Title:

Salicylic acid up-regulates the expression of chloroplastic Cu,Zn-superoxide dismutase in needles of maritime pine (Pinus pinaster Ait.)

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Short Title (running head):

Maritime pine chl Cu,Zn-SOD expression
Abstract

Several studies have supported a major role of salicylic acid in modulating plant response against abiotic and biotic stresses, by induction antioxidant capacity. In this work, a full-length cDNA encoding a Cu,Zn-superoxide dismutase was isolated by screening a Pinus pinaster needle cDNA library. The predicted protein of 215 amino acid residues has a molecular mass of 22.1 kDa and exhibits a N-terminal transit peptide, which putatively targets the protein to the chloroplast. Treatment of pine seedlings with salicylic acid resulted in the increase of chloroplastic Cu,Zn superoxide dismutase transcript levels in needles, suggesting a role of this isoform in salicylic acid-mediated H$_2$O$_2$ increase in chloroplasts.

Key Word Index

Pinus pinaster / salicylic acid / chloroplastic Cu,Zn-SOD
Résumé

Plusieurs études suggèrent le rôle prédominant de l’acide salicylique dans la modulation de la réponse des plantes aux stress abiotiques et biotiques par induction de la capacité antioxidante. Dans ce travail, un ADNc pleine longueur codant pour une Cu,Zn-superoxide dismutase a été isolé par criblage d’une banque d’ADNc d’aiguille de Pinus pinaster. La protéine prédite de 215 acides aminé possède une masse moléculaire de 2 avec s2.1 kDa et présente, en N-terminal, un peptide signal potentiel d’adressage vers chloroplaste. Le traitement de jeunes plantules de pin par l’acide salicylique induit une augmentation du niveau de transcrit de la Cu,Zn-superoxide dismutase dans les aiguilles, ce qui suggère un rôle de cette isoforme dans l’augmentation des niveaux de H2O2 dans le chloroplaste sous l’effet de l’acide salicylique.

Mots Clés

Pinus pinaster / acide salicylique / Cu,Zn-SOD chloroplastique
1. INTRODUCTION

In Portugal, maritime pine (*Pinus pinaster*, Ait.) is an important species for pulp, paper and timber industries, due to good market placement, ecological low demanding and high versatility to stress endurance. It was initially used as a suitable species for reforestation, as centuries of indiscriminate forest use led to the disappearance of the original Atlantic and Mediterranean stances, causing a condition of rapid soil erosion. Maritime pines have also been extensively used in setting dunes as a way of preventing sand invasion on agricultural and urban fields. By the beginning of the last century, it represented over 60% of the Portuguese forest. While it is still the main forest species in Portugal, *P. pinaster* lost 50% of its distribution in only the last thirty years, being progressively replaced by stances of *Eucalyptus* sp. In addition to forest fires, the main cause for this decline lies on biotic stress, which has led to a high mortality rate of seedlings and young trees, thus preventing natural regeneration within the population. Most biotic stress accounted, lies on insect predation and fungi attack, with the relevant role being played by the needle striking fungi *Lophodermium seditiosum*, *Sphaeropsis sapinea*, *Botrytis cinerea* and *Dothistroma septospora*.

Salicylic acid (SA) has been referred as playing a role in modulating plant responses to abiotic and biotic stresses. Accordingly, SA has been reported to increase thermostolerance and heat acclimation [5], chilling...
tolerance [10], salt and osmotic stress responses [2]. SA has also been described as being an endogenous signal for the activation of plant defenses during pathogen attack, mediating the oxidative burst that leads to cell death in the hypersensitive response, and acting as a signal for the development of systemic acquired resistance (SAR) [6, 14]. The transduction of the resistance signal by SA could be achieved by the inhibition of the major \textit{H}_2\textit{O}_2-scavenging enzymes, such as ascorbate peroxidase and catalase, leading to an increased level of \textit{H}_2\textit{O}_2 [reviewed by 6]. More recently, it has been suggested that \textit{H}_2\textit{O}_2 functions upstream rather than, or in addition to, acting downstream of SA, since SA-treated plants showed no differences in catalase or ascorbate peroxidase activity [6]. SA-enhanced \textit{H}_2\textit{O}_2 levels could also be attributed to an increased activity of \textit{H}_2\textit{O}_2–producing enzymes, as reported for Cu,Zn - superoxide dismutase [13].

The superoxide dismutase (SOD) protein family comprises metal containing enzymes responsible for the dismutation of superoxide radical into oxygen and hydrogen peroxide. This family is divided in three groups differing in their active site metal ion (Cu/Zn, Fe and Mn). Cu,Zn-SOD isoforms are structurally unrelated to the remaining groups; most of them form an homodimer and each subunit binds a copper and zinc ion [11]. In plants, Cu,Zn-SODs are mainly located in the cytosol and in the chloroplast stroma, although other locations have been reported [3]. The extracellular
location of this isoform in Scots pine is possibly related to the transmission
of systemic signal in wounding or in pathogen responses [12].

In this work, the effect of SA was studied in what concerns the
chloroplastic form of Cu,Zn-SOD from Pinus pinaster.

2. MATERIALS AND METHODS

2.1. Plant material and growing conditions

Pinus pinaster seedlings were grown in a culture chamber at 26 °C
with a 16-hour photoperiod. Needles from 30-year-old pine trees were
harvested, immediately frozen in liquid nitrogen and stored at -80 °C.

2.2. Salicylic acid treatment

Two month-old Pinus pinaster seedlings were sprayed with 5 mM
salicylic acid. Twenty randomly picked seedlings were removed daily for
one week. The harvested material was immediately frozen in liquid nitrogen
and stored at –80 °C.

2.3. mRNA purification and cDNA library construction
Total RNA from adult needles of *Pinus pinaster* was extracted using a CTAB based method, followed by chloroform extraction, adapted from Chang *et al.* [4]. For cDNA library preparation, poly(A)+ RNA was isolated using Oligo (dT) beads (Dynabeads Oligo (dT)25, Dynal) and used in ZAP Express™ cDNA synthesis/Gigapack III Gold cloning kits (Stratagene) according to the manufacturer's instructions.

2.4. cDNA library screening and sequence analysis of Cu,Zn-SOD cDNA

Screening of *P. pinaster* cDNA library for Cu,Zn-sod cDNA clones was performed using the Cu,Zn-sod cDNA from *Zantedeschia aethiopica* as probe (accession AF054151). Duplicate plaque filters (Hybond-N+; Amersham) were hybridised at 42°C for 16 h with the referred $^{32}$P-labelled probe and successive washings were performed until the final concentrations of 1 x SSC and 0.1% SDS, at 60°C, for 30 min. After a second round of screening, cDNA inserts were excised *in vivo* from positive phage clones as pBK-CMV plasmids.

The inserts of Cu,Zn-sod cDNA clones were sequenced in both directions using universal T3 and T7 primers and BigDye terminator chemistry. Complete sequencing was achieved designing new primers. Nucleotide and amino acid sequences analysis were performed using
DNASTAR package software version 1.58 (Lasergene). The search of databases for sequence similarities was carried out using the BLAST algorithm at www.ncbi.nlm.nih.gov/blast [1].

2.5. Northern analysis

Seedling samples were grinded to a fine powder in a mortar using liquid nitrogen. Total RNA extraction was performed using a CTAB based method with chloroform extraction, adapted from Chang et al. [4]. Formaldehyde gel electrophoresis with ethidium bromide staining allowed for sample normalization and integrity assessment. For Northern blot analysis, 20 μg of RNA were separated by 1,2% formaldehyde agarose gel electrophoresis and transferred to Hybond-N+ nylon membranes (Amersham Biosciences). RNA blots were hybridized with 150 ng of full length 32P-labelled Cu,Zn-sod cDNA from Pinus pinaster. Hybridization was performed overnight at 42°C with 50% formamide, 5 mM EDTA (pH 8.0), 50 mM sodium phosphate, 0.9 M NaCl, 10x Denhardt reagent, 0.1% SDS and 250 μg/ml denatured salmon sperm DNA. The blot was successively washed until the final concentrations of 1x SSC and 0.1% SDS, at 65°C, for 30 min, and then was exposed to BioMax MS film (Kodak) for three days.

RESULTS AND DISCUSSION
3.1 Pinus pinaster Cu,Zn-sod cDNA analysis

A full-length cDNA (961 bp; accession AF434186) containing a putative 648 bp open reading frame was identified after screening a P. pinaster needle cDNA library with a chloroplastic Cu,Zn-sod fragment from Zantedeschia aethiopica (accession AF054151). The predicted protein of 215 amino acid residues has a molecular mass of 22.1 kDa and a 6.41 isoelectric point. Multiple nucleotide sequence alignment using ClustalW indicated the presence of a putative N-terminal signaling peptide (Fig. 1). Indeed, chloroplast targeting of the protein was predicted by TargetP [8], allowing the recognition of the first cDNA encoding a chloroplastic Cu,Zn-SOD in gymnosperms. The amino acid sequence of the corresponding protein was analysed together with other chloroplastic and cytosolic SODs (Fig. 2), using the Jones-Taylor-Thorton model of maximum likelihood as the criteria of inference [7]. PROML and DrawTree from the PHYLIP software package [9] were used for algorithm computation and unrooted tree plotting, respectively. Analysis of the tree clearly established the P. pinaster deduced protein within the chloroplastidic Cu,Zn-SODs, yet indicating the natural divergence from all angiosperm species in the clade (the P. pinaster deduced protein showed between 46.2% and 70.8% similarity to angiosperm sequences). While pointing towards the divergence between cytosolic and chloroplastic Cu,Zn-SODs occurring early in plant evolution [3], branch distance also indicates that chloroplastic Cu,Zn-SOD
have much higher variability in nucleotide substitution rates than their
cytosolic paralogues. This has already been found in other plant gene
families [7]. The increased resistance to H₂O₂ which was found in
chloroplastic Cu,Zn-SODs has been pointed as a possible evolutionary
motor for the higher divergence rate [11].

3.2. Effect of salicylic acid on the expression of *Pinus pinaster*
chloroplastic *Cu,Zn-sod*

The expression of maritime pine chloroplastic *Cu,Zn-sod* was
evaluated in two month-old seedlings by Northern analysis. While high
levels of transcripts were detected in needles, low levels of expression were
observed in roots and stems, corroborating the chloroplastic location of this
protein (Fig. 3A). For studying the effect of SA on chloroplastic *Cu,Zn-sod*
expression, *P. pinaster* seedlings were sprayed with 5 mM salicylic acid and
transcript levels were analyzed along time. The results indicate that
maritime pine needles treated with SA exhibit a transient increase in
chloroplastic *Cu,Zn-sod* transcript levels (Fig. 3B).

In the same time it has been hypothesized that SA could serve as the
long-distance SAR signal that moves from inoculated leaf to uninoculated
portions of the plant, it was also suggested that H₂O₂ produced during the
oxidative burst that occurs in incompatible plant-pathogen interactions.
could be the signal responsible for the induction of SAR [6]. Several studies reported the effects of SA on the activity of H$_2$O$_2$-scavenging enzymes; however its role on the regulation of the expression of enzymes responsible for H$_2$O$_2$ production is not well understood. Our results are in accordance with those reported by Rao et al. [13], in which SA-enhanced H$_2$O$_2$ levels were related to the increased activity of Cu,Zn-SOD. We suggest that due to SA-mediated up-regulation of chloroplastic Cu,Zn-sod expression, chloroplasts might play a role in the increase of H$_2$O$_2$ levels that are associated to the systemic microbursts that occur in uninfected cells.

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salicylic acid decreases the effects of chilling injury in maize (Zea mays L.)


Legends

Figure 1

Amino acid sequence analysis of *P. pinaster* chloroplastic Cu,Zn-SOD with other chloroplastic (chl) and cytosolic (cyt) Cu,Zn-SOD. Multiple alignment was done using ClustalW. See Fig. 2 for accession numbers. (Cu-binding residues are marked with open circles; Zn-binding residues are marked with closed circles).

Figure 2

Unrooted tree for the phylogenetic analysis of Cu,Zn-SOD using maximum likelihood. Inference was determined using the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html).

*Arabidopsis thaliana* chl (AF061519), *Arabidopsis thaliana* (X60935), *Dichanthelium lanuginosum* chl (AF385581), *Homo sapiens* (NP_000445), *Lycopersicon esculentum* chl (M37151), *Lycopersicon esculentum* (X87372), *Marchantia paleacea* chl (AB004870), *Medicago sativa* chl (AF056621), *Oryza sativa* chl (D85239), *Oryza sativa* (D00999), *Pinus sylvestris* (X58578), *Pinus pinaster* chl (AF434186), *Pisum sativum* chl (J04087), *Pisum sativum* (M63003), *Populus tremuloides* chl (U08097), *Saccharomyces cerevisiae* (J03279), *Solidago altissima* (D49485), *Spinacea*
oleracea chl (D10244), Triticum aestivum (U69536), Zantedeschia aethiopica chl (AF054151), Zantedeschia aethiopica (AF054150).

**Figure 3**

Expression analysis of *P. pinaster* chloroplastic Cu,Zn-sod mRNA by Northern analysis. Aliquots (20 µg) of total RNA were separated in formaldehyde-agarose gel, blotted and hybridized with 32P-labelled *P. pinaster* Cu,Zn-SOD probe. The amount of RNA was determined using ethidium bromide-stained RNA. A – Expression analysis in different plant organs: roots (R), stems (S) and needles (N). B – Time course expression analysis in needles after treatment with 5 mM salicylic acid.
Figure 1

Figure 2
Figure 3

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