IDENTIFICATION OF a cDNA ENCODING ASCORBATE PEROXIDASE FROM *PINUS PINASTER* AIT.

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Introduction

The maritime or wild pine (*Pinus pinaster* Ait.) is the conifer with the widest distribution in Portugal, being of an extreme economic importance to wood and paper industries and also as a large source of pitch, turpentine and resin. Pine forest decay is largely associated with abiotic and biotic stresses, which ultimately leads to excessive production of reactive oxygen species (ROS), thus resulting in damage at the cellular level. A regulated balance between oxygen radical production and destruction is required if metabolic efficiency and function are to be maintained in both optimal and stress conditions, because the reactive oxygen species are highly destructive. To counteract the toxicity of active oxygen species a highly efficient antioxidative defence system, composed of both non-enzymatic and enzymatic constituents, is present in all plant cells (**Figure 1**).

Ascorbate peroxidase (APX; EC 1.11.1.11) is a heme-containing protein that efficiently scavenges H_2O_2 and participates in the ascorbate-gluthatione cycle, the major hydrogen peroxide-detoxifying system in plant chloroplasts and cytosol, preventing not only cellular damage but also the inhibition of cytosolic and chloroplastic enzyme activity. Many authors have reported APX activity increase and/or Apx enhanced expression in response to environmental and biotic stresses, suggesting an important role for this enzyme in those situations. In this work, the identification of cDNAs encoding ascorbate peroxidase isoenzymes in *P. pinaster* was attempted. These cDNAs could then be used in genetic expression profiling.

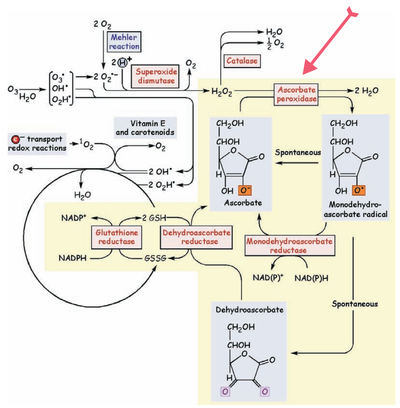

Figure 1- Antioxidant defence system pathways, detailing enzyme and nonenzymatic antioxidants. The ascorbate-glutathione cycle is highlighted in yellow and oxidative stress enzymes represented in red.

PCR Amplification

- APX exists as several isoforms that play an important role in the scavenging of H_2O_2 in higher plants:
  - Chloroplast isoform (chAPX) subdivided in tylakoidal chAPX (chtAPX) and stromal chAPX (chsAPX)
  - Cytosolic membrane isoform (cmAPX)
  - Cytosolic soluble isoform (csAPX)

- Specific a.a. sequences for each isoform were used for designing specific degenerated primers (represented by color arrows in **Figure 7**):
  - CHAPX (GARACIAARTAYACIAAR), CMAPX (ATHMGIAAYGARGARGAR) and CSAPX (GGICCITTYGGIACIAT).

- DNA from a cDNA library was purified by phenol/chloroform extractions followed by ethanol selective precipitation.

- A gradient PCR program (with increasing annealing temperatures) was used with specific degenerated primer (CHAPX or CMAPX or CSAPX) together with T7 universal primer flanking cDNA fragments from 3'end.

Some PCR fragments originated a strong signal after *Southern analysis* using a mixed probe of *csApx* and *cmApx* from *Z. aethiopica* (**Figure 2**).

The most prominent PCR fragments from *chApx*, *cmApx* and *csApx* were reamplified (**Figure 3**) and will be used for *P. pinaster* cDNA library screening.

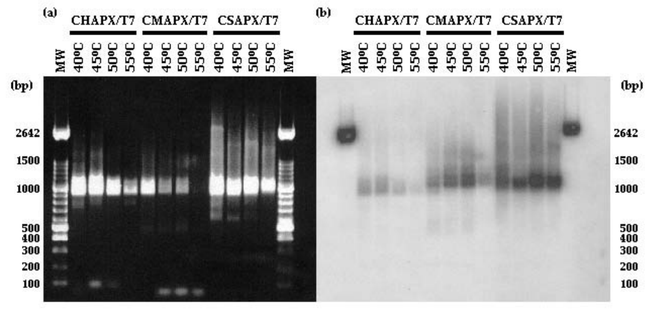

Figure 2 - Electrophoresis (a) and *Southern* analysis (b) of the amplified fragments obtained using the cDNA library as template. The primers and annealing temperatures used in the gradient PCR program are present above the corresponding lane. The PCR program comprised: 1 first cycle of 94°C for 5 min, followed by 40 cycles of a) 94°C for 1.5 min, b) 40°C (or 45°C, or 50°C or 55°C) for 1 min and c) 72°C for 1.5 min. Electrophoretic analysis was performed on 1.2% agarose gel. *Southern* analysis was performed using a mixed *csApx* and *cmApx* probe from *Zantedeschia aethiopica*.

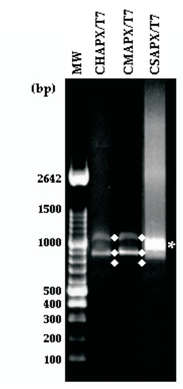

Figure 3 - Electrophoretic analysis of reamplified fragments from the reactions: CHAPX/T7 (using 50°C as annealing temperature), CMAPX/T7 (using 50°C as annealing temperature) and CSAPX/T7 (using 55°C as annealing temperature). The PCR program was the same as in Figure 2. Electrophoretic analysis was performed on 1.2% agarose gel.

Screening of *P. pinaster* cDNA library

- A *P. pinaster* cDNA library prepared from needles of a 30-year-old *P. pinaster* tree (titer of 1.74 x 109 upf/ml) was used.

- 25000 upf were screening using each of *chApx*, *cmApx* or *csApx* fragments as probes (exemplified in **Figure 4**).

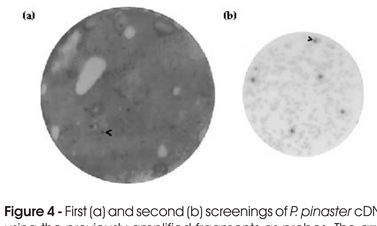

Figure 4 - First (a) and second (b) screenings of *P. pinaster* cDNA library using the previously amplified fragments as probes. The arrow in (a) points to the phage clone used for the second screening shown in (b).

- Homology with *Apx* sequences was confirmed by *Southern* analysis using a mixed probe of *Apx* (**Figure 5b**).

- Homology with amplified fragments was confirmed by *Southern* analysis using *chApx*, *cmApx* or *csApx* fragments as probe (**Figure 5c**).

Only one cDNA clone (pcsApx43) seems to share high homology with *Apx* sequences.

All the other cDNA clones gave a positive signal after *chApx*, *cmApx* or *csApx* hybridization, indicating that they contain fragments homologous to those previously amplified.

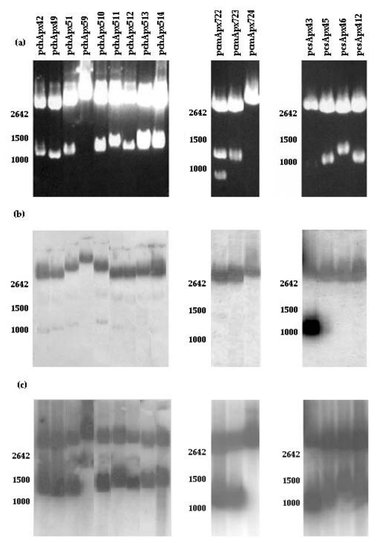

Figure 5 - Electrophoresis (a) and *Southern* analysis (b and c) of putative *chApx*, *cmApx* or *csApx* clones identified after screening *P. pinaster* cDNA library with previously amplified fragments (Figure 3). Minipreps of recombinant plasmids were used for *EcoRI*/*XhoI* double digestion and analyzed by electrophoresis on 1.2% agarose gel. *Southern* analysis was performed using a mixed *csApx* and *cmApx* probe from *Zantedeschia aethiopica* (b) or using the previously amplified fragments as probes (c).

Sequence analysis of *P. pinaster csApx*

- cDNA clone (pcsApx43) revealing high homology with *Apx* probe was completely sequenced using T3 and T7 universal primers and the complete nucleotide and deduced amino acid sequences are shown in **Figure 6**.

- Sequence analysis revealed high identity with cytosolic soluble APX from higher plants (75,5-82,7%). Identity values found with other membrane-bound cytosolic APXs is 47,4-66,7% and with chloroplastidial APXs is 42,2-47,4%.

- csAPX does not seem to have a transit peptide or a membrane-spanning region (**Figure 7**).

- The deduced amino acid sequence of *P. pinaster* csAPX exhibits all the conserved amino acid residues of cytosolic soluble APXs (**Figure 7**).

- *P. pinaster* csAPX clusters together with other plant csAPXs (**Figure 8**).

P. pinaster csApx encodes a cytosolic soluble APX and was released on GenBank database under Acc. No. AY485994

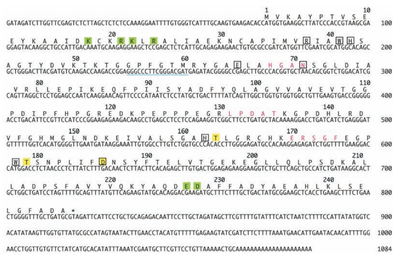

Figure 6 - Nucleotide and deduced amino acid sequences of *P. pinaster csApx* cDNA (Acc. No. AY485994). The predicted amino acid sequence is depicted above the nucleotide sequence in the one-letter code. Numbers on the right refer to nucleotides, and numbers above amino acid sequence refer to amino acids. (shadowed in green - residues involved in electrostatic interaction in the dimer interface; shadowed in yellow - metal-binding residues; boxed - active site residues; pink - sequence of residues forming the active site entrance channel; underlined in blue - nucleotides where CSAPX primer could have hybridized * - stop codon).

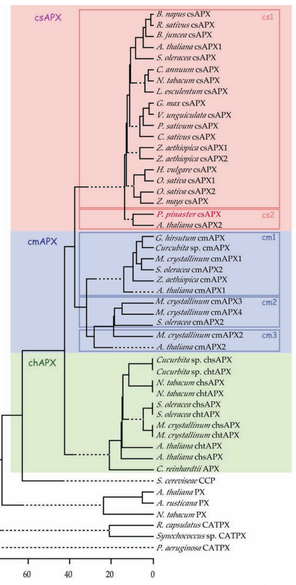

Figure 8 - Phylogenetic tree representing the relationship between *P. pinaster* csAPX and other peroxidases. Amino acid sequences were aligned using the *MegAlign* program (DNASTAR package) using Clustal Method with PAM250 residue weight table. The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree measures the distance between sequences.

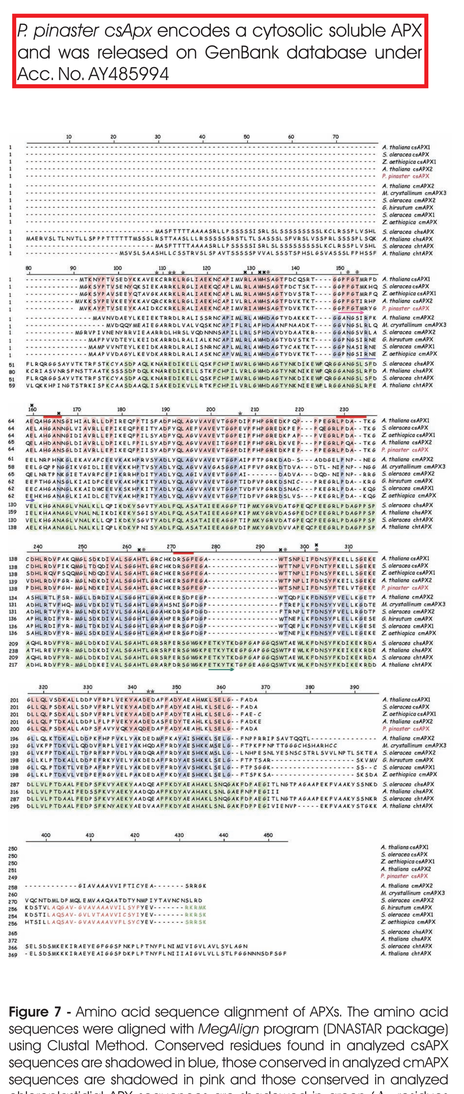

Figure 7 - Amino acid sequence alignment of APXs. The amino acid sequences were aligned with *MegAlign* program (DNASTAR package) using Clustal Method. Conserved residues found in analyzed csAPX sequences are shadowed in blue, those conserved in analyzed cmAPX sequences are shadowed in pink and those conserved in analyzed chloroplastidial APX sequences are shadowed in green (☆- residues involved in electrostatic interaction in the dimer interface; ❄- metal-binding residues; x - active site residues; red bar - sequence of residues forming the active site entrance channel; red - putative membrane binding domain; green - putative mPTS2; pink arrow - CSAPX primer; violet arrow - CMAPX primer; green arrow - CHAPX primer).

Sequence analysis of *P. pinaster csMdh*

- From the cDNA clones responsible for a positive signal in Figure 5.c, six were selected for further sequencing (pchApx42, pchApx51, pchApx514, pcmApx722, pcmApx723, pcmApx724).

- All cDNA clones revealed high homology with plant malate dehydrogenases. The complete nucleotide and deduced amino acid sequences of the largest insert are shown in **Figure 9**.

- Sequence analysis revealed high identity with cytosolic soluble MDH from *Arabidopsis thaliana* (86.4-88.6%). Identity values found with *A. thaliana* chloroplastidial MDH is 38.3%, with *A. thaliana* mitochondrial MDH is 16.9% and with *A. thaliana* peroxisomal MDH is 14.2%.

- *P. pinaster* csMDH does not seem to have a transit peptide and the deduced amino acid sequence exhibits the conserved amino acid residues of other cytosolic MDHs.

- *P. pinaster* csMDH clusters together with other csMDHs (**Figure 10**).

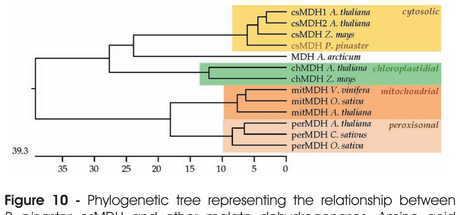

Figure 10 - Phylogenetic tree representing the relationship between *P. pinaster* csMDH and other malate dehydrogenases. Amino acid sequences were aligned using the *MegAlign* program (DNASTAR package) using Clustal Method with PAM250 residue weight table. The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree measures the distance between sequences.

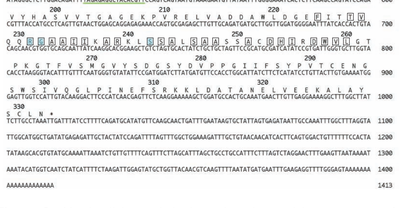

Figure 9 - Nucleotide and deduced amino acid sequences of *P. pinaster csMdh* cDNA (Acc. No. AY485993). The predicted amino acid sequence is depicted above the nucleotide sequence in the one-letter code. Numbers on the right refer to nucleotides, and numbers above amino acid sequence refer to amino acids. (shadowed in pink - residues involved in NADH binding; shadowed in blue - residues involved in oxaloacetate binding; boxed - residues involved in electrostatic interaction in the dimer interface; underlined in yellow - nucleotides where CMAPX primer could have hybridized; underlined in green - nucleotides where CHAPX primer could have hybridized; * - stop codon).

P. pinaster csMdh encodes a cytosolic MDH and was released on GenBank database under Acc. No. AY485993

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