Structural and phylogenetic characterization of a polyphenol oxidase gene in lulo (*Solanum quitoense* Lam.)

Caracterización estructural y filogenética de un gen de polifenol oxidasa en lulo (*Solanum quitoense* Lam.)

Mauricio Antonio Pulido Jiménez[1*], Silvia Gómez Daza[2], and Víctor Núñez[3]

ABSTRACT

The lulo or naranjilla (*Solanum quitoense* Lam.) is one of the most important Colombian native fruits. The sale and industrial processing of fresh fruit is severely limited by enzymatic browning. Until now, there was no knowledge about polyphenol oxidases (PPO) in lulo. The aim of this study was to understand some structural and phylogenetic aspects of the first lulo *ppo* gene that has been characterized. Using two pairs of degenerate primers, two fragments of lulo genomic DNA were isolated by PCR, sequenced and assembled into a partial sequence of 1417 bp (*SquPPO1*) lacking introns. Hybridization of a 920-bp probe generated from a potato *ppo* gene with a 12 kb region of *Bam*HI-*Pst*I, *Bam*HI-*Xba*I and *Xba*I-*Pst*I digested lulo DNA confirmed the presence of at least one *ppo* gene in this species. While two conserved sites (Tyr-1 and Tyr-2) have been identified in the copper-binding domains of other Solanaceae PPOs, no Tyr-2 site was found in lulo PPO because of a conservative substitution DxE in this region. Phylogenetic analysis placed the *SquPPO1* gene in the same cluster as the *SmePPO4*, *SmePPO5,* and *SmePPO6* eggplant (*Solanum melongena* L.) genes. Our results show that *SquPPO1* is phylogenetically closer to eggplant *ppo* genes than to those of potato, tobacco, and tomato and that it exhibits a variation that modifies the distribution of protein-conserved sites. These findings offer new insights into the molecular basis of enzymatic browning in lulo and may inform strategies to reduce postharvest losses.

Key words: Solanaceae, catechol oxidase, browning, phenolic compounds, protein domain, naranjilla.

RESUMEN

El lulo o naranjilla (*Solanum quitoense* Lam.) es una de las frutas nativas colombianas más importantes. La venta y el procesamiento industrial de la fruta fresca son severamente limitados por el pardeamiento enzimático. Hasta ahora, no había conocimiento sobre las polifenol-oxidasas (PPO) en lulo. El objetivo de este estudio fue conocer algunos aspectos estructurales y filogenéticos del primer gen *ppo* de lulo caracterizado. Utilizando dos pares de iniciadores degenerados, dos fragmentos de ADN genómico de lulo fueron aislados por PCR, secuenciados y ensamblados en una secuencia parcial de 1417 pb (*SquPPO1*) que carece de intrones. La hibridación de una sonda de 920 pb generada a partir de un gen *ppo* de papa con una región de 12 kb de ADN de lulo digerido con *Bam*HI-*Pst*I, *Bam*HI-*Xba*I y *Xba*I-*Pst*I confirmó la presencia de al menos un gen *ppo* en esta especie. Mientras que se han identificado dos sitios conservados (Tyr-1 y Tyr-2) en los dominios de unión a cobre de otras PPOs de solanáceas, no se encontró ningún sitio Tyr-2 en la PPO de lulo debido a una sustitución conservativa DxE en esta región. El análisis filogenético situó al gen *SquPPO1* en el mismo grupo que los genes *SmePPO4*, *SmePPO5* y *SmePPO6* de la berenjena (*Solanum melongena* L.). Nuestros resultados muestran que *SquPPO1* es filogenéticamente más cercano a los genes *ppo* de berenjena que a los de papa, tabaco y tomate, y que presenta una variación que modifica la distribución de los sitios conservados de la proteína. Estos hallazgos ofrecen nuevos conocimientos sobre las bases moleculares del pardeamiento enzimático en lulo y pueden servir de base a estrategias para reducir las pérdidas poscosecha.

Palabras clave: Solanaceae, catecol oxidasa, pardeamiento, compuestos fenólicos, dominio proteico, naranjilla.

Introduction

Polyphenol oxidases (PPOs) are a group of metalloenzymes that oxidize phenolic compounds in the presence of molecular oxygen. They are widely distributed in bacteria, fungi, plants, and animals (Hong *et al*., 2024; Sarsenova *et al*., 2023; Taranto *et al*., 2017). The activity of PPOs on phenolic molecules results in the formation of quinones that spontaneously polymerize to form gray, brown or black pigments (Yoruk & Marshall, 2003) responsible for

Received for publication: January 31, 2025. Accepted for publication: March 30, 2025. Doi: 10.15446/agron.colomb.v43n1.118599

[1] Centro de Estudios en Biología Molecular, Gimnasio Campestre, Bogotá (Colombia).
[2] Departamento de Biología, Universidad Pedagógica Nacional, Bogotá (Colombia).
[3] Grupo de Mejoramiento Genético y Biotecnología, Corporación Colombiana de Investigación Agropecuaria (Agrosavia), Centro de investigación Tibaitatá, Bogotá (Colombia).
* Corresponding author: mauricio.pulidojimenez@gmail.com

the browning of plant tissues (Gerdemann *et al.*, 2002; Mayer, 2006). This biochemical phenomenon affects the appearance and the organoleptic properties of fruits and vegetables. These changes significantly reduce the marketing potential of fresh fruit or its use in industrial processing (Hasan *et al.*, 2019; Moon *et al.*, 2020).

PPOs are present in most cell types of higher plants, either free in the cytoplasm or bound to the cell membrane. They perform a variety of functions (Li, 2020; Liu *et al.*, 2023). There is evidence that the PPOs are involved in (1) resistance to biotic (Constabel *et al.*, 2000; Constabel & Barbehenn, 2008; Thipyapong *et al.*, 2007; Zhang & Sun, 2021) and abiotic (Mishra & Singh Sangwan, 2019) stresses, (2) the biosynthesis of pigments (Molitor *et al.*, 2015; Nakayama *et al.*, 2000; Ono *et al.*, 2006), (3) flavonoid oxidation in seed coats (Pourcel *et al.*, 2005) and indirectly in (4) the regulation of cell death (Araji *et al.*, 2014). In addition, PPOs have a key role in plant reproduction control mechanisms (Wei *et al.*, 2023). Their expression is induced by pathogenic and herbivore attacks or by tissue damage (Constabel & Ryan, 1998; Thipyapong *et al.*, 1995; Zhang & Sun, 2021).

PPOs belong to a group of copper-containing proteins that includes hemocyanins and tyrosinases (Lerch, 1983). These enzymes are characterized by the presence of two copper-binding domains (CuA and CuB) at the active site; each has three histidine residues involved in the coordination of a metal ion. Although both domains are very well conserved and define the PPO protein family, the CuA domain is more variable than the CuB domain (Klabunde *et al.*, 1998). Recently, a CRISPR/Cas9 construct with a single guide RNA (sgRNA) targeting the copper-binding domain was used to edit the *ppo1* and *ppo2* genes in wheat 'Fielder' grains. This reduced PPO activity by up to 86.7%. The same construct was transformed into the winter wheat cultivars 'Guardian' and 'Steamboat' and PPO activity was reduced by >90% (Wold-McGimsey *et al.*, 2023).

The *ppo* genes in plants are organized into multi-gene families. The number of *ppo* genes in some species can vary from 5 to 13, while others have only one. Seven *ppo* genes were isolated in tomatoes (Newman *et al.*, 1993), and nine were identified in potatoes (Chi *et al.*, 2014). Despite the progress in genomic analysis techniques, there are many Solanaceae species for which insufficient genetic information is available (Deanna *et al.*, 2022). From an economic point of view, the lulo or naranjilla is one of the most important of these species in Colombia. There are no reports of *ppo* genes in lulo (*Solanum quitoense* Lam.). Lulo is a perennial shrub of the Solanaceae family that grows in the Andean regions of Colombia, Peru, and Ecuador (Heiser, 1985). In these countries, it is considered valuable for agricultural and industrial use. However, in Colombia, where the species has a large market and is widely accepted, industrial processing and the possibilities of selling fresh fruit are very limited due to the damage caused by enzymatic browning during harvest and post-harvest handling.

This study presents the partial sequencing of a lulo *ppo* gene, a characterization of its structural features and an analysis of its evolutionary relationships with homologous genes from other Solanaceae species. Understanding the structural features of PPOs in lulo is crucial for developing strategies to mitigate enzymatic browning, a major limitation in fruit marketing and industrial processing.

Materials and methods

DNA extraction

Lulo (*Solanum quitoense* accession ILS-388) and potato (*Solanum tuberosum* cv. Millenia) seeds obtained from CORPOICA germplasm bank (Colombia) were grown in a greenhouse. Lulo and potato genomic DNAs have been extracted from young leaves (Doyle & Doyle, 1990).

Primers for PCR, PCR amplification, and DNA sequence analysis

The *ppo* gene sequences from species belonging to the Solanaceae, Convolvulaceae, Rosaceae, Fabaceae, Poaceae, Salicaceae and Zygophyllaceae families were retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) (Tab. 1). A multiple sequence alignment was carried out using MUSCLE v5 (Edgar, 2021) to identify regions showing high homology. For the partial amplification of the *SquPPO1* gene by PCR, two pairs of degenerate primers were designed using Oligo v7 (Rychlik *et al.*, 1990, 2007). Primers SupPPO-F (forward) and SupPPO-L (reverse) were designed to amplify a first fragment (f1) corresponding to the half closest to the 5' end of the *SquPPO1* gene. Primers InfPPO-F (forward) and PPO-Rv (reverse) were designed to amplify a second fragment (f2) corresponding to the part closest to the 3' end of the gene (Tab. 2).

TABLE 1. Accession numbers of the genes used in the multiple sequence alignment.

Species	GenBank ID
Eggplant (*Solanum melongena* L.)	GQ246219.1, HQ731444.1, HQ731445.1, HQ731446.1, HQ731447.1, HM015902.1, JQ621950.1, JQ621948.1, JQ621952.1, JQ621949.1
Tomato (*Solanum lycopersicum* L.)	Z12833.1, Z12834.1, Z12835.1, Z12836.1, Z12837.1, Z12838.1
Tomato (*Solanum pennellii* Correll)	XM015229418.1, XM015229419.1, XM027919008.1
Potato (*Solanum tuberosum* L.)	M95196.1, M95197.1, U22921.1, U22922.1, U22923.1
Tobacco (*Nicotiana tabacum* L.)	KC540916.1, Y12501.1
Tobacco (*Nicotiana tomentosiformis* L.)	XM009591772.2
Tobacco (*Nicotiana attenuata* Steud.)	XM019396541.1
Tobacco (*Nicotiana sylvestris* Speg & S. Comes)	XM009769073.1
Tobacco (*Nicotiana benthamiana* Domin.)	HQ245096.1
Chili pepper (*Capsicum annuum* L.)	XM016718336.1
Goldenberry (*Physalis peruviana* L.)	MF682054.1
Sweet potato (*Ipomoea batatas* L. Lam.)	AB038994.1, AY822711.1
Apple (*Malus domestica* Borkh.)	D87670.1, L29450.1
Chinese plum (*Prunus salicina* Lindl.)	AY865623.2
Faba bean (*Vicia faba* L.)	Z11702.1
Wheat (*Triticum aestivum* L.)	HQ228148.1, HQ228150.1, HQ228152.1
Poplar (*Populus euphratica* Olivier)	HQ914443.1
Balsam poplar (*Populus balsamifera* L.)	AY665682.1
Creosote bush (*Larrea tridentata* (DC.) Coville)	AY370019.1

TABLE 2. Sequence of degenerate primers used for PCR amplification of *SquPPO1* gene fragments 1 and 2. Y= C+T; W= A+T; R= A+G; N= A+C+G+T.

Primer name	Sequence (5´- 3')
SupPPO-F	CTC CTA YWC CAY CYC CTG ATC T
SupPPO-L	CAG AAY TCN GAG TTC AAC CAA TC
InfPPO-F	CAA WTG RTN ACT AAK GCT CC
PPO-Rv	TTA ACA ATC CKC AAG CTT GAT

The PCR was carried out in 0.5 ml microcentrifuge tubes containing 60 mM of Tris-SO_4 (pH 8.9), 18 mM $(NH_4)_2SO_4$, 1.5 and 2.0 mM $MgSO_4$ (f1 and f2 respectively), 0.2 mM of each dNTP, 0.4 µM of each primer, 1.0 unit of Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), and 75 ng of lulo and potato genomic DNA. Amplification was carried out in an MJ Research PTC-100 Thermal Cycler using 35-40 cycles of 1.0 min at 94°C, 45 s at 65.5°C (for f1) and 58°C (for f2) and 75 s at 72°C. One additional cycle was carried out at 72°C for 10 min for the complete extension of the PCR products. The negative amplification control was carried out using DNA from *E. coli*. Both PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sequenced without cloning in an ABIPrism 310 sequencer (Applied Biosystems, USA). Both nucleotide and protein BLAST analyses were carried out using tools from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Southern blot analysis

DNA was extracted from lulo and potato young leaves, previously described. Both genomic DNAs were double digested with combinations of restriction enzymes *BamHI*, *XbaI*, and *PstI*. Digestions were carried out for 8 h at 37°C. Enzymatically digested DNAs were electrophoresed on a 0.8% (w/v) agarose gel and subsequently transferred to a Hybond N^+ nylon membrane (# cat. RPN 225B, Amersham Life Science, USA) using saline-sodium citrate (SSC) buffer 20X (pH 7.0) as transfer solution. This procedure was carried out for 16 h. To fix the DNA, the membrane was treated with UV light (254 nm) for 3 min on each side. A 920-bp potato *ppo* gene fragment generated with primers InfPPO-F and PPO-Rv was used as a probe. The probe amplification conditions were the same as those used for the f2 fragment production. The probe was labeled using the Detector Random Primer DNA Biotinylation Kit (KPL, Gaithersburg, MD, USA). The nylon membrane with digested DNA was prehybridized for 1 h at 42°C. The prehybridization solution contained herring sperm DNA (100 µg ml^{-1}). Next, the biotinylated probe was denatured by heating at 95°C for 10 min and added to the hybridization solution. Hybridization was carried out at 42°C for 16 h. The membrane was washed four times (first wash: 2X SSC + 0.5% SDS, room temperature, 15 min; second and third washes: 1X SSC + 0.5% SDS, 55°C, 15 min; last wash: 0.1X SSC, room temperature, 15 min). Labeled probe detection was made with the Detector AP Chemiluminescent Blotting Kit following the manufacturer's instructions (KPL, Gaithersburg, MD, USA).

Phylogenetic analysis

The *ppo* gene sequences mentioned above were aligned using MUSCLE v5 (Edgar, 2021). The MEGA X software (https://www.megasoftware.net) (Kumar *et al.*, 2018) was used to carry out a Maximum Likelihood test with 500 iterations. Wheat *ppo* genes were used as an outgroup since they possess introns (Massa *et al.,* 2007).

Conservation analysis of the Tyr-2 site

The sequences of the PPO proteins of species belonging to the family Solanaceae were obtained from the National Center for Biotechnology Information Protein Database. (https://www.ncbi.nlm.nih.gov/protein/) (Tab. 3). The Tyr-2 site conservation was evaluated using InterPro v 97.0 (https://www.ebi.ac.uk/interpro/release_notes/97.0/) (Paysan-Lafosse *et al*., 2023).

TABLE 3. Accession numbers of the PPO proteins used in the Tyr-2 site conservation analysis.

Species	Protein database ID
Potato (*Solanum tuberosum* L.)	AAA02877.1, AAA02879.1
Tomato (*Solanum lycopersicum* L.)	CAA78299.1, CAA78300.1
Tomato (*Solanum pennellii* Correll.)	XP015084904.1
Eggplant (*Solanum melongena* L.)	ADY18410.1, ADY18411.1, ADY18412.1
Chili pepper (C*apsicum annuum* L.)	XP016573822.1
Tobacco (*Nicotiana tabacum* L.)	CAA73103.1, AGK83468.1
Tobacco (*Nicotiana benthamiana* Domin.)	AEO00529.1
Goldenberry (*Physalis peruviana* L.)	ASW18458.1
Lulo (*Solanum quitoense* Lam.)	ACN78382.1

Results and discussion

SquPPO1 gene sequence

Two PCR products of 950-bp and 920-bp corresponding to the regions close to the 5' and 3' ends of the *SquPPO1* gene, respectively, were amplified with the primers designed for this study (Tab. 2). Each pair of primers amplified a fragment of the same length in the potato genomic DNA (Fig. 1).

The assembly of nucleotide sequences corresponding to f1 and f2 produced a 1417-bp partial sequence (GenBank ID: FJ573257), which has 425 adenines (30%), 318 cytosines (22.44%), 300 guanines (21.17%), and 374 thymines (26.39%). The guanine-cytosine content (%GC) of the *SquPPO1* gene is 43.61%, a value within the established range for Solanaceae genomes, which varies between 40% and 45% (Carels *et al*., 1998; Rensink *et al*., 2005). The %GC found is within the range observed for tomato and potato *ppo* genes, which vary between 41.83% and 43.64%; in eggplant *ppo* genes this value varies between 41.67% and 44.30%, while in tobacco it is slightly higher than in the above species (44.80%).

A comparison of *SquPPO1* with homologous genes from other species revealed similarity values greater than 89% with those of eggplant (*S. melongena* cv. Arka-Shirish); regarding some of the potato, tomato and tobacco *ppo* genes,

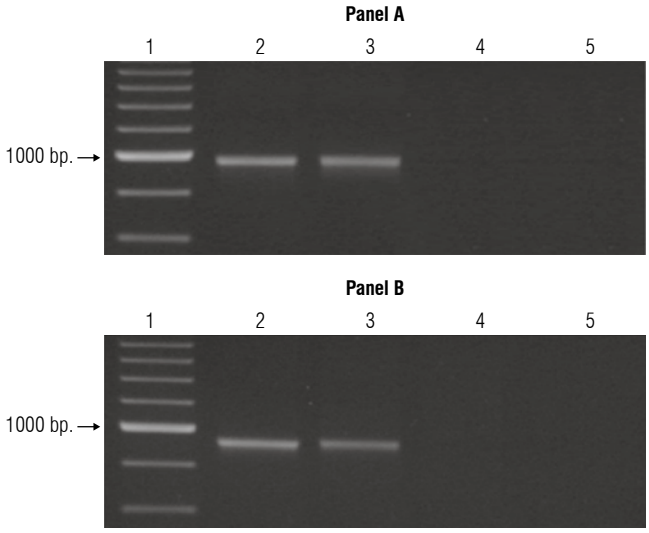

FIGURE 1. Electrophoretic analysis of fragments 1 and 2. Panel A: line 1: molecular size marker; line 2: fragment 1; line 3: positive amplification control (potato genomic DNA); line 4: negative amplification control (*E. coli* DNA); line 5: blank. Panel B: line 1: molecular size marker; line 2: fragment 2; line 3: positive amplification control (potato genomic DNA); line 4: negative amplification control (*E. coli* DNA); line 5: blank.

the similarity ranged between 80% and 82.85%. This range included genes from wild species such as *Solanum pennellii*, a tomato endemic to the Andes, *Nicotiana attenuata*, *N. tomentosiformis*, *N. sylvestris,* and *N. benthamiana* (Tab. 4). Comparison with genes from other Asian eggplant cultivars (such as Azad Kranti, Anupam, Ravaiya and Raveena) showed similarity levels ranging from 74.34% to 76.79%. Although there are similarities with the *ppo* genes from phylogenetically distant families such as Salicaceae (Wang & Constabel, 2004) and Zygophyllaceae, these values are less than 60%. Like the previously characterized Solanaceae *ppo* genes, *SquPPO1* has no introns. Therefore, the presence of two introns in some members of the wheat *ppo* gene family (Chang *et al*., 2007) explains the low percentage of similarity (less than 40%) between Solanaceae *ppo* genes and those of monocots such as wheat.

Southern hybridization analysis

In most plant species, *ppo* genes are part of multigene families, as in the case of tomato (Newman *et al*., 1993; Thipyapong *et al*., 1997), potato (Thygesen *et al*., 1995), faba bean (Cary *et al*., 1992), wheat (Jukanti *et al*., 2004) and others. Southern blot revealed hybridization signals of approximately 12 kb corresponding to lulo and potato genomic DNA (Fig. 2). This result is consistent with previous findings in tobacco (Goldman *et al*., 1998), where a hybridization signal of 11.5 kb was obtained with *BamHI*-digested DNA, and with that of tomato, which showed that the *PPO-E* and *PPO-F* genes are located together in a 12.4

kb region, while the *PPO-A*, *PPO-B* and *PPO-D* genes are located in a second 12.4 kb region (Newman *et al.*, 1993).

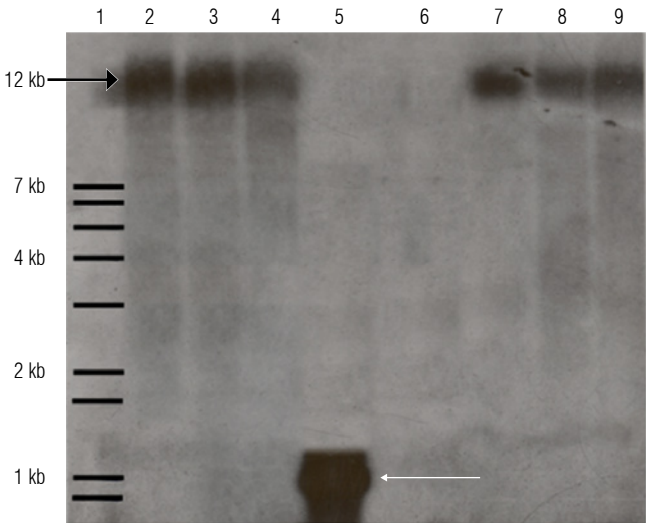

FIGURE 2. Southern analysis: Black arrow shows the Ca.12 kb hybridization signals. Line 1: molecular size marker; lines 2-4: *Bam*HI-*Pst*I, *Bam*HI-*Xba*I and *Xba*I-*Pst*I digested lulo DNA; line 5: 920-bp probe; line 6: blank; lines 7-9: *Bam*HI-*Pst*I, *Bam*HI-*Xba*I and *Xba*I-*Pst*I digested potato DNA.

In monocots like wheat, something similar has been observed: the six *PPO* genes known in this species are organized into two independent clusters, with each one of them having three of these genes (Jukanti *et al.*, 2004). The existence of highly conserved gene clusters in Solanaceae species implies that the location of such clusters on the chromosomes and the positions of some genes relative to others in the same group are also conserved. Using molecular markers, a high degree of collinearity is found between potato and tomato (Bonierbale *et al.*, 1988; Tanksley *et al.*, 1992). In the case of pepper and tomato, 18 common homologous linkage blocks are identified using the same approach (Livingstone *et al.*, 1999). Similarly, tomato markers used in eggplant reveal syntenic regions between these species (Doganlar *et al.*, 2002). The evidence we present, together with the knowledge of the gene family organization in related species allows us to suggest that *SquPPO1* may be accompanied by at least one other *PPO* gene.

Sequence and similarity of lulo PPO to homologous proteins

PPO deduced from the 1417 bp sequence found in lulo has 472 amino acids (GeneBank ID: ACN78382). The BLASTP

TABLE 4. Similarity of the *SquPPO1* gene to related genes from other Solanaceae species.

Species	Gene	Accession number	E-value	Identity (%)
S. melongena cv. Arka Shirish	SmePPO-5	HQ731446.1	0.0	90.28
S. melongena cv. Arka Shirish	SmePPO-4	HQ731445.1	0.0	89.91
S. melongena cv. Arka Shirish	SmePPO-6	HQ731447.1	0.0	89.15
S. tuberosum	ppo-A	M95196.1	0.0	82.85
S. tuberosum	ppo-B	M95197.1	0.0	82.65
S. tuberosum cv. DM 1-3 516 R44	ppo	XM006355177.2	0.0	82.34
C. annuum cv. Zunla-1	ppo-A	XM016718336.1	0.0	82.32
S. lycopersicum cv. Micro-Tom	ppo	AK247410.1	0.0	81.79
S. lycopersicum	ppo-E	Z12837.1	0.0	81.79
S. pennellii	ppo-E	XM015229418.1	0.0	81.72
S. pennellii	ppo	HG975447.1	0.0	81.72
S. pennellii	ppo-F	XM015229419.1	0.0	81.34
S. lycopersicum cv. Tiny Tim LA154	ppo	S40548.1	0.0	81.23
N. tabacum cv. TN90	ppo-E	XM016600648.1	0.0	80.94
N. attenuata	ppo-E	XM019396541.1	0.0	80.87
S. lycopersicum cv. Micro-Tom	ppo-F	NM001331130.1	0.0	80.81
N. tomentosiformis	ppo-E	XM009591772.2	0.0	80.80
N. sylvestris	ppo-E	XM009769073.1	0.0	80.31
N. tabacum cv. Petit Havana SR1	ppo	Y12501.1	0.0	80.31
N. benthamiana	Nbppo-1	HQ245096.1	0.0	80.24
N. tabacum	ppo	KC540916.1	0.0	80.10
S. lycopersicum	ppo-F	Z12838.1	0.0	80.10

analysis showed results consistent with the nucleotide sequence analysis mentioned above (Tab. 5).

Location of copper-binding sites and central domain of tyrosinases in lulo PPO

Lulo PPO has the central domain characteristic of tyrosinases, a protein family that includes all plant PPOs. The region of the lulo PPO where the common central domain would be located extends from P70 to I276. The studies of the three-dimensional structure of sweet potato PPO showed that each one of the two copper-binding sites is coordinated by three histidine residues (Klabunde *et al*., 1998). These six histidine residues are inside the conserved central domain of the protein. The amino acids involved in the coordination with copper ions called $H_{A1,}$ $H_{A2,}$ H_{A3} (for Copper A) and $H_{B1,}$ $H_{B2,}$ H_{B3} (for Copper B) follow the general rule of H_{A1} – x(n) – H_{A2} – x(8) – H_{A3} and H_{B1} – x(3) – H_{B2} – x(n) – $H_{B3,}$ respectively, where *n* is a variable amino acid number (García-Borrón & Solano, 2002).The histidines that would interact with the two copper ions in lulo PPO fit this distribution. The copper atom in binding site A would be coordinated with H80, H98 and H107; the copper atom in binding site B would be coordinated with H229, H233 and H263. The six histidines mentioned above are well-conserved in all plant PPOs (Fig. 3). The PPO regions involved in the interaction with copper atoms show a high degree of conservation (Halaouli *et al*., 2006). The alignment of the PPOs (including that of lulo) allowed these regions to be identified by sequence similarity. The Copper A binding site identified in the lulo PPO would be located between H98 and W134, while the Copper B binding site would be located between H229 and D282.

Variation of an amino acid in the hydrophobic shell containing the active site of the lulo PPO

The active site of tyrosinases has been described as a hydrophilic sphere demarcated by four alpha helixes in which the six histidine residues are included (García-Borrón & Solano, 2002). This hydrophilic sphere would be located inside a hydrophobic shell formed by aromatic amino acids

TABLE 5. Similarity of lulo PPO to other Solanaceae PPOs.

Protein	E-value	% identity	Accession
Chloroplast polyphenol oxidase (*S. melongena*)	0.0	86.64%	QLG20182.1
Chloroplast polyphenol oxidase (*S. melongena*)	0.0	85.84%	ADY18411.1
Chloroplast polyphenol oxidase (*S. melongena*)	0.0	85.41%	ADY18412.1
Polyphenol oxidase F, chloroplastic (*C. chinense*)	0.0	84.36%	PHU29006.1
Polyphenol oxidase F, chloroplastic (*C. baccatum*)	0.0	84.14%	PHT58552.1
Catechol oxidase B, chloroplastic (*C. annuum*)	0.0	84.14%	XP016573822.2
Polyphenol oxidase E, chloroplastic (*S. lycopersicum*)	0.0	83.26%	NP001318057.1
Polyphenol oxidase E, chloroplastic (*S. pennellii*)	0.0	83.05%	XP015084904.1
Catechol oxidase B, chloroplastic (*S. commersonii*)	0.0	82.84%	KAG5592182.1
Catechol oxidase B, chloroplastic (*S. stenotomum*)	0.0	82.63%	XP049377390.1
Catechol oxidase B, chloroplastic (*S. verrucosum*)	0.0	82.63%	XP049361834.1
Polyphenol oxidase B precursor (*S. tuberosum*)	0.0	82.42%	Q06355.1
Propolyphenol oxidase (*S. tuberosum*)	0.0	82.20%	AAA02877.1
Polyphenoloxidase (*S. lycopersicum*)	0.0	82.20%	AAB22610.1
Chloroplast polyphenol oxidase (*S. melongena*)	0.0	81.97%	ADY18410.1
Polyphenol oxidase F, chloroplastic (*S. lycopersicum*)	0.0	81.82%	NP001318059.1
Polyphenol oxidase E, chloroplastic (*N. tabacum*)	0.0	81.72%	XP016456134.1
Polyphenol oxidase (*N. tabacum*)	0.0	81.30%	CAA73103.1
Polyphenol oxidase E, chloroplastic (*N. sylvestris*)	0.0	81.30%	XP009767375.1
Polyphenol oxidase E (*N. tomentosiformis*)	0.0	81.30%	XP009590067.1
Polyphenol oxidase F, chloroplastic (*S. pennellii*)	0.0	81.18%	XP015084905.1
Polyphenol oxidase E, chloroplastic (*N. bombycine*)	0.0	81.09%	XP019252086.1
Polyphenol oxidase (*Nicotiana benthamiana*)	0.0	80.88%	AEO00529.1

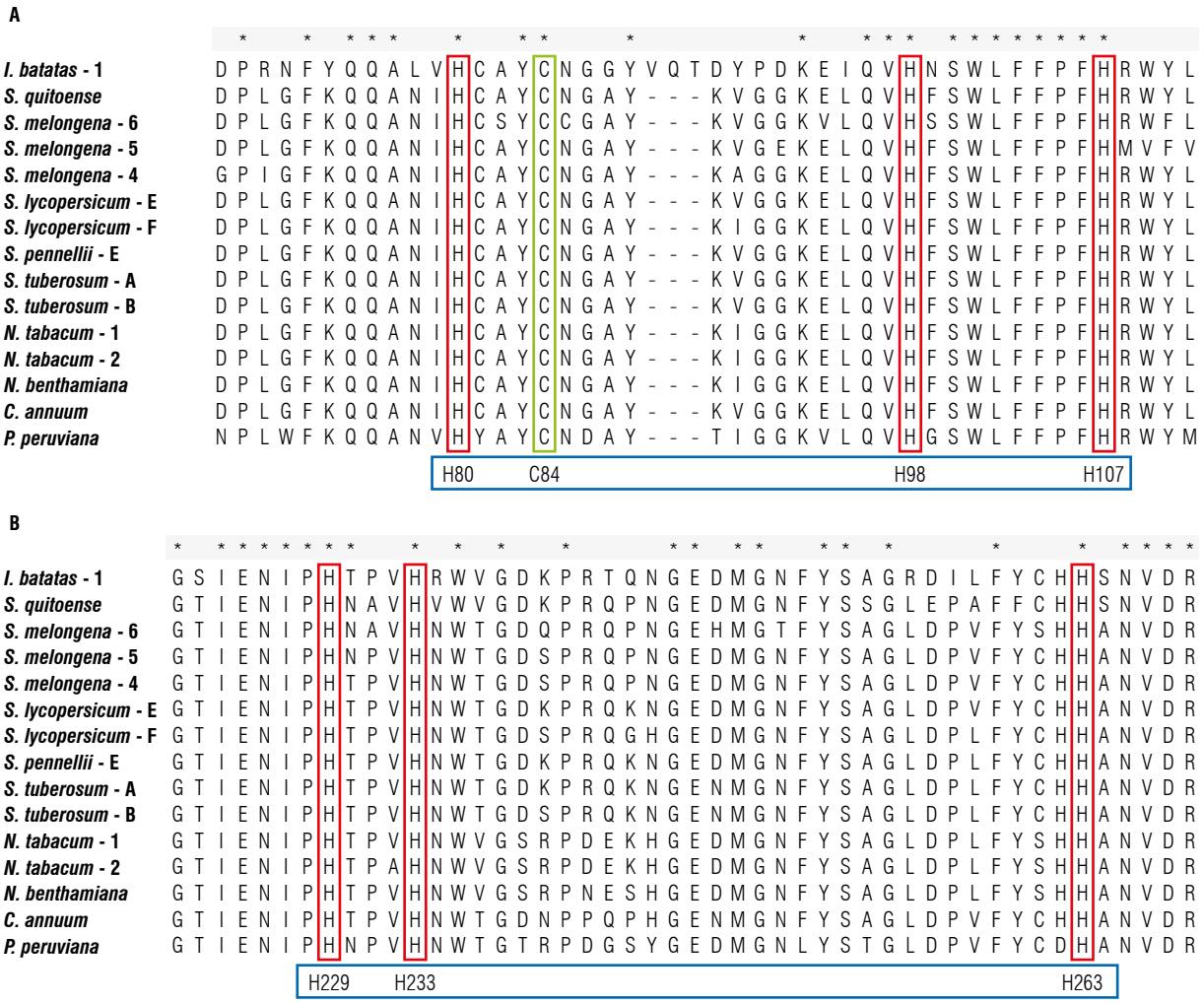

FIGURE 3. Conservation of the histidines involved in the coordination with copper atoms A and B in plant PPOs. Sweet potato PPO was included because its crystal structure has been well studied. A) Histidines coordinated with the Copper A site, B) Histidines coordinated with the Copper B site. Asterisks denote conserved amino acids.

and highly conserved residues. The structure proposed is: $\Phi_{(HA1-7)}$, $R_{(HA3+1)}$, $\Phi_{(HA3+3)}$, $\Phi_{(HA3+7)}$, $E_{(HA3+8)}$, $D_{(HB3-7)}$, $D_{(HB3+4)}$, $\Phi_{(HB3+7),}$ and $W_{(HB3+10)}$ (where "Φ" represents an aromatic residue). The lulo PPO retains the following residues: $\Phi_{(HA1-7)}$ (Φ corresponds to F), $R_{(HA3+1)}$, $\Phi_{(HA3+3)}$ (Φ corresponds to Y), $\Phi_{(HA3+7)}$ (Φ corresponds to Y), $E_{(HA3+8)}$, $D_{(HB3+4),}$ $\Phi_{(HB3+7)}$ (Φ corresponds to W), and $W_{(HB3+10)}$. In the lulo PPO, the $D_{(HB3-7)}$ that is conserved in most of the Solanaceae PPOs, is replaced by E (Fig. 4). This sequence change is conservative because oxidized copper preferentially coordinates with oxygen in aspartic and glutamic acids or with the imidazole nitrogen group in histidines (Festa & Thiele, 2011). Another characteristic trait of fungal tyrosinases and plant PPOs is the presence of a highly conserved cysteine residue, which is involved in a thioether covalent bond with the second conserved histidine at the copper A site. In the lulo PPO, this cysteine is at position 84 (green box in Fig. 3A). As in the previously studied PPOs, the regions of the lulo PPO where the two copper atoms would be coupled are asymmetric concerning the amino acid composition (Fig. 4).

The lulo PPO does not have a Tyr-2 site

One of the characteristic features of PPOs is the presence of three conserved regions: a chloroplast transit peptide (Ctp) at the N-terminal end, the copper-binding domain (dicopper center), and the C-terminal end (Tran *et al.*, 2012; Zhang, 2023). Two conserved sites were identified in the copper-binding domain of all studied Solanaceae PPOs. The conserved site called Tyrosinase 1 (Tyr-1) is 18 residues in size and contains two of the histidines (the second and third) that bind CuA, whereas the conserved site called Tyrosinase 2 (Tyr-2) is 12 residues in size and contains one of the histidines (the third) that binds CuB (Fig. 5).

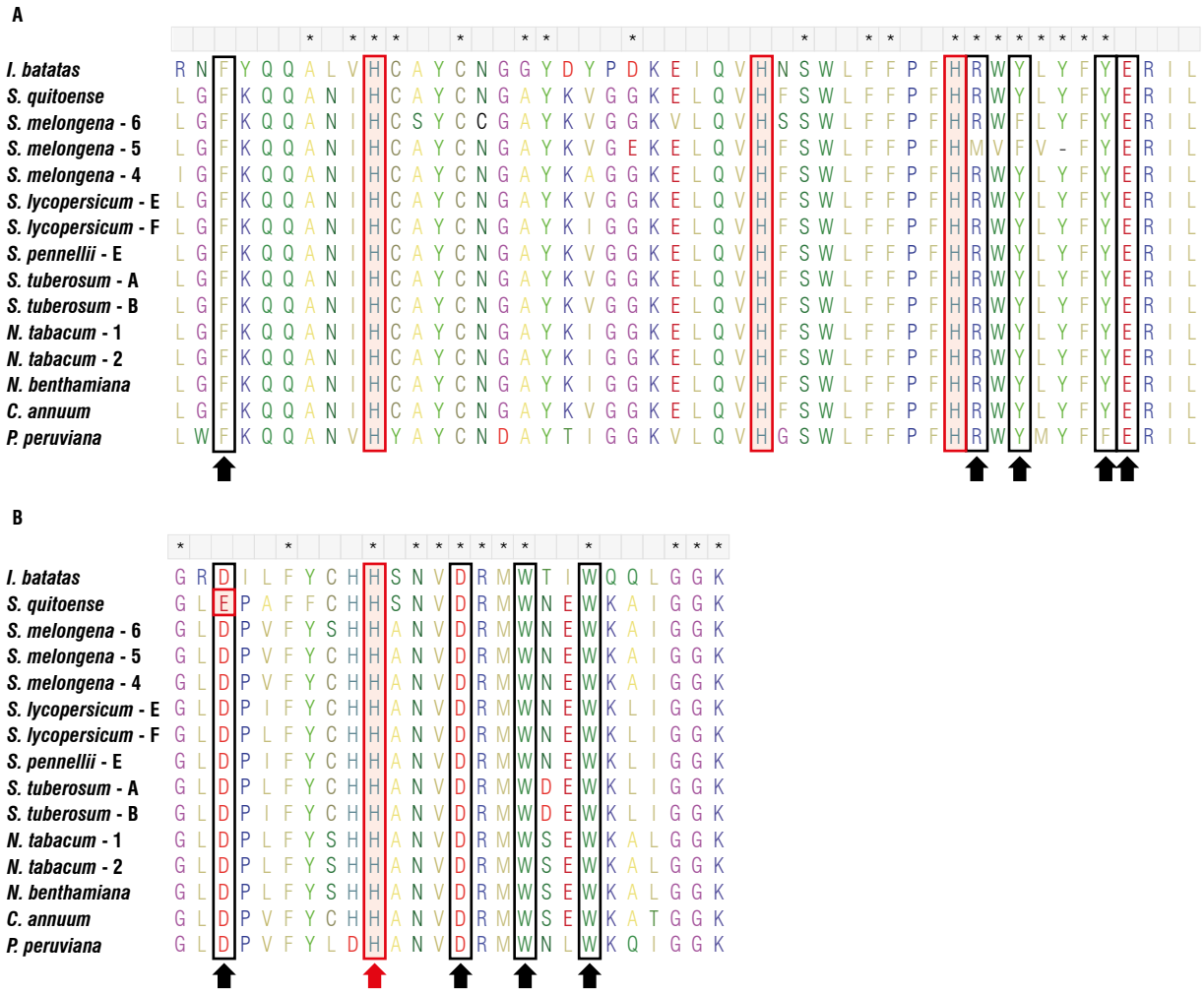

FIGURE 4. Conserved amino acids in the hydrophobic shell containing the active site of plant PPOs (black arrows). A) Copper site A, B) Region near the third histidine (red arrow) of the copper site B. Glutamic acid (E) is located at position HB3-7 of the lulo PPO instead of aspartic acid (D) (red box). Asterisks denote conserved amino acids.

FIGURE 5. The Tyr-2 site (black box) contains the third histidine (pink box) of the CuB-binding site. The red box indicates the DxE substitution that is critical for the conservation of the Tyr-2 site. The sequence of *I. batatas* is included as reference since it is the best characterized plant PPO from a structural point of view. Asterisks denote conserved amino acids.

The absence of the Tyr-2 site in the PPO of lulo (Fig. 6) is due to a conservative DxE substitution, altering the conserved motif found in other Solanaceae PPOs (García-Borrón & Solano, 2002). In most plant PPOs, the conserved Tyr-2 site presents the following sequence pattern: D – P – x – F – [LIVMFYW] – x(2) – H – x(3) – D, as shown in the alignment (Fig. 5). The first residue of the conserved site is D, but in the lulo PPO, D is conservatively substituted by E (red box). In all copper oxidases studied, there are water channels that connect the copper-binding center of the enzyme to the external environment. A conservative substitution D500E in the copper-binding center of a bacterial laccase suggests that acidic groups in the water channel play a key role in the deprotonation events that occur at the copper-binding center (Nasoohi *et al.*, 2013).

On the other hand, the conservative substitution D208E at the copper B-binding site in *S. glaucescens* tyrosinase increases the stability of the oxy-enzyme with respect to the met and deoxy forms, causing a slight perturbation in the kinetic and spectroscopic properties of the enzyme, but without substantially affecting the catalytic activity of the enzyme (Jackman *et al.*, 1992). With the information currently available, it is difficult to precisely establish what effect this amino acid change may have on the function or structure of the lulo PPO. Since crystallography on its own cannot elucidate the enzymatic function, the evaluation of the catalytic activity of this PPO on various *o*-diphenols may offer valuable additional insights.

Phylogenetic relationships between *SquPPO1* and *SmePPO4, SmePPO5* and *SmePPO6*

In our study, a phylogenetic relationship analysis between several Solanaceae *ppo* genes was carried out (Fig. 7). Surprisingly, the phylogenetic tree revealed that *SquPPO1* is more closely related to the eggplant *PPO* genes (*SmePPO4, SmePPO5*, and *SmePPO6*) than to those of potato, tobacco and tomato. This is interesting because lulo, potato, tobacco, and tomato are native to the Andean region of northern South America, while eggplant has as a center of origin the northeastern region of India. Wheat *ppo* genes were used as an outgroup since they possess introns (Massa *et al.*, 2007).

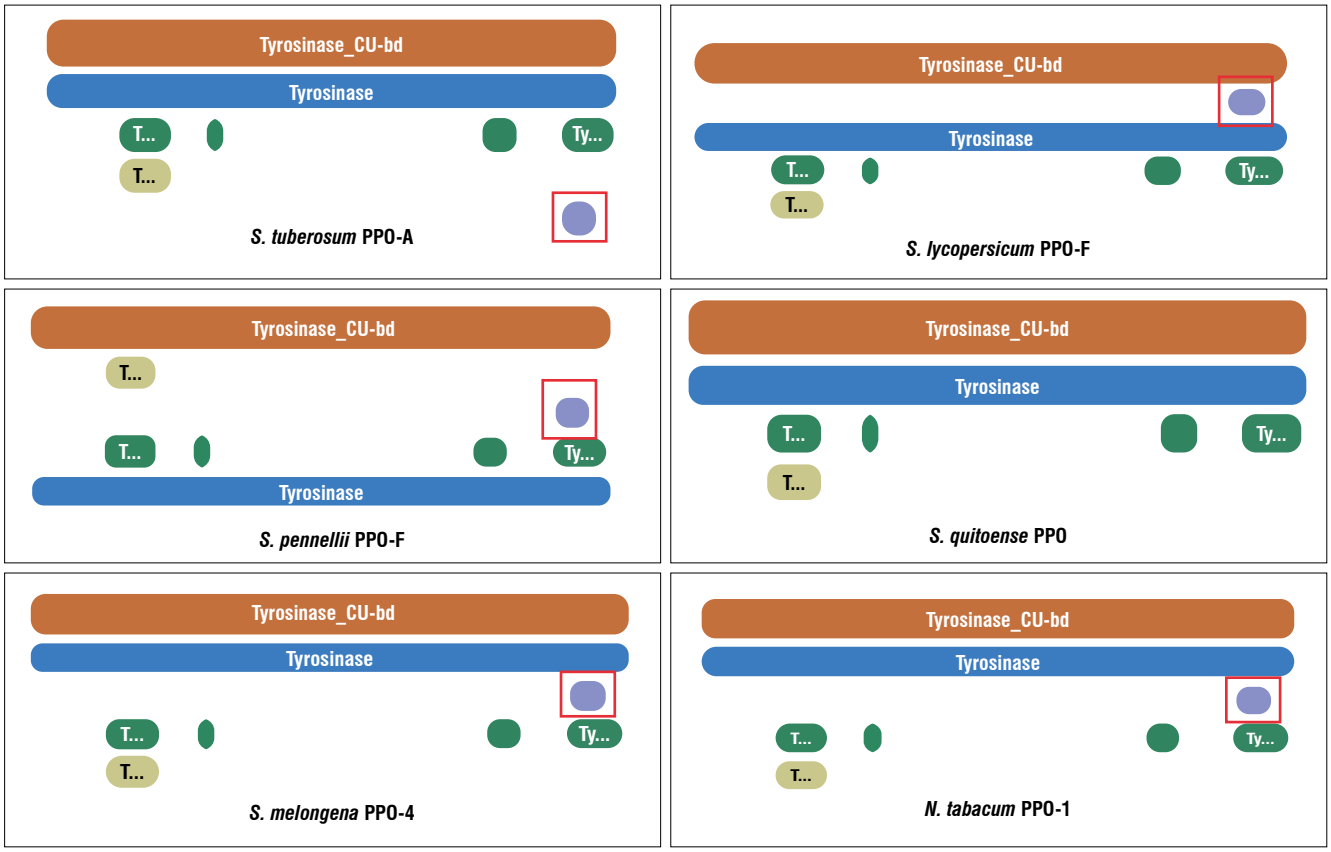

FIGURE 6. The Tyr-2 site (light purple oval inside the red square) is conserved in the Solanaceae PPOs, except in that of lulo (only some of the PPOs are shown). Tyrosinase Cu-bd: copper-binding domain of tyrosinase family; blue oval: common central domain of tyrosinase family. Highly conserved sequence motifs are indicated by the other ovals.

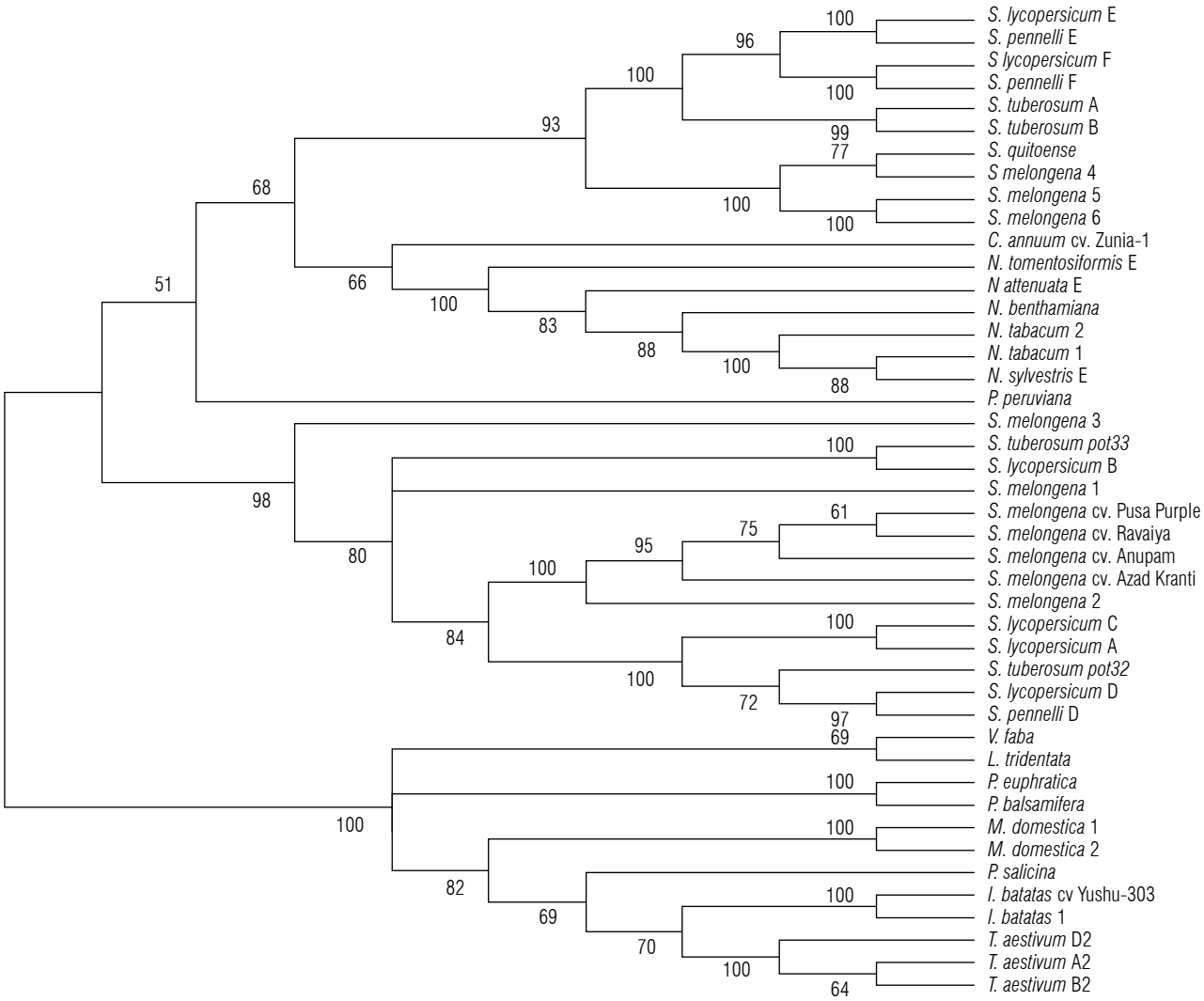

FIGURE 7. Phylogenetic relationships among *ppo* genes are shown as a strict consensus tree. Wheat (*Triticum aestivum*) *ppo* genes were used as an outgroup. Numbers above branches represent bootstrap values.

Depending on the similarity levels between members of the eggplant *ppo* multigene family, the six genes were assigned to two structurally distinct classes: class A (*SmePPO1, SmePPO2,* and *SmePPO3*) and class B (*SmePPO4, SmePPO5,* and *SmePPO6*). This distribution agrees with that observed in our phylogenetic tree. Since the eggplant *SmePPO4* gene is expressed in young and mature leaves, flowers (pre- and post-anthesis), and fruits (Shetty *et al.*, 2011), it is possible to suppose that *SquPPO1* could show a similar pattern of expression and function given the close phylogenetic relationship between the two (Fig. 7).

The close relationship between *SquPPO1* and *SmePPO4* can be interpreted as the consequence of selective pressures of a similar nature acting on them, since they are involved in functions related to the adaptive response of both species to their respective environments. These pressures induce genetic convergence events that maintain the ability of these individuals to oxidize phenolic molecules in certain types of tissues. Similar evolutionary phenomena have been studied in several groups of higher plants. In species of the Iochrominae (Solanaceae) clade, it was observed that flower color transitions are determined by the convergent loss of expression of three genes (*F3'5'h, Dfr,* and *Ans*) of the anthocyanin pathway (Larter *et al.*, 2018). Another well-studied case is that of the furanocoumarin pathway in Moraceae, where the emergence of a new cytochrome P450 is a consequence of convergent evolution (Villard *et al.*, 2021). Although the reactions catalyzed by PPOs are well known, data on the function of these enzymes in cellular or tissue-level processes are still under discussion. The diversity of tissues and conditions in which PPOs are expressed suggests that these enzymes may play a key role in various stress response-related processes.

Conclusions

In this study, the sequence of the lulo *SquPPO1* gene was partially determined. As is the case with other Solanaceae *PPO* genes, the *SquPPO1* gene lacks introns. The results of Southern hybridization suggest that *SquPPO1* may be accompanied by at least one other *PPO* gene, given the size of the genomic region in which it was located. Further studies are required to determine the precise size of the *PPO* gene family in this species. On the other hand, the utilization of molecular markers previously developed in related species (Bonierbale *et al*., 1988; Doganlar *et al*., 2002; Tanksley *et al*., 1992) may be useful in elucidating the degree of collinearity that lulo *PPO* genes share with other Solanaceae species. A comparison of the PPO deduced from the *SquPPO1* gene with PPOs from other species of the Solanaceae family allowed us to determine a DxE substitution (position 256). This substitution involves the disappearance of the Tyr-2 site in the copper-binding domain of the lulo PPO. It remains to be determined whether this amino acid change would confer a structural advantage with evolutionary implications related to the conservation of this gene in the species. Finally, a close phylogenetic relationship was found between *SquPPO1* and the eggplant genes *SmePPO4*, *SmePPO5* and *SmePPO6*. This evolutionary proximity can be interpreted as the result of a genetic convergence event. It is tempting to assume that *SquPPO1* could exhibit a pattern of expression and function similar to that of the eggplant *SmePPO4* gene. The results of our study provide a starting point for subsequent investigations into the effect of DxE substitution on the catalytic activity of lulo PPO. In addition, the use of tools to generate point mutations in the active site of PPO may offer the possibility of reducing its enzymatic activity, thus minimizing the occurrence of enzymatic browning in fruits.

Acknowledgments

We thank the Corporación Colombiana de Investigación Agropecuaria CORPOICA (now AGROSAVIA) for funding.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author´s contributions

MAPJ and SGD: methodology design, research, formal analysis and data curation. MAPJ: writing the initial draft. VNZ: research design, funding acquisition and project management. All authors reviewed the final version of the manuscript.

Literature cited

Araji, S., Grammer, T. A., Gertzen, R., Anderson, S. D., Mikulic-Petkovsek, M., Veberic, R., Phu, M. L., Solar, A., Leslie, C. A., Dandekar, A. M., & Escobar, M. A. (2014). Novel roles for the polyphenol oxidase enzyme in secondary metabolism and the regulation of cell death in walnut. *Plant Physiology, 164*(3), 1191–1203. https://doi.org/10.1104/pp.113.228593

Bonierbale, M. W., Plaisted, R. L., & Tanksley, S. D. (1988). RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. *Genetics, 120*(4), 1095–1103. https://doi.org/10.1093/genetics/120.4.1095

Carels, N., Hatey, P., Jabbari, K., & Bernardi, G. (1998). Compositional properties of homologous coding sequences from plants. *Journal of Molecular Evolution, 46*, 45–53. https://doi.org/10.1007/PL00006282

Cary, J. W., Lax, A. R., & Flurkey, W. H. (1992). Cloning and characterization of cDNAs coding for *Vicia faba* polyphenol oxidase. *Plant Molecular Biology, 20*, 245–253. https://doi.org/10.1007/BF00014492

Chang, C., Zhang, H., Xu, J., You, M., Li, B., & Liu, G. (2007). Variation in two PPO genes associated with polyphenol oxidase activity in seeds of common wheat. *Euphytica, 154*, 181–193. https://doi.org/10.1007/s10681-006-9285-2

Chi, M., Bhagwat, B., Lane, W. D., Tang, G., Su, Y., Sun, R., Oomah, B. D., Wiersma, P. A., & Xiang, Y. (2014). Reduced polyphenol oxidase gene expression and enzymatic browning in potato (*Solanum tuberosum* L.) with artificial microRNAs. *BMC Plant Biology, 14*, Article 62. https://doi.org/10.1186/1471-2229-14-62

Constabel, C. P., & Barbehenn, R. V. (2008). Defensive roles of polyphenol oxidase in plants. In A. Schaller (Ed.), *Induced plant resistance to herbivory* (pp. 253–269). Springer. https://doi.org/10.1007/978-1-4020-8182-8_12

Constabel, C. P., & Ryan, C. A. (1998). A survey of wound- and methyl jasmonate-induced leaf polyphenol oxidase in crop plants. *Phytochemistry, 47*(4), 507–511. https://doi.org/10.1016/S0031-9422(97)00539-6

Constabel, C. P., Yip, L., Patton, J. J., & Christopher, M. E. (2000). Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiology, 124*(1), 285–296. https://doi.org/10.1104/pp.124.1.285

Deanna, R., Acosta, M. C., Scaldaferro, M., & Chiarini, F. (2022). Chromosome evolution in the family Solanaceae. *Frontiers in Plant Science, 12*, Article 787590. https://doi.org/10.3389/fpls.2021.787590

Doganlar, S., Frary, A., Daunay, M. C., Lester, R. N., & Tanksley, S. D. (2002). A comparative genetic linkage map of eggplant (*Solanum melongena*) and its implications for genome evolution in the Solanaceae. *Genetics, 161*(4), 1697–1711. https://doi.org/10.1093/genetics/161.4.1697

Doyle, M., & Doyle, A. (1990). Isolation of DNA from small amounts of plant tissues. *BRL Focus, 12*, 13–15.

Edgar, R. C. (2021). Muscle5. https://github.com/rcedgar/muscle/releases/tag/v5.3

Festa, R. A., & Thiele, D. J. (2011). Copper: An essential metal in biology. *Current Biology, 21*(21), R877–R883. https://doi.org/10.1016/j.cub.2011.09.040

García-Borrón, J. C., & Solano, F. (2002). Molecular anatomy of tyrosinase and its related proteins: Beyond the histidine bound metal catalytic center. *Pigment Cell Research, 15*(3), 162–173. https://doi.org/10.1034/j.1600-0749.2002.02012.x

Gerdemann, C., Eicken, C., & Krebs, B. (2002). The crystal structure of catechol oxidase: New insight into the function of type-3 copper proteins. *Accounts of Chemical Research, 35*(3), 183–191. https://doi.org/10.1021/ar990019a

Goldman, M. H. S., Seurinck, J., Marins, M., Goldman, G. H., & Mariani, C. (1998). A tobacco flower-specific gene encodes a polyphenol oxidase. *Plant Molecular Biology, 36*, 479–485. https://doi.org/10.1023/A:1005914918284

Halaouli, S., Asther, M., Sigoillot, J. C., Hamdi, M., & Lomascolo, A. (2006). Fungal tyrosinases: New prospects in molecular characteristics, bioengineering and biotechnological applications. *Journal of Applied Microbiology, 100*(2), 219–232. https://doi.org/10.1111/j.1365-2672.2006.02866.x

Hasan, M. U., Malik, A. U., Ali, S., Imtiaz, A., Munir, A., Amjad, W., & Anwar, R. (2019). Modern drying techniques in fruits and vegetables to overcome postharvest losses: A review. *Journal of Food Processing and Preservation, 43*, Article e14280. https://doi.org/10.1111/jfpp.14280

Heiser, C. B. (1985). Ethnobotany of the naranjilla (*Solanum quitoense*) and its relatives. *Economic Botany, 39*(1), 4–11. https://doi.org/10.1007/BF02861168

Hong, Q., Chen, Y. L., Lin, D., Yang, R. Q., Cao, K. Y., Zhang, L. J., Liu, Y. M., Sun, L. C., & Cao, M. J. (2024). Expression of polyphenol oxidase of *Litopenaeus vannamei* and its characterization. *Food Chemistry, 432*, Article 137258. https://doi.org/10.1016/j.foodchem.2023.137258

Jackman, M. P., Huber, M., Hajnal, A., & Lerch, K. (1992). Stabilization of the oxy form of tyrosinase by a single conservative amino acid substitution. *Biochemical Journal, 282*(3), 915–918. https://doi.org/10.1042/bj2820915

Jukanti, A. K., Bruckner, P. L., & Fischer, A. M. (2004). Evaluation of wheat polyphenol oxidase genes. *Cereal Chemistry, 81*(4), 481–485. https://doi.org/10.1094/CCHEM.2004.81.4.481

Klabunde, T., Eicken, C., Sacchettini, J. C., & Krebs, B. (1998). Crystal structure of a plant catechol oxidase containing a dicopper center. *Nature Structural & Molecular Biology, 5*(12), 1084–1090. https://doi.org/10.1038/4193

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution, 35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Larter, M., Dunbar-Wallis, A., Berardi, A. E., & Smith, S. D. (2018). Convergent evolution at the pathway level: Predictable regulatory changes during flower color transitions. *Molecular Biology and Evolution, 35*(9), 2159–2169. https://doi.org/10.1093/molbev/msy117

Lerch, K. (1983). Neurospora tyrosinase: Structural, spectroscopic and catalytic properties. *Molecular and Cellular Biochemistry, 52*(2), 125–138. https://doi.org/10.1007/BF00224921

Li, F. (2020). Purification, kinetic parameters, and isoforms of polyphenol oxidase from "Xushu 22" sweet potato skin. *Journal of Food Biochemistry, 44*(2), Article e13452. https://doi.org/10.1111/jfbc.13452

Liu, H., Pan, M., Lu, Y., Wang, M., Huang, S., Li, J., Luo, K., Luo, L., Yao, M., Hua, D., & Wang, H. (2023). Purification and comparison of soluble and membrane-bound polyphenol oxidase from potato (*Solanum tuberosum*) tubers. *Protein Expression and Purification, 202*, Article 106195. https://doi.org/10.1016/j.pep.2022.106195

Livingstone, K. D., Lackney, V. K., Blauth, J. R., van Wijk, R., & Jahn, M. K. (1999). Genome mapping in Capsicum and the evolution of genome structure in the Solanaceae. *Genetics, 152*(3), 1183–1202. https://doi.org/10.1093/genetics/152.3.1183

Massa, A. N., Beecher, B., & Morris, C. F. (2007). Polyphenol oxidase (PPO) in wheat and wild relatives: Molecular evidence for a multigene family. *Theoretical and Applied Genetics, 114*(7), 1239–1247. https://doi.org/10.1007/s00122-007-0514-4

Mayer, A. M. (2006). Polyphenol oxidases in plants and fungi: Going places? A review. *Phytochemistry, 67*(21), 2318–2331. https://doi.org/10.1016/j.phytochem.2006.08.006

Mishra, B., & Singh Sangwan, N. (2019). Amelioration of cadmium stress in *Withania somnifera* by ROS management: Active participation of primary and secondary metabolism. *Plant Growth Regulation, 87*(3), 403–412. https://doi.org/10.1007/s10725-019-00480-8

Molitor, C., Mauracher, S. G., Pargan, S., Mayer, R. L., Halbwirth, H., & Rompel, A. (2015). Latent and active aurone synthase from petals of *C. grandiflora*: A polyphenol oxidase with unique characteristics. *Planta, 242*(3), 519–537. https://doi.org/10.1007/s00425-015-2261-0

Moon, K. M., Kwon, E. B., Lee, B., & Kim, C. Y. (2020). Recent trends in controlling the enzymatic browning of fruit and vegetable products. *Molecules, 25*(12), Article 2754. https://doi.org/10.3390/molecules25122754

Nakayama, T., Yonekura-Sakakibara, K., Sato, T., Kikuchi, S., Fukui, Y., Fukuchi-Mizutani, M., Ueda, T., Nakao, M., Tanaka, Y., Kusumi, T., & Nishino, T. (2000). Aureusidin synthase: A polyphenol oxidase homolog responsible for flower coloration. *Science, 290*(5494), 1163–1166. https://doi.org/10.1126/science.290.5494.1163

Nasoohi, N., Khajeh, K., Mohammadian, M., & Ranjbar, B. (2013). Enhancement of catalysis and functional expression of a bacterial laccase by single amino acid replacement. *International Journal of Biological Macromolecules, 60,* 56–61. https://doi.org/10.1016/j.ijbiomac.2013.05.011

Rychlik, W., Spencer, W. J., & Rhoads, R. E. (1990). Optimization of the annealing temperature for DNA amplification *in vitro*. *Nucleic Acids Research, 18*(21), 6409–6412. https://doi.org/10.1093/nar/18.21.6409

Sarsenova, A., Demir, D., Çağlayan, K., Abiyev, S., Darbayeva, T., & Eken, C. (2023). Purification and properties of polyphenol oxidase of dried *Volvariella bombycina*. *Biology, 12*(1), Article 53. https://doi.org/10.3390/biology12010053

Shetty, S. M., Chandrashekar, A., & Venkatesh, Y. P. (2011). Eggplant polyphenol oxidase multigene family: Cloning, phylogeny, expression analyses and immunolocalization in response to wounding. *Phytochemistry, 72*, 2275–2287. https://doi.org/10.1016/j.phytochem.2011.08.028

Tanksley, S. D., Ganal, M. W., Prince, J. P., de Vicente, M. C., Bonierbale, M. W., Broun, P., Fulton, T. M., Giovannoni, J. J., Grandillo, S., & Martin, G. B. (1992). High density molecular

linkage maps of the tomato and potato genomes. *Genetics, 132*, 1141–1160. https://doi.org/10.1093/genetics/132.4.1141

Taranto, F., Pasqualone, A., Mangini, G., Tripodi, P., Miazzi, M. M., Pavan, S., & Montemurro, C. (2017). Polyphenol oxidases in crops: Biochemical, physiological and genetic aspects. *International Journal of Molecular Sciences, 18*(2), Article 377. https://doi.org/10.3390/ijms18020377

Thipyapong, P., Hunt, M. D., & Steffens, J. C. (1995). Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase. *Phytochemistry, 40*(3), 673–676. https://doi.org/10.1016/0031-9422(95)00359-F

Thipyapong, P., Joel, M. D., & Steffens, J. C. (1997). Differential expression and turnover of the tomato polyphenol oxidase gene family during vegetative and reproductive development. *Plant Physiology, 113*(3), 707–718. https://doi.org/10.1104/pp.113.3.707

Thipyapong, P., Stout, M. J., & Attajarusit, J. (2007). Functional analysis of polyphenol oxidases by antisense/sense technology. *Molecules, 12*(6), 1569–1595. https://doi.org/10.3390/12081569

Thygesen, P. W., Dry, I. B., & Robinson, S. P. (1995). Polyphenol oxidase in potato (a multigene family that exhibits differential expression patterns). *Plant Physiology, 109*(2), 525–531. https://doi.org/10.1104/pp.109.2.525

Tran, L. T., Taylor, J. S., & Constabel, C. P. (2012). The polyphenol oxidase gene family in land plants: Lineage-specific duplication and expansion. *BMC Genomics, 13*, Article 395. https://doi.org/10.1186/1471-2164-13-395

Villard, C., Munakata, R., Kitajima, S., van Velzen, R., Schranz, M. E., Larbat, R., & Hehn, A. (2021). A new P450 involved in the furanocoumarin pathway underlies a recent case of convergent evolution. *New Phytologist, 231*(5), 1923–1939. https://doi.org/10.1111/nph.17458

Wang, J., & Constabel, C. P. (2004). Three polyphenol oxidases from hybrid poplar are differentially expressed during development and after wounding and elicitor treatment. *Physiologia Plantarum, 122*(3), 344–353. https://doi.org/10.1111/j.1399-3054.2004.00403.x

Wei, X. M., Shu, J., Fahad, S., Tao, K. L., Zhang, J. W., Chen, G. L., Liang, Y. C., Wang, M. Q., Chen, S. Y., & Liao, J. (2023). Polyphenol oxidases regulate pollen development through modulating flavonoids homeostasis in tobacco. *Plant Physiology and Biochemistry, 198*, Article 107702. https://doi.org/10.1016/j.plaphy.2023.107702

Wold-McGimsey, F., Krosch, C., Alarcón-Reverte, R., Ravet, K., Katz, A., Stromberger, J., Mason, R. E., & Pearce, S. (2023). Multitarget genome editing reduces polyphenol oxidase activity in wheat (*Triticum aestivum* L.) grains. *Frontiers in Plant Science, 14*, Article 1247680. https://doi.org/10.3389/fpls.2023.1247680

Yoruk, R., & Marshall, M. R. (2003). Physicochemical properties and function of plant polyphenol oxidase: A review. *Journal of Food Biochemistry, 27*, 361–422. https://doi.org/10.1111/j.1745-4514.2003.tb00289.x

Zhang, J., & Sun, X. (2021). Recent advances in polyphenol oxidase-mediated plant stress responses. *Phytochemistry, 181*, Article 112588. https://doi.org/10.1016/j.phytochem.2020.112588

Zhang, S. (2023). Recent advances of polyphenol oxidases in plants. *Molecules, 28*(5), Article 2158. https://doi.org/10.3390/molecules28052158