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Note

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

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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_2O_2 increase in chloroplasts.

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

Résumé – L'acide salicylique induit une augmentation de l'expression de la Cu,Zn-superoxide dismutase du chloroplaste d'aiguille de pin maritime (*Pinus pinaster* Ait.). 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 aminés possède une masse moléculaire de 22,1 kDa et présente, en N-terminal, un peptide signale 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 H_2O_2 dans le chloroplaste sous l'effet de l'acide salicylique.

***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 thermotolerance and heat acclimation [5], chilling tolerance [11], salt and osmotic stress responses [3]. 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, 16]. The transduction of the resistance signal by SA could be achieved by the inhibition of the major H_2O_2-scavenging enzymes, such as ascorbate peroxidase and catalase, leading to an increased level of H_2O_2 (reviewed by [6]). More recently, it has been suggested that H_2O_2 functions upstream rather than, or in addition to, acting downstream of SA, since SA-treated

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plants showed no differences in catalase or ascorbate peroxidase activity [6]. SA-enhanced H_2O_2 levels could also be attributed to an increased activity of H_2O_2-producing enzymes, as reported for Cu,Zn-superoxide dismutase [15].

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 classes (each encoded by a small gene family [14]), 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 [12]. In plants, Cu,Zn-SODs are mainly located in the cytosol and in the chloroplast stroma, although other locations have been reported [4]. The extracellular location of this isoform in Scots pine is possibly related to the transmission of systemic signal in wounding or in pathogen responses [13].

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-h photoperiod. Needles from two month-old seedlings and from 30-year-old pine trees were harvested, immediately frozen in liquid nitrogen and stored at –80 ºC.

2.2. Salicylic acid treatment

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 ºC.

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 [2]. For cDNA library preparation, poli(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 32P-labelled probe and successive washings were performed until the final concentrations of 1 × 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].

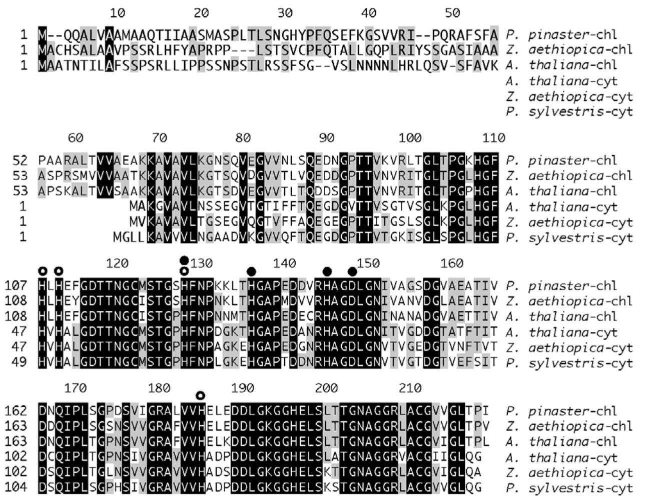

Figure 1. Amino acid sequence analysis of *P. pinaster* chloroplastic Cu,Zn-SOD with other chloroplastic (chl) and cytosolic (cyt) Cu,Zn-SODs. Multiple alignment was done using ClustalW. See Figure 2 for accession numbers. (Cu-binding residues are marked with open circles; Zn-binding residues are marked with closed circles.)

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 [2]. 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 coding region 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, 10 × Denhardt reagent, 0.1% SDS and 250 µg/ml denatured salmon sperm DNA. The blot was successively washed under high stringency conditions, until the final concentrations of 1 × SSC and 0.1% SDS, at 65 °C, for 30 min, and then was exposed to BioMax MS film (*Kodak*) for three days.

3. 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 complete coding region shares 24.7% identity with the full-length *Pinus sylvestris* cytosolic *Cu,Zn-sod* (X58578). 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.

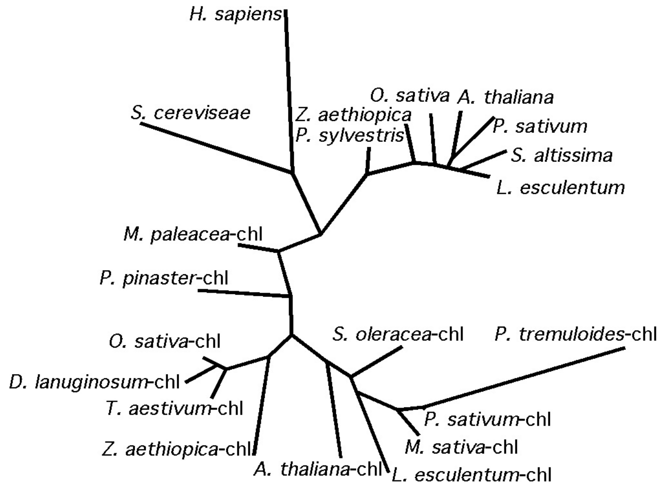

Figure 2. Unrooted tree for the phylogenetic analysis of Cu,Zn-SOD using maximum likelihood. Inference was determined using the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html). *Arabidopsis thaliana* chl (AF061519), *Arabidopsis thaliana* (X60935), *Dichanthelium lanuginosum* chl (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).

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 [4], branch distance also indicates that chloroplastic Cu,Zn-SOD have much higher variability in the amino acid residue substitution rate than their cytosolic paralogues. The increased resistance to H_2O_2 found in chloroplastic Cu,Zn-SODs has been pointed as a possible evolutionary motor for the higher divergence rate [12].

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

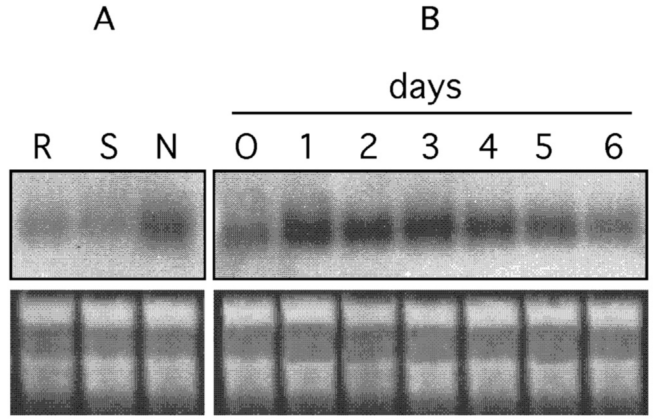

Figure 3. Expression analysis of *P. pinaster* chloroplastic *Cu,Zn-sod* mRNA by Northern analysis. Aliquots (20 μg) of total RNA were separated in formaldeyde-agarose gel, blotted and hybridized with 32P-labelled *P. pinaster* Cu,Zn-SOD probe. The amount of RNA was determined using ethidium bromide-stained RNA on gel. (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.

(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 H_2O_2 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_2O_2-scavenging enzymes; however its role on the regulation of the expression of enzymes responsible for H_2O_2 production is not well understood. Our results are in accordance with those reported by Rao et al. [15], in which SA-enhanced H_2O_2 levels were related to the increased activity of Cu,Zn-SOD. In addition, Fodor et al. [10] also reported an SA-dependent increase in Cu,Zn-SOD activity in tobacco leaves, similar to that observed in uninfected leaves when tobacco was inoculated with tobacco mosaic virus. 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_2O_2 levels that are associated to the systemic microbursts that occur in uninfected cells during SAR.

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