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Report

Manuscript Title: Functional Genetic Studies of the Tarnished Plant Bug

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Abstract: The tarnished plant bug (TPB), *Lygus lineolaris* (Palisot de Beuvois) has become a primary pest of cotton in the Mississippi Delta. To identify new techological and genetic methods to control TPB, studies have begun to focus on genes expressed by the insect. Initial studies on interference of transcription of critical genes showed that double stranded RNA (dsRNA) could decrease transcript amounts and affect longevity, but delivery of the dsRNA required injection. Saliva of TPB digested dsRNA and will be a major hurdle in utilizing genetic control.

Keywords: gene sequencing, transcription, RNA interference, biotechnology

Introduction

The era of genomics has brought new prospects for insect biology and ecology studies, insect-based discoveries, and insect control strategies. Not only are insect genomes being sequenced and analyzed, but the activities of genes within living insects are being studied and interpreted. Over the last ten years, one of the insects that has become a subject of functional gene study is the tarnished plant bug (TPB), *Lygus lineolaris* (Palisot de Beuvois). Focus on this insect has been driven partly by its increasing role in crop damage in cotton crops in the Midsouth region of the United States. TPB is not controlled by current genetic modification (GMO) technologies, and thus infestations must be controlled by chemical pesticides. Many of the available pesticides approved for use against TPB are losing effectiveness because of resistance development (Snodgrass et al. 2009).
The functional genetics of TBP can be investigated by several methods:

1. Sequencing the insect's DNA or RNA and comparing those sequences to genetic materials from other, more intensively studied organisms such as the genetic model insects *Drosophila melanogaster* (fruit fly[1]), *Anopheles gambiae* (malaria mosquito), *Tribolium castaneum* (red flour beetle), *Apis mellifera* (honey bee), *Acyrthosiphon pisum* (pea aphid), *Pediculus humanus* (human louse), and an ever-expanding group of flies, mosquitoes, ants, and other arthropods. Sequence comparisons can be performed using multiple online resources, including those offered by the National Center for Biotechnology Information (NCBI) GenBank (Benson et al. 2013) and the Swiss Institute of Bioinformatics (SIB) (Artimo et al. 2012);

[1] While the common name "fruit fly" is used by entomologists to describe the insect family Tephritidae, the genetics community including GenBank (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=7227) has assigned the common name "fruit fly" to the genus Drosophila and the species *Drosophila melanogaster*.

2. Amplifying TPB sequences from either DNA or RNA samples harvested from specific developmental stages, specific tissues, or from insects subjected to different treatments and comparing presence/absence or quantity of alleles or markers (from DNA) or transcripts (RNA). Variations on this sort of investigation may involve preparing probes and performing hybridization to prepared tissue samples (*in situ* hybridization) or to a blot of size fractionated material.
3. Preparing double stranded RNA (dsRNA) from a TBP sequence of special interest and introducing the dsRNA into the living insect to interfere with the transcription/translation process (RNAi), and observing or measuring (through quantitative reverse translation PCR) the effect. This is called “gene knockdown”, and is a form of transient genetic manipulation.
4. Preparing transgenic insects through germ-line transformation methods. These methods have not yet been successfully developed for TPB, as they have for insects in the genus *Drosophila* (see (Handler and Harrell 1999; Schetelig and Handler 2013; Thummel et al. 1988).

Materials and Methods

To revisit TPB sequences, those sequences published (Allen 2007) and identified as having homology but unknown function were analyzed by NCBI BLASTx (translated sequences compared to GenBank proteins) and by SIB Translate tool (http://web.expasy.org/translate). Searches were limited to insects (Hexapoda:taxid6960) in BLASTx when Translate indicated that top translations were insect proteins.

Results and Discussion

Ten TPB sequences previously described as unknown are now verified as most homologous to other known insect gene sequences, coding for proteins that are being characterized. One sequence appears to be most homologous to a bacterial sequence. These results are shown in Table 1. The bacterial sequence may indicate a symbiont, either contaminating the TPB surface or interior, or incorporated into the TPB normal digestive flora.

Table 1. Revision of "unknown" (Allen 2007) GenBank submitted expressed sequences from the tarnished plant bug, *Lygus lineolaris*, and the most homologous BLASTx and/or EXpasY protein match. Species names are only provided for insect matches.

L. lineolaris GenBank accession No.	Closest GenBank accession No.	Closest GenBank match organism	Closest protein description	similarity (e-value)
DY524596	EU401991.2	*Lygus lineolaris* mitochondrion	Cytochrome c oxidase subunit 3	0
DY470860	AGM32587.1	*Coptotermes formosa*	C-terminal to lisH motif domain containing protein	2e-99
DY470891	EHJ65471.1	*Danaus plexippus*	UPF0605 protein CG18335-like	1e-91
DY470890	XP_623555.2	*Apis mellifera*	nascent polypeptide-associated complex subunit	7e-76
DY470844	XP_974795.2	*Tribolium castaneum*	protein YIF1A	1e-60
DY470846	WP_017478801.1	*Pseudomonas sp.*	peptidoglycan-binding protein	2e-75
DY470885	XP_001950809.2	*Acyrthosiphon pisum*	hexosaminidase D-like	4e-60
DY473212*	XP_001952118.2	*Acyrthosiphon pisum*	thymidine phosphorylase-like	7e-64
DY524523	XP_969699.1	*Tribolium castaneum*	Protein fuzzy homologue	8e-63
DY524566	XP_001946021.2	*Acyrthosiphon pisum*	Sn1-specific diacylglycerol lipase beta-like	6e-83
DY470845	BAN20153.1	*Riptortus pedestris*	natterin-3-like, CG3884 isoform	9e-54

* Identified in Allen 2007 as "human growth factor"

Sequences have been obtained from RNA samples extracted from TPB, and deposited in GenBank (Allen et al. 2007) The purpose of these genes was to establish a foundation of materials for performing functional studies. Initially, gene sequences thought to be associated with digestion of carbohydrates, called polygalacturonase (PG) genes, were identified and examined (Allen and Mertens 2008; Walker and Allen 2010). One of three forms of PG was knocked down by injected dsRNA. Based on the limited success of RNAi using these sequences, another sequence, IAP1 (inhibitor of apoptosis), anticipated to result in insect mortality, was used to test RNAi in TPB (Walker and Allen 2011).

Quantitative PCR confirmed that IAP1 transcripts were decreased by injection of the IAP1 dsRNA. So RNAi worked in *Lygus*. Also, injected insects had convincingly shortened survival. However, preparations of dsRNA introduced to the insects through ingestion appeared to have no similar effect.Examination of the saliva of TPB showed that salivary secretions, and to some lesser degree hemolymph, effectively digested the dsRNA (Allen and Walker 2012). This indicated that the RNAi inducing material was unable to reach the interior of the insect cells, where the RNAi process occurs (see reviews (Agrawal et al. 2003; Huvenne and Smagghe 2010). This result illustrates a difficulty in delivering genetic control products to extraoral feeders such as TPB. Nonetheless, the future of RNAi-based strategies in insect control and control of other pests, for example weeds, plant pathogens, and insect pathogens, remains a topic of enthusiastic interest (Burand and Hunter 2013; Gu and Knipple 2013; Li et al. 2013; Price and Gatehouse 2008; Scott et al. 2013). For insects like TPB that produce digestive enzymes that hinder delivery of the dsRNA through a feeding strategy, other delivery, or perhaps packaging methods must be devised. Because of recent additions to GenBank including the genome of the pea aphid (International Aphid Genomics Consortium 2010) and the bean bug (Futahashi et al. 2013), new comparisons of genes may provide the materials needed to devise new dsRNA products and suggest delivery methods to solve critical agricultural problems.

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