

TECHNICAL NOTE

Longevity of ingested mRNA transcripts in the gut of a homopteran (*Bemisia tabaci*): avoiding experimental artifacts

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Introduction

Bemisia tabaci (Genn.) (Homoptera: Aleyrodidae) biotype B, also known as *Bemisia argentifolii* (Bellows et al., 1994), has been characterized as the main vector of begomoviruses, a virus genus that poses a major threat to the production of many vegetable crops worldwide (Polston & Anderson, 1997; Mansoor et al., 2003). Begomoviruses are transmitted by whiteflies in a persistent circulative manner (Hunter et al., 1998; Rosell et al., 1999; Ghanim & Czosnek, 2001). However, important aspects of virus–vector interactions at the molecular level remain controversial. Replication and/or molecular activity of some begomoviruses in the whitefly have been suggested (Ghanim & Czosnek, 2000; Czosnek et al., 2001; Sinisterra et al., 2005). We have studied the genetic activity of a begomovirus, tomato yellow leaf curl virus (TYLCV), in the whitefly vector by measuring the presence of selected virus transcripts (V1, V2, and C3) using real-time reverse transcription-polymerase chain reaction (RT-PCR) methods (Sinisterra et al., 2005). In those studies, it was important to rid the insect of ingested host derived virus particles and products that do not cross the gut-epithelium. In order to conduct those quantitative studies, we needed to find a method that would guarantee the removal of any virus transcript ingested by the whiteflies and remaining in the alimentary canal, thereby

allowing the quantification of virus transcripts that are either stably acquired or synthesized de novo in the insect. Laboratory manipulation of different RNA species has shown that RNA is very easily degradable due to the ubiquitous presence of RNases. These enzymes can maintain their activity even after being subjected to many forms of harsh treatments including autoclaving and boiling (Sambrook & Russel, 2001). Because high RNase activity has been identified in plant phloem sap, RNase inactivation techniques have been used in studies that intend to detect plant mRNA moieties in samples collected by severing the stylets of aphids that were allowed to feed overnight on barley plants (Doering-Saad et al., 2002). We tested the efficiency of two gut-clearing methods: (i) non-viral host feeding and (ii) artificial diet feeding by monitoring the permanence of ingested plant mRNA transcripts within non-viruliferous and viruliferous insects.

The work presented is to call attention to the finding that typical RNA transcripts are not as unstable in the alimentary canal of phloem feeders such as whiteflies (and potentially other homopterans) as may have been thought. The stability of such molecules should be taken into consideration in the experimental design of studies concerning molecule retention/interaction studies in insects.

Materials and methods

Source and maintenance of insects

Adult male and female whiteflies, *B. tabaci* biotype B, were obtained from mixed age/sex laboratory colonies maintained by the US Horticultural Research Laboratory (Fort Pierce, FL, USA). Whiteflies used in these experiments were originally obtained from Dr. Lance Osborne, University of Florida in Apopka, FL, USA, and have been maintained on

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dwarf cherry tomato (*Lycopersicon esculentum* cv. Florida Lanai) since 1996 by serial transfer. Whitefly biotyping was based on random amplification of polymorphic DNA-PCR (RAPD-PCR) analysis using primers developed by De Barro & Driver (1997). Non-viruliferous and viruliferous whitefly colonies were housed separately in screened plexiglass cages located in separate walk-in environmental growth chambers (Environmental Growth Chambers, Chagrin Falls, OH, USA) at 25 ± 1 °C under a L16:D8 photoperiod and an average light intensity of 700 μ E photosynthetically active radiation (PAR) at the top of plant canopy.

Virus source

In 2001, a TYLCV viruliferous whitefly colony was established using cuttings from field grown tomatoes infected with TYLCV obtained from Dr. David Schuster, University of Florida in Bradenton, FL, USA. Tomato yellow leaf curl virus-infected tomato cuttings were kept in water until roots sprouted. Rooted cuttings were planted in 15.24-cm pots and infested with whiteflies from the non-viruliferous colony. After establishment, serial transfers on dwarf cherry tomato have maintained the virus colony. The presence of the virus was assessed by visual identification of symptom development and PCR amplification with virus-specific primers (Pico et al., 1998) (Table 1).

Whitefly sucrose chamber feeding experiments

Approximately 5000 whiteflies were vacuumed from both non-viruliferous and TYLCV-viruliferous colonies with a

Makita Handy Vac Model 4071D (Makita Inc., La Mirada, CA, USA) (outfitted with plastic vials screened at the bottom for collection). Half of the whiteflies were immediately frozen in liquid nitrogen and stored at -80 °C. The remaining 2500 whiteflies from each colony were placed into different magenta boxes ($66 \times 99 \times 68$ mm). The open end of the box was quickly covered with a thin layer of parafilm stretched uniformly to insure a secure fit. One corner of the parafilm was lifted to produce a depression in the center to accommodate 1–2 ml of 30% sucrose solution. A second layer of parafilm was used to tightly cover and spread the solution evenly. The magenta box was enveloped in aluminum foil leaving the parafilm end with the sucrose exposed to light, which stimulated the whiteflies to move toward the food source. Whiteflies were given access to sucrose solution for 72 h in the feeding chamber and then frozen in liquid nitrogen and stored in a -80 °C freezer until ready for total RNA extraction. Non-viruliferous and viruliferous whitefly experiments were run separately, but simultaneously, and repeated twice.

Whitefly non-viral host plant feeding experiments

Approximately 5000 whiteflies were collected from both non-viruliferous and TYLCV-viruliferous colonies. Half of the adults were immediately frozen in liquid nitrogen and stored at -80 °C, and the remaining whiteflies were placed on 5–6 cotton plants in large screened plexiglass cages for 72 h. After 72 h, whiteflies were vacuumed from the plants, frozen in liquid nitrogen and stored at -80 °C until ready

Table 1 Oligonucleotide primer sequences used in the polymerase chain reaction and reverse transcription-polymerase chain reaction detection of tomato mottle virus, tomato yellow leaf curl virus, and whitefly and plant genes in samples of whiteflies feed on artificial diet or cotton plants following a feeding period on virus-infected tomato plants

Organism	Genes identified by RT-PCR and PCR	Primer sequence
TYLCV	Coat protein gene	5'-CGCCCGTCTCGAAGGTTTC-3' 5'-GCCATATACAATAACAAGGC-3'
TYLCV	V1	5'-GAAGCGACCAGGCGATATAA-3' 5'-GGAACATCAGGGCTTCGATA-3'
ToMoV	AV1	EH 287 5'-GCCTTCTCAAACCTTGCTCATTCAAT-3' EH 288 5'-GTTTCGCAACAAACAGAGTGTAT-3'
ToMoV	AV1	5'-GAGCTTCATGAAAATGGGGA-3' 5'-GACGTCGGAGCTCGATTTAG-3'
Cotton	β -actin	5'-TTCAGAAGATCCGGTTC-3' 5'-ACGACCACTGGCATATAGGG-3'
Tomato	β -actin	5'-GGAAAAGCTTGCCTATGTGG-3' 5'-CCTGCAGCTTCCATACCAAT-3'
Tomato	Rubisco	5'-CCTGATTTGTCTGACGAGCA-3' 5'-GCACCCAAACATAGGCAACT-3'
Whitefly	β -actin	5'-TCTTCCAGCCATCCTTCTTG-3' 5'-CGGTGATTTCTTCTGCATT-3'

for total RNA extraction. Non-viruliferous and viruliferous whitefly experiments were run separately, but simultaneously, and repeated twice.

Nucleic acid extraction

Total RNA extractions were performed using previously frozen samples of virus host tomato plants, non-viruliferous and TYLCV viruliferous whiteflies collected immediately after removal from healthy or TYLCV-infected tomato, respectively, and after 72 h of feeding on sucrose solution or cotton plants. Samples were ground to fine powder using mortar and pestle in the presence of liquid nitrogen, then processed with the RNeasy miniprep Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol for isolation of total RNA. Trace DNA contamination was removed from total RNA preparations by double extraction with one volume of acid phenol : chloroform, 5 : 1, pH 4.7 (Ambion Inc., Austin, TX, USA), followed by precipitation with two volumes of 95% ethanol and 200 mM sodium acetate, pH 4.0, overnight at 20 °C. Ten micrograms of RNA were digested with two units of DNase I (Ambion Inc.), in a 25- μ l reaction, 1 h at 37 °C. After a second precipitation the sample was resuspended in diethyl pyrocarbonate-treated water and digested with 30 units of Rec J[®] (New England Biolabs, Beverly, MA, USA) ssDNA specific exonuclease, 2 units DNase I, 1X RecJ[®] reaction buffer in a 25 μ l reaction, 1 h at 37 °C. These samples were used directly for RT-PCR applications.

Detection of plant transcripts in non-viruliferous and viruliferous whiteflies

Tomato ribulose biphosphate carboxylase (rubisco, 4.1.1.39) and tomato chlorophyll a/b binding protein transcripts were used to determine the survival of ingested plant material in whiteflies. Total RNA from non-viruliferous and viruliferous whiteflies collected immediately after removal from healthy and TYLCV-infected tomato plants, and after 72 h of feeding on a sucrose solution or on cotton plants was analyzed by RT-PCR using primers designed to amplify cDNA segments <200 bp (Table 1). RT-PCR reactions were conducted with 300 ng of total RNA from every sample in a 25- μ l reaction using the Quantitect SYBR Green Real time RT-PCR kit (Qiagen) and recommended reaction conditions. Reverse transcription was performed for 30 min at 50 °C followed by a 15 min denaturation at 95 °C, and 40 cycles of 40 s at 95 °C, 40 s at 58 °C, and 40 s at 72 °C. As positive controls for the detection of plant transcripts, RT-PCR reactions with tomato β -actin were conducted on total RNA from tomato used as the host plant for whitefly colonies. To ensure amplification quality of the whitefly RNA, detection of whitefly β -actin was performed for each whitefly sample. DNA contamination

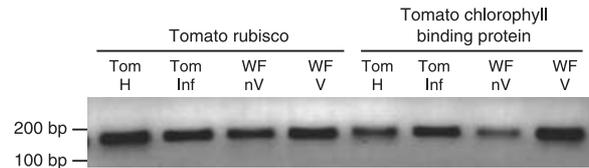


Figure 1 Reverse transcription-polymerase chain reaction detection of tomato rubisco and chlorophyll a/b binding protein transcripts in healthy (H) and TYLCV-infected (Inf) tomato (Tom) and non-viruliferous (nV) and TYLCV-viruliferous (V) *Bemisia tabaci* whiteflies (WF) collected immediately after removal from the corresponding healthy or infected tomato plants.

of the RNA samples was checked by PCR amplification with each primer pair and 300 ng of DNase treated total RNA from all plant and insect samples. Products of the RT-PCR and PCR reactions were visualized by agarose gel separation.

Results and discussion

Reverse transcription-polymerase chain reaction was used to detect the presence of ingested tomato transcripts in whiteflies collected immediately after removal from tomato (Figure 1), and following a 72 h feeding period on either a sucrose solution or cotton plants after removal from tomato (Figure 2). Results are shown for whiteflies removed from healthy tomato (non-viruliferous) and from TYLCV-infected tomato (viruliferous). The primers selected for rubisco and chlorophyll a/b binding protein transcripts were very specific and did not amplify their cotton counterparts. Rubisco primers would periodically produce non-specific primer dimer products that ran as a <100 bp staining region (seen in Figure 2, lanes 2, 3, 4, and 7). This was most common when template availability was low. Melt curve analysis of reactions producing the smaller DNA fragments indicated the products were non-specific, without defined melt-points similar to what is typically observed for primer dimers (data not shown). The 162-bp amplicon of rubisco was only observed in the plant and in whiteflies allowed to feed on sucrose for 3 days after removal from tomato. This was observed with both non-viruliferous and viruliferous whiteflies (Figure 2, lanes 1 and 2). This amplicon was never seen in whitefly fed on cotton for 3 days after removal from tomato (lane 3). Controls of each total RNA preparation included checking for contaminating DNA that would give a false-positive result (Figure 2, lanes 4, 5, and 6) and amplification of a transcript that is present in each sample [tomato β -actin for tomato RNA and whitefly β -actin for whitefly RNA (lanes 7, 8, and 9)]. All results were similar for non-viruliferous and viruliferous whiteflies. The results were identical for tomato chlorophyll binding protein, where the transcript was only successfully

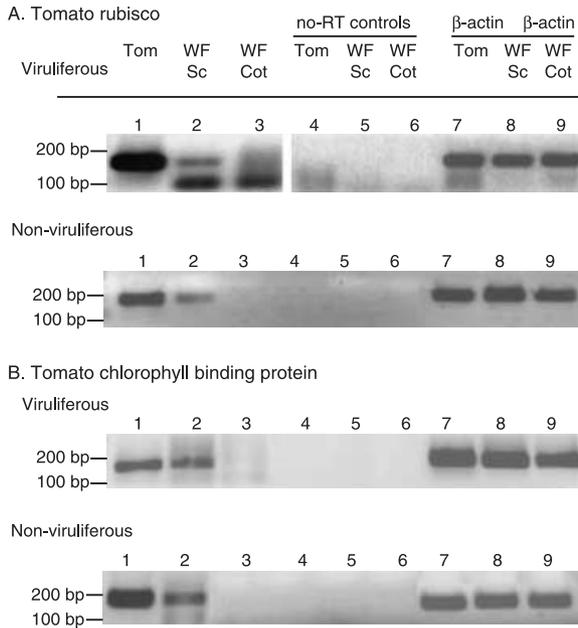


Figure 2 Detection of tomato rubisco and chlorophyll a/b binding protein transcripts in tomato (Tom) plants and TYLCV-viruliferous and non-viruliferous *Bemisia tabaci* whiteflies (WF) by reverse transcription-polymerase chain reaction after 72 h of feeding on sucrose (Sc) or healthy cotton plants (Cot). (A) Tomato rubisco transcripts. Lane 1, tomato plant; lane 2, whiteflies from sucrose feeding chamber; lane 3, whiteflies from cotton; lanes 4, 5, and 6, negative controls (PCR without RT reaction) for tomato rubisco in total RNA samples from tomato, whiteflies from sucrose feeding chamber, and whiteflies from cotton, respectively; lanes 7, 8, and 9, positive controls for tomato β -actin in tomato, whitefly β -actin in whiteflies from sucrose feeding chamber, and whiteflies from cotton, respectively. (B) Chlorophyll a/b binding protein transcripts. Lane 1, tomato plant; lane 2, whiteflies from sucrose feeding chamber; lane 3, whiteflies from cotton; lanes 4, 5, and 6, negative controls (PCR without RT reaction) for tomato chlorophyll a/b binding protein in total RNA samples from tomato, whiteflies from sucrose feeding chamber, and whiteflies from cotton, respectively; lanes 7, 8, and 9, positive controls for tomato β -actin in tomato, whitefly β -actin in whiteflies from sucrose feeding chamber, and whiteflies from cotton, respectively.

amplified in total RNA from tomato and from whitefly feeding on a sucrose artificial diet.

The surprising persistence of plant transcripts in both viruliferous and non-viruliferous whiteflies after 72 h of feeding on a sucrose diet indicates that plant transcript survival is independent of the virus acquisition status of the whiteflies and that virus-dependent factors like coencapsidation of plant transcripts in virus particles were not involved in plant transcript survival. We envision at least three hypotheses to describe this pervasiveness of plant transcripts.

First, there may be a significant reduction in the synthesis and release of insect-derived nucleases due to a decrease in active feeding (or decrease in diet complexity) on the sucrose diet as compared to cotton. Second, lower feeding activity on the sucrose diet may reduce bulk flow through the insect gut and thereby decreased efficiency of waste elimination. Third, plant-derived nucleases in cotton (that would be absent in the artificial diet) that are acquired by whiteflies during feeding may play an active role in nucleic acid degradation.

Our evidence shows the need to feed whiteflies on an alternative host plant to sufficiently clear previously ingested material from the insect. We speculate that similar results might be obtained with other homopteran, and that when experiments require flushing of gut contents, these findings should be taken into consideration. Since the insect feeding dynamic depends on the host and environmental conditions, the appropriate length of required feeding for gut clearing will have to be determined for each insect–host interaction. We believe that detection of selected host derived transcripts from the primary host plants in the insect can be used as a reliable indicator of the progress and successful completion of the clearing process.

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