Molecular Cloning and Functional Characterization of the Dual Oxidase (BmDuox) Gene from the Silkworm Bombyx mori

Xiaolong Hu1, Rui Yang1, Xing Zhang2, Lin Chen1, Xingwei Xiang1, Chengliang Gong3, Xiaofeng Wu1*

1 College of Animal Sciences, Zhejiang University, Hangzhou, China, 2 Institute of Cell Biology, Zhejiang University, Hangzhou, China, 3 National Engineering Laboratory for Modern Silk, Soochow University, Suzhou, China

Abstract

Reactive oxygen species (ROS) from nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and their related dual oxidases are known to have significant roles in innate immunity and cell proliferation. In this study, the 5,545 bp cDNA of the silkworm Bombyx mori dual oxidase (BmDuox) gene containing a full-length open reading frame was cloned. It was shown to include an N-terminal signal peptide consisting of 28 amino acid residues, a 240 bp 5′-terminal untranslated region (5′-UTR), an 802 bp 3′-terminal region (3′-UTR), which contains nine ATTTA motifs, and a 4,503 bp open reading frame encoding a polypeptide of 1,500 amino acid residues. Structural analysis indicated that BmDuox contains a typical peroxidase domain at the N-terminus followed by a calcium-binding domain, a ferric-reducing domain, six transmembrane regions and binding domains for flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD). Transcriptional analysis revealed that BmDuox mRNA was expressed more highly in the head, testis and trachea compared to the midgut, hemocyte, Malpighian tube, ovary, fat bodies and silk glands. BmDuox mRNA was expressed during the developmental stages of the silkworm. Subcellular localization revealed that BmDuox was present mainly in the periphery of the cells. Some cytoplasmic staining was detected, with rare signals in the nucleus. Expression of BmDuox was induced significantly in the larval midgut upon challenge by Escherichia coli and Bombyx mori nucleopolyhedrovirus (BmNPV). BmDuox-deleted larvae showed a marked increase in microbial proliferation in the midgut after ingestion of fluorescence-labeled bacteria compared to the control. We conclude that reducing BmDuox expression greatly increased the bacterial load, suggesting BmDuox has an important role in inhibiting microbial proliferation and the maintenance of homeostasis in the silkworm midgut.

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* E-mail: wuxiaofeng@zju.edu.cn

Introduction

Dual oxidases (Duoxes) and NAPDH-oxidases (Noxes) have various biological and pathological roles in the generation and regulation of reactive oxygen species (ROS). ROS are by-products of mitochondrial respiration or are produced by activation of Duoxes/Noxes in the immune response to pathogen invasion [1,2]. ROS is cytotoxic at concentrations above certain thresholds, which can lead to damage of important cellular building blocks, such as DNA, proteins and lipids [3]. This oxidative damage can be counteracted by the action of enzymes, including superoxide dehydrogenase (SOD), glutathione peroxidase (Gpx), catalase and thioredoxin peroxidase, to detoxify ROS by host cells [4]. Increasing evidence suggests cells can achieve homeostasis by production of ROS in response to diverse environmental signals [5–14].

Generation of ROS and nitric oxide (NO) was suggested to be a potential bactericidal mechanism in the gut epithelial barrier [15]. Duox1 and Duox2 were cloned from human thyroid tissue and highly expressed in mucosal surfaces of the lungs and gastrointestinal tract, respectively [16–18]. The participation of epithelial cells as a primary barrier against microbes and their constant encounter with microbes indicate that epithelial Duox is involved in ROS-dependent activation of immunity [16,19]. The Duox in the zebrafish Danio rerio (DrDuox) epidermal cells is involved in paracrine signaling to resolve wound inflammation [20] and Duox has been shown to have an antimicrobial function in the zebrafish intestinal epithelium [21]. Noxes/Duoxes are present in vertebrates and have been found also in invertebrates, including Drosophila melanogaster, Anopheles gambiae, Aedes aegypti and Marsupena japonica. The Duox and Nox families are distinguished by the presence of an N-terminal extracellular peroxidase-homology domain (PHD), except for the gp91phox-like oxidase domain [19,22]. There is one Duox gene and one Nox gene in the Drosophila genome, and genetic analysis showed infection-induced ROS were not produced in DrDuox-knockout flies but were not influenced in Nox-knockout flies. DrDuox-knockout flies failed to control bacteria in the gut and were highly susceptible to gut infection, confirming Duox is the source of infection-induced ROS as a microbial effector molecule [8]. In A. gambiae, peroxidases and dual oxidase formed a di-tyrosine network between the midgut epithelial and peritrophic membrane that decreased the...

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products were inserted into the pMD-19 T vector for sequencing.

The Adavatage
The 5th instar larvae and various developmental stages with the Kit
2 PCR Kit were purchased from TaKaRa (Dalian, China). The
pUC-18 T vector, PCR primers and DNA sequencing were
in both vertebrates and invertebrates. However, there are few reports for the silkworm Bombyx mori, which is an important economic insect and a model organism in the Lepidoptera family, the majority of which are agricultural pests. We undertook characterization of the silkworm dual oxidase gene (BmDuox) to help us understand the molecular mechanisms underlying the silkworm immune response. In addition, information obtained for the silkworm will provide leads for the development of more efficient strategies for pest control.

Materials and Methods

Materials and Reagents

B. mori strain P50 was used for all experiments. Larvae were fed mulberry leaves (Morus sp.) and kept at 25 ± 1°C with 70–85% relative humidity and a photoperiod of 12 h light:12 h dark. The RNA extraction kit was from Invitrogen (Carlsbad, CA). The pMD-19 T vector, T4 DNA ligase, restriction enzymes, SMART™ RACE cDNA Amplification Kit and Adavatage® 2 PCR Kit were purchased from TaKaRa (Dalian, China). The pUC-18 T vector, PCR primers and DNA sequencing were acquired from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

Primer Design

A candidate silkworm BmDuox gene was identified by TBLASTN analysis using the D. melanogaster Dual Oxidase amino acid sequence (GenBank accession number NP_608715). PCR primers used for cloning BmDuox are given in Table S1.

Total RNA Isolation

Total RNA was isolated from various tissues of the 3rd day of the 5th instar larvae and various developmental stages with the Kit (Invitrogen) according to the manufacturer's instructions and stored at −70°C. The purity of the RNA was determined by the ratio of absorbance at 260 nm and at 280 nm (A260/A280) and by agarose (1%) gel electrophoresis.

Cloning of Full-length BmDuox by EST Analysis and 5' 3' RACE

Two expressed sequence tags (ESTs; GenBank accession numbers CK529174 and CK528166) were found in the silkworm genome database (SilkDB). Full-length BmDuox was cloned with RACE-PCR with the SMART™ RACE cDNA Amplification Kit according to the manufacturer’s instructions. Gene-specific primers BmDuox RACE-F and RACE-R were designed on the basis of the EST fragment sequence (Table S1). The 5’ first-strand cDNA template was synthesized from silkworm head total RNA (1 μg) in the presence of 5’ RACE CDS primer A and a SMARTer II A Oligonucleotide in a standard reverse transcription buffer containing 100 U/μl of SMARTer™ Reverse Transcriptase. The 3’ first-strand cDNA template was obtained from silkworm head total RNA (1 μg) in the presence of 3’ RACE CDS primer A in a similar standard reverse transcription reaction. The Adavatage® 2 PCR Kit was used for RACE-PCR. Amplified products were inserted into the pMD-19 T vector for sequencing.

Sequence Analysis

The structural domains of BmDuox were predicted using the simple modular architecture research tool (SMART; version 7.0) (http://smart.embl-heidelberg.de/) [25]. During analysis, PFAM domains, signal peptides and internal repeats were checked in the SMART screen page. Translation of the gene and characteristic prediction of the deduced protein were done with ExPaSy (http://au.expasy.org). Secretory signal sequences were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Transmembrane domain searches were done with TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Alignments were done with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and MEGA 4 was used to produce a phylogenetic tree (http://www.megasoftware.net/). SilkDB (http://silkworm.genomics.org.cn/silksoft/silkmap.html) was used to predict the chromosomal localization of the BmDuox gene. Similarity and identity were analyzed with MASTGAT 2.0 [26]. Multiple sequence alignment was done by Florence Corpet (http://multalin.toulouse.inra.fr/multalin/multalin.html) [27]. Tertiary structure was predicted using SWISS-MODEL (http://swissmodel.expasy.org/) [28–30].

Phylogenetic Analysis

The nucleotide and deduced amino acid sequences of BmDuox cDNA were analyzed using DNAMAN version 5.2.2. The sequences of Duoxes/Noxes from various species [24] were downloaded from the NCBI database and compared with the NCBI BLAST search program. Multiple sequence alignment was done with ClustalW and the phylogenetic trees of Duoxes/Noxes entire length and oxidase domains from Duoxes were made using the neighbor-joining method in MEGA 4 software. GenBank accession numbers of the Duoxes sequences are given in Table S2.

Expression Patterns of BmDuox mRNA in Various Developmental Stages and Different Tissues of the 3rd day of the 5th Instar Larvae by Real-time Quantitative PCR (RT-qPCR)

RT-qPCR was used to detect and quantify the BmDuox gene expression levels in developmental stages and different tissues in the 3rd day of the 5th instar larvae, using the actin 3 gene as an internal control. Total RNA was isolated from different developmental stages (from egg to adult). First, the samples were freeze-dried, then a portion of the powder was used for total RNA extraction with the RNA extraction kit according to the user's manual. Total RNA was isolated from the head, midgut, silk gland, testis, ovary, trachea, fat body, Malpighian tube and hemocyte of the 3rd day of the 5th instar larvae as described above. A 1 μg portion of total RNA was used as the template for the first-strand cDNA synthesis. RT-qPCR was done using SYBR green dye (SYBR Green PCR Master Mix, TaKaRa) with the ABI 7300 System (Applied Biosystems, USA). The BmDuox gene expression analysis primers are given in Table S1. The relative expression level of BmDuox was determined using the 2ΔΔCt method [31]. Experiments were done in triplicate for each tissue. Melting curve analysis of amplified products was done at the end of each run to confirm the specificity of amplification.

Antibody Generation

A partial fragment of BmDuox was cloned by RT-PCR (the primers used are given in Table S1). The PCR product was ligated into the pEASY-E1 expression vector. The error-free prokaryotic expression cassette pEASY-E1-BmDuox (Figure S1A) was used to transform E. coli strain BL21 cells. After induction for 4 h with isopropyl-β-D-thiogalactopyranoside at a final concentration of
1 mmol/L, 1.5 ml of the transformed BL21 cells was harvested for verification of successful induction of the correct antigen, the His-
BmDuox fusion protein. The cells were suspended in 100 μl of TE
buffer (pH 8.0) and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and western
blotting (Figure S1B). Detection used a primary antibody of rabbit anti-6×His (Sigma) and a secondary antibody of horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Sigma).

To purify the recombinant protein, 11 of BL21 cells was harvested by centrifugation. The pellet was suspended in lysis
buffer (10 mM Tris–HCl pH 8.0, 50 mM NaH2PO4, 100 mM NaCl) and subjected to sonication (5 pulses (200 W), 1 min each) to disrupt the cells. The lysate was centrifuged at 12,000 g for 15 min at 4°C, the pellet was discarded and the supernatant was purified by the use of N-iNTA (Ni2+-nitrilotriacete) resin (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After elution, the concentration of purified protein was determined by the Bradford method using bovine serum albumin (BSA) as the standard. Briefly, antibody was raised in New Zealand rabbits by subcutaneous injection of purified BmDuox (100 μg) expressed in E. coli at to me zero (s) as described above with an equal volume of Freund’s complete adjuvant and made to a total volume of 1 ml/animal. Booster injections were administered at t=7, 14 and 21 days with the same dose of antigen in Freund’s incomplete adjuvant. Final booster injections were administered at t=28 days with the same dose of antigen in 1 ml of sterile PBS. Serum was collected and stored at −70°C.

The specificity of the antibody was verified by western blotting with extracted total proteins from BmN cells (Figure S1C).

Subcellular Localization of BmDuox by Immunofluorescence

BmN cells derived from silkworm ovary were grown at 27°C in TC-100 medium supplemented with fetal bovine serum (FBS) to a final concentration of 10% (v/v). BmN cells were fixed in 4% (v/v) paraformaldehyde at room temperature for 2 h and rinsed with 0.01 M PBST (10 mM Na2HPO4, 1.8 mM KH2PO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.05% (v/v) Tween-20. The fixed cells were blocked with 3% (w/v) BSA at room temperature for 2 h followed by three washes (5 min each) in PBST then incubated overnight at 4°C with BmDuox polyclonal antibody (diluted 1:100 in blocking buffer). After three washes (5 min each) in PBST, cells were incubated with anti-rabbit fluorescein isothiocyanate (FITC)-labeled secondary antibody (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After elution, the concentration of purified protein was determined by the Bradford method using bovine serum albumin (BSA) as the standard. Briefly, antibody was raised in New Zealand rabbits by subcutaneous injection of purified BmDuox (100 μg) expressed in E. coli at to me zero (s) as described above with an equal volume of Freund’s complete adjuvant and made to a total volume of 1 ml/animal. Booster injections were administered at t=7, 14 and 21 days with the same dose of antigen in Freund’s incomplete adjuvant. Final booster injections were administered at t=28 days with the same dose of antigen in 1 ml of sterile PBS. Serum was collected and stored at −70°C.

The specificity of the antibody was verified by western blotting with extracted total proteins from BmN cells (Figure S1C).

Expression Profiles of BmDuox in vitro and in vivo

To further confirm the expression profiles of BmDuox induced by bacterial challenge, the relative expression levels of BmDuox in vitro and in vivo were quantified by RT-qPCR. B. mori BmN cells were incubated in TC-100 medium supplemented with 10% (v/v) FBS at 27°C. Upon reaching 70–80% confluence, soluble microbial extract (SME) was added to the medium to a final concentration of 10 μg/ml. Cells were collected at 5, 10, 15, 20, 25 and 30 min after challenge. To investigate the BmDuox expression pattern in vivo, OD600 = 1. E. coli was spread onto the surface of mulberry leaves, which were then used to feed 5th instar larvae. Midguts were collected at 3, 6, 9, 12 and 24 h after challenge.

Virus Inoculation

BmNPV was suspended in distilled water to a concentration of 106 polyhedra/ml. A 1 ml sample of viral suspension was spread equally onto 10 pieces of mulberry leaves, each ~15 cm², which were later fed to 5th instar larvae and control larvae were fed with the same quantity of leaves but treated with distilled water. Midguts were collected at 12, 24, 48, 72 and 96 h after inoculation.

ROS Detection

The cell-permeant reagent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a molecular probe indicator of ROS. After diffusion into the cell, DCFH-DA was deacetylated by cellular esterases to yield the non-fluorescent compound 2',7'-dichlorofluorescein (DCF), which was later oxidized by ROS to yield 2',7'-dichlorofluorescein (DCF). The highly fluorescentDCF can be detected by fluorescence spectroscopy using an excitation wavelength of 488 nm and an emission wavelength of 550 nm [32]. Briefly, B. mori BmN cells were challenged with SME (10 μg/ml) for 3, 5, 10, 15, 20 or 30 min, then 1 μl of 10 μM DCFH-DA (0.1 ml of 10 mM DCFH-DA in DMSO; Molecular Probes) was added to each plate and incubated at 27°C for 30 min. Cells were washed three times with double-distilled water (2 ml) and then lysed with 3 ml of double-distilled water. Cell lysates were collected and fluorescence was measured. H2O2 (final concentration is 2 μM) was added to the cell medium as a positive control.

Construction of Gene-targeting Vectors and Follow-up Verifications

To construct the gene-targeting vector, the left homologous arm (BmDuox-left), a 1.2 kb fragment, and the right homologous arm (BmDuox-right), a 1.8 kb fragment, were cloned. Reporter gene egfp (1.8 kb) was driven with the A3 promoter and the selection gene neo (1.7 kb) was driven with the ie-1 promoter from the BmNPV. PCR sequencing was used to confirm the recombinant vectors. All these elements were amplified by PCR (the primers are given in Table S1).

Production of the Transgenic Silkworm

Sperm-mediated gene transfer (SMGT) was done as described [33,34]. Briefly, 10 μg of homologous recombination vector was injected into the copulatory pouch of virgin B. mori female adults (strain Quifeng × Baiyu), which were then free to copulate for 3–4 h. The eggs were maintained until hatching at 25°C in a chamber with 85–90% relative humidity.

Screening and Identification of the Targeted Silkworm

The expression of enhanced green fluorescent protein (EGFP) in G0 eggs, larva and pupae was detected using a fluorescence stereomicroscope (SZX12, OLYMPUS). Individuals with GFP-positive G0 offspring were identified as germline-positive transgenic silkworms. Genomes from the G0 generation of the transgenic silkworm moths were monitored by PCR using egfp-specific primers egfp-1/egfp-2 and ie-1-neo-sv40polyA-1/ie-1-neo-sv40polyA-2.

The genome status of the G0 generation transgenic silkworms was confirmed by dot hybridization with a DIG-labeled ie-1 probe according to the Roche protocols (Roche, DIG High Prime DNA Labeling and Detection Starter Kit I, No. 11745832910). Midguts from the G0 generation of transgenic silkworm larvae were collected for total protein extraction for western blotting. Briefly, samples were ground into powder and dissolved in lysis buffer (2.5% (w/v) SDS, 10% (v/v) glycerin, 5% (v/v) β-
mercaptoethanol and 62.5 mM Tris–HCl pH 6.8) overnight at 4 °C. After centrifugation, the supernatant was used for SDS-PAGE and western blotting. The primary antibody used was rabbit anti-GFP (Sigma) and the secondary antibody was HRP-conjugated goat anti-rabbit (Beyotime Institute of Biotechnology). Positive individuals were used for the extraction of total mRNA and total proteins for analysis of the gene silencing effect. The mRNA of BmDuox was identified by RT-PCR, and the mRNA extracted from normal silkworms was used as the control. BmDuox expression was identified by western blotting between normal and positive silkworms.

**In vivo Bacterial Persistence Assay**

The bacterial persistence assay was done as described [8]. Briefly, spectinomycin-resistant *E. coli-GFP* was coated onto the surface of mulberry leaves and used to feed the 3rd day of the 5th instar larvae. *E. coli-GFP* persistence was measured by plating appropriate dilutions of the homogenates of four surface-sterilized midgut contents and samples were collected at 12 h after infection. The microbes were grown on LB agar plates containing spectinomycin (100 μg/ml) and the number of colony-forming units (CFUs) was counted. The results are expressed as mean and standard deviation of three independent experiments.

**Statistical Analysis**

All data are presented as mean ± SD. Statistical differences were evaluated using Student’s t-test for unpaired samples. The level of statistically significant difference was set at *P*<0.05, **P**<0.01 and ***P***<0.001.

**Results**

**Sequence Analysis of the BmDuox Gene**

The full length of BmDuox cDNA was obtained using RACE (Figure S2) and bioinformatics approaches. The BmDuox cDNA was 5,545 bp long with a 4,503 bp open reading frame (ORF) coding for 1,500 amino acids. It contained a 28 amino acid residue signal peptide at the N-terminus, a 240 bp 5′-untranslated region (5′-UTR) and an 802 bp 3′-untranslated region (3′-UTR). The signal for polyadenylation, ATTTA, was found at the 3′-UTR (Figure 1). The deduced amino acid sequence had a predicted molecular mass of 172 kDa and an isoelectric point of 8.96. This cDNA sequence was deposited with GenBank (accession number JQ768349). Six transmembrane domains were identified in BmDuox using TMHMM online software (Figure S3A). A search of SilkDB revealed that BmDuox is localized on the 8th chromosome (Figure S3B).

Blasting the BmDuox cDNA against the NCBI *B. mori* genome database revealed it was distributed on genomic DNA fragments AADK01028014.1, AADK01003202.1, AADK01000925.1 and AADK01010320.1. The BmDuox cDNA was aligned with the silkworm genomic sequence, and sites for exon-intron borders were deduced by comparison. The BmDuox gene was organized into 26 exons and 25 introns. Further comparison with its orthologs from other organisms revealed a generally well-conserved exon-intron organization. All exon–intron splicing junctions matched canonical consensus sequences for donor and acceptor sites (data not shown). Exon 1 contained the start codon, exon 26 contained the termination codon and the 3′-UTR harbored a polyadenylation signal.

**Domain Structure**

The complete domain structures of BmDuox were compared to other Duoxes/Noxes by the SMART program (Figure S3C). The
protein contained six transmembrane regions (at positions 593–615, 887–992, 1027–1049, 1080–1102, 1132–1154 and 1175–1197) and binding domains for FAD (at positions 1218–1319) and NAD (at positions 1325–1481) known to be functionally important in various electron transport systems. The arrangement of these domains was found to be common to Duox and Nox proteins (Figure 2). In addition to these domains, BmDuox harbored a peroxidase domain (at positions 36–556), a ferric–reductase domain (at positions 1035–1183) and an N-terminal calcium-binding domain containing 3 EF-hand motifs (at positions 822–850, 858–886 and 903–931), which was found in Duoxes/Noxes of many species; e.g. D. melanogaster DmDuox, D. rerio DrDuox, Arabidopsis thaliana AtDuox and Homo sapiens Duox2/1. The deduced BmDuox protein sequence had a signal peptide of 28 amino acid residues in the N-terminus with an excision site located at Cys28/Asp29. Seven tertiary structure models were predicted using SWISS-MODEL (Figure S4). Compared to BmDuox, DmDuox and DrDuox, the structure domains showed conserved similarity in these tertiary models (data not shown).

Sequence Homology
The BmDuox amino acid sequence was aligned with published sequences of Duoxes of other species (Figure S5). BmDuox contained a peroxidase domain, a calcium-binding domain, six transmembrane regions, a ferric-reductase domain, an FAD-binding domain and an NAD-binding domain with 521, 87, 43, 149, 102 and 157 amino acid residues, respectively. Sequence alignment was used to determine percentage homology with other Duoxes. BmDuox shared 72.6% sequence homology with DmDuox, 75% with AaDuox1, 71.7% with AmDuox and more than 37% with other species Duoxes (Table 1). The BmDuox peroxidase domain shared 67.3% sequence homology with DmDuox, 62.3% with AmDuox and 68.6% with AaDuox1 (Table S3A). Duoxes/Noxes of all species contained the transmembrane region and the FAD-binding domain (Figure 2). For the transmembrane region, BmDuox shared 57.3% homology with DmDuox and 71.4% with AaDuox1 but only 24.1% with DrDuox (Table S3B). For the calcium-binding motif, BmDuox shared 89.7% homology with DmDuox, 88.5% with AmDuox and 89.7% with AaDuox1 (Table S3C). The BmDuox ferric-reductase domain shared 77.9% homology with DmDuox, 76.5% with AmDuox and 83.2% with AaDuox1 (Table S3D). For the BmDuox FAD-binding domain, there was 86.3% sequence homology with DmDuox, 47.1% with DrDuox, 88.3% with AmDuox and 88.2% with AaDuox1 (Table S3E). Finally, the BmDuox NAD-binding domain shared 96.2% homology with DmDuox, 93% with AmDuox and 96.8% with AaDuox (Table S3F). The amino acid sequences (SGQQWVR, FTLSAPHEH and GIGVTPYAS) in the NAD-binding domains of vertebrates and invertebrates were all conserved (Figure S6). These results showed that BmDuox has a high level of amino acid sequence homology with Duoxes from various species. The analysis of individual domain homology

Figure 2. Domain organizations of Duox/Nox proteins. The calcium-binding region contains EF-hand motifs in BmDuox. The ORFs from BmDuox and Duox/Nox of other species are compared: Bombyx mori Duox, BmDuox (Q768349); Drosophila melanogaster Duox, DmDuox (NP_608715.2); Aedes aegypti Duox, AaDuox1 (XP_001658452.1); Danio rerio Duox, DrDuox (BAF33370.1); Homo sapiens Duox2, HsDuox2 (NP_0547994); Homo sapiens Duox1, HsDuox1 (NP_059130.2); Drosophila melanogaster Nox, DmNox (NP_001097336); Arabidopsis thaliana Nox, AtNox (O81212.2); Tribolium castaneum Nox, TcNox (XP_972375.2); Aedes aegypti Nox, AaNox (EAT37894); Homo sapiens Nox1, HsNox1 (CAI42337.1).

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among various species revealed high levels of similarity and conservation.

Phylogenetic Analysis
To better elucidate the evolutionary relationships between BmDuox and other sequenced Duoxes proteins, a molecular taxonomy was constructed by aligning the oxidase domains of the BmDuox and other species of Duoxes. The accession numbers, symbols and nomenclature used in the phylogenetic analysis of sequences are given in Table S2. BmDuox formed a cluster with insect Duoxes, including those of D. melanogaster, A. mellifera and A. aegypti (Figure 3). D. rerio Duox and H. sapiens HiDuox2 formed another cluster. The whole sequence of BmDuox and other species of Duoxes/Noxes was used to determine the evolutionary relationships (Figure S7).

Expression Profile of BmDuox in various Developmental Stages and Different Tissues by RT-qPCR
Our results indicated that stained nuclei emit blue fluorescence upon stimulation by UV. under a laser confocal scanning microscope (TCS SP5). DAPI-BmIgG were used as primary and secondary antibodies, respectively, to examine the localization of BmDuox in various Developmental Stages and Different Tissues by RT-qPCR (Figure S8B).

Subcellular Localization of BmDuox
BmDuox polyclonal antibody and FITC-labeled goat anti-rabbit IgG were used as primary and secondary antibodies, respectively, to examine the localization of BmDuox. Cells were examined under a laser confocal scanning microscope (TCS SP5). DAPI-stained nuclei emit blue fluorescence upon stimulation by UV. Our results indicated that BmDuox was present mainly in the periphery of the cells. Some cytoplasmic staining was detected, with rare signals in the nucleus, suggesting this protein is localized in the cell membrane (Figure S9).

Table 1. Amino acid identity and similarity of the silkworm BmDuox gene compared to other known Duoxes/Noxes sequences using the entire Duoxes/Noxes protein.

| Full-length | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 |
|-------------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1. BmDuox   | 58 | 72 | 38 | 39 | 38 | 38 | 38 | 38 | 37 | 71 | 71 | 75 | 70 |
| 2. DmDuox   | 62 | 36 | 54 | 55 | 55 | 55 | 55 | 55 | 55 | 55 | 55 | 55 | 55 |
| 3. DrDuox   | 84 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 4. HiDuox2  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 5. HiDuox1  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 6. RnDuox   | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 7. RnDuox1  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 8. MmDuox2  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 9. MmDuox1  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 10. BtDuox  | 59 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 11. OdDuox2 | 85 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 12. AmDuox  | 85 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 13. AdDuox1 | 85 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |

BmDuox Induction, Expression Analysis and Generation of ROS
To further explore the possible BmDuox expression pathways at the molecular level, BmN cells were treated with SME and the mRNA expression of BmDuox was later examined. Our results showed the level of ROS in BmN cells was increased significantly by SME (Figure S10A). In addition, the initial level of BmDuox mRNA expression was basal and was later induced by SME (Figure S10B). The expression pattern of BmDuox upon challenge by bacteria (DH5α, Gram-negative bacteria) (Figure S10C) and virus (BmNPV) (Figure S10D) in vivo was examined and the results showed expression of BmDuox was induced at specific time points in the silkworm midgut.

Screening and Identification of Transgenic Silkworms
To investigate the role of the BmDuox gene in vivo, we generated a knockout silkworm with the BmDuox gene silenced by transgenic methods. The BmDuox gene was disrupted by insertional mutagenesis. A vector containing A3-egfp-neo40polyA and ie1-neo-40polyA expression cassettes was integrated into the silkworm genome through homologous recombination (Figure 4). Fluorescence was detected in fertilized eggs (Figure 5A and 5B), third instar larvae (Figure 5C) and pupae (Figure 5D and 5E), demonstrating the egfp gene was expressed continuously in vivo.

Disruption of the BmDuox gene was verified using PCR analysis of genomic DNA extracted from transgenic silkworms that were positive for egfp fluorescence. Specific bands of the ie1-neo-40polyA (~1800 bp) expression cassette and the egfp gene (~720 bp) were amplified (Figure 6A). All the PCR products amplified from G1 transgenic silkworms were recovered from agarose gel after separation by electrophoresis and further verified by DNA sequencing (data not shown). In addition, the foreign protein expression was verified by western blotting analysis of total proteins extracted from transgenic silkworms that were positive for egfp fluorescence. An expected band of ~26 kDa was identified in fluorescent silkworms with the GFP-labeled antibody (Figure 6B). Dot hybridization with an ie1 gene probe, which was used to verify the presence of the foreign gene fragment in fluorescent silkworms, showed the genomes of fluorescent moths of generation

Table 1. Amino acid identity and similarity of the silkworm BmDuox gene compared to other known Duoxes/Noxes sequences using the entire Duoxes/Noxes protein.

| Full-length | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 |
|-------------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1. BmDuox   | 37 | 72 | 38 | 39 | 38 | 38 | 38 | 38 | 37 | 71 | 71 | 75 | 70 |
| 2. DmDuox   | 62 | 36 | 54 | 55 | 55 | 55 | 55 | 55 | 55 | 55 | 55 | 55 | 55 |
| 3. DrDuox   | 84 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 4. HiDuox2  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 5. HiDuox1  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 6. RnDuox   | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 7. RnDuox1  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 8. MmDuox2  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 9. MmDuox1  | 60 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 10. BtDuox  | 59 | 71 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 11. OdDuox2 | 85 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 12. AmDuox  | 85 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |
| 13. AdDuox1 | 85 | 60 | 37 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 | 38 |

Upper triangle: identity, lower triangle: similarity.
doi:10.1371/journal.pone.0070118.t001
G1 contained the *ie-1* gene (Figure 6B). Endogenous gene expression was verified by RT-PCR and western blotting analysis of mRNA and total proteins extracted from transgenic silkworms positive for *egfp* fluorescence. The levels of expression of *BmDuox* mRNA and *BmDuox* protein were reduced significantly compared to control silkworms (Figure 6C).

**In vivo Bacterial Persistence Assay**

To investigate the relationships between *BmDuox* activity and microbial persistence *in vivo*, we examined the midgut of *BmDuox*-silenced larvae during natural infection, using the GFP-tagged pathogen *E. coli-GFP*. Dramatic differences in midgut loads were observed between wild type (wt) and *BmDuox* knockdown (kd) silkworms. *BmDuox* kd larvae harbored 100-fold more CFUs.
Figure 5. Identification of BmDuox knockout silkworms by fluorescence microscopy. (A) Egg laid by non-transgenic silkworm. (B) Fluorescence of eggs laid by G1 generation of the BmDuox knockout silkworm. (C) Third instar larva of the G1 generation of BmDuox knockout silkworm. (D) Normal pupa (left) and targeted pupa (right) under natural light. (E) Fluorescent pupa of the G1 generation of BmDuox knockout normal (left) and targeted pupa (right). doi:10.1371/journal.pone.0070118.g005

Figure 6. Identification of transgenic silkworms by molecular biology methods. (A) Amplification with ie-1-neo-sv40polyA-1/ie-1-neo-sv40polyA-2 primers (left) and egfp-1/egfp-2 primers (right) from fluorescent silkworms. (B) Identification of fluorescent silkworms with western blotting using GFP antibody (up) and by dot hybridization with a DIG-labeled ie-1 probe (down). (C) RT-PCR (up) and western blotting (down) analysis of the knockout silkworms (fluorescent silkworms) and normal silkworms. doi:10.1371/journal.pone.0070118.g006
compared to wt silkworm controls 12 h after bacterial feeding (Figure 7).

**Discussion**

The roles of ROS from Duox and Nox have been studied extensively in vertebrates and invertebrates but the role of ROS released during activation of the *BmDuox* gene in the silkworm, an important economic insect and a model organism in the Lepidoptera family, is not known. The *BmDuox* gene is expected to be of great importance in the response of silkworm innate immunity upon challenge by pathogens. In this study, the full length of the *BmDuox* gene was cloned. The ORF is 4,503 bp long and encodes a 1,500 amino acid protein with a theoretical mass of 172 kDa, which is to *M. japonicus* (~173 kDa), *D. rerio* (~172 kDa) and *D. melanogaster* (~178 kDa). The amino acid sequence of *BmDuox* has a high level of identity with Duoxes and some Noxes of other species, indicating Duoxes/Noxes are highly conserved among species. Furthermore, the signal peptide in the N-terminus was found only in *BmDuox* and *H. Duox2.*
Analysis of the BmDuox amino acid sequence revealed a peroxidase domain and a calcium-binding domain at the N-terminus and conserved FAD- and NAD-binding domains at the C-terminus. The structure of BmDuox was similar compared to Duoxes from invertebrates [35,36] and vertebrates [37,38,39]. There is a high level of identity among Duoxes, suggesting the role of these domains is conservative and BmDuox might have multifunctions that distinguish it from other organisms [40].

The peroxidase domain present in the N-terminus of Duoxes from various species is absent from Noxes. DmDuox has an additional peroxidase domain that could produce ROS in a regulated manner [41]. Although tissues are likely to be damaged by excessive ROS, over-proliferation of intestinal microbes is limited, indicating the role of ROS in vivo is controlled mainly by the interactions of multiple genes [8].

N-terminal EF-hand motifs are present in the calcium-binding domain. However, except for human HsNox1 and A. aegypti AaNox, a calcium-binding domain was found in all the aforementioned Duoxes/Noxes. The most diverse characteristic of the calcium-binding domain among various species is the number of EF-hand motifs. The BmDuox, DmDuox, HsDuox and DrDuox calcium-binding domains include three EF-hand motifs. It has been shown that intracellular concentrations of Ca^{2+} can moderate Drosophila Duox enzymatic activity via the EF-hand motifs [15]. Human Duox1 and Duox2 have a calcium-binding domain and Ca^{2+} is considered a regulatory factor for the activation of Duox via a pair of EF-hands [9]. Therefore, we suggest that the EF-hands of BmDuox are involved in the response to Ca^{2+} in a manner similar to those of the Drosophila and human Duoxes.

The transmembrane region of BmDuox contains six transmembrane sites, whereas DmDuox and AmDuox possess seven such sites. BmDuox has three phenylalanine and four glycine residues in transmembrane sites that are well conserved among the aforementioned Duoxes/Noxes. The role of these conserved sites in the transmembrane site warrants further study.

Homology analysis showed both the FAD- and NAD-binding domains of BmDuox share similar homologies with other species. The amino acid sequences SGQWVR, FTLTSAPHEN and 25 min. Similarly, expression of BmDuox mRNA expression level was initially dramatic differences in midgut loads were observed between wt and BmDuox knockdown [kd] silkworms after bacterial feeding, suggesting BmDuox has an important biological defense factor preventing pathogen infection because BmDuox mRNA was highly expressed after pathogenic stimuli. On the contrary, down-regulation of BmDuox mRNA expression would lead to increased bacterial load in the midgut. In D. melanogaster, Duox has an important role in host-microbe homeostasis [8,44]. Duox was shown to have an antimicrobial function in the intestinal epithelium [21]. A peroxidase/Duox system in A. gambiae was used to protect microbiota and malaria parasites in the midgut microbiota by preventing the activation of epithelial immunity to release immune elicitors [23].

We conclude the silkworm BmDuox gene has an important role in the innate immunity system. More studies are needed to fully understand the mechanism underlying the action of the BmDuox gene in innate immunity at the molecular level.

Supporting Information

Figure S1 Expression BmDuox in E. coli, BmDuox antibody preparation from New Zealand rabbits and the specificity of polyclonal antibody detection using extracted BmN cells. (A) Identification of pEASY-E1-BmDuox with BamHI and Hind III enzymes. (B) BmDuox samples were resolved by SDS/12% polyacrylamide gel electrophoresis (SDS-PAGE) under normal conditions. Left, SDS-PAGE; 3, protein molecular mass marker; 4, recombinant BmDuox expression in E. coli. Right, western blotting; 5, protein molecular mass marker; 6, recombinant BmDuox expression in E. coli. (C) The specificity of the antibody was verified by western blotting with extracted BmN cells. 7 and 8, BmDuox protein band; 9, protein molecular mass marker.

(TIF)

Figure S2 PCR analysis. (A) The 3’ RACE product of ~1,000 bp (containing the C-terminal partial CDS sequence). (B) The 5’ RACE product of ~1,300 bp (containing the N-terminal partial CDS sequence); M, Trans2K DNA marker.

(TIF)

Figure S3 Transmembrane region analysis and chromosomal localization of BmDuox. (A) Six transmembrane regions in BmDuox. (B) Chromosomal localization of BmDuox, three red-shaded stars indicate the best hit. (C) Predicted domain organizations of BmDuox from the silkworm.

(TIF)

Figure S4 Tertiary structure of BmDuox predicted by SWISS-MODEL.

(TIF)

Figure S5 Comparison of the deduced amino acid sequence of NADPH oxidase (BmDuox) from B. mori with other Duoxes. BmDuox was aligned with Duoxes from other species; BmDuox (JQ678349), DmDuox (NP_608715.2), DsDuox (BAF33370.1), HsDuox2 (NP_054799.4), HsDuox1 (NP_059130.2), RdDuox2 (NP_077055.1), RdDuox1 (NP_714961.1), MnDuox2 (NP_808278.2), MnDuox1 (NP_001029767.1), BrDuox (XP_002690988.1), OoDuox2 (NP_001177321.1), AnDuox (XP_624353.3) and AeDuox1 (XP_001658452.1) using the online multiple sequence alignment tool [http://multalin.toulouse.inra.fr/multalin/multalin.html]. Red, high consensus; blue, low consensus; black, neutral consensus. Consensus levels: high, 90%; low, 50% must be less than the first value.
Figure S6 Comparison of NAD-binding domain amino acid sequence of BmDuox with Duoxes of other species using the online multiple sequence alignment tool (http://multalin.toulouse.inra.fr/multalin/multalin.html). Red, high consensus; blue, low consensus; black, neutral consensus. Consensus levels: high, 90%; low, 50% must be less than the first value.

Figure S7 Phylogenetic relationship of the Duoxes and Noxes from the silkworm, other insects, vertebrates and plants. The deduced amino acid sequences were analyzed with ClustalW and MEGA 4 software. GenBank accession numbers of the other Duoxes/Noxes sequences are given in Table S2.

Figure S8 RT-PCR analysis of BmDuox expression in different developmental stages and different tissues. (A) Spatio-temporal distribution of BmDuox mRNA from egg to adult. (B) Tissue distribution of BmDuox mRNA in 3rd day of 5th instar larva. The actin 3 gene was used as the internal control.

Figure S9 Subcellular localization of BmDuox in BmN cells by immunofluorescence. BmN cells were fixed, probed with rabbit polyclonal BmDuox antibody to detect BmDuox, and visualized by FITC-labeled goat anti-rabbit IgG (green). Additionally, cells were stained with DAPI to visualize nuclear DNA (blue) directly. Cells were examined under a laser confocal scanning microscope (TCS SP5).

Figure S10 Induction expression of BmDuox and ROS generation in BmN cells and midgut. (A) ROS generation after SME treatment in BmN cells. (B) BmDuox expression pattern after SME treatment in BmN cells. (C) BmDuox expression pattern after DH5α treatment in the midgut. (D) BmDuox expression pattern after BmNPV treatment in the midgut. Results are expressed as the mean and standard deviation of three different experiments. Statistical differences were evaluated using Student’s t-test for unpaired samples. *P<0.05, **P<0.01, ***P<0.001.

Table S1 PCR primers used for BmDuox gene analysis.

Table S2 The symbols, GenBank accession numbers and nomenclature used in the phylogenetic analysis.

Table S3 Amino acid identity and similarity of the silkworm BmDuox gene compared to other known Noxes/Duoxes sequences using each domain. Upper triangle, identity; lower triangle, similarity. A, Peroxidase domain; B, transmembrane region; C, calcium-binding region; D, ferric-reductase; E, FAD-binding domain; F, NAD-binding domain.

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Author Contributions

Conceived and designed the experiments: XH XW. Performed the experiments: XH RY XZ LC XX. Analyzed the data: XH XW. Contributed reagents/materials/analysis tools: XH RY XZ LC XX CG. Wrote the paper: XH XW.

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