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A catechol oxidase AcPPO from cherimoya (*Annona cherimola* Mill.) is localized to the Golgi apparatus

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ABSTRACT

Cherimoya (*Annona cherimola*) is an exotic fruit with attractive organoleptic characteristics. However, it is highly perishable and susceptible to postharvest browning. In fresh fruit, browning is primarily caused by the polyphenol oxidase (PPO) enzyme catalyzing the oxidation of o-diphenols to quinones, which polymerize to form brown melanin pigment. There is no consensus in the literature regarding a specific role of PPO, and its subcellular localization in different plant species is mainly described within plastids. The present work determined the subcellular localization of a PPO protein from cherimoya (AcPPO). The obtained results revealed that the AcPPO- green fluorescent protein co-localized with a Golgi apparatus marker, and AcPPO activity was present in Golgi apparatus-enriched fractions. Likewise, transient expression assays revealed that AcPPO remained active in Golgi apparatus-enriched fractions obtained from tobacco leaves. These results suggest a putative function of AcPPO in the Golgi apparatus of cherimoya, providing new perspectives on PPO functionality in the secretory pathway, its effects on cherimoya physiology, and the evolution of this enzyme.

1. Introduction

The *Annonaceae* family is comprised of more than 100 genera and 2400 species [1]. The cherimoya (*Annona cherimola* Mill.), the most cultivated species of this family [2], is an exotic subtropical fruit native to South America [3]. The organoleptic characteristics of this fruit make it very attractive for use in fresh-cuts, but, like many other fruits, the cherimoya is extremely susceptible to browning caused mainly by polyphenol oxidase (PPO) enzyme activity [4]. This browning reduces the nutritional and sensory properties valued in this fresh fruit [5]. Therefore, study of cherimoya PPO function is of great importance to the fruit industry.

Polyphenol oxidases, classified according to tyrosinase or catecholase activity, are copper-containing enzymes that catalyze the oxidation of different plant metabolites [6–10]. Cherimoya PPO (AcPPO) catalyzes two different reactions, the hydroxylation of monophenols to diphenols and the oxidation of these diphenols to quinones in the presence of oxygen, which spontaneously polymerize to form melanin pigments [11,12]. The role of PPO in plants remains unclear, but PPO has been largely associated with plant defense systems against pathogens and herbivores [13–17]. Polyphenol oxidase has also been related to oxidative stress protection [18], cellular differentiation [19,20], fermentation [21], and protein preservation [22,23].

Protein function is closely related to localization within the cell. Plant PPOs are nuclear-encoded proteins, and most PPOs have an N-terminal chloroplast transit peptide that targets them to the thylakoid lumen [24–26]. However, there are examples of PPO proteins existing in other organelles, such as the vacuole. Specific exceptions include PtrPPO13 from *Populus trichocarpa* and aureusidin synthase 1, a PPO-like protein from *Antirrhinum majus*. These vacuolar PPOs are directed

Abbreviations: AcPPO, *Annona cherimola* polyphenol oxidase; BFA, brefeldin A; BiP, binding immunoglobulin protein; CEF, chloroplast enriched fraction; GEF, Golgi-enriched fraction; GFP, green fluorescent protein; PCC, Pearson's correlation coefficient; PPO, polyphenol oxidase; RGP1, reversibly glycosylated peptide 1; SDS, sodium dodecyl sulfate

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by an N-terminal signal peptide to the secretory pathway and, ultimately, the vacuole [27,28]. Recently, Tran et al. [29] suggested that several plant PPO genes also possess a signal peptide instead of a chloroplast transit peptide, but the subcellular localization of these genes remains undetermined.

Currently, AcPPO is the only PPO gene cloned from *A. cherimola,* and it is predominantly expressed in the leaves, although it is also present in flowers and fruit tissue [4]. This study characterized a Golgi apparatus-localized PPO from *A. cherimola* using a combined biochemical and confocal microscopy approach.

2. Materials and methods

2.1. Plants

Cherimoya (*A. cherimola* 'Concha Lisa') leaves were collected from adult plants grown in a greenhouse on a 16:8 h light:dark cycle at 21 °C. *Nicotiana benthamiana* plants were grown for 3–4 weeks in a greenhouse on a 16:8 light:dark cycle at 21 °C.

2.2. Cloning of full length AcPPO cDNA

A. cherimola leaves were individually ground to a fine powder in a mortar containing liquid nitrogen. Using the cetrimonium bromide protocol [30,31], total RNA was isolated from 1 g of tissue. RNA integrity was verified on a 1.2% (w/v) agarose gel under denaturing conditions. RNA quality and concentration were measured according to spectrophotometric absorbance at 260 and 280 nm. After treatment with DNase I (Invitrogen™, Carlsbad, CA, USA), cDNA was generated from 1 μg of total RNA by reverse transcription using the Super Script II First-Strand Synthesis System (Invitrogen™, Carlsbad, CA, USA). The polymerase chain reaction was performed using primers for the full-length PPO coding sequence without the stop codon: forward primer 5′-ATGGGACGACCAAGGCTACAG-3′ and reverse primer 5′-CCGCATGTATTCAACGCGTATCC-3′ (GenBank Accession No. DQ990911.1). The polymerase chain reaction product was cloned into the pGEM®-T Easy Vector System (Promega Corp., Madison, WI, USA) and sequenced (Macrogen Inc., Seoul, Korea).

2.3. Plasmid construction and transient expression assay

The AcPPO coding sequence was fused to the green fluorescent protein (GFP) by C-terminal fusion into the pGWB505 binary plant vector under the expression of the 35S promoter [32]. Organelle markers that target the Golgi apparatus (α-1,2 mannosidase 1 [Man1]) and endoplasmic reticulum (wall associated kinase 2 [WAK2]) were used, as described in Nelson et al. [33]. A transient expression assay was carried out as described in Sparkes et al. [34] using *N. benthamiana* leaves 48 h after *Agrobacterium*-mediated infiltration. Brefeldin A treatment was performed as described by Baldwin et al. [35]. Confocal fluorescent images were obtained using an IX 81 inverted microscope (Olympus Corp., Tokyo, Japan) coupled with a FluoView FV 1000 confocal laser scanning microscope with a UPLSAPO 60X NA: 1.35 objective (Olympus Corp., Tokyo, Japan). The colocalization index is represented by Pearson's correlation coefficient calculated as described in [36] using the Jacop plug-in from ImageJ software.

2.4. Computational analysis of AcPPO sequence

Signal peptide prediction was conducted using the bioinformatics tools SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) [37] and iPSORT Prediction (http://ipsort.hgc.jp/) [38]. Analysis of subcellular localization was carried out using the TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/) [39], MultiLoc (https://abi.inf.uni-tuebingen.de/Services/MultiLoc) [40] and ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/) [41]. The transmembrane domain was predicted using the TMHMM 2.0 Server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) [42]. A glycosylation analysis was performed using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) for *N*-glycosylation and using and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) for *O*-glycosylation [43].

2.5. Subcellular fractionation and enrichment for Golgi apparatus vesicles

Fractionation and enrichment of Golgi apparatus vesicles derived from *A. cherimola* and *N. benthamiana* leaves were conducted as described by Muñoz et al. [44] with some modifications. Briefly, fresh leaves were homogenized by a blender and in the presence of 0.5 M sucrose, 100 mM Tris-HCl (pH 7.5), and 5 mM $MgCl_2$, obtaining a crude extract (CE). The suspension was filtered through two layers of gauze and Miracloth (EMB Millipore, Billerica, MA, USA), and chloroplasts and nuclei were sedimented for 20 min at 1000 × *g* by a fixed-angle rotor. The supernatant was loaded onto a 1.3 M sucrose, 100 mM Tris-HCl (pH 7.5), and 5 mM $MgCl_2$ cushion and centrifuged for 100 min at 100,000 × *g* by a swinging bucket rotor. The upper phase was discarded without disturbing the interphase fraction, and sucrose layers of 1.1 M and 0.25 M were overlaid on the membrane pad. The gradient was then centrifuged for 100 min at 100,000 x *g* by a swinging bucket rotor. Fractions of 250 μL were collected from the top of the gradient and used in enzymatic and immunodetection assays.

2.6. PPO activity

Polyphenol oxidase activity was assayed using pyrocatechol as a substrate, following an adapted procedure from Prieto et al. [4]. The reaction mixture contained 50 mM phosphate buffer (pH 6.5) and 20 mM pyrocatechol. Quinone formation was measured in a spectrophotometer at 420 nm for 3 min. Units for PPO activity corresponded to 0.001 absorbance value changes under these conditions.

2.7. PPO activity inhibition and activation assay

The inhibitory dose-response curve was constructed using 1, 5, 10, 25, 50, 100, and 200 μM tropolone (Sigma-Aldrich, St. Louis, MO, USA), and PPO activity was measured as described above. The inhibition assays were performed using Golgi-enriched fractions (GEFs) from *A. cherimola* and *N. benthamiana* expressing AcPPO:GFP leaves. GEFs from control *N. benthamiana* and heat denatured GEFs from *N. benthamiana* expressing AcPPO:GFP were utilized as a negative control. Additionally, PPO activity was measured in absence of pyrocatechol as substrate. PPO activation was carried out using sodium dodecyl sulfate (SDS) [45] and PPO activity was measured as described above plus the addition of 0.1% (w/v) of SDS.

2.8. Measurement of enzyme activity markers

The activity of UDPase, a Golgi apparatus marker, was assayed as described by Nagahashi et al. [46] with modifications. Briefly, a solution containing 6 mM UDP, 6 mM $MgSO_4$, 0.1% (v/v) Triton X-100, and 60 mM Tris-MES (pH 6.5) was mixed with an equal volume of fraction. The released inorganic phosphate was measured at 650 nm according to the method described by Ames [47].

The activity of NADH-cytochrome c oxidoreductase, a mitochondria marker, was measured as described in Wienecke et al. [48] with modifications. For this, the reaction assay mix contained 20 mM phosphate buffer (pH 7.2), 0.02 mM cytochrome c, 0.2 mM NADH, 0.4 mM sodium dithionite, and 0.04% (v/v) Triton X-100. The absorbance decrease at 550 nm was measured by a spectrophotometer for 3 min. In turn, the activity of hydroxypyruvate reductase, a peroxisome marker, was measured as described in Titus et al. [49] with modifications. The assay mixture contained 60 mM phosphate buffer (pH 6.2), 0.2 mM NADH,