

Browning in Annona cherimola fruit: role of polyphenol oxidase (PPO) and characterization of a coding sequence of the enzyme

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Cherimoya (Annona cherimola Mill.) fruit is an attractive candidate for food processing applications as fresh cut. However, along with its desirable taste, cherimoya shows a marked susceptibility to browning. This condition is mainly attributed to polyphenol oxidase activity (PPO). A general lack of knowledge regarding PPO and its role in the oxidative loss of quality in processed cherimoya fruit requires a better understanding of the mechanisms involved. The work carried out included the cloning of a full-length cDNA, an analysis of its properties in the deduced amino sequence, and linkage of its mRNA levels with enzyme activity in mature and ripe fruits after wounding. The results showed one gene different at the nucleotide level when compared with previously reported genes, but a well-conserved protein, either in functional and in structural terms. Cherimoya PPO gene (Ac-ppo, GenBank DQ990911) showed to be present apparently in one copy of the genome, and its transcripts could be significantly detected in leaves, and less abundant in flowers and fruits. Analysis of wounded matured and ripen fruits revealed an inductive behavior for mRNA levels in the flesh of mature cherimoya after 16 h. Although the highest enzymatic activity was observed on rind, a consistent PPO activity was detected on flesh samples. A lack of correlation between PPO mRNA level and PPO activity was observed, especially in flesh tissue. This is probably due to the presence of enzyme

Figure 1. Complete coding sequence of Annona cherimola polyphenol oxidase and its 5'- 3' - UTR regions. Nucleotide (Ac-ppo) and deduced amino acid (Ac-PPO) sequences of a fruit polyphenol oxidase from A. cherimola is shown. 5' and 3' RACE on cDNA templates were also determined and sequence of nucleotides after and before CDS is shown. Intron sequence at 5' end of the CDS, between nucleotides 234 and 317, is indicated (underline).

1	2	ATG	GACTO	GAAG	GGAG	TAGA	AAATO	CTCA	СССТИ	AGAG	AAACO	CTG	ATCCO	TATO	TCTC	CACG	GTG	TGTG	CTG	TAGO	CTTAC	CCTT	ACTTO	CATI	CTTT	rccco	CAGCO	CCAT	CCTG	ATG	GGA	CGA	CCA	AGG	CTA
1																														м	G	R	Р	R	L
130	c	CAG	TTC	TCA	GCC	ATC	TCG	CTC	GCT	TTT	TGC	CTG	GTT	GCT	GTA	GCC	TTC	CCA	TCG	AAT	стс	CTT	GTT	AGC	TGC	TGC	CAT	GCT	GCA	GAC	CAA	TCC	GTG	CTG	CTC
7		Q	F	s	A	I	s	L	A	F	с	L	v	A	v	A	F	Р	s	N	L	L	v	s	с	с	н	A	A	D	Q	s	v	L	L
232	C	GG-	<u>GTA</u>	TGTT	ITGA	ATTC	CTCT	TATG	ATCT	GTGG!	ICAG	TAT	GCTAZ	AGCG	GTA	IGTC	TTT	TACT	TCT	IGTG	CTGA	ATGT	TCAG	G	GAA	AAC	AAT	AAC	ATC	TAC	GCT	TCT	AAT	GGA	TTT
41		G																							Е	N	N	N	I	Y	A	s	N	G	F
351	c	GGA	TGG	CTG	AAG	AAT	TTG	ATC	TAC	GAA	ATG	GGT	GGT	GGG	TGG	CTC	TCC	GGC	ACT	CAC	GTC	ССТ	GCT	GTT	GGT	GAG	GCA	GAG	ААА	ACG	AGA	AGC	AGC	TTG	GCC
53		G	W	L	к	N	L	I	Y	Е	м	G	G	G	W	L	s	G	т	н	v	Р	A	v	G	Е	A	Е	к	т	R	s	s	L	A
453	c	ccc	AAC	CTT	ACC	ACC	TGC	CAC	AGA	TCG	CTC	TCC	GAC	GCC	GAC	CGA	CCA	GTC	TAC	TGC	TGC	ccc	ccc	AAG	CCA	GCA	TCC	GAA	GAG	AGC	GTG	ATT	GAT	TTC	CAG
87		Р	N	L	т	т	С	н	R	s	L	s	D	A	D	R	P	v	Y	с	с	Р	Р	к	Р	A	s	Е	Е	s	v	I	D	F	Q
555	2	гтс	ccc	AGC	CCT	TCG	TCG	ccc	CTC	CGC	ATC	CGC	CGC	CCG	GCC	CAC	CTC	CTC	GAC	GAA	GAG	TAC	ATC	GCC	AAG	TAC	GAG	ATG	GCC	GTG	GCC	AAG	ATG	AAG	CAG
121		F	Р	s	Р	s	s	P	L	R	I	R	R	Р	A	н	L	L	D	Е	Е	Y	I	A	к	Y	Е	м	A	v	A	к	м	к	Q
657	c	CTG	AGC	TAC	GAC	CAT	ccc	CAC	AGC	TTC	ATG	CGC	CAA	GCA	AAC	ATC	CAC	TGC	ATC	TAC	TGC	ACC	GGC	GCC	TAC	AAC	CAA	GAG	AAC	TCC	ACC	TCT	CTC	стс	AAG
155		L	s	Y	D	н	Р	н	s	F	м	R	Q	A	N	I	н	С	I	Y	с	т	G	A	Y	N	Q	Е	N	s	т	s	L	L	к
759	2	ATC	CAC	CGC	тсс	TGG	CTC	TTC	TTT	ccc	TTC	CAC	CGC	ATG	ATG	ATC	TAC	TTC	CAC	GAG	CGC	ATC	стс	GGT	AAG	CTC	ATG	GGC	GAC	GAC	ACC	TTC	GCG	TTG	CCG
189		I	н	R	s	W	L	F	F	P	F	н	R	м	м	I	Y	F	н	Е	R	I	L	G	к	L	м	G	D	D	т	F	A	L	P
861	1	FAC	TGG	AAC	TGG	GAC	GCG	CCG	ccc	GGC	ATG	GTG	ATT	CCG	GCC	ATG	TAC	TCA	AAC	GGG	тсс	стс	CGG	GAG	GAG	CAG	CGC	GAT	CGC	GCC	CAC	стс	CGG	CCG	CAG
223		Y	W	N	W	D	A	Р	Р	G	м	v	I	P	A	м	Y	s	N	G	s	L	R	Е	Е	Q	R	D	R	A	н	L	R	P	Q
963	c	GCG	GCG	GAC	ATT	GAC	TTC	GAC	TAC	GTG	GAA	AGC	GGG	CTG	GGG	CGG	GAG	GAG	CAG	ATC	AGC	AAG	AAC	CTG	GCC	TTC	ATG	TAT	CAC	CAG	ATG	GTA	TCG	GGT	GCG
257		A	A	D	I	D	F	D	Y	v	Е	s	G	L	G	R	Е	Е	Q	I	s	к	N	L	A	F	м	Y	н	Q	м	v	s	G	A
1065	2	AAG	AAG	ACC	GAA	стс	TTC	ATG	GGC	TGC	AAG	TAC	AGG	GCG	GGA	GAG	GAC	GGC	TTC	TGC	GAT	GGG	CCG	GGG	ACG	ATC	GAG	CTG	GCT	ccc	CAC	AAC	GCG	стс	CAC
291		к	к	т	Е	L	F	м	G	с	к	Y	R	A	G	Е	D	G	F	с	D	G	Р	G	т	I	Е	L	A	P	н	N	A	L	н
1167	2	ACG	TGG	GTC	GGG	AGC	GAT	CTG	AAC	CCA	GAG	CGG	GAG	AAC	ATG	GGC	GCC	TTC	TAT	тсс	GCC	GCG	CGC	GAT	ccc	ATC	TTC	TAC	GCA	CAC	CAC	GCC	AAC	ATC	GAC
325		т	W	v	G	s	D	L	N	Р	Е	R	Е	N	м	G	A	F	Y	s	A	A	R	D	Р	I	F	Y	A	н	н	A	N	I	D
1269	c	CGC	ATG	TGG	ACC	GTG	TGG	AAG	AAG	CTG	AGG	GGC	CAC	GAG	CCG	GAG	TAC	GTG	GAC	CCG	GAG	TGG	CTT	AAC	тсс	TAT	TTC	TAT	TTC	CAC	GAC	GAG	AAC	GCG	CAG
359		R	м	W	т	v	W	к	к	L	R	G	н	Е	P	Е	Y	v	D	P	Е	W	L	N	s	Y	F	Y	F	н	D	Е	N	A	Q
1371	c	CTG	GTC	CGG	GTT	AGG	ATC	CGC	GAC	GTG	стс	GAC	TTC	TCC	AAG	стс	AGA	TAC	GCT	TAC	GAG	GAG	GCG	GAT	CTT	ccc	TGG	стс	AAC	GCG	CGC	ccc	AAG	CCG	TCC
393		L	v	R	v	R	I	R	D	v	L	D	F	s	к	L	R	Y	A	Y	Е	Е	А	D	L	Р	W	L	N	A	R	Р	к	Р	s
1473	c	GTC	ССТ	ccc	GAG	ATC	GCT	CGC	CGC	GTG	TTG	AAG	ATG	CGC	GAG	тст	CAG	CAG	AAT	CTG	CTG	CAG	ACG	CGG	GGC	TAC	AGC	GCT	TCT	ccc	GAC	TTC	GGT	ccc	CAG
427		v	Р	Р	Е	I	А	R	R	v	L	к	м	R	Е	s	Q	Q	N	L	L	Q	т	R	G	Y	s	A	s	P	D	F	G	Р	Q
1575	c	GGC	CGG	CGT	СТС	GAT	GCC	ACG	GTC	AGG	GTC	AAG	GTC	CAA	AGG	CCG	AAG	ATT	CAG	AGA	AAG	GCA	GAG	GAA	GAG	GAG	GTG	TTG	GTG	GTC	TAT	GGG	ATT	GAT	ATC
461		G	R	R	L	D	A	т	v	R	v	к	v	Q	R	P	к	I	Q	R	к	A	Е	Е	Е	Е	v	L	v	v	Y	G	I	D	I
1677	2	AAG	AAG	GAC	ATG	TAT	GTG	AAG	TTC	GAC	GTG	TTC	GTG	AAC	CTG	GTG	GCG	GAT	GAG	AGC	AGC	GTG	GGA	ССТ	GAG	GCT	CGG	GAG	TTC	GCG	GGG	ACG	TTC	GTG	AAC

Figure 3. PPO mRNA quantification by qPCR in different cherimoya organs. Basal PPO mRNA levels is observed in growing (GF) and unripe fruits rind (RMF) or flesh (FMF) and higher level of expression was obtained in leaves (L) followed by flowers (F). Triplicate quantitative PCR experiments were performed for each sample and the expression values were normalized against 18S ribosomal gene indicated as expression in arbitrary units (A.U.). Bars atop SE. Bars followed by different small letter are significantly different at $P \le 0.05$.



Figure 4. PPO activity determined in unripe (A) and ripe (B) fruits after wounding on time course analysis at 20°C (0 to 16 h). Whole unripe and ripe fruits were wounded and then split into rind and flesh tissues and enzymatic activity measured. Bars atop SE.





Figure 2. Functional and structural comparison of deduced A. cherimola PPO. Based on pipeline proposed by Marusek et al.(15), bioinformatic tools were selected to analyze N- (A) and C- (B) terminal segments of currently described and A. cherimola PPOs. A. Nterminal analysis showing the two active sites (copper A and B) are shown (squares CuA and CuB), including their conserved histidines (H); cysteine of the proposed structural thioether bridge (C) and a tyrosine motif (square) with a couple of residues (Y) marking domain termini. Arginine (R) and aspartic (D) residues that interact with Y motif, throughout of π -cation and hydrogen bond, respectively, were also detected. Gate residues (F or L) inside of copper B active site are also found. Transit peptides have been also included in this comparison, showing the proposed signal (underlined) for the A. cherimola PPO and its putative cleavage site (arrow). In all the included sequences, processed proteins are postulated to start at residue 107, which is still undefined in case of an A. cherimola unripe protein. B. C-terminal region analysis, showing the proposed linker area (linker), rich in α -helix structure (n); followed of six strands of β -sheet structure (n)(square I-VI), in which an inserted α-helix could be found between strands IV and V. Active site regulator residue, ending strand III is also showed (n).

A Ananas comosus Ipomea batatas Ipomea batatas Ipoopersicon esculentum Nicotiana tabacum Nicotiana tabacum Oryra sativa Solanum tuberosum Spinacia oleracea Driticum aestivum Erifolium pratense Vicia faba Vitis vinifera Annona cherimola	10 2 MATLSKLASQ PITPPL SPL MATLSKLASQ PITPPL SPL MESINVAPGT TATPEMAPP -MASL CN	30 30 40 I I I I P PLHAPSLTKS FTTTFLSPV6 VPN S SFTNTNTNSS FFANPSQLFL HGR S SFTNTNTNSS FFANPSQLFL HGR P P-PPCITNLQSTLR YNN T PTTSSSTSLS STPNPSQLFI HGK N PFLPNTPQLS AHHHR5VRSV NGKV3 N FTLPNTPQLS AHHHR5VRSV NGKV3	50 60 7 	0 80 90 4 PT3LRAASPA ATY30ALGGL 5 LDAVDR RNVLLGLGGF 6 LDAVDR RNVLLGLGGF 7 LDAVDR RNVLLGLGGF 9 LDAVDR RNVLLGLGGF 9 LDAVDR RNVLLGLGGF 9 LDAVDR RNVLLGLGGF 9 ONVETNSVDR RNVLLGLGGGF 9 NTNTPYLDR RNILLGLGGGF 9 NTNTPYLDR RNILLGLGGF 9 DDLULRRIDR RDVLLGLGGF 9 QENPSPR RNVLIGLGGF 9 RET 33 GKLDR RNVLIGIGGL 1 YASNGFG0L NNLIVEMG GG	B490500Aranas comosusDV-EIFOLXA NPTPXSALONINSKVIpomea batatasDV-EIFOLXA NPTPXSALONINSKVLycopersicon esculentumNicotiana tabacumPM-PTPURNF NPIRRSSSGKNicotiana tabacumPM-PTPURNF NPIRRSSSGKFM-PTPURNF NPIRRSSSGKSolanum tuberosumPM-PTPURNF NPIRKSNAGKFM-PTPURNF NPIRKSNAGKSpinacia oleraceaDV-ELPUNSI TQQQQQQRQ QUROPITriticum aestivumEXETLEULDK RPKPATGIDRFM-PTPURNF XPIRKSSGKTriticum aestivumEXETLEULDK RPKPATGIDRFM-PTPURNF XPIRKSSGKVicia fabaDV-PIPUEXA KPVPRTKVQ KLVEVVicis viniferaDI-PIPULPK NTKAXANTT XSSKSQAnnona cherimolaEA-DLPULNA RPKPSVPPEI	510 TIXA -YA3
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Ananas comosus Ipomea batatas Lycopersicon esculentum Nicotiana tabacum Oryza sativa Solanum tuberosum Spinacia oleracea Triticum aestivum Trifolium pratense Vicia faba Vitis vinifera Annona cherimola	460 4' SFLFYDENAQ LVRVKVKDCJ TFLFYDENGQ AVKVRIGDSJ EFFFYDENGR PYRVKVRDCJ EFFFYDENGR PYRVKVRDCJ SFFFYDENGR PYRVKVRDCJ SFVFYDENAR PVRVVVRDSJ SFVFYDENAR PVRISVRDVJ EFLFYDENAR LVKVKVKDGJ GFLFYDENAR LVRVNVRDSJ TFVFYDENAQ LVRVRIRDVJ	CuB 70 480 490 1			Reaction buffer + 100 µL rind crud Reaction buffer + 100 µL rind crud ¹ Means followed by different small	e extrac e extrac e extrac e extrac <u>e extrac</u> 11 letter

Figure 5. PPO mRNA quantification by qPCR on time course analysis at 20°C (0 to 16 h) after wounding in unripe (A) and ripe (B) fruits in rind (exocarp) and flesh (mesocarp). Triplicate quantitative PCR experiments were performed for each sample and the expression values were normalized against 18S ribosomal gene indicated as expression in arbitrary units (A.U.). Bars atop SE. Bars followed by different small letter are significantly different at P ≤ 0.05.



ripe cherimoya rind crude extract and its ition of flesh crude extract in the reaction

K WATCHER AIN ELLEDIGAED

Reaction mixture	PPO activity U min ⁻¹ mg ⁻¹ total
	protein
Reaction buffer + 100 μ L rind crude extract	$32520 c^{1}$
Reaction buffer + 100 μ L rind crude extract + 100 μ L dd H ₂ O	33900 c
Reaction buffer + 100 μ L rind crude extract + 100 μ L flesh crude extract	17870 b
Reaction buffer + 100 μ L rind crude extract + 600 μ L flesh crude extract	230 a
Reaction buffer + 100 μ L rind crude extract + 600 μ L dd H ₂ O	32440 c

are significantly different at PS 0.05.

Remarks

•Our analyses showed that Ac-PPO keeps remarkable similarities to other PPOs (Type 3 copper protein).

•qPCR indicated that leaves showed significantly higher amount of PPO transcript followed by flowers and fruit tissue.

•PPO activities did not correlate with abundance of **PPO mRNA.**

•The possibility of existence of an inhibitory agent should be considered.

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