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ABSTRACT

Western corn rootworm, *Diabrotica virgifera virgifera*, is the major agronomically important pest of maize in the US Corn Belt. To augment the repertoire of the available dsRNA-based traits that control rootworm, we explored a potentially haplolethal gene target, *wings up A (wupA)*, which encodes Troponin I. Troponin I, a component of the Troponin-Tropomyosin complex, is an inhibitory protein involved in muscle contraction. *In situ* hybridization showed that feeding on *wupA*-targeted dsRNAs caused systemic transcript knockdown in *D. v. virgifera* larvae. The knockdown of *wupA transcript,* and by extension Troponin I protein, led to deterioration of the striated banding pattern in larval body muscle and decreased muscle integrity. Additionally, the loss of function of the circular muscles surrounding the alimentary system led to significant accumulation of food material in the hind gut, which is consistent with a loss of peristaltic motion of the alimentary canal. In this study, we demonstrate that *wupA* dsRNA is lethal in *D. v. virgifera* larvae when fed via artificial diet, with growth inhibition of up to 50% within two days of application. Further, *wupA* hairpins can be stably expressed and detected in maize. Maize expressing *wupA* hairpins exhibit robust root protection in greenhouse bioassays, with several maize transgene integration events showing root protection equivalent to commercial insecticidal protein-expressing maize.

1. Introduction

The corn rootworm species that are mainly represented by the western corn rootworm (WCR), *Diabrotica virgifera virgifer*a and the northern corn rootworm (NCR), *Diabrotica barberi* are the major below ground pests of maize and are believed to cause in excess of $1 billion in control costs and crop loss in the US (Gray et al., 2009; Metcalf, 1986). Current traits for below ground insect control include insecticidal proteins from *Bacillus thuringiensis* (Bt) Cry3Bb1, mCry3A, eCry3.1Ab, and Cry34Ab1/35Ab1. Field-evolved resistance to Cry3-derived traits (Gassmann et al., 2011, 2012) as well as cross resistance between Cry3Bb1 and mCry3a (Gassmann et al., 2014; Wangila et al., 2015) and Cry3Bb1 and eCry3.1Ab (Zukoff et al., 2016) has been documented. While Cry3-resistant *D. v. virgifera* does not show cross-resistance to Cry34Ab1/35Ab1 (Zukoff et al., 2016), emerging evidence of what has been described as incomplete resistance to Cry34Ab1/35Ab1 highlights the vulnerability of this trait (Gassmann et al., 2016).

RNA interference (RNAi) is lethal to *D. v. virgifera* when provided as double-stranded RNA (dsRNA) in a diet (Baum et al., 2007; Bolognesi et al., 2012; Rangasamy and Siegfried, 2012) or expressed transgenically in a plant (Baum et al., 2007; Hu et al., 2016; Knorr et al., 2018; Li et al., 2018; Moar et al., 2017). RNAi trait that is closest to commercialization is *D. v. virgifera* Snf7 (a long RNA hairpin transcript DvSnf7 expressed in MON 87411), which is a component of SmartStax® PRO[1] maize. Snf7 (also called Vps32 or shrub) is a vacuolar sorting protein that is a constituent of Endosomal Sorting Complex Required for Transport-III (ESCRT-III). Neither the DvSnf7 trait or the *DvSnf7* dsRNA showed cross resistance in field-derived Cry3Bb1 resistant *D. v. virgifera* (Moar et al., 2017); thus, RNAi provides a novel mode of action with respect to Cry3Bb1. Recent evaluation of SmartStax® maize and

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[1] SmartStax® is a registered trademark of Monsanto Technology LLC. SmartStax® is a multi-event technology developed by Dow AgroSciences and Monsanto.

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Abbreviations

Bt	*Bacillus* thuringiensis
BLAST	Basic local alignment search tool
dsRNA	Double-stranded RNA
GI	Growth inhibition
hpRNA	Hairpin RNA
IRM	Insect resistance management
ISH	*In situ* hybridization
NCR	Northern corn rootworm
T_0	Regenerated transgenic plants
RNAi	RNA interference
SpecR	Spectinomycin-resistance gene
ssRNA	Single-stranded RNA
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
WCR	Western corn rootworm
wupA	Wings up A

SmartStax® PRO[1] maize, expressing Cry3Bb1 and Cry34Ab1/Cry35Ab1 toxins alone or the above toxins with the *DvSnf7* RNAi trait estimated that the RNAi trait reduced rootworm emergence by 80–95% (Head et al., 2017). Multiple studies have proved that in addition to the reduction in adult emergence in the field, RNAi can cause lethality in *D. v. virgifera* (Baum et al., 2007; Bolognesi et al., 2012; Hu et al., 2016; Li et al., 2015; Rangasamy and Siegfried, 2012). It was reported that DvSnf7 induces mortality in *D. v. virgifera* but on a slower time scale than Bt insecticidal proteins (Bolognesi et al., 2012). These observations suggest that more potent RNAi traits can bring even greater benefit to the pyramided Bt rootworm control products.

One of the key predictors of a successful RNAi target in Insect Resistance Management (IRM) models for agricultural traits is that the targeted gene is essential for the survival of the insect. While about 25%–35% of genes in insect genomes are estimated to be essential (Dietzl et al., 2007), in the vast majority of cases one copy of the gene is sufficient to sustain viability. Thus while many genes products are essential, few are dose-sensitive. One functional manifestation of gene dose sensitivity is Haplolethality: organisms heterozygous (50% dose) at a haplolethal locus cannot survive. Another manifestation of gene dose insufficiency includes haplosterile insects that produce minute phenotypes and no progeny. In *Drosophila*, the most genetically amenable insect, researchers have identified only 49 loci that are demonstrably haplolethal or haplosterile (Cook et al., 2012). Any surviving heterozygous mutants in these haploinsufficient genes exhibit minute phenotypes, which is small body size presumably due to a decrease in protein production. Interestingly, 40 of the haplolethal or haplosterile loci in *Drosophila* correspond to ribosomal proteins. The non-ribosomal loci that are mapped to a single gene or a gene region include *Fs(1)*10A region, *Hdl* region, *wupA* region, *dpp*, Haplolethal region 67D10-E1, *Tpl* region, *Ms(3)*88C region, *Su(var)3–9*, and *Abd-B* (Cook et al., 2012).

In this study, we selected an ortholog of the *Drosophila* non-ribosomal haplolethal *Troponin I (TnI)/wings up A (wupA)* gene (FlyBase). The *Drosophila wupA* encodes Troponin I protein (Barbas et al., 1991, 1993). Troponin is an actin binding protein that performs a regulatory role in muscle contraction. In vertebrates and insects, Troponin I is part of the heterotrimeric Troponin complex that includes the inhibitory subunit TnI, the $Ca2^+$ binding subunit Troponin C (TnC), and the Tropomyosin binding subunit Troponin T (TnT). In turn, Troponin interacts with Tropomyosin (Tropomyosin 1 (Tm1) and Tropomyosin 2 (Tm2)) to generate a Tn-Tm complex. The Tn-Tm complexes attach to actin filaments, blocking actin-myosin interactions between thin (actin) and thick (myosin) filaments in the relaxed muscle state. Upon Ca^{2+} activation, Tn-Tm changes conformation, allowing for muscle contraction. In mammals, Troponin I is encoded by three distinct genomic loci: slow skeletal muscle TnI (TNNI1), fast skeletal muscle TnI (TNNI2), and cardiac TnI (TNNI3) reviewed in *e.g.,* (Hastings, 1997; Sheng and Jin, 2016; Wei and Jin, 2016). In *Drosophila, wupA* is encoded by a single gene locus with multiple transcripts and at least ten protein isoforms (Barbas et al., 1991). While it is a key regulator of myosin in cardiac and skeletal muscles, Troponin is largely absent from smooth muscle and non-muscle cells (Somlyo and Somlyo, 2000). In smooth muscle and in non-muscle cells myosin, including its cytoskeletal functions, myosin is regulated by tropomyosin, caldesmon, and calponin (Rozenblum and Gimona, 2008); contraction is initiated by intracellular Ca2+ via Ca2+-calmodulin-regulated myosin light chain kinase (MLCK), myosin phosphatase (MLCP), and RhoA/Rho family proteins (Somlyo and Somlyo, 2000).

Our hypothesis was that haplolethal gene targets may lead to greater potency and dose sensitivity of the RNAi effect. We identified nine potential alternative transcripts/splice variants of the *wupA* open reading frame in *D. v. virgifera*. DsRNA targeting *wupA* in *D. v. virgifera* produced high levels of larval lethality when tested on artificial diet and showed significant levels of growth inhibition within two days of feeding. Morphological phenotypes after *wupA* dsRNA consumption by *D. v. virgifera* larvae include perturbations in body muscle, muscle Z-bands/Z-discs, fat body, and hindgut that are consistent with the loss of Troponin I function. These observations indicate that *wupA* hairpin RNAs may be suitable RNAi traits for protection of maize roots from rootworm feeding damage. In agreement with this hypothesis, several transgenic maize lines expressing *wupA* hairpins provided high levels of root protection in the greenhouse.

2. Materials and methods

2.1. Insect rearing

Non-diapausing wild type *D. v. virgifera* eggs were purchased from Crop Characteristics, Inc. (Farmington, MN). *D. v. virgifera* eggs were washed from soil with water and surface-sterilized with 10% formaldehyde for ten minutes (Števo and Cagáň, 2012). The eggs were then rinsed with water, hatched on artificial diet and held at 28 °C, ~40% Relative Humidity, 16:8 (Light:Dark), as described earlier (Pleau et al., 2002; Tan et al., 2016).

2.2. Identification of wupA orthologs within D. v. virgifera transcriptome

D. v. virgifera de novo transcriptome of 69,840 transcripts that was described by Knorr et al. (2018) was used for transcript analyses. To identify proteins in *D. v. virgifera*, TBLASTN searches using candidate protein coding sequences were run against the above transcriptome database. Significant hits to a *D. v. virgifera* sequence (defined as e-value lower than 1×10^{-20} for contig homologies) were confirmed using BLASTX against the NCBI non-redundant database. The results of this BLASTX search confirmed that the *D. v. virgifera* ortholog candidate gene sequences identified in the TBLASTN search corresponded to the *D. v. virgifera wupA* gene, which were assigned GenBank accession numbers MH001571, MH001572, MH001573, MH001574, MH001575, MH001576, MH001577, MH001578, and MH001579.

2.3. Template preparation and dsRNA design and synthesis

D. v. virgifera contigs were annotated with the location of the open reading frame (ORF) for the ortholog, based on BLASTX results from NCBI non-redundant database. Using the ORF location dsRNA sequence was designed with a target range between approximately 200 and 500

base pairs, a %GC between 40 and 60, and a distance from the ATG and stop codon of greater than seventy base pairs. *WupA* dsRNA is 254 bp in size and fully or partially covers all splice forms of *D. v. virgifera wupA* (Supplementary Sequence 25) and the control *YFP* dsRNA is 503 bp in size (Supplementary Sequence 26). Primer sequences for *wupA* and *YFP* dsRNA amplification are listed in Supplementary Table 1. DsRNA was synthesized as described by Knorr et al. (2018).

2.4. Artificial diet bioassays

Nine-day, five-day, and two-day *D. v. virgifera* feeding bioassays were conducted with neonate larvae (two to three larvae per well) in 128-well plastic bioassay trays, as earlier described (Knorr et al., 2018). Briefly, dsRNA was diluted in 0.1X TE and surface-applied at 500 ng/cm^2. Trays were held at 28 °C and approximately 40% Relative Humidity at 16:8 light:dark photoperiod. The total number of insects exposed to each sample, the number of dead insects, and the weight of surviving insects were recorded after two, five, or nine days. YFP dsRNA, 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} (concentration at which 50% of the insects are dead) and GI_{50} (concentration that causes 50% growth inhibition or GI) were estimated using a dilution series bioassays (*i. e.,* four-fold serial dilutions of dsRNA). LC_{50} and GI_{50} values were calculated using log-logistic regression analysis in JMP.

2.5. RNA in situ hybridization

An improved embedding and sectioning method for RNAscope *in situ* hybridization (ISH) is described by (Bowling and Pence, 2018). In brief, insects were collected after 48 h on diet that contained dsRNAs targeted to the *wupA* gene. Once collected, insects were fixed in 4% formaldehyde (from sealed ampoules; Ted Pella, Inc., Redding, CA, USA) in 10 mM phosphate buffered saline that contained 1:10,000 Silwet L-77 (Lehle Seeds, Round Rock, TX, USA), vacuumed until specimens sank, dehydrated in a graded series of ethanol (25%, 50%, 75%, and 100%), and infiltrated in a graded series of 3:1 butyl:methyl methacrylate resin (Polysciences, Inc., Warrington, PA, USA). Samples were transferred into catalyzed resin in flat-bottom polyethylene embedding capsules (Ted Pella, Inc., Redding, CA, USA) and polymerized on a UV light source at 4 °C. Specimens were then sectioned (1 μm) on a diamond histoknife (Delaware Diamond Knives, Inc., Wilmington, DE, USA), collected onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA), deplasticized for 10 min in 100% acetone, and processed for ISH. Slides were analyzed with the Advanced Cell Diagnostics (ACD) Automated RNAscope VS Assay – Reagent Kit Brown (Hayward, California, USA) on a Ventana Discovery Ultra automated IHC/ISH research slide staining system (Roche Diagnostics, Indianapolis, Indiana, USA). Proprietary experimental probes for *wupA* sequences that were common to all identified transcript variants and outside of dsRNA target region were designed and generated by ACD (Hayward, California, USA). The standard *DapB* negative control probe was also purchased from ACD. Probed slides were washed in soapy water, air dried, dipped in xylene, and mounted with Polymount-Xylene (Polysciences, Inc., Warrington, PA, USA).

2.6. D. v. virgifera histopathology

Insects were collected after 96 h on diet that contained dsRNAs targeted to the *wupA* gene. Larvae were processed as above, but then infiltrated with a graded series of LR White resin (Polysciences, Inc., Warrington, PA, USA), transferred into flat-bottom polyethylene embedding capsules (Ted Pella, Redding, CA, USA), and heat polymerized for 3 h at 50 °C. Specimen blocks were then trimmed and sectioned as above (but at 500 nm thickness), stained with Toluidine Blue O, and mounted with Polymount-Xylene.

2.7. Microscopy

Image data were captured using LAS software (version 4.6) on a Leica DM5000B upright microscope, equipped with a Leica DFC 7000T camera (Leica Microsystems, Buffalo Grove, IL, USA). Figure panels were created with GIMP (v2.8.16); minor contrast and color balance adjustments were also done with GIMP.

2.8. Construct design and the development of transgenic plants

Standard cloning methods were used to construct binary transformation plasmids that were based on pTI15955 plasmid from *Agrobacterium* (Barker et al., 1983), as described by Knorr et al. (2018). Briefly, the expression of *wupA* hairpin, containing 254 bp *wupA* target region, was cloned under maize *ubiquitin1* gene promoter (Quail et al., 1996) and terminated by the 3′ untranslated region of the potato, *Solanum tuberosum*, *proteinase inhibitor II* gene (StPinII 3′UTR) (Ainley et al., 2004). Binary expression vectors also contained an herbicide tolerance gene (aryloxyalknoate dioxygenase; AAD-1 v3), under the regulation of a maize *ubiquitin1* promoter 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 (Merlo et al., 2015). The transformation of immature embryos inbred line, *Zea mays* c.v. B104 was performed, embryos were selected, and calli and shoots regenerated as described by (Miller, 2013), with modifications (Knorr et al., 2018).

Rooted plantlets about 6 cm or taller were transplanted into soil and placed in growth chambers. Plants were transplanted from Phytatrays to small pots (T. O. Plastics, 3.5″ SVD, 700022C) filled with growing media (Premier Tech Horticulture, ProMix BX, 0581 P) and covered with 7.5″ humidity domes (Hydrofarm, Petaluma, CA) to help acclimate the plants. Plants were 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 (2 7/8″ x 9″) (Grower's Nursery Supply, Inc., Portland, OR) containing Metro Mix (Sun Gro Horticulture, Quincy, MI) for bioassay. One plant per transgene insertion/integration event (hereafter referred to as event) was bioassayed.

2.9. Transgene copy number analysis

Transgene copy number was determined using probe hydrolysis quantitative PCR (qPCR) to detect a portion of the AAD herbicide tolerance gene as described by Knorr et al. (2018). To determine if the transgenic plants contained extraneous plasmid backbone, qPCR assays to detect a portion of the Spectinomycin-resistance gene (SpecR; from the binary vector plasmids outside of the T-DNA) were also performed as previously described (Knorr et al., 2018). The relevant primer and probe sequences are listed in Supplementary Table 2.

2.10. T_1 seed generation and plant care

T_0 plants containing 1–3 copies of the transgene (see Table 2) were transplanted into 5-gallon pots and grown to maturity. T_0 transgene integration events were reciprocally crossed to inbred B104 plants to generate T_1 seed. Ears were peeled back at 21 days after pollination to enhance dry down followed by complete harvest (ear removed from plant) at 42 days after pollinations. Ears were placed in the dryer for 1

week prior to processing for seed. Forty-five T_1 seeds from each T_0 event were planted in Metro Mix soil in individual pots and moved to the greenhouse. The plants were maintained at 16:8 h light:dark regime and watered when necessary. After one week, the T_1 seedlings were sprayed with 35 g ai/ha of quizalofop-P-ethyl (Assure II). Four to five days later, ten healthy plants per event that survived the herbicide spray were transplanted into Rootrainers (2 7/8″ x 9″) (Grower's Nursery Supply, Inc., Portland, OR) containing Metro Mix.

2.11. Root protection assays

The whole plant maize bioassays were conducted by following the protocol described in (Dönitz et al., 2015). In brief, the transgenic corn plants (T_0, one plant per event) were planted into root trainer pots containing Metro Mix soil after reaching V3 to V4 stage. On the 3rd day after transplanting, the T_0 plants were infested with approximately 250 *D. v. virgifera* eggs and allowed to grow for two weeks. If the rootworm hatch rate was lower than 50%, the plants were re-infested three days after the initial infestation. During infestation, two holes approximately 1″ deep were made using a pencil approximately ½" away from the stem of the plant and the *D. v. virgifera* eggs suspended in agar solution were injected into these holes. Two weeks after infestation, the plant roots were washed and *D. v. virgifera* feeding damage was scored using node-injury scale (NIS) ranging from 0 to 1 as compared to 0–3, as described by (Oleson et al., 2005). T_1 plants were infested with approximately 125 eggs per plant 14 days after transplanting, with an additional 125 eggs per plant 17 days after transplanting. Infestation at three-day intervals provides opportunity for extended rootworm hatching that better mimics field conditions. T_1 maize pants were graded three weeks after the first infestation; the NIS scale used for T_1 was 0–3. Plants expressing Cry34Ab1/Cry35Ab1 in 7sh382 and B104 genetic background served as Bt positive controls. The negative controls included non-transformed B104, B104 plants expressing YFP protein, non-transgenic isoline 7sh382 (Johnson, 2000). Both positive and negative controls were included in each bioassay experiment.

2.12. Plant expressed hairpin transcript size and integrity via northern blot assay

Plant RNA extractions from leaf and root tissues were carried out using TRIzol reagent (Thermo Fisher Scientific, Wilmington, DE, USA) following the manufacturer's protocol. The isolated RNA was digested with RNase I_f (New England Biolabs, Ipswich, MA, USA) per the manufacturer's protocol at 37 °C for 10 min followed by heat inactivation at 70 °C for 20 min. The digested RNA samples were then precipitated using 2.5X (V/V) 100% ethanol and 0.35X (V/V) 3M ammonium acetate, and subsequently washed 1X with 75% ethanol, then re-suspended in RNA sample loading buffer (Sigma-Aldrich, St. Louis, MO, USA).

A 1.5% Roche Mp agarose gel (Roche Diagnostics, Indianapolis, Indiana, USA) with the addition of 6.5% formaldehyde in 1X MESA buffer (MOPS-EDTA-sodium acetate) was cast and run on electrophoresis equipment capable of running samples at 65 V/30 mA for ~ 150 min. Following electrophoresis, the gel was rinsed in 2X SSC for 15 min. The gel was passively transferred to a nylon membrane (Millipore, Billerica, MA, USA) overnight at room temperature, using 10X SSC as the transfer buffer. Following the transfer, the membrane was rinsed in 2X SSC for 5 min, subsequently crosslinked (Stratagene, San Diego, CA), and allowed to dry at room temperature for up to two days.

The membrane was prehybridized in UltraHyb buffer (Ambion) for two hours at 65 °C. Blots were probed with DIG digoxigenin-11-UTP labelled probe using the Roche DIG RNA labeling Kit (Roche Diagnostics, Indianapolis, Indiana, USA) according to manufacturer's protocols. The appropriate amount of probes (80 ng/ml of hybridization solution) were denatured at 95 °C for 5 min and flash frozen on ice before adding to fresh hybridization solution. In this study, blots were probed with a 170 bp DIG-labelled single-stranded RNA complementary to the sense-strand RNA of the *wupA* hpRNA. Hybridization was performed in hybridization tubes at 65 °C, overnight. Following hybridization, the blots were processed following manufacturer's instructions, and included DIG immunological washes and detection (DIG wash and block buffer set, Roche Diagnostics, Indianapolis, Indiana, USA).

2.13. Statistical analysis

Diet bioassay percent mortality and GI comparisons of the treatment means were performed on logit-transformed data in JMP Pro 12.2.0 using Tukey-Kramer HSD test. For the two-day and five-day diet bioassays, the statistical comparisons were performed in Excel using Student's *t*-test.

3. Results

wupA in *D. v. virgifera* was identified via BLAST (tblastn) homology searches using *Drosophila* wupA/TnI protein isoforms A-M, GenBank IDs NP_523398, NP_728137, NP_728138, NP_728139, NP_728140, NP_728141, NP_728142, NP_001245734, NP_001245732, and NP_001245733 as the queries. The *wupA* ortholog was identified from a *D. v. virgifera* transcriptome that was assembled from multiple life-stages including eggs, larvae, pupae and adults. TBLASTN sequence searches to *D. v. virgifera* transcriptome databases at Dow AgroSciences produced high homology hits. Top matching *D. v. virgifera* contigs appeared to be multiple transcript isoforms derived from a single locus. The identified *D. v. virgifera wupA* gene locus encodes nine potential protein splice isoforms, designated Dvv_wupA-PA – Dvv_wupA-PI (Supplementary Sequences 1–9). These proteins share 63.6%–85% identity with known isoforms of *Drosophila* TnI (see alignment in Supplementary Fig. 1 and Supplementary Table 3). Apart from an amino terminal extension present in two *Drosophila* isoforms, the *Drosophila* and *Diabrotica* TnI have similar protein lengths. Both species also contain an alternative exon in the middle of TnI (amino acids 151–183 in Supplementary Fig. 1). The highest identity of *D. v. virgifera* TnI/wupA in the nr database is 98% between Dvv_wupA-PF and *Tribolium castaneum* Troponin I isoforms X3 and X11. Open reading frames of the putative *D. v. virgifera wupA* transcripts are Supplementary Sequences 10–18. Two additional TnI homologs were also identified from the *D. v. virgifera transcriptome* (wupA homolog 1 and a partial wupA homolog 2, isoforms A and B; Supplementary Sequences 19–24). Unfortunately, we were not able to PCR-amplify sequences corresponding to either of these homologs. WupA homolog 1 and 2A/B have the highest identity of 93% and 84%/82%, respectively, to cabbage white, *Pieris rapae,* Troponin I. Without experimental support for their expression, we speculate that these sequences may represent low-expressed transcripts or contamination with extraneous genetic material.

To test whether dsRNA targeting *wupA* may control *D. v. virgifera* larvae, we selected a dsRNA target within the open reading frame. *WupA* dsRNA region (Supplementary Sequence 25) was successfully amplified by PCR from a cDNA pool that contained multiple developmental stages (see **Methods**). In the initial experiments, *in vitro* transcribed dsRNA was applied to artificial insect diet. Diet overlay bioassays at 500 ng/cm^2 showed *D. v. virgifera* larval mortality of 94.6% and growth inhibition (GI) of 0.99 at the end of a nine-day treatment (Table 1).

A dilution series of dsRNA tested in diet-overlay bioassays approximated (concentration that leads to 50% lethality) LC_{50} of 3.6 ng/cm^2 (LC_{50} limits 2.6–4.8 ng/cm^2) and a (50% growth inhibition) GI_{50} of 0.49 ng/cm^2 (GI_{50} limits 0.39–0.61 ng/cm^2) for *wupA* dsRNASince the initial bioassays were scored after nine days of continuous dsRNA exposure, we inquired whether the mortality and growth inhibition phenotypes were present earlier. To test this, we conducted two-day and

Table 1
dsRNA feeding bioassay results of *wupA* target in *D. v. virgifera* larvae. Percent mortality and growth inhibition (GI; seem Methods for calculation) at nine days after exposure to diet treated with dsRNA. N: number of replicates (8 wells; 16 insects per replicate). SEM: standard error of the mean. After the data was logit transformed, ANOVA was performed in JMP Pro 12.2.0, followed by means separation using Tukey-Kramer HSD test at $p < 0.05$: treatments with the same letter designation are not significantly different. The non-transformed values are reported below.

treatment	dose (ng/cm^2)	N	Avg. % mortality ± SEM	Avg. GI ± SEM
Dvv-wupA dsRNA	500	13	94.6 ± 1.2 **A**	0.99 ± 0.003 **A**
TE buffer	0	28	8.5 ± 1.6 **B**	0.04 ± 0.032 **B C**
YFP dsRNA	500	24	8.0 ± 1.5 **B**	0.10 ± 0.040 **B**
WATER	0	24	6.9 ± 1.6 **B**	−0.14 ± 0.036 **C**
ANOVA			$F_{3,85} = 41.4$ $p < 0.0001$	$F_{3,85} = 84.3$ $p < 0.0001$

five-day bioassays. The *wupA* dsRNA activity was compared to dsRNA that targeted RNA polymerase II subunit *RpII140,* which shows high levels of mortality in *D. v. virgifera* (Knorr et al., 2018). Whereas in the nine-day bioassays both the growth inhibition and the mortality induced by *wupA* and *RpII140 v1* dsRNAs were similar, *wupA* caused average growth inhibition of 0.48 and 0.77 at 2 days and 5 days, respectively (Fig. 1). These growth inhibition values were statistically different from the *YFP* dsRNA and 0.1X TE (buffer) negative controls. Unlike *wupA*, *RpII140* dsRNA did not produce significant growth inhibition within two days (Fig. 1).

To characterize *wupA* transcript knockdown in *D. v. virgifera*, we performed qPCR and RNAscope *in situ* hybridization (ISH). qPCR analysis revealed transcript knockdown of 77% and 89% after 24 h and 48 h of dsRNA application, respectively, at 500 ng/cm^2 (Fig. 2). The native expression pattern of the *wupA* gene was determined via ISH using longitudinal sections (longisections) of entire *D. v. virgifera* larvae. *WupA* was found to be most abundant in muscle cells; specifically, those surrounding the alimentary system and the body muscle fibers (Fig. 3A and B). After 48 h on diet overlaid with *wupA*-specific dsRNA, *D. v. virgifera* larvae showed obvious knockdown of *wupA* transcript both from the circular and longitudinal muscles surrounding the midgut (Fig. 3C) and the body muscle fibers (Fig. 3D). Furthermore, this knockdown of *wupA* transcript was observed in body muscle fibers throughout the body of the larvae, including those near the posterior

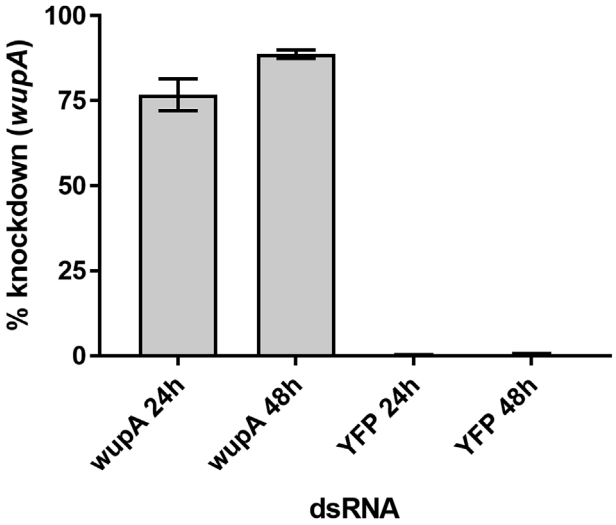

Fig. 2. Diet-applied *wupA* dsRNA leads to knockdown of the *D. v. virgifera wupA* gene transcript. Insects were collected at 24 h and 48 h after dsRNA application (500 ng/cm^2 dose) on artificial diet. Percent knockdown was calculated as described in the Methods section. The graph generated in GraphPad Prism 7.03 shows mean measurements with standard error of the mean (SEM).

end of the larva, which are very distant from the anterior midgut where the dsRNAs first entered the insect. And finally, the presence of a very small amount of wupA transcript was observed around Malpighian tubules in untreated larvae (Supplementary Fig. 4), and this transcript was lost following ingestion of *wupA*-specific dsRNA (Fig. 3C).

The histopathological impact of the loss of *wupA* transcript on the tissues and organs of *D. v. virgifera* larvae was investigated by light microscopy of resin sections. Since larval mortality was prevalent after approximately five days on *wupA* dsRNA-containing diet, the insects were collected for embedding at four days (96 h) on diet. At 96 h, significant histopathological phenotypes were observed. The body muscle fibers in *D. v. virgifera* larvae showed typical striated muscle banding patterns. In the negative control (*YFP* dsRNA-fed) larvae, these striated muscle patterns were clearly visible (Fig. 4A). *WupA* dsRNA-treated larvae at the same time point showed a clear loss of banding pattern and overall muscle fiber integrity (Fig. 4B). However, knockdown of *wupA* transcript had no apparent negative effects on the morphology of midgut enterocytes (Fig. 4C and D). The morphology of the hindgut in untreated *D. v. virgifera* larvae has a distinctive six-pointed shape in cross section (Fig. 4E). A buildup of material in the hindgut lumen and

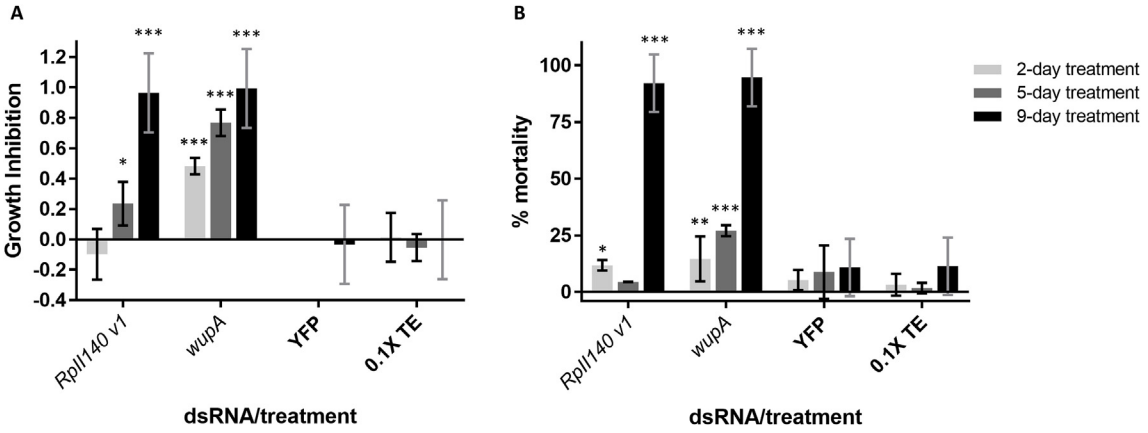

Fig. 1. *wupA* dsRNA confers significant growth inhibition in short-term and long-term larval bioassays. Two-, five-, and nine-day *D. v. virgifera* diet overlay bioassays at 500 ng/cm^2 dsRNA application. Mortality and Growth Inhibition were calculated as described in the **Methods** section. The graph generated in GraphPad Prism 7.03 shows mean measurements with standard error of the mean (SEM). Statistical comparisons to the *YFP* dsRNA treatment at corresponding time points were performed using Student's *t*-test; * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$.

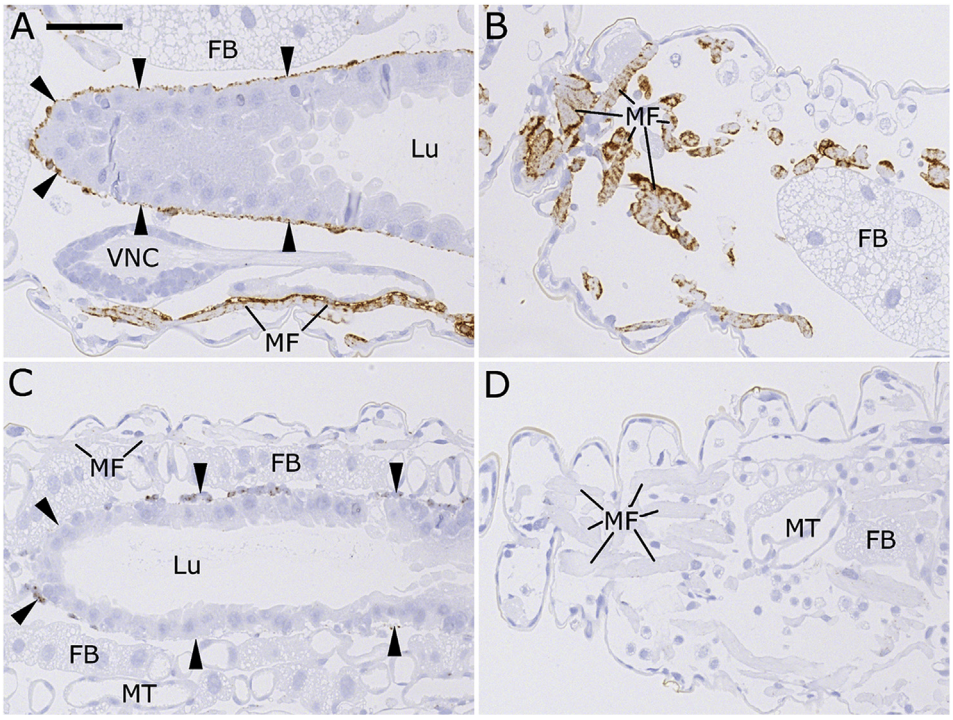

Fig. 3. RNA *in situ* hybridization on long-isections of *D. v. virgifera* reveals the native expression pattern of *wupA* and RNAi-mediated knockdown by ingestion of *wupA*-specific dsRNA. *WupA* transcripts were detected with DAB chromogen (brown), and the sections were counterstained with hematoxylin (blue). Scale bar = 50 μm; all panels are at the same magnification. **A.** Native expression pattern of *wupA* mRNA in the anterior midgut region of a first instar *D. v. virgifera* larva. *WupA* transcripts appear predominantly in body muscle fibers (MF) and in circular and longitudinal muscles surrounding the midgut (arrowheads). **B.** Native expression pattern of *wupA* mRNA in the posterior region of a larva, showing high levels of *wupA* mRNA in the body muscle fibers. The transcripts are concentrated in the Z-bands of the fibers, which appear as distinct banding patterns along the fibers. **C.** Anterior midgut region of a larva after 48 h on diet overlaid with *wupA*-specific dsRNA showing significant loss of *wupA* transcripts in the midgut circular muscles (arrowheads) and body muscle fibers (MF). **D.** Posterior region of a *D. v. virgifera* larva after 48 h on diet overlaid with *wupA*-specific dsRNA showing dramatic loss of *wupA* transcripts in the body muscle fibers. VNC = ventral nerve cord ganglion; FB = fat body; Lu = midgut lumen; MT = Malpighian tubule; MF = body muscle fiber. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

distortion of the hindgut was observed in *wupA* dsRNA-fed larvae (Fig. 4F).

For expression in *Zea mays* B104, single hairpin RNA transcript that targets 254 nt of *D. v. virgifera wupA* was cloned downstream from a maize *ubiquitin1* constitutive promoter. Utilizing an *Agrobacterium* binary vector, maize was transformed with target DNA (T-DNA) that contained *wupA* target sequence (Supplementary Sequence 25) in a hairpin confirmation. Fourteen T_0 maize plants, with a one to three estimated transgene insertions (Table 2 and Supplementary Table 4), were subjected to a two-week greenhouse root protection bioassay (Fig. 5A). Of the fourteen plants, six showed significant levels of root protection (≤0.5 on a node-injury scale [NIS] of 1.0); these passing plants were saved to generate seed. Of T_0 maize plants that passed the greenhouse bioassay, four had root ratings of ≤0.1 NIS (Table 2), compared to typical values of 0.75–1.0 for B104 in this assay, suggesting that *wupA* dsRNA can confer high levels of root protection. In T_1 generation, the greenhouse bioassays included four of the transformation events tested in T_0 generation (126165[1]-014, 126165[1]-017, 126165[1]-023, 126165[1]-026) plus three previously-untested events. All of the tested events showed high levels of root protection in a three-week greenhouse bioassay (Fig. 5B), as benchmarked against YFP-transformed maize (126944[2]-006), line B104 that was used for transformation, and line 7sh382 (Johnson, 2000). In this longer bioassay, the roots were measured on NIS of 3.0, with a maximum number of three full nodes of roots removed. All *wupA* hpRNA-expressing lines had median root rating of ≤0.5 NIS (Fig. 5B). Median root ratings of the negative controls YFP-transformed maize, B104 line that was used for transformation, and 7sh382 were over 2.5 NIS, indicating that the bioassay provided high level of insect pressure (for visual comparison see Fig. 5C–E). Moreover, median root scores for 126165[1]-014 and 126165[1]-026 were similar to those of Cry34Ab1/35Ab1-expressing maize lines B104rw (hemizygous for Cry34Ab1/35Ab1) and 7sh382rw (homozygous for Cry34Ab1/35Ab1).

Data from previous studies indicate that long double-stranded RNA rather than siRNA mediate oral/environmental RNAi in *D. v. virgifera* (Bolognesi et al., 2012; Li et al., 2015). To determine if maize expressing *D. v. virgifera wupA* sequences accumulate intact RNA hairpins, we performed northern blot analysis. Northern blots of material that had been treated with RNase I_f to remove single-stranded RNA (ssRNA) show that the predominant dsRNA species is of the expected size (Fig. 6). Transcript regions that are presumed to be removed by RNase I_f include the hairpin loop and UTR regions. A northern blot experiment performed without ssRNA depletion showed two dominant bands that are larger than those in RNase I_f-treated samples (Fig. 6). The presence of two bands on a northern blot, under strong denaturing conditions (*i. e.,* 6.5% formaldehyde) may represent mRNA variants (possibly alternative UTRs). Both mRNA variants (*i. e.,* hpRNAs) appear to contain the intact dsRNA, since the corresponding samples that were subjected to RNase I_f show a single band of the expected size (Fig. 6).

4. Discussion

While the most efficacious RNAi targets for insect control are also expected to be lethal as mutant alleles, the essential nature of the gene does not guarantee mortality via RNAi application. Whereas the lethal outcome of essential mutations is a cumulative effect over the entire life-span of an insect, the best RNAi targets are expected to be lethal within days of RNAi treatment. In this study, we examined an RNAi target that is anticipated to be haplolethal (insufficient for viability in haploid state) in insects. We hypothesized that this additional criterion may provide greater dose sensitivity to the RNAi effect. Indeed, the selected *wupA* gene target was shown to be lethal in a diet-based *D. v. virgifera* larval assay (Table 1). Moreover, *wupA* dsRNA produced growth inhibition (GI) of 0.48 (or 52%) within two days of treatment (Fig. 1A). These results indicate that *wupA* RNAi response may not only be lethal, but also fast-acting.

The product of the *wupA* gene is the Troponin I protein, which plays an important role in muscle function (Barbas et al., 1993). In contrast to mammals, where three distinct genomic loci encode Troponin I, in *Drosophila* wupA is encoded by a single gene locus (Barbas et al., 1991). Likewise, we identified a single gene locus in *D. v. virgifera* that encodes up to nine protein isoforms of TnI/wupA. The singularity of *wupA*

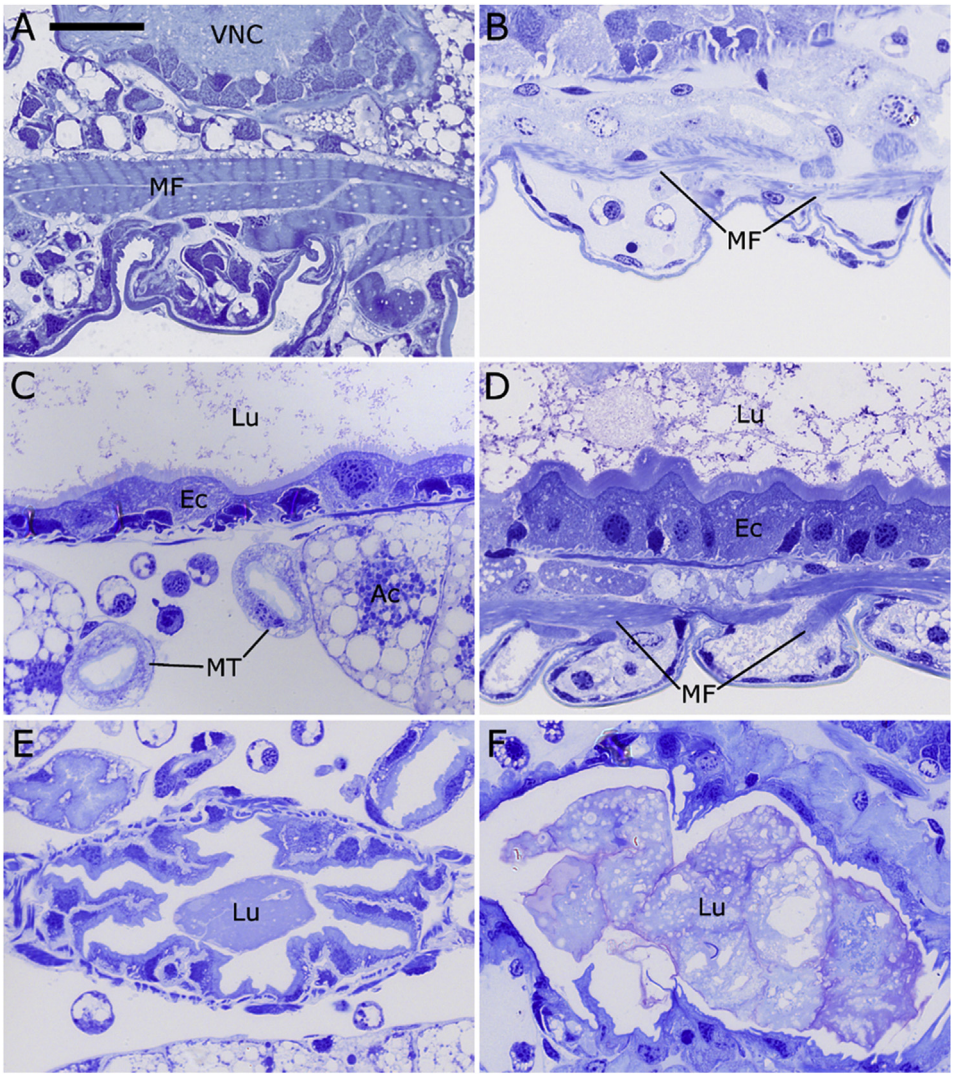

Fig. 4. Morphological defects in the anterior midgut of *D. v. virgifera* larvae that result from feeding on *wupA* dsRNA. Larvae were treated for four days with *wupA* dsRNA. Scale bar = 25 μm; all panels are at the same magnification **A.** Overview montage at low magnification of an entire larval longisection. **B.** Anterior midgut region showing relatively intact enterocytes in the AMG; dense/normal microvilli; circular muscles appear loose; food material and peritrophic membrane visible in lumen. **C.** Median region of larva showing MMG enterocytes intact; fat body is dense/low in lipid content; Malpighian tubules are swollen, with some pink/acidic material present in some lumens; body muscles appear swollen in some regions, with disordered Z-bands. **D.** Posterior region of larva showing swelling in hindgut; hindgut lumen is filled with material. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Root ratings of the six *wupA* RNAi-expressing events that had a NIS of ≤0.5. The insertion copy number was approximated based on the AAD1 selectable marker via quantitative RT-PCR.

Event	T_0 Root Rating	Copy#
126165[1]-014	0.1	2.2
126165[1]-017	0.1	1.7
126165[1]-020	0.5	1.9
126165[1]-023	0.25	1.9
126165[1]-026	0.1	2.2
126165[1]-029	0.05	1.0

within *D. v. virgifera* likely allows for the severe and lethal phenotypes associated with RNAi response to this gene target. Using RNAscope ISH, we have demonstrated that the native expression of the *wupA* gene is primarily in muscle fibers of both the body and alimentary system in *D. v. virgifera* larvae. This expression pattern is consistent with the reported function of tropomyosin in the striated muscle in other insects and animals (*e.g.* (Farah and Reinach, 1995),).

Ingestion of *wupA*-specific dsRNA resulted in a clear knockdown of this transcript that is consistent with qPCR data (Fig. 3C and D). The systemic nature of this knockdown throughout the body of WCR larvae is also consistent with previous reports (Li et al., 2018). After four days of treatment, the knockdown of *wupA* causes a range of phenotypes observable by resin histopathology. Knockdown of this protein results in the loss of the highly-ordered striated structure of the body muscle fibers (Fig. 3B and Supplementary Figs. 2 and 3). This is consistent with reports showing that mutants of *wupA* with lower TnI levels show disorganized Z-disks in the muscle fibers in *Drosophila* (Marin et al., 2004). This loss of body muscle fiber structure presumably negatively impacts their function, and thus reduces the voluntary movement capabilities of the larvae. Obviously, if the insects are not able to move normally, their ability to forage, evade predation, molt, *etc.*, will be hindered. And finally, the small amount of *wupA* transcript that was observed around Malpighian tubules may indicate that they are surrounded by thin muscle fibers that are critical for their proper function (*e.g.,* Coast, 1998; Crowder and Shankland, 1972; Supplementary Fig. 4). If these muscle fibers associated with Malpighian tubules are important for their function, knockdown of *wupA* may also negatively impact the function of the Malpighian tubule system.

RNA *in situ* hybridization revealed that all of the major regions of the alimentary system (foregut, midgut, and hindgut) are completely surrounded with a layer of small bands of muscle (Fig. 3A). These muscles are responsible for generating peristalsis, which is required to move food material through the alimentary system of the *D. v. virgifera* larva. Though morphological changes such as were observed in the body muscles are not obvious in these small alimentary muscles, the ISH data clearly show that the *wupA* transcript is lost from them; it is therefore likely that their function is also negatively impacted. In fact, the large amount of food material in the lumen of the hindgut, and the

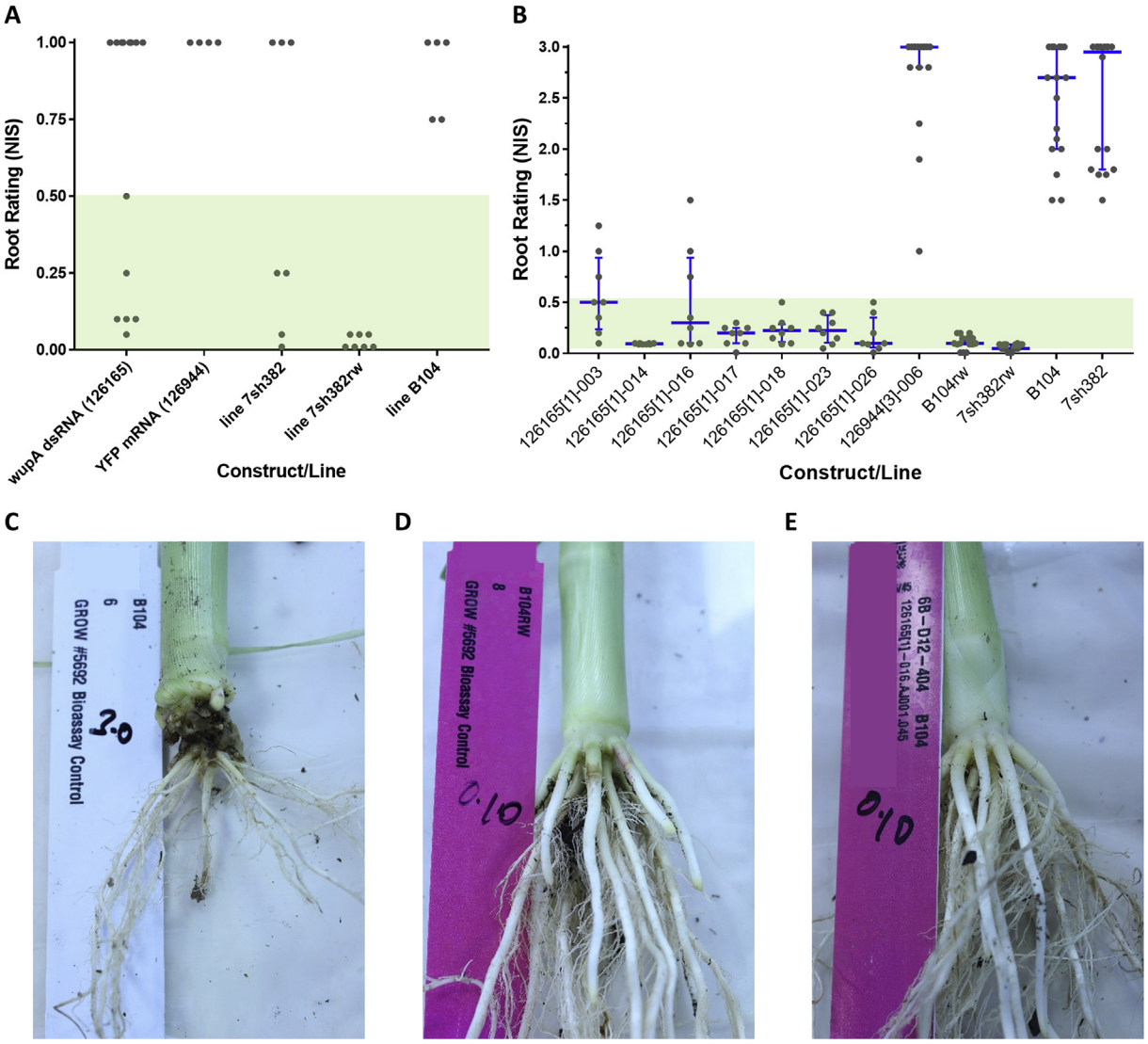

Fig. 5. T_0 and T_1 maize plants expressing wupA RNA hairpins exhibit root protection in greenhouse bioassay. A. T_0 maize root rating in a two-week bioassay, rated on scale from 0 to 1 on a node injury scale (NIS). Root ratings of ≤0.5 are highlighted in green. **B.** T_1 maize expressing *wupA* exhibits root protection in greenhouse bioassay. T_1 maize root rating in a three-week bioassay were rated on scale 0–3 on NIS. Root ratings of ≤0.5 are highlighted in green. NIS plot generated in GraphPad Prism 7.03 shows median and upper and lower quartiles (horizontal blue lines). **C-E.** Photos of representative maize roots at the conclusion of a three-week T_1 bioassay. **C.** B104 (negative control) with root rating of 3.0. **D.** B104rw (hemizygous for Cry34Ab1/35Ab1) with root rating of 0.1. **E.** plant form *wupA* hpRNA event 126165[1]-016 with root rating of 0.1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

swelling and loss of the normal ridges of this region, indicate that these muscles are no longer able to move food through the alimentary system. Although the alimentary muscles are impaired, and the hindgut is showing signs of poor function, the midgut region, specifically the midgut enterocytes, appear normal. Although the alimentary muscles are impaired, and the hindgut is showing signs of poor function, the midgut region appears largely unaffected overall, and the midgut enterocytes, in particular, appear intact. This lack of obvious lysis and death of the midgut enterocytes is in contrast to the damage to these cells caused by Bt insecticidal proteins (Bowling et al., 2017).

WupA hairpin sequence that was transformed into maize, provided root protection in T_0 and T_1 generations (Fig. 5A and B). Although not all T_0 events showed root protection (Fig. 5A), T_1 progeny of those T_0 events that did show root protection were themselves highly insect-resistant (Fig. 5B). Median T_1 root ratings of *wupA* maize lines 126165[1]-014 and 126165[1]-026 were similar to those of maize lines expressing Cry34Ab1/35Ab1 (Fig. 5B), indicating that RNAi may provide root protection that is equivalent to a Bt protein.

An accumulation of intact RNA hairpins in maize leaves indicated that maize can accumulate long dsRNA hairpins without significant processing into siRNA. Our northern blot analysis also indicated that various levels of dsRNA accumulation could translate to root protection (Fig. 6). For example, both 126165[1]-003 plants, which contained relatively low hpRNA levels and 126165[1]-023 plants, which contained relatively high hpRNA levels provided root protection of ≤0.5 NIS (Fig. 5). A comparison of northern blot and T_1 root protection bioassay data also suggests that there may be correlation between accumulation of dsRNA in the leaf and root protection. For instance, 126165[1]-003 plants that showed lowest level of accumulation of the intact hpRNA in the leaf (Fig. 6), also showed highest median root rating of the tested events in greenhouse bioassay [lowest root protection] (Fig. 5). These observations also call for additional work to determine the level of correlation between the accumulation of leaf and root transcripts, and the minimum effective dose for *wupA* hpRNA in maize.

This study demonstrates that *D. v. virgifera wupA*, which was

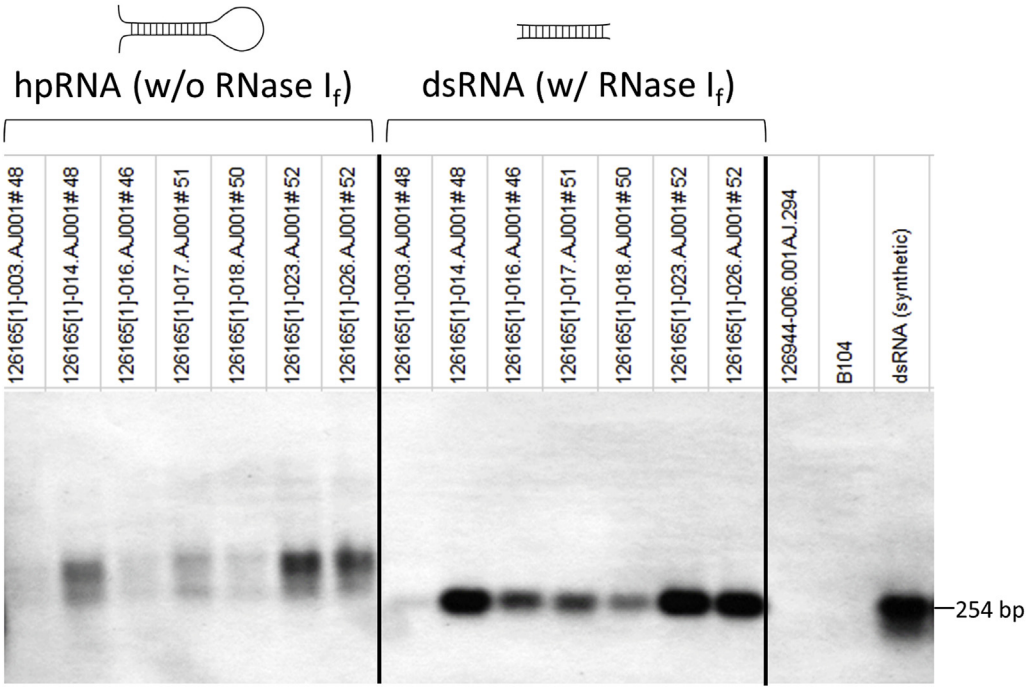

Fig. 6. Northern blot analysis for plant expressed *wupA* hpRNA. The leaf RNA from T_1 maize transgenic events expressing *wupA* hpRNA was isolated and processed for northern blot analysis using either RNase I_f –treated or RNase I_f-non-treated RNA and probed with a 170 bp *wupA* probe complementary to the sense *wupA* transcript. The non-*wupA* transgenic maize and wild-type maize (B104) RNA were used as negative controls. The synthetic *wupA* dsRNA (254 bp) identical to the *wupA* sequence in transgenic maize was used as positive control. The size of 254 bp dsRNA duplex is indicated on the right.

selected on the basis of its *Drosophila* ortholog being a known haplolethal gene, is a lethal RNAi target when tested as a dsRNA on artificial diet bioassay and can confer root protection against *D. v. virgifera* when expressed as a hairpin in maize. Further, because wupA in *D. v. virgifera* is likely to be haplolethal/dose-sensitive, its dsRNA may provide stronger cumulative level of control.

Several insect RNAi targets have been extensively described in the literature. In *D. v. virgifera* these include the subunits of vacuolar ATPase complex *v-ATPase A* (Baum et al., 2007; Vélez et al., 2016) and *v-ATPase C* (Li et al., 2015), *Snf7* (Bolognesi et al., 2012) septate junction proteins snakeskin (ssk) and mesh (Hu et al., 2016), as well as *Rop*, *ncm*, *dre4*, and RNA Polymerase II subunit RpII140 (Knorr et al., 2018). Several of these proteins (*e. g.,* v-ATPase, Snf7, or Rop) participate in cellular membrane traffic that includes endocytosis and exocytosis. Since it is involved in muscle contraction, *wupA* may provide an RNAi function that is complementary to the gene products that are involved in membrane transport. And while we may assume that multiple RNAi act as a single mode of action, there is an opportunity to increase the efficacy of RNAi traits by combining two or more RNA hairpins from different cellular pathways within the same maize plant. These RNAi targets may be expressed as different molecules or be a part of a single fusion molecule.

To summarize, dsRNA targeting *wupA* in WCR causes several biological effects that are potentially advantageous for development of highly efficacious, pyramided WCR control traits. First, as a haplolethal trait the improved speed to kill, compared here to the essential gene RNA polymerase II, subunit *RplII140*, demonstrates that fast acting dsRNA triggers can be identified with the goal to improve overall efficacy in terms of reduced feeding damage and possibly lower levels of adult emergence. Further, the impact of *wupA*-mediated RNAi on muscle fibers resulting in reduced mobility might also provide added benefit by reducing larval migration to new feeding sites. Next, genes for muscle as RNAi targets are different from the targets of many other candidate dsRNA traits aimed at essential cellular mechanisms such as transcription or cellular trafficking (Fishilevich et al., 2016). While dsRNAs are not differentiated at the level of dsRNA uptake (Khajuria et al., 2018) *wupA* dsRNA combined with other dsRNA triggers affords the possibility to achieve additive or potentiated trait efficacy by targeting independent biological functions. Last, *wupA* dsRNA is not anticipated to be antagonistic of Bt proteins; RNAi of *wupA* does not disrupt the insect midgut, leaving Bt protein sites of action intact and available for binding, thereby maintaining the benefit of two different modes of action traits.

Author contributions

PG assembled the *D. v. virgifera* transcriptome. MLFF, PG, and EF identified and cloned *wupA*. MLFF, EF, and SW synthesized dsRNA. MR and WL implemented insect diet-based and maize bioassays. EK and LT designed and performed 2- and 5-day diet-based rootworm bioassays. KDS and EF performed statistical analyses. AJB and HEP carried out ISH and histopathology analysis and image interpretation. SEW designed maize constructs and coordinated maize transformation. JRL oversaw greenhouse activities. SLW performed insect qPCR and maize analytics. GS and PHW carried out northern blots. EF, RLH and MR analyzed the data. EF, KEN, and SN coordinated the research activities. EF drafted the manuscript; all authors contributed to manuscript writing and review. All authors approved the manuscript.

Declaration of interest

AJB, P-HW, WL, SEW, HEP, PG, SLW, JRL, RLH, KDS, and KEN are employees of Corteva Agriscience™, Agriculture Division of DowDuPont™. EF, MLLF, MR, GS, and SN are former employees of Agriculture Division of DowDuPont™.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.09.006.

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