Characterization of a Pathogen-Induced Potato Catalase and Its Systemic Expression Upon Nematode and Bacterial Infection

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We have isolated a cDNA encoding a catalase (Cat2St) by differential screening of a cDNA library constructed from potato roots infected with the cyst nematode Globodera pallida. Expression analysis confirmed the local induction of Cat2St and showed that it was highest at the adult stage of the parasite. It also revealed that Cat2St was induced in uninfected roots, stems, and leaves of infected plants. Localized and systemic induction of Cat2St was also observed upon root-knot nematode (Meloidogyne incognita) and root bacteria (Erwinia carotovora, Corynebacterium sepedonicum) infections. Based on sequence and expression analysis, Cat2St was found to belong to the recently described class II of dicotyledonous catalases, suggesting that these catalase isoforms could also be pathogen induced. Plant-parasitic nematodes are known to induce, in the roots of their hosts, highly metabolic feeding cells that function as nutritional sinks. Whereas the local induction of Cat2St is probably a consequence of an oxidative stress of metabolic nature, the systemic induction of Cat2St shows striking similarities with the induction of systemic acquired resistance (SAR) genes. The possible role of catalase in compatible plant-pathogen interactions is discussed.

Additional keywords: cyst nematode.

Cyst and root-knot nematodes are obligate parasites of plant roots that cause severe damage to agriculture, both in temperate and in tropical climates. After penetration of the roots, they induce the formation of sophisticated feeding cells, specialized in nutrient withdrawal from the vascular system (Jones 1981). Root-knot nematodes additionally induce hyperplasia in the tissues around feeding cells, leading to the formation of root-knots or galls.

The nematodes depend strictly on these feeding structures for food supply during the entire duration of their life cycle (1.5 to 3 mo). Consequently, these parasites do not usually kill their host, although they severely affect their growth and development. Recently, the use of molecular techniques has allowed the isolation of plant genes whose expression is up-regulated after nematode infection (for a review, see Niebel et al. 1994; Sijmons et al. 1994). However, only few nematode-induced genes have been studied and characterized in detail. Here, we present the isolation of a novel potato catalase cDNA, by differential screening of a cDNA library constructed from potato roots infected with the potato cyst nematode Globodera pallida (pathotype Pa3). Further, we present a detailed expression analysis of this gene upon compatible nematode (Globodera pallida, Meloidogyne incognita) and bacterial (Corynebacterium sepedonicum, Erwinia carotovora) infection.

Catalase is a tetramer heme protein, occurring in almost all aerobic organisms, that catalyses the dismutation of hydrogen peroxide (H₂O₂) into water and oxygen

\[ 2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2 \]

Together with peroxidases it provides protection against the toxic effects of H₂O₂, such as damage to membrane lipids, proteins, or DNA (Halliwell and Gutteridge 1989).

Plant catalases are predominantly peroxisomal enzymes and most of them contain a carboxy terminal consensus sequence for peroxisomal import (Gould et al. 1988, 1990). In plants, photorespiration (Salisbury and Ross 1992), the β-oxidation of fatty acids (Beevers 1979), and several other metabolic pathways contain H₂O₂-producing steps that thus require the presence of catalase for detoxification (Tolbert 1981).

Catalases are present as multiple isoforms in plants and the recent isolation of catalase cDNA clones from several species has shown that catalases exist as small gene families (Scandalias 1994). Expression analysis of catalases in dicotyledonous plants showed that each of these genes is associated with a specific H₂O₂-producing process. Su2 (cotton) and Cat1 (Nicotiana plumbaginifolia) appear to be specifically involved in the scavenging of H₂O₂ produced during photorespiration. Su1 (cotton) and Cat3 (N. plumbaginifolia), however, were shown to be glyoxysomal proteins (involved in

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the scavenging of \( \text{H}_2\text{O}_2 \) produced during the \( \beta \)-oxidation of fatty acids) (Ni and Trelease 1991; Willekens et al. 1994a). Cat2 (\textit{N. plumbaginifolia}) transcripts are found in nearly the entire plant, with a preference for stems, and could play a role in protection against environmental stress (Willekens et al. 1994c). Interestingly, a DNA sequence comparison analysis defined three groups, within dicotyledonous catalases, that match this functional classification (Willekens et al. 1994a, 1994b). Whereas the expression of catalases upon various abiotic stresses has been shown, their expression pattern during plant-pathogen interactions has, to our knowledge, not been reported on so far.

RESULTS

Isolation of a potato cDNA by differential screening.

Potato plants were inoculated with potato cyst nematodes and their roots collected at the adult stage of the parasite, 9 weeks after inoculation. RNA was extracted from this tissue and used to construct a cDNA library. With the help of probes obtained from infected and uninfected roots, we performed a differential screening of this library and isolated several cDNA clones showing up-regulated mRNA levels upon cyst nematode infection. After sequence analysis and database searches, one of them (named Cat2St) showed high identity scores to plant catalase cDNAs. Cat2St is a partial cDNA containing an open reading frame of 448 bp (Fig. 1).

Isolation of a genomic clone encoding a potato catalase.

Several genomic clones were isolated using the Cat2St cDNA as a probe and a 4.4-kb EcoRI fragment, called Gcat2St, was subcloned and entirely sequenced (Fig. 1). Comparison with the deduced amino acid sequences from other plant catalase cDNAs, in particular with a tomato catalase cDNA (Droy and Woodson 1992), led to the identification of seven introns in the coding region. One particularly large intron (1.4 kb) is situated close to the translation start. Amino acids essential for catalytic activity and for heme binding (Murthy et al. 1981) are present in the deduced Cat2St protein sequence. A carboxy-terminal peroxisomal targeting sequence (SRL), found in almost all plant catalases, is also present (Fig. 1).

Sequence comparison further showed that this catalase is a class II catalase as defined by Willekens et al. (1994a) for dicotyledons. Class II catalases include, among others, a tomato catalase (Droy and Woodson 1992), showing 99.2% identity with Cat2St on amino acid level and Cat2 of \textit{N. plumbaginifolia} (Willekens et al. 1994b) (92.2% identity) (Fig. 2). We thus decided to name this potato catalase Cat2St (\textit{Solanum tuberosum}).

Expression analysis.

To confirm that higher levels of Cat2St steady-state mRNA can be found upon cyst nematode infection, we performed Northern analysis experiments. In control plants, as shown in Figure 3, the 1.85-kb Cat2St mRNA is most abundant in stems as compared to leaves, roots, and especially tubers. Upon cyst nematode infection, a slight but reproducible induction can be seen in infected roots at the infection site but also in uninfected parts of the infected root system. An induction of Cat2St can also be seen in stems and leaves of infected plants. Cat2St is thus systemically induced in almost the entire plant after cyst-nematode infection in the roots.

To test whether Cat2St induction was a specific response to cyst nematode infection, we inoculated potato plants with the compatible root-knot nematode \textit{Meloidogyne incognita} as well as with two compatible bacterial root pathogens, \textit{Erwinia carotovora} and \textit{Corynebacterium sepedonicum}. As seen in Figure 3, \textit{Meloidogyne incognita} strongly induces Cat2St at the infection site (in galls) and systemically in uninfected roots, stems, and leaves. \textit{Corynebacterium sepedonicum} infection also led to a strong induction of Cat2St in infected roots and to a less pronounced, but still significant, induction in stems. Upon \textit{Erwinia carotovora} infection, elevated levels of Cat2St steady-state mRNA were observed in stems and tubers, and, to a lesser extent, in roots.

To investigate whether the observed Cat2St induction was a specific response to pathogen attack, a number of other stress conditions were tested. Figure 4 shows that wounding or salicylic acid treatment only affect Cat2St mRNA levels in roots very weakly, whereas in other organs a strong induction can be seen upon these treatments. This result indicates that the induction of Cat2St upon bacterial infection in the roots, is most probably not due to the inoculation procedure (see Materials and Methods) but rather to the pathogen attack. Cat2St steady-state mRNA levels are particularly high upon salicylic acid treatment in stems (the control tissue in which Cat2St is most abundant).

Nematode-induced feeding cells have been shown to function as nutritional sinks efficiently concentrating nutrients from the rest of the plant (Bird and Loveys 1975; Mc Clure 1977). The development of the feeding cells inside or close to the vascular system of the plant also seriously affects water transport (Wilcox-Lee and Loria 1987; Dorhout et al. 1988). To try to partly mimic the stress caused by nematode infection, two other conditions, drought and starvation (see Materials and methods) were also tested. Neither treatment significantly affected Cat2St mRNA levels (data not shown). Induction of Cat2St after nematode infection was generally higher in vitro than in pots. This is probably due to the higher inoculum levels and the more synchronized infections achieved in the in vitro system.

To establish the timing of the Cat2St induction during the life cycle of the potato cyst nematode inside roots, a time course experiment was performed using the inoculation system in Petri dishes set up by Migniéry and Person (1977). This system allows synchronous infections and a precise determination of the stage of the infection. Induced Cat2St mRNA levels can be seen in infected roots starting from 2 to 5 days after cyst nematode inoculation, depending on the experiment (Fig. 5). However, Cat2St induction becomes maximal only during later stages (about 25 days after inoculation). Time course experiments in pots (where cysts and not larvae are used as inoculum, thus leading to delayed and unsynchronized infections) showed detectable systemic induction of Cat2St starting from 10 days after inoculation. Highest induction levels were only observed 4 weeks after inoculation (data not shown).

Finally, we performed in situ hybridizations on infected roots 7, 14 (data not shown), and 25 days after inoculation. A gradual increase of Cat2St mRNA was observed with time in infected roots, confirming the results obtained using Northern
Fig. 1. Sequence of Gcat2St (Gnem2; EMBL Accession no. Z37106) and of the deduced amino acids. Note the presence of seven introns in the coding region and of a putative 3'-exon junction sequence (boxed SRL). The 3' end (●) as well as the 5' end (○) of the partial cDNA we isolated are indicated. Amino acid numbering starts at the first methionine (●), which is not indicated because Gcat2St does not contain an ATG codon. However, because the sequence of plant catalases are very conserved in this region it is likely to also contain an ATG at this position. The shaded boxes correspond to the exons. The underlined sequence is from the polymerase of the phage vector EMBL4; the dots highlight the EcoRI sites.
analysis. As seen on Figure 6, 25 days after infection a clear difference in the amounts of Cat2St mRNA present in infected and control tissue can be observed. Induced Cat2St mRNA levels can be seen throughout the infected roots. Cat2St mRNA is very abundant in emerging lateral roots, which are commonly formed at nematode feeding sites. Particularly high levels of mRNA were detected in the root cortex and in the vascular system. No expression was seen in the epidermis. Induced levels of Cat2St mRNA were detected around the feeding parasites and in the nurse cells (syncytia). However, the induced mRNA was not most abundant in these cells and also not restricted to the tissues in direct contact with the nematode. No significant label was detected in control slides hybridized with a Cat2St sense probe (data not shown).

### DISCUSSION

We have isolated a potato catalase, Cat2St, whose expression pattern is comparable to that of Cat2 of N. plumbaginifolia, and to that of a tomato catalase, being expressed in most tissues but predominantly in stems. In addition, the nucleotide sequence of Cat2St is also most similar with the class II of dicotyledonous catalases which includes Cat2 of N. plumbaginifolia and the tomato catalase. Cat2 has been shown to be induced by various abiotic stresses such as ozone, UV-B, and SO2 (Willekens et al. 1994c), which are agents known to induce pathogenesis-related responses. We could show that Cat2St mRNA levels increase upon compatible nematode and bacterial infections, which gives experimental evidence that class II catalases are also pathogen-

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Fig. 2. Comparison of the amino acid sequences of Cat2St (potato), a tomato catalase, and Cat2 of Nicotiana plumbaginifolia. Cat2St shows a very high sequence identity (99.2%) with the tomato catalase, only three amino acid residues of the 492 are different. There is also a high sequence identity (92.9%) between Cat2St and Cat2 from N. plumbaginifolia.
induced isoforms. This is, to our knowledge, the first report of a pathogen-induced catalase in plants.

It was interesting to see that Cat2St gene expression was not only locally induced at infection sites. mRNA in situ hybridization experiments showed that induced Cat2St mRNA levels were not limited to the immediate surroundings of the parasites or the feeding cell, but were found throughout the infected root. Northern analysis experiments performed on different tissues of infected and control plants, showed in addition that Cat2St was systemically induced. Indeed increased levels of Cat2St steady-state mRNA were also detected in uninfected root sectors of Globodera-infected roots, as well as in lower and upper parts of the stem, and even in leaves. Similarly all the tissues analyzed (except for tubers) after root-knot nematode infection showed elevated Cat2St mRNA levels. Upon Erwinia infection, induction was observed locally in infected tissues, i.e., tubers and roots. Systemic induction was only found in the stems in this case. Similarly, no induction was found in leaves after Corynebacterium infection. Cat2St induction was strongest in the infected roots and appears weaker in tissues more distant from the infection site. The difference observed between the two types of bacteria in their ability to induce Cat2St in infected roots and tubers, might be due to differences in pathogenic behavior. Whereas Erwinia carotovora is a rotting agent, Corynebacterium sepedonicum is a parasite of the vascular cylinder. In general, with the root pathogens we have used, the induction of Cat2St was systemic, even if it seemed less dispersed after bacterial infections. A diffusible factor that propagates the information of a local stress in the roots towards the other parts of the plant may thus be involved in the systemic Cat2St induction.

It is important to distinguish between the systemic Cat2St induction and the induction observed at the infection site. Locally, increased levels of Cat2St mRNA were observed starting from approximately 1 week after inoculation with cyst nematodes, using both Northern analysis and mRNA in situ hybridization. The induction was, however, maximal only at later stages, 3 to 5 weeks after inoculation. At that time, the parasites have undergone three molts and are at the adult stage. The feeding cells (syncytia) are fully developed and have reached their stage of highest activity in order to feed the gravid adult females. Induced catalase steady-state mRNA levels at the infection site are thus highest when the stress due to the presence of heavily feeding nematodes is maximal. As previously mentioned, feeding cells have been shown to function as metabolic sinks, showing a very high metabolic activity (Rubinstein and Owens 1964). These results suggest that the local nematode-induced oxidative stress could be of metabolic nature. We propose that the plant would induce catalase (Cat2St) to protect itself from local damage.

Locally, an oxidative burst, consisting of two successive O₂ and H₂O₂ bursts, and possibly mediated by a plasma membrane-bound NADPH oxidase, has been shown to occur upon pathogen attack (Doke 1985). However, this phenomenon is too rapid (the first oxidative burst takes place within minutes, while the second one was shown to occur after a few hours) to account for the prolonged Cat2St induction observed. Additionally, the second oxidative burst has exclusively been characterized in incompatible plant-pathogen interactions (Doke et al. 1994).

Little is known about the mechanisms leading to the transcriptional activation of catalase in plants, even if circumstantial evidence points to a direct role for H₂O₂ in inducing its own scavenging mechanisms (Prasad et al. 1994; Scandalios 1994; Willekens et al. 1994b). It is possible that H₂O₂ would, for example, act by activating transcription factors as was shown for NF-κB in humans (Schreck et al. 1991).

Striking similarities exist between the expression of Cat2St and the expression of systemic acquired resistance (SAR) genes (Ward et al. 1991). Both are induced systemically, thus are expressed in tissues devoid of pathogens. In both cases a diffusible signal is probably released at the infection site and transported to uninfected parts of the plant. In both cases, the response is nonspecific in that it can be induced by different pathogens (Kuc1982; Ryals et al. 1994). In addition mRNA levels of both SAR genes and Cat2St can be induced by salicylic acid (SA) treatment, suggesting that SA is involved in the signal transduction pathway leading to the transcriptional activation of these genes (Ward et al. 1991; Vermooy et al. 1994a, 1994b). An important difference, however, is that SAR is usually associated with an incompatible (resistance) interaction, whereas Cat2St was shown to be systemically induced after compatible interaction. Interestingly, there are tissue-specific responses to salicylic acid treatment (Fig. 4). Only very weak Cat2St induction was observed in roots. In stems, however, in which systemic induction of Cat2St had been observed upon pathogen attack, SA induced Cat2St steady-state.

![RNA gel blot analysis of Cat2St in different organs upon attack by various compatible root pathogens. The tissues of the Globodera- and the Meloidogyne-infected plants were collected 6 weeks after inoculation. The bacterial-infected plants were harvested when the first symptoms of the infection were clearly visible, 3 days after Erwinia and 7 days after Corynebacterium infection. In nematode-infected plants, samples were taken both from infected root sectors (infection site for Globodera and galls for Meloidogyne) and uninfected root sectors of infected plants (roots). All panels from this figure are derived from one experiment and hybridization intensities are thus directly comparable.](image-url)
mRNA levels. These results suggest that the mechanisms leading to local and systemic Cat2St induction might be different.

The above-mentioned correlation is particularly interesting in the light of the recent finding that a catalase would play a crucial role in mediating SAR in tobacco (Chen et al. 1993; Dempsey and Klessig 1994). These authors propose that this catalase (SABP) would function as a kind of receptor for SA. The binding of SA to SABP would lead to an inactivation of its catalase activity thus allowing the onset of H₂O₂-mediated defense mechanisms.

Although the induction of SAR has not been tested in our case, it is tempting to compare the two systems and to assume that inoculation by root pathogens also leads to systemic increases in SA, that would bind and inactivate catalases in these tissues. The resulting increase in H₂O₂ would then not only lead to induction of defense-related genes but also, directly or indirectly, activate the transcription of pathogen-reactive genes such as Cat2St.

Taking into account the proposed role of H₂O₂ as a secondary messenger for the induction of defense mechanism (Apostol et al. 1989), another more mechanistic interpretation can be formulated. If H₂O₂, generated during plant-pathogen interactions can induce defense mechanisms, it would be critical for a pathogen seeking to establish a compatible interaction to interfere with the endogenous levels and hence the diffusion of this secondary messenger. During evolution, pathogens would thus have evolved mechanisms to control levels of H₂O₂ inside plant tissues. One such mechanism may consist in controlling, directly or indirectly, the systemic expression of plant catalases.

**MATERIALS AND METHODS**

Cyst nematode infections.

Sixty cysts of Globodera pallida pathotype 3 (Pa3) were allowed to rehydrate for =10 days in water and subsequently placed (inside 100-μm nylon mesh bags) in a pot containing a
1/1 mix of sand and soil, under a sprouting tuber of the cultivar 'Bintje'. Plants were grown at 20°C ± 2°C and 14 h of light/day.

Nine weeks after infection, root sectors, containing numerous white females, were isolated. After removing soil and protruding nematodes RNA was extracted.

For precise expression analysis at the infection site, we essentially used the culture and inoculation system worked out by Mugnier and Person (1977) in agar medium-containing plates. In this case, cysts were rehydrated for 7 days and subsequently allowed to hatch in potato root exudate for 3 to 7 days. Approximately 80 infective larvae were inoculated per root tip. Infected root sectors were then precisely cut out at different time points after inoculation and further processed.

### Root-knot nematode infections.

Approximately 50 egg masses of the root-knot nematode *Meloidogyne incognita* were placed in pots, under a sprouting tuber just as for cyst nematode infections. Infected tissues were harvested 6 weeks after inoculation.

### Bacterial infections.

For both *Corynebacterium sepedonicum* NCPPB 2137 and *Erwinia carotovora* spp. *carotovora* NCPPB 312 infections, cultures in the logarithmic phase were diluted to a concentration of 10⁶ bacteria per milliliter. The soil was washed away from roots of 3-week-old potato plants that were subsequently placed in the bacterial solutions for 2 h, before being replaced in pots. Roots were wounded with a razor blade to assure a good infection. Tissues were harvested after appearance of the first symptoms, 3 days for *Erwinia* infection, 1 week for *Corynebacterium*.

### Stress conditions.

For salicylic acid treatments, plants were sprayed until saturation and the roots were soaked with a 5 mM solution of salicylic acid sodium salt (Sigma, St. Louis, MO). Tissues were collected after 2 days. For wounding experiments, tissues from different organs were sectioned, using razor blades, into pieces of about 1 cm in length and subsequently left in a moist environment for 2 days. For drought experiments, plants were not watered for approximately 3 weeks. Tissues were harvested when the first wilting symptoms became visible. For “starvation” experiments, plants were grown in sand and supplied only with deionized water. Tissues were harvested on 4-week-old plants.

During all infections and treatments, control plants were handled in the same way and harvested at the same developmental stage as the infected or treated plants.

### cDNA library synthesis and differential screening.

From approximately 20 g of infected root tissue, 5 mg of total RNA was extracted essentially according to Jones et al. (1985). Using an oligo-dT-cellulose column, 38.7 µg of poly(A)⁺ RNA could be isolated from this total RNA, of which 5 µg was used to construct a cDNA library. Double-stranded cDNA was first obtained using the cDNA synthesis plus system (Amersham, Aylesbury, UK). *EcoRI* adapters from the λgt10 cDNA cloning system kit (Amersham) were subsequently ligated to them, and finally the library was cloned into the plasmid vector pUC18. From the approximately 36,000 recombinant clones containing inserts, of an average length of 900 bp, 3,000 were isolated in microtiter plates and replicas were made on nylon membranes. The replicas were then hybridized with ³²P-labeled first-strand cDNA synthesized from 5 µg of infected and control poly(A)⁺ RNA. After several screening rounds, differentially expressed clones were isolated and further characterized.

### Sequencing.

Sanger sequencing was performed on Cat2St and Gcat2St using the deaza sequencing kit from Pharmacia (Uppsala, Sweden).

### Screening of a genomic library.

A potato genomic library from the cultivar Datura constructed in the phage vector EMBL4, kindly provided by E. Kombrink (Max-Planck-Institut für Zuchtforschung, Köln, Germany), was screened using the partial *Cat2St* cDNA as a probe. Overlapping clones (18) were isolated and finally a suitable 4.3-kb fragment was subcloned and entirely sequenced. None of the other isolated genomic clones extended further 5’.

### Northern analysis.

RNA concentrations were determined spectrophotometrically and 10 µg of total RNA was denatured in 10% formaldehyde, electrophoresed, and transferred to nylon membranes according to Sambrook et al. (1989) in the presence of ethidium bromide to check equal loading and integrity of the samples. To obtain highly specific probes, the 800-bp partial potato catalase cDNA was subcloned into pGem2 (Promega, Madison, Wisconsin), and ³²P-labeled single-stranded riboprobes were synthesized using the Riboprobe kit (Promega). Using these probes, Northern hybridizations were performed overnight at 68°C in 50% formamide, 3x SSC [1x SSC is 150 mM NaCl, 15 mM Na₃-citrate, pH 7.0], 0.25% nonfat milk powder, 0.5% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 20 µg/ml denatured salmon sperm DNA. Under these stringent conditions, no cross-hybridization with other catalase gene family members was observed (Willekens et al. 1994b). The filters were washed at 68°C for 20 min each, twice with 3x SSC, 0.5% SDS, once with 1x SSC, 0.5% SDS, once with 0.1x SSC, 0.5% SDS, and autoradiographed on Kodak XAR-5 films with intensifying screens at -70°C. Each figure containing the result of a Northern analysis was made from a single Northern blot.

### mRNA in situ hybridizations.

³⁵S-Labeled riboprobes were prepared using the Riboprobe kit (Promega). Full-length transcripts were reduced to an average length of 0.1 kb by alkaline hydrolysis (Cox et al. 1984; Martineau and Taylor 1986). Infected and noninfected root pieces were fixed using a 0.1 M cacodylate buffer containing 0.25% glutaraldehyde and 4% paraformaldehyde, pH 7.4 for 3 h, and again overnight at 4°C. The tissue was dehydrated and embedded in paraffin. Ten-micrometer, longitudinal sections were made with an ultratc microtome 2000 (Reichert-Jung GmbH, Nußloch, Germany) pretreated according to the method of Cox et al. (1984) and hybridized with the hydroxylated riboprobes essentially as described by Barker et al. (1988). Control sections were hybridized with a sense potato *Cat2Sr* probe. Autoradiography was performed as described by Angerer and Angerer (1981).
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LITERATURE CITED


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