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Control of western corn rootworm *via* RNAi traits in maize: Lethal and sublethal effects of *Sec23* dsRNA

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Abstract

Background: RNA interference (RNAi) triggered by maize plants expressing RNA hairpins against specific western corn rootworm (WCR) transcripts have proven to be effective at controlling this pest. To provide robust crop protection, mRNA transcripts targeted by double-stranded RNA must be sensitive to knockdown and encode essential proteins.

Results: Using WCR adult feeding assays, we identified *Sec23* as a highly lethal RNAi target. *Sec23* encodes a coatomer protein, a component of the coat protein (CO-PII) complex that mediates ER-Golgi transport. The lethality detected in WCR adults was also observed in early instar larvae, the life stage causing most of the crop damage, suggesting that WCR adults can serve as an alternative to larvae for dsRNA screening. Surprisingly, over 85% transcript inhibition resulted in less than 40% protein knockdown, suggesting that complete protein knockdown is not necessary for *Sec23* RNAi-mediated mortality. The efficacy of *Sec23* dsRNA for rootworm control was confirmed *in planta*; T_0 maize events carrying rootworm*Sec23* hairpin transgenes showed high levels of root protection in greenhouse assays. A reduction in larval survival and weight were observed in the offspring of WCR females exposed to *Sec23* dsRNA LC_{25} in diet bioassays.

Conclusion: We describe *Sec23* as RNAi target for *in planta* rootworm control. High mortality in exposed adult and larvae and moderate sublethal effects in the offspring of females exposed to *Sec23* dsRNA LC_{25}, suggest the potential for field application of this RNAi trait and the need to factor in responses to sublethal exposure into insect resistance management programs.

Keywords: coat protein complex; COPII; *Diabrotica*; RNAi; rootworm; Sec23; vesicle coat

1 Introduction

Western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) remains a major challenge to maize growers in the United States, and more recently in Europe. Costs of management and yield losses exceed US $ 1 billion annually,[1–4] and historically WCR has been a difficult pest to manage given its high adaptability to multiple management strategies.[4] The adoption of chemical pesticides and subsequently crops expressing *Bacillus thuringiensis* (Bt) insecticidal proteins over large geographic areas has led to high selection pressure against this insect pest. Consequently, WCR has developed resistance to conventional insecticides,[5–10] crop rotation,[11] and commercial maize hybrids expressing the Bt insecticidal proteins Cry3Bb1, mCry3A, and Cry3A.1Ab.[12–14] More recently there are indications of WCR resistance maize hybrids that express the Bt toxin Cry34/35Ab1.[15]

Over the past decade, RNA interference (RNAi)[16] has been explored as an additional control measure for WCR.[17] During the RNAi response, small interfering RNAs (siRNAs) guide the degradation of the target transcript mRNA in a sequence-specific manner. In certain insect species such as WCR, this response can be triggered by orally-supplied

double-stranded RNA (dsRNA).[17] While the sensitivity of insects to dsRNA varies greatly among insect orders,[18–20] rootworms are very sensitive to dsRNA applied to artificial diet[17,21–24] or transgenically expressed in maize plants.[18,23–26] Target sequences that have demonstrated plant protection against WCR include the vacuolar proton pump, *V-ATPase A*,[17] the *Snf7/Vps32* subunit of the ESCRT-III (endosomal sorting complex required for transport III),[27] septate junction proteins *snakeskin* (*dvssj1*) and *mesh* (*dvssj2*),[26] *Troponin I*,[24] SNARE-binding protein *Ras opposite/Sec1*, RNA polymerase II subunit *RpII140*, and FACT complex proteins dre4/spt16.[23]

The most common method to initially assess the efficacy of RNAi targets is by applying dsRNA to artificial diet to determine if the insect is susceptible to orally administered dsRNA and the efficacy of the selected target gene. This approach provides a high throughput strategy to screen a large number of targets. Diet-based bioassays also allow evaluating multiple concentrations to generate various measures of toxicity such as the LC_{50} (concentration that generates 50% lethality), GC_{50} (concentration necessary to generate 50% growth inhibition), as well as the 'time to kill' or LT_{50} (time to 50% lethality). To date, RNAi target identification has been performed using larvae.[17,21,23,25,26,28] However, WCR adults are also sensitive to orally fed dsRNA.[22,29,30] Feeding of *V-ATPase A* dsRNA to adults *via* artificial diet resulted in mortality and reduction of transcript levels suggesting that adult WCR bioassays may serve as an alternative screening method to identify novel RNAi targets. A study evaluating the susceptibility of larvae and adult WCR to *V-ATPase A* and *dvSnf7* dsRNA reported that the ratio of sensitivity between larvae and adults was 387X and 23X for *V-ATPase A* and *dvSnf7* dsRNA, respectively, indicating that WCR adults are less sensitive to dsRNA compared to larvae.[31] The above observations suggest that screening in the less sensitive life stage may ensure a high level of response in larvae, which is the targeted life stage and the cause of most yield loss. Furthermore, this approach might also be extended to other species for which diet-based larval feeding assays are difficult or not available.[23]

The susceptibility of WCR adults to dsRNA enables the evaluation of candidate RNAi target genes, which are generally easier to maintain on artificial diet compared to larvae. However, reduced susceptibility of adults has the potential to cause sublethal exposure of WCR adults through feeding on above ground dsRNA-expressing maize

tissues, which could contribute to resistance evolution. For example, adult exposure to *dvSnf7 and V-ATPase A* dsRNAs adult LC$_{50}$ led to a reduction in fecundity and larval survival in southern corn rootworm, *Diabrotica undecimpunctata howardi* (Coleoptera: Chrysomelidae).[31] In contrast, Fridley *et al.*[32] reported that the maize event MON 87411 expressing the WCR RNAi trait *dvSnf7* did not generate lethal or sublethal effects in WCR adults. However, the lack of observed effects in this study may be due to the low concentration of dsRNA present in maize pollen and silks where feeding is concentrated in WCR adults. Such effects should be approached on a gene-by-gene basis.

The present study describes RNAi of WCR *Sec23*, a subunit of the coat protein complex II (COPII) vesicle transport complex which promotes the formation of transport vesicles in the endoplasmic reticulum(ER).[33–35] *Sec23* was initially identified as a lethal RNAi target in WCR adults.We showthat a robust dose–response observed in WCR adults translates well to larval lethality. Furthermore, we demonstrate that this target also provided root protection when an RNA hairpin was expressed in transgenic maize. Given that this target gene was identified in adults, laboratory studies were performed to evaluate potential sublethal effects in adults. The results of these experiments are discussed in terms of potential impacts on the refuge strategy used for insect resistance management (IRM) programs.

2 Materials and Methods

2.1 Insects and diet

Non-diapausing WCR eggs and newly emerged adults were purchased from Crop Characteristics, Inc. (Farmington, MN, USA). Eggs were washed from soil with water and surface-sterilized with 10% formaldehyde for 10 min, rinsed with water, hatched on artificial diet, and held at 28 °C and 40% RH, at 16:8 (Light : Dark) photoperiod, as previously described.[36,37] Newly emerged adults were placed in cages at arrival with untreated artificial diet modified from Branson and Jackson.[38] The diet consisted of 6 g of the dry ingredients,[38] 12.5 mL of water, 0.365 g of agar, 0.7 mL of glycerol and 27.5 mL of a solution of 47% propionic acid and 6% phosphoric acid to reduce microbial contamination. The diet was dispensed into a Petri dish and allowed

to solidify. A Petri dish was provided in the cages, and for bioassays, diet plugs were cut using a cork borer (approximately 4mm diameter × 2mm height) and treated with dsRNA or water. WCR adults used for bioassays were approximately 48 h old and were kept in a growth chamber at 23±1 °C, 75±5% RH with 16:8 photoperiod.[30]

2.2 Identification of Sec23 in WCR

WCR transcriptome sequencing and gene identification were described previously.[30,39] The amino acid sequence of *Sec23* from Colorado potato beetle, *Leptinotarsa decemlineata*,[40] was used as a query sequence to search the WCR transcriptome. BLAST algorithm identified the WCR transcript with significant homology to the *L. decemlineata Sec23* gene, which has been submitted to Gen- Bank (Accession number: MK474471). The amino acid sequence of WCR Sec23 is listed as Supporting information Sequence 1 in Appendix S1.

2.3 RNAi target design and dsRNA preparation

The open reading frame of the contig with the highest level of homology to *L. decemlineata Sec23* was used for dsRNA design (Supporting information Sequence 2 in Appendix S1).[40] A region of 383 bp was selected as *Sec23* dsRNA (Supporting information Sequence 3 in Appendix S1) for initial screening in adults. To minimize the probability of matches to non-target organisms, shorter regions of the *Sec23* sequence were selected for dsRNA evaluation in larvae and plant transformation, designated as *Sec23 v1* (204 bp, Supporting information Sequence 4 in Appendix S1) and its subsequence *Sec23 v2* (104 bp, Supporting information Sequence 5 in Appendix S1). Negative controls included *GFP* dsRNA of 376 bp (Supporting information Sequence 6 in Appendix S1) for adult assays and *YFP* dsRNA of 503 bp (Supporting information Sequence 7 in Appendix S1) for larval assays. All *Sec23* dsRNAs were amplified from WCR cDNA, while *GFP* was amplified from the pIZT/V5-His expression vector (Invitrogen, Carlsbad, CA, USA). A previously-described *YFP* dsRNA was used.[24] All PCR amplification products were sequenced to confirm the identity and specificity of the sequence before dsRNA synthesis, which was performed using the MEGAscript high-yield Transcription Kit (Applied Biosystems Inc., Foster City, CA, USA) as previously described.[23,30] Synthesized

dsRNAs were purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), and examined by agarose gel electrophoresis to determine their purity and integrity.

2.4 Bioassays with WCR adults

RNAi lethal target screening in WCR adults was conducted by feeding artificial diet plugs treated with *Sec23* dsRNA (383 bp) (Supporting information Sequence 3 in Appendix S1) or controls (*GFP* dsRNA or water) using the methodology described by Vélez *et al.*[41] Specifically, an initial experiment to determine if *Sec23* generated mortality was performed using mixed-sex WCR adults (approximately 48 h old) fed on artificial diet plugs surface-treated with either *Sec23* dsRNA (500 ng/diet plug), and the same concentration of *GFP* dsRNA or water as negative controls. Freshly-treated artificial diet was provided every other day for up to 10 days and mortality was recorded every day for 15 days. Adults were reared using the conditions previously described (23±1 °C, 75%R H and 16:8 photoperiod). Once mortality was detected, a subsequent experiment was performed to determine the LC_{50}, for this purpose, beetles were exposed to six concentrations of *Sec23* dsRNA including a water control (*i.e.*, 1000, 100, 10, 1, 0.1, and 0 ng/diet plug). Newly treated diet was provided every other day for 10 days and mortality was recorded every day for 15 days. Insects were maintained under the conditions previously described. Using the same methodology, adults were exposed to a single concentration of 100 or 50 ng per diet plug for 48 h, to determine the minimum dsRNA exposure time for achieving mortality. We then selected 50 ng and evaluated the mortality of a single exposure for 3 h, and 6 h and then moved to untreated artificial diet. Adults were reared for 15 days and mortality was recorded daily. Rearing conditions were used as mentioned above. All adult experiments included three biological replicates (*i.e.*, three generations) with 10 mixed-sex WCR adults (approximately 48 h old) per treatment for a total of 30 insects per treatment. Two adults per biological replicate were flash-frozen after 6 h, 1 day, 3 days, and 5 days of exposure for a total of six insects per treatment per time point for transcript knockdown verification with two technical replicates per sample.

2.5 Larval WCR bioassays

WCR larval bioassays were conducted with neonate larvae, approximately 24 h old, on 128-well plastic bioassay trays (BIO-BA-128, C-D International, Pitman, NJ, USA). Two to three larvae were introduced per well, with eight wells per replicate for 9 days, as previously described.[23] The number of replicates per experiment is captured in Supporting information Table S3 in Appendix S1. Briefly, dsRNA was diluted in 0.1X TE and the artificial diet was surface treated with 500 ng cm^{-2} *Sec23* dsRNA (Supporting information Sequence 3 in Appendix S1) and two shorter dsRNA versions of 204 bp (*Sec23 v1* dsRNA, Supporting information Sequence 4 in Appendix S1) and its subregion (*Sec23 v2* dsRNA, Supporting information Sequence 5 in Appendix S1). Trays were held at 28 °C and approximately 40% RH at 16:8 photoperiod. The total number of insects exposed to each sample, the number of dead insects, and the weight of surviving insects were recorded after 9 days. *YFP* dsRNA eluted in 0.1X TE buffer and water were used as negative controls. Growth Inhibition (GI) was calculated based on the average weights of all controls, as follows: $GI = [1 - (TWIT/TNIT)/(TWIBC/TNIBC)]$, where TWIT is the Total Weight of live Insects in the Treatment; TNIT is the Total Number of Insects in the Treatment; TWIBC is the Total Weight of live Insects in the Background Check (Buffer control); and TNIBC is the Total Number of Insects in the Background Check (Buffer control). The LC_{50} and GC_{50} were estimated using six concentrations of *Sec23* dsRNA and a water control (*i.e.,* 500, 125, 31.3, 7.81, 1.95, 0.41, and 0 ng cm^{-2}); six experimental replicates in 128-well format, as described above, were performed at each concentration.

2.6 Transcript knockdown verification in WCR adults and larvae

Total RNA was isolated from flash-frozen WCR adults using the RNeasy Mini Kit (Qiagen, Valencia, CA,USA). The quantity and quality of RNA was validated by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen) using 500 ng of RNA, following the manufacturer's instructions. The Real-Time quantitative PCR (RT-qPCR) reactions included 1 µL of cDNA diluted 50X, 5 µL of Fast SYBR® Green Master Mix (Applied Biosystems), 0.2 µL at

10 μM of each primer, and 3.6 μL of nuclease-free water, for a total volume of 10 μL. The primers were designed with Primer3Plus[42,43] and validated by RT-qPCR amplification efficiencies (E) (Supporting information Table S1 in Appendix S1). For larvae, total RNA was isolated from flash-frozen WCR larvae treated with 500 ng cm^{-2} *Sec23* dsRNA (Supporting information Sequence 3 in Appendix S1) at 0, 2, 4, 6 and 8 days after treatment (n = 15; 3 replicates) using Mag-MAX™ mirVana™ total RNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA). The quantity and quality of RNA was validated by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). cDNA was synthesized from 500 ng of RNA with oligo(dT) and random hexamer primers with the First Strand cDNA Synthesis Kit (ThermoFisher Scientific).

Both primer efficiency tests and RT-qPCR reactions for adults were performed on a 7500 Fast RT-PCR System (Applied Biosystems). The thermocycler conditions were one cycle at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/ extension at 60 °C for 30 s. At the end of each PCR reaction, a melting curve was generated to confirm a single peak and rule out the possibility of primer-dimers and nonspecific product formation. RT-qPCR for larvae was performed on a Roche Light Cycler 480 system using 2X Roche master mix and 2 μL of the resulting cDNA per reaction (Roche Diagnostics, Basel, Switzerland). The expression of the genes was calculated using the $2^{-\Delta\Delta CT}$ method[44]; *β-actin* was used as the reference gene for adults (Supporting information Table S1 in Appendix S1), while *β-tubulin* and *β-actin* were used as endogenous controls for larvae (Supporting information Table S2 in Appendix S1).[45] Using *β-actin* as the only reference gene in adults has previously demonstrated to be stable under exposure to dsRNA and the most stable reference gene between larvae and adults.[45] Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method,[44] based on three biological replicates with approximately 20–30 larvae per replicate and two to three adults per biological replicate for a total of six to nine adults, with three and two technical replicates for larvae and adults, respectively. Transcript levels were normalized to water or an untreated control to calculate percentage knockdown of the target gene in each treatment.

2.7 Construct design and development of transgenic plants

Construct design and development of transgenic plants were performed as described by Fishilevich *et al.*[24] Briefly, standard cloning methods were used to construct binary transformation plasmids based on pTI15955 from *Agrobacterium*.[23,46] *Sec23* hairpins, containing 204 bp (*Sec23v1*) and 104 bp (*Sec23v2*) target regions, were cloned under maize *ubiquitin1* gene promoter[47] and terminated by the 3′ untranslated region (UTR) of *Zea mays peroxidase 5* gene (ZmPer5 3′UTR).[48] Binary expression vectors also contained an herbicide tolerance gene (aryloxyalknoate dioxygenase; AAD-1 v3), expressed from a sugarcane bacilliform badnavirus promoter,[49,50] combined with the maize streak virus leader 5′ untranslated region,[51] which is interrupted by the *alcohol dehydrogenase I intron 6* from maize[52] (SCBV(MAM)) and a fragment containing a 3′ untranslated region from a maize lipase gene (ZmLip 3′UTR). The plasmid was transformed into *Agrobacterium tumefaciens RecA*-deficient ternary strain, DAt13192.[53] The transformation of immature embryos inbred line, *Zea mays* c.v. B104 was performed as described by Knorr *et al.*[23] Rooted plantlets were transplanted into soil and placed in a Conviron growth chamber (28 °C/24 °C, 16-h photoperiod, 50–70% RH, 200 µmol m^{-2} s^{-1} PAR) until reaching V3-V4 stage. Plants were then moved to the greenhouse (Light Exposure Type: Photo or Assimilation; High Light Limit: 1200 µmol m^{-2} s^{-1} PAR; 16-h day length; 27 °C day/24°C night) and transplanted from the small pots into Rootrainers (27/8′′ × 9′′) (Grower's Nursery Supply, Inc., Portland, OR, USA) for bioassay. One plant per event was bioassayed.

2.8 Transgene copy number analysis

Transgene copy number was determined using probe hydrolysis RT-qPCR to detect a portion of the AAD herbicide tolerance gene as described by Knorr *et al.*[23] RT-qPCR assays to detect a portion of the Spectinomycin-resistance gene (SpecR; from the binary vector plasmids outside of the T-DNA) to determine if the transgenic plants contain extraneous plasmid backbone were also performed as previously described.[23]

2.9 Root protection assays

Whole plant maize bioassays were conducted using the protocol described in Fishilevich *et al.*[54] In brief, the transgenic maize T_0 plants were planted into root trainer pots containing Metro Mix soil after reaching V3 or V4 stage. The plants were infested with 125–150 WCR eggs and allowed to grow for 2 weeks. Since WCR larvae feed primarily on growing root tips, we adjusted the commonly used node-injury scale[55] described by described by Oleson *et al.*[56] to rate 2nd node injury within the 2-week assay. Two weeks after infestation, the plant roots were washed and WCR feeding damage was scored on the second root node only, using a node-injury scale[55] ranging from 0 to 1 as compared to 0 to 3 described by Oleson *et al.*[56] A NIS of 0 signifies no damage; a NIS of 1 signifies an entire node of roots removed; a NIS of 0.5 signifies half of a root node removed; a NIS or 0.25 signifies one quarter or the node eaten; pruning of root tips resulted in 0.1 and 0.01 ratings. The negative controls included non-transformed B104, B104 plants expressing either *YFP* hairpin dsRNA or YFP protein, and non-transgenic isoline 7sh382 (null). Isoline 7sh382 expressing Cry34Ab1/Cry35Ab1 (7sh32rw) served as a positive control where complete root protection was expected. When maize events expressing *Sec23* dsRNA sequences were bioassayed on different days, both positive and negative controls were included in each experiment. All T_0 events and control plants that showed a root rating of ≤0.5 were designated as 'pass' and the events with root ratings >0.5 to 1.0 were called 'fail'.

2.10 Sec23 protein expression quantification

Protein expression was measured using untargeted mass spectrometry of tandem mass tagged (TMT6plex) isobaric labeled protein digests[57] of treated larvae. Treated WCR larvae (*n* =100 for each treatment)were collected at 2, 4, 6 and 8 days after treatment with either buffer (untreated), dsRNA for *YFP* or *Sec23* at 500 ng cm^{-2} and flash-frozen in liquid nitrogen. Larvae were extracted with protein extraction reagent type 4 (Sigma Aldrich, St Louis, MO, USA) supplemented with 25mM TCEP and Protease Inhibitor Cocktail (Promega, Madison, WI, USA). A single stainless-steel bead (1/8″ diameter) was added to each tube and the samples were homogenized at room temperature

using a GenoGrinder (SPEX Sample Prep, Metuchen, NJ, USA) for 3 × 2 min at 1000 Hz. After homogenization, cysteine alkylation was carried with iodoacetamide, excess iodoacetamide was quenched with the addition of DTT. Sodium dodecyl sulfate (SDS, 2% final concentration) was added to each sample to assist in solubilization of membranes and the fat body. Extracts were digested using a modification of filter assisted sample preparation (FASP)[58] using 10 kD molecular weight cut-off filters (Pierce Biotechnology, Waltham, MA, USA). Buffer exchange was carried out with multiple washes of 8 M urea in 100mM triethylammonium bicarbonate (TEAB). The final concentration of urea in the spin filter concentrator was adjusted to 4 M.

Digestion was carried out using LysC/trypsin (Promega) using a two-stage digestion for 3 h in 4 Murea, followed by dilution of the urea to <1 M for a total digestion time of 16 h at 37 °C. The peptides were subsequently removed from the spin filter concentrator by centrifugation and labeled with TMT6plex labeling reagents (ThermoScientific) according to the manufacturer's directions. After labeling, the samples were mixed, and the resulting multiplexed sample was prefractionated at pH 10[59] using a Waters Acquity UPLC peptide (Waters Corporation, Milford, MA, USA) BEH C18 column (1 mm× 50 mm, 1.7 µm particle size, 300 Å pore) operated at 150 µL min^{-1} at 50 °C. The solvent gradient used 10 mM ammonium formate pH 10 (solvent A) and acetonitrile (solvent B). Two-minute fractions were collected throughout the gradient. Ten fractions from the linear portion of the gradient were evaporated to dryness *in vacuo* at room temperature and redissolved in 20 µL of 0.1% formic acid for analysis by MS.

The buffer, *YFP* control and *Sec23* dsRNA – treated samples from 2 and 4-day treatments were combined and analyzed as a single six-plexed sample. Similarly, the buffer, *YFP* control and *Sec23* dsRNA – treated samples from 6 and 8-day treatments were combined and analyzed as a single six-plexed sample. For both the 2 and 4-day treatments, and the 6 and 8-day treatments, triplicate experiments were carried out. Each multiplexed sample was analyzed by mass spectrometry on a Thermo Fusion using 60 k MS1 and top speed 30 k MS2 with HCD at 35% using an Eksigent model 425 nanoLC trap and elute separation with a 3-h gradient separation.

Raw data files were searched using ProLuCID[60] on an IP2 server (Integrated Proteomics Applications, San Diego, CA, USA) using a Corteva Agrisciences-generated sequence database of WCR sequences

(189 312 sequences). The search results and raw data files were imported into GeneData Refiner (Lexington, MA, USA) and subsequently analyzed in GeneData Analyst. TMT6plex reporter ion intensities for each identified peptide were summed across the high pH fractions for peptides identified in more than one fraction. Protein values were calculated using the geometric mean of all peptides identified for each protein (six peptides for Sec23 and more than 30 peptides for *β*-tubulin). The protein values for Sec23 were normalized to *β*-tubulin within each sample. Results are reported as the ratio of either *YFP* dsRNA or *Sec23* dsRNA to the untreated buffer control.

2.11 Effects of sublethal dsRNA exposure on WCR beetles

The effects of exposure to a sublethal concentration of *Sec23* dsRNA were evaluated in WCR adults to determine the potential impact on adult fitness, which may be important to refuge design and modeling of resistance evolution. For this purpose, we used the 204 bp dsRNA1 sequence (*Sec23 v1*, Supporting information Sequence S4 in Appendix S1) expressed in plants. Since the earlier experiments with adults evaluated a 383 bp sequence, we tested a new concentration range to estimate the 10-day LC_{25} and LC_{50} for the 204 bp dsRNA. Five concentrations plus the control were tested (*i.e.*, 90, 30, 9.9, 3.3, 1.1 and 0 ng/diet plug) in WCR adults, using the same methodology described for the initial adult bioassays. Three replications of 15 adults per concentration per replication for a total of 36 beetles per replication were used for this experiment.

Once the LC_{25} (3 ng/diet plug), and LC_{50} (8.5 ng/diet plug) were estimated, multiple fitness parameters were evaluated in WCR adult females exposed to *Sec23 v1* dsRNA LC_{25}. Two negative controls, *GFP* dsRNA at 3 ng/diet plug, and the same volume of water (3 µL) were tested. For each treatment, 15 females and 15 males (24–48 h old) were maintained on untreated diet and allowed to mate for 4 days[30,61] in containers (7.5 cm wide cm × 6 cm long) with vented lids. Containers were held in a growth chamber at 23±1 °C, RH >80%, and 16:8 L:D photoperiod.[30] Four days after mating, males were removed from the experiment and females were transferred to containers with artificial diet treated with the respective treatments (*i.e.*, *Sec23* dsRNA, *GFP* dsRNA, or water). Surviving females (7–15) were exposed to dsRNA for 10 days with freshly-treated diet that was provided every other

day for a total of five exposures. The experiment was repeated two times, for a total of 14–30 females per treatment. On day 10 of exposure, females were transferred to polystyrene oviposition egg boxes (7.5 cm × 5.5 cm× 5.5 cm) (ShowMan box, Althor Products, Wilton, CT, USA) using the design of Campbell and Meinke.[62] The boxes contained silty clay loam soil, pre-sifted through a 60-mesh sieve and autoclaved.[63] Females were allowed to lay eggs for 4 days then removed and flash-frozen for RT-qPCR. Eggs were incubated in the soil for 7 days at 27 °C, RH>80% and 24 h dark. Eggs were removed from the soil by washing through a 60-mesh sieve. Harvested eggs were held in Petri dishes on moistened filter paper at 28°C, RH >80% and 24 h dark. The Petri dishes were photographed and the total number of eggs was counted using the cell counter function of ImageJ software.[64] The number of larvae hatching from each plate was recorded daily for 15 days to determine egg viability.[30,61]

To evaluate the sublethal effects of adult exposure on larval offspring, the day after the WCR eggs were collected, 50-mL Falcon tubes (Corning, NY, USA) containing vermiculite were sowed with four untreated maize seeds. The tubes were transferred to a growth chamber with temperature 23±1 °C, RH >80%, and 16:8 photoperiod to allow for germination and kept in the growth chamber until larval emergence. Three replications of 30 tubes were sowed staggered over 3 days to allow the evaluation of larvae hatched on three different days. Six days after the eggs were collected, larvae started to emerge, and on days six, seven and eight after egg collection, larvae were transferred to seedlings. Four neonates were added to each Falcon tube with seedlings for a total of 30 tubes per treatment (*i.e.*, *Sec23 v1* dsRNA, *GFP* dsRNA, or water). Twelve days after the larvae were transferred to the Falcon tubes with seedlings, survival and weight of larvae were evaluated for each tube. Surviving larvae were stored in 70% ethanol for subsequent identification of larval instar and measurement of head capsules.[31] Larval weight, instar identification, and head capsule measurements were evaluated for 100–122 larva per treatment.

2.12 Statistical analysis

Adults lethal concentrations (LC_{50}), their 95% confidence intervals, slopes and standard errors were estimated using probit analysis[65] with POLO-PC.[66] Larval LC_{50} and GC_{50} values with their 95% confidence

Table 1. *Sec23* v1 and v2 dsRNAs concentration response in WCR larvae matured in a nine-day bioassay *via* surface-treated diet

Biological stage (units)	dsRNA	LC_{50}	LC_{50} 95% CI	GC_{50}	GC_{50} 95% CI
Larvae (ng cm^{-2})	*Sec23 v1*	53.6	33.8–88.0	2.5	1.3–4.9
	Sec23 v2	36.1	22.5–58.7	5.8	2.9–11.6
Adults (ng/diet plug)	*Sec23 v1*	8.5	1.2–22.1	-	-

LC_{50} (approximated concentration that leads to 50% lethality) and GC_{50} (approximated concentration that leads to 50% growth inhibition), with 95% confidence intervals (CI), were calculated in JMP. Six experimental replicates in 128-well plates, with eight wells per concentration with two to three larvae per well for a total of 96–144 larvae per concentration. Adults *Sec23v1* LC_{50} in a 10 day bioassay was estimated using POLO-PC. Three replications of 15 adults per concentration per replication were used for this experiment.

intervals (**Table 1**) were calculated using log-logistic regression analysis in JMP® Pro 12.2.0 from SAS Institute Inc.[67] Since the T_0 root damage ratings are not normally distributed; the ratings were converted into categorical data which follows a binomial distribution. All T_0 events and control plants that showed a root rating of ≤0.5 were designated as 'pass' and the events with root ratings >0.5 to 1.0 were called 'fail'. To identify the constructs that provided better root protection, the proportion of plants which passed the bioassay was analyzed with the generalized linear mixed model procedure ($\eta^{ij} = \eta + Construct^i + Test\ date^j$ with observations binomially distributed, $y^{ij} \square Binomial$ (N^{ij}, π^{ij})) using PROC GLIMMIX (SAS 2013).[68] The link function for the binomial distribution was the logit function $\eta^{ij} = log[\pi^{ij}/1 - \pi^{ij}]$. The construct/ genotype was modeled as a fixed effect and the test date as a random effect. Cry34/35Ab1, and the two YFP genotypes had low variability and were eliminated from the statistical analysis.

Experiments with adults were analyzed with an analysis of variance (ANOVA) using PROC GLIMMIX with the least-square estimated means procedure to determine differences between treatments for mortality, RT-qPCR and sublethal effects (*i.e.,* number of eggs per female, percent larval hatch, percent larval survival, percent larval instar, larval weight, and head capsule size). RT-qPCR of insects collected at different time points (Figs 1(b) and 3(b)) were analyzed with repeated measures ANOVA using PROC GLIMMIX with the least-square estimated means procedure to determine differences between treatments. Statistical analyses were performed using SAS software version 9.3.[69] Mortality and growth inhibition of larvae after 9 days of feeding with 0

and 500 ng cm^{-2} (Supporting information Table S3 in Appendix S1) were analyzed with a one-way ANOVA and the Tukey–Kramer test in JMP® Pro 12.2.0.[67]

3 Resuls

3.1 Identification of Sec23 and RNAi response in WCR beetles

Secretory 23 (*Sec23*) was selected as an RNAi target gene for WCR based on its efficacy on Colorado potato beetle, *Leptinotarsa decemlineata*.[40] A *Sec23* transcript was identified from the WCR transcriptome, as previously described.[30] A 383 bp region of WCR *Sec23* ORF was selected as the dsRNA target for initial testing (Supporting information Sequence 3 in Appendix S1). Continuous exposure of WCR beetles to *Sec23* dsRNA for 5 days with treated diet provided every other day, resulted in significant beetle mortality by day six at exposures of 10, 100 and 1000 ng/diet plug, per insect, but no mortality at the 1.0 and 0.1 ng/diet plug treatments (**Fig. 1**(a)). The 6-day LC$_{50}$ was estimated at 44.2 ng/diet plug. By day 10, 100% mortality was observed at 1000 ng and nearly complete mortality at the 100 ng treatment, although a few beetles remained even at 15 days when the experiment was terminated. In contrast, only 50% mortality was achieved at the 10 ng treatment by day 15 (Fig. 1(a)). To determine the time course of *Sec23* transcript knockdown, we used a concentration of 500 ng/diet plug per insect per day as described above, and samples were collected at 6 h, 1, 3 and 5 days. Adult rootworms exhibited significantly reduced gene expression within 24 h after initial exposure to *Sec23* dsRNA-treated diet (Fig. 1(b)).

Based on the 6-day LC$_{50}$ of 44.2 ng/diet plug, we chose to perform a single exposure experiment at 50 and 100 ng/diet plug plus controls to evaluate the effectiveness using a single exposure. Both concentrations 50 and 100 ng/diet plug generated 93% and 97% mortality, respectively, demonstrating the effectiveness of this gene with a single exposure for 48 h (Supporting information Figure S1A in Appendix S1). Given that 100% mortality was achieved with 50 ng/diet plug at 48 h, we evaluated exposure for 3 and 6 h to estimate the length of exposure required to generate significant mortality at this concentration. We observed mortality of exposed beetles by day six, with as little as 3 h of exposure (Supporting information Figure S1B in Appendix S1).
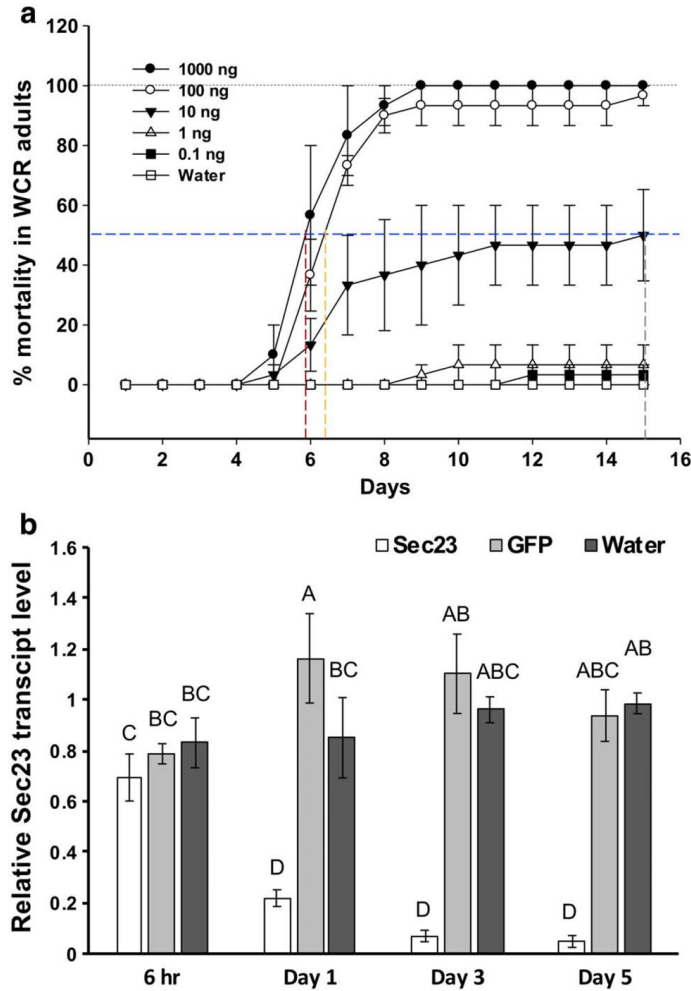
Figure 1. Concentration-response in WCR adults to *Sec23* dsRNA. (a) Five concentrations of a 383 bp *Sec23* dsRNA (Supporting information Sequence 3 in Appendix S1) were used to determine the LC_{50} value in WCR adults. New dsRNA-treated diet was provided every other day for 10 days; mortality was recorded every day. LC_{50} at day 6 was estimated at 44.2 ng/diet plug. Error bars indicate standard error of the mean from three biological replicates (*i.e.*, three generations) of 10 mixed-sex WCR adults (approximately 48 h old) per treatment per replications for a total of 30 insects per treatment. (b). Relative *Sec23* transcript level in WCR adults exposed to 500 ng/diet plug of *Sec23* dsRNA, the same amount of *GFP* dsRNA (Supporting information Sequence 6 in Appendix S1, 376 bp) or the same volume of water as negative controls. Freshly treated diet was provided on days 1 and 3. Two adults per biological replicate were collected after 6 h, 1 day, 3 days, and 5 days of exposure for a total of six insects per treatment per time point. Error bars indicate standard error of the mean from three biological replicates, represented by two adults per biological replicate with and two technical replicates per sample, bars with different letters indicate significant statistical differences ($P < 0.05$).

Although mortality never reached 100%, 47 and 50% mortality were observed with both 3 and 6 h exposures, respectively by day 13. However, 97% mortality was achieved with the single 48 h exposure (Supporting information Figure S1A in Appendix S1). These results show that relatively short exposure to dsRNA in adult WCR may cause significant mortality with *Sec23* dsRNA.

3.2 RNAi target validation using larval diet-overlay bioassays

Once the 383 bp dsRNA target region was demonstrated to cause mortality in WCR adults, a shorter dsRNA of 204 bp (*Sec23 v1* dsRNA, Supporting information Sequence 4 in Appendix S1) and its subregion (*Sec23 v2* dsRNA, Supporting information Sequence 5 in Appendix S1) were tested in larvae. When dsRNA was surface applied to artificial WCR diet at 500 ng cm^{-2}, all three dsRNAs produced over 50% mortality and growth inhibition within 9 days of treatment (**Fig. 2**(a) and (b), and Supporting information Table S3 in Appendix S1). Mortality
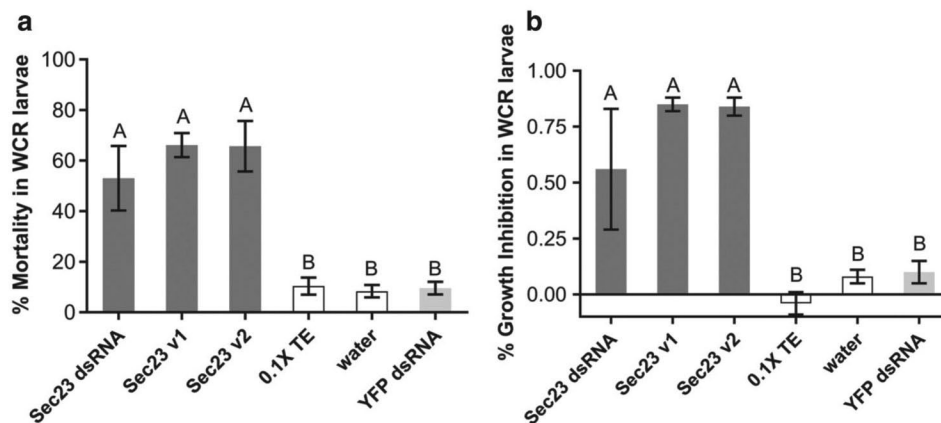

Figure 2. RNAi response in WCR larvae to *Sec23* dsRNA at 500 ng cm^{-2}. Neonate WCR larvae were placed on artificial larval diet treated with *Sec23* (383 bp, Supporting information Sequence 3 in Appendix S1), *Sec23 v1* (204 bp, Supporting information Sequence 4 in Appendix S1), *Sec23 v2* (104 bp, Supporting information Sequence 5 in Appendix S1), or *YFP* dsRNA (503 bp, Supporting information Sequence 7 in Appendix S1). Water and buffer (0.1X TE) were included as additional negative controls. Error bars indicate standard error of the mean from 6 to 12 experiments (N); N are captured in Supporting information Table S3 in Appendix S1. Means were separated using the Tukey–Kramer test, bars with different letters indicate statistical differences ($P < 0.05$) (a) Percent larval mortality 9 days after dsRNA treatment. (b) Growth inhibition plotted on a scale of 0–1, 9 days after dsRNA treatment.

rates induced by *Sec23* dsRNA were statistically different from water, *YFP* dsRNA, and buffer (0.1X TE) controls (Fig. 2(a) and (b), and Supporting information Table S3 in Appendix S1). The 204 and 104 bp dsRNA target regions were further tested to determine concentration-response curves. The LC_{50} values were approximated at 54 ng cm^{-2} for *Sec23 v1* and 36 ng cm^{-2} for *Sec23 v2* dsRNA (Table 1). The GC_{50} values were approximated at 2.5 ng cm^{-2} for *Sec23 v1* and 5.8 ngcm^{-2} for *Sec23 v2* dsRNA (Table 1).

3.3 dsRNA treatment effect on Sec23 transcript and protein levels in WCR larvae

Feeding WCR larvae with *Sec23* dsRNA at 500 ng/cm^2 (Supporting information Sequence 3 in Appendix S1) resulted in a significant level of gene knockdown that ranged from 85.1% to 88.7% (**Fig. 3**(a)). Interestingly, within 2 days of dsRNA exposure, the transcript levels were suppressed and stable through day eight (Fig. 3(a)). Sec23 protein level was measured by untargeted mass spectrometry of TMT-6plex isobaric-labeled protein digests at days two, four, six and eight
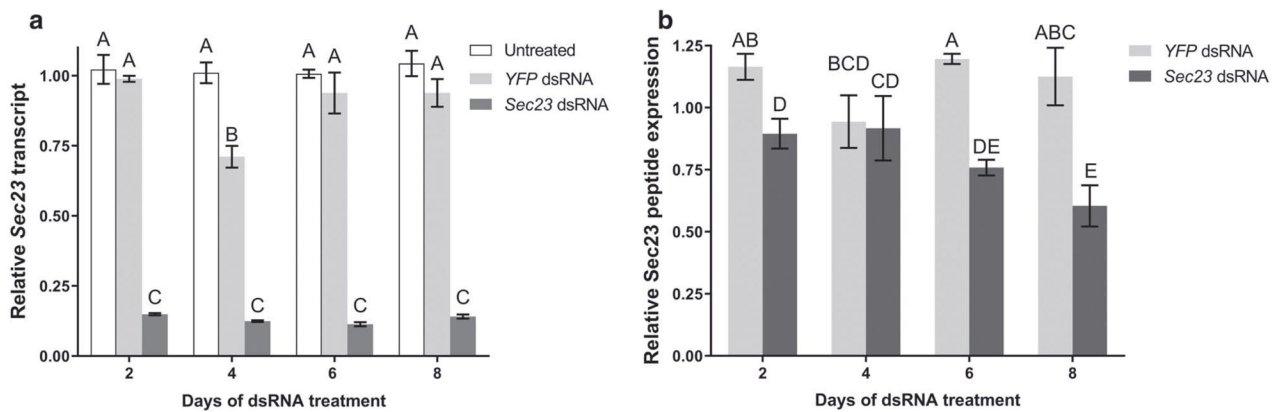
Figure 3. Levels of *Sec23* transcript and Sec23 protein during diet-based dsRNA treatment of WCR larvae. WCR samples collected at 2, 4, 6, and 8 days after initiation of diet surface overlay treatment with *Sec23* dsRNA at 500 ng cm^{-2} (Supporting information Sequence 3 in Appendix S1). Three biological replicates were performed. (a) Relative *Sec23* transcript levels were measured by probe hydrolysis RT-qPCR. (b) Relative peptide levels were measured using untargeted mass spectrometry of TMT-labeled digests. The protein amounts were normalized to β-tubulin (>30 detected peptides). Relative Sec23 protein abundance was calculated as a geometric mean of six detected Sec23 peptides. Error bars indicate standard error of the mean from three biological replicates; bars with different letters indicate significant statistical differences (P <0.05).

after exposure. Sec23 protein levels were consistently reduced only at 6 days after *Sec23* dsRNA application (Supporting information Sequence S3 in Appendix S1, Fig. 3(b)). After 8 days of treatment, the level of protein knockdown reached approximately 40% (Fig. 3(b)).

3.4 Sec23 hairpins, expressed in maize, protect roots against WCR damage

Sec23 v1 and *Sec23 v2* were incorporated as hairpins into binary Agrobacterium transformation vectors. Multiple T_0 maize plants transformed with hpSec23 v1 and hpSec23 v2 expression cassettes showed root protection in the greenhouse (**Fig. 4**(a), (b), (c), and (h)). The plants that showed root protection had root damage ratings and appearance that were similar to Cry34/35Ab1-expressing plants (Fig. 4(d)) compared to the control plants (Fig. 4(e), (f), (g) and (i)). To further quantify root protection of T_0 events, the root damage ratings were converted into binomial pass/fail frequency at ≤0.50 root rating on 0–1 NIS. The construct/genotype was modeled as the fixed effect in a generalized linear model procedure (JMP® Pro 12.2.0[67]) and showed to be significantly different (*P* <0.05). hpSec23 v1 had 0.78 pass frequency and hpSec23 v2 a pass frequency of 0.69. The positive control 7sh382rw (null), homozygous for Cry34Ab1/35Ab1, had a pass rate of 0.97 (**Table 2**). Predicted fail frequencies with 95% confidence intervals showed that B104 did not overlap with hpSec23 v1 or v2. Hence B104 had a higher fail rate.

Table 2. T_0 maize plants expressing WCR *Sec23* hairpin RNAs show robust root protection compared to negative controls

| Maize genotype	Construct	# of tested events	Pass frequency
7sh382rw	Positive control	15	0.97
7sh382	Negative Control	10	0.02
B104	B104	12	0.02
hpSec23 v1	pDAB117241	15	0.78
hpSec23 v2	pDAB117243	20	0.69
YFP protein	pDAB101556	11	0.02
hpYFP	pDAB110853	10	0.02

The root damage rating data estimated on 0–1.0 NIS scale was converted into binomial categorical data and the pass frequency (≤0.50 root rating) for each construct was calculated using a generalized linear model (JMP®Pro 12.2.0). Constructs were found to be significantly different (*P* <0.05).

Figure 4. T$_0$ maize expressing *Sec23* RNA hairpins protects against WCR in greenhouse bioassay. (a) T$_0$ maize root rating in a 15-day bioassay rated on a scale from 0 to 1 on a node-injury scale.[55] Root ratings of ≤0.5 are highlighted in green. The positive control is an inbred 7sh382 maize line that expresses Cry34Ab1/35Ab1. Negative controls include null (7sh382 isoline), B104, which was used as transformation material, as well as T$_0$ B104 plants transformed with *YFP* mRNA construct and *YFP* hairpin. (b–g) Photos of representative maize roots from a 15-day WCR greenhouse bioassay of T$_0$ plants (NIS 0–1). (b) B104 expressing WCR *Sec23 v1* hairpin with a root rating of 0.05. (c) B104 expressing WCR *Sec23 v2* hairpin with a root rating of 0.1. (d) 7sh382 maize line that expresses Cry34Ab1/35Ab1 with a root rating of 0.01. (e) B104 expressing *YFP* transcript (negative control) with a root rating of 1.0. (f) B104 (negative control) with a root rating of 1.0. (g) null: 7sh382 inbred maize line with a root rating of 1.0.

3.5 Effects of sublethal exposure on WCR fitness

To accurately quantify the effects of *Sec23 v1* sublethal concentrations on adults, we examined several fitness parameters in adult rootworms exposed to *Sec23* dsRNA LC$_{25}$. For sublethal concentration, we selected the LC$_{25}$, given that the LC$_{50}$ of other dsRNA targets (*i.e.*, *vATPase-A* and *dvSnf7*) varies between 23-fold to 387-fold between WCR larvae and adults.[31] In these experiments, we used *Sec23 v1* (Supporting information Sequence 4 in Appendix S1). To determine the LC$_{25}$ for *Sec23 v1* we evaluated a new concentration range (**Fig. 5**(a)) and confirmed *Sec23* transcript knockdown at the concentrations tested (Fig. 5(b)). The LC$_{50}$ at day 10 for *Sec23v1* was 8.5 ng/diet plug, and the LC$_{25}$ was 3 ng/diet plug.
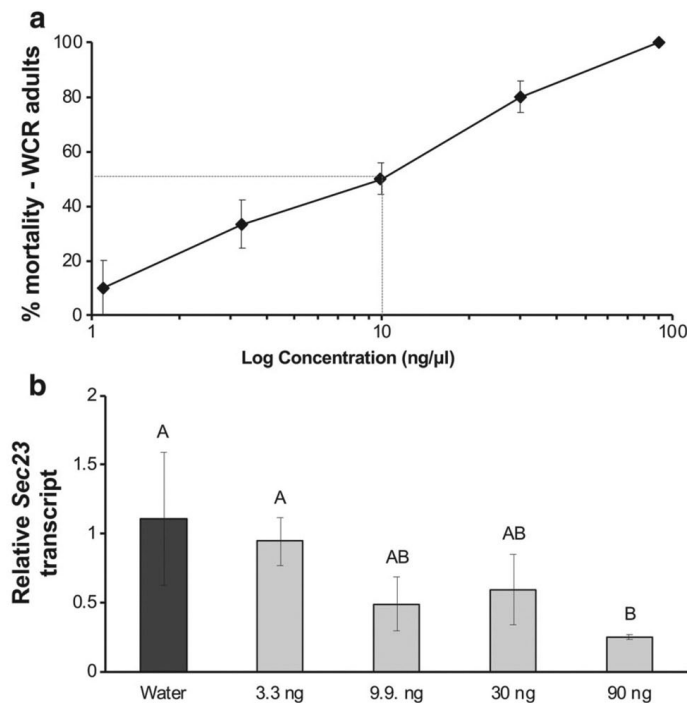
Figure 5. Concentration-response in WCR adults to the *Sec23 v1* dsRNA. (a) Five concentrations of *Sec23 v1* dsRNA (Supporting information Sequence 4 in Appendix S1) were used to determine the 10-day LC$_{25}$ and LC$_{50}$ in WCR adults. Fresh dsRNA-treated diet was provided every other day for 10 days and mortality was recorded every day. LC$_{50}$ at day 10 was estimated at 8.5 ng/diet plug and the LC$_{25}$ at 3 ng/diet plug. (b) Relative *Sec23* transcript levels in WCR adults collected on day 10 of treatment for four *Sec23 v1* concentrations. Error bars indicate standard errors of the mean from three biological replicates, bars with different letters indicate significant statistical differences with water (P <0.05).

Sec23 knockdown was evaluated in females treated with *Sec23 v1* dsRNA LC$_{25}$ and their offspring (*i.e.,* neonates) (**Fig. 6**(a) and (b)).We observed an average of 56% *Sec23* knockdown in females treated with *Sec23 v1* dsRNA at LC$_{25}$, compared to the water control (*P* =0.054) (Fig. 6(a)), while no knockdown was observed in their offspring (Fig. 6(b)). The numbers of eggs per female originating from females exposed to *Sec23 v1* dsRNA LC$_{25}$ (Fig. 6(c)) showed no significant differences when compared to females treated with water and *GFP* dsRNA. We observed significant differences between the percent of larvae hatching from females exposed to *Sec23 v1* dsRNA LC$_{25}$ and the water control (*P* =0.0255) (Fig. 6(d)). However, the percent of larvae hatching from females exposed to *Sec23 v1* dsRNA LC$_{25}$ was 64.5%, while the *GFP* dsRNA and water controls were 51.9% and 38.5%, respectively, suggesting no detrimental effects of *Sec23* dsRNA on larval hatching.
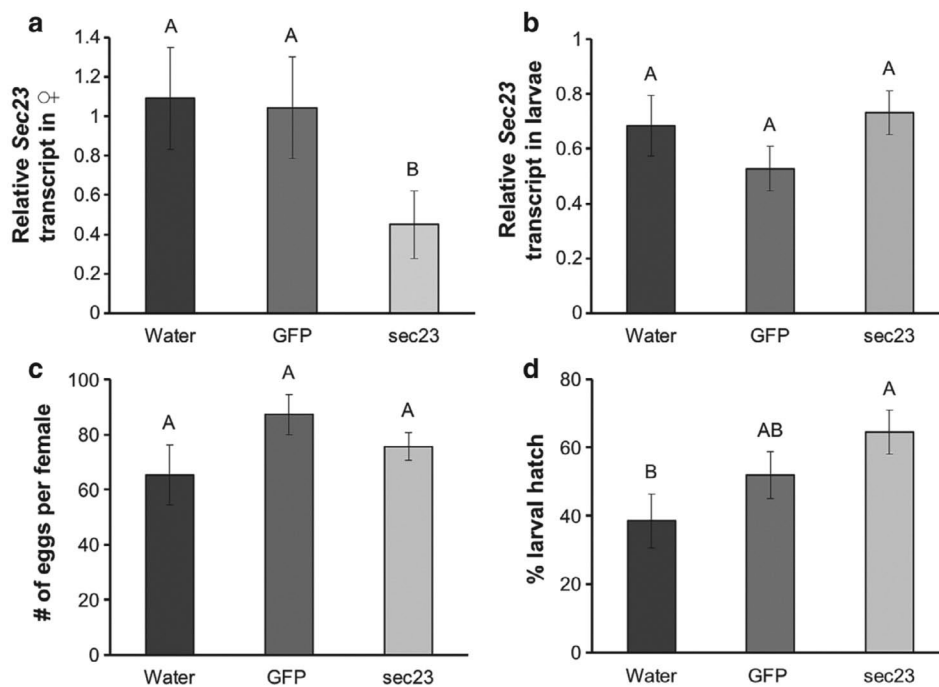
Figure 6. Effects on the offspring of females feeding on a sublethal *Sec23 v1* dsRNA concentration of 3 ng/plug. (a) Relative *Sec23* transcript levels in females fed with 3 ng dsRNA/diet plug. (b) Relative Sec23 transcript levels in the offspring of females fed with 3 ng dsRNA/diet plug. (c) Number of eggs per female. (d) Percent of larvae hatching. Error bars indicate standard errors of the mean from three biological replicates, bars with different letters indicate significant statistical differences with water (*P* <0.05).

Larval survival (**Fig. 7**(a)), instar (%) (Fig. 7(b)), larval weight (Fig. 7(c)) and head capsule size (Fig. 7(d)) was also measured in the offspring of females treated with *Sec23 v1* dsRNA LC_{25}, *GFP* dsRNA and water. We observed no differences in the percent of larvae in first, second and third instar between the controls (water and *GFP* dsRNA) and the offspring of females treated with *Sec23* dsRNA LC_{25} (Fig. 7(b)) or in head capsule size (Fig. 7(d)). However, we observed a 15% decrease in the percent of larval survival in larvae originating from females treated with *Sec23 v1* dsRNA at LC_{25}, compared to the water control (P =0.0409) (Fig. 7(a)). However, there were no significant differences between the *GFP* dsRNA control with the water control and *Sec23* (Fig. 7(a)).We also found a significant reduction in the larval weight of larvae originated from females treated with *Sec23* dsRNA LC_{25} compared to both controls (P =0.0235) (Fig. 7(c)). The weight of the offspring of females in the controls after 12 days was on average 4.9 mg and 4.64mg for water and *GFP* dsRNA, respectively, while the weight of the offspring of females treated with *Sec23 v1* dsRNA LC_{25} was 4.11mg.
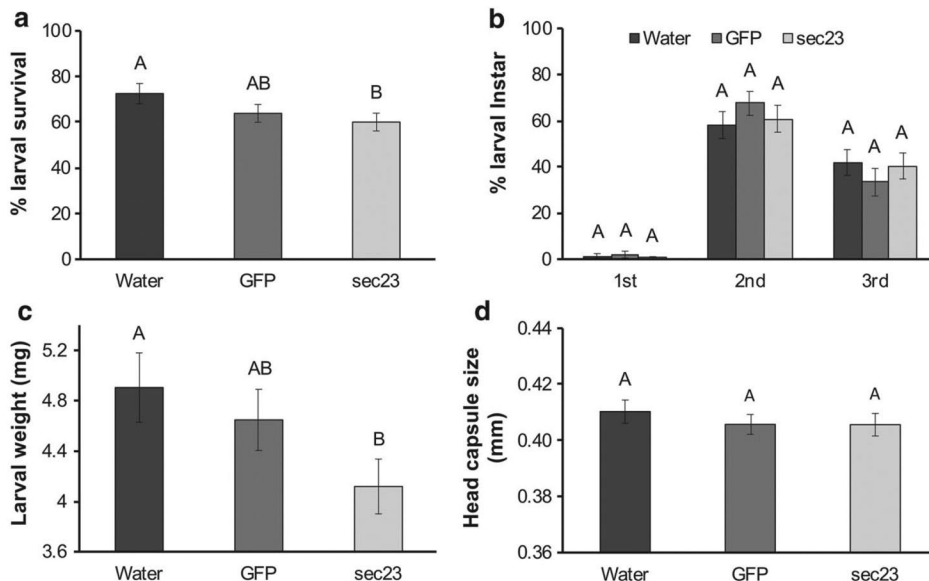
Figure 7. Life history parameters of the offspring of adults feeding on a sublethal (LC_{25}) *Sec23 v1* dsRNA concentration of 3 ng/plug. (a) Percent larval survival. (b.) Percent of each larval instar. (c) Larval weight (mg). (d) Head capsule size (mm). Error bars indicate standard errors of the mean from three biological replicates (N = 100–122), bars with different letters indicate significant statistical differences with water (P <0.05).

4 Discussion

RNAi has become well established as an effective technology to control WCR with several lethal targets documented.[17,23,24,26,27] The first commercial maize product expressing insecticidal dsRNA for WCR management will have three modes of action, *dvSnf7* dsRNA and two Bt proteins Cry3Bb1 and Cry34/35Ab1.[27,70] The combination of Bt and RNAi technology is expected to delay resistance development and extend the durability of existing traits.[71] Despite the recent demonstrations of field selected resistance to dsRNA in WCR,[72] identification and characterization of additional RNAi lethal targets will contribute to further innovation around RNAi-mediated control of WCR once RNAi resistance determinants and mechanisms of resistance are better understood.

In this report we describe a new RNAi target lethal to WCR, *Sec23*, that was selected using a knowledge-based approach and demonstrate the potential use of WCR adults for initial screening of RNAi targets. Mutations in *Sec23* and *Sec24CD* (also known as *haunted* and *ghost* in *Drosophila melanogaster*) perturb cuticle differentiation in *D. melanogaster*.[73] These proteins are necessary for the maintenance of cell polarity and deposition of extracellular matrix/chitin.[73] The insect midgut is a primary producer of chitin,[74] one can speculate that the loss of Sec23 would affect the secretion and maintenance of the peritrophic membrane, the major chitinous extracellular matrix of WCR midgut.[54] While the RNAi response in WCR is systemic, the midgut is likely to be the first site of action for orally-delivered dsRNA. Therefore, the key role of Sec23 in epithelial cell polarity and the maintenance of the peritrophic membrane may contribute to the efficacy of *Sec23* dsRNA. One should also note that known potent RNAi targets such as *v-ATPase A* are enriched in the midgut epithelium.[26,75] The loss of *Sec23* in *Drosophila* perturbs epidermal adherents and septate junctions.[73] As described by Hu *et al.*,[26] the depletion of adherent junction transcripts, *mesh,* and *snakeskin*, *via* dsRNA feeding in WCR also leads to strong lethal phenotypes. *Sec23* and *Sec24CD* mutations in *Drosophila* also disrupt the structure of ER and Golgi, affecting the expression and the localization of resident ER and Golgi proteins,[73] thereby further perturbing the secretion and deposition of the extracellular matrix.[73]

RNAi efficacy depends on selecting targets that are sensitive to perturbation of protein levels. Ideal gene targets for RNAi are thought to encode proteins with short half-lives. However, in screening for sensitive RNAi targets, transcript knockdown and phenotype are routinely used as a measure of RNAi, while knockdown of the encoded protein is seldom investigated by quantitative measurements. In this work, we measured both transcript and protein knockdown of WCR Sec23. To determine the level of protein depletion after *Sec23* dsRNA treatment, we employed untargeted mass spectrometry of the protein digests. Our results show that the maximum knockdown of Sec23 protein is only about 40% at day 8 (Fig. 3(b)). The lack of a more pronounced protein knockdown is somewhat unexpected, given >85% knockdown of *Sec23* transcript after only 2 days of exposure (Fig. 3(a)). The slow depletion of Sec23 protein may reflect its long half-life in WCR. The half-life of Sec23 in budding yeast, *Saccharomyces cerevisiae*, was reported to be 10.8 h, and in fission yeast, *Schizosaccharomyces pombe*, the half-lives of Sec23 homologs Sec23-1 and Sec23-2 are 83.8 and 22.0 h, respectively.[76] If the half-life of Sec23 in WCR is long, such as in *e.g. S. pombe*, it would be reflected in the levels of detected protein during the timeframe of our assay. Another explanation for the observed low levels of protein knockdown is based on *Sec23* dsRNA causing 50%–60% larval mortality within 9 days of treatment (Fig. 2(a)), which is similar to other RNAi targets,[21,24,26] and it is possible that the surviving larvae have the highest levels of remaining Sec23 protein.

Sec23 serves multiple functions within the COPII complex. Sec23 is a component of the inner shell of the COPII vesicle coat complex.[77–80] COPII vesicles facilitate (anterograde) transport from ER to Golgi.[77] Sec23 heterodimerizes with Sec24 to form a structural and binding platform for the vesicle cargo.[78] Sec23 also functions as a GTPase-activating protein for membrane invaginating protein Sar1.[81] The Sec23/Sec24 dimer also recruits the outer layer of the COPII coat. It is therefore quite possible that the high levels of lethality induced by *Sec23* dsRNA, despite incomplete knockdown at the protein level, are due to pleiotropic effects that imbalance processes essential for membrane traffic. Multiple potent RNAi targets in WCR and other insect species are involved in membrane traffic/vesicle transport pathways.[82] Along with *Sec23*, such targets include vacuolar H^+ ATPase subunits (*e.g.,*

v-ATPase A, *v-ATPase C*, and *v-ATPase D*),[17,25,41,83–85] components of the endosomal sorting complexes required for transport (ESCRT; *e.g., Snf7*), and vesicle coat proteins that facilitate (retrograde transport) from Golgi to the endoplasmic reticulum (COPI complex; *e.g., αCOP, βCOP, β′COP, γCOP*).[17,83–86] The mortality generated by *Sec23* was comparable to that observed in previous studies evaluating *v-ATPase A* and *dvSnf7* dsRNA in WCR larvae and adults.[31] In adults, the six-day LC_{50} of the *Sec23* dsRNA sequence initially tested was 44.2 ng/diet plug, and the 10-day LC_{50} of *Sec23 v1* was 8.5 ng/diet plug. The 14-day LC_{50} for *v-ATPase A* and *dvSnf7* were 7.56 ng/diet plug and 82.56 ng/diet plug, respectively,[31] suggesting that *Sec23* dsRNA had adult efficacy that was similar to *dvSnf7*. For larvae, Baum *et al.*[17] reported the LC_{50} for *v-ATPase A* and *dvSnf7* as 1.2 and 1.82 ng/cm^2, respectively. Pereira *et al.*[31] reported the larval LC_{50} for *v-ATPase A* and *dvSnf7* as 2.62 and 1.7 ng/cm^2, respectively. The *Sec23* LC_{50} values for larvae were 54 ng/cm^2 for *Sec23 v1* and 36 ng/cm^2 for *Sec23 v2* dsRNA suggesting higher LC_{50} values compared to those reported for *v-ATPase A* and *dvSnf7*. Larval mortality was evaluated on day 14 in Baum *et al.*,[17] and Pereira *et al.*[31] studies, while for *Sec23*, mortality was assessed 9 days after initial exposure. Furthermore, the sequences tested in Baum *et al.*,[17] and Pereira *et al.*[31] were >250 bp, while the *Sec23 v1* and *Sec23 v2* were 204 and 104 bp, respectively. The time of evaluation and the sequence length are factors known to affect the response to dsRNA.[21]

Preliminary experiments with lethal RNAi targets suggest that reduced egg production is observed in adult rootworms that have survived exposure to *v-ATPase A* and *dvSnf7* dsRNAs LC_{50} exposure in *D. undecipunctata*. The reduction in egg production was more pronounced with *dvSnf7*, indicating that the extent of sublethal effects varies between gene targets.[31] As a consequence, sublethal exposure to dsRNA in adults may affect egg production and further suppress rootworm populations. Furthermore, delays in larval development may lead to reproductive isolation, causing assortative mating of exposed adults and contributing to the emergence of resistance. Since exposure to *Sec23* would likely involve a plant-incorporated protectant (PIP), it is especially important to consider all the factors that could influence resistance evolution. With PIPs, insects are constantly exposed to the toxin and more likely to evolve resistance. Therefore, it is critical to anticipate the probability of resistance to develop adequate Insect Resistance Management (IRM) strategies. One of the assumptions of

current IRM strategies for PIPs is that mating between susceptible and individuals carrying resistant alleles should be random.[87] For this reason, we examined the sublethal effects of *Sec23* dsRNA on WCR larvae and adults. Knowing that adult WCR are less sensitive to dsRNA than larvae,[31] adult LC_{25} was selected to evaluate fitness parameters. In choosing LC_{25} as the testing concentration, we also assumed that adults might be exposed to lower dsRNA concentrations in the field. As shown in Figures 6 and 7, knockdown of *Sec23* transcript in adult female WCR did not have a significant impact on the number of eggs oviposited or hatched (Fig. 6(c) and (d)). While a slight reduction in larval survival and weight was observed (Fig. 7(a) and (c)), fitness parameters such as larval development and head capsule size were unaffected (Fig. 7(b) and (d)). A reduction in larval survival and weight suggest moderate sublethal effects for sublethal concentrations of *Sec23* dsRNA, information that should be considered in mitigating resistance to dsRNA. However, it is necessary to recognize that the sublethal dsRNA concentrations at which WCR females will be exposed, will depend on the expression in maize pollen and silk, the tissues consumed by WCR adults.

With the introduction of any insecticidal compound that exhibits a typical concentration-response, the evolution of resistance is expected, given that there is a fraction of the population that can survive a higher concentration of the compound due to a genetic component to survival. A robust WCR management program requires multiple control strategies, including plant traits, with different modes of action. Highly lethal RNAi targets such as *Sec23* may provide high dose or close to high dose plant protection, generating an insignificant number of rootworm survivors, thereby slowing down the evolution of resistance to RNAi. Based on this work, it will be important to determine the dsRNA concentrations expressed in maize pollen and silks, to estimate the potential exposure of WCR adults to sublethal concentrations and factor them into the future IRM strategies for PIPs.

5 Conclusion

This paper describes *Sec23* dsRNA, a subunit of the coat protein complex II (COPII) vesicle transport complex,[33–35] as a new RNAi target for WCR. *Sec23* was initially identified as a lethal RNAi target in WCR

adults and translated into robust larval lethality. This adult screening approach may be leveraged to dsRNA screening in other insects where continuous lab rearing has not been established and/or where there are no well-formulated larval diets. In addition to transcript abundance, we measured protein knockdown and observed that WCR lethality was achieved under partial depletion of Sec23. We also demonstrated that targeting WCR *Sec23* provides root protection when a *Sec23* RNA hairpin was transgenically expressed in corn. Sec23 dsRNA-induced WCR lethality is consistent with known cuticle/extracellular matrix secretion phenotypes of Sec23 in *Drosophila*. When sublethal effects were examined in the offspring of WCR females exposed to *Sec23* dsRNA LC_{25}, a slight reduction in larval survival and weight was observed. The observed moderate sublethal effects in adults suggest that dsRNA exposure in adult WCR will have minimal impact on the refuge strategy used in IRM programs. However, determining the expression of RNA hairpins in maize silks and pollen, preferred feeding sites of adults, is necessary to better characterize the potential effect of these moderate sublethal effects on IRM programs.

Supporting information follows the **References**.

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Control of western corn rootworm via RNAi traits in maize: lethal and sublethal effects of *Sec23* dsRNA

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SUPPLEMENTARY TABLES

Supplementary Table 1. Primer sequences used to quantify transcript knockdown in WCR adults.

| Gene name	Primer sequences for RT-qPCR	Product Length (bp)
Sec23	Forward: AGCTCCATTCAACCTGACAGA Reverse: TGTGCATCATCTACTGGAGCC	161
Sec23 (plant experiments)	Forward: TGCAAACTAGGTTCCCAATG Reverse: TATGCTGTGGACGAAACTGC	211
β-actin	Forward: TCCAGGCTGTACTCTCCTTG Reverse: CAAGTCCAAACGAAGGATTG	134

Supplementary Table 2. Primer sequences used to quantify transcript knockdown in WCR larvae.

Gene name	Primer sequences for RT-qPCR	Product Length (bp)
β-tubulin	Forward: TTGAGTTGCCGATGAAAGTG Reverse: GATCCCAGACACGGAAGGTA	205
β-actin	Forward: TCCAGGCTGTACTCTCCTTG Reverse: CAAGTCCAAACGAAGGATTG	134
Sec23	Forward: CTGTTGTTGCACCAGGAAGC Reverse: CATAACTCGGGCGCCAGTAT	200

Supplementary Table 3. Mortality and growth inhibition of WCR larvae after nine days of feeding with *Sec23* dsRNA. GC = Growth Inhibition; SEM = standard error of the mean; Replicates = 8 wells per replicate, 2-3 insects per well. Means were separated using the Tukey-Kramer test in JMP Pro. Letters in parentheses designate statistical levels: levels with different letters are significantly different ($p < 0.05$).

Treatment/ dsRNA	Concentration (ng/cm^2)	Replicates	% mortality ± SEM	GC ± SEM
Sec23	500	6	53.0 ± 12.8 **(A)**	0.56 ± 0.27 **(A)**
Sec23 v1	500	6	66.1 ± 4.8 **(A)**	0.85 ± 0.03 **(A)**
Sec23 v2	500	6	65.7 ± 10.0 **(A)**	0.84 ± 0.04 **(A)**
0.1X TE	0	12	10.4 ± 3.3 **(B)**	-0.04 ± 0.05 **(B)**
water	0	12	8.4 ± 2.5 **(B)**	0.08 ± 0.03 **(B)**
YFP dsRNA	500	12	9.6 ± 2.5 **(B)**	0.10 ± 0.05 **(B)**

SUPPLEMENTARY FIGURE

A. Single concentration 48 hours

B. Single concentration 3 and 6 hours
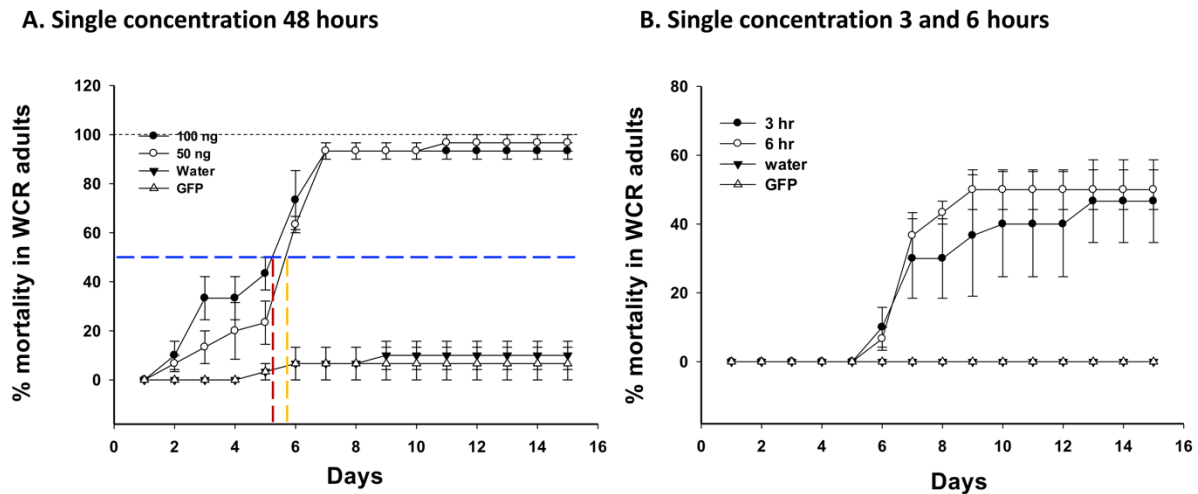
Supplementary Figure 1. Effect of a single *Sec23* dsRNA exposure in WCR adults. A. Adult mortality after a single *Sec23* dsRNA (383 bp, Supplementary Sequence 3) feeding for 48 h at 50 and 100 ng/diet plug; artificial diet and 100 ng of *GFP* dsRNA were used as controls. **B.** Adult mortality after single *Sec23* dsRNA (383 bp, Supplementary Sequence 3) feeding for 3 h and 6 h at 50 ng/diet plug; artificial diet and 100 ng of *GFP* dsRNA were used as controls. Error bars indicate standard errors of the mean from three biological replicates (*i.e.*, three generations) of ten mixed sex WCR adults (~48 h old) per treatment for a total of 30 insects per treatment.

SUPPLEMENTARY SEQUENCES

Sequence 1
> *D. v. virgifera* Sec23 protein

MSTYEEYIQQNEDRDGIRFTWNVWPSSRIEATRLVVPLACLYQPIKERLDLPPIQYDPVLCTRNTCRAILNPLCQVDYRA
KLWVCNFCFQRNPFPPQYAAISEQHQPAELMPMFSTIEYTITRAQCLPPIFLYVVDTCMDDEELGSLKDSLQMSLSLLPP
NALIGLITFGKMVQVHELGTEGCSKSYVFRGTKDLSAKQVQEMLGIGKVALGQQAPQQPGQPLRPGQMQPTVVAPG
SRFLQPVSKCDMNLTDLIGEQQKDPWPVHQGKRYLRSTGVALSIAIGLLECTYSNTGARVMLFVGGPCSQGPGQVVN
DDLKQPIRSHHDIQKDNAKYMKKGIKHYDALAMRAATNGHSVDIYSCALDQTGLMEMKQCCNSTGGHMVMGDSFN
SSLFKQTFQRVFTRDQKSDLKMAFNGTLEVKCSRELKVQGGIGSCVSLNVKSPLVSDTEIGMGNTVQWKMCTLTPSTT
MSLFFEVVNQHSAPIPQGGRGCIQFITQYQHSSGQRKIRVTTVARNWADATANIHHISAGFDQEAAAVIMARMAVYR
AESDDSPDVLRWVDRMLIRLCQKFGEYNKDDPNSFRLGQNFSLYPQFMYHLRRSQFLQVFNNSPDETSFYRHMLMRE
DLTQSLIMIQPILYSYSFNGPPEPVLLDTSSIQPDRILLMDTFFQILIFHGETIAQWRSLKYQDMPEYENFRQLLQAPVDDA
QEILQTRFPMPRYIDTEQGGSQARFLLSKVNPSQTHNNMYSYGGDSGAPVLTDDVSLQVFMDHLKKLAVSSTA

Sequence 2
> *D. v. virgifera Sec23* open reading frame
ATGAGCACATATGAAGAGTATATACAACAAAATGAAGATCGAGATGGGATTAGATTTACCTGGAATGTATGGCCT
TCAAGCAGAATTGAAGCTACCCGTCTCGTAGTACCCTTAGCTTGTCTGTACCAGCCTATAAAGGAACGTCTGGATC
TTCCACCAATACAATATGACCCTGTTTTATGTACTAGAAATACTTGTAGAGCAATATTAAACCCACTGTGTCAGGTA
GATTATCGAGCAAAACTCTGGGTATGCAACTTTTGTTTCCAGAGAAATCCATTTCCACCTCAATATGCTGCTATTTC
AGAACAACATCAACCAGCGGAATTGATGCCTATGTTTTCCACCATTGAATACACAATAACTAGAGCTCAATGTTTAC
CACCAATATTTTTGTATGTTGTTGACACCTGCATGGATGATGAAGAACTGGGTTCCCTGAAAGACTCATTGCAAAT
GTCCCTTAGTTTGTTGCCACCTAATGCGTTAATAGGACTAATAACATTTGGGAAAATGGTTCAAGTTCATGAACTTG
GCACTGAAGGTTGTAGTAAGTCATATGTGTTCAGAGGTACAAAAGATCTTAGTGCTAAACAGGTTCAAGAAATGC
TGGGAATAGGCAAAGTGGCTTTAGGTCAGCAAGCCCCTCAACAGCCAGGGCAGCCTCTAAGACCTGGGCAAATGC
AACCTACTGTTGTTGCACCAGGAAGCAGGTTTCTACAACCTGTATCCAAATGCGATATGAATCTAACAGACCTAAT
AGGAGAACAACAGAAAGATCCTTGGCCTGTTCATCAGGGTAAAAGGTATTTAAGATCTACAGGTGTAGCTTTATC
GATTGCCATTGGTTTGTTAGAATGTACATATTCCAATACTGGCGCCCGAGTTATGCTATTTGTTGGAGGACCTTGCT
CACAAGGACCTGGTCAGGTAGTTAATGATGATTTAAAACAGCCTATTAGATCACATCATGATATTCAGAAAGATAA
TGCAAATATATGAAGAAAGGTATTAAACATTATGATGCGTTAGCAATGAGAGCCGCAACTAATGGTCACTCTGTT
GATATTTATTCTTGTGCTTTGGATCAGACAGGTCTGATGGAAATGAAGCAATGCTGTAATTCTACTGGGGGACACA
TGGTAATGGGGGATTCATTTAATTCTTCCTTGTTTAAGCAAACTTTCCAACGTGTGTTTACCAGAGATCAAAAAAGT
GATCTGAAAATGGCATTTAACGGTACTTTGGAAGTGAAGTGTTCCCGAGAATTAAAAGTTCAAGGAGGTATCGGT
TCGTGTGTATCACTTAACGTGAAGAGCCCCTTGGTTTCCGACACAGAAATAGGAATGGGTAATACTGTGCAATGG
AAAATGTGTACTTTAACGCCAAGTACTACCATGTCTTTATTCTTTGAGGTCGTAAATCAACATTCTGCTCCCATACCT
CAAGGTGGTAGAGGTTGTATACAATTTATTACGCAGTACCAGCATTCAAGTGGTCAAAGAAAAATCAGAGTAACA
ACAGTGGCTCGAAATTGGGCTGACGCAACTGCTAATATACACCATATCAGTGCCGGATTCGATCAAGAAGCTGCT
GCTGTAATAATGGCTAGGATGGCCGTTTATAGGGCAGAATCTGATGATAGTCCAGATGTTCTTAGATGGGTTGAC
AGAATGCTGATTAGATTGTGTCAAAAATTCGGAGAATACAATAAGGACGACCCCAATTCATTCAGACTTGGTCAAA
ACTTCAGTCTTTACCCACAGTTCATGTATCACTTAAGAAGATCTCAATTTCTTCAAGTATTCAATAATTCTCCGGACG
AGACTTCATTCTACAGACACATGTTGATGAGGGAAGATCTTACTCAATCTTTGATAATGATTCAACCTATTTTGTAT
AGTTATAGTTTCAATGGTCCACCAGAGCCTGTATTACTAGATACTAGCTCCATTCAACCTGACAGAATATTACTTAT
GGATACTTTCTTCCAAATATTAATTTTCCATGGAGAGACTATCGCCCAATGGCGTAGTTTAAAATATCAAGACATGC
CAGAATATGAAAACTTTAGACAGCTACTACAGGCTCCAGTAGTGATGCACAAGAAATTTTGCAAACTAGGTTCCC
AATGCCGAGATATATTGATACCGAACAAGGCGGATCCCAAGCCAGATTTTTGTTGTCGAAAGTAAATCCAAGTCAA
ACTCATAACAACATGTATTCCTACGGAGGTGATTCTGGAGCTCCAGTTTTGACAGATGATGTATCCCTTCAAGTATT
CATGGACCATCTAAAGAAATTGGCAGTTTCGTCCACAGCATAA

Sequence 3
> *D. v. virgifera Sec23* dsRNA

AGGACGACCCCAATTCATTCAGACTTGGTCAAAACTTCAGTCTTTACCCACAGTTCATGTATCACTTAAGAAGATCT
CAATTTCTTCAAGTATTCAATAATTCTCCGGACGAGACTTCATTCTACAGACACATGTTGATGAGGGAAGATCTTAC
TCAATCTTTGATAATGATTCAACCTATTTTGTATAGTTATAGTTTCAATGGTCCACCAGAGCCTGTATTACTAGATAC
TAGCTCCATTCAACCTGACAGAATATTACTTATGGATACTTTCTTCCAAATATTAATTTTCCATGGAGAGACTATCGC
CCAATGGCGTAGTTTAAAATATCAAGACATGCCAGAATATGAAAACTTTAGACAGCTACTACAGGCTCCAGTA

Sequence 4

> *D. v. virgifera Sec23 v1* dsRNA
AGGTTCCCAATGCCGAGATATATTGATACCGAACAAGGCGGATCCCAAGCCAGATTTTTGTTGTCGAAAGTAAATC
CAAGTCAAACTCATAACAACATGTATTCCTACGGAGGTGATTCTGGAGCTCCAGTTTTGACAGATGATGTATCCCTT
CAAGTATTCATGGACCATCTAAAGAAATTGGCAGTTTCGTCCACAGCATAA

Sequence 5

> *D. v. virgifera Sec23 v2* dsRNA
ATTCCTACGGAGGTGATTCTGGAGCTCCAGTTTTGACAGATGATGTATCCCTTCAAGTATTCATGGACCATCTAAAG
AAATTGGCAGTTTCGTCCACAGCATAA

Sequence 6

>*GFP* dsRNA
GGGAGTGATGCTACATACGGAAAGCTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAA
CACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTTCA
AGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACTACAAGACGCGTG
CTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAA
CATTCTCGGACACAAACTCGAGTACAACTATAACTCACACAATGTATACATCACGGCAGACAAACAACCCA

Sequence 7

>*YFP* dsRNA
CACCATGGGCTCCAGCGGCGCCCTGCTGTTCCACGGCAAGATCCCCTACGTGGTGGAGATGGAGGGCAATGTGG
ATGGCCACACCTTCAGCATCCGCGGCAAGGGCTACGGCGATGCCAGCGTGGGCAAGGTGGATGCCCAGTTCATCT
GCACCACCGGCGATGTGCCCGTGCCCTGGAGCACCCTGGTGACCACCCTGACCTACGGCGCCCAGTGCTTCGCCA
AGTACGGCCCCGAGCTGAAGGATTTCTACAAGAGCTGCATGCCCGATGGCTACGTGCAGGAGCGCACCATCACCT
TCGAGGGCGATGGCAATTTCAAGACCCGCGCCGAGGTGACCTTCGAGAATGGCAGCGTGTACAATCGCGTGAAG
CTGAATGGCCAGGGCTTCAAGAAGGATGGCCACGTGCTGGGCAAGAATCTGGAGTTCAATTTCACCCCCCACTGC
CTGTACATCTGGGGCGATCAGGCCAATCACGGCCTGAAGAGCGCCTTCAAGATCT