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ORIGINAL ARTICLE

Enzymatic browning and genome-wide polyphenol oxidase gene identification in three contrasting avocado accessions

Jesús M. Higuera-Rubio[1] · Enrique Ibarra-Laclette[2] · Miguel A. Reyes-López[1] · Eduardo Sandoval-Castro[5] · Abraham Cruz-Mendívil[3] · Misael O. Vega-García[4] · Carlos L. Calderón-Vázquez[5]

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Abstract

This study aims to disentangle avocado enzymatic browning by identifying and analyzing the PPO coding genes. Two avocado accessions (AVO48 and San Miguel) and the Hass cultivar with contrasting browning kinetics and enzyme activity levels were selected for gene characterization. Upon 90 min of light exposure, Hass and San Miguel showed a greater decrease in luminosity retention (closer to 40% of initial luminosity) compared to AVO48 (85% of luminosity). PPO activity in crude extracts was significantly higher ($P < 0.05$) in San Miguel (696 U μg^{-1} protein) than Hass (174 U μg^{-1} protein) and AVO48 (46–56 U μg^{-1} protein). San Miguel showed a higher V_{max} K_m^{-1} ratio (20.88 min^{-1}), followed by Hass (14.29 min^{-1}) and AVO48 (1.64 min^{-1}), suggesting that San Miguel and Hass have higher substrate affinity. Four PPO coding genes: *PamPPO1*, *PamPPO2*, *PamPPO3* and *PamPPO4* were identified in the Hass genome, all of them containing the main features of plant PPOs, but with specific amino acid combinations in the catalytic pocket of the tyrosinase domain; suggesting that *PPO1*, *PPO2* and *PPO4* have monophenolase activity, whereas *PPO3*, has o-diphenolase activity. The evidence of transcription of *PPO3* in fruit of the three genotypes suggests an important role for this gene in avocado pulp browning. *PPO2* expression was only found in AVO48. This research provides gene candidates for selective silencing to reduce enzymatic browning.

Keywords Avocado · Browning · Enzymatic activity · Polyphenol oxidase · PPO gene

Introduction

Avocado (*Persea americana* Mill.) is a very important fruit in the international market, because of its flavor and consumption as guacamole. Mexico is the world's leader in the production and exportation of avocado. Pulp browning is a serious issue for avocado processing, affecting its sensory properties and reducing commercial value (Martinez and Whitaker, 1995; Bustos et al. 2015). Browning is one of the most devastating reactions for many fruits and vegetables, with estimations that over 50% of losses occur because of enzymatic browning (Mesquita and Queiroz, 2013). The PPOs (polyphenol oxidases) are enzymes widely distributed among plants, animals, fungi and bacteria. In plants and fungi, PPOs are implicated in different biological roles, including defense against insects and other pathogens, and enhancing the flavor in many food products (Panadare and Rathod, 2018). However, these enzymes are best known for their association with undesirable browning of fruits and vegetables (Constabel et al. 1995; Thipyapong et al. 1997). PPOs are copper-containing enzymes that perform

✉ Carlos L. Calderón-Vázquez
ccalderon@ipn.mx

1 Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Blvd. del Maestro S/N Esq. Elías Piña. Col. Narciso Mendoza, 88710 Reynosa, Tamaulipas, México

2 Instituto de Ecología (INECOL), Carretera antigua a Coatepec 351, El Haya, 91070 Xalapa, Veracruz, México

3 CONACYT - Instituto Politécnico Nacional CIIDIR Unidad Sinaloa, Blvd. Juan de Dios Bátiz-Paredes 250, San Joachín, 81101 Guasave, Sinaloa, México

4 Posgrado en Ciencia y Tecnología de Alimentos, Facultad de Ciencias Químico-Biológicas, Universidad Autónoma de Sinaloa, Av. de las Américas y Josefa Ortiz S/N, 80010 Culiacán, Sinaloa, México

5 Instituto Politécnico Nacional, CIIDIR Unidad Sinaloa, Blvd. Juan de Dios Bátiz-Paredes 250, San Joachín, 81101 Guasave, Sinaloa, México

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the conversion of monophenols (cresolase/monophenolase activity, EC 1.14.18.1) and *o*-diphenols (catecholase/diphenolase activity, EC 1.10.3.1), with the presence of molecular oxygen, into o-quinones, which are subsequently polymerized, producing the undesirable dark brown color (Weemaes et al. 1999; Mayer, 2006), a major problem in fruit and vegetable processing (Putnik et al. 2017; Marszałek et al. 2018). One of the main characteristics of PPOs enzymes is the presence of an active site with two copper atoms located in the CuA and CuB sites in the central domain (Pourcel et al. 2007).

The PPOs in avocado fruit have been studied for several decades, however, to date, the genetics controls in avocado remains uncharacterized. Differences in browning rates among avocado cultivars such as Hass, Lerman, Horeshim and Fuerte have been reported (Kahn, 1975). It is known that Hass and Fuerte show similar browning behaviors and relative luminosity ($L^* = 40\%$). Until now, the browning process in avocado has mainly been attributed to PPO enzymes, since positive correlations exist between darkening levels and activity of these enzymes (Kahn, 1975; Quevedo et al. 2011). PPO activity depends on several factors including the accession, biotic and abiotic stress as well as the stage of development (Mishra et al. 2013). Avocado PPOs can also exhibit different enzyme activity and affinity, depending on the type of phenolic substrate and its concentration (Dizik and Knapp, 1970; Kahn, 1976; Golan et al. 1977; Lelyveld and Bower, 1984

To date, the PPO coding genes in avocado have not been described. In other plant species that show browning, including *Salvia miltiorrhiza*, *Populus trichocarpa*, *Physcomitrella patens*, *Solanum lycopersicum* and *Threobama cacao*, multiple copies of PPO coding genes are present in their genomes (Tran et al. 2012; Li et al. 2017). Pericarp browning in litchi (*Litchi chinensis* Sonn.) is positively correlated with both differences in the PPO coding gene sequence and differences in PPO activity, resulting in contrasting levels of pericarp browning among cultivars (Wang et al. 2014a). The identification of the PPO coding genes has allowed the use of gene silencing and CRISPR/Cas9-based mutagenesis approaches to generate apple and eggplant varieties with reduced flesh browning (Maioli et al. 2020; Stowe and Dhingra, 2021). Based on the prediction of PPO amino acid sequences in plants, previous studies have proposed specific amino acids that are key to understanding PPO activity and affinity (Goldfeder et al. 2014; Kanteev et al. 2015). The first amino acid that functions as a "gate keeper" in plants is usually a phenylalanine, and it is considered a stabilizer for the substrate orientation through hydrophobic T-shaped π-π interactions of this residue with the aromatic rings of the substrate (Magnus et al. 1994; Bijelic et al. 2015; Molitor et al. 2016; Solem et al. 2016). The second amino acid is the "water keeper", a conserved glutamic acid, located at the entrance of the active site, which presumably assumably stabilizes a conserved water molecule responsible for the deprotonation of incoming monophenolic substrates (Bijelic et al. 2015; Molitor et al. 2016; Solem et al. 2016). The third includes two non-conserved residues (named as "activity controllers", located immediately after the first and second CuB coordinating histidines), which in combination with gate and water keeper residues, are strongly directing either monophenolase or diphenolase activities (Kampatsikas et al. 2017). In this sense, the combination of the water keeper residue with an asparagine as the first activity controller, favors monophenolase activity (Kampatsikas et al. 2017). Considering that plant species prone to fruit browning contain large families of PPO coding genes, we hypothesized that differences in gene sequences among members of the PPO family correlate with contrasting browning patterns and enzyme activity. Aiming to elucidate the molecular basis of the genetic family of PPO associated with pulp browning in avocado, this research combines the analysis of PPO enzymatic activity through the identification and analysis of the PPO coding genes in three avocado accessions with contrasting browning patterns (AVO48, San Miguel and the Hass cultivar). Our results contribute to understand the genetic basis of the PPO enzyme family in avocado, which would help to create future strategies for silencing or mitigate the browning without affecting the metabolism of the fruit.

Materials and methods

Plant materials

To identify avocado fruit with contrasting pulp browning, nine non-commercial accessions were evaluated. Fruit with at least 20% dry matter (as an indicator of physiological maturity) were selected and collected in Guasave, Sinaloa, Mexico, during August-November 2018. Additionally, fruit of the commercial Hass cultivar were obtained from a local market (dry matter of 23 %). Fruit of uniform size and dry matter were washed and disinfected with a 10 % solution of commercial bleach. After 3 days, the pulp of five fruit per accession was homogenized and frozen with liquid nitrogen and stored at −80 °C.

Color measurement

Enzymatic browning index was determined by a Computer Vision System (CVS) according to Agudelo-Laverde et al. (2013) and Bustos et al. (2015) with some modifications. Briefly, 5 g of avocado pulp (in triplicates) were homogenized and distributed in a Petri dish, leaving a thin layer of 0.3 cm. The dishes were placed in a white chamber and exposed to fluorescent light (D65 Philips lamps, 36 W

and 0.55 Klx) with a temperature of 60 °C. Images were recorded at 0, 15, 45, 75, and 90 min of light exposition using a professional camera (Nikon DX AF=S 18–55 mm 1:3.5–5.6 G) with an objective of 35 mm. The color changes were quantified by the CIE L* (luminosity), a* (green-red coordinate) and b* (yellow-blue coordinate) values using Adobe Photoshop Software CS4 2016, and the total color difference (ΔE) was calculated with the expression $\Delta E = \sqrt{(\Delta L^2+\Delta a^2+\Delta b^2)}$ according to Papadakis et al. (2000). Furthermore, the relative luminosity (RL) was calculated with the expression $RL = (L_0/L^*)*100$, where L_0 is the initial luminosity and L* is the luminosity recorded at every analyzed time. Statistical analyses were performed in StatGraphics Centurion XVIII (Statgraphics Technologies). The data were previously normalized and then an ANOVA was performed. Means comparison was done using the LSD Fisher method with a degree of significance of $P \leq 0.05$.

Enzymatic activity and kinetic parameters

Avocado pulp samples were collected at 0, 15, 45, 75, and 90 min during light exposure. A crude enzymatic extract was obtained according to Pizzocaro et al. (1993). First, 2 g of avocado pulp were homogenized into 10 mL of McIlvaine buffer at 25 °C (0.2 M sodium phosphate, 0.1 M citric acid, 2% w/v of polyvinylpyrrolidone, 1% v/v triton X-100, pH 6.6) using 15-mL tubes. Samples were then centrifuged at 2000 g (Eppendorf, Germany) during 40 min at 4 °C, then, the supernatant was passed through a filter paper #1 and aliquoted in 1.5 mL centrifuge tubes. Samples were centrifuged at 2900 g for 20 min at 4 °C. Finally, the supernatant was used as a crude extract for PPO activity assays. All extractions were performed in triplicates.

For PPO enzyme activity assay, 0.15 mL of the crude extract were mixed with 0.25 mL of catechol (0.1 M, Sigma Aldrich, United States of America) and 0.5-mL sodium phosphate (0.05 M, pH 6.6). Samples were analyzed in a spectrophotometer (Thermo Scientific, United States of America) at 420 nm, recording the absorbance every 2 min during 20 min. All the assays were performed in triplicates. To determine the specific activity, the protein quantification was performed according to Bradford (1976), using a BSA stock solution at 2 g L^{-1}. Units of specific activity are given as ΔABS min^{-1} μg^{-1} total protein (Kahn, 1976).

K_m (Michaelis constant) and V_{max} (maximum reaction velocity) values for PPO were determined by the Michaelis-Menten equation using the nonlinear regression method described by Olp et al. (2020). A catechol concentration gradient (10 to 300 mM) was used. The initial velocity (V_0) of PPO at different substrate concentrations was determined. The catalytic power (V_{max} K_m^{-1} ratio), was taken as an evaluation criterion of the catalytic efficiency.

Gene model prediction and transcriptional evidence

The plant PPO sequences previously analyzed by Tran et al. (2012) were used as templates to identify PPO coding genes sequences in the genome of Hass cultivar (Genome ID: 29302) (Rendón-Anaya et al. 2019). This genome is available from the repository of sequenced genomes through the Comparative Genomics (COGE) database (Lyons and Freeling 2008). Once homologous and syntenic regions were identified through BLASTn searches (E-value of $1e^{-10}$) and the SynFind tool, respectively, the sequences were manually curated, as follows: the exon-intron gene structure was determined by comparing the genomic sequence with their corresponding predicted gene-model and translated amino acid sequence. *PPO*-like sequences with incomplete domains or with premature stop codons were discarded from the candidate sequences. Predicted *PPO* gene models were then used as templates for searching transcriptional evidence in avocado transcriptomes of San Miguel, Hass and AVO48.

Total RNA was extracted from pulp samples taken at 0, 15, 45 and 75 minutes of light exposure of each genotype (in triplicates). Each transcriptome was assembled from 12 RNA-Seq libraries sequenced with the NextSeq Illumina platform. Over 205 million paired-end (2x150 bp) reads were generated, and at least 162 million clean reads were kept for each assembly. 50,088, 52,723 and 48,107 contigs were assembled for AVO48, San Miguel and Hass transcriptomes, respectively, using the Trinity Software v2.6.6 (Grabherr et al. 2011). The AlignWise software (Evans and Loose, 2015) was used to identify protein-coding sequences and to correct frame shifts in the transcriptomes. Previously, the software was trained with a database built from 80 angiosperm species phylogenetically close to *Persea americana*. To annotate the transcriptomes, sequence similarity searches were performed with the BLASTX algorithm (E-value 10^{-3}, bit score ≥25) against *Arabidopsis thaliana* (TAIR v11; www.arabidopsis.org/), *Amborella trichopoda* (v1.0; www.amborella.org/), and plant proteins from other species available in the Reference Sequence (RefSeq) collection (NCBI; ftp.ncbi.nlm.nih.gov/refseq/release/plant/). Top protein matches from the databases were assigned to each avocado contig. The genes were identified using Hidden Markov Model (HMM) based on searches against the Pfam database (E-value 10^{-3}) (Finn et al. 2014). The expression profiles of *PamPPO3* were determined using pseudoalignments of each RNA-Seq library against their corresponding transcriptome. Gene expression levels were calculated by log-2FoldChange with respect to samples taken at 0 minutes of light exposure using DESeq2 (Love et al. 2014).

Identification of functional domains and phylogenetic analysis

The conserved elements that characterize PPO enzymes (Tyrosinase domain, CuA and CuB sites, PPO1_KFDV and PPO1_DWL domains and water-keeper, gate-keeper and activity controller amino acids) were predicted in the avocado genes. The theoretical isoelectric point (pI) and molecular weight (Mw) were calculated using the Compute pI/Mw tool on the ExPASy server (Artimo et al. 2012). The transit peptide was predicted using ChloroP 1.1 and TargetP 2.0 (Emanuelsson et al. 2007; Almagro-Armenteros, 2019). Aiming to examine the phylogenetic relationship among PPOs of *Persea americana* (*PamPPOs*), *Sorghum bicolor* (*SbiPPOs*), *Oryza sativa* (*OsaPPOs*), *Spirodela polyrhiza* (*SpoPPOs*), *Solanum lycopersicum* (*SlyPPOs*), *Vitis vinifera* (*VviPPOs*) and *Populus trichocarpa* (*PtrPPOs*) (Jaillon et al. 2007; Ouyang et al. 2007; Tomato Genome Consortium, 2012; Wang et al. 2014b; Du et al. 2015; McCormick et al. 2018), the corresponding amino acid sequences were downloaded from Phytozome version 13 (Goodstein et al. 2012) and NCBI databases (https://www.ncbi.nlm.nih.gov). The *PPO* gene orthologs and paralogs from these species were identified using the OrthoMCL pipeline, which identifies any potential orthologues pairs using the reciprocal best BLAST hit method. Translated PPO amino acid sequences were checked to corroborate that these sequences presented the Tyrosinase, C-terminal and N-terminal domains using BLASTP and PFAM (Mistry et al. 2021). The sequences lacking some of these domains or with premature stop codons were discarded. Multiple sequence alignments were performed using MUSCLE with the default parameters (Edgar, 2004). Finally, the alignment file was used as input for phylogenetic tree construction using MEGA X (Tamura et al. 2007), with 1000 bootstrap replicates.

Results

Browning patterns and color parameters

The results showed an intermediate browning rate in Hass cultivar (Fig. 1a). The browning was more evident at 45 min increasing markedly until 90 min of exposure. In AVO22 and AVO55 accessions, the browning rate remained stable during the first four sampling points (0, 15, 30, and 45 min) of light exposure. Browning started at 60 min, reaching a brown coloration thereafter. Meanwhile, the accessions AVO28, AVO36, AVO56 and AVO58 showed a slow browning rate during the first 60 min, reaching an intense browning after 90 min of light exposure. The accession AVO60 showed a slow browning rate during the first 75 min of exposure but increased markedly after 90 min. San Miguel showed the fastest browning rate, displaying an intense browning just after 30 min of light exposure. Interestingly, AVO48 showed no browning during the 90 min of light exposure (Fig. 1a).

Due to differences in the initial values of L* between the pulp of each accession, this color parameter was normalized to the RL, considering 100% of L* at time zero for each accession (Fig. 1b). San Miguel and Hass showed similar patterns in RL with a rapid decrease after 15 min of exposure, reaching the lowest RL values in all accession at 90 min (40%). The RL values of AVO22 and AVO55 showed no notable decrease during the first 45 min, but decreased drastically after 60 min. Meanwhile, RL values of AVO28, AVO36, AVO56, AVO58 and AVO60 remained stable during the first 75 min, but decreased drastically at the end. Accession AVO48 was the only one that retained a high RL value (85%) after 90 min (Fig. 1b). ΔE was then determined. Hass, AVO36, AVO56 and AVO60 started with a slow change in ΔE but increased at the end. ΔE in AVO22, AVO28 and San Miguel increased, reaching the highest values (35–40) along with Hass (Fig. 1c). In contrast, the accession AVO48 showed the lowest ΔE value (25) of all samples, with a moderate change during the first 30 min of the experiment, which means that color parameters (L*, a*, b*) remained more stable across time (Fig. 1c).

PPO enzymatic activity and kinetic parameters

Based on their contrasting browning patterns, San Miguel, Hass and AVO48 accessions (with high, medium and low browning levels, respectively) were selected for evaluating PPO enzymatic activity. The results showed that PPO activity in crude extracts was significantly higher ($P \leq 0.05$) in San Miguel compared to Hass and AVO48 along the time assayed, with a marked increase (33 %) of PPO activity only after 15 min of light exposure, which precedes the decrease in RL and the increase of ΔE (Fig. 1), reaching a maximum peak of 696 U μg^{-1} protein, after 45 min (Fig. 2). Hass extracts showed moderate levels of PPO activity during the time of experiment, with a slight trend to increase across time, with the highest activity (174 U μg^{-1} protein) at 90 min (Fig. 2). The PPO activity trend observed in Hass did not correlate with the way that RL decreased and ΔE increased. Finally, AVO48 presented the lowest PPO activity levels (46 U μg^{-1} protein) without significant changes across time. This PPO activity trend in AVO48 was similar to that of RL, but not to that of ΔE (Fig. 1).

A catechol concentration gradient was used for determining the PPO enzyme kinetic parameters, obtaining differences in the initial rates of PPO reaction. As expected, the substrate was oxidized following a Michaelis–Menten kinetics. The kinetic parameters are summarized in Table 1. Regarding the catalytic power (V_{max} K_m^{-1} ratio), this was higher in San Miguel (20.88 min^{-1}) followed by Hass (14.29

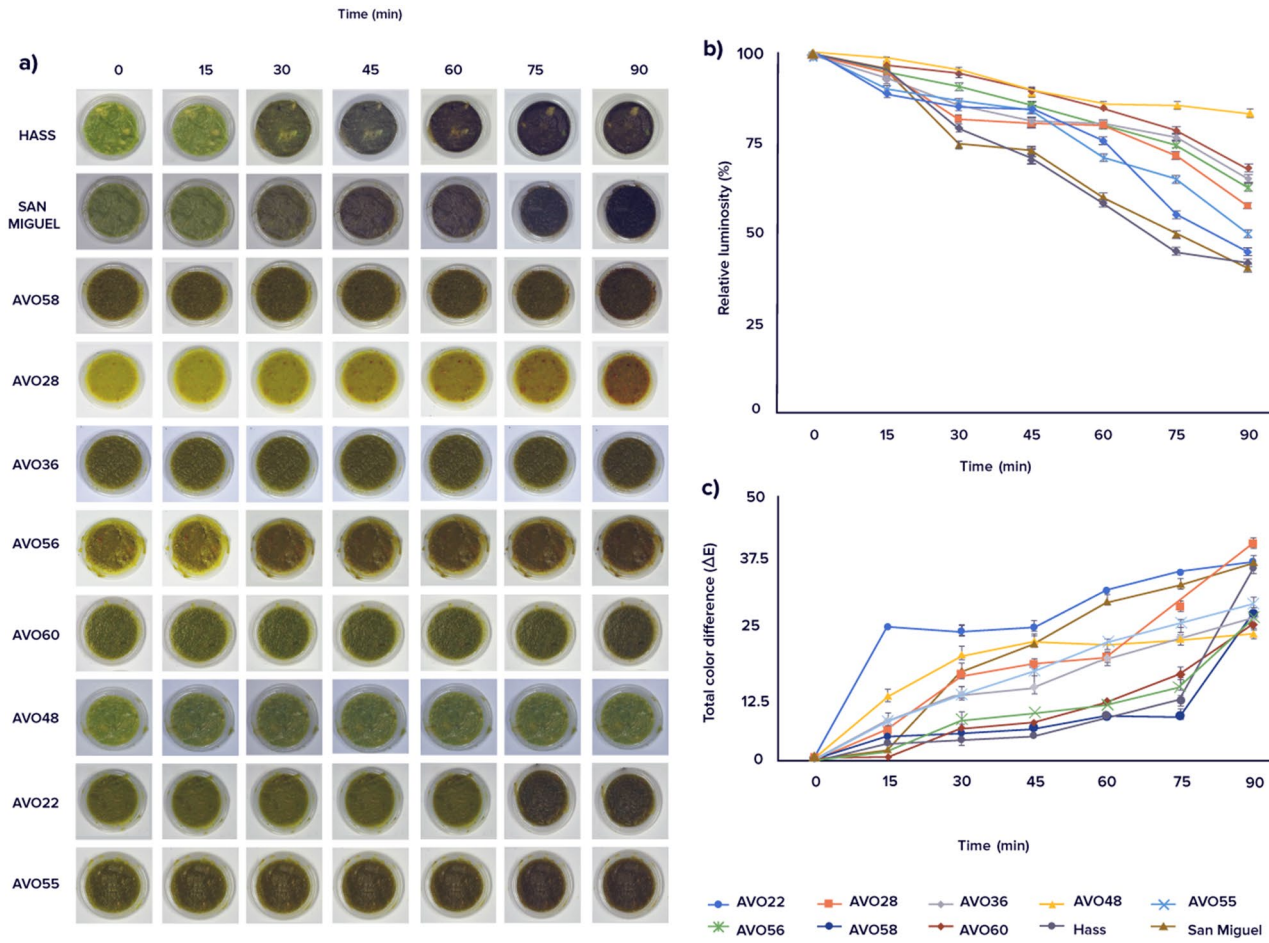

Fig. 1 Browning patterns of ten avocado accessions in an accelerated browning chamber, exposing fruit pulp to 0.55 klx of fluorescent light during 90 min at 60°C. **a** Images of avocado pulp samples recorded every 15 min. **b** Relative luminosity (RL, the percentage of retained luminosity related to the initial luminosity of each sample at time 0 of pulp samples). **c** Total color difference (ΔE, a parameter that takes into account the luminosity, but also the green-red and yellow coordinates) of pulp samples. The normalized data were plotted and compared by the LSD fisher method with a degree of significance of $P \leq 0.05$. Error bars represent the Standard Error

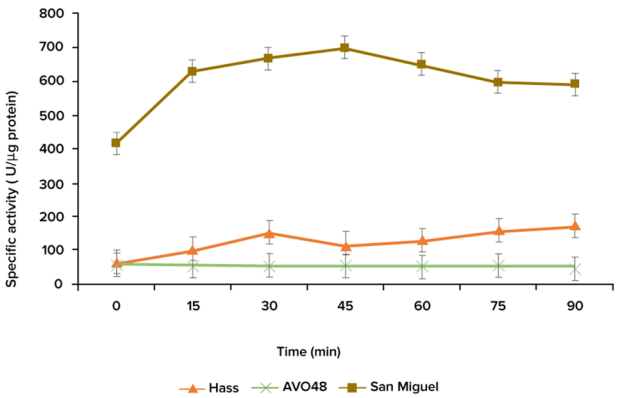

Fig. 2 Specific activity of PPO enzyme in crude extracts of pulp of avocado accessions with contrasting browning patterns, evaluated every 15 min during 90 min. The data points are the mean of three biological replicates. The vertical bars represent the Standard Error of three samples, with a degree of significance of $P \leq 0.05$

Table 1 Michaelis-Menten kinetic parameters toward varying concentrations of catechol as action of PPO enzyme in three different avocado accessions, determined using the non linear regression model

Sample	V_{max} (mM/min)	K_m (mM)	V_{max} K_m^{-1} ratio (min^{-1})
Hass	113.21 ± 1.35	7.92 ± 0.26	14.29
AVO48	50.11 ± 1.53	30.43 ± 1.57	1.64
San Miguel	485.25 ± 5.03	23.23 ± 1.31	20.88

K_m the Michaelis-Menten constant, V_{max} maximum velocity

min^{-1}) whereas the cultivar AVO48 showed the lowest V_{max} K_m^{-1} ratio (1.64 min^{-1}). On the other hand, The K_m value of Hass showed major affinity (7.92 mM) between the enzyme-substrate complex in contrast to San Miguel (23.23 mM) and AVO48 (30.43mM), suggesting that recognition between two elements is slower when compared to Hass.

Identification of PPO coding genes

PPO genes were identified in the Hass genome using as a query the plant *PPOs* reported by Tran et al. (2012). Predicted *PPO* gene models suggest the presence of a family of four members in the *P. americana* species: *PamPPO1* (chromosome 4, location: 8569699-8576304), *PamPPO2* (chromosome 5, location: 30235900-3024355), *PamPPO3* (chromosome 7, location: 5933148-5939681) and *PamPPO4* (chromosome 9, location: 21060043-21067560) (Table 2). Putative deduced proteins have a range of 524 to 611 amino acids, isoelectric points of 6.11 to 8.28, and molecular weights of 58.36 to 67.95 kDa. A signal transit peptide was found in the sequences of both *PamPPO2* and *PamPPO3*, suggesting that they are located in the chloroplast.

The *PamPPO* genes here identified contain the main features of *PPO*s reported in other plant species (Fig. 3), including the three main domains: Tyrosinase (CuA and CuB sites), C-terminal (PPO1_DWL and PPO1_KFDV domains). Furthermore, they also possess own a thioether bridge between an adjacent cysteine residue and the second histidine of CuA site, that keeps the structure of the active site, as well as the glutamic acid residue Glu346 (water keeper), located four amino acids upstream of the first conserved histidine of CuB site. On the other hand, there are differences in the activity controllers between *PamPPOs*, with Asn351-Glu355 and Asn351-Thr355 (polar uncharged-polar uncharged) in *PamPPO1* and *PamPPO2* (respectively), Gly351-Ile355, (polar-nonpolar) in *PamPPO3* and Asn351-Met355 (non-polar-nonpolar) in *PamPPO4*. In terms of post-translation activation, *PamPPO2* and *PamPPO3* sequences possess sites of proteolytic activation.

Phylogenetic reconstruction

To examine the relation of avocado PPO coding genes with respect to other plant PPOs, a phylogenetic reconstruction of 27 PPO proteins of *P. americana, P. trichocarpa, O. sativa, S. lycopersicum, V. vinifera* and *S. bicolor* was created (Fig. 4). The phylogenetic analysis revealed species-specific PPO-subgroups. *PamPPO1* and *PamPPO4* were clustered with little homology to *P. trichocarpa*, *S. bicolor*, *O. sativa*, *S. lycopersicum* and *V. vinifera PPOs*. *PamPPO2* was clustered separately from *PamPPO3*, but together with *PtrPPO3* and *PtrPPO5*. *PamPPO3* was clustered with *SlyPPO1* in a subgroup of several *P. trichocarpa PPO* sequences (Fig. 4).

Evidence of PamPPOs expression

To find evidence of *PamPPO* expression, their corresponding transcripts were searched in AVO48, San Miguel and Hass pulp transcriptomes, but also in an independent Hass transcriptome (Kilaru et al. 2015) (Table 3). No evidence

Table 2 Features of PPO sequences found in the avocado genome var. Hass

PPO Gene	Locus	Gene length (nt)	ORF (nt)	Protein length (aa)	Isoelectric point	Molecular weight (kDa)	Transit Peptide	cTp-Lenght (aa)	Secretory Pathway	Loc
PamPPO1	Chromosome: 4, Strand: 1, Location: 8569699-8576304	2948	1833	611	6.13	67.95	0.024	NF	0.717	S
PamPPO2	Chromosome: 5 Strand: -1, Location: 30235900-3024355	2608	1773	591	6.11	67.29	0.864	59	0.023	C
PamPPO3	Chromosome: 7, Strand: -1, Location: 5933148-5939681	4007	1572	524	6.46	58.36	0.893	45	0.017	C
PamPPO4	Chromosome: 9, Strand: 1, Location: 21060043-21067530	2366	1791	597	8.28	66.93	0.017	NF	0.848	S

ORF open reading frame, isoelectric point theoretical isoelectric point, *cTp* Lenght chloroplast transit peptide length, *Loc* protein prediction localization, *C* chloroplast, *S* secretory pathway, *NF* not found

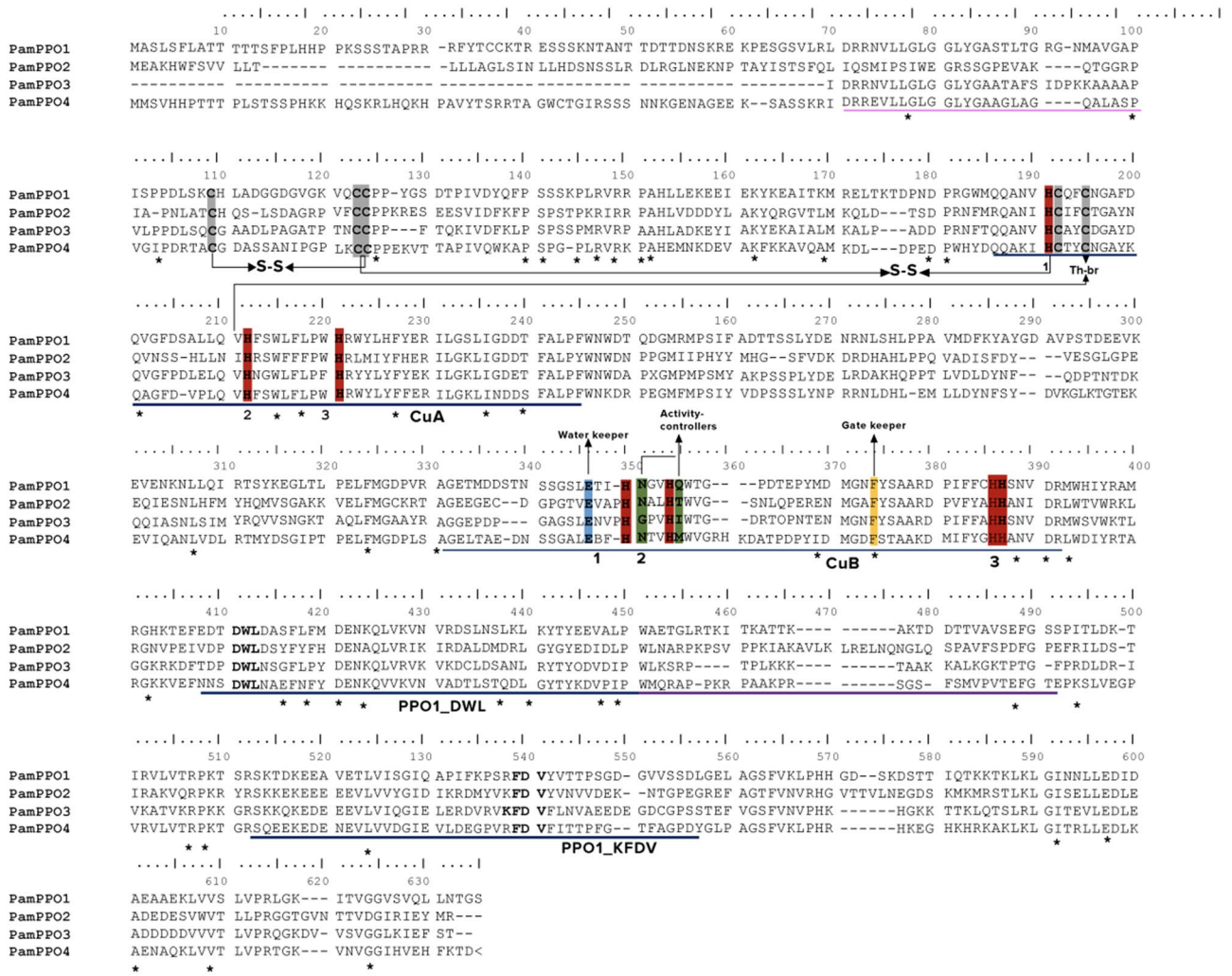

Fig. 3 Phylogenetic relationships of PPO protein sequences of six plant species. The phylogenetic tree was built using the neighbor-joining method implemented in MEGA X with 1000 bootstrap replicates. PPOs from *Oryza sativa* (*Osa*), *Populus trichocarpa* (*Ptr*), *Solanum lycopersicum* (*Sly*), *Vitis vinifera* (*Vvi*), *Sorghum bicolor* (*Sbi*) and *Persea americana* (*Pam*) are indicated by bullet points with different colors. The *PamPPOs* (green) are divided into three subgroups

of expression was found for *PamPPO1*. For *PamPPO2*, a highly homologous (99% of identity) transcript of 1728 bp was found in the AVO48 transcriptome. For *PamPPO3*, a transcript of 1461 bp, with 99% homology was found in all 3 transcriptomes. Interestingly, the quantification of *PamPPO3* expression along the sampling times showed a slight but not satistically significant up-regulation in San Miguel and Hass (Table 4). Finally, for *PamPPO4*, a 1623 bp transcript was found in San Miguel and AVO48 pulp transcriptomes, but with only a 90% of identity and with several premature stop codons.

To gain more detail about *PamPPO3* role in browning, the putative transcripts from Hass, AVO48 and San Miguel were aligned (Fig. 5). Despite all three *PPO3* transcripts contained the functional domains, five-point variants were found. One located downstream the CuA and four in the PPO1_DWL and PPO1_KFDV domains. Whether these variations could affect the enzyme activity still needs to be analyzed.

Discussion

The CVS allowed identification of avocado accessions with contrasting browning (Fig. 1). Various authors have reported an intermediate browning rate (L=40%) in Hass Cultivar, consistent with the results presented here (Bustos et al. 2015; Quevedo et al. 2011, Quevedo et al. 2016). Also, showed that although most pulp samples presented close ΔE values at the end of the assay, each sample followed different patterns along sampled time points. On the other hand, the RL value was more consistent with the observed browning and

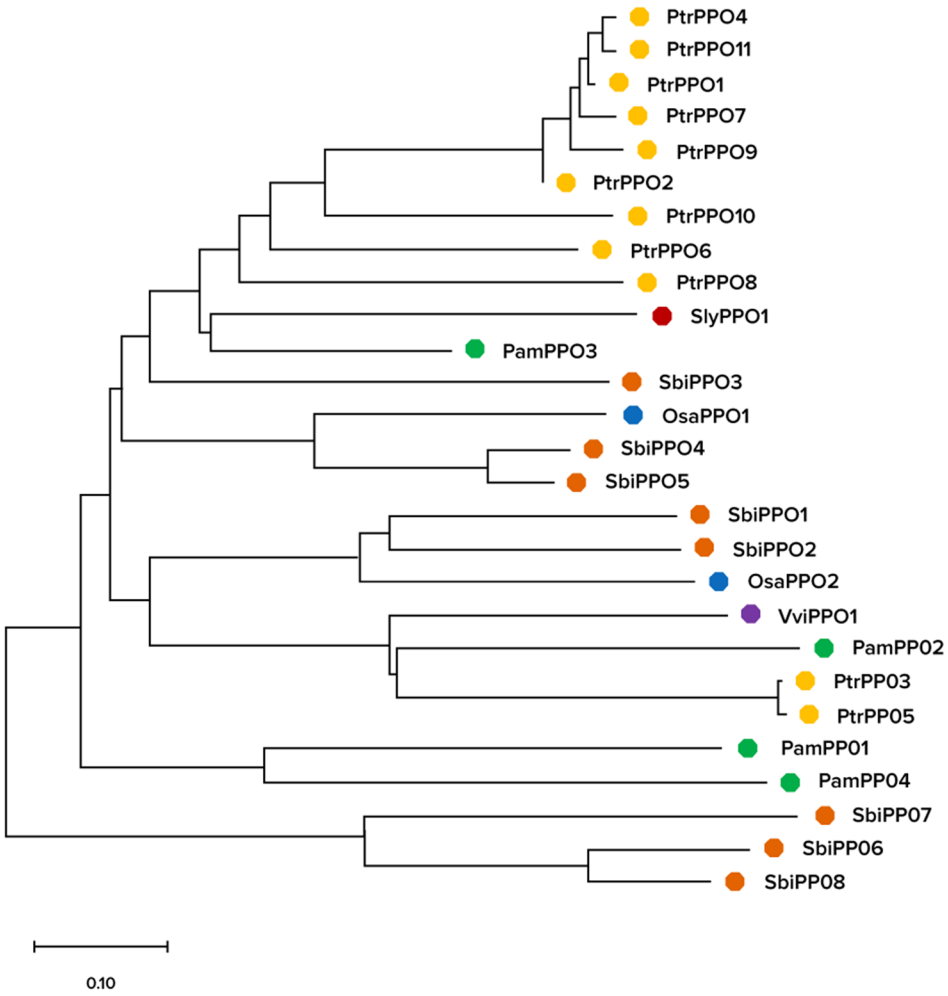

Fig. 4 Sequence components found in the deduced avocado PPO proteins. Typical PPO domains: Tyrosinase (CuA and CuB sites) and C-terminal (PPO1_DWL and PPO1_KFDV domains) are underlined. Red, conserved histidine residues that coordinate the Cu center (CuA and CuB). Pink, transit peptide to chloroplast (cTP). Gray, conserved cysteines that form disulfide bonds. Black arrows, thioether bridge between the second cysteine of the HCxxC motif and the second CuA histidine. Blue, water keeper (conserved glutamic acid). Green, activity controllers: Asparagine (N), glycine (G), glutamine (Q), threonine (T), isoleucine (I), and methionine (M) between the first and second histidines of CuB. Yellow, gate keeper residue (phenylalanine). Purple, site of proteolytic cleavage

Table 3 Transcriptional evidence of *PamPPO1*, *PamPPO2*, *PamPPO3* and *PamPPO4* genes in fruit pulp of AVO48, San Miguel and Hass

Gene	AVO48			San Miguel			Hass		
	Contig	Length (bp)	% Identity	Contig	Length (bp)	% Identity	Contig	Length (bp)	% Identity
PamPPO1	NF	NF	NF	NF	NF	NF	NF	NF	NF
PamPPO2	UN001999	1728	99	NF	NF	NF	NF	NF	NF
PamPPO3	UN001030	1461	99	UN001369	1461	99	UN005633	1461	99
PamPPO4	UN001499	1623	90	UN001187	1623	90	NF	NF	NF

NF not found, *% identity* percentage of identity of transcript to the corresponding genomic sequence

thus would help to differentiate pulp browning along the sample points.

The PPO enzymatic activity of the three selected avocado accessions with contrasting browning patterns was determined (Fig. 2). San Miguel showed higher PPO activity than previously reported (100 to 150 U μg^{-1} protein) in other avocado cultivars (Kahn, 1977; Gomez-Lopez, 2002), but similar to those observed in crude extract of mushrooms (530 U μg^{-1} protein) (Kahn, 1985; Colak et al. 2007; Morosanova et al. 2020). On the other hand, the PPO activity levels observed in Hass (Fig. 2) were like those reported by George and Christoffersen (2016). Additional factors to the enzyme specific activity must be considered to explain the observed browning patterns, possibly affecting the accessions in a different way. The presence of other molecules, including antioxidants, proteases or fatty acids could also affect K_m values impacting in enzymatic activity of PPO (George and Christoffersen, 2016).

To better understand the behavior of the PPO enzyme in avocado, it is important to look at three indicators: the

Table 4. Changes in *PamPPO3* gene expression in avocado pulp exposed to fluorescent light

Accesion	Contrast	Fold Change	Adjusted *P* value
AVO48	T0_VS_T15	1.10	0.99
	T0_VS_T45	1.34	0.76
	T0_VS_T75	1.50	0.78
San Miguel	T0_VS_T15	2.71	0.64
	T0_VS_T45	2.72	0.80
	T0_VS_T75	3.21	0.69
Hass	T0_VS_T15	2.03	0.88
	T0_VS_T45	2.05	0.92
	T0_VS_T75	3.71	0.89

substrate binding (low K_m values mean a high affinity), the catalytic efficiency (given by high V_{max} values) and the V_{max} K_m^{-1} ratio, referred to as catalytic power. This last variable indicates how fast the recognition between the enzyme-substrate complex occurs, therefore a high ratio indicates that the enzyme quickly recognizes the substrate and increases its reaction velocity (Brannan, 2016). San Miguel and Hass showed the highest catalytic power, with 20.88 min^{-1} and 14.29 min^{-1}, respectively, suggesting that PPOs in the crude extracts have a high affinity to catechol (Table 1). The lowest catalytic power was found for AVO48 (1.64 min^{-1}) suggesting a low affinity to catechol, possibly affecting its enzymatic activity and thus explaining in part its slow browning rate (Table 1, Fig. 1). The highest catalytic efficiency and thus the highest catalytic power was observed in San Miguel, impacting its activity. This study suggests that the San Miguel PPO has a higher browning intensity and activity, compared to other plant PPOs. San Miguel PPO could be promising for further use in the industry (Atrooz et al. 2020).

The presence of four PPO coding gene variants in the avocado genome could indicate a certain degree of functional redundancy and explain the different browning patterns and enzymatic activity observed among accessions. However, only *PamPPO2* and *PamPPO3* possess sites of proteolytic activation that allow the enzyme to proceed from a latent state to an active state, suggesting that both PPO2 and PPO3 activity could be regulated by this way (Fig. 3). PPO2 and PPO3 proteins also possess a thioether bridge between an adjacent cysteine residue and the second histidine of CuA, that preserves the structure of the active site (Fig. 3) (Bijelic et al. 2015; Kanteev et al. 2015; Prexler et al. 2019) as well as the glutamic acid residue Glu346 (water keeper), located four amino acids upstream of the first conserved histidine of CuB (Fig. 3). According to previous studies (Kanteev et al. 2015 and Solem et al. 2016), PPO1, PPO2 and PPO4 proteins of avocado could have monophenolase activity, while for PPO3 the o-diphenolase activity could be favored (Panis and Rompel, 2020).

PamPPO4 could be coding for small peptides, or its putative translated PPO may not be functional, although there is also the possibility that these nonsense mutations could be related to errors during the transcriptome assembly process. The role of these putative peptides should be determined, but there is evidence that some micropeptides are translational regulators of protein members of the same family (Eguen et al. 2015; Lauressergues et al. 2015; Ruiz-Orera and Albà, 2019). Based on this, we suggest that *PamPPO4* is either a pseudogene or a gene that might have regulatory roles.

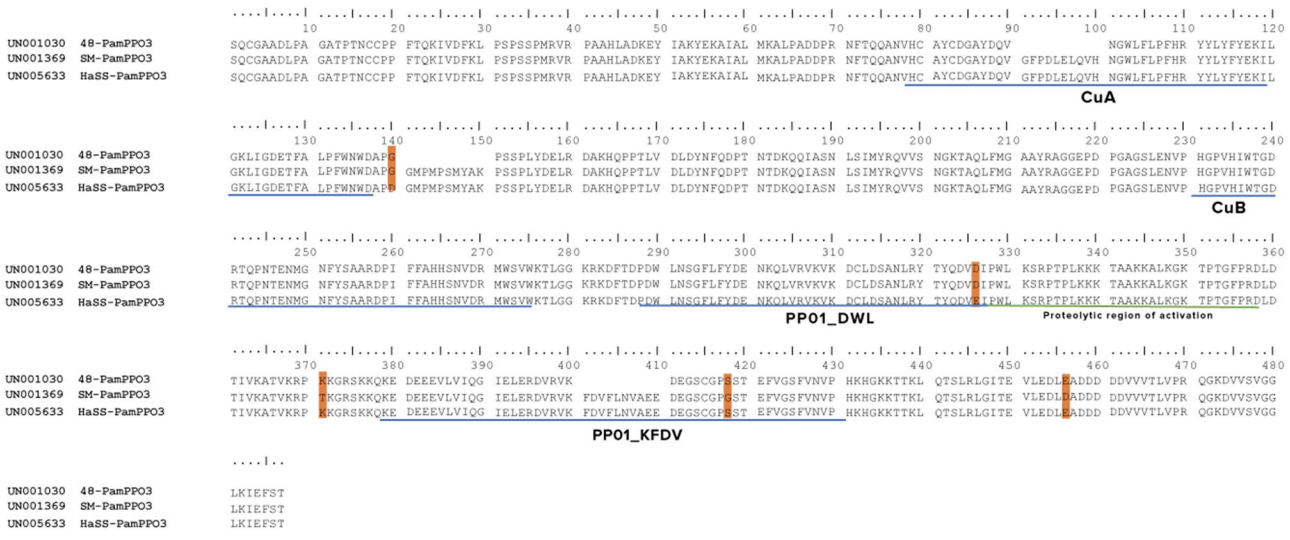

Fig. 5 Multiple alignment of translated *PamPPO3* sequences extracted from AVO48, San Miguel and Hass transcriptomes. The sequences were aligned using MUSCLE. Five variations were identified (orange): (1) glycine (G) x aspartic acid (D); (2) aspartic acid (D) x glutamic acid (E); (3) lysine (K) x threonine (T); (4) serine (S) x glycine (G); (5) glutamic acid (E) x aspartic acid (D)

Based on expressed *PPO* genes in each accession and considering that catechol is a diphenol, it is possible that we measured mainly PPO3 (o-diphenolase) activity and not PPO2 (monophenolase) activity (Fig. 1), thus explaining the low K_m values observed in vitro (Fig. 2), in agreement with a previous report (Taranto et al. 2017). Gene promoter variations and their transcriptional regulation have also been reported as relevant in PPO activity (Zhou et al. 2003; Xiao-Bo et al. 2008). The *PamPPO3* gene clustered (Fig. 4) with genes that have been involved in ascorbate and gluthanione metabolism and are major mediators of oxidative browning reactions in *P. trichocarpa*, *M. domestica*, *S. lycopersicum* and *S. tuberosum* during defense responses (Thipyapong et al. 1997; Yoruk and Marshall, 2003; Yan et al. 2013). Therefore, we suggest a main role of *PamPPO3* gene in pulp browning, as is the only variant expressed in Hass pulp and it contains the typical domains of plant PPO enzymes, but also a region of proteolytic activation and a transit peptide, a signal sequence in chloroplast interior proteins. This work contributes to an understanding of the genetic bases of the pulp browning in avocado, and our results suggest that *PamPPO3* can be a candidate for gene editing and silencing to decrease PPO activity and thus browning in pulp.

Conclusions

This work identified three avocado accessions with contrasting pulp browning patterns: San Miguel, Hass and AVO48, with high, intermediate, and low browning rates, respectively. These patterns correlated with PPO enzyme activity and substrate affinity. Avocado has at least four variants of *PPO* genes, which showed the main characteristic PPO enzyme domains. PPO1 and PPO3 could favor the o-diphenolase activity, with PPO*2* favoring monophenolase activity. *PamPPO4* could probably be a pseudogene, but further analyses are required. Transcriptional evidence of *PamPPO3* was found in the analyzed accessions (San Miguel, Hass and AVO48), suggesting a major role of this gene in enzymatic pulp browning. AVO48 also expressed *PamPPO2* but might not be related to the browning, thus its function still needs to be elucidated.

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