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Viral resistance in shrimp that express an antisense Taura syndrome virus coat protein gene

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Abstract

Taura syndrome virus (TSV) is a major cause of mortality and morbidity in shrimp, and has a profound economic impact on commercial U.S. shrimp farming. This paper describes the stable expression of an antisense Taura syndrome virus-coat protein (TSV-CP) gene construct in shrimp zygotes, via transfection using jetPEI reagent, over a period of at least 236 days. The transgenic shrimp showed no statistically significant difference from normal control shrimp in terms of weight gain or their appearance, morphology, swimming and eating activities. When challenged with live TSV, the transgenic shrimp exhibited increased resistance to the TSV infection (83% survival rate) as compared to control animals (44% survival rate). This work demonstrates that transgenic shrimp, which stably express an antisense transcript from the TSV-CP gene, are partially resistant to TSV infection. These data may have an important implication for commercial shrimp farming.

Keywords: Virus resistance; Transgenic shrimp; Taura syndrome virus; Antisense RNA; TSV coat-protein; Transfection

1. Introduction

In the last decade, world shrimp aquaculture has suffered significant losses due to serious virus outbreaks (Chamberlain, 1994; MacMillan, 1996; Brock, 1997; Lightner and Redman, 1998). Over 22 shrimp viruses have been described, several of which are highly pathogenic—including white spot baculovirus, yellow head virus and Taura syndrome virus (TSV). Collectively, these agents pose a serious problem to global shrimp production (You et al., 2004). However, there are currently no effective chemicals or drugs to treat viral diseases in shrimp. Strategies generally used in combating shrimp diseases include immuno-stimulation, vaccination, quarantining, and environmental management (Xiang, 2001). These strategies are non-specific in combating infectious diseases and cannot boost the shrimp's ability to cope with future infection even with the same pathogen. However, vaccine-based approaches are likely to prove difficult in shrimp since these organisms lack the ability to produce antibodies (Reichhart et al., 1992; Bachere et al., 1995; Glinski and Jarosz, 1997). Thus, controlling viral diseases clearly represents a great challenge in the shrimp aquaculture industry. The serious impact of viral disease on cultured shrimp, which is coupled with a decline in natural fisheries of healthy shrimp (Pullin et al., 1998), has led to a critical demand for advanced biotechnological applications.

In the present study, we have used genetic transfer technology to produce a TSV-resistant shrimp strain, through the expression of an antisense RNA that corresponds to a 493-bp fragment of the TSV coat protein (CP) gene. The expression vector, consisting of the chimeric shrimp beta-actin promoter (Sun et al., 2002) from *Penaeus vannamei* and 493 bp partial sequence of the target gene, TSV-CP, was constructed by the method described previously (Sun, 1997). Briefly, the pSV-Galactosidase vector (Promega) was used as the base vector; through PCR, *Nco*I and *Hin*dIII restriction enzyme sites were created at the 5′ end and 3′ end, respectively, of the beta-actin promoter, actP2. The SV40 promoter and enhancer of the pSV-Galactosidase vector were excised through restriction enzyme digestion with *Nco*I and *Hin*dIII, and the βactP2 was inserted into the vector to construct the expression vector, pβactP2-β-Gal. In addition, *Hin*dIII and *Sal*I restriction enzyme sites were added to the 493-bp TSV-CP target gene.

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Again through PCR, the *lac* Z gene of the pβactP2-Gal vector was replaced with the TSV-CP (AS) target gene in antisense orientation, thus producing the expression vector, pβactP2-TSV-CP (AS). The orientation of the strand of TSV-CP gene was confirmed by DNA sequencing in both directions using an automated DNA sequencer (model 373A, Applied Biosystems Inc., Foster City, CA, USA). General procedures of ligation, cloning, and plasmid DNA purification followed the methods of Sambrook et al. (1989).

An established jetPEI-based transfection protocol in this laboratory was employed for gene delivery into shrimp eggs (Calderon and Sun, 2003). Briefly, 800–1000 fertilized shrimp eggs approximately 5 min post-spawning were transferred into a Petri dish (35 mm × 10 mm) containing 1 μg of the expression vector, pβactP2-TSV-CP (AS), and 1.2 μL of the transfecting reagent, jetPEI (7.8 mM) in 2.0 mL of sterile seawater. Following a 50-min incubation at room temperature, the reaction mixture of approximately 2.5 mL was transferred into a 1-L beaker filled with sterile aerated seawater at 28 °C for the hatching process. After recording the hatching rate at 24–36 h after treatment, the transfected shrimp were transferred to 55-gallon tanks for further development and growth. The control shrimp were treated in the same manner except that no plasmid DNA was introduced into the shrimp eggs. The putative transgenic shrimp and control animals were raised in aerated seawater at 28 °C and fed with commercial pelleted shrimp feed twice a day, and screened for the transgene expression by reverse transcription-polymerase chain reaction (RT-PCR) or by genomic PCR assay at various developmental stages (130, 190, and 236 days). For TSV-CP screening, two pleoplods (swimmerets) from experimental shrimp were excised and homogenized with a mortar and pestle pre-chilled in liquid nitrogen. RNA or genomic DNA was isolated using the Purescript RNA or DNA isolation kits (Gentra). For RT-PCR reaction, total RNA (0.2–1.0 μg) isolated from jetPEI/DNA transfected shrimp and normal control animals was used as a template, with TSV-CP gene-specific primer pair A (primer 1: 5′-CTTAATTAATGCCTGCTAACCC-3′ and primer 2: 5′-ATTGATGTCTGCTTAGCATTCA-3′). In order to confirm the presence of the target gene in the transgenic shrimp, a second round PCR reaction was performed using 1 μL of the RT-PCR product as a template and the TSV-CP nested gene-specific primer pair (primer 3: 5′-TGATACAACAACCAGTGGAGGAC-3′ and primer 4: 5′-TGTCATCAGGTAGGGAAATTTC-3′). These two sets of oligonucleotide PCR primers were designed basing on the published genomic sequence of the TSV-CP (GenBank Accession No. AF277378). The RT-PCR procedure was as described by Sun (1995) using the GeneAmp PCR Kit (PE Biosystems). For genomic PCR assay, 1 μg of genomic DNA isolated from the pleopods, was used as template. Either gene-specific oligonucleotide pairs of primer 1 and primer 2 or primer 3 and primer 4, that are complementary to the TSV target genes were used as primers in the PCR reactions. Genomic DNA amplification was performed by following the instruction suggested by the vendor (Perkin Elmer). In brief, reaction mixtures were initially denatured at 94 °C for 5 min, then programmed for 1 min at 95 °C and 1.5 min at 55 °C for 35 cycles, followed by 7 min at 67 °C for 1 cycle as a final extension in a DNA Thermal Cycler (Perkin-Elmer 9600). PCR products were subjected to 1.0% agarose gel electrophoresis and the expected DNA band of either 493 bp (when primer 1 and primer 2 were used) or 302 bp (when primer 3 and primer 4 were used) was revealed following ethidium bromide staining, indicating the presence of the TSV-CP target gene in the shrimp.

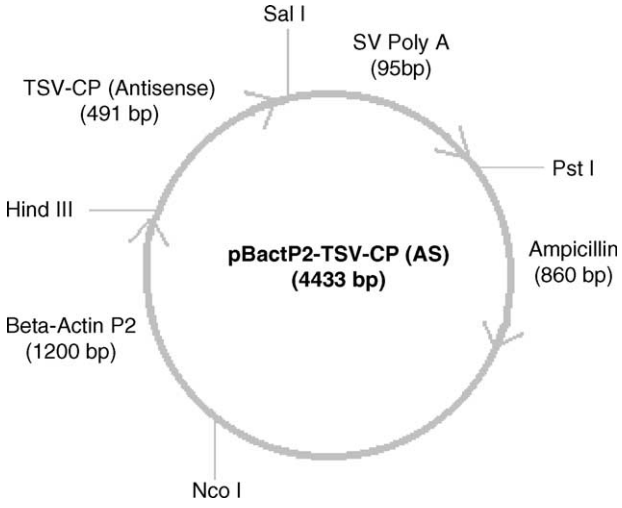

Fig. 1. Schematic structure map of the expression vector pβactP2-TSV-CP (AS).

As expected, the fertilized eggs of *Litopenaeus vannamei*, transfected with the expression vector, pβactP2-TSV-CP (AS) (Fig. 1) were found to be positive for the presence of the transgene. Under the described laboratory conditions, shrimp zygotes that were transfected with plasmid DNA via jetPEI showed a 50–60% hatchability, 35–50% post-hatching survival rate, and a 64% gene transfer efficiency as determined by the RT-PCR method (Table 1). This rate of gene transfer efficiency was substantially greater than that obtained using other gene transfer techniques including microinjection and electroporation (Sun et al., 2005). As such, this finding is consistent with the recent reports of successful use of transfection reagents for transferring desirable genes into mammalian

Table 1
A summary of gene transfer into shrimp zygotes

Experimental[a] shrimp	Hatchability (%)	Post-hatching survival (%)	Transfection[b] efficiency (%)
Test group	50–60	35–50	64 (64/100)
Control group	50–60	40–60	0 (0/25)

N/A: not applicable.

[a] Delivery of plasmid DNA into fertilized shrimp eggs (test group) by jetPEI-based transfection as described in the materials and methods; control animals were untreated.

[b] Transfection efficiency was determined by PCR screening of genomic DNA isolated from the swimming legs of individual juvenile and adult shrimp for the presence/absence of the target gene, TSV-CP.

cells and other vertebrates (Abe et al., 1998; Carballada et al., 2000; Wall, 2002). In addition to remarkable gene transfer efficiency, the current application of jetPEI reagent for gene transfer in shrimp represents a new, rapid and simple way of delivering exogenous DNA into shrimp.

To determine the constitutive expression of the target gene in transgenic shrimp, total RNA was isolated from various organs and tissues including gills, eyestalks, stomach, heart, hepatopancrease, pereiopods (walking legs), pleopods (swimmerets), body muscles, and hemolymph from transgenic shrimp at day 236 by Purigene RNA isolation kits (Gentra) and evaluated by the RT-PCR. As shown in Fig. 2, the TSV-CP gene was detected in the majority of tissue specimens (6/9) tested from transgenic shrimp, whereas all the RNA isolated from different organs/tissues of normal control shrimp was negative for TSV-CP RNA. The fact that the target gene was not detected in pereiopods and gills is difficult to interpret, but not surprising, since very little RNA can be extracted from these very small tissue specimens (thus making detection of any transcript very difficult). However, we have no explanation for the absence of TSV-CP gene expression in heart tissue, especially because expression of this gene was detected in hemolymph. Additional studies will be needed in the future, using large groups of animals, to clarify if the transgene is truly not expressed in heart, gill and pereiopods of transgenic shrimp.

Shrimp tested positive for the target gene, TSV-CP, were transferred to a growth tank (55-gallon) where they were reared further to about 5–10 g in weight and then tested for TSV infection. Viral challenge bioassay was conducted by using two different infection routes: (1) oral-feeding of 20% (w/v) tissue homogenate of TSV-infected shrimp and (2) direct intramuscular (i.m.) injection of a purified virus stock. A total of 18 test shrimp were equally divided into two groups: for oral-feeding infection, nine animals were transferred to two 1-L beakers containing 350 mL of the seawater (four to five animals per beaker) and fed with 0.5 mL/beaker of the tissue homogenate. The animals were kept in the beaker for 1.5 h, then poured together with the seawater back to a 38-L aquarium. For control, 10 normal shrimps were treated the exactly same way. For the IM infection, both test (9) and normal (10) shrimps received an IM inoculation of a purified TSV stock (5×10^6 LD_{50}/mL) at 0.1 mL/animal. A third group of 10 normal shrimp was injected (IM) with the same volume of PBS (pH 7.4) as negative control. Moribund/dead shrimp were collected and recorded daily for 12 days and cumulative mortality was plotted as percentage (%) of total shrimp. All experimental shrimp were housed in 38-L glass aquaria with approximately 15–20 parts per thousand (ppt) seawater at room temperature (22–23 °C) and fed with commercial pelleted shrimp feed every other day during the course of the experiments. Except for water circulation through a filter, no change of water was done during the period of the experiment as described previously (Lu et al., 1994). Water discarded from the experimental tanks was decontaminated with 50 parts per millions (ppm) chlorine overnight or longer before being discharged into the municipal sewage line.

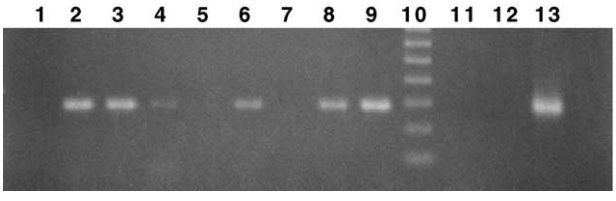

Fig. 2. Detection of transgene expression in different organs/tissues from transgenic shrimp at growth day 236, using RT-PCR. Lanes: 1, gill; 2, stomach; 3, hemolymph; 4, hepatopancrease; 5, heart; 6, body muscle; 7, pereiopods; 8, pleopods; 9, eyestalk; 10, 100-bp DNA ladder; 11–12, negative controls (H_2O and RNA from body muscle of normal control shrimp, respectively); and 13, positive control (pβactP2-TSV-CP).

Following exposure to infectious TSV, the initial appearance of moribund shrimp was detected at day 3–4 post-infection for both control and test groups. However, more shrimp died in the control group as compared to the transgenic animals (Table 2). Statistical analysis using paired *t*-test revealed that adjusted death rates between test and positive control groups were significantly different ($p < 0.05$) and these data suggest that the transgenic shrimp are at least partially protected from TSV infection. We confirmed that the TSV-CP antisense RNA was expressed in the surviving transgenic shrimp (data not shown), which also exhibited the typical dark-pigment deposition on their back shell, which is a characteristic of TSV-infected survivors (data not shown). Notably, these animals were negative for the sense RNA and for viral genomic RNA—indicating that they had successfully cleared their viral infection.

To determine if the introduction of the TSV-CP gene leads to any adverse effect on shrimp growth and development, transgenic shrimp were raised and maintained at the same laboratory conditions as control animals for a period of 236 days. Comparative analysis of the growth kinetics between test and control shrimp revealed that there was no statistically significant difference in the growth rate of transgenic shrimp as compared to normal control animals, as measured by body weight. In addition, long-term observation revealed that the transgenic shrimp were active and healthy, with a normal

Table 2
Summary of mortality data for test and control shrimp infected with TSV by both oral-feeding and intramuscular (i.m.) injection

Infection	Group	Number of shrimp seeded	ADR	Death rate (%)
Oral-feeding	T	9	1/6	16.7
	C+	10	5/9	55.6
IM Injection	T	9	1/7	14.3
	C+	10	5/10	50.0
	C−	10	0/8	0

ADR: adjusted death ratio where death due to injection trauma or molting-predation was excluded; T: transgenic shrimp; C+: positive control shrimp (normal); and C−: negative control shrimp (normal) with no TSV infection. *p*-value of comparing T and C+ (in both groups) using paired *t*-test is less than 0.05.

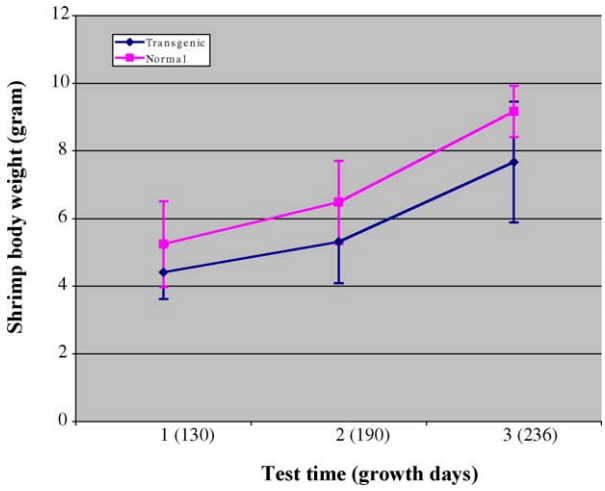

Fig. 3. Comparative analysis of growth kinetics between transgenic and normal control shrimp at three selected growth time. Data presented correspond to mean values of five individual test and error bars represent the standard error of mean values. Paired *t*-test indicated that these two growth curves are similar.

appearance, during the entire course of this study. However, there was a slight trend towards a reduction in weight gain in the transgenic animals (Fig. 3). We speculate that target gene integrated into the specific site of the shrimp genome may affect the early growth of transgenic animals. We also documented apparent variation in body size of transgenic shrimp and we conclude that this difference may be a reflection of differences in the chromosomal integration site with some of the transgenic shrimp—since some transgenic animals reached a normal size, while others appeared noticeably smaller (Fig. 4). These data suggest that it is possible to obtain good trait of transgenic shrimp for commercial application through screening test and selection.

Overall, our experimental data clearly suggest that introduction of an antisense TSV-CP transgene under the transcriptional control of the shrimp-β-actin promoter could provide transgenic shrimp with a substantial protection from TSV infection. This protective effect most likely reflects the sequence-specific degradation of viral TSV transcripts, which are predicted to form double stranded RNA (dsRNA) duplexes in the presence of the antisense RNA (Ahlquist, 2002; Tang et al., 2002). These duplex RNAs are targeted by dsRNA-specific nucleases (Dalmay et al., 2000; Mourrain et al., 2000), and may also interfere with protein translation and viral genomic replication. Antisense RNA is a well-established nucleic acid-based antiviral strategy. This technique based on the recognition of specific target in a highly sequence-specific matter to down regulate target gene expression or to combat viral replication has been widely documented in vitro and in vivo tests (Sczakiel, 1995; Abe et al., 1998; Blair et al., 2000; Barnor et al., 2004; Friedrich et al., 2004; Li et al., 2004; Liu et al., 2004; Lu et al., 2004). Our data represent the first report of the application of antisense RNA technique in crustacean and our finding extends and substantiates current test and development of viral-resistance in animals and plants through the production of viral genes, particularly viral coat protein RNA (Han et al., 1991; Sasaki et al., 1993; Gaines et al., 1996; Johnson et al., 1999; Pang et al., 2000; Gitlin et al., 2002). A recent publication by Robalino et al. (2004) reported the sequence-independent protection against viral infection in marine shrimp by dsRNA, which provides the first evidence for the existence of an inducible, innate antiviral defense mechanism in invertebrates. Thus, we cannot exclude the possibility that the partial antiviral activity of the transgenic shrimp may also be attributed to the sequence-independent effect of the antisense TSV-CP gene construct. Further studies will examine the underlying mechanism of virus

(A)

(B)

	Shrimp weight (g) 130	190	236 (day)
Transgenic shrimp	3.68	4.36	8.03
	4.02	6.82	7.51
	4.93	5.39	5.57
	5.55	6.12	10.4
	3.89	3.86	6.83
AV	**4.41**	**5.31**	**7.67**
Normal shrimp	6.21	4.54	10.3
	6.58	5.99	8.46
	5.59	7.13	9.26
	4.01	7.25	8.48
	3.82	7.49	9.33
AV	**5.24**	**6.48**	**9.17**

Fig. 4. A comparison of transgenic shrimp with normal control shrimp in their morphological appearance (A) and body weight (B). AV: average body weight.

inhibition, but the present data clearly establish that one can readily generate transgenic shrimp that resist infection by a major viral pathogen. This may have important commercial implications in the world shrimp farming industry.

Acknowledgements

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