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Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte

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Abstract

RNA interference (RNAi) is being developed as a potential tool for insect pest management and one of the most likely target pest species for transgenic plants that express double stranded RNA (dsRNA) is the western corn rootworm. Thus far, most genes proposed as targets for RNAi in rootworm cause lethality in the larval stage. In this study, we describe RNAi-mediated knockdown of two developmental genes, *hunchback* (*hb*) and *brahma* (*brm*), in the western corn rootworm delivered via dsRNA fed to adult females. dsRNA feeding caused a significant decrease in *hb* and *brm* transcripts in the adult females. Although total oviposition was not significantly affected, there was almost complete absence of hatching in the eggs collected from females exposed to dsRNA for either gene. These results confirm that RNAi is systemic in nature for western corn rootworms. These results also indicate that *hunchback* and *brahma* play important roles in rootworm embryonic development and could provide useful RNAi targets in adult rootworms to prevent crop injury by impacting the population of larval progeny of exposed adults. The ability to deliver dsRNA in a trans-generational manner by feeding to adult rootworms may offer an additional approach to utilizing RNAi for rootworm pest management. The potential to develop parental RNAi technology targeting progeny of adult rootworms in combination with Bt proteins or dsRNA lethal to larvae may increase opportunities to develop sustainable approaches to rootworm management involving RNAi technologies for rootworm control.

Keywords: RNAi, pRNAi, hunchback, brahma, Western corn rootworm

1. Introduction

First described almost 20 years ago in the nematode, *Caenorhabditis elegans*, RNA interference (RNAi) refers to a set of related processes in which small regulatory double-stranded RNAs (dsRNAs) direct sequence-specific repression of gene expression (Fire et al., 1998). This pathway has been implicated as a mechanism of defense against invasive nucleic acids from viruses or from mobile genetic elements, and has been conclusively shown to regulate gene expression in virtually all eukaryotic organisms (Fire, 2007; Hussain et al., 2010; Huvenne and Smagghe, 2010; Scott et al., 2013; Terenius et al., 2011).

In insects, the effectiveness of RNAi has been confirmed in a number of species but varies across different taxa and among different tissues (Burand and Hunter, 2013; Terenius et al., 2011). Most of the studies with insects have involved injection of long dsRNA directly in the insect hemocoel to achieve silencing which has become a routine method for assessing gene function. While injection of dsRNA for functional genomics studies has been successful in a variety of insects, uptake of dsRNA from the gut environment through oral exposure to dsRNA and subsequent down-regulation of essential genes is required in order for RNAi to be Terenius et al., 2011). Systemic RNAi through oral administration effective as a pest management tool (Auer and Frederick,

2009 has been documented in a number of different species representing seven different orders (Huvenne and Smagghe, 2010).

The ability to achieve systemic RNAi by oral exposure to dsRNA and to genetically engineer crop plants to express dsRNA led to the first report of *in planta* RNAi in corn plants targeting the western corn rootworm, *Diabrotica virgifera*, (Baum et al., 2007) a devastating pest of corn production throughout North America. Baum et al. (2007) described a high-throughput *in vivo* dietary RNAi system to screen potential target genes for developing transgenic RNAi corn. A total of 14 genes from an initial gene pool of 290 exhibited potential for control based on larval mortality. One of the most effective double-stranded RNAs (dsRNA) targeted a gene encoding vacuolar ATPase subunit A (v-ATPase A), resulting in a rapid suppression of corresponding endogenous mRNA and triggering a specific RNAi response with low concentrations of dsRNA. Importantly, the authors also demonstrated that corn plants expressing dsRNA directed against the *v-ATPase A* gene effectively protected the plants from root damage, documenting for the first time the potential for *in planta* RNAi as a possible pest management tool.

Rangasamy and Siegfried (2012) designed dsRNA for the same *v- ATPase* described by Baum et al. (2007) and documented that oral delivery to adult rootworms could also induce reduced gene expression and protein synthesis and that mortality in the exposed beetles could be achieved within 14 days. The authors suggest that adults may provide a more effective developmental stage to screen for activity of dsRNAs because they are easier to manipulate and can be induced to feed compulsively on artificial diet by incorporating a natural feeding stimulant. The potential to target both adults and larvae may provide increased protection over technologies that target only larvae by minimizing egg deposition and larval damage in the subsequent growing season.

Another potential application of RNAi for insect control involves parental RNAi (pRNAi). First described in *C. elegans*, pRNAi was identified by injection of dsRNA into the body cavity or application of dsRNA via ingestion causing gene inactivity in offspring embryos (Fire et al., 1998; Timmons and Fire, 1998). Bucher et al. (2002) described a similar process in the model coleopteran, *Tribolium castaneum* whereby female pupae injected with dsRNA corresponding to three unique genes that control segmentation during embryonic development resulted in knock down of zygotic genes in offspring embryos. Nearly all offspring larvae displayed gene-specific phenotypes one week after injection.

Since this early report, parental RNAi has been used to describe the function of embryonic genes in a number of other insect species including the milkweed bug, *Oncopeltus fasciatus* (Liu and Kaufman, 2004), the cricket, *Gryllus bimaculatus* (Mito et al., 2006), the springtail, *Orchesella cincta* (Konopova and Akam, 2014), the sawfly, *Athalia rosae* (Yoshiyama et al., 2013), the German cockroach, *Blattella germanica* (Piulachs et al., 2010), the silkworm, *Bombix mori* (Nakao, 2012), and the pea aphid, *Acyrthosiphon pisum* (Mao et al., 2013). The pRNAi response in all these instances was achieved by injection of dsRNA into the hemocoel of the parental female.

In the present study, we examined the potential for parental RNAi in the western corn rootworm by administering dsRNA for genes that potentially affect embryonic development through oral ingestion. Given the potential to achieve systemic RNAi in rootworm adults, we tested whether parental RNAi could be achieved by administering dsRNA in treated artificial diet to gravid *D. v. virgifera* females for two genes previously identified as important to embryonic development. The *brahma* gene (*brm*) was selected based on the report of Brizuela et al. (1994) who described both maternal and zygotic functions of *brahma* (*brm*) during embryogenesis in *Drosophila melanogaster*. Brm is an ATP-dependent remodeling enzyme of the SWI2/SNF2 family (mating type switch/sucrose non-fermenting); it has been associated with nucleosome remodeling that is essential for regulated gene expression (Clapier and Cairns, 2009; Mohrmann and Verrijzer, 2005; Zraly et al., 2004). The second gene, *hunchback (hb)*, is a gap gene which encodes a zinc-finger-containing transcription factor known be important for axial patterning in a number of insects (Jurgens et al., 1984; Lehmann and Nusslein-involhard, 1987; Patel et al., 2001; Schröder, 2003; Tautz et al., 1987). Injection-based pRNAi phenotypes for *hb* have been observed in insects that include the milkweed bug, *O. fasciatus* (Liu and Kaufman, 2004), the oriental migratory locust, *Locusta migratoria manilensis* (He et al., 2006), and pea aphid, *A. pisum* (Mao et al., 2013). A feeding-based lethal (non parental) phenotype for *hb* has also been described in pea aphid nymphs (Mao and Zeng, 2012). Our results extend the parental RNAi effect to western corn rootworms and show that the response can be achieved by oral administration of dsRNA to adult females.

2. Material and methods

2.1. Sequence identification

Transcriptome sequencing of *D. v. virgifera* has been previously described (Eyun et al., 2014). Using Illumina paired-end as well as 454 Titanium sequencing technologies, ~700 gigabases (700 billion bases) were sequenced from cDNA prepared from eggs (15,162,017 Illumina paired-end reads after filtering), neonates (721,697,288 Illumina paired-end reads after filtering), and midguts of third instars (44,852,488 Illumina paired-end reads after filtering). *De novo* transcriptome assembly was performed using Trinity (Grabherr et al., 2011) for each of three samples as well as for the pooled dataset and the pooled assembly resulted in 163,871 contigs (the average length: 914 bp). The estimated coverage of this transcriptome is 28× and is similar to the reported coverage for *T. castaneum*, a Coleoptera with published genome. The transcriptome for *T. castaneum* was 700 million bp, corresponding to ~30× transcriptome coverage (Altincicek et al., 2013). The amino acid sequences of *hb* (Accession Number: NP_001038093.1) and *brm* (Accession Number: XP_008198809.1) from *Tribolium* were used as query sequences to search the rootworm transcriptome with tBlastn using a cut-off E value of 1×10^{-5}. Amino acid alignments of open reading frames were performed in Vector NTI Advance 11.0 using AlignX tool.

2.2. Protein domain identification

Protein domains within Brm homologs were identified using Pfam database sequence search http://pfam.xfam.org. The SnAC domain of *D. v. virgifera* Brm was identified by InterProScan http://www.ebi.ac.uk/interpro/. The C2H2-type zinc fingers of Hunchback proteins were annotated using SMART database within InterProScan.

2.3. dsRNA synthesis

Total RNA was isolated from the whole bodies of *D. v. virgifera* adults using RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. Total RNA (1 µg) was used to synthesize first strand cDNA using the Quantitech reverse transcription kit (Qiagen, Valencia, CA). Primers were designed using Beacon designer software (Premier Biosoft International, Palo Alto, CA) and T7 polymerase promoter sequences were placed in front of both forward and reverse primers (Table 1). For a negative control, a non-specific *GFP* (green

Table 1. Primer sequences used for dsRNA synthesis and qPCR analysis.

Gene name	Primer sequences for dsRNA synthesis	Product length (bp)
brahma	Forward: TAATACGACTCACTATAGGGAACCTTCTTCATCTTCTG Reverse: TAATACGACTCACTATAGGGCTCTCCTAATACAGTTCAA	352
hunchback	Forward: TAATACGACTCACTATAGGGAAGTGTAAGCAATGTGATT Reverse: TAATACGACTCACTATAGGGTTATGGTACAAGGAGAGGA	405
GFP	Forward: TAATACGACTCACTATAGGGGGTGATGCTACATACGGAAAG Reverse: TAATACGACTCACTATAGGGTTGTTTGTCTGCCGTGAT	370

Gene name	Primer sequences for qRT-PCR	Product length (bp)	Slope	R^2	Primer efficiency (%)
brahma	Forward: TCGCTTGATTCTGCTTGTGGA Reverse: AGAACGAAGCGACAGGGTCT	166	–3.266	0.996	100.41
hunchback	Forward: TGCCCCAAGTGTCCTTTTGT Reverse: CAGTCAGAACAGCGGTATTGGT	179	–3.348	0.997	98.94
β-actin	Forward: TCCAGGCTGTACTCTCCTTG Reverse: CAAGTCCAAACGAAGGATTG	134	–3.419	0.999	96.1

Underlined sequence corresponds to T7 promoter

fluorescent protein) gene was amplified from the pIZT/V5-His expression vector (Invitrogen) using the gene-specific primers provided in Table 1. The PCR product amplified for *brm*, *hb* and *GFP* were used as a template for *in vitro* synthesis of dsRNAs using the MEGAscript high-yield transcription kit (Applied Biosystems Inc., Foster City, CA). All the synthesized dsRNAs were purified using the RNAeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. All dsRNA preparations were quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Franklin, MA) at 260 nm and analyzed by gel electrophoresis to determine purity.

2.4. Parental RNAi (pRNAi) bioassay

Parental *RNA* interference in *D. v. virgifera* adults was conducted by feeding dsRNA corresponding to the gene of interest to adult females after mating. Test insects were purchased from a commercial vendor (Crop Characteristics, Farmington, MN), and 6 males and females (24–48 h old) were maintained on untreated artificial diet adapted from Branson and Jackson (1988) and allowed to mate for 4 days in 16-well trays (5.1 cm long × 3.8 cm wide × 2.9 high) with vented lids. The diet was modified slightly to provide the consistency necessary to cut diet plugs that could be treated with dsRNA. The dry ingredients as described by Branson and Jackson (1988) were added to distilled water at a rate of 48 gm/100 ml with 3% agar, and 5.6 ml of glycerol. In addition, 0.5 ml of a mixture of propionic acid:phosphoric acid (47%:6%) was added to inhibit microbial growth. The agar was dissolved in boiling water and the dry ingredients and microbial inhibitor solution added, mixed thoroughly and poured to a depth of approximately 0.5 cm. After solidification, the diet plugs (ca. 0.4 cm in diameter) were cut from the diet with a #1 cork borer.

On day five of the bioassay, males were removed from the container, and the remaining females were provided artificial diet surface treated with gene specific dsRNA (2 mg/diet plugs, 4 mm diameter × 2 mm height). Freshly treated artificial diet was provided every other day throughout the exposure period. Control treatments consisted of gravid females exposed to diet treated with either *GFP* dsRNA or water. After six days of exposure to each treatment, females were transferred to polystyrene oviposition egg boxes (7.5 cm × 5.5 cm × 5.5 cm) (ShowMan box, Althor Products, Wilton, CT) using the design of Campbell and Meinke (2010) and held at 23 ± 1 °C, relative humidity >80% and L:D 16:8. Boxes contained moistened silty clay loam soil pre-sifted through a 60-mesh sieve and autoclaved (Jackson, 1986). Females were allowed to lay eggs for four days, and the eggs were incubated in soil within the oviposition boxes for 10 days at 27 °C. Eggs were removed from the ovipositional soil by washing through a 60-mesh sieve. Both females and a subsample of eggs from each treatment were removed from the oviposition boxes and flash frozen in liquid nitrogen for subsequent expression analyses by quantitative qRT-PCR (see below).

Harvested eggs were held in Petri dishes on moistened filter paper at 28 °C, relative humidity >80%, 24 h dark and monitored for 15 days to determine egg viability. The Petri dishes were photographed with a Dino-Lite Pro digital microscope (Torrance, CA) and total eggs counted using the cell counter function of Image J software (Schneider et al., 2012). The number of larvae hatching from each plate was recorded daily until no further hatching was observed. Embryos from unhatched eggs were dissected from each treatment to examine embryonic development and to estimate phenotypic responses to the pRNAi effect.

2.5. Baseline expression

The baseline expression of *brahma* and *hunchback* was determined by qRT-PCR with six *D. v. virgifera* life stages. Three biological replications per developmental stage were used in this experiment. Three sets of ~50 eight-day-old eggs, ~30 first instars, ~25 second instars, and ~10 third instars were collected and flash frozen for RNA extraction as previously described. For pupae, mated females and males, one individual per replication was used for RNA extraction. RNAs were used to synthesize cDNA for qRT-PCR as described below.

2.6. Quantitative real-time PCR

Total RNA was isolated from the whole bodies of both adult females and eggs using RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. Before the initiation of the transcription reaction, total RNA was treated with DNase to remove any genomic DNA using Quantitech reverse transcription kit (Qiagen, Valencia, CA). Total RNA (500 ng) was used to synthesize first strand cDNA as a template for real-time quantitative reverse transcriptase-PCR (qRT-PCR). The RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Franklin, MA) at 260 nm and purity evaluated by agarose gel electrophoresis.

qRT-PCR was performed using the Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) and 7500 Fast System real-time PCR detection system (Applied Biosystems, Grand Island, NY). Primers used for qRT-PCR analysis were designed using Beacon designer software (Premier Biosoft International, Palo Alto, CA) and are provided in Table 1. The 7500 Fast System SDS v2.0.6 Software (Applied Biosystems Grand Island, NY) was used to determine the slope, correlation coeffi-

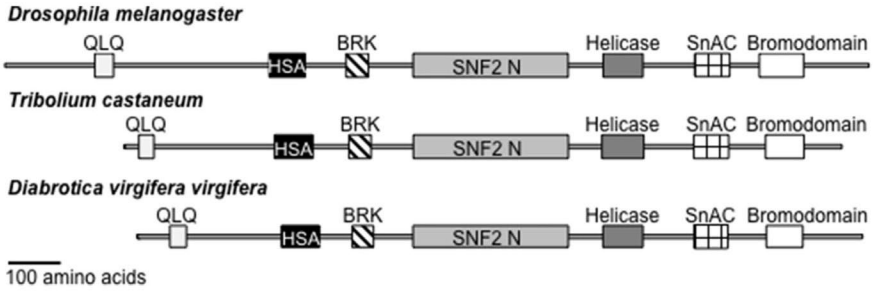

Figure 1. Domain organization of the *Drosophila* (Accession Number: AAA19661.1), *T. castaneum* (Accession Number: XP_008198809.1), and *D. v. virgifera* (Accession Number: KR152260) Brahma protein sequences. A combination of Pfam and InterProScan database analyses identified the following domains: QLQ (PF08880), which contains a conserved Gln, Leu, Gln motif, is found at the N-terminus of SWI2/SNF2 proteins, and is involved in protein–protein interactions; HSA (PF07529), a predicted DNA-binding domain found in helicases; BRK (PF07533), a domain called after Brahma and Kismet that is found in SWI2/SNF2 and CHD family chromatin remodelers; SNF2 family N-terminal (PF00176) and Helicase conserved C-terminal (PF00271) domains, a combination of which is characteristic to chromatin remodeling ATPases. SNF2 N-terminal domain also appears in chromatin repair proteins. SnAC (PF14619) Snf2-ATP coupling, chromatin remodeling complex, a functional domain with nucleosome remodeling activity in SWI/SNF proteins. The bromodomain (PF00439), a chromatin interaction domain that recognizes acetylated lysine residues on the N-terminal tails of histones.

cients, and efficiencies (Table 1). The efficiencies of primer pairs were evaluated using 5 fold serial dilutions (1: 1/5: 1/25:1/125: 1/625) in triplicate. Amplification efficiencies were higher than 96.1% for all the qRT-PCR primer pairs used in this study (Table 1). All primer combinations showed a linear correlation between the amount of cDNA template and the amount of PCR product and all correlation coefficients were larger than 0.99. qRT-PCR analysis was performed with three biological replications for the eggs and six for the adults, each biological replication had two technical replications. qRT-PCR cycling parameters included 40 cycles each consisting of 95 °C for 3 s, 58 °C for 30 s, as described in the supplier's protocol. At the end of each PCR reaction, a melting curve was generated to confirm single peaks and rule out the possibility of primer-dimer and nonspecific product formation. Relative quantification of the transcripts were calculated using the comparative 2-ddCT method (Livak and Schmittgen, 2001) and were normalized to b-actin (Rangasamy and Siegfried, 2012).

2.7. Statistical analysis

Statistical analyses were performed using SAS software version 9.3 (SAS-Institute, 2011). An analysis of variance (ANOVA) was performed using PROC GLIMMIX with the least-square estimated means procedure to determine differences between treatments (water, *GFP*, *dvvbrm,* and *dvvhb*) for the number of eggs per female, percent of total emerged larvae and relative expression of eggs and adults.

3. Results

3.1. Gene identification

Open reading frame nucleotide sequences and protein alignments for *brm* and *hb* sequences are provided in Supplemental Figures 1 and 2, respectively. The western corn rootworm *brahma* (ATP-dependent chromatin remodeler *brm*; *dvvbrm*) represents a sequence of 4768 bp and a predicted peptide sequence of 1375 amino acids (Accession Number: KR152260) (Supplemental Figure 1). Within this sequence, several domains were predicted including QLQ (59–95), HSA (275–347), BRK (409–453), DEXDc (516–708), HELICc (876–960), SnAC (1056–1120), and BROMO (1169–1279) (Figure 1), which are characteristic of genes associated with members of the SWI2/SNF2-family ATPase subunit chromatin remodeling complexes (Clapier and Cairns, 2009; Mohrmann and Verrijzer, 2005). When searched in the NCBI database using the BLASTx algorithm, the most similar sequence was from *T. castaneum* with 74% sequence identity.

The western corn rootworm *hunchback* (*dvvhb*) represents a sequence of 1955 bp and 573 amino acids (Accession Number: KR152261) (Supplemental Figure 2). Within this sequence, six C2H2 type zinc finger domains were predicted at positions 226–248, 255–277, 283–305, 311–335, 520–542, and 548–572 (Figure 2) in agreement with its role as a zinc finger transcription factor (Jurgens et al., 1984; Lehmann and Nussleinvolhard, 1987; Patel et al., 2001; Schröder, 2003; Tautz et al., 1987). When searched in the NCBI database using the BLASTx algorithm, the most similar sequence was from *T. castaneum* with 53% sequence identity.

3.2. Bioassay results

The administration of dsRNA for *dvvbrm* and *dvvhb* through treated artificial diet did not significantly affect adult longevity over the 15 days of mating, ovariole maturation and oviposition (data not shown). In addition, the mated females exposed to dsRNA *for dvvbrm* and *dvvhb* produced approximately equal number of eggs to females exposed to untreated diet or diet treated with *GFP* dsRNA (Figure 3). However, the eggs collected from females that were exposed to *dvvbrm* and *dvvhb* dsRNA were not viable (Figure 4). In the case of adult females exposed to *dvvbrm* dsRNA, none of the collected eggs hatched and for females exposed to *dvvhb* dsRNA, 2.4% of the eggs hatched. Each oviposition box produced approximately 200 eggs which were evaluated for hatching. Unhatched eggs were dissected to examine content, there was no evidence of embryonic development in the eggs from the *dvvbrm* treatment as only undifferentiated cytoplasm was present (Figure 5). In contrast, the eggs obtained from adult females exposed to *dvvhb* dsRNA exhibited some development (Figure 6) but were generally shortened in comparison to controls and appeared to be missing a number of abdominal and thoracic segments and appendages (Figure 6), although the response was variable among individual larvae (Figure 6).

3.3. Gene expression

We evaluated the expression of *dvvbrm* and *dvvhb* genes in both the adult females exposed to dsRNA and in the eggs oviposited by these females (Figs. 8 and 7). We observed 86.0% and 84.6% reduction in the expression of *dvvbrm* in the adult females exposed to the *dvvbrm* dsRNAs compared to beetles exposed to water and *GFP* dsRNA (Figure 8A) and for females exposed to *dvvhb* dsRNA, 63.5% and 59.3% reduced expression of *dvvhb* relative

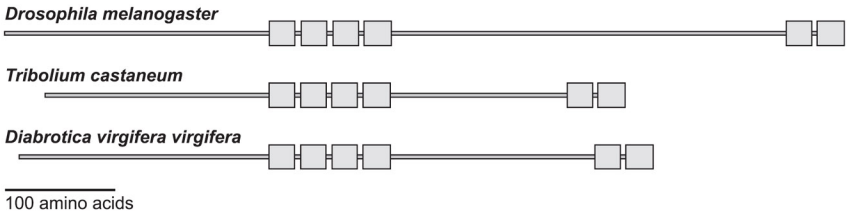

Figure 2. Domain organization of *D. melanogaster* (Accession Number: NP_731267.1), *T. castaneum* (Accession Number: NP_001038093.1), and *D. v. virgifera castaneum* (Accession Number: KR152261) Hunchback protein sequences. *D. melanogaster, T. castaneum,* and *D. v. virgifera* Hunchback proteins contain six C2H2-type zinc fingers (represented by shaded boxes), annotated using SMART database within InterProScan http://www.ebi.ac.uk/interpro/.

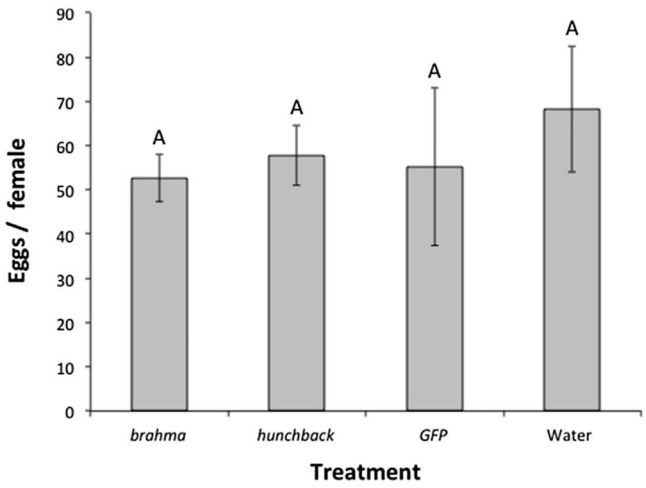

Figure 3. Effect of *dvvbrm* and *dvvhb* dsRNA on egg production relative to *GFP* and water controls in adult females exposed to treated artificial diet. Bars with the same letter are not significantly different (P > 0.05; N = 6 replications of 5 females/rep).

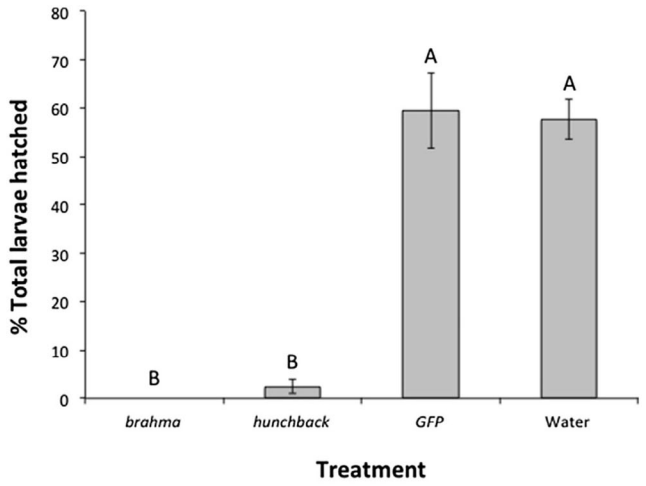

Figure 4. Effect of *dvvbrm* and *dvvhb* dsRNA on egg viability relative to *GFP* and water controls in eggs collected from adult females exposed to treated artificial diet. At least 820 total eggs were evaluated per treatment. Bars with the same letter are not significantly different (P > 0.05; N = 6 replications of 5 females/rep).

to water and *GFP*, respectively was observed (Figure 8B). In eggs collected from the exposed females, we observed 98.9% and 99.0% reduction in *dvvbrm* expression relative to the water and *GFP* dsRNA treatments (Figure 7A).We confirmed that the eggs used for qRT-CR from females exposed to *dvvbrm* dsRNA were not dead by evaluating RNA quality on a 1% agarose gel. In addition, the CT values for the reference gene were compara-

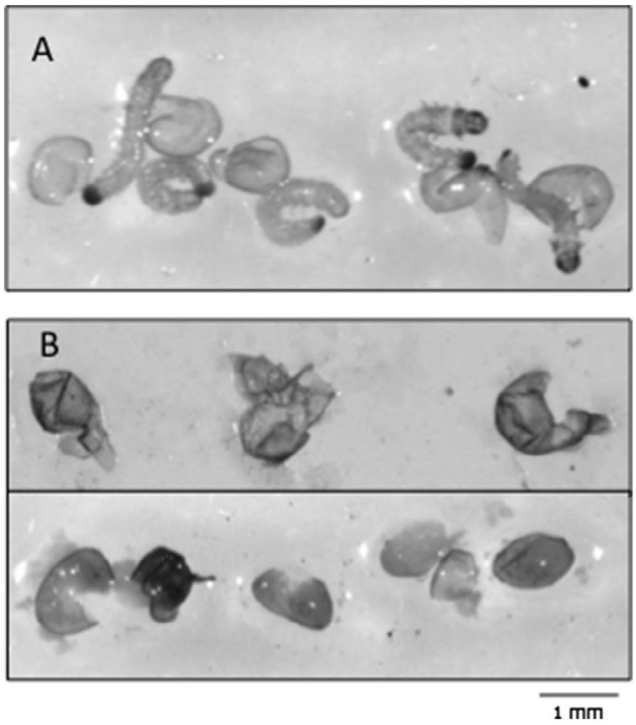

Figure 5. Phenotypic response to *brahma* parental RNAi; A) larvae dissected from eggs of *GFP* dsRNA treated females exhibiting normal development; B) lack of embryonic development and undifferentiated cytoplasm in eggs oviposited by females exposed to *dvvbrm* dsRNA.

ble between the controls and eggs from *dvvbrm* dsRNA treated females. Similarly, we observed 86.0% and 84.6% reduction in the expression of *dvvhb* in eggs obtained from females treated with *dvvhb* dsRNA compared to water and *GFP* controls, respectively. The mRNA level for the *GFP* control was unusually high which we have previously observed with expression analysis with other genes (Figure 7B).

The baseline expression of *dvvbrm* and *dvvhb* was measured at different developmental stages (Figure 9). Relative to eggs, the highest *dvvbrm* expression level was observed in second instar larvae and females (around 100%), and, the expression was relatively low in third instar larvae and pupae with 35% and 23% expression, respectively (Figure 9A). The expression level of *dvvhb* was high in eggs and gradually decreased from first instar larvae (59%) to pupae (7.3%). Relative to eggs, the highest *dvvhb* expression was observed in adult females and males (Figure 9B).

4. Discussion

The results of this study clearly document the systemic nature of RNAi in western corn rootworm adults and the potential to achieve a parental RNAi effect where genes associated with em-

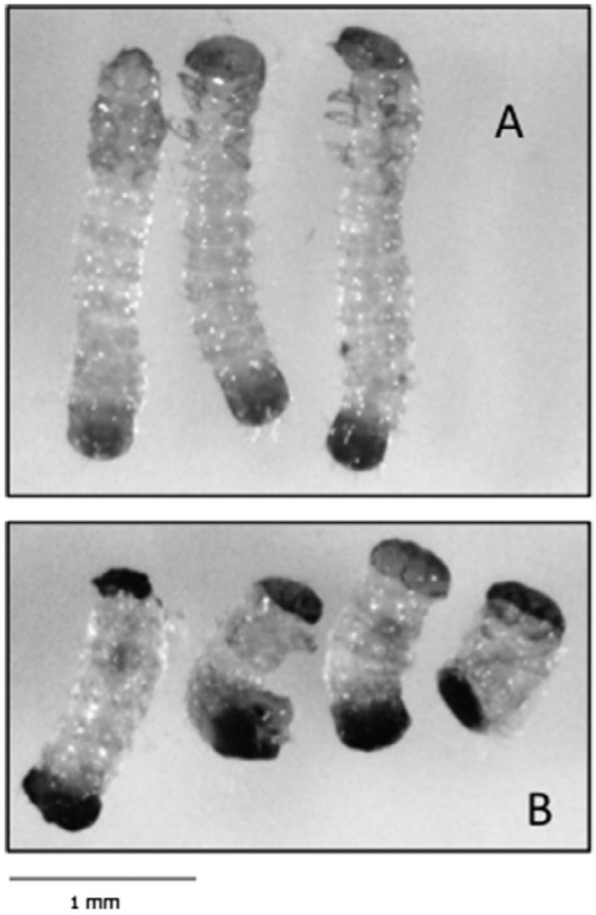

Figure 6. Phenotypic response to *hunchback* parental RNAi; A) normal development of larvae dissected from eggs of *GFP* dsRNA treated females; B) morphological anomalies (*e.g.,* reduced segmentation, loss of appendages and deformed head) in larvae dissected from eggs of females exposed to *dvvhb* dsRNA.

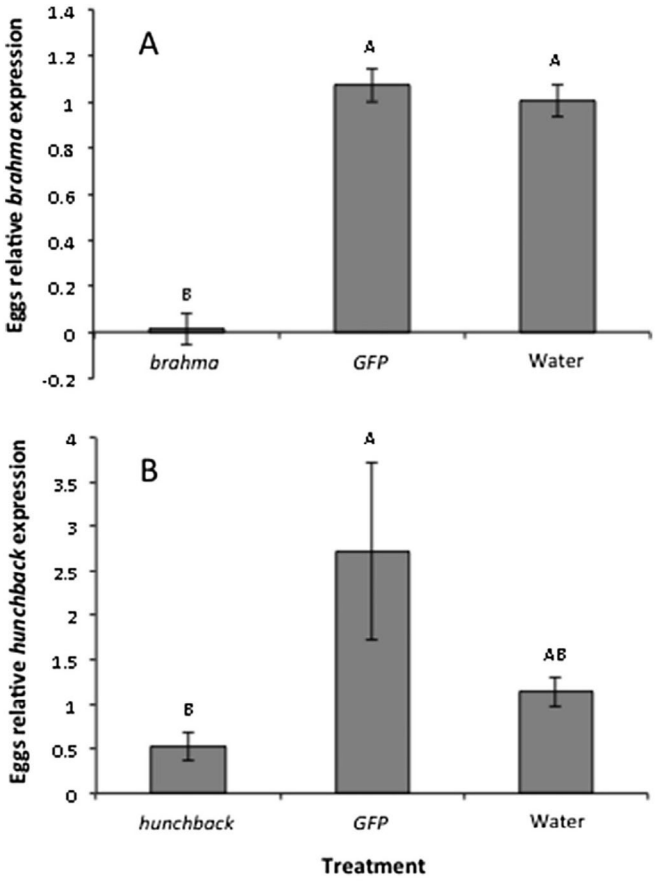

Figure 7. Relative expression of *dvvbrm* (A) and *dvvhb* (B) in eggs collected from females exposed to dsRNA in treated artificial diet relative to *GFP* and water controls. Bars followed by the same letter are not significantly different (P > 0.05; N = 3 biological replications of 10 eggs/replication with 2 technical replications/sample).

bryonic development are knocked down in the eggs or ovaries of females that are exposed to dsRNA. Importantly, this is the first report of a pRNAi response to ingested dsRNA in western corn rootworms. A systemic response is indicated based on the observation of knockdown in tissues other than the alimentary canal where exposure and uptake of dsRNA occurs.

The mechanism by which systemic RNAi response in insects is achieved is not yet clear (Gatehouse and Price, 2011). Our results confirm that the dsRNA can be taken up by gut tissue and translocated to other tissues (*e.g.,* developing ovarioles). It is uncertain whether the response is the result of cell-to-cell transport of dsRNA or siRNAs generated by the activity of the endonuclease, Dicer-2, within the cell initially exposed to the dsRNA. It is also uncertain whether the parental RNAi effect observed in rootworms is restricted to transcripts that are maternally supplied or whether it is possible to achieve robust pRNAi effect with transcripts that have only zygotic expression. This question may be best answered using genes that have exclusively maternal or zygotic expression profiles in WCR, rather than *hb* and *brm* that are known to have both maternal and zygotic expression in other insects (Brizuela et al., 1994; Lehmann and Nussleinvolhard, 1987). Furthermore, while we investigated genes that are associated with embryonic development, it may be possible to achieve knockdown of genes that function in late larval development or that have completed development and are important to other aspects of rootworm biology. Further studies are necessary to determine if larval feeding of dsRNA can affect larval survival and/or if the RNAi response persists through larval development to adulthood.

The precise mechanisms by which *dvvbrm* and *dvvhb* disrupt embryonic development in western corn rootworms are also unclear and warrant further consideration. In the case of *hb*, its role has been described as a key regulatory gene in the anterior-posterior patterning of insect embryos that can be provided both maternally and zygotically (Jurgens et al., 1984; Lehmann and Nussleinvolhard, 1987; Patel et al., 2001; Schröder, 2003; Tautz et al., 1987). A gap phenotype has been identified in *Drosophila* mutants with deletions of the labial through third thoracic segments and the eighth abdominal segment (Doe et al., 1991; Finkelstein and Perrimon, 1990; Tautz et al., 1987). In *Tribolium*, the reduced expression of *hb* by parental RNAi has been shown to cause disruption of head and thoracic segmentation (Schröder, 2003). In western corn rootworms, the phenotype associated with *dvvhb* RNAi generally involves disruption of abdominal and thoracic segmentation rather than the head, which appeared to be mostly developed in the individuals dissected from unhatched eggs. However in some individuals, the mouthparts also seemed to be somewhat deformed.

In contrast to the developmental phenotype associated with the *hb* RNAi described above, eggs collected from females treated with *brm* dsRNA exhibited a complete absence of development. The SWI/SNF ATP-dependent chromatin remod-

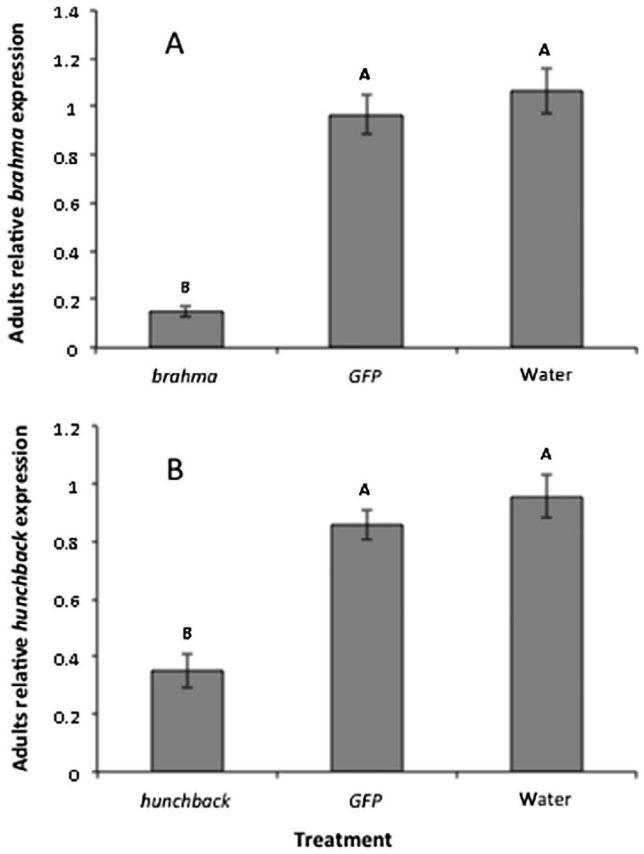

Figure 8. Relative expression of *dvvbrm* (A) and *dvvhb* (B) in adult females exposed to dsRNA in treated artificial diet relative to *GFP* and water controls. Bars followed by the same letter are not significantly different (P > 0.05; N = 6 biological replications with 2 technical replications/sample).

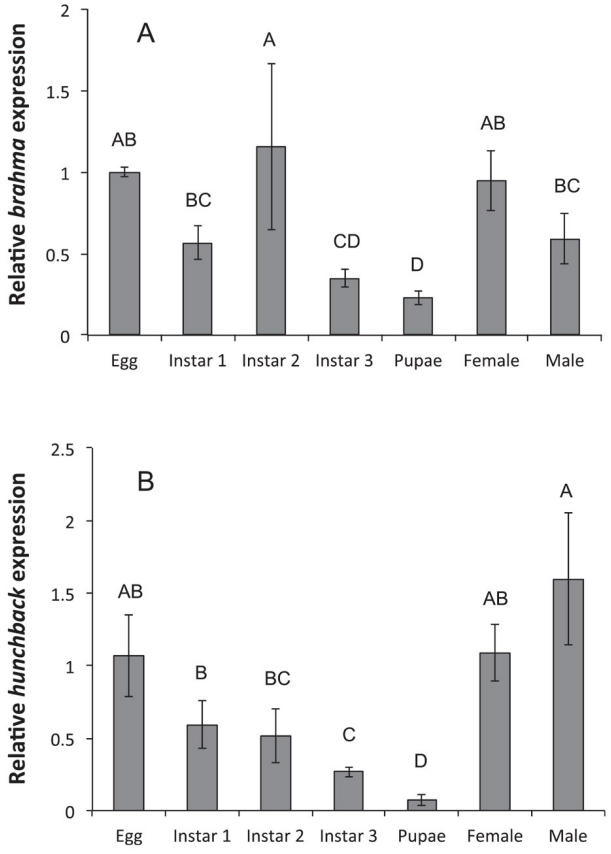

Figure 9. Relative expression of *dvvbrm* (A) and *dvvhb* (B) in different *D. v. virgifera* life stages. Bars followed by the same letter are not significantly different (P > 0.05; N = 3 biological replications with 2 technical replications/sample).

eling assists transcription events by interaction with nucleosomal substrates to alter DNA:histone contact and facilitates DNA translocation (Clapier and Cairns, 2009). The loss of *Drosophila brahma* impairs overall transcription by RNA polymerase II (Pol II), suggesting a broad function for the Brahma complexes (Armstrong et al., 2002). Such roles and the clear impact of RNAi-mediated knockdown of *dvvbrm* suggest strongly that disruption of the *dvbrm* expression causes effects very early in embryonic development. In *Drosophila*, *brahma* is also involved in gametogenesis (Brizuela et al., 1994; Elfring et al., 1998).

In addition to embryonic expression, both *brm* and *hb* transcripts and Brm and Hb proteins have been detected in other life stages in insects other than *D. v. virgifera*. For example, low levels of *brm* transcript are present in *Drosophila* larvae, pupae, and adult females (Tamkun et al., 1992). Likewise, Brm protein is present in all stages of development, with the lowest levels of expression in larva and adult females (Elfring et al., 1998). The results from our baseline expression experiment suggest that *dvvbrm* and *dvvhb* are expressed in all developmental stages. The lowest expression was observed in pupae, and the highest in eggs, females and males. It is possible that in both *Drosophila* and *D. v. virgifera* the detected transcript in adult females is from the ovaries. Second instar larvae showed a 15% higher expression of *dvvbrm*. Immunohistochemical analysis of the *Drosophila* Brm protein indicates that the pattern changes from ubiquitous expression in early embryogenesis, to enrichment in the central nervous system in late embryogenesis, to broad expression in imaginal discs in larvae (Elfring et al., 1998). Moreover, an inducible dominant-negative allele of *Drosophila brm* has been used to identify wing, leg, bristle, and other homeotic transformations in adults, when turned on in larval stages (Elfring et al., 1998). While we did not observe increased mortality in adult *D. v. virgifera* females fed with *dvvbrm* and *dvvhb* dsRNA, it is possible that exposure to these dsRNAs could cause phenotypic effects in the adults and other life stages. Since pRNAi is likely to be deployed in combination with a lethal RNAi trait, additional long-term phenotypic effects may not affect the overall utility of *dvvbrm* and *dvvhb* RNAi for parental control.

More complete examination of the effects of *dvvbrm* and *dvvhb* RNAi on embryology may shed further light onto the role of these genes in regulating development of rootworm embryos. Targeting the functions of *brahma* and other chromatin remodeling and chromatin modifying proteins may be of particular utility for the parental RNAi control of rootworms. Since the functions of chromatin remodelers confer epigenetic effects that lead to stable changes in gene expression, short-term exposure to dsRNA in the ovary or in the embryo may lead to long-lasting RNAi-induced phenotype. Mutations in other members of the chromatin remodeler gene group are known to have gametogenic and/or embryonic effects in fruit flies (Daubresse et al., 1999; Deuring et al., 2000; Kehle et al., 1998; McDaniel et al., 2008); it remains to be seen if these genes would also serve as efficacious pRNAi targets for pest insect control. The ability of western corn rootworm to develop resistance to crop protection chemistries (Gray et al., 2009; Narva et al., 2013) and transgenic insect resistance traits based on Bt proteins (Gassmann et al., 2011) creates the need for new approaches to corn rootworm control to combine with existing technology. Regardless

of the precise mechanisms, the ability to knock down the expression of genes involved with embryonic development to prevent rootworm eggs from hatching offers a unique opportunity to achieve control of western corn rootworms. Adults readily feed on above ground reproductive tissues such as silks and tassels, so it may be possible to expose adult rootworms by transgenic expression of dsRNA and achieve corn root protection in the subsequent generation by preventing eggs from hatching. This technology could be deployed with larval control technologies including Bt and/or RNAi lethal genes, and potentially be used to increase the durability of transgenic traits for rootworm pest management. However, because the adult females were exposed continuously throughout ovariole development and oviposition, the timing and level of exposure necessary to achieve the pRNAi response are uncertain and a more thorough investigation of dose–response relationships for pRNAi targets is necessary to fully evaluate their utility for pest management.

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Appendix A. Supplementary data — Supplementary data related to this article can be found following the **References**, or at http://dx.doi.org/10.1016/j.ibmb.2015.05.011

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SUPPLEMENTAL FIGURES

Supplemental Figure 1. *Diabrotica virgifera virgifera brahma* sequences

Supplemental Figure 1A. *Diabrotica virgifera virgifera cDNA* sequence containing *brahma* open reading frame (underlined). dsRNA sequence is highlighted in grey. Accession No. KR152260.

CAAGTGGCCATGGCATGCCACAGGGTCCCCCTGGACAACCAGGTCAGCAACACCAAGGCCGAAC
TGCTGATAATTTACATGCCTTACAAAAAGCAATAGATACAATGGAAGAAAAAGGTATGCAAGAA
GATCAGAGGTATTCACAGTTACTGGCGTTACGTGCTAGATCCAGTGGTCAACCATCTAACGGAG
TTCTTACACCGCTGCAAATGAATCAACTTAGAAATCAAATTATGGCATACAGGTGCCTAGCGAG
GAGCCAACCAATTCCTCCTTCAATAATGTTGGGGCTGCAAGGAAAGAGGCCTGACGGTTCACCA
CAGTTTCCTACACCTCCGTCAAGTCCGTTTCAACCACAAGGACCTGGTGCACCCCCTGGTCCGG
AACAACCACCAGCTAATGCAGAAAACGTAGCAGAGCCAGCAGCACCAGTAGGACCGCAAGGTGC
ACAAGGACCTCCTAACCAACAGAGAGCTCAAACTAGCCAGTTAGTCCCCAATAAGCAAACTCGT
TTCACTACCATGCCCAAACCATCTGGACTAGATCCACTAGTTCTTCTTCAAGAGAGGGAAACTA
GGGTGGCAGCTAGAATCGCTGCTAGAATAGAACAATGTAGTAACTTACCTACCAATCTTTCAGA
CAAAGTCCGCATGCAAGCACAGATAGAATTGAGAGCTTTGCGGTGCCTTAATTTCCAAAGGCAA
CTAAGAAGCGAAATTTTGAACTGTATTAGGAGAGATATAACGCTTGAATCTGCTGTAAATTTTA
AAGCATATAAAAGAACGAAGCGACAGGGTCTAAAAGAATCGAGAGCTACAGAGAAGTTAGAAAA
ACAACAGAAGTTAGAAGCAGAAAGAAAGAGAAGACAGAAGAACCAAGAATTTTTGAATGCTGTA
TTGAACAATGGAAAAGAATTCAAGGAATTCCACAAGCAGAATCAAGCGAAATTAGCTAAGATTA
ATAAAGCTGTTATTAATTATCACGCTAATGCTGAAAGAGAGCAAAAGAAAGAAGCAGAAAGGAG
AGAGAAGGAACGTATGATCAGATTGATGGCAGAAGATGAAGAAGGTTATAGACAGTTGATCGAT
CAAAAGAAAGACAAACGTCTAGCGTTCTTGCTTTCCCAAACAGATGAATATATCAGTAACTTAA
CAGAGATGGTGAAAATGCACAAAGTCGAACAAAGTAACAAGAAGCGGGAAGAAGAACGACGGAA
GAGAAGGCAAGACAAAATGCAGCAGCCTGACAGGAAAGTCACAGTTATCGAAATGGCTACTGGG
AATAAGGTTAGTGGAGAAAACGCTCCGACTGTCCAGGAACTTCCTGAATGGTTACAGACTCATC
CTGGTTGGGAGATGATAGATACAGAAGACGAGGACGAGAATGACGAATATAGAATGGACGATTA
TGAAGAAAATAATCAAGTCGATGCTACAGAAATCATTCAGAAAGCCAAGGTTGAGGATGACGAA
TATCACAAGAATGCCACAGAGGAACAGACGTACTACGGTATTGCACATACAGTGAGCGAGTCAG
TATCAGAACAGGCCTCCATTATGATAAACGGTGAACTGAAAGAGTACCAGGTCAAAGGACTGGA
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AAGACTATTCAAACCATTGGCCTGATCACCTACTTGATGGAGAAAAAAAAGTTGAATGGGCCAT
TTTTGATCATTGTGCCGTTATCCACTATATCTAATTGGATGTTGGAGTTCGAAAAATGGGCTCC
TTCTGTTGTGGTCGTCTCCTACAAAGGCTCACCTGGTCACAGGAAATTGCTTCAGGGTCAGATG
AAGTCAGCAAAATTCAATGTTCTTCTTACTACTTATGAATATATCATTAAAGATAAGGGAATTC
TTTCAAAAGTACCGTTTAAGTATATGATCGTGGACGAGGGTCACAGAATGAAGAACCATCATTG
CAAGTTGACCCAGACTTTGAACACTCACTACGCAGCTCCTTTCCGCCTTCTCTTAACCGGTACT
CCTCTACAAAACAAACTACCAGAACTGTGGGCGTTGCTTAACTTCTTACTTCCGTCTATTTTCA
AGAGTTGTTCCACTTTCGAGCAATGGTTCAACGCCCCTTTCGCAACCACGGGAGAAAAGGTTGA
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ATGTAGGCATGGGTGGGGGACTCACATCAGGGCCGGATATATACAGATCTTCTGGTAAATTTGA

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ATGACGACGTTGATGAACATCATGGAAGACTACTTCATTTGGAGAGGTTACAAATATCTTCGTC
TGGATGGTATGGTAAAAGCGGAAGATCGGGCGGAACTACTCAAGAAGTTCAATGACAAACAAAG
CGAATATTTTGTGTTTCTATTGTCAACAAGAGCAGGAGGTCTTGGACTCAACTTGCAAAGTGCT
GATACTGTTATCATCTTTGATTCTGACTGGAATCCTCACCAGGATTTACAAGCTCAAGATCGTG
CCCATCGTATAGGCCAGCAAAATGAAGTCAGGGTCCTACGTTTAATGACAGTTAATTCAGTGGA
AGAAAGAATCTTAGCTGCAGCTAAATACAAACTTATAATGGACGAGAAAGTAATCCAAGCTGGT
ATGTTCGATCAGAAGTCTACAGGCTCAGAGAGACATCAGTTTTTGCAGAGTATTTTACACCATG
ACGGAAGCGACGAAGAAGAGGAAAACGAAGTTCCTGATGACGAAACAGTGAACCAGATGTTGGC
CCGAAGGGAAAACGAATTTCAGCTTTTCCAGAAGATGGATCAGGAAAGAAAGGAAGAAGATGAA
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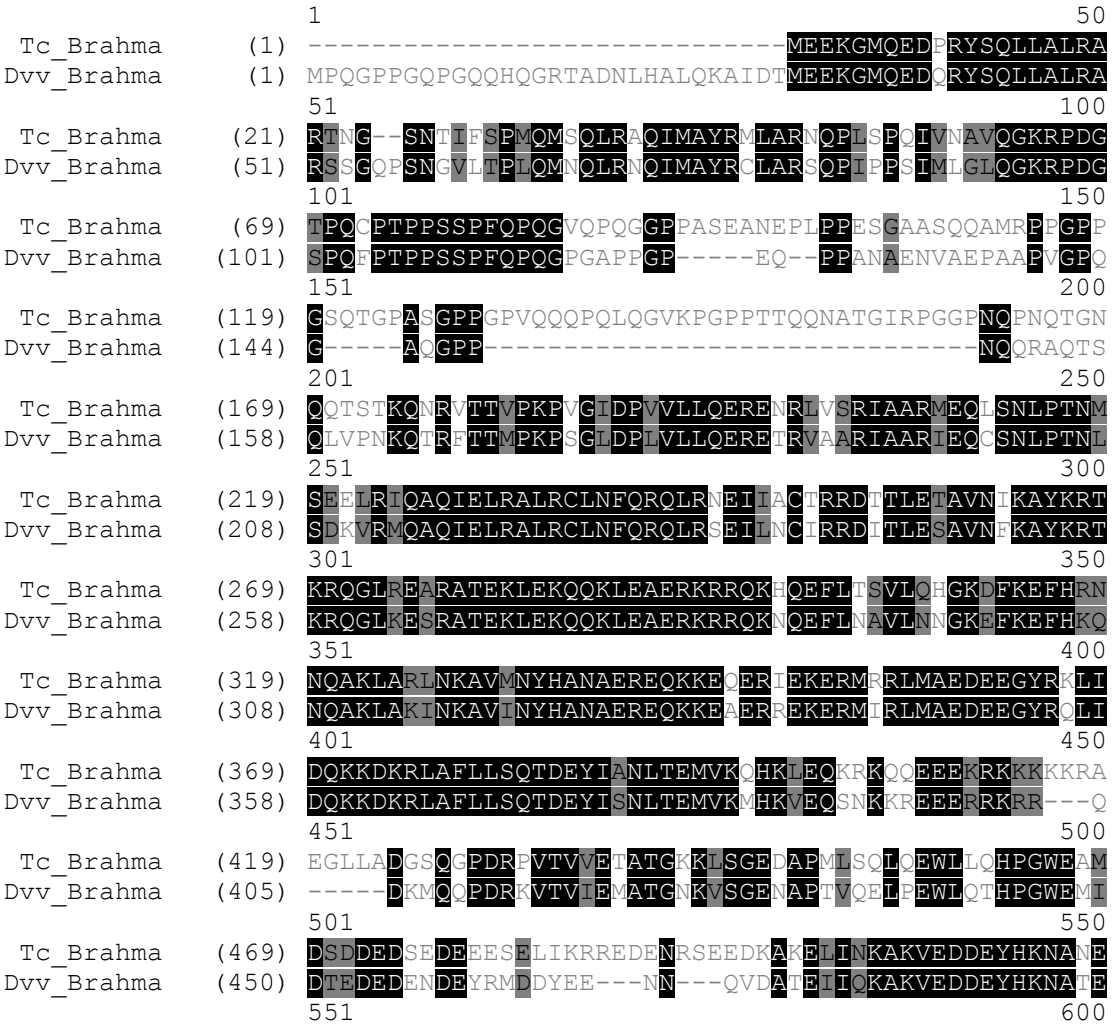
AAAAGAAGTTGATTATGTTGACAGCCTGACGGAGAAAGAGTGGCTTAAGGCTATTGACGAAGAG
GGAGAATTTGAGGAAGAACAAGAAGGTGATAAAGAAGGTCTCAGAAAGAAAAGAGGGAGGAAGA
GGAAGAAGCGCGATGATGACGAAGAGGCAAGCCAAATTAAGAGAAGAAAGGTGCATCTAGCCGA
GATCAAGATGAAGAAAAAGATGAAGAGGCTTATGGAAGTTGTTGTGAACTACAGGGATAGGGAT
GGTAGAGTATTGAGCGAACCGTTTATGAAACTTCCATCAAAGAAGGAGTTACCTGAATATTACG
ATACGATTAAGAAACCTATTGATATTGAAAAAGTCGTTGCCAACGTAGAAGAAGGAAAATATTT
CACGATGCACGATTTGGAAAGAGATTTCGACTTGCTGTGCCAAAACGCCCAACAATACAACGAA
GAAGACTCCATGATCTACGAGGACAGCCTCGTTCTTCGACAGGTGTTTAGAAGCGCGAGGGAAA
AGATCGACGGTACCTCAGACCACGACGACAACGCCGATGGACCGGCGGTGGCTCAGATCAAACG
ACCTCGTGGTAGACCTCGAAAACACAAGAGACCCGAAGAGATCGAGGCCGAAGCGGCGGCTCAG
AAAGCTATGGAGGAGGCATCGAAGCTGAGAGCTCAAGCTGAGGCGGAAGAGCTTAGATCTAAGG
TGGAGGAGGCATCTCAGAGAGCCAAAGAGGAAGCGAAAGCAAGGGAGGAAGCCAAAGCTAGGGA
AGAAGCCGAAATCGAGAACATGGAGGAGATTCCCACAAGCACATGATCTATAGAGCAACCGGAA
ACAAAAAGGCAAAAAAGAAATATTATATAGAAAAGATGTACATGTTCAATGGAGATACATTTTC
GCTGAGTTACAACGGGTAATGCTTTTACAACGGATATTTTGACGTATGAATGTTGACGTTCAGA
TGAAGTATATTTATAAAATAATCCAGACCTTTACGTTTTGGTTGATTTGTTTTCTGTATTGTTC
AGTTTATTGAACAACCATTAATAGCAGCTTACCTAAATGATTTAGAAAAGCATCTGAGTTATTT
AGATAAGTTTTGAGATTATATTTATTAACTTTAATATTACTATCTTTATTATAGCATATTGTAA
TTATTTTTTCCTGTCCTTCTTTCGTTGTGTGGTAGATAATCCGAGAGTCAACAGTTATAAGCAA
ATGAAATTCAGTTAAACCTCAAATGTACAAAATGATCAAATTAATGTTTACAATTTATTTTTTT
ACCACGCACATCCACTATTACTATTGTCAGTCATTGAGATATCATTTTATATAGCTCCATGTCT
GTCTTCCTCAATTTACAGAGAAGCAATTAGACAAGTAATGACATAATATGGTGCTGAAATAATG
TGCTTGATAGTGATGTTGAAAAAGTAACTATT

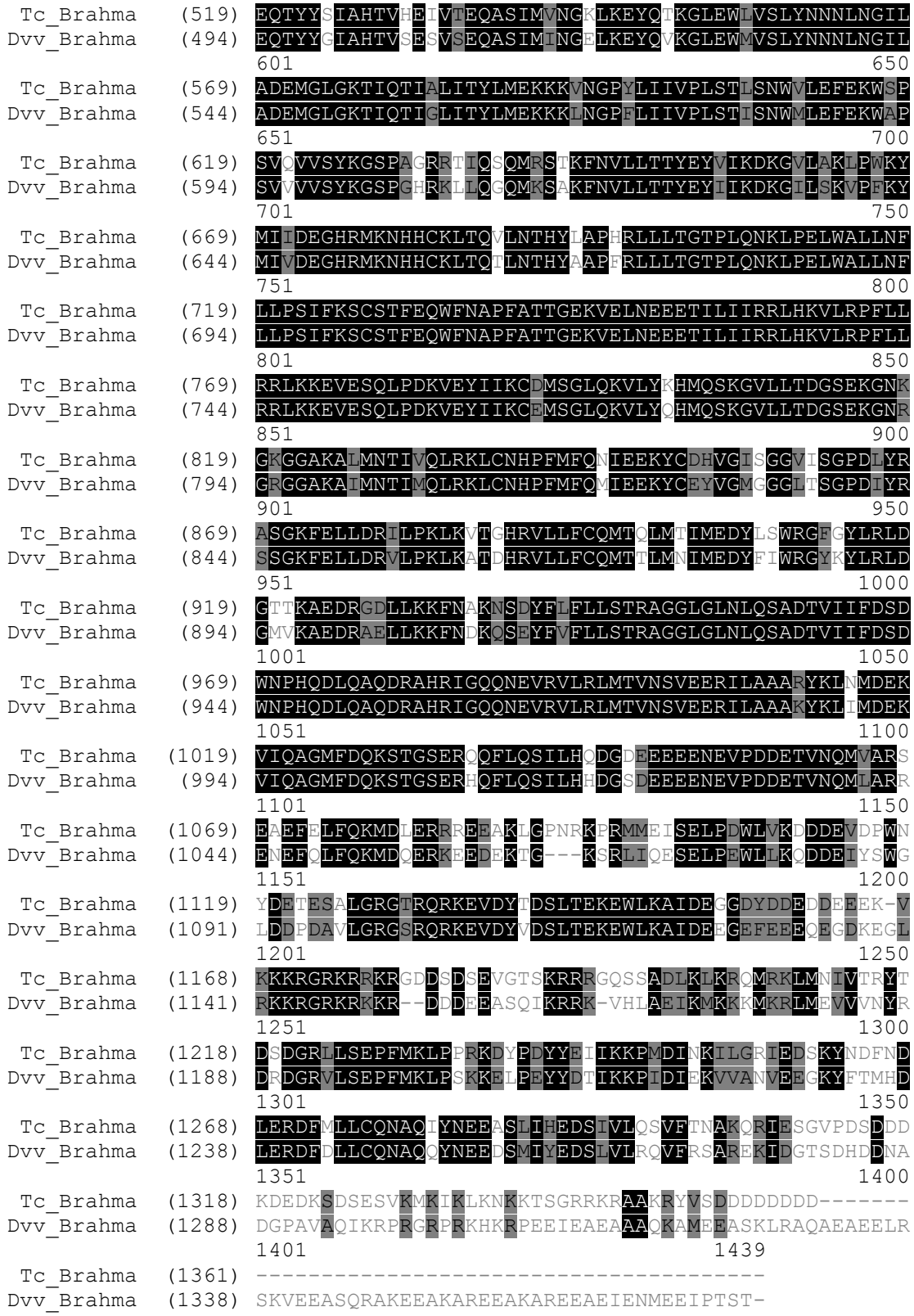
Supplemental Figure 1B. Protein translation of *brahma* sequence from *Diabrotica virgifera virgifera cDNA*.

MPQGPPGQPGQQHQGRTADNLHALQKAIDTMEEKGMQEDQRYSQLLALRARSSGQPSNGVLTPL
QMNQLRNQIMAYRCLARSQPIPPSIMLGLQGKRPDGSPQFPTPPSSPFQPQGPGAPPGPEQPPA
NAENVAEPAAPVGPQGAQGPPNQQRAQTSQLVPNKQTRFTTMPKPSGLDPLVLLQERETRVAAR
IAARIEQCSNLPTNLSDKVRMQAQIELRALRCLNFQRQLRSEILNCIRRDITLESAVNFKAYKR
TKRQGLKESRATEKLEKQQKLEAERKRRQKNQEFLNAVLNNGKEFKEFHKQNQAKLAKINKAVI
NYHANAEREQKKEAERREKERMIRLMAEDEEGYRQLIDQKKDKRLAFLLSQTDEYISNLTEMVK
MHKVEQSNKKREEERRKRRQDKMQQPDRKVTVIEMATGNKVSGENAPTVQELPEWLQTHPGWEM
IDTEDEDENDEYRMDDYEENNQVDATEIIQKAKVEDDEYHKNATEEQTYYGIAHTVSESVSEQA
SIMINGELKEYQVKGLEWMVSLYNNNLNGILADEMGLGKTIQTIGLITYLMEKKKLNGPFLIIV

PLSTISNWMLEFEKWAPSVVVVSYKGSPGHRKLLQGQMKSAKFNVLLTTYEYIIKDKGILSKVP
FKYMIVDEGHRMKNHHCKLTQTLNTHYAAPFRLLLTGTPLQNKLPELWALLNFLLPSIFKSCST
FEQWFNAPFATTGEKVELNEEETILIIRRLHKVLRPFLLRRLKKEVESQLPDKVEYIIKCEMSG
LQKVLYQHMQSKGVLLTDGSEKGNRGRGGAKAIMNTIMQLRKLCNHPFMFQMIEEKYCEYVGMG
GGLTSGPDIYRSSGKFELLDRVLPKLKATDHRVLLFCQMTTLMNIMEDYFIWRGYKYLRLDGMV
KAEDRAELLKKFNDKQSEYFVFLLSTRAGGLGLNLQSADTVIIFDSDWNPHQDLQAQDRAHRIG
QQNEVRVLRLMTVNSVEERILAAAKYKLIMDEKVIQAGMFDQKSTGSERHQFLQSILHHDGSDE
EEENEVPDDETVNQMLARRENEFQLFQKMDQERKEEDEKTGKSRLIQESELPEWLLKQDDEIYS
WGLDDPDAVLGRGSRQRKEVDYVDSLTEKEWLKAIDEEGEFEEEQEGDKEGLRKKRGRKRKKRD
DDEEASQIKRRKVHLAEIKMKKKMKRLMEVVVNYRDRDGRVLSEPFMKLPSKKELPEYYDTIKK
PIDIEKVVANVEEGKYFTMHDLERDFDLLCQNAQQYNEEDSMIYEDSLVLRQVFRSAREKIDGT
SDHDDNADGPAVAQIKRPRGRPRKHKRPEEIEAEAAAQKAMEEASKLRAQAEAEELRSKVEEAS
QRAKEEAKAREEAKAREEAEIENMEEIPTST*

Supplemental Figure 1C. Alignment of *brahma* amino acid sequences from *Diabrotica virgifera virgifera (Dvv)* (Accession number: KR152261) and *Tribolium castaneum (Tc)* (Accession number: XP_008198809.1). Amino acid identities are highlighted in black and similarities are in grey.




Tc_Brahma (519) EQTYYSIAHTVHEIVTEQASIMVNGKLKEYQTKGLEWLVSLYNNNLNGIL
Dvv_Brahma (494) EQTYYGIAHTVSESVSEQASIMINGELKEYQVKGLEWMVSLYNNNLNGIL
601 650
Tc_Brahma (569) ADEMGLGKTIQTIALITYLMEKKKVNGPYLIIVPLSTLSNWVLEFEKWSP
Dvv_Brahma (544) ADEMGLGKTIQTIGLITYLMEKKKLNGPFLIIVPLSTISNWMLEFEKWAP
651 700
Tc_Brahma (619) SVQVVSYKGSPAGRRTIQSQMRSTKFNVLLTTYEYVIKDKGVLAKLPWKY
Dvv_Brahma (594) SVVVVSYKGSPGHRKLLQGQMKSAKFNVLLTTYEYIIKDKGILSKVPFKY
701 750
Tc_Brahma (669) MIIDEGHRMKNHHCKLTQVLNTHYLAPHRLLLTGTPLQNKLPELWALLNF
Dvv_Brahma (644) MIVDEGHRMKNHHCKLTQTLNTHYAAPFRLLLTGTPLQNKLPELWALLNF
751 800
Tc_Brahma (719) LLPSIFKSCSTFEQWFNAPFATTGEKVELNEEETILIIRRLHKVLRPFLL
Dvv_Brahma (694) LLPSIFKSCSTFEQWFNAPFATTGEKVELNEEETILIIRRLHKVLRPFLL
801 850
Tc_Brahma (769) RRLKKEVESQLPDKVEYIIKCDMSGLQKVLYKHMQSKGVLLTDGSEKGNK
Dvv_Brahma (744) RRLKKEVESQLPDKVEYIIKCEMSGLQKVLYQHMQSKGVLLTDGSEKGNR
851 900
Tc_Brahma (819) GKGGAKALMNTIVQLRKLCNHPFMFQNIEEKYCDHVGISGGVISGPDLYR
Dvv_Brahma (794) GRGGAKAIMNTIMQLRKLCNHPFMFQMIEEKYCEYVGMGGGLTSGPDIYR
901 950
Tc_Brahma (869) ASGKFELLDRILPKLKVTGHRVLLFCQMTQLMTIMEDYLSWRGFGYLRLD
Dvv_Brahma (844) SSGKFELLDRVLPKLKATDHRVLLFCQMTTLMNIMEDYFIWRGYKYLRLD
951 1000
Tc_Brahma (919) GTTKAEDRGDLLKKFNAKNSDYFLFLLSTRAGGLGLNLQSADTVIIFDSD
Dvv_Brahma (894) GMVKAEDRAELLKKFNDKQSEYFVFLLSTRAGGLGLNLQSADTVIIFDSD
1001 1050
Tc_Brahma (969) WNPHQDLQAQDRAHRIGQQNEVRVLRLMTVNSVEERILAAARYKLNMDEK
Dvv_Brahma (944) WNPHQDLQAQDRAHRIGQQNEVRVLRLMTVNSVEERILAAAKYKLIMDEK
1051 1100
Tc_Brahma (1019) VIQAGMFDQKSTGSERQQFLQSILHQDGDEEEEENEVPDDETVNQMVARS
Dvv_Brahma (994) VIQAGMFDQKSTGSERHQFLQSILHHDGSDEEEENEVPDDETVNQMLARR
1101 1150
Tc_Brahma (1069) EAEFELFQKMDLERRREEAKLGPNRKPRMMEISELPDWLVKDDDEVDPWN
Dvv_Brahma (1044) ENEFQLFQKMDQERKEEDEKIG---KSRLIQESELPEWLLKQDDEIYSWG
1151 1200
Tc_Brahma (1119) YDETESALGRGTRQRKEVDYTDSLTEKEWLKAIDEGGDYDDEDDEEEK-V
Dvv_Brahma (1091) LDDPDAVLGRGSRQRKEVDYVDSLTEKEWLKAIDEEGEFEEEQEGDKEGL
1201 1250
Tc_Brahma (1168) KKKRGRKRRKRGDDSDSEVGTSKRRRGQSSADLKLKRQMRKLMNIVTRYT
Dvv_Brahma (1141) RKKRGRKRKKR--DDDEEASQIKRRK-VHLAEIKMKKKMKRLMEVVVNYR
1251 1300
Tc_Brahma (1218) DSDGRLLSEPFMKLPPRKDYPDYYEIIKKPMDINKILGRIEDSKYNDFND
Dvv_Brahma (1188) DRDGRVLSEPFMKLPSKKELPEYYDTIKKPIDIEKVVANVEEGKYFTMHD
1301 1350
Tc_Brahma (1268) LERDFMLLCQNAQIYNEEASLIHEDSIVLQSVFTNAKQRIESGVPDSDDD
Dvv_Brahma (1238) LERDFDLLCQNAQQYNEEDSMIYEDSLVLRQVFRSAREKIDGTSDHDDNA
1351 1400
Tc_Brahma (1318) KDEDKSDSESVKMKIKLKNKKTSGRRKRAAKRYVSDDDDDDDD-------
Dvv_Brahma (1288) DGPAVAQIKRPRGRPRKHKRPEEIEAEAAAQKAMEEASKLRAQAEAEELR
1401 1439
Tc_Brahma (1361) ----------
Dvv_Brahma (1338) SKVEEASQRAKEEAKAREEAKAREEAEIENMEEIPTST-

Supplemental Figure 2. *Diabrotica virgifera virgifera hunchback* sequences
Supplemental Figure 2A. *Diabrotica virgifera virgifera cDNA* sequence containing *hunchback* open reading frame (underlined). dsRNA sequence is highlighted in grey. Accession No. KR152261

GTTAGATAGTGGTGGTCACATGACATTGTTATCAGTGATTTTAATACGTGTTTTTGAGGAATGA
AAATAATAGTTGGATTATTTCTAATACAGACTTTGATTCTTACCGTGAAATGAGAGGAGGTGTT
TCTGACGATATGACTTCAACTTGCGTTCAAGGAGGAATTAGACCAATTGGACGATATCAACCAA
ACATGCTTATGGAACCATCGTCTCCTCAATCTGCCTGGCAGTTTCACCCAGCCATGCCGAAACG
AGAACCCGTCGATCATGATGGCAGAAATGACTCCGGCTTAGCATCTGGAGGTGAATTTATTTCA
TCTTCACCAGGAAGTGACAATAGTGAACACTTCAGCGCTTCCTATTCATCTCCAACCAGTTGCC
ATACAGTAATTTCTACTAATACTTATTATCCCACCAATCTAAGAAGACCTTCACAGGCGCAGAC
GAGTATTCCAACGCACATGATGTACACCGGCGATCACAACCCCTTAACTCCCCCGAATTCGGAA
CCTATGATTTCGCCCAAAAGCGTGTTATCAAGAAACAACGAAGGTGAACATCAAACTACTCTGA
CGCCTTGTGCGTCTCCTGAGGATGCTTCTGTTGATGCTACAGACAGCGTTAATTGCGACGGTGC
TTTAAAAAAATTACAAGCGACTTTTGAAAAAAATGCTTTTAGTGAAGGTTCTGGGGATGACGAT
ACCAAATCTGATGGAGAGGCAGAAGAATACGACGAACAAGGACTAAGAGTTCCAAAAGTTAACT
CTCATGGAAAAATTAAAACTTTCAAGTGTAAGCAATGTGATTTTGTGGCCATTACTAAACTAGT
CTTCTGGGAACATACCAAGTTACATATTAAAGCTGACAAACTCCTTAAATGCCCCAAGTGTCCT
TTTGTCACCGAATATAAGCACCATTTAGAATATCACCTTAGAAATCATTATGGTTCAAAACCAT
TTAAATGTAACCAGTGTAGTTACTCTTGTGTAAACAAATCAATGCTTAATTCACATTTAAAATC
TCACTCTAATATTTACCAATACCGCTGTTCTGACTGCAGTTATGCCACAAAATATTGTCATTCG
CTGAAATTGCATCTTAGAAAATACTCGCACAAACCTGCTATGGTACTAAACCCAGATGGAACAC
CAAATCCGTTGCCCATAATCGATGTTTATGGTACAAGGAGAGGACCAAAGATGAAGTCAGAACA
AAAATCATCTGAGGAAATGTCTCCGAAACCCGAACAAGTTCTACCATTCCCATTTAACCAGTTT
CTACCCCAAATGCAGTTACCATTCCCAGGATTTCCATTATTTGGAGGTTTTCCAGGTGGCATTC
CAAATCCTTTGTTATTGCAAAACTTGGAAAAACTAGCCCGAGAAAGGCGTGAATCCATGAACTC
TTCAGAACGTTTTTCTCCCGCACAATCAGAACAAATGGATACCGATGCAGGCGTTCTTGATCTC
AGTAAACCAGATGACTCTTCCCAGACAAACCGACGAAAAGATTCAGCTTACAAACTTTCAACTG
GTGATAATTCTTCAGATGAAGAAGACGATGAGGCAACTACAACAATGTTCGGTAATGTTGAAGT
TGTTGAAAATAAAGAACTAGAAGATACTTCATCGGGGAAACAGACACCAACTAGTGCTAAAAAG
GATGACTACTCGTGCCAATACTGTCAGATAAATTTCGGGGACCCCGTTTTGTATACTATGCATA
TGGGTTACCACGGATACAAGAATCCATTTATTTGCAACATGTGCGGTGAGGAATGTAATGATAA
AGTGTCTTTCTTCTTGCACATTGCACGAAATCCTCATTCTTAAAAATATCAATAAGACTGAATT
CAAGGTTAGCATTTTTATATATTATATTCACACTGAAACTTTTTTAATATTCAATATTTGGTTG
CGTAACATTTACGCATATCTATACTTTATTTCACG

Supplemental Figure 2B. Translation of *hunchback* sequence from *Diabrotica virgifera virgifera* cDNA.

MRGGVSDDMTSTCVQGGIRPIGRYQPNMLMEPSSPQSAWQFHPAMPKREPVDHDGRNDSGLASG
GEFISSSPGSDNSEHFSASYSSPTSCHTVISTNTYYPTNLRRPSQAQTSIPTHMMYTGDHNPLT
PPNSEPMISPKSVLSRNNEGEHQTTLTPCASPEDASVDATDSVNCDGALKKLQATFEKNAFSEG
SGDDDTKSDGEAEEYDEQGLRVPKVNSHGKIKTFKCKQCDFVAITKLVFWEHTKLHIKADKLLK
CPKCPFVTEYKHHLEYHLRNHYGSKPFKCNQCSYSCVNKSMLNSHLKSHSNIYQYRCSDCSYAT
KYCHSLKLHLRKYSHKPAMVLNPDGTPNPLPIIDVYGTRRGPKMKSEQKSSEEMSPKPEQVLPF
PFNQFLPQMQLPFPGFPLFGGFPGGIPNPLLLQNLEKLARERRESMNSSERFSPAQSEQMDTDA

GVLDLSKPDDSSQTNRRKDSAYKLSTGDNSSDEEDDEATTTMFGNVEVVENKELEDTSSGKQTP
TSAKKDDYSCQYCQINFGDPVLYTMHMGYHGYKNPFICNMCGEECNDKVSFFLHIARNPHS*

Supplemental Figure 2C. Alignment of *hunchback* amino acid sequences from *Diabrotica virgifera virgifera (Dvv)* (Accession number: KR152261) and *Tribolium castaneum (Tc)* (Accession Number: NP_001038093.1). Amino acid identities are highlighted in black and similarities are in grey.