Journal of Invertebrate Pathology 99 (2008) 136–140

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/yjipa

Infection characteristics of *Solenopsis invicta* virus 2 in the red imported fire ant, *Solenopsis invicta*

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ARTICLE INFO

Article history:
Received 25 January 2008
Accepted 11 June 2008
Available online 19 June 2008

Keywords:
Solenopsis invicta virus 2
Positive-strand RNA virus
Prevalence
Tissue tropism
Transmission
QPCR

ABSTRACT

Solenopsis invicta virus 2 (SINV-2) is the second virus identified from the fire ant, *S. invicta* Buren. SINV-2 is unique among positive-strand RNA viruses from insects by possessing four cistrons in a monopartite genome. Fire ant colonies testing positive for SINV-2 by RT-PCR did not exhibit any discernable symptoms. RT-PCR-based surveys for SINV-2 among 688 fire ant mounds in Alachua County, Florida, sampled during the period January, 2006 through December, 2007 showed that the prevalence of SINV-2 among nests ranged from 1.6% to 16.4%. Unlike *S. invicta* virus 1, no seasonal-associated prevalence was observed with regard to SINV-2 infection among fire ant colonies. No social form specificity was evident; SINV-2 was found in both monogyne and polygyne *S. invicta* ants. Real-time quantitative PCR experiments showed that SINV-2 genome equivalents per individual ant ranged from 1.9×10^7 in. pupae to 4.3×10^{11} in. inseminated queens. The SINV-2 infection was detected in all ant stages examined (eggs, larvae, pupae, workers, and queens). Tissue tropism studies indicated that the alimentary canal (specifically the midgut) is most likely the susceptible tissue. SINV-2 was successfully transmitted to uninfected *S. invicta* ants by feeding a partially purified homogenate of SINV-2-infected ants. The SINV-2 transmission rate ranged from 30% to 80%, and both positive (genomic) and negative (replicative) SINV-2 RNA strands accumulated in recipient ants over the course of the experiment. These results indicated that SINV-2 replicates within *S. invicta*.

1. Introduction

Red imported fire ant, *Solenopsis invicta* Buren, was introduced into the United States from South America in the early 1900s (Creighton, 1950). Damage attributed to this aggressive, territorial ant species is wide-ranging and includes physical damage to agricultural commodities and equipment, roads, electrical equipment, and livestock, decreased biological diversity, and human health concerns associated with stings (Williams et al., 2001). Nearly all of the natural enemies of *S. invicta* identified in South America are absent from the US population which is likely the reason *S. invicta* has become such a major pest in the US (Porter et al., 1992, 1997). Thus, identification and utilization of natural enemies specific to *S. invicta* are considered to be crucial in achieving sustainable control (Williams et al., 2003).

We reported recently the discovery of a new virus (*S. invicta* virus 2 [SINV-2]) from *S. invicta* (Hashimoto and Valles, 2008; Valles et al., 2007a). SINV-2 is only the second virus known to infect this ant species. Molecular characterization of SINV-2 revealed a single-stranded positive sense RNA genome comprised of 10,303 nucleotides and a polyadenylated tail at the 3′ terminus. The SINV-2 genome is unique among other insect-infecting RNA viruses in that it possesses four open reading frames (ORFs) in a monopartite genome. Protein domains consistent with positive-strand RNA viruses (i.e., an RNA-dependent RNA polymerase [RdRp], helicase, and protease) were present in the ORF 4 of the SINV-2 genome. Phylogenetic analysis of SINV-2 RdRp and helicase amino acid sequences indicated unique placement of SINV-2 exclusive from the *Dicistroviridae* (including the first identified fire ant virus, SINV-1), iflaviruses, and plant small RNA viruses. Although an initial molecular characterization of SINV-2 has been completed (Valles et al., 2007a), information concerning the infection characteristics of this virus in the ant host is lacking. Therefore, descriptive studies focused on the infection characteristics (tissue tropism, stage-specificity, transmission, replication, and prevalence) of SINV-2 in the fire ant were conducted.

2. Materials and methods

2.1. Field surveys and social form specificity

A field survey was conducted to examine the extent of SINV-2 infection among *S. invicta* mounds in Alachua County, Florida. Samples of workers were retrieved from the field and RNA

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0022-2011/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2008.06.006

was extracted from the ants with Trizol reagent (Invitrogen, Carlsbad, CA). SINV-2-specific oligonucleotide primers (p64, 5′-ATTTGTTTGGCCACGGTCAAC, genome position 10,758→10,780, and p65, 5′-GATGATACAAAGCATTAGCGTAGGTAAACG, genome position 11,047←11,076) were used in an RT-PCR reaction to determine the presence of infection. In an effort to determine possible ant social form specificity of SINV-2, each mound that tested positive for SINV-2 was also genotyped at the *Gp-9* locus to determine the social form of the nest (Valles and Porter, 2003).

2.2. Stage specificity and tissue tropism

Experiments were conducted to examine the developmental stages of *S. invicta* for SINV-2 infection. Samples of queens ($n = 4$ individuals), workers ($n = 5$ groups of 10 individuals), larvae ($n = 5$ groups of 10 individuals), sexual larvae ($n = 12$ individuals), eggs ($n = 2$ masses of an unknown number of eggs), and pupae ($n = 5$ groups of 10 individuals) were sampled directly from the field from SINV-2-positive colonies. RNA was extracted from the ants with Trizol reagent. The RNA concentration was determined spectrophotometrically.

Tissue tropism of SINV-2 was examined by dissecting different tissues and quantifying the number of SINV-2 genome equivalents in each respective preparation. Ice-chilled fire ant workers from a SINV-2-positive colony were placed on a glass microscope slide, and head, thorax, and abdomen were separated with a surgical blade under a Leica MS5 dissecting microscope. The head and thorax were placed directly in Trizol and the abdomen was immersed in 10 mM Tris–HCl, pH 8.0, for further dissection. Tissues were isolated and removed from the abdomen with micro-dissecting forceps in the following order: crop, poison sac, alimentary canal (comprised of the midgut, hindgut, and Malpighian tubules), and the remaining abdominal carcass (comprised of the Dufour's gland, ovary, fat body, muscle, and cuticle). The alimentary canal was later examined further by separating the midgut, hindgut, and Malpighian tubules. Larvae from SINV-2 positive colonies were dissected to the following groups: Malpighian tubules, alimentary canal, and remaining carcass. Five replicates were conducted. RNA was extracted with Trizol.

The number of SINV-2 genome equivalents in different developmental stages and tissues were quantified by QPCR. cDNA was synthesized from the SINV-2 genome region corresponding to an area between the helicase and RdRp with total RNA isolated from dissected tissues and stages using SuperScript III Reverse Transcriptase (SsRT; Invitrogen) and a gene-specific primer (p514, 5′-TA CACTTGGGTCTCAGGAACC, genome position 8816←8836). In a 0.5 ml PCR tube, 2 μl of primer p514 (1 μM), 1 μl of a dNTP mix (10 mM), and 10 μl of total RNA (50 ng) were mixed and heated to 65 °C for 5 min in a PTC 100 thermal cycler, followed by incubation on ice for 1 min. Then, 4 μl of first-strand buffer (250 mM Tris–HCl, pH 8.3; 375 mM KCl, 15 mM $MgCl_2$), 2.75 μl of DEPC-treated water, and 0.25 μl of SsRT (200 U/μl) were added. The mixture was incubated at 55 °C for 30 min, followed by inactivation of SsRT by heating to 70 °C for 15 min.

QPCR was performed on an ABI PRISM 7000 Sequence Detection System interfaced to the ABI prism 7000 SDS software (Applied Biosystems, Foster City, CA) in a 25 μl reaction volume. The reaction contained 12.5 μl of SYBR Green SuperMix (with UDG and ROX, Invitrogen), 0.4 μl each of 10 μM SINV-2-specific primers (p511, 5′-CGGAGACACTGAGCCTTTCTGGACTCCATAG, genome position 8677←8707 and p515, 5′-TGTATCGCGGAAATTACCCAACATCA CAAC, genome position 8584→8613), 3 mM $MgCl_2$, 1 μl of the cDNA synthesis reaction, and 10.7 μl of DEPC-water. QPCR conditions consisted of one cycle at 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 64 °C for 15 s, 72 °C for 1 min. The non-template control for QPCR included a complete cDNA synthesis reaction devoid of RNA template. A standard curve was constructed from a plasmid clone of the corresponding SINV-2 genome region using a copy number range of $5–5 \times 10^7$ copies. Reaction efficiencies were determined by regressing C_T values against the template copy number (log) and calculated according to the formula $[E = (10^{-1/\text{slope}}) - 1]$ (Klein et al., 1999). Reaction efficiencies routinely exceeded 96%.

QPCR was also conducted to separately quantify the plus (genomic) and minus (replicative) RNA strands of SINV-2. cDNA was synthesized from the SINV-2 plus strand with oligonucleotide primer p511 and minus strand with oligonucleotide primer p515 as described above. After cDNA synthesis, the RNA templates were digested with RNase A and RNase H at 37 °C for 30 min. After RNA digestion, QPCR was conducted as described above with oligonucleotide primers p511 and p515.

2.3. SINV-2 transmission to uninfected fire ants

SINV-2-uninfected laboratory-reared monogyne colonies were identified by RT-PCR and divided into four equivalent fragment colonies comprised of 0.5 g of brood and 4 ml of workers. Colonies were infected by a modified method described by Ackey and Beck (1972). Workers and brood (0.15 g) from a SINV-2-infected colony were homogenized in an equal volume of 10% sucrose with a Potter-Elvehjem Teflon pestle and glass mortar. The homogenate was filtered through four layers of cheesecloth. Approximately 4 ml of the homogenate/sucrose solution was placed into a cotton-stopped test tube and presented to three of the four fragment colonies; one fragment colony was provided a homogenate of uninfected ants and served as control. After 2 days, the homogenate was removed and replaced with unadulterated 10% sucrose, water, frozen crickets (*Acheta domesticus*), and egg yolk (hard-boiled). Three experiments were conducted comprised of three colonies that had been fragmented into four sub-colonies (one control and three treated for a total of nine experimental units experiencing treatment). Fragment colonies were examined for the presence of SINV-2 by extracting total RNA from 20 worker ants and conducting RT-PCR at 0, 7, 14, 21, and 28 days after exposure to the homogenate. Percent infection, by day, among recipient fragment colonies was analyzed by analysis of variance (SAS Institute, 1988). Samples testing positive for SINV-2 by RT-PCR were analyzed by strand-specific QPCR to quantify the genome equivalents (plus and minus strand).

3. Results and discussion

3.1. Field prevalence and fire ant social form

Among 688 *S. invicta* nests sampled from January, 2006 through December, 2007, 38 (5.5%) were found to be infected with SINV-2 by RT-PCR (Table 1). Sampling during the first quarter of 2006 yielded the lowest SINV-2 prevalence (1.6%) and the fourth quarter of 2006 the highest (16.4%). The nest infection rate for SINV-2 was lower than either *S. invicta* virus 1 (SINV-1) genotype; SINV-1 and SINV-1A were found previously in 22.9% and 55% of *S. invicta* nests sampled, respectively (Valles et al., 2004; Valles and Strong, 2005). The prevalence of the SINV-2 infection was independent of collection time. In other words, the presence of the SINV-2 infection in *S. invicta* nests did not exhibit any apparent pattern with respect to season. SINV-1 and SINV-1A infections of fire ants were found in significantly greater frequency during the late spring, summer, and early fall in Alachua County, Florida (Valles et al., 2007b). Further examination revealed that the SINV-1/SINV-1A infection prevalence exhibited a significant correlation with temperature (Valles et al., 2007b). A number of other insect-infecting, positive-strand RNA viruses have been shown to exhibit either seasonal fluctuations (Bailey et al., 1981, 1983; Tentcheva et al., 2004) or greater