ORIGINAL PAPER

Two Oleosins Expressed in the Mesocarp of Native Mexican Avocado, Key Genes in the Oil Content

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Abstract

Intracellular lipid droplets (LD) provide the oil storage mechanism of plants. They are found within seeds as individual structures, even under conditions of cold stress and dehydration, due to the protein that covers them. This protein, called oleosin, is found exclusively in plants and has been widely studied in seeds. Avocado fruits (*Persea americana* Mill.) are rich in oil, which is stored in the mesocarp, not in the seeds. The presence of oleosin in the mesocarp tissue of avocadoes has been reported, but its physiological role is still unknown. In this study, we identify two genes that code for oleosin in the mesocarp of the native Mexican avocado. These sequences are very different from those of seed oleosins. Both genes are expressed during fruit ripening, while one, *PaOle1*, has the highest expression in the green fruit stage. The protein of PaOle1 is stable during the fruit ripening process and covers all the mesocarp LDs. The expression of PaOle1 gene and protein is organ specific to avocado mesocarp. Among avocadoes varieties oleosin abundance is directly related to oil content.

Keywords Avocado · *Drymifolia* · Oleosin · Lipid droplet

Introduction

Oleosins are small proteins measuring 17 to 25 kDa, found exclusively in plants. They are involved in stabilizing lipid storage and have a hydrophobic hairpin with a proline knot in the middle. The carboxyl and amino terminal regions give them an electrostatic charge density that is transferred to the lipid droplets. This charge is responsible for avoiding coalescence of LDs [1]. Oleosins have been thoroughly described in seed tissues, where they are the principal element responsible for maintaining the integrity and the size of LDs through dehydration, vernalization and rehydration before germination

[2]. Oleosins evolved from similar oleo-like proteins that are present in unicellular algae. The first oleosins are characterized by the presence of low-expression codifying oleosin transcripts in chlorophytes (*Chlamydomonas reinhardtii*), and its protein are detectable in the LDs of charophytes (*Spirogyra grevilleana*) [3]. Research has identified five well-differentiated lineages of these proteins: 1) primitive oleosins (P), from algae, mosses and ferns; 2) universal oleosins (U), found in all terrestrial plants; 3) low molecular weight oleosins (SL), on seeds from gymnosperms to angiosperms; 4) high molecular weight oleosins, in seeds from angiosperms (SH); and 5) oleosins in tapetum (T), expressed in anthers from plants of the Brassicaceae family and *Olea europaea* [4, 5]. Recent studies report a new lineage of oleosins expressed in the mesocarp that is exclusive to the Lauraceae family [6]. Analyses of the mesocarp of Hass avocadoes show that mesocarp oleosins covers only the smallest LDs [6]. The Hass avocado (*Persea americana* cv. Hass) is a hybrid cultivar that comes from Guatemalan (*P. americana* var. *guatemalensis*) and Mexican (*P. americana* var. *drymifolia*) varieties [7]. The Mexican native avocado variety is 42% of the gene source of the Hass cultivar [8]. The fruit of this cultivar has recently become very popular, and an important component of human

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nutrition. The Mexican avocado variety has a mid-size fruit, with the highest oil content [9, 10], a thin peel, and a large seed which is over 50% of the weight of the fruit. They are adapted to growth at high altitudes (>1000 masl) and cold climates. The fruits and leaves are used as food and traditional medicine, and this is the only variety with an aniseed scent [11]. Recent work has shown that the Mexican avocado has mid-size LDs that bring a higher oil yield with a greater polyunsaturated fatty acid oil profile. This differs from the Hass avocado, which has bigger LDs but a lower oil yield and more saturated fatty acids in its oil profile [10]. In this work, we describe and analyze two oleosin genes found in the mesocarp of the native Mexican avocado (*P. americana* var. *drymifolia*). We measured gene expression in different tissues to identify were oleosins occur. Then we used western blot analysis to determine the relative quantity of oleosins between mesocarp from different avocadoes, Finally we used flow cytometry and confocal microscopy to locate oleosin on isolated LDs.

Materials and Methods

Oleosin Sequences and Phylogeny

Two publicly available transcriptome (ESTs) databases [12, 13] for Mexican avocadoes were used to determine if there was more than one oleosin gene. BLAST searches were performed using a UGENE V 33, BLASTn algorithm V 2.2.26, and a 477 nt EST sequence (GenBank access no. KF006324.1 *PaOle1*). The top 10 best-hit sequences were reviewed, to obtain *PaOle1* and a 730 nt candidate sequence. Oleosin amino acid sequences from Lauraceae were obtained from oleosin transcripts from organs and tissues of avocado and other Lauraceae species (*Lindera* and *Litsea*) [6]. The Entrez direct tool (Edirect V 7.4) was used to retrieve oleosin amino acid sequences from the RefSeq database [14]. Entrez sequences were depurated by hand eliminating duplicates and non-oleosin sequences. Oleosin amino acid sequences were aligned with ClustalW V 2.1, using standard parameters (Gap Open penalty: 10.0, Gap extension penalty: 0.1). These data were used in SeaView software [15] to construct a neighbor-joining and bootstrap phylogenetic tree of 1000 replicates. Oleosin amino acid sequences from *Spirogyra grevilleana* were obtained from the NCBI database and used for rooting the tree.

Sample Collection

Samples of *P. americana* var. drymifolia, at six and eight months postanthesis (green stage and physiological maturity, respectively), were collected from Petembo, Tacámbaro, Michoacán, Mexico (lat. 19°07′29″N, long. 101°29′15″W). The fruits of eight months were allowed to ripen at room temperature (ripe stage). Ripe samples of *P. americana* var. americana were purchased in a market in Mérida, Yucatán, Mexico (lat. 20°41′49″N, long. 89°48′4″W), and ripe *P. americana* cv. Hass avocadoes were acquired in a market in Morelia, Michoacán (lat. 19°41′46″ N, long. 101°11′18″ W). All fruits were separated into peel (pericarp), pulp (mesocarp) and seed (embryo and cotyledon). Leaves and roots were obtained from *in vitro* seedlings. Tissues were finely powdered in liquid nitrogen and stored at −80 °C until use.

Primer and Antibody Design

PCR primers for the variable regions of the *PaOle1* and *PaOle2* oleosins, as well as for ACP-Δ9-desaturase (*PaFad*) and ribosomal 60S (*Pa60S*) genes, were designed using UGENE V 33 (Table 1 Supplementary Material). In addition, antibodies were designed against the 14 variable amino acids from the amino terminal region of the PaOle1 protein (NH3-MADQPKTIKQTERA-CO2H) chosen by its high antigenicity and low hydrophobicity according to Antibody Epitope Prediction (http://tools.immuneepitope.org/bcell/), polyclonal antibodies were produced by Biomatik™, CA.

RNA Isolation and Semiquantitative PCR

Total RNA was isolated from flower, embryo, cotyledon, and mesocarp from green and ripe stages using the LiCl precipitation method [16], and then digested with DNase I (Invitrogen™ USA) for use in cDNA synthesis (SMART™MMLV Reverse Transcriptase Clontech™ USA), following the manufacturer's instructions. Gene expression was analyzed by semiquantitative PCR (SQPCR) from 100 ng of cDNA using primers described above and respective conditions (start with 5 min, 95 °C; then 35 cycles of: 45 s, 95 °C; 30s at primers Tm; 45 s, 72 °C; and finish with 5 min, 72 °C; ∞, 4 °C). PCR products were measured in 1.2% TBE-agarose gel with 0.1% ethidium bromide stain.

Protein Isolation and Western Blot Assay

Total protein extracts from leaf, root, seed, peel, and mesocarp from *Persea americana* var. *drymifolia*; and, from mesocarp from *P. americana* var. americana and *P. americana* cv. Hass, were obtained using the phenol-ammonium acetate precipitation method [17]. Bradford quantification was applied to the protein extracts. Total protein extracts were resolved in 13% denaturing polyacrylamide gel electrophoresis (SDS-PAGE), and then stained with Coomassie blue. Proteins were transferred to a PVDF membrane (Immobilon-P™ 0.45 μm Millipore™ USA). The quick method of the western blot assay was performed following the membrane manufacturer's protocol, using anti-PaOle1 as the primary antibody, a goat anti-rabbit IgG-AP (catalog no. 7054, Cell Signaling Technology™ USA) as the

Table 1 Properties of avocado mesocarp oleosins (*Persea americana* var. *drymifolia*),with data from the NCBI GenBank. * Isoelectric point

Name	Id Gen	CDS (bp)	Id Protein	Size aa/kDa	pI*	Hairpin (aa)
PaOle1	KF006324	477	AGT63296.1	159/17.47	10.02	15–91
PaOle2	KX682343	513	ARO50424.1	171/17.71	10.14	42–121

secondary antibody, and NBT/BCIP (catalog no. 00–2209 Invitrogen™ USA) as the enzyme substrate.

Lipid Droplet Isolation, Flow Cytometry and Confocal Microscopy

The lipid droplets were isolated by flotation in neutral detergent (Tween 20) and ionic solution (2 M NaCl), then rinsed twice with phosphate saline buffer (PBS; 10 mM phosphates, pH = 7.4, 9% NaCl) and re-suspended in PBS (modified from Tzen [18]). The isolated LDs were diluted to an optical density of 0.5 at 600 nm for use, and then analyzed by flow cytometry (BD Accuri™ C6,Becton Dikinson™ USA), using BD Accuri™ C6 Analysis Software, and data acquisition from 10,000 events. The fluorescence channels (FL1 533/30, FL2 585/40, FL3 670LP) were set at logarithmic gain. The oleosin

on the LDs surfaces was detected using anti-PaOle1 as the primary antibody and conjugated anti-rabbit-fluorescein (catalog no. F2765 Life technologies™ USA) as the secondary antibody. Laser confocal scanning microscopy (LCSM) was used to visualize the tagged LDs. Fluorescein signaling, with a 494 nm excitation line and 512 nm emission line was observed under a confocal microscope (Olympus™ JP 1200, series 4 K43415) equipped with a − 525 BA505 filter.

Statistics

The electrophoresis gels and western blot membrane images were analyzed by densitometry with ImageJ software [19]. For gene expression the data was normalized dividing by *Pa60s* expression, then analyzed through Kruskall-Wallis test for each gene among tissues using R software [20].
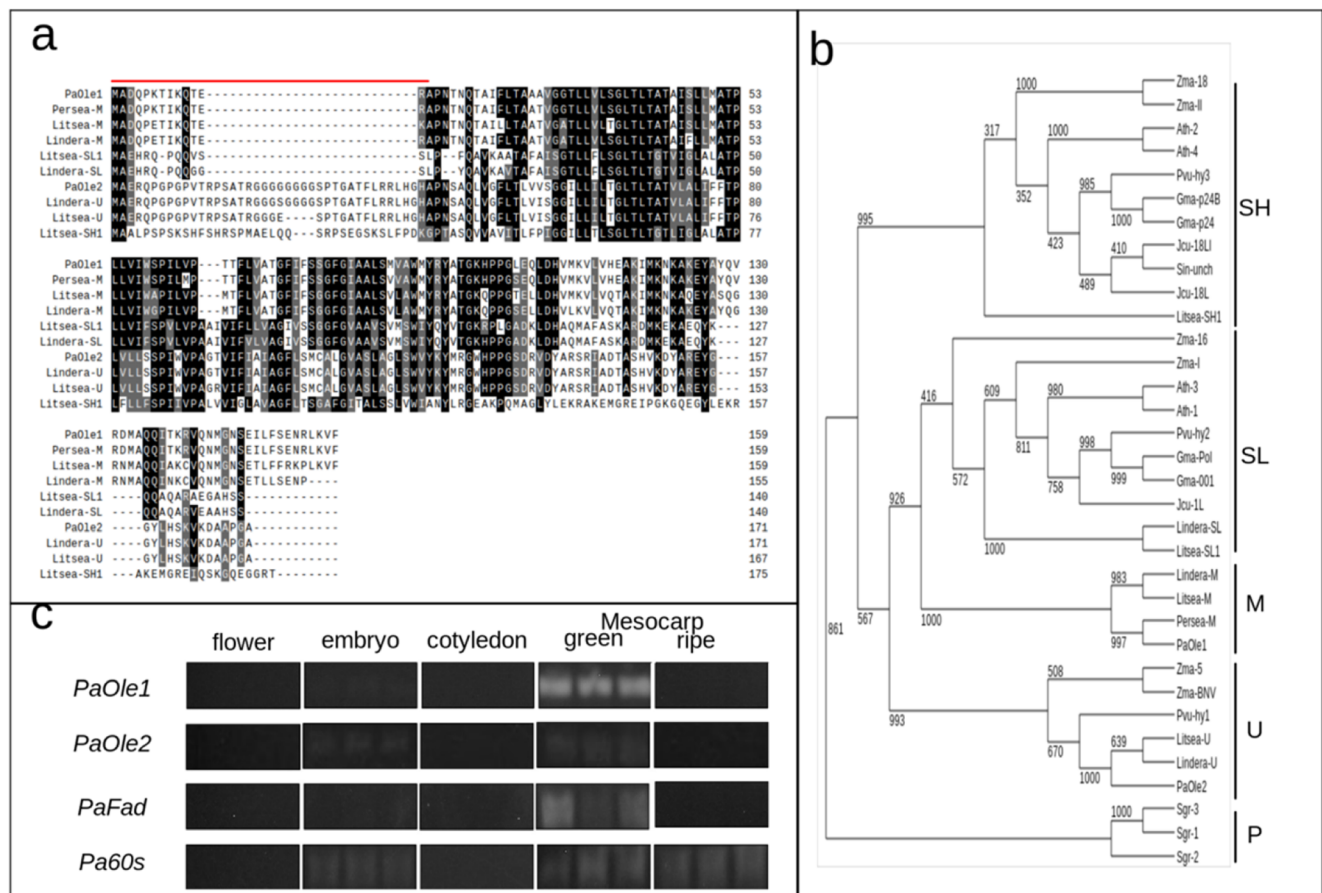

Fig. 1 **a** Aminoacid sequences Alignment from PaOle1, PaOle2 and LAURACEAE oleosins from [6]. Red line covers the antigenic region used for antibody design. **b** Neighbour joining phylogenetic tree from aminoacid sequences from refseq data base (Table 2 Supplementary Material). Five oleosin lineages are shown. **c** SQPCR gels for four genes and five tissues from *Persea americana* var. *drymifolia*

Results and Discussion

Two Oleosins Genes Expressed in the Mesocarp of Mexican Avocadoes

The blast search revealed the presence of a closely related sequence that shares the hydrophobic hairpin and proline knot from oleosins. We reported this new sequence, named *PaOle2*, and we uploaded to the NCBI database (GenBank accession number KX682343; Table 1).

These sequences were aligned with Lauraceae oleosins [6], which shows that *PaOle1* belongs to the mesocarp oleosins, an exclusive lineage of oleosins found only in mesocarp tissues from Lauraceae plants. *PaOle2*, however, possess an ~AAPGA termini and a high homology (>95%) with universal oleosins [4, 6] (Fig. 1a).

An alignment and a phylogenetic tree were constructed using oleosin amino acid sequences (Table 2 Supplementary Material) from mono and dicotyledonous plants from Reference sequence data base (NCBI-RefSeq) [14] (Fig. 1b). This confirms that mesocarp oleosins are more closely related to low molecular weight seed oleosins than to other lineages. The universal oleosin lineage was developed earlier in oleosins evolution and are the basal ancestors for all other oleosin lineages [4]. We found that avocado have just two oleosin genes, one from the universal lineage and one from the mesocarp lineage.

PaOle1 Is Expressed Only in Mesocarp Tissue

SQPCR was used to detect oleosin gene expression in mesocarp at two ripening stages (green and ripe) and in flower, embryo, and cotyledon tissues (Fig. 1c). We found that only two genes (*PaOle1* and *PaFad*) are strongly expressed in the mesocarp in the green stage. There was no expression in the mesocarp in the ripe stage. During climacteric stage the ethylene production increase and this increase halts the lipid biosynthesis [13]. If oleosins gene expression is also inhibited by ethylene production during the climacteric stage has to be elucidated. Recent research has also shown that an oleosin transcript is highly expressed in the mesocarp of Hass avocadoes, and that expression increases with the age of the fruit, prior to the climacteric and harvesting stages [21]. We detected *PaOle2* at low but significant levels in the green mesocarp and embryo tissues (Table 3 Supplementary Material), and lower levels in flower, cotyledon, and ripe mesocarp. This expression pattern is consistent with housekeeping activity, as reported by Huang universal oleosins have low expression [4]. Since oleosins stabilizes lipid storage molecules, we evaluated the expression of mesocarp *PaFad*, as an indication of lipid synthesis. We found that the expression of *PaFad* is related to the expression pattern of *PaOle1*, suggesting that the expression of these genes is associated with lipid metabolism. Other studies have shown M-oleosin expression during developmental stages where triacylglycerols (TAGs) accumulation occurs [6],

this is consistent with the co-expression observed between *PaFad* and *PaOle1* found in this study.

PaOle1 Protein Avocado Tissue Accumulation and Mesocarp Lipid Droplets

Using total protein extracts from the mesocarp (Fig. 1 Supplementary Material) and other tissues (leaf, root, seed and peel), we made a western blot assay using anti-PaOle1 antibodies. We only detected the PaOle1 protein in fruit mesocarp; no oleosin was detected in the other avocado tissues (Fig. 2a). To understand the role of the oleosins in the creation and size control of lipid droplets, we isolated LDs from ripe mesocarp of *P. americana* var. *drymifolia* and analyzed them by flow cytometry (Fig. 2b–d). There was a significant variation in size. We previously reported that *drymifolia* LDs have a Feret's diameter of between 11.90 and 27.96 μm [10]. Therefore, they produce a frontal scattering (FSC) of approximately 5×10^4 to 6.5×10^5. For the purpose of our analysis,
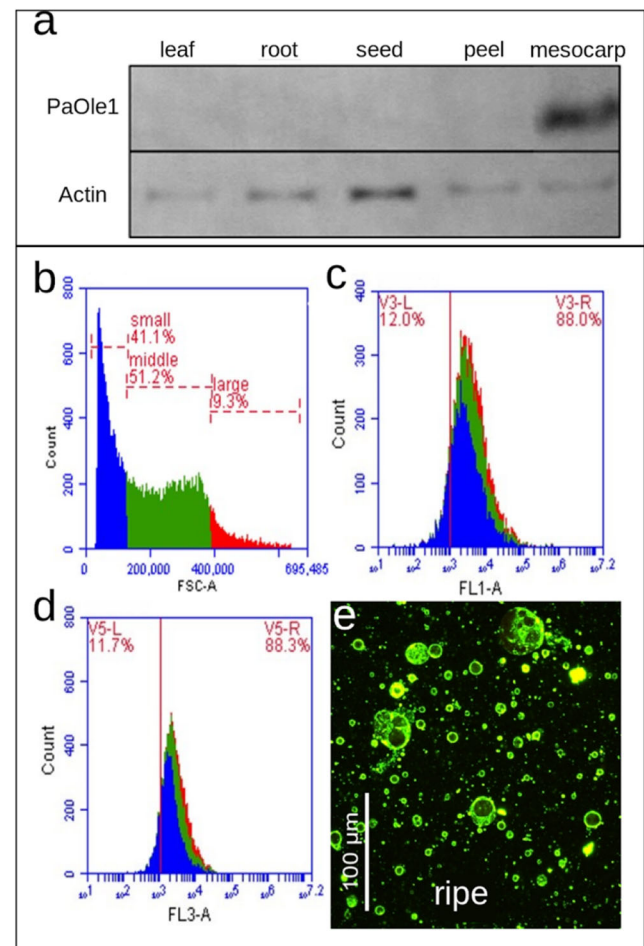

Fig. 2 a Western blot from *Persea americana* var. *drymifolia* tissues. **b** Flow citometry frontal scattering (FSC) for *drymifolia* isolated LDs, size distribution. **c** Flow citometry fluorescence (FL1 filter), fluorescein signal. **d** Flow citometry fluorescence (FL3 filter), autofluorescence signal. **e** LCSM for *drymifolia* LDs, full oleosin covers are seen in all LD sizes

Fig. 3 **a** Western blot from avocadoes varieties and cultivar. **b** Correlation between oleosin accumulation and oil content, oil content data from [10]
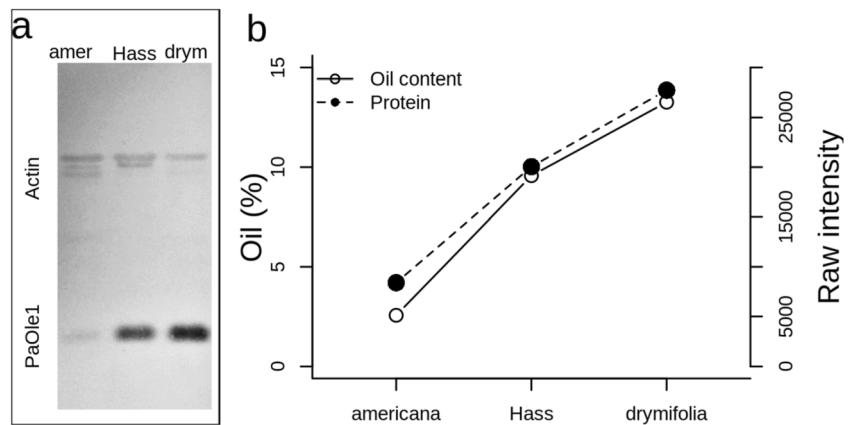
this range was divided into small (5×10^4–1.5×10^5 FSC, blue), medium (1.5×10^5–3.9×10^5 FSC, green), and large (>3.9×10^5 FSC, red) LDs (Fig. 2b). The small and medium LDs accounted for >90% of the total sample, while the large LDs represented <10%. These LDs exhibited auto fluorescence in FL3 (Fig. 2d), meaning a red auto fluorescence. Next, the LDs isolated from the mesocarp of ripe *P. americana* var. *drymifolia* were marked with anti-PaOle1 + antirabbit-fluorescein antibodies and the signal was read in FL1 (Fig. 2c). Regardless of size, all the LDs had a signal of equal intensity. The isolated *drymifolia* LDs with fluorescein were observed by LCSM to confirm the presence of oleosin. All the LDs were completely covered by PaOle1, regardless of their size (Fig. 2e). Another study has reported mesocarp LDs sizes ranging from 5 to 20 μm, where, once isolated and let to sit, the mesocarp LDs coalesce, also demonstrated the presence of a large LD (approximately 40 μm) that is not covered by oleosins, while the surrounding small LDs (>1 μm) have an oleosin envelope [6]. We previously reported a mean of 41.53 μm for Hass avocado LDs, and the presence of LD clusters in the *drymifolia* mesocarp. Those clusters were dense groups of smaller LDs (approximately 20 μm) that have a protein layer [10]. These results suggest that in *P. americana* cv. Hass, the oleosin only covers the small LDs, while the large LDs are uncovered and may depend on other proteins for stability [22, 23]. However, *P. americana* var. *drymifolia* possess a full oleosin coating, despite the size of LDs. This coating allows *drymifolia* LDs to remain as individuals when clustered, which may improve the stability of oils during the ripening process.

Relation of PaOle1 Protein Accumulation and Oil Content of Avocado Fruit Type

PaOle1 protein accumulation is correlated with oil content in three different type of avocado fruits. *P. americana* var. americana showed the lowest value in the raw band intensity, and

P. americana var. *drymifolia* the highest value, Hass shown an intermediate abundance (Fig. 3a). This correlates with the oil content of these avocado fruits type: Hass only yields 72.12% of the oil of *drymifolia,* and 72.25% of the oleosin; americana yields 19.29% of the oil than does *drymifolia* and has 30.29% of the oleosin of *drymifolia* [10] (Fig. 3b). This correlation between oleosin protein accumulation and oil yield has been demonstrated in other studies [24, 25], where the accumulation of oleosins is related to oil synthesis. The oleosin abundance impacts on oil yield, therefore, the effect of oleosin expression on oil yield is worthy of further study.

Conclusion

The Mexican avocado has two oleosin genes, however only one, *PaOle1*, is expressed abundantly in the mesocarp. All lipid droplets are fully covered by this protein and are clustered, improving stability during ripening process. The expression of *PaOle1* gene is specific to the mesocarp and is associated to fruit ripening. Our data suggest that the oil mesocarp content of different varieties of avocadoes are related to the abundance of PaOle1. If oleosin from *drymifolia* is improving the stability of oil storage, it may be the responsible of increasing the oil content of this variety and then understanding how it is done may help to improve the avocado cultivation. The lipid droplets and oleosins in both Hass and native Mexican avocadoes present different physicochemical behaviors. These differences, and the previously reported oil profiles, highlight the need of further studies about the dietary and biochemical differences between Hass and *drymifolia* avocadoes. More studies are necessary to elucidate the physiological function of *PaOle1* during avocado fruit ripening, and its potential biotechnological uses.

Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflict of interest.

Code Availability Not applicable.

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