In Vitro Cellular & Developmental Biology - Animal (2021) 57:849–855
https://doi.org/10.1007/s11626-021-00633-y

REPORT

Cell lines derived from the small hive beetle, *Aethina tumida*, express insecticide targets

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Received: 23 September 2021 / Accepted: 5 November 2021 / Published online: 18 November 2021 / Editor: Tetsuji Okamoto

The small hive beetle (SHB), *A. tumida* (Murray; Coleoptera, Nitidulidae), is a significant agricultural pest because its larvae seriously damage honey bee and bumble bee colonies by eating bee larvae, pollen and honey (Ellis Jr *et al.* 2002). SHB is native to Africa but has spread to Europe, Australasia and North America over recent decades, and SHB invasions into new regions are forecast to increase with impending climate change (Cornelissen *et al.* 2019). Adult SHBs follow olfactory cues that are produced by bees or present in bee products (*e.g.*, wax) to locate bee colonies they invade for egg deposition (Graham *et al.* 2011; Bobadoye *et al.* 2018). Adult SHBs cause minimal colony damage other than potentially vectoring some honey bee viruses such as Deformed wing virus (Eyer *et al.* 2009). SHB larvae contribute to bee colony collapse, thereby reducing the ecological services of bees as pollinators in agricultural systems (Klein *et al.* 2007).

Bee colonies display varying resiliency to SHB invasions depending on their genetic background, with African subspecies tending to be more successful in defending their colonies than European subspecies (Elzen *et al.* 2001). Various tools and techniques have been used to control SHB infestations, including mechanical intervention, odorant-based trapping, application of fungal pathogens, entomopathogenic nematodes, and other biological control agents (Cuthbertson *et al.* 2013). SHB are also susceptible to several classes of insecticides (Kanga and Somorin 2012), however there are only two products currently approved in the U.S. for SHB control. Permethrin is a pyrethroid that functions as a sodium channel modulator and is used as a soil drench in the immediate area surrounding bee colonies to kill SHB larvae that have left to pupate underground; coumaphos, an organophosphate that inhibits acetylcholinesterase, is used within hives under strict regulatory guidance to prevent carry over into food products (*i.e.*, honey and beeswax). Both chemicals also harm bees, making it difficult, if not counterproductive, to use them to protect bee colonies from SHB. SHB populations in Florida have developed resistance to both classes of insecticides currently labeled for SHB control (Kanga *et al.* 2021). As such, more efficient and effective control tools are needed to protect honey bee colonies from SHB.

Here we report establishing several continuously dividing *A. tumida* cell lines that may facilitate the development of improved insecticides for SHB control. The cell lines were derived from SHB eggs and larvae, and they have favorable growth characteristics for routine culture. Transcriptomic analysis suggests that these lines might express proteins that could be targeted by insecticides. These cell lines could be adopted for high-throughput screening for identification and optimization of SHB-targeting insecticides.

We established SHB cell lines by taking eggs and neonates from *A. mellifera* hives maintained at USDA – ARS in Columbia, Missouri. Briefly, beetle eggs and/or neonate larvae were collected using a Chinese grafting tool (Mann Lake Ltd., Hackensack, MN) and allowed to float on the surface of calcium-magnesium-free phosphate-buffered-saline (CMF-PBS; pH 7.1, 137 mM NaCL, 4 mM KCl, 0.26 mM Na_2HPO_4 (7 H_2O), 0.15 mM KH_2PO_4, 11 mM Glucose). The CMF-PBS was poured off and replaced with 0.8% sodium hypochlorite, incubated for 2 min (inverted every 30 s), washed twice with CMF-PBS via pipetting, and centrifuged (800×*g*, 5 min, 4°C). Egg/larval pellets were gently ground using a pestle on the side of an Eppendorf tube in 1.5 mL of cell culture medium ([1:1] Leibovitz's L-15 medium (Caisson Labs, Smithfield, UT) and EX-CELL 420 medium

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containing 9% heat-inactivated FBS, 50 U/mL penicillin and 0.05 mg/mL streptomycin (Sigma-Aldrich, Inc., St. Louis, MO)). The cell/tissue suspension was transferred into a $T_{12.5}$ flask and maintained at 33°C to simulate the average internal temperature of a bee colony (Stabentheiner *et al.* 2010). Cultures were observed weekly and fed every seven to ten days (either by replacing half or all culture medium). Once confluent, cells were dissociated from flasks using 0.05% trypsin +0.02% EDTA initially or with trypsin alone for subsequent passages. These efforts produced two continuously replicating cell lines, BCIRL-AtumE-1127 from eggs and BCIRL-AtumEN-1129 from eggs and neonate larvae. Each line is comprised of a variety of cell morphotypes (Fig. 1). The AtumE-1127 line contains firmly attached elongated and multi-sided cells, loosely attached spherical cells, and some cell clumps growing in the medium (Fig. 1*a*, *b*). The AtumEN-1129 line also contains attached elongated and multi-sided cells, with some cell clumps (Fig. 1*c*, *d*). The cell lines were tested for mycoplasma (MycoAlert™, Lonza, Basel, Switzerland) prior to freezing, in which the cell lines were placed in a cell freezing container (CoolCell™, Corning, Corning, NY) and kept at −80°C for 2 days prior to transfer to liquid nitrogen for long-term storage.

Sublines from the two cell lines, AtumE-1127 and AtumEN-1129, were then generated using a previously described limiting dilution method (Reall *et al.* 2019). Briefly, 96-well plates were seeded at 20–50 cells/well in (7:3) or (1:1) conditioned:fresh medium. As cultures became confluent, wells containing cells with uniform morphologies were selected for further culture. Cells were detached as described above and transferred first to a 24-well plate, then to $T_{12.5}$ flasks, and finally to T_{25} flasks. Cells were fed every 7 d initially with conditioned/fresh medium mixtures while in 96-well plates and with fresh medium thereafter. Ultimately, 20 subcultures were selected for continued maintenance and cryopreservation. Of these, six AtumE-1127 derivatives and three AtumEN-1129 derivatives were selected for further characterization based on morphological properties. The AtumE-1127 sublines A3, B7, and C10 consist of attached elongated cells and cell clumps (Fig. 2*a-c*). Similarly, the C11 and D5 sublines exhibit attached elongated cells, but also spherical cells that are either loosely attached or remain in suspension (Fig. 2*d*, *e*). Subline H3 contains attached multi-sided and elongated cells, as well as some spherical cells in suspension (Fig. 2*f*). The AtumEN-1129 sublines consist primarily of firmly attached monolayers of cells with either spherical, multi-sided or slightly elongated morphologies (Fig. 2*g-i*), however each subline displays varying amounts of loosely attached cell clumps.

Growth curves were generated for each of the nine derivative sublines by seeding cells into 24-well plates (0.5 to 2×10^5 cells/mL, depending upon time to confluency) and gently shaking the plates on an orbital shaker for 1 h to facilitate even attachment. Plates were then placed in a live cell imager (Lionheart FX, BioTek, Winooski, VT) and cell counts were generated every 8 h for a minimum of 104 h. The linear portion of the growth curves were used to determine the doubling times (http://www.doubling-time.com/compute.php) which ranged from 29 to 68 h (Table 1).

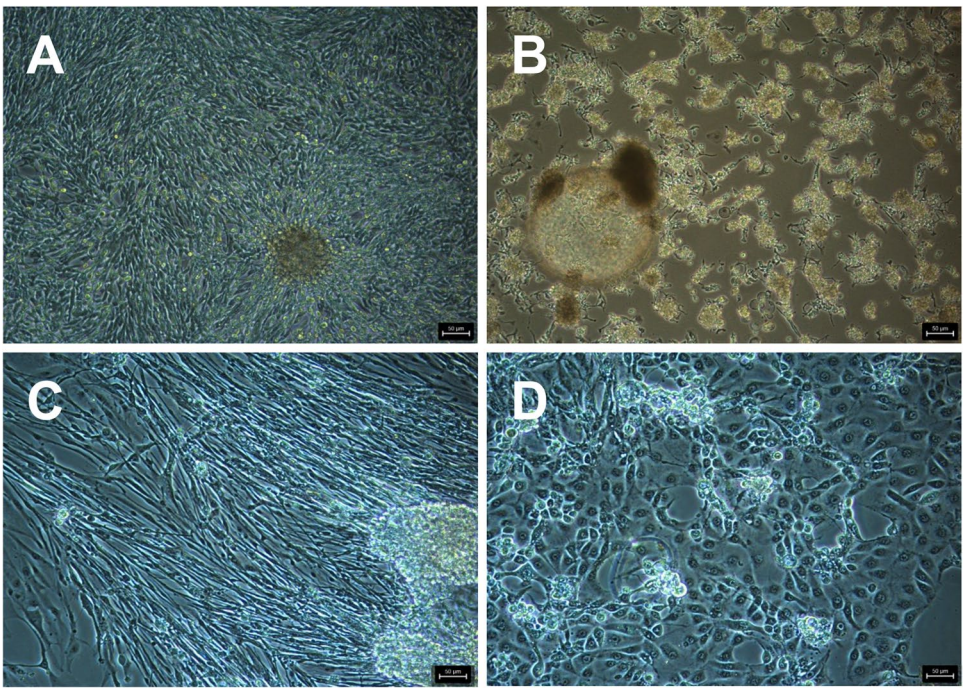

Figure 1. Images of parental *Aethina tumida* cell lines. (*a*) AtumE-1127, passage (p) 7; (*b*) AtumE-1127, p11; (*c*) AtumEN-1129, p7; (*d*) AtumEN-1129, p11. Magnification = 200X, *scale bar* = 50 µm.

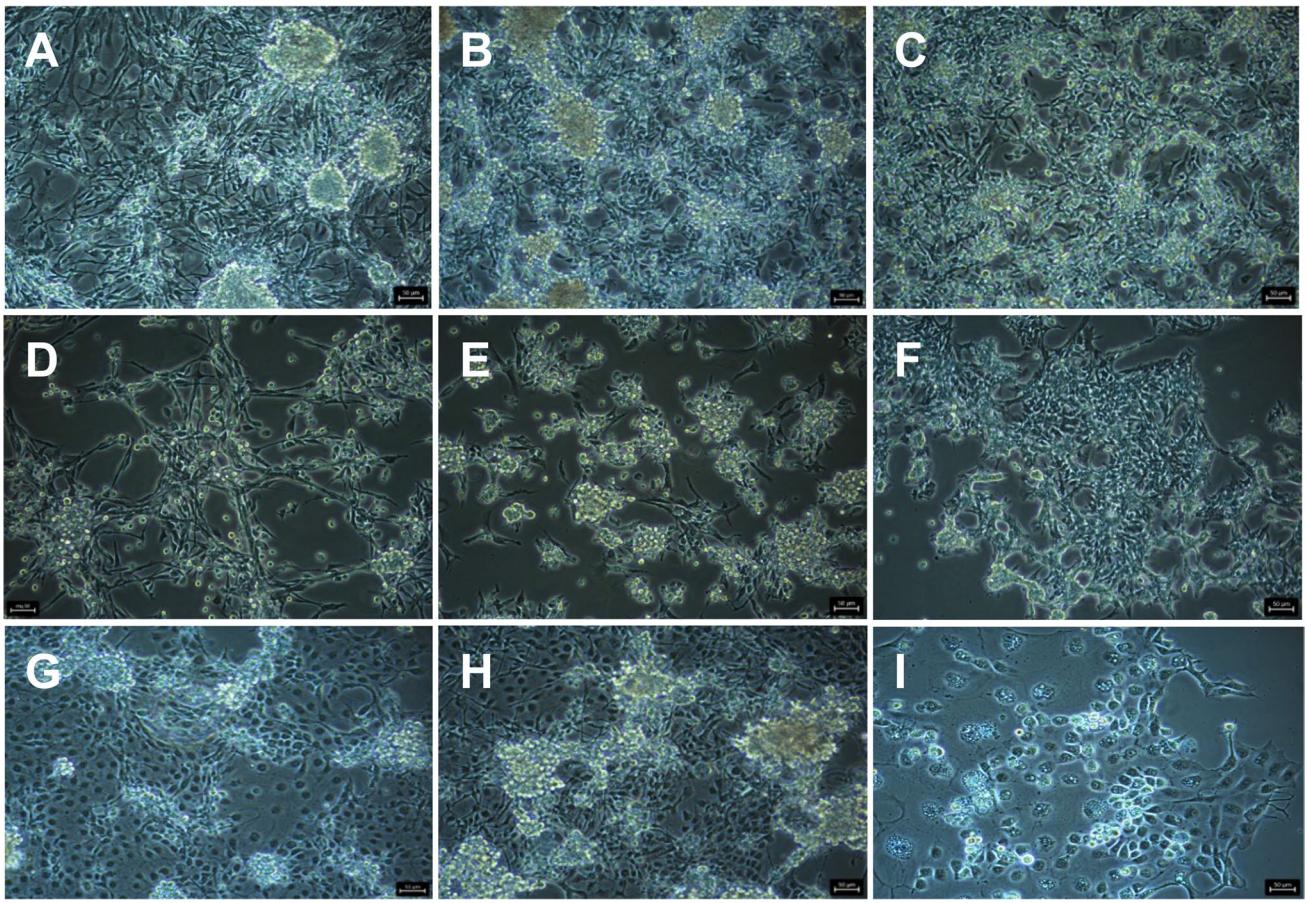

Figure 2. Images of sublines produced from AtumE-1127 and AtumEN-1129 parental cell lines. (*a*) AtumE-1127-A3, passage (p) 12.3; (*b*) AtumE-1127-B7, p6.5; (*c*) AtumE-1127-C10, p13.4; (*d*) AtumE-1127-C11, p12.4; (*e*) AtumE-1127-D5, p12.3; (*f*) AtumE-1127-H3, p12.2; (*g*) AtumEN-1129-D6, p6.5; (*h*) AtumEN-1129-D12, p8.6; (*i*) AtumEN-1129-F10, p8.2. Magnification = 200X, *scale bar* = 50 µm.

The doubling times were not related to cell morphology, as AtumE-1127 sublines D5 and C11 display similar morphologies yet different doubling times (64 and 27 h, respectively).

We then confirmed the genetic identity of the nine derivative sublines by DNA barcoding, in which the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene was amplified by PCR, sequenced, and compared to CO1 sequences in publicly available databases. Briefly, DNA was extracted from SHB larvae and cell pellets using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) and used as a template in PCRs with Vent (exo-) DNA Polymerase (New England Biolabs, Ipswich, MA), primers (For: 5'-CGACCCTCAGGCATA ACCTT-3′, Rev.: 5'-AGGCTCGAGTATCAACGTCTA-3′) optimized to amplify CO1 from *A. tumida* (Silacci *et al.* 2018), and previously described cycling parameters (Hebert *et al.* 2004). PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen, Valencia, CA), visualized by standard DNA electrophoresis, and Sanger sequenced at the University of Missouri DNA Core Facility (Columbia, MO). Resulting CO1 sequences from SHB larvae and each of the nine sublines were used to search GenBank via BLASTn analyses. As expected, the CO1 sequences obtained from SHB larvae and the nine sublines each shared >97% identity (Table 1) to a *bona fide* SHB CO1 sequence in GenBank (MK286579.1), confirming that the nine sublines were derived from *A. tumida*.

Our goal in making SHB cell lines was to create a resource to support fundamental and applied research programs to develop novel SHB control technologies. Towards this end, we conducted RNA-Seq analyses on three of the SHB sublines that displayed desirable growth-related characteristics (*e.g.*, adherence, morphology, doubling time) for high-throughput screening campaigns. The sublines AtumE-1127-B7, AtumEN-1129-D6 and -D12 were each plated into a T_{25} flask and allowed to grow for 2–3 d until they reached approximately 70% confluency, at which point they were lifted using trypsin-EDTA, resuspended in medium, centrifuged (1000×*g*, 5 min, 4°C), washed in PBS and re-centrifuged. The supernatant was removed, and cell pellets were frozen at −80°C. Total RNA was purified from each cell pellet using Trizol (ThermoFisher, Waltham, MA) reagent following the manufacturer's protocol. Purified RNA was

Table 1. Growth rates and genetic authentication of nine SHB cell sublines. For passage number, the first number represents the passage at which the line was isolated from the parental line, while the second number represents the number of passages since that subline was generated and the point at which doubling times were determined

Sample/Cell line	Passage Number	Doubling Time (h)	Identity (%)	E-value
SHB larvae	n/a	n/a	99.7	0.0
AtumE-1127-A3	12.19	68	97.4	$3.00e^{-108}$
AtumE-1127-B7	**6.22**	**51**	**99.7**	**$4.00e^{-179}$**
AtumE-1127-C10	13.21	44	100	$2.00e^{-122}$
AtumE-1127-C11	12.18	64	98.7	$2.00e^{-72}$
AtumE-1127-D5	13.18	27	98.0	$2.00e^{-119}$
AtumE-1127-H3	12.10	44	98.1	$1.00e^{-122}$
AtumEN-1129-D6	**6.21**	**32**	**98.6**	**$7.00e^{-177}$**
AtumEN-1129-D12	**8.23**	**29**	**99.4**	**$1.00e^{-166}$**
AtumEN-1129-F10	8.16	41	99.6	$1.00e^{-119}$

For authentication, the DNA sequence of COI amplified from each subline, as well as SHB larvae, was compared to a known SHB COI sequence (MK286579.1) in Genbank, resulting similarities are expressed as percent identity. E-values $<1e^{-5}$ are considered statistically significant. Sublines used in RNA-Seq analyses are indicated in bold

used to prepare separate, stranded mRNA libraries using a TruSeq RNA Library Prep Kit (Illumina, San Diego, CA) for sequencing on a NovaSeq SP Flow Cell (PE50) at the University of Missouri DNA Core Facility (Columbia, MO). Raw sequencing data was then submitted to Novogene, Inc. (Sacramento, CA) where it was filtered to remove adapters and low-quality sequences prior to transcriptomic assembly and bioinformatic analyses. A total of 102,589,132 clean reads were obtained from AtumE-1127-B7, 84,996,308 clean reads were obtained from AtumEN-1129-D6, and 109,472,846 clean reads were obtained from AtumEN-1129-D12, which were assembled into a single transcriptome that contained 27,331 unigenes with an N_{50} of 2816 bp. The genome of *A. tumida* has recently been sequenced (Evans *et al.* 2018) and used to identify the SHB orthologues of genes that are commonly targeted by insecticides (Rinkevich and Bourgeois 2020). We evaluated the transcription of these insecticide targets in the three SHB sublines via tBLASTn analyses of the SHB cell line transcriptomes using Geneious software (Biomatters, Auckland, New Zealand). Of the 47 candidate insecticide targets identified by Rinkevich and Bourgeois (2020), only five were expressed (FPKM >1) in any of the three SHB sublines. Transcripts were detected for three different acetylcholine receptor subunits, α1, α5 and α9, in the sublines derived from SHB eggs and neonates (AtumEN1129-D6 and -D12) but not in the subline derived from eggs alone (AtumE1127-B7). A similar pattern was observed for transcripts encoding the octopamine receptor subtype β2. Transcripts encoding the ryanodine receptor were detected in all three sublines (Table 2).

We then searched the SHB cell subline transcriptome for transcripts of other candidate insecticide target genes that were not identified in Rinkevich and Bourgeois (2020). Two calcium pumps, plasma membrane Ca^{2+} -ATPase (PMCA) and sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), have been proposed as novel insecticide targets due to their critical roles in calcium homeostasis in eukaryotic cells (Lümmen 2013). The SHB orthologues of PMCA and SERCA were identified from the *A. tumida* genome available in NCBI using *Drosophila melanogaster* sequences of these proteins (NP_001188516.1 and NP_001286796., respectively) as queries in tBLASTn analyses. The SHB genome-derived PMCA and SERCA sequences were then used to evaluate the transcription of these genes in the sublines. Interestingly, three PMCA transcript variants were detected in each subline which all differed from the genome-predicted SHB PMCA sequence in NCBI (XM_020009951.1). The three PMCA transcripts in the sublines encoded proteins with alternatively spliced C-terminal exons, as evidenced by intron/exon boundaries present in the transcripts at the precise location where the divergence occurs. Two SERCA transcripts with differing 5'UTRs were detected in the subline transcriptome, both of which differed at the 3′ end from the genome-predicted sequence in NCBI, presumably due to alternative splicing as the difference coincided with an intron/exon boundary in the transcript.

Some G protein coupled receptors (GPCRs) have also been promoted as targets for novel insecticide development due to their critical roles as neurotransmitter, neuropeptide and hormone receptors in insects (Verlinden *et al.* 2014; Audsley and Down 2015), however, there currently exists only one GPCR-targeting insecticide in the market. This compound, Amitraz, targets octopamine receptors and is currently labeled for use in controlling *Varroa* mites. The octopamine receptor is a particularly attractive target for insecticide development as it is only found in invertebrates, meaning compounds that target this molecule are less likely to have off-target effects on vertebrate animals. As mentioned above, we found transcripts for one type of octopamine receptor in two of our SHB sublines, however many other GPCRs were also expressed in the cell lines based on automated functional annotation results. Two of these, the neuromedin U-like (NumR) and moody-like receptors (MoodyR), were studied more closely in the sublines due to their roles as neuropeptide receptors in insects (Ahn

Table 2. Transcript abundance, measured by fragments per kilobase of transcript per million mapped reads (FPKM), of housekeeping and select insecticide target genes in AtumE-1127-B7 (B7), AtumEN-1129-D6 (D6) and AtumEN-1129-D12 (D12) cell sublines

Gene	Gene Product	Function	B7	D6	D12
Housekeeping genes					
SDHA	succinate dehydrogenase, subunit A	Krebb's cycle & cellular respiration	74.77	66.95	82.27
Rpn2	26 s proteasome regulatory unit	ubiquitinated protein degradation	75.9	84.81	106.44
Rpt6	Regulatory particle AAA ATPase, subunit 8	protein degradation	144.65	193.31	101.43
Insecticide targets					
nACh-R-α1	acetylcholine receptor, subunit α1	neurotransmitter receptor	0.04	6.2	1.58
nACh-R-α5	acetylcholine receptor, subunit α5	neurotransmitter receptor	0.03	0.93	2.71
nACh-R-α9	acetylcholine receptor, subunit α9	neurotransmitter receptor	0.29	2.82	2.77
Oct-R-β2	octopamine receptor, subtype β2	neurotransmitter receptor	0	2.18	2.2
Ry-R	ryanodine receptor	intracellular calcium signaling	5.87	7.56	20.62
PMCA	plasma membrane calcium ATPase	calcium signaling/transportation	28.44	7.44	22.56
SERCA	sarco/endoplasmic reticulum calcium ATPase	calcium signaling/transportation	29.2	23.45	51.32
NueromedinU-l	NeuromedinU-like receptor	neuropeptide receptor	4.2	10.68	9.54
Moody-l	moody-like receptor	blood-brain barrier function	6.96	43.27	23.58
MDRP1L-a1	ABC transporter, C-family	cellular detoxification	0.23	1.81	6.52
MDRP1L-a2	ABC transporter, C-family	cellular detoxification	0.36	0.91	4.25
MDRP1L-b	ABC transporter, C-family	cellular detoxification	5.08	1.6	2.26
MDRP2L	ABC transporter, C-family	cellular detoxification	2.36	0.08	0
MDRP4L-a	ABC transporter, C-family	cellular detoxification	4.46	13.04	14.93
MDRP4L-b	ABC transporter, C-family	cellular detoxification	15.63	14.49	21.4

The three housekeeping genes, SDHA, Rpn2 and Rpt6 are included to convey the relative expression of insecticide target genes within a given cell line. Note: FPKM analyses allow for comparison of gene transcription levels within a sample, not between samples

et al. 2020; Ma *et al.* 2020). The SHB genome-predicted open reading frames of these genes were used to evaluate their transcription in cell sublines via tBLASTn analyses. Both receptors were expressed in all three SHB sublines (Table 2), particularly in those derived from SHB eggs and neonates. The transcripts for NumR and MoodyR present in the sublines matched the predicted sequences available on Genbank (XM_020018468.1 and XM_020014900.1, respectively), except that the predicted sequence for NumR was incomplete, lacking 243 C-terminal residues, relative to the transcript expressed in sublines.

Finally, ATP-binding cassette (ABC) transporters represent another attractive class of physiologically relevant molecules for insecticide development due to their roles in transporting molecules, including insecticides, out of cells. Genomic analyses identified 56 ABC transporter family members in *A. tumida* (Evans *et al.* 2018), approximately half of which were present in the SHB cell subline transcriptomes, based on automated functional analysis results. Members of the ABC-C transporter subfamily (a.k.a, multidrug resistance-associated proteins, MDRPs) were examined more closely in the SHB cell sublines due to their documented role in insecticide resistance (Merzendorfer 2014) and Cry protein toxicity in the Lepidoptera (Heckel 2021). Six ABC-Cs/MDRPs were expressed at various levels in the SHB sublines however it is difficult to speculate their putative function based on homology to other ABC-Cs with known function because there is high intra- and interspecific sequence diversity within this subfamily of transporters. For example, three of the SHB MDRPs (MDRP1L-a1, MDRP1L-a2 and MDRP1L-b) share 91% identity and are most closely related to MDRP1 in *D. melanogaster* (NM_205982.2) and to ABCC1 in humans (NM_004996.4). However, the other three MDRPs expressed in the cell sublines (MDRP4L-a, MDRP4L-b and MDRP4L-c) share only 48% identity yet are each most closely related to an uncharacterized ABC transporter in *D. melanogaster* (NM_001259014.2) and ABCC4 in humans (NM_005845.5), indicating the types of gene expansion events that occur within a species for this transporter family. Nevertheless, it appears that the SHB sublines express orthologues (plus additional paralogues) of the human ABCc1 and ABCc4 transporters.

Here, we identified several candidate insecticide targets that are expressed in the SHB cell sublines at a transcriptional level. While it is tempting to assume that transcript levels for these insecticidal targets in the cell lines equates to protein expression, evidence suggests that transcript abundance does not always correlate with protein expression levels (Gry *et al.* 2009; Edfors *et al.* 2016). In addition, even

if the insecticide targets are expressed at the protein level, it remains to be determined whether they exhibit normal functionality in the cell lines. To test for functionality, cell lines could be exposed to agonistic or antagonistic molecules that interact with the targets of interest and monitor the cellular response. For example, octopamine-induced octopamine receptor activation causes calcium influx into cell lines, which can readily be monitored using calcium-sensitive fluorescent dyes (Grohmann *et al.* 2003; Hana and Lange 2017). Similarly, the SHB cell lines could be exposed to any of the diamides that interfere with ryanodine receptor activity and evaluated for changes in, for example, growth rates and viability. While such evaluations were beyond the scope of this study, the SHB cell sublines are available upon request for research purposes.

Acknowledgements We thank Marilise Stamps for her help in generating the subcultures reported in this paper.

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Author contributions CG, SS, JR, and YG generated, cultured, and/or characterized cell lines. BB and DS provided financial support and developed experimental design. JC generated and evaluated transcriptomic libraries and drafted the manuscript. All authors edited and contributed to the final version of this manuscript.

Funding This research was supported in part by the U.S. Department of Agriculture, Agricultural Research Service under CRIS project: 5070–22000-037-00D, and by The National Science Foundation IUCRC, the Center for Arthropod Management Technologies under Grant No. IIP-1821914, and by industry partners.

Data availability The SHB cell sublines, AtumE-1127-B7, AtumEN-1129-D6 and -D12, as well as the transcriptomic assembly described herein are available upon request for research purposes.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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