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Molecular identification of catalases from *Nicotiana plumbaginifolia* (L.)

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Abstract We have isolated three different catalase cDNAs from *Nicotiana plumbaginifolia* (*cat1*, *cat2*, and *cat3*) and a partial sequence of a fourth catalase gene (*cat4*) that shows no discernible expression based on Northern analysis. The catalase sequences were used to determine the similarity with other plant catalases and to study the transcriptional response to paraquat, 3-aminotriazole, and salicylic acid. 3-Aminotriazole induces mRNA levels of *cat1*, *cat2* and *cat3*, indicating that a reduction in catalase activity positively affects catalase mRNA abundance. Salicylic acid that binds catalase in vitro, had no effect on catalase transcript levels at physiological concentrations. Paraquat resulted in the induction of *cat1*.

Key words: Aminotriazole; Oxidative stress; Paraquat; Peroxisome; Salicylic acid

1. Introduction

H_2O_2 is formed during cellular processes such as photorespiration [1] and β-oxidation of fatty acids [2], and additionally as a consequence of biotic and abiotic stress [3]. The biological effects of H_2O_2 are dual. First, H_2O_2 is known to cause cellular damage, either directly or after conversion to the hydroxyl radical [4]. Alternately, H_2O_2 can be used by peroxidases in several biochemical processes, e.g. lignin biosynthesis [5], polysaccharide cross-linking [6] and protein insolubilization in the cell wall [7]. Recently, evidence has been presented that H_2O_2 may additionally have a signalling function in the cell. Prasad et al. [8] have shown that a transient accumulation of low levels of H_2O_2 during cold acclimation may induce antioxidant enzymes such as catalase, which subsequently could provide an effective protection against enhanced H_2O_2 production at lower temperatures. This induced protection was also observed after chemical treatment with H_2O_2, but was prevented by 3-aminotriazole (3-AT), a specific inhibitor of catalase [9]. Hydrogen peroxide can also induce PR proteins and is possibly part of the salicylic acid-mediated pathway of systemic acquired resistance (SAR) of plants [10]. Salicylic acid (SA) was shown to specifically inhibit catalase activity in vitro, and to induce an increase in H_2O_2 concentrations in vivo. It is conceivable that inactivation of catalases contributes to the increase in reactive oxygen species during the PR response. This would imply however that peroxisomes are directly involved in the PR response, because thus far plant catalases have only been found in peroxisomes and, in a single case, in mitochondria [11].

These data indicate that both H_2O_2 and H_2O_2-removing enzymes such as catalase may be intimately involved in the regulation of stress responses in plants. Studying catalase expression during stress conditions could therefore contribute to our understanding of stress-induced resistance in plants. Plants generally contain several isoforms of catalase, which are differentially expressed during development and by environmental conditions [12,13]. As a first step to study the physiological function of catalases in *Nicotiana plumbaginifolia*, we have isolated three different catalase cDNAs and a partial genomic sequence of a fourth catalase. These were used to investigate the effect of paraquat, a generator of superoxide radicals, of 3-AT, a catalase inhibitor, and of SA. SA, in the mM range, inactivates catalase in vitro and raises H_2O_2 levels in vivo [10].

2. Materials and methods

2.1. Cloning of catalases

Total RNA was isolated by the guanidine hydrochloride/phenol/chloroform method [14] from leaf, stem, and flowers of *N. plumbaginifolia*. One μg total RNA of each organ was used to synthesize first strand cDNA by priming with an oligo-dT (SuperScript Preamplification System, Gibco/BRL). This cDNA was used in a polymerase chain reaction (PCR) [15] with two degenerated primers ol-1 and ol-2. ol-1 (sense) and ol-2 (antisense) were designed based on highly conserved domains of plant catalases (corresponding to the amino acid regions AKGFFEV and FHWKPTC) and were made with tails containing an *Eco*RI site (ol-1) and a *Bam*HI site (ol-2). PCRs consisted of 30 amplification cycles under the following conditions: 94°C, 1 min; 50°C, 1 min; 72°C, 2 min. PCR products were digested with *Eco*RI and *Bam*HI, separated on 1% agarose gels, purified and ligated in the *Eco*RI/*Bam*HI-digested pGEM2 vector (Promega, Madison, WI). Plasmids were analyzed by restriction enzyme mapping and automatically sequenced using an automatic DNA sequencer model 370A (Applied Biosystems Inc., Foster City, CA). Three different partial catalase cDNA fragments were identified. Vectors containing these fragments were denoted pCat1A, pCat2A and pCat3A. 3′ RACE was performed on the same first-strand cDNA with the degenerated ol-1 combined with a poly(A) primer, according to Frohman et al. [15]. In second PCR amplifications, 1/50 of the product was used as template using specific sense primers for *cat1* (ol-3), *cat2* (ol-4), and *cat3* (ol-5). 5′ RACE was done according to the instructions of the manufacturer (5′ RACE System; Gibco/BRL, Gaithersburg, MD), using primer ol-2 for the first-strand cDNA synthesis. The amplification was performed with the degenerated antisense primer ol-2 in combination with a universal sense primer supplied by the manufacturer. The product of the first amplification was used as a template in second reactions with antisense-specific

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Abbreviations: 3-AT, 3-aminotriazole; *cat*, gene (DNA, RNA) encoding catalase; PCR, polymerase chain reaction; PR, pathogenesis related; RACE, rapid amplification of cDNA ends; SA, salicylic acid; SAR, systemic acquired resistance; SSC, 0.15 M NaCl, 0.015 M sodium citrate.

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primers for *cat1* (ol-6), *cat2* (ol-7), and *cat3* (ol-8), that are complementary to the specific primers used in the 3′ RACE. PCR products were ligated using the pGEM-T vector system (Promega) and automatically sequenced. To discern eventual mutations in the sequences due to reading mistakes of the *Taq* polymerase during the PCR, sequences were confirmed twice with fragments obtained from independent PCR amplifications.

The following oligonucleotides were used (added restriction site in lower case): ol-1, ggaattcGCIAAGGGITT$^{C}_{T}$TT$^{C}_{T}$GA$^{A}_{G}$GTIAC; ol-2, cgggatcc$^{A}_{G}$CAIGTIGGITTCCA$^{A}_{G}$TG$^{A}_{G}$AA; ol-3, TCTGGTGTCCACACATTCAC; ol-4, TATGGTGTCCACGCCTATCA; ol-5, TCCGGTGTTAATACCTACAT; ol-6, TGTGAATGTGTGGACACCAGA; ol-7, TTGATAGGCGTGGACACCAT; ol-8, CATGTAGGTATTAACACCGG.

2.2. DNA extraction and DNA gel blot analysis

Genomic DNA from *N. plumbaginifolia* was prepared according to Dellaporta et al. [16] and used for PCR. PCR products were separated on 0.8% agarose gels and transferred to nylon membranes (Hybond-N; Amersham, Aylesbury, UK) by standard techniques [17]. ^{32}P-labelled DNA fragments (Megaprime DNA Labelling System; Amersham), from pCat1A, pCat2A and pCat3A were used as probes. Hybridizations were carried out in 3 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 20 μg/ml denatured salmon sperm DNA at 55°C for 24 h. The filters were washed at low stringency (55°C with 2 × SSC) and at 68°C with 0.1 × SSC for gene-specific hybridization.

2.3. Catalase similarity analysis

The DNA and protein sequences were compared to other plant catalases, using the GCG Software Package (Madison, WI, USA), version 7 (April 1991).

2.4. Treatment with paraquat, aminotriazole, and salicylic acid

Leaves of mature *N. plumbaginifolia* plants, cultivated in a 14-h light (100 μmol/m^2/s)/10-h dark cycle, 23°C and 70% humidity, were vacuum infiltrated with 10 μM paraquat, 2 mM 3-AT, 50 μM SA, and water until saturation. Treated leaves were kept in Petri dishes floating on water until sampling after 3, 8, and 24 h.

2.5. RNA extraction and Northern analysis

RNA from frozen leaf tissue was extracted as described by Logemann et al. [14]. Total RNA (10 μg) of each sample was separated on agarose gels containing 6% (v/v) formaldehyde and transferred to nylon Hybond-N filters (Amersham) by capillary blotting [17]. Filters were hybridized at 68°C in 50% formamide, 5 × SSC, 0.5% SDS, 10% dextran sulfate, and 0.1 mg/ml denatured salmon sperm DNA with ^{32}P-labelled antisense RNA probes (Riboprobe Gemini II Core System; Promega) of pCat1A, pCat2A, and pCat3A.

2.6. Reverse transcriptase PCR and DNA gel blot analysis

Conditions for first-strand cDNA synthesis and PCRs were as described in section 2.1. Catalases were amplified with ol-1 and ol-2 and the acidic chitinase with ol-9 (CAT AAG AAA GTA CAG AGG AAA ATG G) and ol-10 (CTA GGC TTC GTT ACA TAG AAT GC). PCR products were separated, blotted, and hybridized at 55°C with a DNA fragment from pCat3A and at 68°C with a DNA fragment containing the coding sequence of the acidic chitinase from *N. plumbaginifolia*.

3 Results and discussion

3.1. Isolation of four different catalases from N. plumbaginifolia

Single-stranded cDNA, synthesized from total RNA from *N. plumbaginifolia* leaf, stem, and flower, was used for PCR amplification with a pair of degenerated primers, ol-1 and ol-2, which correspond to two highly conserved regions of plant catalases (section 2; Fig. 1A). DNA fragments of the expected size (± 500 bp) were ligated into pGEM2 and 75 clones were analyzed by restriction enzyme mapping. Three distinct groups

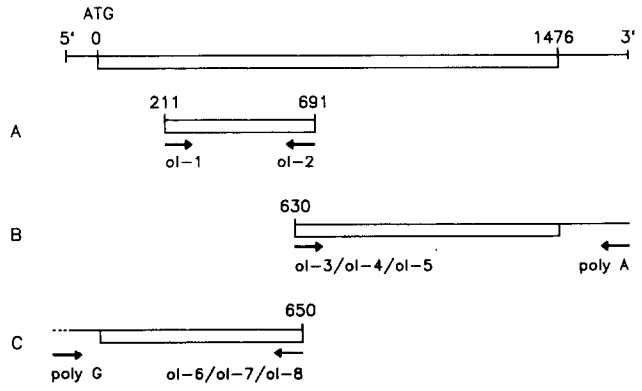

Fig. 1. Isolation of catalases by means of PCR. (A) Amplification with the degenerated primers ol-1 and ol-2 results in the isolation of internal fragments of ± 500 bp from three different catalases. (B) 3′ RACE was performed with specific sense primers for *cat1* (ol-3), *cat2* (ol-4), and *cat3* (ol-5) in combination with an antisense poly A primer. (C) 5′ RACE was performed with specific antisense primers for *cat1* (ol-6), *cat2* (ol-7), and *cat3* (ol-8) in combination with a universal sense primer.

were identified and from each group several clones were sequenced. Sequence data confirmed that we isolated three different partial catalase cDNAs, denoted *cat1*, *cat2* and *cat3*.

Subsequently, specific primers were designed for each catalase in order to isolate the 5′ and 3′ ends using RACE techniques. In both RACE protocols a double PCR was performed. In the first reaction, a degenerated primer (ol-1 or ol-2) was used to amplify catalase sequences, and a second amplification was performed with gene-specific primers to specifically enrich for *cat1*, *cat2*, and *cat3* (Fig. 1B and C). In case of *cat2* and *cat3*, DNA fragments were obtained that covered the complete coding region, whereas for *cat1*, 20 bases of the coding sequence are missing at the 5′ end.

DNA gel blot analysis was carried out to examine whether additional catalase genes are present in the genome of *N. plumbaginifolia*. The degenerated primers, ol-1 and ol-2, were used for PCR amplification of catalase genes from genomic DNA of *N. plumbaginifolia*. Four fragments of approximately 600,

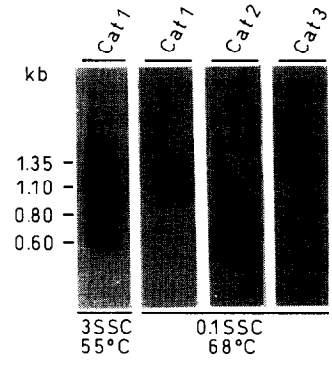

Fig. 2. PCR analysis of the catalase gene family in *N. plumbaginifolia*. Four fragments were obtained by amplification of catalase genes with the degenerated primers ol-1 and ol-2. Hybridization with *cat1*, *cat2* and *cat3* and washing under non-stringent conditions (3 × SSC, 55°C) revealed four hybridizing bands (data not shown for *cat2* and *cat3*, but identical to *cat1*), indicating that in the genome of *N. plumbaginifolia* four catalases with differently sized introns are present. After stringent washing (0.1 × SSC, 68°C), *cat1*, *cat2* and *cat3* were identified.

Fig. 3. Protein sequence comparison of catalases from *N. plumbaginifolia*. Amino acids that are conserved between at least two of the sequences are shaded. Amino acid residues involved in catalytic activity (|), proximal heme binding (▼), and distal heme binding (*) are indicated.

800, 1100, and 1300 bp were amplified and identified as catalases by Southern analyses. Non-stringent washing revealed four hybridizing bands, indicating that in the genome of *N. plumbaginifolia* four catalase genes are present with introns of different sizes (Fig. 2). After stringent washing, *cat1*, *cat2* and *cat3* were identified.

The unidentified fragment of 800 bp was isolated and cloned into pGEM-T. Sequence analysis revealed that this catalase (designated *cat4*) was most similar to *cat3* (data not shown). By Northern analysis no Cat4 mRNA could be detected in any of the organs tested (root, leaf, stem and different flower organs) (data not shown). This result and the inability to amplify *cat4* by PCR suggest that *cat4* mRNA is low abundant or absent in most plant organs.

3.2. *Sequence comparison of the catalases from* N. plumbaginifolia

A comparison of the deduced amino acid sequence of catalases from *N. plumbaginifolia* is presented in Fig. 3. Amino acids involved in catalytic activity (His-65, Ser-104, and Asn-138) and in proximal (Pro-326, Arg-344, and Tyr-348) and distal (Val-64, Arg-102, Thr-105, Phe-143, and Phe-151) heme binding are conserved in all catalases [18]. Nine amino acids from the carboxy terminus, a putative targeting sequence (SRL) to the peroxisomes was identified. Gould et al. [19,20] have shown that the presence of the consensus C-terminal tripeptide C-R-L is sufficient to direct proteins to the peroxisomes. The S-$\begin{smallmatrix}R\\H\end{smallmatrix}$-L motif in plant catalases is most likely a functional transit sequence because plant catalases, with exception of Cat3 from maize, are generally localized in peroxisomes.

The catalases of *N. plumbaginifolia* are highly similar in sequence. Sequence identity of Cat1, Cat2, and Cat3 ranges between 87 and 93% (see Table 1). Cat1 and Cat3 are most similar, whereas Cat2 is most divergent from the other catalases. In agreement with plant phylogeny, catalases from *N. plumbaginifolia* are more closely related to catalases from cotton than from maize. Cat1 and Cat3 from *N. plumbaginifolia* show 94–95% sequence identity with the cotton catalases and 88–90% with Cat1 and Cat2 from maize. Cat2 from *N. plumbaginifolia* and Cat3 from maize are more divergent and thus may correspond either to older or to faster evolving isozymes.

The catalase cDNA from *N. tabacum* that was recently isolated by screening an expression cDNA library with monoclonal antibodies against a SA-binding protein [10] is most similar to Cat2 from *N. plumbaginifolia* (98%). However, the peptides that were sequenced from the originally purified protein appear to correspond to Cat1. This finding illustrates that due to the

Table 1
Amino acid sequence similarity of catalases from *N. plumbaginifolia*, maize, cotton and tobacco

	N. plumbaginifolia				Cotton		Maize			Tobacco
	Cat1	Cat2	Cat3	Cat4	SU1	SU2	Cat1	Cat2	Cat3	
N. plumbaginifolia Cat1	100	87.0	93.1	90.1	95.0	95.3	88.4	90.1	81.3	87.1
N. plumbaginifolia Cat2		100	88.8	82.0	87.4	87.6	85.0	82.9	78.9	98.0
N. plumbaginifolia Cat3			100	91.3	95.5	94.5	90.5	89.6	81.1	89.0
N. plumbaginifolia Cat4				100	93.2	91.9	90.1	90.1	84.5	87.2
Cotton SU1					100	95.7	90.7	90.2	80.5	87.5
Cotton SU2						100	90.1	90.1	81.1	87.5
Maize Cat1							100	86.8	78.7	85.1
Maize Cat2								100	77.8	82.9
Maize Cat3									100	79.0
Tobacco										100

Similarity determinations of Cat4 were performed with the 160-amino acid internal peptide.

high sequence similarity between catalases of one species, discrimination between different isozymes during screening procedures may be particularly difficult. This is nevertheless primordial because catalase isozymes in plants are differentially expressed during development and in response to stress and thus seem to have distinct cellular functions [3,21].

3.3. Effect of paraquat, 3-AT, and SA on catalase transcripts

To gain insight into the regulation of these different catalases, different compounds that increase the cellular H_2O_2 concentration were analyzed for their effect on catalase transcript abundance.

3-AT is an inhibitor of catalase [9]; therefore, it will increase the H_2O_2 levels specifically in the parts of the cell that are normally protected by catalase. The effect of 3-AT on catalase transcript levels was tested by vacuum infiltration of 3-AT in leaves of *N. plumbaginifolia*. Treatment with 3-AT caused an induction of all three catalase transcripts 3 h after application (Fig. 4A). This result shows that inactivation of catalase leads to a general induction of catalase transcription.

Paraquat accepts electrons from electron transport chains in the chloroplasts, mitochondria, and cytosol, and reduces oxygen to the superoxide radical. Superoxide in turn is converted to H_2O_2, either spontaneously or enzymically by superoxide dismutases. Hence, H_2O_2 production during paraquat stress is thought to occur mainly or exclusively outside the peroxisomes. Yet, paraquat may increase peroxisomal H_2O_2 concentration by diffusion from other compartments. The effect of paraquat on catalase transcript levels was analyzed after 3 and 8 h. While after 3 h no effects on catalase mRNA levels were discernible (data not shown), *cat1* mRNA was specifically induced 8 h after paraquat infiltration (Fig. 4B). We have evidence that Cat1 is

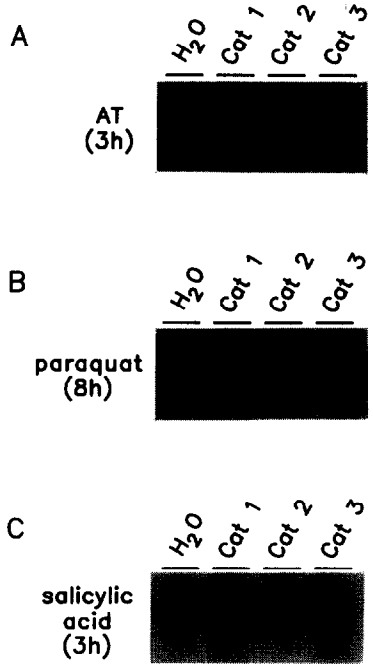

Fig. 4. Response of catalases to 3-AT (A), paraquat (B), and SA (C). Filters used in each hybridization contained RNA of leaf samples treated with H_2O, 3-AT, SA, and paraquat. To facilitate the comparison of catalase response, exposure times after hybridizations were adapted in such a manner that the water control on each film had the same density. The shown H_2O controls are hybridized with *cat1*.

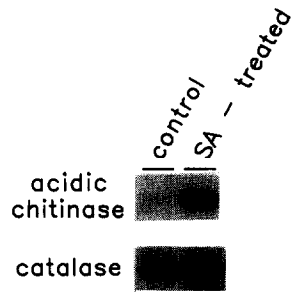

Fig. 5. Response of catalases and the acidic chitinase 3 h after infiltration with water (control) and 50 μM SA.

involved in the removal of H_2O_2 that is produced during photorespiration [21]. Because chloroplasts are the major source of paraquat-induced H_2O_2 production in photosynthesizing cells, the induction of *cat1* by paraquat may be attributed to the fact that Cat1 is localized in peroxisomes that are in close association with chloroplasts.

In contrast to 3-AT, SA had no effect on catalase transcription levels after 3 h (Fig. 4C). Recently, Chen et al. [10] have shown that SA can bind and inactivate catalase in vitro. Whether this interaction also occurs in vivo is difficult to assess, because no methods are available to quantify catalase activity in situ on intact tissues. However, our data indicate that in vivo, catalase is not inactivated by SA, as it is by 3-AT. Two plausible explanations can be given for this observation. First, the increase of H_2O_2 that was observed in vivo after SA application may not be due to catalase inactivation. Paraquat is well known to increase H_2O_2 levels, yet it only induced the catalase isoform that is localized in close proximity to the chloroplasts (Fig. 4B). Along the same line, induction of H_2O_2 production by salicylic acid, distantly from the peroxisomes, may not affect the transcriptional regulation of any of the catalases.

Second, it should be noted that we applied non-toxic doses of SA, that are 10-fold less than what was used by Chen et al. [10] but at least 3-fold higher than the natural leaf concentrations after infection. Fifty μM SA was sufficient to increase mRNA levels of the acidic chitinase within 3 h (Fig. 5), indicating that the PR response was induced in our experimental conditions. Nevertheless, it cannot be excluded that physiological levels of SA give a sufficient decrease in catalase activity for induction of the PR response, but that a larger decline is required for induction of catalase transcription.

A salient observation of this and previous studies is the drastic effect that modulation of H_2O_2 or catalase levels has on various processes such as pathogen defense [10], cold tolerance [8], as well as gene expression ([10]; this study). This suggests that cellular control mechanisms for the production and scavenging of H_2O_2 may not only prevent oxidative damage, but may also regulate different defense responses. The availability of the different catalase cDNAs from *N. plumbaginifolia* should allow a much clearer picture of the role of catalases in stress responses to be obtained.

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