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Evaluation of some potential target genes and methods for RNAi-mediated pest control of the corn earworm *Helicoverpa zea*

Jinda Wang[a,b,c], Liuqi Gu[b,*], Douglas C. Knipple[b]

[a] National Engineering and Research Center of Sugarcane, Fujian Agricultural and Forestry University, Fuzhou, Fujian 350002, PR China
[b] Department of Entomology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456, USA
[c] College of Plant Protection, Nanjing Agricultural University, Nanjing, Jiangsu 210095, PR China

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ABSTRACT

In this study, we explored the efficacy of knockdown four genes required for proper nervous system function by RNAi, in the corn earworm *Helicoverpa zea* (Boddie). Three of these genes encode components of validated insecticide target sites. We synthesized cDNA sequences orthologous to the *Drosophila melanogaster* genes *Para* (*paralytic*^{ts}), *TipE* (*temperature-induced paralysis locus E*), *GluCl* (*glutamate-gated chloride channel*), and *Notch*, and used these fragments to synthesize double-stranded RNAs (dsRNAs). We then performed experiments in an attempt to induce RNAi-mediated effects on gene expression and viability using three modes of delivery of the dsRNAs: microinjection of eggs, soaking of eggs and feeding of larvae. Microinjection of dsRNAs into eggs induced reduced hatch rates and knockdown of target gene expression for *GluCl*, *para* and *TipE*, but not for *Notch*. However, neither feeding nor soaking eggs in dsRNA solutions resulted in discernable RNAi effects. These results demonstrated the susceptibility to RNAi effects of the expression of *H. zea* genes encoding insecticide target sites, which suggests future avenues of research toward practical applications.

1. Introduction

The corn earworm, *Helicoverpa zea* (Boddie), is an important agricultural pest widely distributed throughout the western hemisphere. This polyphagous insect has a wide host range of over 100 crop species, including corn, cotton, soybean, tomato, and peppers [1–3]. In the United States, *H. zea* is a key pest of sweet corn, and its damage has increased dramatically over the past two decades [4], which can cause severe yield reduction and up to $250 million in costs for insecticide applications [5, 6]. While the widespread use of chemical insecticides has led to development of resistance to many of them [7, 8], genetically modified sweet corn and cotton plants expressing *Bacillus thuringiensis* (Bt) toxins have had considerable success in the control of this pest [9]. However, there are some limitations to the continued use of this technology, such as the development of BT resistance and regulatory constraints [10, 11]. It is therefore highly desirable to develop alternative pest control strategies for this insect.

RNA Interference (RNAi) is a naturally occurring mechanism of post-transcriptional gene silencing in eukaryotic cells [12, 13]. When double-stranded RNA (dsRNA) is introduced experimentally into cells, it is processed into small interfering RNAs (siRNAs) that form a complex with nucleases that specifically degrade the endogenous mRNAs containing complementary sequences. Since its discovery, RNAi has become an invaluable tool for reverse genetic studies. Recently, the development of transgenic plants expressing dsRNAs targeting the expression of specific insect genes suggests the potential of this approach for next-generation pest management [14]. However, a significant limitation to the practical application of this approach is that the susceptibility to experimentally induced RNAi varies considerably across difference insect species [15–18]. In particular, while the order Lepidoptera contains the most agriculturally important pest species worldwide, lepidopteran species are notoriously insensitive to systemic RNAi compared to other insect groups [19]. Although the efficiency of experimentally induced RNAi depends on intrinsic properties of individual species, a number of other factors also come into play, most important among them being target gene selection and mode of delivery of dsRNA [20].

In this study, we conducted RNAi experiments in an attempt to disrupt the expression of four *H. zea* genes that play important roles in nervous system function and development. Three of these genes encode subunits of neuronal ion channels initially identified in *Drosophila melanogaster*: *para* (*paralytic*^{ts}) [21] and *TipE* (*temperature-induced paralysis locus E*) [22–24] encode subunits of voltage-gated sodium channels, while *GluCl* (*glutamate-gated chloride channel*) encodes a

* Corresponding author.
E-mail address: lg356@cornell.edu (L. Gu).

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subunit of inhibitory glutamate-gated chloride channels [25]. Both of these neuronal ion channels are sites of action of commercially useful chemical insecticides. Several mutations have been identified in orthologs of the *para* gene that confer target site resistance to DDT and pyrethroid insecticides in agriculturally important species [26, 27]. Demonstration of the effective knockdown of the expression of these genes encoding components of validated insecticide target sites could lead to practical application of RNAi approaches to overcome target site resistance. In contrast to the above three genes, the *Notch* gene encodes a protein that plays a crucial role in the initiation and maintenance of cell surface interactions in differentiating cell lineages, including neuronal lineages [28]. Here we identified sequences of *H. zea* orthologs of these four genes, used them to construct dsRNAs and evaluated their effects on survival and the targeted gene expression using three different modes of delivery: microinjection of eggs, soaking of eggs, and feeding of larvae.

2. Materials and methods

2.1. Insect rearing

H. zea eggs were purchased from Benzon Research, Inc. (Carlisle, PA, USA) and maintained in plastic cups containing 15 mL artificial diet (based on wheat diet developed by USDA, Stoneville, MS). Those insects were reared in growth chambers at 28 ± 2 °C with 75 ± 5% relative humidity (Light: Dark = 14:10).

2.2. RNA isolation, library construction and sequencing

Embryonic development of *H. zea* under the above rearing conditions was about four days. A total of 300 late stage embryos (eggs that start to show a black spot corresponding to the head capsule) and 100 neonate larvae (within 1 h of hatching) were collected separately, preserved in Ambion RNAlater ® (Thermo Fisher Scientific, USA), and stored at −70 °C until further processing. Total RNA was extracted from the two samples using RNAeasy Mini kit (Qiagen, USA) according to manufacturer's protocol. Sequencing libraries were constructed according to the High-Throughput Illumina Strand-Specific RNA Sequencing Library protocol. Briefly, the protocol involves the following steps: PolyA$^+$ RNA was captured from 2 to 5 μg of total RNA with magnetic oligo (dT) beads (Invitrogen, USA), fragmented at 95 °C for 5 min and then eluted from the beads. Cleaved RNA fragments were primed with random hexamer primers to synthesize first-strand cDNA using reverse transcriptase SuperScript III (Invitrogen, USA) and dNTPs. Second-strand cDNA was generated by DNA polymerase I (Enzymatics, USA) with dUTP mix. Following end-repair (Enzymatics, US), dA-tailing (Klenow 3′-5′, Enzymatics, USA) and adapter ligation (T4 DNA Ligase HC Enzymatics, US), the second dUTP-strand was digested by uracil DNA glycosylase (Uracil DNA Glycosylase, Enzymatics, US). The resulting paired-end adaptor ligated-cDNA tags at the 3'end were amplified by PCR for 15 cycles using PCR primers with barcodes to enrich the final libraries.

The double-stranded cDNA libraries were evaluated by electrophoresis in 2% agarose gels to verify mean size of ~250 bp. The libraries were pooled together (20 ng/library), and sequenced by the 250 bp paired-end sequencing on an Illumina HiSeq™ 2000 platform at the Biotechnology Resource Center of Cornell University (Ithaca, USA).

2.3. Bioinformatics

Trimmomatic (V0.32) [29] was used to filter out low-quality reads and trim off adaptors sequences. The remaining reads from four libraries were pooled for de novo assembly of the transcriptomes on the Trinity 2.2.0 [30, 31]. The finished assembly contained all “Trinity components” (or contigs) greater than or equal to 200 bp in length and represents the combined transcriptome of *H. zea* embryonic and early neonatal stages.

The assembled sequences were annotated using Blastx against the NCBI nr database with *E*-value cut-off of 10^{-5}. Functional annotation with gene ontology terms (GO, http://www.geneontology.org) was performed by Blast2Go (3.0) software [32]. The orthologous and pathway analyses were performed against Cluster of Orthologous Groups (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

Gene expression intensity was measured as fragments per kilobase per million mappable reads (FPKMs). Read count were generated using RSEM [33] by mapping filtered read sets from each of the four libraries individually to the transcriptome assembly. FPKM values were normalized by Trimmed Mean of M-values (TMM) method [34]. Differentially-expressed transcripts were identified and clustered according to the expression profiles using “EdgeR” software (http://www.bioconductor.org/packages/2.11/bioc/html/edgeR.html) bundled with the Trinity package.

2.4. Molecular cloning and sequence analysis

Specific primers (Table S1) were designed to amplify *notch*, *glucl*, *para* and *tipE* gene and cDNA made from total RNA was isolated from 3rd instar larvae using the Qiagen RNA Extraction kit (Qiagen, Valencia, CA). RNA was converted to cDNA using the cDNA synthesis kit (QuantiTect® Reverse Transcription Kit, Qiagen, CA). Taq DNA Polymerase with standard Taq Buffer (New England Biolabs, USA) was used for PCR reactions under general four-step amplification cycles: 94 °C for 30 s, 65–50 °C for 30 s (according to the primer annealing temperature), 50 °C for 30 s, 72 °C for 30 s. The predicted sizes of the PCR products were confirmed by electrophoresis in 1.5% agarose gels. Excised PCR products were purified with Amicon® Ultra-0.5 Centrifugal Filter Devices (Millipore, MA) and then individually cloned into pEASY-T3 plasmid vector. Cloned PCR products were verified by DNA sequencing, and the sequences were further validated using BLASTX.

The obtained sequences were then aligned to their respective orthologs from nine representative insect species (*H. armigera*, *Bombyx mori*, *Plutella xylostella*, *Apis mellifera*, *Nasonia vitripennis*, *Tribolium castaneum*, *Leptinotarsa decemlineata*, *Drosophila melanogaster* and *Musca domestica*,) for checking sequence identity using DNAMAN (https://www.lynnon.com/pc/framepc.html).

2.5. dsRNA synthesis and procedures for delivery of dsRNAs

The dsRNAs were synthesized using the cDNA templates and the Promega T7 Express kit (Promega, USA) according to the manufacturer's protocol. Double stranded RNA encoding green fluorescence protein (dsGFP) was also synthesized and used as a negative control. The primers are listed in Table S3. The dsRNAs were administered via microinjections and soaking of embryo and feeding of newly hatched neonates. Each of the three experiments included six treatments: four target dsRNAs (dsNotch, dsGluCl, dsPara, dsTipE) and two controls (dsGFP and miliQ water). All five dsRNA solutions were diluted with nuclease-free water to the same concentration of 200 ng μL^{-1} prior to use. Microinjection was performed using a nanoliter injector (WPI, China) and a dissection microscope (Nikon, Japan). *H. zea* eggs were collected within 8 h of oviposition. After piercing the lateral side of egg chorion, 0.2–0.3 μL containing 40-50 ng dsRNA was injected into each embryo. Each treatment had five replicates; for each replicate, 50–60 eggs were injected. After injection, the eggs were reared under standard conditions as described above and survival rates were counted.

For the soaking method, eggs were collected in the same way as described above. Approximately 400 eggs were randomly divided into six groups and placed inside Petri dishes. Each egg was incubated in a droplet of 1 μL (200 ng) dsRNA solution for 2 h before being transferred to standard rearing conditions; the Petri dish lids were then removed to allow the eggs to be air dried for 1 h. Each treatment was replicated

three times.

The feeding treatments were carried out in sterile 24-well cell culture plates (Becton, USA). One milliliter of fresh artificial diet was filled into each well and then coated on top with 50 µl (1000 ng) of dsRNA. One neonate (within 12 h of hatching) was placed on the dsRNA-coated diet inside each well. Every 24 h, an additional 20 µl (~400 ng) of the dsRNA was pipetted into each well for four consecutive days. For survival observation, 20–30 larvae were analyzed for each treatment group and counted every day. At Day 5, ten larvae from each treatment group were selected at random and individually weighed. Three replications were performed for each experiment. Significant differences between assay groups and controls were assessed using one-way ANOVA.

2.6. Quantitative real-time PCR

Neonate larvae were collected from dsRNA-injected embryos within two hours of hatching. Total RNA samples were extracted from 10 to 15 individuals with Direct-zol™ RNA MiniPrep Kit (ZymoResearch, USA), and four replicates were made for each treatment group. cDNA was synthesized using the iScript™ cDNA Synthesis Kit (BIO-RAD, USA), *β-actin* was used as the reference gene, and the primers used were listed in Table S4. Real-time PCR was conducted with SsoAdvanced™ universal SYBR® Green supermix (BIO-RAD, USA) on the BioRad CFX96 qPCR System. Data were analyzed using one way ANOVA followed by Tukey' HSD for multiple comparisons by the SPSS 18.0 software (SPSS, 2001).

3. Results

3.1. Amplification of candidate genes

Low coverage transcriptomes were generated from late stage embryos and neonate larvae in order to ensure that the cDNAs used to produce dsRNAs exactly matched the target sequences and did not contain any regions of developmentally regulated splice variants [35] or sequence polymorphisms. The four genes selected for investigation here were found to be among the class of genes with the highest levels of increase in expression levels between the two developmental stages sampled. Partial cDNA sequences of *HzNotch*, *HzGlucl*, *HzPara* and *HzTipE* were obtained by PCR using gene specific primers (Table S1) designed based on the transcriptomic data we generated. When compared with insects representing seven orders, these sequences showed 95%–100% identify on the amino-acid level, the highest being with *H. armigera* (Table 1). The amplified cDNA sequences were deposited to NCBI GenBank with specific accession number (Table 1).

3.2. Microinjection of embryos induced a significant RNAi effect in H. zea

The efficacy of dsRNA to induce RNAi effects on the expression of *H. zea para*, *TipE*, *GluCl*, and *Notch* genes was evaluated for three different methods of administration, i.e., microinjection and soaking of embryos, and feeding of neonate larvae. For the microinjection method, there was no significant difference in hatch rates between eggs injected with water and eggs injected with dsGFP ($p = 0.167$). In contrast, injection of three experimental dsRNAs (*GluCl*, *Para* and *TipE*) significantly reduced the hatch rate compared to the water or dsGFP controls ($p < 0.001$, Fig.1), indicating a phenotypic RNAi effect. The most notable effect was induced by dsGluCl, which reduced the hatch rate by half (HR = 17%, $p = 0.010$). However, dsNotch did not induce a significant RNAi effect ($p = 0.354$), even when the dsRNA concentration was increased to 1 µg/µL^{-1} (data not shown).

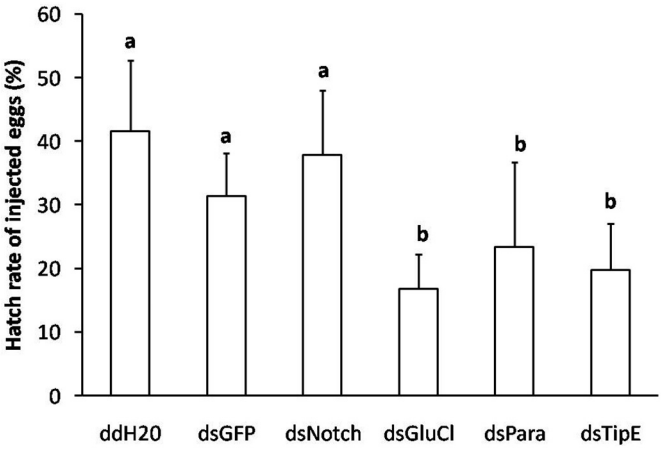

Fig. 1. Hatch rates of eggs injected with different solutions.

We next measured the expression levels of the four target genes in the surviving neonates that hatched from dsRNA-injected eggs, in order to confirm that the observed phenotypic effects (reduced hatch rates) in dsRNA-injected embryos, were the direct consequence of RNAi knockdown (Fig.2). The results were consistent with the observations of hatch rates. Significant gene knockdown effects were detected in the groups treated with dsGluCl, dsPara and dsTipE, where the target gene expression levels decreased by ~40% to 70%. The most dramatic reduction resulted from treatment with dsGluCl, with *GluCl* expression levels of only 33% compared to the controls ($p = 0.005$). In contrast, no significant gene silencing effect was detected for *Notch* ($p = 0.732$).

3.3. Soaking and feeding treatments produced no RNAi effects

In contrast to the effectiveness of RNAi via microinjection, neither soaking nor feeding treatments induced a significant RNAi effect (Figs. 3 and 4). For the soaking method, not even dsGluCl, which induced the greatest RNAi effect via microinjection, produced a reduction in hatch rate (Fig.3, hatch rate = 94.4% and $p = 0.269$). For the feeding method, none of the four dsRNAs were able to cause significant

Table 1
Percentage amino acid sequence identities between amplified *H. zea* candidate genes and orthologs in different insect species.

Species	*HzNotch* (MG820034)	*HzGluc* (MG820033)	*HzPara* (MG820035)	*HzTipE* (MG820036)
H. armigera[a]	100	100	95	100
B. mori	98	95	99	88
P. xylostella	90	97	89	84
A. mellifera	89	95	71.7	56
N. vitripennis	89	88.8	69.5	57
T. castaneum	88	90	69	52
L. decemlineata	88	89	70	[b]
D. melanogaster	84.6	95.2	78	41.7
M. domestica	77.3	89.9	75.8	54.4

[a] The amino acid sequences used here were listed in Table S2.
[b] No homologues were found in L. *decemlineata*.

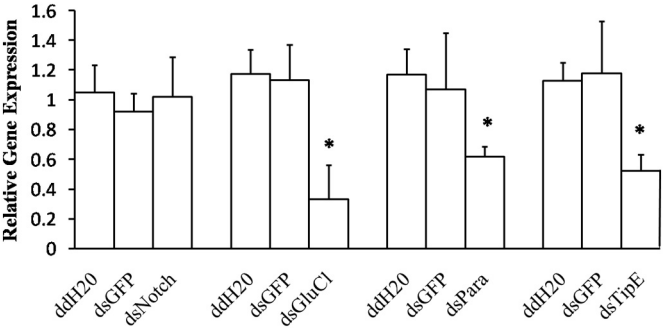

Fig. 2. Steady state transcript levels in neonate larvae of four genes after injection of embryos with dsRNA.

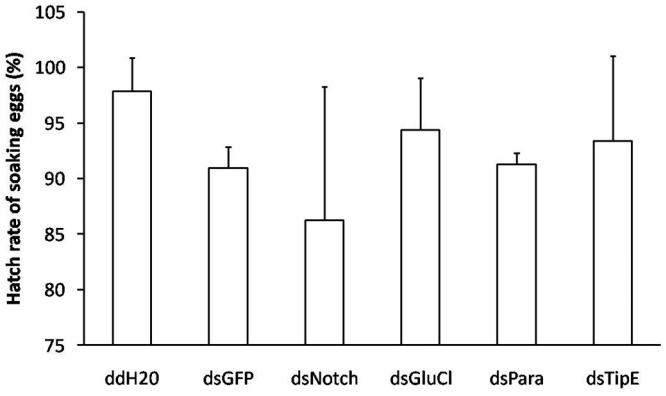

Fig. 3. Hatch rates of eggs soaked in different solutions.

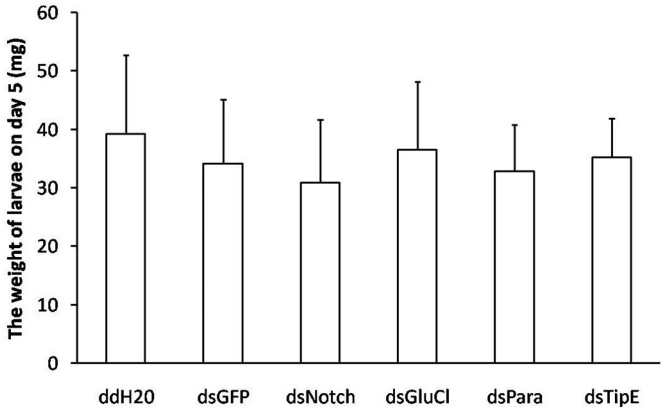

Fig. 5. Weights of dsRNA-fed larvae five days after hatching.

differences in mortality compared to the two control groups (Fig. 4). At the end of the trial, the weight of larvae did not show a significant difference among treatment groups ($p = 0.133$, Fig. 5).

4. Discussion

Lepidopteran species are notoriously refractory to experimentally induced RNAi effects [36]. The latter paper summarized results obtained from studies of 16 lepidopteran species: only 38% of the 130 genes tested could be silenced at high levels, while 48% and 14% of the genes failed to be silenced or were silenced at low levels, respectively. It thus would appear that in this insect order the type of gene selected for gene silencing significantly influences the outcome of RNAi experiments.

In the present investigation, we selected four genes presumed to play essential roles in *H. zea* nervous system function or development. Injection of embryos with dsRNAs targeting the expression of the three insecticide target site genes, *para, GluCl, and TipE*, caused significant reductions in survival (hatch rate) (Fig. 1), and expression of the corresponding genes among the survivors (Fig. 2).

It is worth noting that transcript levels of the target genes were measured in larvae that survived the experimental treatments. We suggest that the RNAi-induced knockdown of gene expression could be more substantial in those embryos that failed to hatch. The survival of some embryos and the failure of others to hatch could reflect differences in the sites of injection and the specific cell lineages exposed to the dsRNA. This interpretation would be consistent with the absence of systemic RNAi in Lepidopterans [36].

In contrast to the results obtained by injecting embryos with dspara, dsGluCl and dsTipE, embryos injected with dsNotch failed to show RNAi effects (Figs. 1 and 2). This result could be explained by sufficient quantities of Notch protein being already present in early embryos prior to injection. Additionally, the *Notch* gene expression that we measured could largely reflect transcripts sequestered in the cell nucleus or that otherwise are inaccessible to the RNAi cellular machinery.

In general, microinjection is considered the most direct way to deliver dsRNA into insects and produces more consistent RNAi effects [37]. Microinjection of dsRNA into embryos induces RNAi effects in several Lepidoptera species [38–40]. From an experimental perspective, the mechanical damage caused by microinjection is significant, as shown in the present study by the low hatch rate in the two injected control groups (Fig. 1, water 41.6% and dsGFP 31.4%), compared to the natural hatch rate (> 99%). Moreover, this mode of administration is obviously impractical for application in pest management. It is therefore important to explore alternative methods to deliver dsRNA into the organisms if RNAi is to be used for practical purposes.

In light of promising advances of transgenic plants expressing dsRNAs that target genes of insect feeders, a preferred approach to inducing RNAi effects in insects is via oral administration [20, 41]. Feeding insects with diet containing dsRNA [42–45] or plants expressing dsRNA [46, 47] is effective in inducing RNAi effects in a number of lepidopteran species. In the present study, we focus on the transition from the late embryonic stage to the neonate larval stage, which we consider the ideal period to target for practical purposes, not only

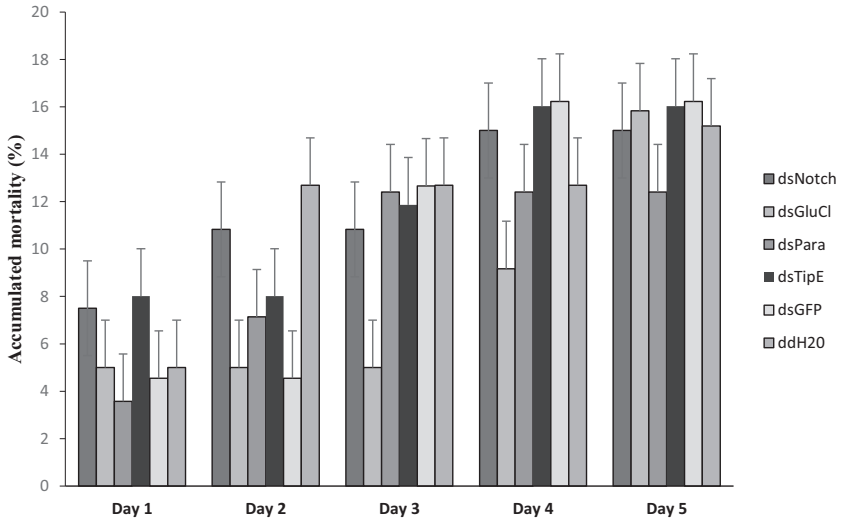

Fig. 4. Accumulated mortality of larvae among different feeding treatments.

because effective RNAi induction targeting essential gene expression would minimize larval feeding damage, but also because this period might be more vulnerable to the disruption of essential gene expression than later developmental stages. However, we observed no RNAi effects following oral administration of dsRNAs for the same target genes whose expression was effectively knocked down following microinjection of dsRNAs into embryos.

There are several possible explanations for the ineffectiveness of oral delivery of dsRNAs in the present investigation. First, we cannot exclude the possibility that the expression of genes selected in the present study was essential during the late embryonic stages but less so or not at all during the first instar larval stage. Another explanation is that *H. zea* is more recalcitrant to RNAi effects via feeding, as has been suggested for the desert locust *Schistocerca gregaria* because the dsRNA is degraded in vivo [48]. After being ingested, dsRNA must be absorbed through the gut lumen and enter the hemolymph to induce RNAi. Thus key factors influencing an insect's susceptibility to dsRNA-induced RNAi is the lability of the dsRNA after entering the midgut and/or hemolymph, perhaps as a consequence of differences in extracellular enzymes capable of degrading dsRNA in different species [49], and the uptake efficiency of midgut epithelial cells [14, 50]. We note that strong phenotypic RNAi effects are induced in *Helicoverpa armigera*, a species closely related to *H. zea*, when fed transgenic cotton expressing dsRNA targeting P450 monooxygenase gene expression [47]. This could be attributable to an idiosyncratic (gene specific) effect, or to more stable (less labile) structures of dsRNAs produced *in planta* compared to those synthesized in vitro as in the present study. In light of these arguments, it is worth exploring whether dsRNA uptake can be improved in recalcitrant species by incorporating dsRNA in nanoparticles or liposomes, or by chemically modifying the dsRNA molecules to decrease their lability in vivo [50, 51].

A third approach to inducing RNAi effects in insects is to soak embryos in dsRNA solutions, which was initially demonstrated in *Drosophila melanogaster* using dechorionated embryos [52]. Subsequently it was reported that RNAi effects are induced by soaking adult mites [53], eggs (in their native state) of the Asian corn borer *Ostrinia furnacalis* [54] and *D. melanogaster* larvae [55] in dsRNA solutions. In the present investigation, no RNAi effects were induced by soaking *H. zea* eggs in dsRNA solutions, although the same dsRNAs induced RNAi via microinjection. The differences in experimental outcomes of the *O. furnacalis* study and the present investigation could reflect differences in the structure and permeability of the egg chorion between these two lepidopteran species. It is worth noting that, to the best of our knowledge, *O. furnacalis* remains the only reported instance where RNAi effects have been induced in a lepidopteran species by soaking embryos in dsRNA solutions. This suggests that the application of dsRNA to insect eggs has limited potential for practical application in pest management, at least for lepidopteran species.

In conclusion, we have demonstrated the RNAi-induced knockdown of the expression of *H. zea* genes encoding neuronal ion channel subunits of economically important insecticide target sites. Unfortunately, the present study also shows that delivery of dsRNA by immersion of embryos and feeding fails to produce discernable effects on the expression of these same genes, suggesting that the latter two routes of delivery may be of limited value for developing future control technologies based on RNAi in this species. Nevertheless these results suggest that it may be worthwhile to investigate the susceptibility to RNAi effects of these and other known sites of insecticide action in other insect pest species, particularly as this approach could be used to overcome target site resistance.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2018.05.012.

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