**NOTE** *Parasitology*

# Gene Silencing of a Cubilin-Related Serine Proteinase from the Hard Tick *Haemaphysalis longicornis* by RNA Interference

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ABSTRACT. RNA interference (RNAi) has been recently exploited to determine gene function by degrading specific mRNAs in several eukaryotic organisms. We constructed a double stranded RNA (dsRNA) from a previously cloned *Haemaphysalis longicornis* serine proteinase (HlSP) gene to test the importance of the function of the HlSP gene product during blood-feeding. Growth of unfed ticks treated with HlSP dsRNA was significantly inhibited compared to that of PBS-treated ticks. This inhibition was supported by the level o f HlSP mRNA. HlSP may play a crucial role for blood-feeding in these ticks. This is the first report on gene silencing of a functional serine proteinase in hard ticks.

KEY WORDS: *Haemaphysalis longicornis*, RNA interference, serine proteinase.

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Ticks are ectoparasites that are well known as vectors for diseases of medical and veterinary importance [3]. Reliance of acaricides for tick control has led to serious problems including the development of tick resistance and harmful effects on the environment. In general, there has been a recent shift in parasite control from targeting the whole organism to manipulating drugs directed against specific molecules playing key roles in parasite survival [9, 20]. Proteolytic enzymes may represent effective target molecules for drug design because of its involvement in the mediation of a wide range of celluar processes such as protein metabolism and the processing of precursor proteins in living organisms [18]. In haematophagous ticks, studies have reported that aspartic and cysteine proteinases exert a proteolytic role in the midgut [15, 19] of the hard tick *Boophilus microplus*.

Recently, we cloned and partially characterized a gene that encodes a functional serine proteinase from the hard tick *Haemaphysalis longicornis* [16]. The ixodid *H. longicornis* is the primary vector of human and animal Babesia parasite in Japan [7, 10]. The *H. longicornis* serine proteinase (HlSP) was composed of complement C1r/C1s, Uegf, and bone morphogenic protein-1 domain, low-density lipoprotein receptor class A domain and catalytic domain. The endogenous form of the enzyme was intensely localized in midgut epithelial cells of the tick and demonstrated to be upregulated during blood-feeding. These data suggest that HlSP may play an important role in the digestion of host blood components.

RNA interference (RNAi) is becoming an increasingly powerful post-transcriptional gene silencing technique that is providing insight into gene function [5, 12, 14] and consequently increases the reliability of gene identification by homology search of databases. The RNAi process involves ATP-dependent production of small interfering RNA molecules from double strand RNA (dsRNA) and is thought to be responsible for targeting and destroying specific mRNAs facilitated by the formation of RNA-induced silencing complex (RISC). The RISC RNA molecules complementary to the mRNAs seem to work as a guide recruiting a ribonuclease that consequently cleaves only specific mRNAs [8, 11]. The RNAi process was studied in several eukaryotic organisms including ticks in the past few years by introducing a dsRNA derived from a determined gene sequence *in vivo* by injecting the dsRNA in the whole organism [1, 2, 13, 17].

In the present study, we introduced an HlSP dsRNA into *H. longicornis* by injection. Adult females of *H. longicornis* (parthenogenetic Okayama strain) were obtained as described previously [6]. The nucleotide sequence encoding the catalytic domain of HlSP was cloned into pBluescript II SK+ plasmid (Toyobo, Osaka, Japan) and the inserted sequence was amplified by PCR using the oligonucleotides T7 (5'-GTAATACGACTCACTATAGGGC-3') and CMo422 (5'-GCGTAATACGACTCACTATAGGGAACAAAAGCTGGAGCT-3') to attach T7 promoter recognition sites in both of the 5' and 3' end. The PCR products were gel purified using the gel extraction kit (QIAGEN, Maryland, U.S.A.) to synthesize RNA by *in vitro* transcription with T7 RNA polymerase (Ribomax™ Express Large Scale RNA Production Systems, Promega, Madison, WI) according to the manufacturer's protocol. Approximately 2 $\mu$g of dsDNA was used as a template and 50–100 $\mu$g of dsRNA was synthesized. We injected 1 $\mu$g of HlSP dsRNA in 0.5 $\mu$l of phosphate buffered saline (PBS) from the fourth coxae into the haemocoel of unfed adult *H. longicornis* females fixed on a glass slide with adhesive tape. The injections were carried out using 50 $\mu$l microcap-

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illaries (MICROCAP(r), Drummond Scientific, Broomall, PA) drawn to fine-point needles by heating. The needles were connected to an air compressor. Control ticks were injected with 0.5 $\mu l$ PBS alone. The ticks were allowed to rest for 1 day at 25°C. There was no mortality resulting from the injection alone as both control and HlSP dsRNA-treated ticks survived after injection while being held in an incubator prior to placement on the host.

Ticks were fed simultaneously on Japanese White rabbit as described previously [6]. The pattern of the control ticks injected with the buffer alone was comparable to that of un-injected ticks infested simultaneously on the same host. Obvious reductions in the body size were observed in the ticks treated with HlSP dsRNA (Fig. 1A, B). The body weights of ticks injected with HlSP dsRNA were significantly lower ($p=0.026$; 39% reduction in the average) than those of ticks injected with PBS alone (Fig. 1C). The group of dsRNA-treated ticks remaining attached with small sizes represents a clear example of an aberrant phenotype possibly resulting from distorting the mechanism by which ticks digest the host blood components. To confirm the gene disruption of HlSP, mRNA expression level was evaluated in HlSP RNAi treated ticks. Tick mRNA was isolated using QuickPrep™ Micro mRNA Purification Kit (Amersham Pharmacia, Piscataway, U.S.A.) as described by the manufacturer. Thirty micrograms of mRNA were submitted to reverse transcription (RT) before PCR. The RT reaction was done using RNA PCR Kit (AMV) Ver.3.0 (Takara Bio Inc., Otsu, Shiga, Japan) following the manufacturers instructions. A series of PCRs was carried out using 500 ng of each cDNA synthesis reaction and either HlSP specific oligonucleotides (5'-AATCGTCGGAGGCCGGGAGGCG-3' and 5'-TCAGAGGGTGCGCACTGCGTTCGC-3') or oligonucleotides specific for a control cDNA encoding a widely expressed *H. longicornis* antigen, troponin I [21] in a final volume of 50 $\mu l$. PCR was performed for 5 min at 95°C and for 25 cycles at 95°C and 55°C for 30 s and 72°C for 1.5 min, followed by elongation at 72°C for 5 min. We found a considerable decrease in the HlSP transcript in the HlSP dsRNA-treated group compared with that in the control as detected by the RT-PCR using HlSP and control (troponin I) gene specific primers (Fig. 1D). These findings suggest that posttranscriptional gene silencing had been achieved in *H. longicornis* treated with sequence-specific dsRNA. Thus, the present results establish a clear *in vivo* function of HlSP in blood-feeding of the hard tick. Our previous findings and present observations together may indicate that HlSP is a key enzyme in the blood-digestion of the tick. We are currently investigating tick bioactive molecules (TBM) established during the evolution of ticks and the function of TBM that *H. longicornis* has acquired as a babesiosis vector to clarify the physiological system involved in blood-feeding and transmission of the etiologic protozoa of babesiosis at the molecular level [4]. We have shown the usefulness of RNAi for examing the physiological role of TBM *in vivo* in the babesial vector *H. longicornis*.

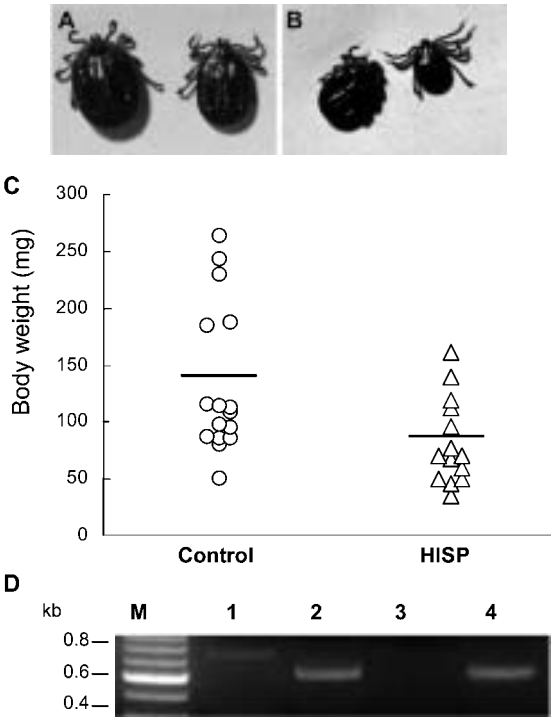


Fig. 1. Effect of dsRNA treatment on HlSP gene disruption. dsRNA complementary to HlSP was injected into the female *H. longicornis* adults. The injected ticks (20 individuals for each group) were allowed to feed, and 14 and 16 ticks (that were successfully attached to the host) were recovered from control and HlSP dsRNA-treated group, respectively, after 6 days of feeding. (A) Control ticks injected with PBS. (B) Ticks injected with HlSP dsRNA. (C) The body weights were recorded and plotted to assess feeding efficiency. Ticks in the HlSP dsRNA-treated group were significantly smaller in comparison with the control group. Bars indicate the average value for each group. (D) Reverse transcription-PCR analysis. PCR was performed using cDNA synthesized either from PBS-treated controls (lanes 1, 2) and HlSP dsRNA-treated ticks (lanes 3, 4) with the primer sets for HlSP (lanes 1, 3) and troponin I (lanes 2, 4). A troponin cDNA [21] remained unaffected in both HlSP dsRNA-treated and PBS-treated ticks. Lane M indicates molecular size in kb.

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