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siRNA-mediated knockdown of two tyrosinase genes from *Schistosoma japonicum* cultured *in vitro* ☆

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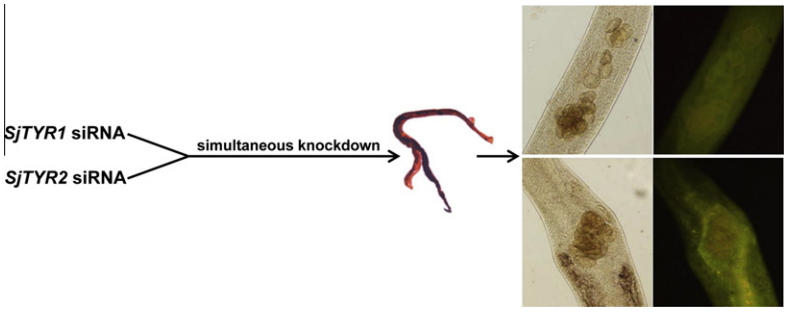
HIGHLIGHTS

- Two *SjTYR* chromosomal gene segments were sequenced.
- Knockdown of single and both *SjTYR* genes.
- The reductions in *SjTYR* enzyme activities followed the reductions in *SjTYR* transcript levels.
- Irregular eggs emerged in female uteri after *SjTYR* gene knockdown.
- Knockdown of both *SjTYR* genes has a greater effect than single knockdown of the *SjTYR* genes.

GRAPHICAL ABSTRACT



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ABSTRACT

The cross-linking process of eggshell proteins in helminths is dependent on the activities of tyrosinases (TYRs), which can be inhibited by phenol oxidase inhibitors. Two genes encoding TYRs, *SjTYR1* and *SjTYR2*, have been identified in *Schistosoma japonicum*. In this study, siRNA-mediated RNA interference (RNAi) was performed to silence these two *SjTYR* genes to evaluate their roles in eggshell formation. The effects of individual or double knockdown of the *SjTYR* genes were compared by determining *SjTYR1*/*SjTYR2* transcript levels, enzyme activities, and by observing the morphology and amounts of intrauterine eggs. Results showed that *SjTYR* transcript levels were significantly reduced on the 3rd day post-RNAi. Significant reductions in TYR enzyme activities, as well as obvious changes in morphology and the number of intrauterine eggs followed the reductions in *SjTYR* transcript levels. On the 8th day after simultaneous knockdown of both *SjTYR* genes, which effected a 40% reduction in *SjTYR1* transcript level and a 59% reduction in *SjTYR2* transcript level, we observed an 80% reduction in diphenol oxidase (DPO) activity of TYRs, and a 74% reduction in the number of normal eggs in female uteri. Knockdown of both *SjTYR* genes has a greater effect than single knockdown of the *SjTYR* genes. These results demonstrate that both *SjTYR*s play an important role in eggshell sclerotization of *S. japonicum*, and that their enzyme activities depend on the transcript levels of two *SjTYR* genes.

1. Introduction

Schistosoma japonicum infects humans in addition to other mammals, and is a major species of schistosomes in East Asia. The ovulation rate of *S. japonicum* is approximately 2200 eggs daily per female adult worm (Cheever et al., 1994). Schistosome eggs can be deposited in the host organs or be emitted into water with the host feces, the latter process representing the beginning of schistosomiasis transmission. Host immune responses are initiated by egg antigens secreted by the von Lichtenberg's envelope (Ashton et al., 2001; Dewalick et al., 2011) through microscopic pores within eggshells (Stenger et al., 1967). Subsequently, granuloma and fibrosis form, and represent the major risk factors of schistosomiasis (Smithers and Terry, 1969). Schistosome eggshells mainly

☆ Nucleotide sequence data reported in this paper are available in the GenBank database under accession numbers FJ617452 and JQ002653.

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consist of cross-linked proteins (Waite, 1995). The cross-linking of eggshell proteins is considered to be essential for helminth eggshell sclerotization, and is dependent on the activities of tyrosinases (TYRs) (Smyth and Clegg, 1959; Smyth and Halton, 1983; Nollen, 1971; Colhoun et al., 1998), which are copper-containing enzymes that catalyze the *ortho*-hydroxylation of monophenols to *o*-diphenols (monophenol oxidase activity, MPO, EC 1.14.18.1), and the oxidation of *o*-diphenols to *o*-quinones (diphenol oxidase activity, DPO, EC 1.10.3.1) (Garcia-borron and Solano, 2002). Schistosome TYR activities can be inhibited by phenol oxidase inhibitors. When cultured *in vitro* for 48 h in the presence of kojic acid, a DPO inhibitor, *Schistosoma mansoni* female adult worms have been shown to produce phenotypically abnormal eggs (Fitzpatrick et al., 2007). The activities of *S. japonicum* tyrosinases (*SjTYR*s) can also be inhibited by the phenol oxidase inhibitor, allyl thiourea. After treatment with allyl thiourea *in vivo*, the uteri of *S. japonicum* adult females were filled with granules instead of normal eggs due to a complete inhibition of the eggshell formation process (Cai et al., 2009).

Genes encoding two forms of TYRs in *S. japonicum* (*SjTYR1* and *SjTYR2*) and in *S. mansoni* (*SmTYR1* and *SmTYR2*) have been reported by Fitzpatrick et al. (2004, 2007). This group also examined the localization of DPO activities in these two trematodes using a modified *in situ* enzyme assay, and found that proteins containing DPO activities were mainly localized to the female eggshell-producing vitelline cells (Fitzpatrick et al., 2004, 2007). Previous work in our group showed that *SjTYR1* and *SjTYR2* share 38% of identity in amino acid sequence, and that *SjTYR* activities are not only localized to the vitelline cells but are present also in the shells of intrauterine eggs (Cai et al., 2009). We also found that transcript levels of both *SjTYR*s were higher in adult female worms than in adult males, and that *SjTYR1* was more highly expressed than *SjTYR2* (Cai et al., 2009).

RNA interference (RNAi), which results in post-transcriptional gene silencing (PTGS) (Bosher and Labouesse, 2000) in a wide variety of organisms, was originally identified in *Caenorhabditis elegans* (Tabara et al., 1998), and has since been used in human parasitic worms, including schistosoma (Boyle et al., 2003; Mourão et al., 2009; Ndegwa et al., 2007; Cheng et al., 2009; Tchoubrieva et al., 2010; Zhao et al., 2008). In eukaryotic cells, small interfering RNA (siRNA) can be recognized by the RNA induced silencing complex (RISC), which effects degradation of the target mRNA, and abolishes expression of the corresponding protein (Zamore et al., 2000). siRNA duplexes, which can be synthesized in large quantities and transfected into target cells, are the most commonly used reagents for RNAi in cultured cells (Milhavet et al., 2003).

In the present study, we wished to define the roles of two *SjTYR*s in eggshell formation. *SjTYR* chromosomal gene segments were amplified according to the published mRNA sequences of two *SjTYR* genes (Fitzpatrick et al., 2004), and siRNA duplexes, respectively targeting *SjTYR1* and *SjTYR2*, were used in the *in vitro* RNAi experiments. We compared the effects of knockdown of either the *SjTYR1* or *SjTYR2* genes, or of both *SjTYR* genes in tandem, by determining *SjTYR* transcript levels and DPO activities of female adult worms, and by observing the morphological characteristics and amounts of intrauterine eggs.

2. Materials and methods

2.1. Parasites

The life cycle of *S. japonicum* (Chinese mainland strain) was maintained in *Oncomelania hupensis* snails and specific pathogen-free (SPF) *BALB/c* mice (female) from the Department of Animal, Hubei Academy of Medical Science, China. The mice were artificially infected with cercaria, 40 ± 2 cercariae per mouse. 42 days post-infection, the adult worms were collected from the mice by dissecting the portal vein and mesenteric vein. A portion of the parasites was stored at −80 °C for further manipulation. The adult parasites for RNAi study were cultured in 6-well plates, which had 50 mated worms in each well with 4 ml of RPMI 1640 medium containing 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin G sulfate, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, at 37 °C and 5% CO_2. During cultivation, the media along with siRNAs were replaced every 2 days.

2.2. Amplification of the chromosomal segments of SjTYR1 and SjTYR2

Genomic DNAs were extracted from the *S. japonicum* adult worms using the Universal Genomic DNA Extraction Kit (Takara, Japan) according to the manufacturer's instructions. Chromosomal segments corresponding to *SjTYR1* and *SjTYR2* were amplified using the TaKaRa LA PCR™ Kit (Takara, Japan). PCR products were purified and sent to Sunny (Shanghai, China) for sequencing. The gene-specific primers for amplification of *SjTYR1* and *SjTYR2* are listed in Table 1.

2.3. siRNAs

SjTYR1 (GenBank accession No. AY815264) and *SjTYR2* (AY812 904) were selected as the target genes for RNAi. One negative control (NC) siRNA against Firefly Luciferase, and three siRNAs targeting each of two *SjTYR* genes, were designed and chemically synthesized by Ribobio (Guangzhou, China). The species-appropriate genome database (www.ncbi.nlm.nih.gov/BLAST/) was used to confirm that the sequences share no significant homology with other genes. The sequences of siRNAs are shown in Table 2.

2.4. Evaluation of efficiency of siRNA transfection into schistosomes cultured in vitro

Cy3-labeled NC siRNA, also synthesized by Ribobio, at a final concentration of 200 nM, was added into 4 ml of culture medium, in which adult worms were incubated for 3 h. After incubation, worms were washed 5 times with phosphate buffered saline (PBS, 140 Mm NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.2), then placed on a glass slide and observed by a confocal laser scanning microscope (CLSM) (Leica, Germany).

Table 1
Sequences of primers.

Assay		Primer sequences	Size (bp)
Amplification of chromosomal segments			
SjTYR1	Forward	5′-GAT TAC ATC ACC AAC AAT AAT GTT C-3′	10,175
	Reward	5′-GGA CAA TAA TAT GCA CGT TGT CCA T-3′	
SjTYR2	Forward	5′-CAT TCT ACG TAA CAA TCA CTT CTG T-3′	6002
	Reward	5′-CTC TAT ATT TAG GAC CAC ATC TCA TG-3′	
Semi-quantitive RT-PCR analysis			
SjTYR1	Forward	5′-CAA CGT TCA ATT AAA GTA CGA CGT G-3′	618
	Reward	5′-CGT CTA TAT CTG GTA GAT CAC TTC-3′	
SjTYR2	Forward	5′-GAA TAT TGC TCT CCG CCA AC-3	238
	Reward	5′-GAC GTA TTA GAA CCA GGA ATA G-3′	
Sj-tubulin	Forward	5′-CTA CTG TAG TGG ATG AAG TGC GAA C-3′	620
	Reward	5′-CAG CTG AAA TTA CTG GTG CAT AAG-3′	
Quantitative RT-PCR analysis			
SjTYR1	Forward	5′-GAA GTG ATC TAC CAG ATA TAG AC-3′	150
	Reward	5′- CTC ATA TTT GGA TAA GCA CCT G-3′	
SjTYR2	Forward	5′-ACC GAT AAC CTC TTG TT-3′	122
	Reward	5′-AGA ACC AGG AAT AGA AC-3′	
Sj-tubulin	Forward	5′-GTG CCT TCA TGG TAG ACA AC-3′	99
	Reward	5′-TGA CCA ATC AAG CGA TTC AG-3′	