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***Biomphalaria glabrata* peroxiredoxin: effect of *Schistosoma mansoni* infection on differential gene regulation**

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Abstract

To identify gene(s) that may be associated with resistance/susceptibility in the intermediate snail host *Biomphalaria glabrata* to *Schistosoma mansoni* infection, a snail albumen gland cDNA library was differentially screened and a partial cDNA encoding an antioxidant enzyme thioredoxin peroxidase (*Tpx*), or peroxiredoxin (*Prx*), was identified. The 753 bp full-length, single-copy, constitutively expressed gene now referred to as *BgPrx4* was later isolated. *BgPrx4* is a 2-Cys peroxiredoxin containing the conserved peroxidatic cysteine (C_P) in the N-terminus and the resolving cysteine (C_R) in the C-terminus. Sequence analysis of *BgPrx4* from both resistant and susceptible snails revealed the presence of several (at least 7) Single Nucleotide Polymorphisms (SNPs). Phylogenetic analysis indicated *BgPrx4* to resemble a homolog of human peroxiredoxin, PRDX4. Northern analysis of hepatopancreas RNA from both resistant and susceptible snails showed that upon parasite exposure there were qualitative changes in gene expression. Quantitative real-time RT-PCR analysis showed differences in the levels of *BgPrx4* transcript induction following infection, with the transcript up-regulated in resistant snails during the early phase (5 h) of infection compared to susceptible snails in which it was down-regulated within the early time period. While there was an increase in transcription in susceptible snails later (48 h) post-infection, this never reached the levels detected in resistant snails. A similar trend - higher, earlier up-regulation in the resistant snails but lower, slower protein expression in susceptible snails - was observed by Western blot analysis. Enzymatic analysis of the purified, recombinant *BgPrx4* revealed the snail sequence to function as *Prx* but with an unusual ability to use both thioredoxin and glutathione as substrates.

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Note: Nucleotide sequence data reported in this paper are available in GenBank under the accession numbers: *BgPrx4*-BS-90: FJ176938; *BgPrx4*-LAC2: FJ176940; *BgPrx4*-M-line: FJ176941; *BgPrx4*-NMRI: FJ176942; *BgPrx4*-δNMRI: FJ176939

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Keywords

Snail; Parasite; Resistant; Susceptible; Peroxiredoxin; *BgPrx4*

1. Introduction

Schistosomiasis remains a disease of major global public health concern and is rated second only to malaria in posing chronic and debilitating illness to populations that endure poor sanitation, inadequate health infrastructure and poverty. Recognized as one of the major neglected tropical diseases, schistosomiasis was recently referred to as a ‘silent pandemic’ [1]. Humans are susceptible to infection by a larval stage shed from intermediate host snails. Until an effective vaccine against the causative parasites (human blood flukes, species within the genus *Schistosoma*) becomes available, there remains a need for alternative control methods. As a means to reduce the transmission of these parasites, efforts must be sustained to more fully understand the molecular basis of the snail-schistosome relationship. Such knowledge may lead to better tools to detect these parasites or to block transmission at the vector (i.e. snail host) stage of parasite development [2].

The outcome of each snail-parasite encounter is governed by the genetics of both organisms. Exposing snail stocks genetically selected for various susceptibility/resistance phenotypes to different strains of parasites showed that the genetics of susceptibility in the snail *Biomphalaria glabrata* to *Schistosoma mansoni* is complex and variable [3-4]. Snails are categorized as resistant if, upon exposure, as either adults or juveniles, they show an active defense reaction (mediated by hemocytes possibly aided by soluble factors in the hemolymph) against the invading miracidia. Without a similar defense response in susceptible snails, miracidia develop through several larval stages, eventually yielding cercariae that are infective to humans.

To determine the molecular basis of these opposing phenotypes, snails displaying either resistance or susceptibility to infection are being utilized to search for genes that may be involved in parasite destruction. Characterization of anti-parasite genes are important if genetically modified (GM) snails carrying such genes are to be realistically considered as part of an integrated control effort aimed at the cessation of transmission of schistosomiasis.

Using various gene-discovery methods, several genes that may be involved in resistance/susceptibility of the snail host in its relationship to trematodes such as *S. mansoni* and *Echinostoma caproni* have been described [5-11]. In addition, a rational, targeted approach to identify genes with deterministic roles in the snail/schistosome interaction has also proven fruitful [12]. An ongoing genome project aimed at deciphering the 931 megabase (Mb) sequence of *B. glabrata* will facilitate further progress in both gene discovery and targeted approaches [13].

Through studies of the innate defense mechanism(s) involved in the snail's ability to destroy incoming parasites, it has been found that the hemocytes of *B. glabrata* exploit reactive oxygen (ROS) and nitrogen species (RNS) [14-16]. As part of an effort to understand the roles of ROS and RNS in this system, we [17] previously identified cDNA transcripts (Accession number AAK26236) encoding peroxiredoxin, one of several enzymes responsible for maintaining redox balance. Several clones encoding this enzyme were found by differential antibody screening of an albumen gland cDNA expression library. The antibodies were raised against soluble albumen gland proteins prepared from a non-susceptible snail stock (LAC 2). This stock was derived through several generations (by self-fertilization) from a generally susceptible stock (NMRI).

Peroxiredoxins (Prxs), formerly known as thioredoxin peroxidases, are a family of conserved antioxidant enzymes that play protective roles by neutralizing reactive oxygen and nitrogen species that can damage cellular function. If not neutralized, reactive oxygen and nitrogen species cause protein oxidation, lipid peroxidation, DNA base modification and DNA strand breaks [18]. The enzymes catalyze the reaction *thioredoxin* + H_2O_2 = *thioredoxin disulfide* + H_2O . Prxs are also involved in cell signaling, protein phosphorylation, transcriptional regulation, and apoptosis [19-20].

Three classes of Prx have been characterized: 1-Cys, typical 2-Cys, and atypical 2-Cys. All three classes catalyze a two-step reaction that is centered on a redox-active cysteine called the peroxidatic cysteine. The first step - the attack of the peroxide substrate by the peroxidatic cysteine and its oxidation to S-hydroxycysteine - is common to all three peroxiredoxins, whereas the second step - the regeneration of cysteine from S-hydroxycysteine - is different in the three classes.

The functions and mechanisms of action of Prxs in protecting against sulfur-containing radicals (but not against oxidation systems lacking thiols) have been recognized [21], but remain poorly understood. However, the involvement of other antioxidant enzymes (*e.g.* catalase, superoxide dismutase, and glutathione peroxidase) in enabling organisms to adapt and survive otherwise lethal oxidative environments has been well documented.

Schistosome parasites lack catalase and utilize a glutathione-thioredoxin peroxidase to reduce hydrogen peroxide that is known to be lethal for the parasite [14,22]. Prxs have now been described in a variety of parasitic worms in which they are believed to play a role in the removal of toxic radicals, thus helping to circumvent the host immune response [23].

The pathways that generate superoxide and NO and that catalyze their further conversion to more or less damaging species are highly varied and complex. In particular, potential roles for Prx in snails remain speculative. Possibilities include contributing to the hemocyte's antagonistic attack on parasites, and protection of the host from peroxides of either host or parasite origin.

Here, we report a full-length cDNA encoding a *B. glabrata* 2-Cys peroxiredoxin gene (*BgPrx4*), its molecular characterization, and the temporal regulation of its transcript upon exposure of resistant and susceptible snails to parasites. Using Western blot analysis, we compared the protein expression profiles of the 30 kDa *BgPrx4* protein in tissues of the resistant and susceptible snails in response to parasite infection. Finally, we report functional enzymatic analysis of the recombinant (rBgPrx4) enzyme, including its capacity to utilize either thioredoxin or glutathione as substrate.

2. Materials and Methods

2.1. Snails

The non-susceptible LAC2 snail stock was developed from the susceptible NMRI stock of *B. glabrata* by self-fertilization for several generations (F_0 - F_{12}) as described previously [24]. At each successive generation, progeny snails displayed progressively less (25%) susceptibility to parasite infection. This was determined by taking a sample of each progressive generation of LAC2 and following their infection rates (cercarial shedding) post infection by *S. mansoni* miracidia. In our laboratory, “non-susceptibility” is defined as the inability of the snail to sustain schistosome infection and on histological examination parasites are seen surrounded by a minimal host reaction consisting primarily of amoebocytes. Lack of parasite development may be due to a weak form of resistance or biochemical unsuitability [24-25]. In contrast, in snails that are classified as “resistant”, the larval stage is engulfed by hemocytes within 3-4

days of parasite infection in a very strong immune mediated reaction. In our laboratory, the *B. glabrata* BS-90 [26] snails display invariant 100% resistance to *S. mansoni* infection as both juveniles and adults. The susceptible *B. glabrata* NMRI snails exhibit both juvenile and adult susceptibility when exposed to the *S. mansoni* NMRI strain. Likewise, the M-line snails are also highly susceptible to *S. mansoni* infection [27]. Within this study, snails were either juveniles (up to 4 mm in diameter) or adults (6-10 mm in diameter), and were maintained in aged, dechlorinated tap water at room temperature and fed on Romaine lettuce. 13-16-R1 snails were maintained at Oregon State University (OSU) and are predominantly resistant to *S. mansoni* [28].

2.2. Copy number analysis

Copy number of the *BgPrx4* gene was determined by slot blot analysis by comparing the hybridization signal of *BgPrx4* to that of the single copy *B. glabrata ferritin* gene using the procedure previously described [29]. Briefly, 10-fold serial dilutions of DNA from 10 µg to 1 ng in a total volume of 150 µl TE buffer, pH 8.0 were pipetted into microcentrifuge tubes containing 12 µl of 5M NaOH and 3 µl 0.5M EDTA. Samples were boiled (10 min), snap-chilled on ice, and neutralized with an equal volume of 2M Ammonium Acetate prior to application. Duplicate strips of the membrane were probed with either ³²P-labeled *ferritin* or ³²P-labeled *BgPrx* under standard hybridization conditions. Membranes were washed at moderate stringency (1× SSC, 0.5% SDS at 60°C) before being subjected to autoradiography on X-ray film with intensifying screens at -70°C for 7 days. Densitometric analyses of the autoradiographs were performed using the public domain NIH Image program (available at <http://rsb.info.nih.gov/nih-image>).

2.3. DNA and RNA isolation

DNA was isolated from individual snails as previously described [5]. For RNA isolation, juvenile snails (15 snails for each stock) of *B. glabrata* (resistant BS-90, non-susceptible LAC 2, and susceptible NMRI and M-line) were treated overnight with autoclaved water at room temperature containing 100 µg/ml ampicillin to minimize bacterial contamination. To assess possible responses to infection, individuals were exposed to five *S. mansoni* miracidia. Exposures were for 5 different time periods (0, 5, 10, 24, 48 h). At the end of each time period, total RNA was extracted immediately from individual snails (whole body) using RNA-Bee according to the manufacturer's protocol (Tel-test, Friendswood, TX). RNA was also isolated from snail tissue (albumen gland, hepatopancreas, cerebral ganglia, hemocytes and ovotestis) as previously described [8,30]. For performing Random Amplification of cDNA Ends (RACE) reactions, RNA was prepared from snail head-foot using an RNeasy® kit from Qiagen (Valencia, CA) as described [28].

2.4. Northern and Southern blot analysis

Northern blots (transfer, hybridization and washes) were performed using total RNA (5µg/lane) with the MOPS/formaldehyde method as described [30]. Southern blots (transfer, hybridization and washes) were done on digested DNA or PCR samples according to a previously described method [31]. DNA probes were either the intact recombinant plasmids [partial cDNA (pBGC2) or full-length cDNA (*BgPrx4*)] or PCR amplified products synthesized from the aforementioned recombinant plasmids using *B. glabrata Prx4* gene specific primers F: 5-ATGGCATCCTCTCTGCAAACCGGG-3' and R: 5'-TTAGAGTTCATCGTTAGATTGC-3'. For Southern and Northern hybridizations, radiolabeled probes were prepared using the random primed DNA labeling kit to incorporate the ³²P - dCTP according to the manufacturer's instructions (Roche, Indianapolis, IN). Hybridized blots were washed twice at room temperature, for 30 min in 0.2× SSC, and with

the same stringency twice for 30 min at 60°C. Washed filters were subjected to autoradiography on Kodak-X-Omat XAR-2 film with intensifying screens at -70°C for between 1 and 4 weeks.

2.5. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA isolated from albumen glands, hepatopancreases, hemocytes, ovotestes and cerebral ganglia of unexposed snails was treated with RNase-free DNase (RQI, Promega, WI). First strand cDNA was synthesized using the treated RNA in the presence of an oligo (dT) primer and MuLV reverse transcriptase at 42°C according to the manufacturer's instructions (Roche Molecular Systems, Branchburg, NJ). Control reactions (with no reverse transcriptase) were performed to preclude any DNA contamination. Second strand reactions were performed using *BgPrx4* gene-specific primers mentioned in section 2.4. The PCR products were analyzed on 1% TBE-agarose gels and the bands were visualized using ethidium bromide. As a reference, the constitutively expressed *B. glabrata* myoglobin gene [32] was amplified in parallel using the same conditions as described above [7].

2.6. Real time RT- PCR analysis of the *B. glabrata* *BgPrx4* transcript

Real time PCR analysis was performed with *BgPrx4* gene-specific primers as described previously using whole, juvenile snail RNA. RT-PCR of the constitutively expressed *myoglobin* gene (*B. glabrata* specific *myoglobin* primers F: 5'-GATGTTTCGCCAATGTTCCCC-3'; R: 5'-AGCGATCAAGTTTCCCCAG-3') was used to assess the comparability of samples and confirm that template cDNA was used in equivalent amounts for each amplification reaction. The RT-PCR reactions were performed using an Applied Biosystems 7300 Real Time PCR System (Applied Biosystem, Foster City, CA). The reaction was performed in a one-step format with 80 ng of DNase treated-total RNA. DNase treatment was done with RNase-free DNase (RQI,) according to the manufacturer's (Promega, Madison, WI) suggested protocol. First strand cDNA reactions and PCR amplifications were performed in triplicate with the Full Velocity SYBR Green QRT-PCR Master mix according to the manufacturer's instructions (Stratagene, La Jolla, CA). A validation assay and melting curve using four different input RNA samples with gene-specific primers corresponding to *BgPrx4* (with an expected product size of 753 bp) and *myoglobin* (with an expected product size of 322 bp) was performed at the beginning of the study according the manufacturer's instructions (Applied Biosystems). The amplification showed efficiencies of these primers were equal and a single peak was obtained at the expected temperature indicating target-specific amplification (data not shown). Each 25 µL of the final amplification reaction volume contained 200 nM of *BgPrx4* forward and reverse primers. A parallel amplification reaction was performed using the same concentrations of starting RNA but with 50 nM of *myoglobin* primers. This reaction was performed for adjusting the differences in concentration of the reverse transcribed RNA starting material. All reactions also contained 300 nM of reference dye, 1× of Full Velocity SYBR Green QRT-PCR master mix containing RT-PCR buffer, SYBR green I dye, MgCl₂ and nucleotides (G, A, U, C). The amplification protocol included an initial incubation at 48°C, 45 min for cDNA synthesis and a 95°C initial denaturation for 10 min followed by 40 cycles with 95°C denaturation for 10 sec, and annealing/ amplification at 58°C for 1 min. Fold increases of gene expression were calculated by comparative Ct method with the formula indicated below [33]:

$$\text{Fold change} = 2^{-\Delta\Delta C_t} \\ = 2^{-\left[(C_{t_{\text{Prx, exposed}}} - C_{t_{\text{myoglobin, exposed}}}) - (C_{t_{\text{Prx, unexposed}}} - C_{t_{\text{myoglobin, unexposed}}}) \right]}$$

In order to determine the significance of differences ($P < 0.05$ or $P < 0.01$) in gene expression for the different time points the P -value was calculated by comparing delta delta Ct values

using the Student's *t*-test and one-way ANOVA between the exposed and non-exposed snails within each stock.

2.7. RACE reaction and isolation of clones encoding the full-length *BgPrx4* cDNA from the 13-16-R1 stock of *B. glabrata*

The original cDNA insert identified from the recombinant plasmid clone pBGC2 contained only the partial cDNA (666 bp) of the *B. glabrata* *Prx* (GenBank Acc. No. AY026258) ortholog [17]. To extend this sequence, the head-foot of an adult 13-16-R1 snail was placed in 200 μ L of TRIzol (Invitrogen, Carlsbad, CA) and immediately frozen at -80°C. RNA was extracted by disruption of the tissue, followed by sequential phenol: chloroform, chloroform:isoamyl alcohol extractions, and isopropanol precipitation. To ensure the removal of any contaminating DNA, extracts from head-foot were digested with DNase (Qiagen, Valencia, CA) and eluted using RNeasy® columns (Qiagen, Valencia, CA). Reverse transcription (1 h) was by MMLV reverse transcriptase (Promega, Madison, WI) in the presence of Prime RNase inhibitor (Eppendorf, Westbury, NY) and Oligo (dT)15 primer (Promega, Madison, WI). The cDNAs were heated at 95°C for 10 min to inactivate reverse transcriptase. After centrifugation, cDNA products were used as templates for PCR. The 5'-ready cDNA was prepared using Ambion's (Austin, TX) FirstChoice® RLM RACE Kit, and the *BgPrx4* sequence was amplified using a gene-specific primer and the primers supplied with the kit. The amplicon was sequenced by the Central Services Laboratory of Oregon State University's Center for Genome Research and Biocomputing.

2.8. Full-length sequences for additional snail stocks and sequence analysis

The *B. glabrata* peroxiredoxin sequence previously submitted to GenBank (Accession No. AY026258, previously named *BgTpx*) lacked 31 amino acids at the 5' end of the transcript. Consequently, using the existing sequence data and mRNA from the 13-16-R1 snail stock (see previous section), the 5' end of this gene was deduced by 5' RACE. Subsequently, gene-specific primers were designed to deduce full-length open reading frames (ORFs) for the BS-90 (resistant), LAC2 (non-susceptible) and the NMRI and M-line (susceptible) snail stocks. The snail *BgPrx4* sequences have been submitted to GenBank (Accession No. *BgPrx4*-BS-90: FJ176938; *BgPrx4*-LAC2: FJ176940; *BgPrx4*-M-line: FJ176941; *BgPrx4*-NMRI: FJ176942; *BgPrx4*- δ NMRI: FJ176939). DNA sequences were analyzed using EMBOSS [34]. The computer program ClustalW (version 2.0.5) was used to generate the multiple sequence alignment [35-36]. Comparisons of the sequences were made with sequences in the protein and nucleic acid public databases using the BLAST algorithm [37]. The deduced amino acid sequence of *BgPrx4* was further analyzed using the NCBI conserved domain database [38] to classify and characterize the type of protein domains encoded within it. These data were also used to query the Interpro EMBL-EBI [39], a database of protein families, domains and functional sites in which identifiable features found in known proteins can be applied to unknown protein sequences, and the Sanger MEROPS release 7.80 [40] database. Signal sequence prediction was performed using the Signal P program version 3.0 [41].

To ascertain possible orthology between potential molluscan *Prx* genes and the more fully characterized mammalian genes, we identified 14 *B. glabrata* EST sequences from GenBank with annotation that included 'thioredoxin peroxidase' or 'peroxiredoxin'. Using Sequencher (Gene Codes Corporation, Ann Arbor, MI), these were assembled, yielding 4 non-redundant contigs (**BxPrx-contig A**: AY026258, EE722991, EE723209, EE723214; **BgPrx-contig B**: FC858724, DW474756; **BgPrx-contig C**: EX003613, FC857164, FC858504, EX003036, EW996752, ES488429, ES482777; **BgPrx-contig D**: EW997425) each encoding the peroxiredoxin active site motifs. The assignment of names for members of gene families in non-model species is non-trivial, in part because of erroneous annotation of many sequences in public databases, in part because of gene duplications and/or loss over evolutionary time,

and in part because changes are, in some cases, so extensive that ancestry is no longer obvious. Taking a prudent approach, we decided to use the human database, as this is a high quality, thoroughly curated and universally available benchmark for the assignment of names, and because conservation of peroxiredoxin sequences is sufficient for meaningful comparisons. The phylogenetic analyses of various Prxs were performed using MEGA4 program that uses a Maximum Composite Likelihood (MCL) method formerly known as Simultaneous Expression (SE) method based on the maximum likelihood principle [42]. It has been shown that the use of this method substantially improves the accuracy of NJ trees.

2.9. Expression of the snail BgPrx4 in *E. coli*: purification and preparation of antibody against the recombinant enzyme

Insert DNA (666 bp) from the recombinant plasmid clone pBGC2 containing the partial cDNA of the *B. glabrata* BgPrx4 ortholog [17] was sub-cloned into the prokaryotic expression vector pRSETB (Invitrogen, Carlsbad, CA) and expressed as a His-tagged fusion protein using the method of Ghosh *et al.* [43]. Briefly, the Open Reading Frame (ORF) was amplified by PCR using a 5' primer 5'-CGCGGATCCTTCGGCAGCAGC-3' containing a recognition site for BamHI, and a 3' primer 5'-CGGAATTCTCAGAAGTTCATCGTTAG-3' containing a recognition site for EcoRI and a stop codon. A three step-cycle PCR program of 94° C for 1 min, 65° C for 45 s and 72° C for 1.5 min was employed for 30 cycles. The gel-purified PCR products were digested with BamHI and EcoRI and ligated into the expression vector pRSETB (Invitrogen, Carlsbad, CA) in frame. Recombinant plasmids were used to transform *E. coli* (DH10B). In order to produce recombinant protein from the BgPrx4-pRSETB constructs, purified recombinant DNA from the transformants propagated in *E. coli* DH10B were checked for the correct size and orientation (in-frame) and verified by PCR before transformation into *E. coli* B strain BL21 (DE3)pLYsS (Invitrogen, Carlsbad, CA). Protein expression in the transformants containing the BgPrx4 insert was induced with 0.4 mM IPTG for 2 hr at 37° C according to manufacturer's instructions (Invitrogen, Carlsbad, CA). The expressed BgPrx4-His-tagged fusion protein was purified using a nickel-NT agarose column. For elution, buffer contained varying concentrations of imidazole (60 mM-500 mM) according to the manufacturer's suggested protocol (Novagen/EMD Biosciences, Madison, WI). The recombinant protein, eluted with 300 mM imidazole, was dialyzed against Tris-Buffered Saline (TBS, pH 8.0), and quantitated by the bicinchoninic protein assay (Pierce, Rockford, IL). Antibodies were produced in six Swiss albino mice (Charles River Laboratories Inc., Wilmington, MA) using purified recombinant BgPrx4 protein. The Biomedical Research Institute's (BRI) animal care program complies with the guidelines adopted by the Office of Laboratory Animal Welfare (OLAW) and maintains an OLAW Assurance. No experimental animals were used unnecessarily and all were well -treated to avoid distress. All animal procedures were conducted by protocols approved by the BRI's animal care and use committee (IACUC). Approximately 50 µl of blood was obtained from each mouse (pre-bleed) by collecting blood from the tail vein by a needle-prick procedure. For primary immunizations, 10 µg of purified BgPrx4 protein was mixed with 40 mg/ml alum (1:1) in a total volume of 300 µl and injected subcutaneously in the right flank. At three-week intervals, two subsequent boosts (300 µl volume) were administered subcutaneously in separate sites, with 5 µg of the purified protein/alum mixture (as above). Following collection of blood as above, antibody titers were determined by Western blotting throughout the course of immunization, prior to each booster immunization, and at 3 weeks after the last boost. Mice were then euthanized by an IACUC-approved CO₂ inhalation procedure, and blood was collected by cardiac puncture.

2.10. Western Blotting

Two individual snails from adult resistant (BS-90) and susceptible (NMRI) stocks were either exposed (12-15 miracidia/ snail) for 5 h and 24 h, or left unexposed (0 h). At the end of each exposure, the hepatopancreas and albumen gland tissues were dissected from the snails, snap

frozen in liquid nitrogen and kept frozen at -70°C . For soluble protein extractions, fresh and/or frozen adult snail tissue samples were homogenized in sterile PBS, pH 7.5 on ice using a mechanized Kontes pestle (VWR, West Chester, PA). Soluble protein was separated from the membrane-bound insoluble material by centrifugation at $10,000 \times g$ for 20 min at 4°C . Extracts were either analyzed immediately or aliquoted and stored at -70°C until required. Long-term storage (up to 6 months) at -70°C had no effect on enzyme activity. The protein concentration in the supernatant was determined by the BCA method [44]. Soluble extracts (50 μg) were resolved under reducing conditions by 15% Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE). Western Blot [45] was performed by transferring resolved protein onto a nitrocellulose membrane using a semi-dry blotter in a buffer containing 0.192 M glycine, 0.025M Tris pH 8.3, 0.0013 M SDS and 10-20% methanol. After transfer for 1 h at 10-15 V/200-300 mA, proteins transferred to the membrane were visualized using 0.4% Ponceau S stain in 2% trichloroacetic acid prior to blocking and antibody addition. The membrane was then blocked using blocking buffer (TBS, 0.1% Tween-20, 0.05% Triton X-100 and 3% Carnation milk powder) for 2 h at room temperature, followed by incubation with mouse anti-*BgPrx4* antibodies (1:500 dilution) in blocking buffer overnight at 4°C as described previously [46]. The second antibody was a 1:2000 dilution of goat anti-mouse IgG Fc conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in blocking buffer. Following three washes with the wash buffer (TBS, 0.1% Tween-20, 0.05% Triton X-100) the antigen-antibody complexes were visualized using the BCIP/NBT phosphatase substrate system according to the manufacturer's instructions (KPL Protein Research Products, Gaithersburg, MD).

2.11. Functional Analysis of *Biomphalaria glabrata* Peroxiredoxin 4

The activity of the recombinant *B. glabrata* peroxiredoxin was determined with the following substrates: H_2O_2 , cumene hydroperoxide, tertiary butyl hydroperoxide, recombinant *Escherichia coli* thioredoxin and glutathione by monitoring the oxidation of NADPH in reactions coupled to recombinant *E. coli* thioredoxin reductase (22) or yeast glutathione reductase (Sigma-Aldrich, St. Louis, MO). All assay reactions were performed in 1.0 ml 0.1 M potassium phosphate (pH 7.4), 10 mM EDTA using 100 μM NADPH and 0.15 μM thioredoxin reductase or 0.3 unit/ml yeast glutathione reductase. Kinetic parameters were determined using Lineweaver-Burk plots using KaleidaGraph 4 (Synergy Software, <http://www.synergy.com>) least squares best fit of data and varying concentrations of one substrate while maintaining the concentration of the other substrate. In thioredoxin-coupled assays, thioredoxin concentrations ranged from 4 to 120 μM and in the glutathione-coupled assays glutathione concentrations ranged from 0.05 to 1 mM at a fixed concentration of 0.5 mM H_2O_2 . To determine kinetic parameters for hydroperoxide substrates, H_2O_2 , cumene hydroperoxide and tertiary butyl hydroperoxide concentrations ranged from 0.01 to 1 mM, with fixed concentrations of either thioredoxin (16 μM) or glutathione (0.7 mM). All assays were done in triplicate and each determination was repeated three times.

3. Results

3.1. Copy number analysis of *BgPrx4*

The copy number of the gene encoding *BgPrx4* was deduced by slot blot analysis. We compared the signal intensities of a known single copy gene, *ferritin* [47], with that of the *BgPrx4* gene, both hybridized to the same blot sequentially. Ten-fold serial dilutions of denatured and neutralized *B. glabrata* BS-90 genomic DNA from 10 μg -1 ng were spotted and probed with either the labeled *ferritin* gene (Fig. 1A) or *BgPrx4* as probes (Fig. 1B) respectively as described previously [29]. Probes for both *ferritin* and *BgPrx4* detected signals with 10 μg and 100 ng of *B. glabrata* DNA and not any of the lower dilutions. Since *ferritin* is present as a single copy

in the snail genome, signals of the same intensities indicated that *BgPrx4* also probably exists as a single copy gene.

3.2. *BgPrx4* is transcribed in all snail stocks examined

Northern blot analysis of *BgPrx* was performed using total RNA from the snail hepatopancreas since this is the most abundant tissue sample that can be easily isolated from a snail. Total RNA from hepatopancreas was isolated from unexposed (Figs. 2A and B, lanes 2 and 5) and *S. mansoni* exposed 5 h (Figs. 2A and B, lanes 3 and 6) and 24 h (Figs. 2A and B, lanes 4 and 7) resistant BS-90 and susceptible NMRI snail stocks. This was done in order to determine if transcripts differed in size between the different strains and if exposure to parasites modulates the transcription of the snail-host *Prx* gene. The ethidium bromide stained gel prior to blotting is shown in Fig. 2A which shows the predominantly staining snail ribosomal RNA (21S) band. Upon hybridization with the radiolabeled *BgPrx4* PCR product, transcript sizes of ~1.8 Kb, ~1.2 Kb and 0.7 Kb were detected in both normal BS-90 (Fig. 2B, lane 2) and NMRI (Fig. 2B, lane 5) unexposed snails. The ~1.8 Kb, ~1.2 Kb size transcripts were also detected in these snails following exposure to parasites for 5 h or 24 h. However, after infection, smaller transcripts of 0.6 Kb and 0.3 Kb (indicated by dark arrows) not seen in the unexposed snails appeared in both BS-90 (Fig. 2B, lanes 3 and 4) and NMRI snails (Fig. 2B, lanes 6 and 7). Mouse liver RNA was loaded in lane 8 as marker (note positions of 28S and 18S rRNA in Fig. 2A) to which there appears to be a signal at ~4 Kb that is unrelated to *Prx* since the expected size of the mouse *Prx* transcript ranges from approximately 1-2 Kb.

3.3. Tissue specificity of expression of the *BgPrx4* transcript

To determine the tissue specificity of the *BgPrx4* in normal snails, we performed RT-PCR assays using gene-specific primers designed from the *BgPrx4* sequence. The ethidium bromide stained gel of the amplified 753 bp product using cDNA template from various tissues is shown in Fig. 3A. *BgPrx4* sequence was amplified from the albumen gland (Lane 2), hepatopancreas (lane 3), ovotestis (lane 5) and cerebral ganglia (lane 6). No signal was discernable in hemocytes (Lane 4) despite several attempts of repeating the assay. Failure to detect the *BgPrx4* transcript in hemocytes was not due to the lack of template, as evidenced from RT-PCR performed instead with gene specific primers corresponding to the constitutively expressed housekeeping gene *myoglobin* (data not shown). Amplicons representing *BgPrx* have been found in hemocytes from other laboratories (Bayne *et al.* personal communication). This anomaly may be due to differences in hemocyte tissue processing and RNA extraction methods in the different laboratories. Lane 7 shows the absence of product with the *BgPrx4* primers in the absence of cDNA template. Southern blot analyses of the above gel using ³²P-labeled *BgPrx4* probe showed products from all tissues examined (Fig. 3B, lanes 2, 3, 5 and 6) other than the hemocytes (Fig. 3B, lane 4).

3.4. Real-time RT-PCR reveals the temporal modulation of gene expression of *BgPrx4* upon *S. mansoni* infection of *B. glabrata* snails

To examine quantitative differences in the amounts of *BgPrx4* transcript between resistant (BS-90) non-susceptible (LAC2) and susceptible (NMRI and M-line) snails following exposure to *S. mansoni*, RNA was isolated from juvenile whole snails before and after exposure to miracidia and analyzed by real time RT-PCR. As shown in Fig. 4, a 13-fold increase in expression occurred in resistant (BS-90) snails early (5 h) post-exposure, followed by a decline at 10 h and 24 h and a 16-fold increase at 48 h in these snails. Similarly, LAC2 snails when infected showed a 5-fold and 8-fold change in induction at 5 h and 10 h, respectively. As seen in the BS-90 snails, expression levels in LAC2 snails also decreased at 24 h followed by 8.5-fold increase at 48 h. Thus, in both stocks that are capable of resisting infection, the levels of induction of the *BgPrx4* encoding transcript was significantly up-regulated during the early

(5-10 h) stages of infection, followed by a decline at 24 h and another peak of expression at 48 h. In contrast, in the same early stages of infection (5-10 h), induction of *BgPrx4* in both susceptible snails (NMRI and M-line) remained weak (≤ 2.3 fold Fig. 4); higher levels of induction were evident later (24-28 h) but never reached levels seen in the resistant snails.

3.5. Sequence and phylogenetic analyses of the full-length cDNA encoding *B. glabrata* peroxiredoxin

From the albumen gland library of the LAC2 snail as mentioned above, a partial cDNA insert (666 bp) with sequence similarity to thioredoxin peroxidase but with an ORF lacking the 5' end of 31 amino acids (*Tpx*- GenBank Accession No. AY026258) was identified [17]. Subsequently, 5' RACE reactions yielded the missing 5' end of the gene and this is denoted by a horizontal arrow below the multiple sequence alignment shown in Fig. 5A. Gene-specific primers designed based on the original, partial ORF and the 5' RACE product yielded the full-length ORF now referred to as *BgPrx4*. *BgPrx4* was cloned and sequenced from resistant (BS-90), non-susceptible (LAC2), and susceptible snails (NMRI and M-line). Nucleotide sequence analyses confirmed that all strains expressed a thiol-specific antioxidant enzyme, peroxiredoxin (*Prx*) (also known as thioredoxin peroxidase [*TPx*]). Constructs from the four snail stocks were completely sequenced in both directions multiple times ($\times 3$). While cDNAs from BS-90, M-line and LAC2 snails each contained a 753 bp sequence that encoded a protein of 250 amino acids (28 kDa), cDNAs derived from NMRI snails were of two forms, the same 753 bp sequence seen in both the resistant and susceptible stocks and a second shorter sequence of 516 bp encoding a smaller peptide of 171 amino acids (19 kDa). In the various constructs, we detected 7 single nucleotide polymorphisms (SNPs), with two resulting in an amino acid change (amino acid 119 was either a lysine or glutamic acid and amino acid 217 was either a valine or methionine). Multiple sequence alignment of the deduced amino acid sequence of the snail enzyme (*BgPrx4*) to that of the parasites *S. mansoni*, *S. japonicum* and the vertebrates human, mouse and rat showed the peroxiredoxins to be highly conserved among snails, humans and schistosomes [17]. The inferred amino acid sequences for the five full-length ORFs (see section 2.8 for GenBank accession numbers) from BS-90, LAC2, M-line and NMRI snail stocks including the second smaller sequence found in the NMRI snail, (δ NMRI) and the original partial *B. glabrata* *Tpx* are aligned (Fig. 5A), in which the numbering system represents the amino acid position of the first full length sequence in the alignment, *i.e.* the BS-90 stock of *B. glabrata*. The variant NMRI *BgPrx4* sequence (δ NMRI) possesses a large deletion (79 amino acids) resulting in a smaller peptide (171 amino acids) compared to the other *BgPrxs* sequences (250 amino acids), and lacks the peroxidatic cysteine (C_p) in the N-terminus. The longer sequences show this highly conserved peroxidatic cysteine (C_p) in the N-terminus in addition to the resolving cysteine (C_R) in the C-terminal arm thus indicating that these sequences are "2-Cys peroxiredoxins" [48]. Deprotonation of the catalytic cysteine thiol is facilitated by H-bonding of the threonine or serine hydroxyl group, and the positive charge of the arginine side chain that together form the invariant active site residues which are shown in Fig. 5A (bold uppercase "T" and "R" and highlighted "•"). Like the mosquito and *Drosophila* 2-cys peroxiredoxins, this *BgPrx* contains the conserved GG(V/I/L)G-(X)_n-YF (Fig. 5A, shaded in gray) sequence that is present in *Prxs* sensitive to inactivation by hyperoxidation [48]. All sequences also contain a signal peptide and the first 21 amino acids encompass the highly hydrophobic signal sequence that is present in secretory proteins. The arrow between amino acids 20 and 21 denotes the putative cleavage site of the signal peptide. An unrooted, phylogenetic analysis of this *BgPrx* sequence revealed a closer evolutionary relationship between the vertebrate orthologs and the snail in contrast to the parasite [17].

In metazoans *Prxs* are typically encoded by multiple genes *e.g.* chicken and *Drosophila* have 4 and 5 different genes, respectively. To ascertain if *B. glabrata* is consistent in this regard and to investigate possible orthologs between potential molluscan *Prx* genes and the more fully

characterized mammalian genes, we identified 14 *B. glabrata* EST sequences from GenBank with annotation that included 'thioredoxin peroxidase' or 'peroxiredoxin'. Using Sequencher (Gene Codes Corporation, Ann Arbor, MI), these were assembled, yielding 4 non-redundant contigs (A, B, C and D) each encoding the peroxiredoxin active site motifs (data not shown). For assignment of contig names for members of gene families in a non-model species such as the snail we decided to use the human database as the benchmark for the assignment of names (for details see section 2.8). Phylogenetic analyses of the various contigs by means of MEGA4 [42] show that the sequences we report in this study are orthologs of human PRDX4 (Figure 5B). Accordingly, the sequence reported in this paper and one of the 4 EST contigs identified from GenBank (data not shown) is named *BgPrx4*. Another of the *B. glabrata* contigs is orthologous (99% - 100% certainty after 1000 iterations of bootstrap analyses) to human PRDX6 (not shown), and the two remaining *B. glabrata* contigs, while they are clearly 2-Cys Prxs, are of uncertain orthologies. When the three unnamed *B. glabrata* genes represented by the contigs are fully sequenced, definitive names can be assigned on the basis of evolutionary relationships with mammalian genes.

3.6. *BgPrx4* protein expression varies between tissues and through time pre- and post exposure to *S. mansoni*

Since Western blot analyses using protein extracts prepared from whole snail bodies of either resistant or susceptible snails (with and without infection) failed to reveal differential protein expression of *BgPrx* (data not shown), we therefore chose, based on our earlier results from real-time RT-PCR studies, to look for any changes in the protein expression profiles using, instead, specific tissues (hepatopancreas and albumen glands). Based on several experiments to assess variations in this enzyme between individual resistant (BS-90) and susceptible (NMRI) snails before and after infection, a representative blot is shown in Figure 6 (A and B). Hepatopancreas and albumen gland tissues were dissected from all individual snails pre- (0 h) and post- exposure (5 h and 24 h) to miracidia. Recombinant *BgPrx4* protein was included in the analysis as positive control (lane 1, Figs. 6A and B). The molecular size of the recombinant protein was ~ 28 kDa (including the 6-His-tag) while the native protein from the tissues expressed a slightly higher molecular size product of ~ 29-30 kDa, probably due to post-translational modifications. In the hepatopancreases (Fig. 6A) from two individual unexposed resistant BS-90 snails there was a low level of expression (0 h) followed by up-regulation at both 5 h and 24 h post- exposure. In the same tissue (hepatopancreas) of individual, susceptible NMRI snails, we detected some individual variation in basal enzyme levels but unlike in the resistant snail, there was no increase during the early stage of infection (5h). However, an increase was evident in both individual susceptible snails analyzed later (24 h) during infection.

Western blot analyses of the albumen gland extracts (Fig 6B) indicated a different pattern of protein expression. In the BS-90 snails there appeared to be only a marginal increase in expression compared to levels detected in the hepatopancreas at both early (5h) and late (24 h) time point post-exposure. In the susceptible NMRI snails however there appeared to be a higher basal level of enzyme expression in the albumen gland compared to the constitutive level of expression found in the same gland of resistant snails. Interestingly, this higher basal level of protein expression in the susceptible snails was down -regulated with parasite exposure (5 h and 24 h).

3.7. Functional analysis of the *B. glabrata* recombinant peroxiredoxin

Steady state kinetic analysis of the recombinant *BgPrx* protein indicated that the enzyme has a wide substrate range and is active with H₂O₂ and with model lipid hydroperoxides (cumene hydroperoxide and tertiary butyl hydroperoxide) (Fig. 7 and Table 1). Somewhat unusually, *B. glabrata* Prx is active with both thioredoxin and glutathione and is indeed slightly more active with glutathione than with thioredoxin. The apparent *K_m*s for the biological substrates

thioredoxin and glutathione are in the range for the expected cellular concentrations of these two important thiol reductants of low micromolar and low millimolar, respectively (Table 1). The apparent catalytic rates (k_{cat}/K_m) for BgPrx ($0.27 - 5.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) are similar to if slightly lower than those seen for Prx from other organisms ($\sim 10^5 \text{ M}^{-1} \text{ s}^{-1}$) and, as expected, significantly lower than those of GPx ($\sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and catalase ($\sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$) [49].

4. Discussion

Peroxioredoxins comprise a family of ubiquitous peroxidases that play important roles as antioxidant enzymes and in cell signaling [49,50]. They have been described in a variety of organisms [19,22,49,51-53] in which they can protect cells from a variety of oxidative and nitrosative stress challenges. They are also involved in redox balance and signal transduction pathways that use hydrogen peroxide as a second messenger, and can affect phosphorylation, transcriptional regulation and apoptosis (48, 49, 54-56). They are often present in abundant quantities since they have relatively lower catalytic activities compared to catalases and glutathione peroxidases [49]. While both ROS and RNS are produced to defend hosts against pathogens, both can also inflict self-damage within the host [57].

Here, we have identified and characterized the cDNA sequence containing the full-length ORF for *BgPrx4* encoding a peroxiredoxin in *B. glabrata*, a snail host of *S. mansoni*. Regulation of this important enzyme was also evaluated following parasite infection of resistant and susceptible snails at both RNA and protein levels. As scavengers of H_2O_2 , these enzymes are pivotal in the varied pathways involved in reactive oxygen species (ROS) detoxification. In the hemolymph of another snail host of this parasite, *B. alexandrina*, the free radical scavengers, catalase and glutathione peroxidase (GPx), were found to be higher in the hemolymph of individual snails that were more resistant to infection, while superoxide dismutase was lower in those snails susceptible to infection [58]. These enzymes varied in expression in both resistant and susceptible *B. alexandrina* snails after infection. In the present study, the abundance of transcripts in resistant and susceptible *B. glabrata* whole, juvenile snails (with and without exposure) (Fig. 4), while not identical to the amount of the enzyme in the hepatopancreas (Fig. 6A), showed the same general trend - infection in the resistant BS-90 snail triggered up-regulation of both RNA and protein expression during the early infection phase (5 h) followed by a decrease at 24 h. In contrast, susceptible NMRI snails infected for the same period showed 2-fold up-regulation of RNA but no discernable change in protein (especially in the hepatopancreas) within the early stages (5-10 h) of infection, followed by further up-regulation at 24 h. However, the protein expression profile in the albumen gland (with infection) within the same period (Fig. 6B) differed from that seen by real time RT-PCR.

Thus far, while Northern blot analysis (Fig. 2) has revealed the occurrence of four putative transcription isoforms corresponding to *B. glabrata* Prx, sequence analysis of cDNAs reverse transcribed from mRNA (from the 4 different snail stocks) have shown only two diverse forms of *BgPrx4* (Fig. 5A), one of 753 bp (250 amino acids) and the other of 516 bp (171 amino acids). Paradoxically, despite this degree of diversity at the RNA level, multiple-sized protein products were not observed in either the hepatopancreas or albumen gland protein extracts of both NMRI and BS-90 snails, indicating that the multiple transcription isoforms observed in the Northern blot might correspond to alternatively spliced transcripts that are not translated but may be involved in regulation. A similar phenomenon has been reported for the *Drosophila* peroxiredoxin I (*dPrx I*) ortholog where there are two alternatively spliced transcripts containing distinct 5' UTRs (Ia and Ib) that leads to an identical coding sequence for a single protein product (59). In a study of the human neuronal nitric oxide synthetase (nNOS) that is encoded by 5 different transcription isoforms, it has been shown that while they share an identical coding sequence they differ in the 5' mRNA leader sequence. Elaborate splicing patterns that involve alternatively spliced leader exons and exon skipping have been

superimposed on this diversity (60). Likewise, the human peroxiredoxin 5 (PRDX5) also has alternative splice variant transcripts with no corresponding proteins. Although the single coding sequence is in frame with the two ATGs of the alternative splice forms, no evidence of translation was seen in Western blots. In fact, attempts at expressing these shorter proteins resulted in insoluble forms of proteins due to misfolding and are in agreement with the absence of expression of these proteins *in vivo*. It is thought that the complexity of regulation of the PRDX5 gene and the presence of variable mRNA sequences might be associated with pathological situations. Such splicing variants have also been shown in baboons and African green monkeys, and thus far there is no evidence of translation of these primate mRNA variants. It has been speculated that the splice variants of the PRDX5 could be involved in gene regulation and have been predicted to have stable secondary structures that could induce specific post-transcriptional gene silencing (61). Furthermore, extensive diversification of genes of *B. glabrata*, for example the Ig superfamily has also been described (63). We plan to express the shorter ORF (516 bp) of *BgPrx4*, if possible, to ascertain whether or not this smaller version is functional. Typically, as stated above, *Prxs* have been shown to be multigenic in a variety of organisms but in this study we have shown that *BgPrx4* exists as a single copy gene. We have recently further validated the single-copy character of *BgPrx4* in this snail by physically mapping a corresponding BAC clone onto metaphase chromosome spreads prepared from the *Bge* snail embryonic cell line [64].

Schistosomes have been shown to express elevated levels of antioxidant enzymes in the presence of hemocytes from susceptible snails while hemocytes from resistant snails may interfere with this detoxification process by down-regulating the schistosome's antioxidant enzymes, thereby enabling parasite killing [65]. In the snail, the enzyme was expressed in a tissue-specific manner (Fig. 4). Thus, Southern blots of RT-PCR products (Figs. 3A and B) indicated abundant transcripts of *BgPrx4* in the albumen gland, hepatopancreas, ovotestis and cerebral ganglia tissue. Its apparently variable expression in hemocytes remains enigmatic; further experiments will be needed to clarify this observation.

Molecular characterization and sequence analysis of the *B. glabrata peroxiredoxin* described in this work implies that it is a homolog of human *PRDX4*, and that it is properly classified as a 2-cys peroxiredoxin because of the presence of 2 cysteines, one preceding the first alpha helix and the other on the C-terminal tail.

Prior to obtaining the complete sequence at the 5' end of the ORF of *BgPrx4*, we had sequenced a 666 bp insert cDNA from an albumen gland library of the LAC2 stock of snails with sequence similarity to *thioredoxin peroxidase (Tpx)* [17]. Despite missing the first 31 amino acids at the N-terminus, the inferred protein had all the elements of a functional enzyme (the peroxidatic and resolving cysteines, invariant active site residues threonine and arginine, etc). To resolve the sulfenic acid intermediate formed upon reduction of peroxide substrates typical 2-Cys *Prxs* function as homodimers and form an intermolecular disulfide bond between the peroxidatic cysteine of one subunit and the resolving cysteine of the other subunit which can then be reduced by thioredoxin. Thus in 2-Cys *Prxs*, the C_P-SOH and C_R-SH react and form a disulfide (C_P-S-S-C_R). 2-Cys *Prxs* have been further subdivided into either "typical" (the C_P-SOH reacts with the C_R residue located in the C-terminal arm from the other subunit of a homodimer) or "atypical" (the C_R residue resides within the same subunit) types depending on the location of the C_R residue [66-68]. Typical 2-Cys *Prxs* function as homodimers, utilizing a unique intermolecular redox-active disulfide center for the reduction of peroxides. The functional homodimer is formed through beta strands at one edge of the monomer (B-type interface). Typical 2-cys *Prx* also form stable decamer (pentamer of dimers) structures. Thus, this sequence also shows the conserved regions I (loop-helix active-site motif) and II that together play a role in decamer formation, consistent with its being a typical 2-Cys peroxiredoxin. We therefore used this partial sequence to express a recombinant protein and later obtained the

complete ORFs from the various snail stocks (Fig. 5A). The recombinant BgPrx4 (rBgPrx4) protein (~28 kDa) was used both to produce mouse antibodies and to characterize its activity. Kinetic analysis of the rBgPrx4 protein indicated that the enzyme has a wide substrate range for peroxides including H₂O₂ (Table 1). An unusual attribute of BgPrx4 is its ability to use both thioredoxin and glutathione (Fig. 7 and Table 1). This ability to utilize both thiol reductants is also seen with the schistosome *Prx* [22]. The preference we observed for glutathione may be the result of using a heterologous (bacterial) thioredoxin in the assay, for which the snail *Prx* may have a lower affinity; confirmation would depend on conducting the assays with *Biomphalaria* thioredoxin. The *K_m*s for the biological substrates are in the range for the expected cellular concentrations and the catalytic activities are similar to if slightly lower than those seen for *Prx* from other organisms [49]. This could be due to improper protein folding due to the missing 31 amino acids at the N-terminus of the rBgPrx4.

In conclusion, while it remains to be seen how peroxiredoxins may influence resistance and susceptibility in *B. glabrata* to invading *S. mansoni*, our studies reveal that the structure of the enzyme is similar in resistant and susceptible snails but that the induction of these enzymes is both time- and tissue-specific at both RNA and protein levels, with higher levels of expression in the resistant compared to the susceptible snail. The presence of variant forms of the transcript but a single, expressed protein product resembling that which has previously been observed for drosophila and human peroxiredoxin orthologs indicates that a complex transcriptional/post-transcriptional regulation and splicing mechanism may also exist for BgPrx4 in *B. glabrata*. Thus, early higher expression of *BgPrx4* RNA and its corresponding protein seems to be a hallmark of snails that are resistant to parasite infection while lower and slower levels of expression are observed in susceptible snails.

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Figure 1. Copy number analysis of *BgPrx4* in the diploid genome of *B. glabrata* (BS-90)
 Single copy gene (ferritin) was used for the copy number estimation of *BgPrx4*. Serial 10 fold dilutions of *B. glabrata* genomic DNA (10 µg-1 ng) was probed with either 100 ng of *ferritin* (429 bp panel A) or *BgPrx4* ORF (753 bp, panel B).

Figure 2. Northern analysis of *B. glabrata* using *BgPrx4* probe

5 µg of total RNA from *B. glabrata* resistant (BS-90) and susceptible (M-line) snail stocks unexposed and exposed to *S. mansoni* miracidia for either 5 h or 24 h and probed using the ³²P-labeled 753 bp insert from *BgPrx4*-BS-90 (GenBank Acc. No. FJ176938). **Panel A** shows the ethidium bromide staining of the agarose-formaldehyde gel prior to transfer. Lane 1: 0.5-8 Kb RNA molecular size ladder; lane 2: BS-90 unexposed; lane 3: BS-90 5 h exposed; lane 4: BS-90 24 h exposed; lane 6: NMRI unexposed; lane 7: NMRI 5 h exposed 24 h exposed; lane 8: mouse RNA. **Panel B** shows the northern blot of the gel transferred onto a nitrocellulose membrane and probed using labeled *BgPrx4* DNA. Black arrows indicate the lower molecular size transcripts seen in BS-90 and NMRI snails exposed to the parasite.

Figure 3. RT-PCR analysis of *B. glabrata* tissue specific distribution of *BgPrx4*

Snail RNA was converted to cDNA by reverse transcription using *BgPrx4* cDNA GSP primers (Panels A and B) or the putative housekeeping gene myoglobin (data not shown). Panel A shows the ethidium bromide stained gel of the 753 bp PCR amplified product. Lane 1: 100 bp DNA ladder; lane 2: albumen gland cDNA; lane 3: hepatopancreas cDNA; lane 4: hemocyte cDNA; lane 5: ovotestis cDNA; lane 6: cerebral ganglia cDNA and lane 7: no template control. **Panel B** shows the Southern hybridization of the gel from panel A probed using the ³²P-labeled 753 bp insert from *BgPrx4*.

Figure 4. Relative abundance of *BgPrx4* transcripts in BS-90, LAC2, NMRI and M-line stocks of *B. glabrata*

The differential expression of *BgPrx4* transcripts by real-time Q-RT-PCR in resistant (BS-90), non-susceptible (LAC2) and susceptible (NMRI and M-line) *B. glabrata* snails following no exposure (0 h) or exposure to *S. mansoni* miracidia for 5 h, 10 h, 24 h or 48 h. The fold-change of expression of the transcripts was calculated by comparison between unexposed and exposed snails within each snail stock by the comparative Ct method and the formula as described in Materials and Methods, with the normalized basal level of the expression in unexposed normal snails as 1.0. Error bar = S.D. ($n = 0.0977$). The significant P -value < 0.05 and < 0.01 were indicated by * and ** at the top of each bar, respectively.

Fig. 5A

Fig. 5B

Figure 5.

A) Alignment of the deduced amino acid sequences of peroxiredoxins (*BgPrx4*) from the snail *B. glabrata*. The amino acid sequences of the *BgPrx4* from the individual snail stocks BS-90 (Acc. No. FJ176938), M-line (Acc. No. FJ176941), LAC2 (Acc. No. FJ176940), NMRI (Acc. No. FJ176942) and the NMRI with the deletion (δ NMRI, Acc. No. FJ176939) have been aligned along with the partial *Tpx* cDNA sequence (Acc. No. AY026258) from *B. glabrata* [17]. The computer program ClustalW (version 2.0.5) was used to generate this alignment. The numbering system used in the alignment represents the amino acid position in the complete ORF of *B. glabrata* BS-90. The horizontal arrow at the bottom of the alignment represents the region determined by 5' RACE from which GSPs were designed to determine the complete ORFs. The two conserved redox-active cysteines (residues 99 and 220) are highlighted by a bold "C" and down arrows (▼). The invariant active site residues threonine (T) and the arginine (R) are shown in bold and highlighted with a "•". Also identified in this alignment are conserved regions I (loop-helix active-site motif) and II. The vertical arrow represents the cleavage site for the signal peptide between residues 21 and 22. Residues highlighted in gray indicate the

conserved GG(V/I/L)G-(X)_n-YF sequence that is sensitive to hyperoxidation. Boxed regions represent variant amino acid residues in the different snail stocks.

B) Alignment of human peroxiredoxins versus *BgPrx4*. Cladogram resulting from MEGA4 analysis of the relationships between the *BgPrx4* sequence from BS- 90 *Biomphalaria glabrata* (this study) and the 6 peroxiredoxin genes (*PRDX1-6*) of humans (GenBank Acc. Nos. PRDX1: NP_002565; PRDX2: NM_005809; PRDX3: NM_006793; PRDX4: NP_006397; PRDX5: NM_181652; PRDX6: NP_004896). This provides the basis for designating the *B. glabrata* peroxiredoxin reported here as *BgPrx4*.

Figure 6. Western blot analysis of *B. glabrata* protein expression

Protein extracts from hepatopancreas (**Panel A**) and albumen gland (**Panel B**) of BS-90 and NMRI snails were analyzed using mouse antisera to rBgPrx4 protein. Fifty μ g of total protein extract from the hepatopancreases and albumen glands were resolved using 15% SDS-PAGE. After transfer, the blots were probed with a 1:500 dilution of mouse polyclonal antisera to rBgPrx4, and a secondary antibody of goat anti-mouse IgG-Fc (1:2000 dilution) conjugated to alkaline phosphatase. Protein extracts from each of the snail stocks comprised of 0 h (unexposed), 5 h and 24 h exposed (to miracidia) samples.

Figure 7. Specific activities of recombinant *Biomphalaria glabrata* peroxiredoxin

Assays were performed as described in Materials and Methods with 35 nM *B. glabrata* peroxiredoxin 1 plus 0.15 μ M recombinant *Escherichia coli* thioredoxin reductase (TrxR) and 16 μ M recombinant *E. coli* thioredoxin (Trx) or 0.3 unit/ml yeast glutathione reductase (GR) and 0.7 mM glutathione (GSH) with 0.5 mM H₂O₂ (black bars), 0.5 mM cumene hydroperoxide (gray bars), or 0.5 mM tertiary butyl hydroperoxide (open bars).

Apparent saturation kinetic constants for *B. glabrata* peroxiredoxin

Kinetic parameters K_m (μM) and k_{cat} (s^{-1}) were determined using kinetic NADPH consumption assay at 340 nm. Reactions contained 35 nM *B. glabrata* peroxiredoxin 1 and either 0.15 μM recombinant *Escherichia coli* thioredoxin reductase or 0.3 unit/ml yeast glutathione reductase. Substrate concentrations were varied and activities were determined with saturating concentrations of the second substrate (GSH = 0.7 mM; recombinant *E. coli* thioredoxin = 16 μM ; H_2O_2 = 0.5 mM) and kinetic constants were determined as described in Materials and Methods. Cum-OOH, cumene hydroperoxide; t-Bu-OOH, tertiary butyl hydroperoxide; Trx, *E. coli* thioredoxin; GSH, glutathione.

Table 1

Substrate	H_2O_2		Cum-OOH		t-Bu-OOH		Trx		GSH	
	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}
GSH	125	5.52	121	6.54	180	2.19	-	-	-	-
Trx	259	4.21	312	3.18	391	1.05	-	-	-	-
H_2O_2	-	-	-	-	-	-	5.2	4.75	309	7.09