-CTGTTTATGCTGTATTTCTGACTG-3′, *PamACCase-BC* (1r) 5′-CCAGAGGGCACGTATGCAGTT-3′; *PamACCase-BC* (2r) 5′-TTTCCCTCGCAGTAGCTTTATC-3′, *PamACCase-BCCP* (1f) 5′-GGCAGGAACATTCTATCGGAGC-3′; *PamACCase-BCCP* (1r) 5′-TCAATGATGCACAACACTTGTCC-3′, *PamACCase-BCCP* (2r) 5′-ATAGCTGCTGGAGGGGAGGGT-3′; and *PamACCase-BCCP* (3r) 5′-ATGGTTCATTGGCATCCTTGTCT-3′. The obtained fragments were cloned using a TA cloning strategy into pGEM T-EASY vector (Promega) according to the manufacturer's recommendations. The positive clones were purified using a Plasmid Mini Kit II (OMEGA, USA), sequenced at Macrogen (Seoul, Korea) and compared to sequences from the National Center for Biotechnology Information (NCBI) using the BLAST alignment programs (http://www.ncbi.nlm.nih.gov/BLAST/).

The nucleotide sequences of *PamACCase-BC* and *PamACCase-BCCP* were translated, and the ORFs were identified using ORF Finder (Wheeler et al., 2003) and Swiss-Model Tools (Arnold et al., 2006). Multiple sequence alignments were performed using the ClustalW algorithm with Geneious 4.8 software. The subcellular localization of the *PamACCase-BC* and *PamACCase-BCCP* proteins was predicted using TargetP software (http://www.cbs.dtu.dk/services/TargetP/).

2.5. Real-time quantitative PCR assays (qPCR)

The transcript abundance of the genes in this study was analyzed by real-time PCR with the LightCycler Real-Time PCR