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

Humberto Prieto[a], Daniella Utz[b], Álvaro Castro[a], Carlos Aguirre[a], Mauricio González-Agüero[a], Héctor Valdés[c], Nicolas Cifuentes[b], Bruno G. Defilippi[a], Pablo Zamora[b], Gustavo Zúñiga[b] and Reinaldo Campos-Vargas[a]*

[a] Instituto de Investigaciones Agropecuarias, INIA, Santa Rosa 11610, Santiago, Chile.
[b] Facultad de Química y Biología, U. de Santiago de Chile, Av. Bernardo O'Higgins 3363, Santiago, Chile.
[c] Facultad de Ciencias, Escuela de Agronomía, U. de La Serena, Benavente 980, La Serena, Chile.

* Corresponding author: rcampos@inia.cl

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 inhibitors. To our knowledge this is the first report of a complete PPO sequence in cherimoya.

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     ATGGACTGAAGGGAGTAGAAAATCTCACCCTAGAGAAACCCTGATCCGTATCTCTCACGAGTGCTGTGCTGCTAGCTTACCTTACTTGCATTCTTTCCCCAGCCCATCCTG ATG GGA CGA CCA AGG CTA
1                                                                                                                       M  G  R  P  R  L
130   CAG TTC TCA GCC ATC TCG CTC GCT TTT TGC CTG GTT GCT GTA GCC TTC CCA TCG AAT CTC CTT GTT AGC TGC TGC CAT GCT GCA GAC CAA TCC GTG CTG CTC
7       Q  F  S  A  I  S  L  A  F  C  L  V  A  V  A  F  P  S  N  L  L  V  S  C  C  H  A  A  D  Q  S  V  L  L
232   GG- GTATGTTTTGAATTCCTCTTATGATCTGTGGTCAGGTATGCTAAGCGGGTATGTCTATTTACTTTCTTGTGCTGAATGTTCAG --G GAA AAC AAT AAC ATC TAC GCT TCT AAT GGA TTT
41    G                                                                                          E  N  N  N  I  Y  A  S  N  G  F
351   GGA TGG CTG AAG AAT TTG ATC TAC GAA ATG GGT GGT GGG TGG CTC TCC GGC ACT CAC GTC CCT GCT GTT GGT GAG GCA GAG AAA ACG AGA AGC AGC TTG GCC
53     G  W  L  K  N  L  I  Y  E  M  G  G  G  W  L  S  G  T  H  V  P  A  V  G  E  A  E  K  T  R  S  S  L  A
453   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     P  N  L  T  T  C  H  R  S  L  S  D  A  D  R  P  V  Y  C  C  P  P  K  P  A  S  E  E  S  V  I  D  F  Q
555   TTC 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  P  S  P  S  S  P  L  R  I  R  R  P  A  H  L  L  D  E  E  Y  I  A  K  Y  E  M  A  V  A  K  M  K  Q
657   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 CTC AAG
155    L  S  Y  D  H  P  H  S  F  M  R  Q  A  N  I  H  C  I  Y  C  T  G  A  Y  N  Q  E  N  S  T  S  L  L  K
759   ATC CAC CGC TCC TGG CTC TTC TTT CCC TTC CAC CGC ATG ATG ATC TAC TTC CAC GAG CGC ATC CTC GGT AAG CTC ATG GGC GAC GAC ACC TTC GCG TTG CCG
189    I  H  R  S  W  L  F  F  P  F  H  R  M  M  I  Y  F  H  E  R  I  L  G  K  L  M  G  D  D  T  F  A  L  P
861   TAC TGG AAC TGG GAC GCG CCG CCC GGC ATG GTG ATT CCG GCC ATG TAC TCA AAC GGG TCC CTC CGG GAG GAG CAG CGC GAT CGC GCC CAC CTC CGG CCG CAG
223    Y  W  N  W  D  A  P  P  G  M  V  I  P  A  M  Y  S  N  G  S  L  R  E  E  Q  R  D  R  A  H  L  R  P  Q
963   GCG GCG GAC ATT GAC TTC GAC TAC GTG GAA AGC GGG CTG GGC 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  E  S  G  L  G  R  E  E  Q  I  S  K  N  L  A  F  M  Y  H  Q  M  V  S  G  A
1065  AAG AAG ACC GAA CTC 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 CTC CAC
291    K  K  T  E  L  F  M  G  C  K  Y  R  A  G  E  D  G  F  C  D  G  P  G  T  I  E  L  A  P  H  N  A  L  H
1167  ACG TGG GTC GGG AGC GAT CTG AAC CCA GAG CGG GAG AAC ATG GGC GCC TTC TAT TCC GCC GCG CGC GAT CCC ATC TTC TAC GCA CAC CAC GCC AAC ATC GAC
325    T  W  V  G  S  D  L  N  P  E  R  E  N  M  G  A  F  Y  S  A  A  R  D  P  I  F  Y  A  H  H  A  N  I  D
1269  CGC ATG TGG ACC GTG TGG AAG AAG CTG AGG GGC CAC GAG CCG GAG TAC GTG GAC CCG GAG TGG CTT AAC TCC TAT TTC TAT TTC CAC GAC GAG AAC GCG CAG
359    R  M  W  T  V  W  K  K  L  R  G  H  E  P  E  Y  V  D  P  E  W  L  N  S  Y  F  Y  F  H  D  E  N  A  Q
1371  CTG GTC GGC GTT AGG ATC CGC GAC GTG CTC GAC TTC TCC AAG CTC AGA TAC GCT TAC GAG GAG GCG GAT CTT CCC TGG CTC AAC GCG CCC AAG CCG TCC
393    L  V  R  V  R  I  R  D  V  L  D  F  S  K  L  R  Y  A  Y  E  E  A  D  L  P  W  L  N  A  R  P  K  P  S
1473  GTC CCT CCC GAG ATC GCT CGC CGC GTG TTG AAG ATG CGC GAG TCT CAG CAG AAT CTG CTG CAG ACG CGG GGC TAC AGC GCT TCT CCC GAC TTC GGT CCC CAG
427    V  P  P  E  I  A  R  R  V  L  K  M  R  E  S  Q  Q  N  L  L  Q  T  R  G  Y  S  A  S  P  D  F  G  P  Q
1575  GGC CGG CGT CTC 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  T  V  R  V  K  V  Q  R  P  K  I  Q  R  K  A  E  E  E  V  L  V  V  Y  G  I  D  I
1677  AAG AAG GAC ATG TAT GTG AAG TTC GAC GTG TTC GTG AAC CTG GTG GCG GAT GAG AGC AGC GTG GGA CCT GAG GCT CGG GAG TTC GCG GGG ACG TTC GTG AAC
495    K  K  D  M  Y  V  K  F  D  V  F  V  N  L  V  A  D  E  S  S  V  G  P  E  A  R  E  F  A  G  T  F  V  N
1779  ATG CGG CGG GGG GTG AGG TTG GTG ATG AAC GAA GGG GAT TCG TGG AGG AAG AGC AAG AGC ACC CTC AAG TTG GGG ATT TCA GAA CTG TTG GAG GAT TTG GAG
529    M  R  R  G  V  R  L  V  M  N  E  G  D  S  W  R  K  S  K  S  T  L  K  L  G  I  S  E  L  L  E  D  L  E
1881  GCA GAC CGA GAT GAG AGC ATC TGG GTG ACT GTT GTG CCC AAA GGA GGA ACA GGC GTC AAC ACC ACG ATT CAA GGG ATA CGC GTT GAA TAC ATG CGG TGA GTT
563    A  D  R  D  E  S  I  W  V  T  V  V  P  K  G  G  T  G  V  N  T  T  I  Q  G  I  R  V  E  Y  M  R  *
1983  CTGGGGTTTGGAACCTGGAGTACAGTGACTTTTGCAAAATTTATTTATTTATTTGTGTAAGCTGTGTGAGCTGGGTCAGAGGAAATCCCATTGGATAGAATTGCCGCTGTCTAATTACTTTGTCTGAGCT
2118  TGTATTTAAACAAAGGATTATTCTTTTTACAAAAATAAACAGTGATGAGGAGTTGTCTTGAACGCAAAAAAAAAAAAAAAAAAAAA
```

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. N-terminal 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).

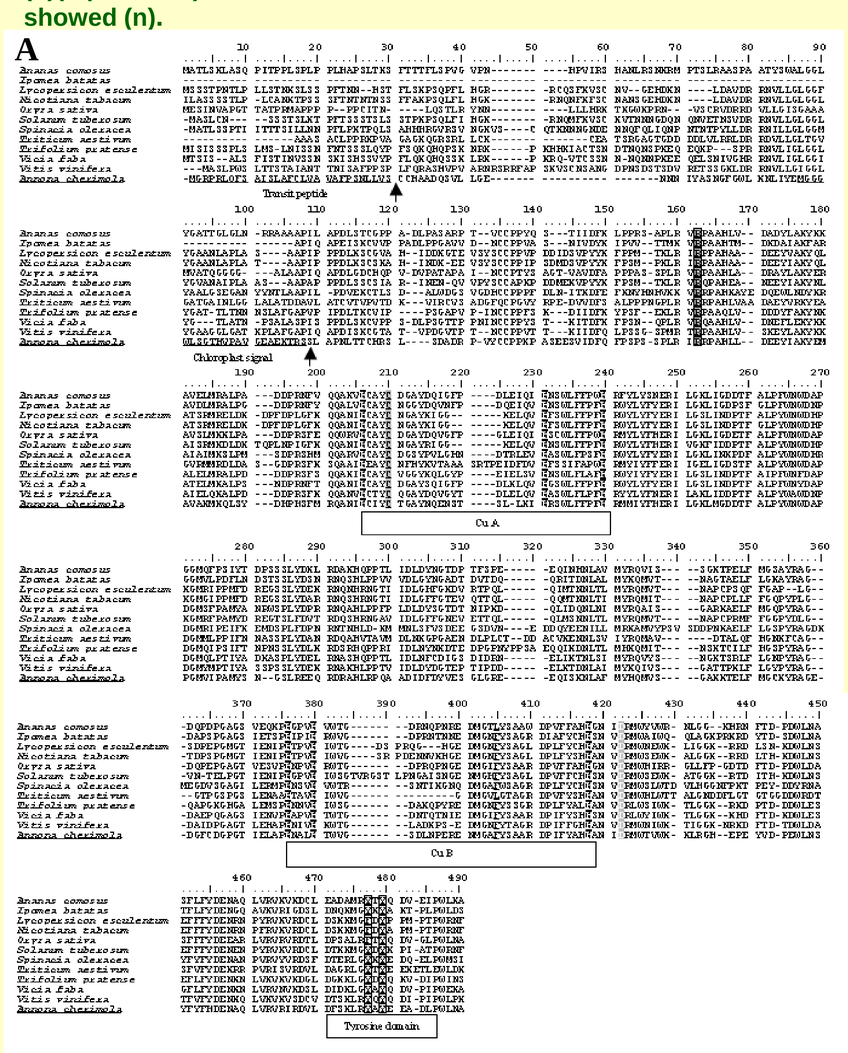

Figure 3. PPO mRNA quantification by qPCR in different cherimoya organs. Basal PPO mRNA levels is observed in growing (GF) and unripe fruits rind (RMF) or flesh (FMF) and higher level of expression was obtained in leaves (L) followed by flowers (F). Triplicate quantitative PCR experiments were performed 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 ≤ 0.05.

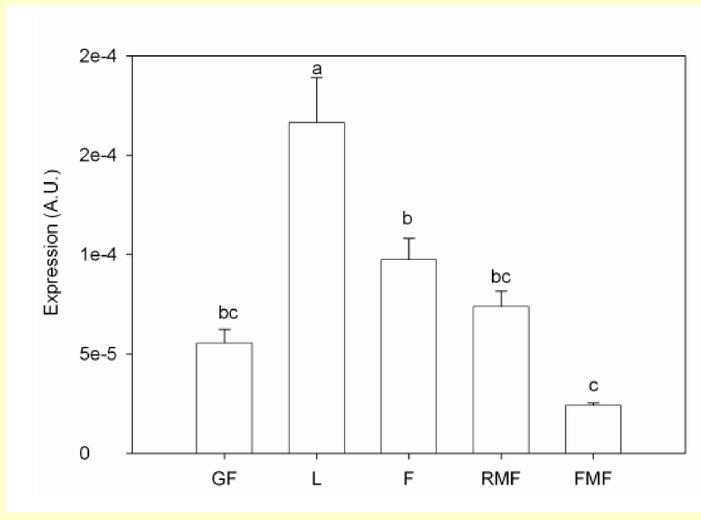

Figure 4. PPO activity determined in unripe (A) and ripe (B) fruits after wounding on time course analysis at 20˚C (0 to 16 h). Whole unripe and ripe fruits were wounded and then split into rind and flesh tissues and enzymatic activity measured. Bars atop SE.

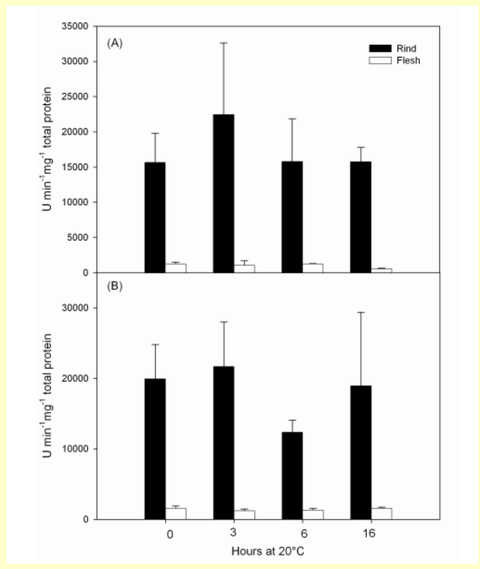

Figure 5. PPO mRNA quantification by qPCR on time course analysis at 20˚C (0 to 16 h) after wounding in unripe (A) and ripe (B) fruits in rind (exocarp) and flesh (mesocarp). Triplicate quantitative PCR experiments were performed for each sample and the expression values were normalized against 18S ribosomal 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.

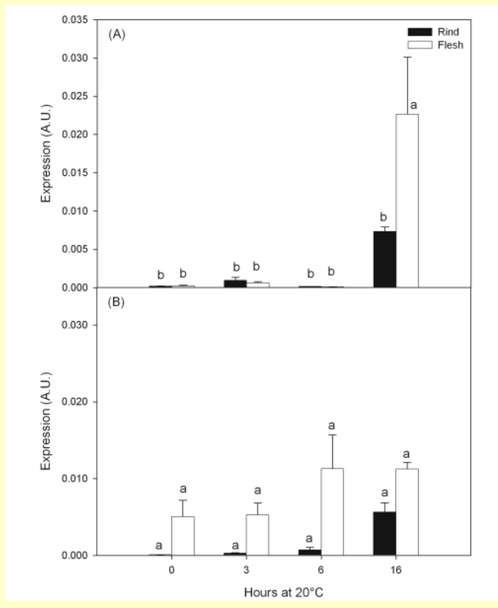

Table 1. PPO activity of unripe cherimoya rind crude extract and its changes as function of addition of flesh crude extract in the reaction mixture.

Reaction mixture	PPO activity U min^{-1} mg^{-1} total protein
Reaction buffer + 100 µL rind crude extract	32520 c[1]
Reaction buffer + 100 µL rind crude extract + 100 µL dd H_2O	33900 c
Reaction buffer + 100 µL rind crude extract + 100 µL flesh crude extract	17870 b
Reaction buffer + 100 µL rind crude extract + 600 µL flesh crude extract	230 a
Reaction buffer + 100 µL rind crude extract + 600 µL dd H_2O	32440 c

[1]Means followed by different small letter are significantly different at PŠ 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.

This work was funded by Fondecyt 1040011