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ORIGINAL PAPER

Characterization of one typical 2-Cys Peroxiredoxin gene of *Taenia solium* and *Taenia crassiceps*

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Abstract The *Taenia* genus is capable of living for long periods within its hosts. Reports have shown that this successful establishment is related to its efficient defense mechanisms against host immune response and its high tolerance to oxidative stress. In this work, we describe the genomic sequences of one *Taenia solium* and *Taenia crassiceps* typical 2-Cys peroxiredoxins (*Ts2-CysPrx*, *Tc2-CysPrx*) genes, which are 94% identical in primary sequence with the typical 2-Cys Prxs catalytic motifs. Both genes have the same genomic architecture, showing a TATA box and Initiator (Inr) sequence in their proximal promoter, two exons split by a 67-bp type III intron and one unique transcription start site located inside the Inr. We show that *T. crassiceps* cysticerci are highly tolerant to H_2O_2 presenting a lethal concentration 50 of 3.0 mM and demonstrate that the typical *Tc2-CysPrx* gene is not induced by H_2O_2, showing a behavior of an antioxidant housekeeping gene. This study describes for first time the gene structure of a typical 2-Cys Prx in the *Taenia* genus.

Introduction

The cestode *Taenia solium* is the causal agent of cysticercosis in humans and pigs. This parasite is present in most non-developed countries and massive human migration has spread it to developed countries as well. The most severe form of the disease, neurocysticercosis, is of worldwide importance due to its impact in human health and economy and although many efforts have been oriented to parasite control or eradication, it is still a public health problem; therefore, more studies are needed to fully accomplish this goal (Carabin et al. 2006).

Several reports have shown that the successful establishment of this taeniid and other helminths in the host is related to its evasion of the immune response and its antioxidant defense (Alvarez et al. 2008; Vaca-Paniagua et al. 2008; Batra et al. 1993) It is known that in the Taeniidae family of parasitic worms the enzymatic antioxidant system is composed by a Cu/Zn superoxide dismutase, two glutathione transferases, a thioredoxin glutathione reductase, and one typical 2-Cys Peroxiredoxin (Vaca-Paniagua et al. 2008; Torres-Rivera and Landa 2008; Rendón et al. 2004; Leid and Suquet 1986; Salinas et al. 1998; Salinas and Cardozo 2000; Li et al. 2004; Bonilla et al. 2008; Chalar et al. 1999). Peroxiredoxins (Prx) are antioxidant enzymes that reduce hydrogen peroxide (H_2O_2) to water and a wide range of hydroperoxides to the corresponding alcohol (Rhee et al. 2005). They are classified in 1-Cys Prxs and 2-Cys Prxs depending on if they use one or two cysteines for catalysis. The 2-Cys Prxs are further divided in typical and atypical regarding if they are dimeric or monomeric, respectively. In human helminth parasites, only typical 2-Cys Prxs have been found and they have been characterized principally in trematodes and nematodes, but in cestodes their studies are limited. They are involved in redox state balance (Sayed et al. 2006), signal and transcriptional regulation (Wood et al. 2003), and antioxidant and parasite defense (Li et al. 2004). For example, in the trematode parasites *Schistosoma mansoni* and *Schisto-*

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soma japonicum, three typical 2-Cys Prxs have been characterized, of which Prx1 is induced under oxidant conditions, while Prx2 and Prx3 are housekeeping genes (Kumagai et al. 2006; Sayed and Williams 2004; Sayed et al. 2006). The silencing of typical 2-Cys Prxs genes with dsRNAi drastically increased parasite sensibility against H_2O_2 (Sayed et al. 2006). The lack of catalase and the fact that no high H_2O_2-reducing activity glutathione peroxidase has been found in the Platyhelminthes phylum (Callahan et al. 1988; Mei and LoVerde 1997) highlight the biological role of typical 2-Cys Prx as a major source of H_2O_2 detoxification in these parasites (Pérez-Torres et al. 2002; Lu et al. 1998). Moreover, the localization of *T. solium* 2-Cys Prxs on the parasite tegument suggests that they are in direct contact with the host immune response (Molina-López et al. 2006).

Here, we describe the genomic structure of a *T. solium* and a *Taenia crassiceps* typical 2-Cys Prx gene (*Ts2-CysPrx*, *Tc2-CysPrx*) and show that *Tc2-CysPrx* is not induced under oxidant conditions. Additionally, we show that *T. crassiceps* is highly resistant to H_2O_2.

Materials and methods

Biological materials

T. solium cysticerci were dissected from naturally infected pork, washed three times with sterile phosphate-buffered saline (PBS), and stored at −70°C until use. *T. crassiceps* WFU strain was extracted from the peritoneum of infected BALB/cAnN female mice killed with CO_2 and washed three times with sterile PBS (Everhart et al. 2004).

Cloning of *Ts2-CysPrx* and *Tc2-CysPrx* genes

T. solium and *T. crassiceps* genomic DNA was extracted as described previously (Campos et al. 1990). Briefly 1.5 g cysticerci was digested with Proteinase K for 2–3 h at 55°C in TRIS 50 mM, EDTA 1 mM, and sarcosyl 0.5%, followed by centrifugation at 1,000 × g for 15 min, phenol/chloroform extractions, and isopropanol precipitation. *Eco*RI-digested *T. solium* genomic DNA was used for the construction of a λ-ZAP library using the Uni-ZAP$^{@}$XR vector System (Stratagene, La Jolla, CA, USA). One hundred and twenty thousand clones were screened overnight at 60°C with a [α-^{32}P]dCTP-labeled *Ts2-CysPrx* probe comprising the complete complementary DNA (cDNA) sequence of the gene labeled by nick-translation with random primers (Amersham Biosciences). After secondary and tertiary screenings, phage-positive clones were converted to Bluescript plasmids using ExAssist helper phage (Stratagene). Bacterial colonies containing the plasmid Bluescript were grown overnight in LB ampicillin (100 μg/mL) medium. Plasmid DNA was prepared with alkaline lysis standard method and sequenced on an automated fluorescent dye DNA sequencer ABI Prism model 373 (Perkin-Elmer, Applied Biosystems). *T. crassiceps 2-Cys Prx* gene cloning was done by polymerase chain reaction (PCR) amplification using 100 ng of parasite DNA and primers designed from 5′ and 3′ non-coding sequences of *Ts2-CysPrx* (forward: 5′-$^{-88}$GCCAATGTGTT TAAGGCTAGG^{-68}-3′ and reverse: 5′-^{705}CAACCAGTT CAAAGAGTGGC^{685}-3′) with the following program: 1 cycle of 94°C, 30 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min; and one final extension of 72°C 5 min. The PCR product was cloned into pCRII dual promoter (Invitrogen) and the plasmid preparation was sequenced as mentioned before. Putative transcription factor binding sites were determined with the sequence analysis program PROMO: http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

Transcription start site determination

T. solium and *T. crassiceps* total RNA was prepared with TRIzol (Invitrogen) and used as template for transcription start site (TSS) determination using the Smart RACE cDNA Amplification Kit and Advantage 2 Polymerase Mix (Clontech). Both 5′ parasite Prx RACE fragments were amplified by PCR using reverse primer Prx6R (5′-AACA TCTTTGAGTTCACCATCGACAA-3′) and forward primer SMARTII (5′-AAGCAGTGGTATCAACGCAGAGTAC GCGGG-3′), following the manufacturer's directions. The 5′ fragment of *T. solium* actin *pAT6* gene was done using the reverse primer PAT6R (5′-AGGGAGGGGAAGACAG CACGAGG-3′) designed from the pAT6 gene (Campos et al. 1990) and SMARTII. The resulting band of each PCR reaction was cloned into pCRII and sequenced.

Determination of *T. crassiceps* viability under oxidative conditions

After a preincubation of 4 h in RPMI 1640 (Sigma) with 5% CO_2 at 37°C, parasites were immediately incubated in medium with 1–7.5 mM of H_2O_2 for 1 h at the same conditions. Afterwards, parasites were washed and incubated in 0.2 mL of pig bile diluted 1:3 in RPMI 1640 for 30–60 min to evaluate scolex evagination, which was observed in an inverted microscope (Nikon Eclipse TS100).

Tc2-CysPrx messenger RNA expression

Three different groups of *T. crassiceps* cysticerci were used for the expression studies: (1) in RPMI 1640 medium for 0, 1, 4, and 24 h; (2) in medium with H_2O_2 (0.25, 0.5, 1, and 2 mM) for 30 min; and (3) in medium with H_2O_2 1 mM for

0, 0.5, 1, 2, 3, and 24 h. Groups 2 and 3 were preincubated 4 h as mentioned before the addition of the medium with H_2O_2. Messenger RNA expression was determined by reverse transcriptase (RT)-PCR with One Step RT-PCR kit (Invitrogen) using 1 μg of *T. crassiceps* total RNA template and primers PRX-3 (5′-CTCCGTGGTCTCTTTATCA-3′) and PRX-9R (5′-CTATCTTGAGCTCATGAACG-3′) to amplify 2-Cys Prx. Likewise, β-actin amplification was done with primers PAT6-5′ (5′-TCCGGTATGTGCAAAGCC-3′) and PAT6-3′ (5′-GTGATGCCAGATCTTCTCC-3′). Empirically, we determined that in 30 cycles of PCR amplification all the amplicons were within the linear range of product formation and did not plateau as a saturated product. The program used in all reactions was 50°C for 30 min for reverse transcriptase reaction and 30 cycles of 94°C for 30 s, 50°C for 1 min, of 72°C for 1 min; and final extension of 72°C 5 min for PCR reaction. Amplicons were visualized by electrophoresis in 2% agarose gels stained with ethidium bromide.

Tc2-CysPrx protein expression by Western blot

Preincubated *T. crassiceps* cysts were incubated in RPMI medium with H_2O_2 (0, 1, and 2 mM for 30 min) and used to prepare crude protein extracts. Approximately 60 mg of tissue was sonicated four times at 40 W for 1 min leaving 1 min on ice between each pulse in 500 μL of lysis buffer (urea 8 M, CHAPS 0.5 M, pepstatin 1 μM, leupeptin 0.6 μM, phenylmethanesulfonyl fluoride 0.2 mM, DTT 0.5 mM). One hundred microliters of the parasite suspension was purified with 2-D Clean-Up Kit (Amersham) following the manufacturer's instructions. The resulting pellets containing the total crude proteins were resuspended in 100 μL and centrifuged at 12,000×*g* for 5 min at 4°C. The supernatant was quantified by the Bradford method, aliquoted, and stored until use at −20°C. Protein extract (15 μg/lane) integrity was determined in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 2-mercaptoethanol and stained with Coomassie blue. For Western blot, 12% SDS-PAGE gels with 2 μg of protein extracts per millimeter of lane were transferred to PVDF membranes (Towbin et al. 1979). Membranes were incubated with rabbit serum anti-*Ts2-CysPrx* (1:1,000) or rabbit anti-β-actin (Abcam, 1:2,000), washed, and incubated with peroxidase-conjugated anti-rabbit IgG. Bound antibodies were revealed with 3,3′-diaminobenzidine and 1% H_2O_2.

Results

Analysis of 2-Cys Prx gene structure

The *Ts2-CysPrx* and *Tc2-CysPrx* genes were cloned from a genomic λ-ZAP library and by PCR using genomic DNA, respectively. The genomic sequences of both genes were deposited in GenBank under accession numbers FJ621569 and FJ621570, respectively. Both Prxs have the typical 2-Cys Prxs catalytic motifs (^{47}FVCP^{50}, ^{168}EVCP^{171} for *Ts2-CysPrx*; and ^{46}FVCP^{49}, ^{167}EVCP^{170} for *Tc2-CysPrx*), where amino-terminal catalytic and resolving carboxy-terminal cysteines are located. They also have residues and motifs similar to that reported for phosphorylation in S^{88} and overoxidation in ^{92}GGVQ^{95} and ^{191}FM^{192} in *Ts2-CysPrx* and in *Tc2-CysPrx* in S^{87}, ^{91}GGVQ^{94}, and ^{190}FM^{191} (Wood et al. 2003). As seen in Fig. 1, sequence analysis showed that both proximal promoters have a TATA box and an Initiator (Inr) sequence, as well as putative binding sites for NF-1 (at −62 and −65 pb for *Ts2-CysPrx* and *Tc2-CysPrx*, respectively), Nrf-2 (at −46 and −153 for *Ts2-CysPrx* and at −49 for *Tc2-CysPrx*), and Sp1 (at −275 for *Ts2-CysPrx*). It is interesting to note that both genes contain the −3 and +4 guanines (in respect to the translation start codon; GNNATGG) described by Kozak to be translation enhancers (Kozak 1987). Both genes had two exons separated by one small type III intron of 67 bp that has NGT-AGN donor-acceptor sites placed in codon 102 for *Ts2-CysPrx* and 101 for *Tc2-CysPrx*. Sequence analysis of both introns showed a putative U1 recognition sequence (*Ts2-CysPrx*: ^{155}GTGAGT^{160}; *Tc2-CysPrx*: ^{160}GTGACT^{165}; numbering from the first transcribed nucleotide, see below) spanning the donor site (underlined), and a pyrimidine-rich tract for U2 Associated Factor (U2AF) binding (*Ts2-CysPrx*: ^{204}TTACGTTGCTCTTCCTAG^{221}; *Tc2-CysPrx*: ^{208}TAG CGTTGCTCTTCTTAG^{225}) positioned in the acceptor site (underlined). For *Ts2-CysPrx,* exon 1 is 134 pb and exon 2 is 454 pb, while exon 1 and 2 of *Tc2-CysPrx* are 131 and 454 nt.

Transcription start site

Analysis of the sequences located upstream of the ATG in *Ts2-CysPrx* and *Tc2-CysPrx* indicated the presence of an Inr sequence and a TATA box. To localize the TSS in both genes, we performed 5′ RACE experiments and sequenced of the amplified products of *Ts2-CysPrx* and *Tc2-CysPrx.* The TSS in *Ts2-CysPrx* was mapped 20 nt upstream of the translation start codon (ATG), whereas in *Tc2-CysPrx* it was located 27 nt upstream of the ATG. In both cases, the TSS corresponds to an A located within Inr sequence (TGAATTCC, for *Ts2-CysPrx* and TGAATCC for *Tc2-CysPrx*; where the A is the first transcribed nucleotide; Fig. 1). Further sequence analysis showed that the Inr and TATA sequences of *Ts2-CysPrx* and *Tc2-CysPrx* are conserved in the Cestoda genes, such as *T. solium* actin genes *pAT5* and *pAT6*, as well as to the *Echinococcus granulosus* actin genes *EgactI* and *EgactII* (Campos et al. 1990; da Silva et al. 1993); moreover, the nucleotide distance between both elements is conserved in all the

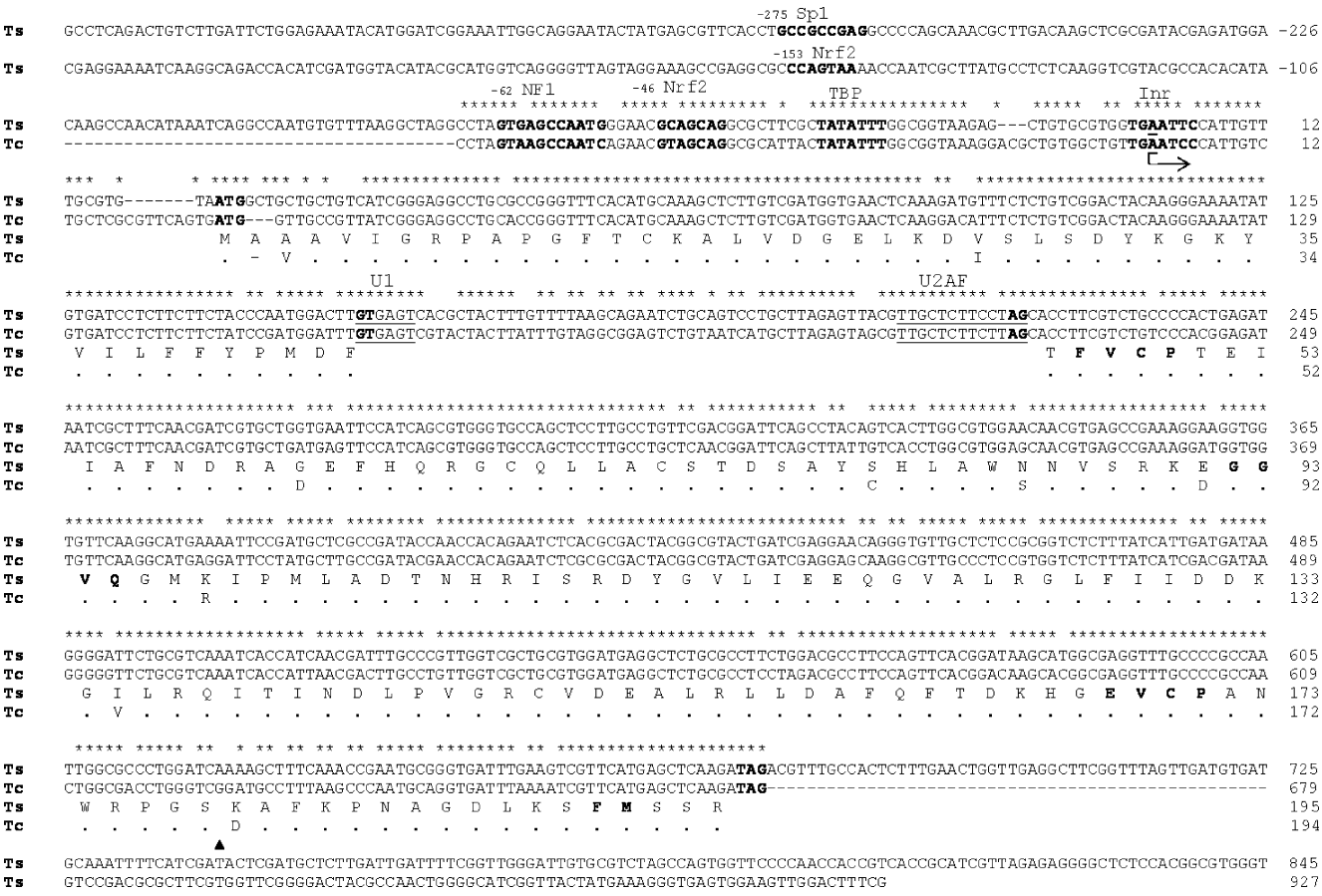

Fig. 1 Gene structure of *T. solium* (*Ts*) and *T. crassiceps* (*Tc*) 2-Cys Prxs. The transcription start point is marked with an *arrow*. Initiator (Inr) sequence, TATA box, start (ATG) and stop (TAG) codons, donor and acceptor intron sequences, and protein regulatory and catalytic motifs are in *bold*. Nucleotide and amino acid identity is denoted with *asterisks and dots*, respectively. The *Ts2-CysPrx* polyadenylation site is denoted with a *triangle*. Putative splicing factor sites for U1 and U2AF are *underlined*. Putative transcription factor binding sites are written above their corresponding motifs

sequences analyzed (Fig. 2). In order to know if the TSS located in the Inr sequence of the Prxs genes studied is conserved in *T. solium pAT6*, we conducted more 5′ RACE experiments, which mapped actin gene TSS also inside the Inr sequence (Fig. 2). This result demonstrates sequence conservation between different taeniids gene proximal promoters. The *T. solium* splice leader sequence reported for a group of parasite genes was absent in all transcripts analyzed (Brehm et al. 2002).

Viability of *T. crassiceps* and *Tc2-CysPrx* expression under oxidant conditions

In order to know if *T. crassiceps 2-Cys Prx* gene is induced under oxidant conditions, expression experiments were conducted using *T. crassiceps* cysticerci in controlled conditions. First, we established a concentration curve to determine viability in cysticerci incubated in medium with H_2O_2 for 1 h. Viability of the parasites was assumed as the

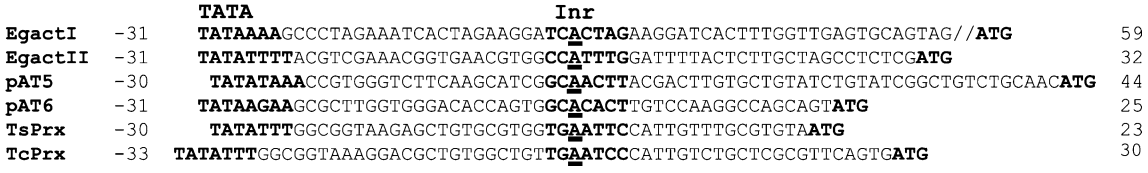

Fig. 2 Multiple alignment of Cestoda promoter nucleotide sequences. Non-coding 5′ upstream sequences of *E. granulosus* actin I and actin II (*EgactI*, *EgactII*) (da Silva et al. 1993), *T. solium* actins *pAT5* and *pAT6* (M28996, M28997), *Ts2-CysPrx* (*TsPrx*, FJ621569), and *Tc2-CysPrx* (*TcPrx*, FJ621570) were manually aligned. TATA box and Inr sequence, and ATG start codon are in *bold*. The TSS is *underlined*. The symbol // denotes the lacking nucleotides 5′-AGAAGA-CAAATCCTTTGGTGAGCC-3′

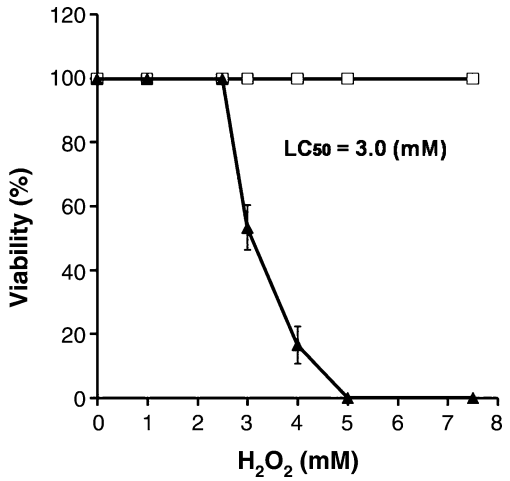

Fig. 3 Determination of the lethal concentration (LC_{50}) of *T. crassiceps* to H_2O_2. Cysticerci were exposed to different H_2O_2 concentrations for 1 h (*triangles*) prior viability determination (see "Materials and methods"). Parasites incubated in medium without H_2O_2 were used as a control (*open boxes*). Data are mean ± SD (n=8)

capacity of cysticerci scolex evagination, to follow its life cycle to adult worm. We found that parasite viability is reduced when H_2O_2 concentration is increased. Parasite viability remains unaffected up to 2 mM of H_2O_2, and after this concentration, it begins to decrease until it reaches zero at 5 mM. This viability kinetics showed that lethal concentration 50 (LC_{50}) of H_2O_2 is 3.0 mM (Fig. 3). These data were used to determine conditions for *Tc2-CysPrx* messenger RNA (mRNA) and protein expression assays. We evaluated the expression profile of the gene in parasites incubated in medium without oxidative insult. These experiments showed that *Tc2-CysPrx* mRNA expression levels remained unchanged in cysticerci incubated in medium for up to 24 h (Fig. 4a). Therefore, we used 4 h of preincubation prior to the incubation of parasites with H_2O_2. As seen in Fig. 4b, *Tc2-CysPrx* mRNA expression level did not change in parasites incubated for 30 min with H_2O_2 concentrations ranging from 0 to 2 mM. Also gene mRNA expression level was not changed in parasites incubated with 1 mM of H_2O_2 for 0.5, 1, 2, 3, and 24 h (Fig. 4c). In these experiments, the expression level of *Tc2-CysPrx* mRNA was constant and the intensity of the bands was similar throughout time and concentrations of H_2O_2 used. On the other hand, *Tc2-CysPrx* protein expression level remained the same in parasites incubated with 0, 1, and 2 mM of H_2O_2 for 30 min (Fig. 4d).

Discussion

We have cloned one gene of a typical 2-Cys Prx in *T. solium* and *T. crassiceps*. Their genomic architecture and high identity at the level of primary and nucleotide sequence suggest that both genes are homologous. Computational analyses showed two putative sites for Nrf2 in their promoter sequence, a factor involved in the regulation of antioxidant genes (Lee and Johnson 2004). Besides we found putative sites for the transcription factors NF1 and Sp1, it is known that these transcription factors can interact with members of the basal transcription factor machinery, such as TBP and TFIIB (Xiao et al. 1994; Kim and Roeder 1994; Emili et al. 1994). However, functional studies should be done to corroborate these findings.

The proximal promoters of both genes have a TATA box, an Inr sequence, and a single TSS that corresponds to an A

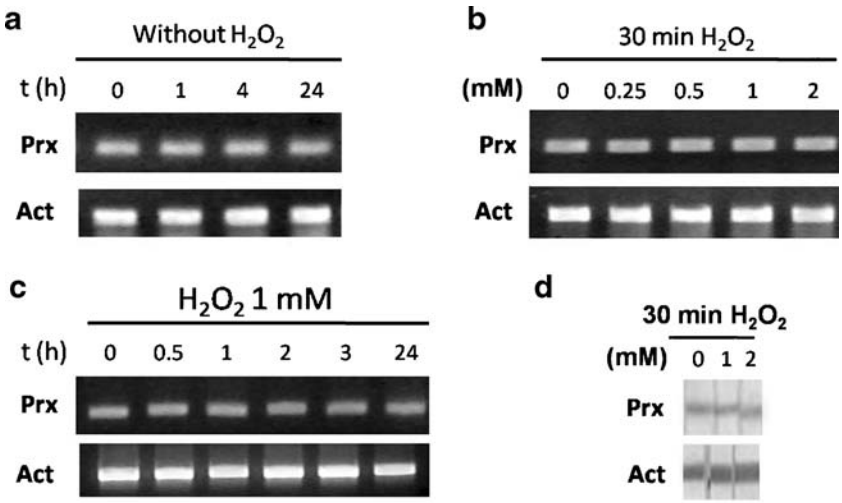

Fig. 4 Gene expression profile of *Tc2-CysPrx* from cysticerci exposed to H_2O_2 determined by RT-PCR and WB. Determination of expression of *Tc2-CysPrx* mRNA in cysticerci: **a** incubated in RPMI medium without H_2O_2 at 0 to 24 h; **b** exposed to different H_2O_2 concentrations for 30 min and **c** exposed to H_2O_2 1 mM for different times. **d** Determination of Tc2-CysPrx protein expression in parasites incubated 30 min in RPMI with H_2O_2 1 and 2 mM. Expression experiments were done by triplicate and β-actin was used as a control. A representative gel and blot of each experiment is shown

located within the Inr sequence. This result is consistent with the data reported for a subset of mammalian genes where TSS is located in the Inr consensus sequence (YYANWYY) comprising −2 to +5 and which has a TATA box located at −28 to −34 from the TSS (Smale and Kadonaga 2003; Sandelin et al. 2007). Additional sequence analysis of other Cestoda genes showed that *pAT5*, *pAT6*, *EgactI*, and *EgactII* proximal promoters present strong similarities to the ones reported in this work, since all have a TATA and an Inr sequence with a conserved A placed in the mapped TSS on *Ts2-CysPrx* and *Tc2-CysPrx*. Therefore, we mapped the TSS of *pAT6* and found it matches to the TSS of *Ts2-CysPrx* and *Tc2-CysPrx*, which suggests that the Inr present in the genes analyzed could be functional and the TSS of *pAT5*, *EgactI*, and *EgactII* is conserved. The structural region of *Ts2-CysPrx* and *Tc2-CysPrx* has two exons split by one intron with known splicing sequences (Padgett et al. 1986; Schellenberg et al. 2008). These analyses showed that proximal promoter sequences, such as Inr and TATA boxes, characterized in mammalian genes are also present in Cestoda genes and that splicing signals in *Taenia* genus are not different from other eukaryotic organisms.

Besides, we showed in vitro that *T. crassiceps* cysticerci have a LC_{50} to H_2O_2 of 3 mM. This extreme concentration of H_2O_2 is never reached in the host. Therefore, the lack of catalase and probably the presence of a low GPx activity toward H_2O_2 in these parasites suggests that resistance to high H_2O_2 concentration in the medium could be conferred mainly by the typical 2-Cys Prxs. In this context, reports on *S. mansoni* and *S. japonicum* show that there are three typical 2-Cys Prxs isoforms in schistosomatids: a cytosolic overoxidation insensitive Prx1, which is overexpressed under oxidant conditions and which could participate in responsive antioxidant defense against exogenous H_2O_2; and a cytosolic Prx2 and a peptide-targeted mitochondrial Prx3, which are both housekeeping genes. The latter two are prone to overoxidation by the presence of a C-terminal overoxidation FM motif, which is similar to the YF motif of the *Taenia* genes studied (Sayed and Williams 2004; Molina-López et al. 2006). The absence of a mitochondrial signal peptide and the presence of the C-terminal YF motif suggest that *Ts2-CysPrx* and *Tc2-CysPrx* are cytosolic overoxidation susceptible Prxs, such as schistosomal Prx2. We found that at the RNA and protein levels *Tc2-CysPrx* is not overexpressed under oxidant conditions. This expression pattern has been observed in other typical 2-Cys Prxs, such as human Prx2 (Diet et al. 2007), the nematode *Haemonchus contortus* 2-Cys Prx (Bagnall and Kotze 2004), the *S. mansoni* Prx2 (Sayed et al. 2006) and *Plasmodium falciparum* PfTPX-1 (Yano et al. 2005). Lack of induction of *Tc2-CysPrx* suggests that this protein could be an antioxidant housekeeping gene for endogenous H_2O_2 that possibly participates as a redox regulator, rather than a responsive gene against exogenous oxidative stress. This is in accordance with our previous observations made in *Ts2-CysPrx*, where gene expression persists through all the life cycle of *T. solium*, even in the adult stage which is not subject to oxidative stress (Molina-López et al. 2006). It is likely that other non-enzymatic antioxidant systems could act to protect the parasite, as seen in *S. mansoni*, where oxidant conditions induce albumin silent gene expression as a sacrificial protein prone to oxidation (Sayed et al. 2006).

The shared proximal promoter architecture found in the Cestoda genes presented here suggests that the transcription machinery in these parasites is similar to their mammalian counterpart, and that Cestoda genes possess TATA and Inr sequences that serve for TSS positioning. This is the first report which describes a proximal promoter sequence of a gene in cestodes. Our findings provide new insights for further investigations of genes in taeniids of medical interest, which could contribute for their eradication.

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