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Control of coleopteran insect pests through RNA interference

James A Baum[1], Thierry Bogaert[2], William Clinton[1], Gregory R Heck[1], Pascale Feldmann[2], Oliver Ilagan[1], Scott Johnson[1], Geert Plaetinck[2], Tichafa Munyikwa[1], Michael Pleau[1], Ty Vaughn[1] & James Roberts[1,3]

Commercial biotechnology solutions for controlling lepidopteran and coleopteran insect pests on crops depend on the expression of *Bacillus thuringiensis* insecticidal proteins[1,2], most of which permeabilize the membranes of gut epithelial cells of susceptible insects[3]. However, insect control strategies involving a different mode of action would be valuable for managing the emergence of insect resistance. Toward this end, we demonstrate that ingestion of double-stranded (ds)RNAs supplied in an artificial diet triggers RNA interference in several coleopteran species, most notably the western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte. This may result in larval stunting and mortality. Transgenic corn plants engineered to express WCR dsRNAs show a significant reduction in WCR feeding damage in a growth chamber assay, suggesting that the RNAi pathway can be exploited to control insect pests via *in planta* expression of a dsRNA.

Eukaryotic organisms, including insects, possess a common machinery for sequence-specific gene silencing that is triggered by the presence of dsRNA[4]. This process is called RNA interference (RNAi) in animals[4] and post-transcriptional gene silencing in plants[5]. dsRNA-mediated silencing of essential genes in insects can induce cessation of feeding and ultimately morbidity, but requires efficient uptake of dsRNA either by feeding or by topical application. Ingestion of dsRNA can silence specific genes in the nematode *Caenorhabditis elegans*[6,7], planarian flatworms[8] and ticks[9]. Micro-injection of dsRNA into adults and late-instar larvae has been used to silence genes and study gene function in the coleopteran *Tribolium castaneum*[10,11]. Similarly, dsRNA induced an RNAi response in the lepidopteran *Spodoptera litura* upon injection, but not after ingestion[12]. In contrast, oral delivery of highly concentrated (4,000 p.p.m.) dsRNA reduced target mRNA levels in the lepidopteran *Epiphyas postvittana*, but did not cause mortality[13].

To assess coleopteran sensitivity to ingested dsRNAs, candidate target genes from several WCR cDNA libraries[14] were selected (**Supplementary Fig. 1** online) and dsRNAs prepared for testing in a WCR feeding assay. We reasoned that genes encoding proteins with essential functions would be the best RNAi targets for causing lethality. In the initial bioassays, dsRNAs were applied to the surface of the WCR agar diet at concentrations from 520 ng/cm^2 to 780 ng/cm^2. As we anticipated a slower response to dsRNAs than to *B. thuringiensis* insecticidal proteins, the WCR bioassay incubation period was extended from 5 d to 12 d. Indeed, 7 d after infestation, little if any effect was observed. However, numerous dsRNAs exhibited significant activity 12 d after infestation, resulting in both larval stunting and mortality (**Supplementary Table 1** online).

Subsequent feeding assays demonstrated that certain dsRNA samples, including dsRNAs targeting putative genes encoding vacuolar ATPase (V-ATPase) subunit A, D and E, as well as α-tubulin, were active at applied concentrations well below 52 ng/cm^2. We identified additional WCR genes that caused mortality when targeted for suppression using dsRNAs in the WCR feeding assay. A two-tiered screen was implemented in which dsRNAs targeting different genes were tested at 52 and 5.2 ng/cm^2. Of the 290 dsRNAs tested, 125 showed significant ($P < 0.05$) larval mortality and/or stunting at 52 ng/cm^2. Of these, 67 showed significant mortality and/or stunting at 5.2 ng/cm^2. To quantify this activity more precisely, we tested serial twofold dilutions of the most active dsRNAs in the WCR feeding assay to derive LC_{50} values, defined here as the concentration causing 50% mortality after 12 d. Fourteen dsRNAs were identified that yielded LC_{50} values $\leq$ 5.2 ng/cm^2 (**Table 1**), with the most active dsRNAs registering LC_{50} values near 0.52 ng/cm^2. Concentration-response curves for several dsRNAs are shown in **Supplementary Figure 2** online.

To confirm that ingestion of dsRNA triggered a specific RNAi response in WCR larvae, we analyzed total RNA from dsRNA-fed and untreated larvae on northern blots to demonstrate downregulation of specific target genes. WCR larvae that ingested dsRNA directed against the V-ATPase A exhibited a dramatic suppression of the endogenous V-ATPase A mRNA within 24 h of ingestion, with reduced mRNA levels apparent as early as 12 h (**Fig. 1f**, 0.5 d). In contrast, levels of α-tubulin mRNA appeared unaltered in larvae that had ingested the V-ATPase A dsRNA (**Fig. 1e**). Likewise, WCR larvae that ingested the α-tubulin dsRNA exhibited a gradual suppression of the endogenous α-tubulin mRNA over 6 d (**Fig. 1c**). The time course of V-ATPase A mRNA accumulation in these larvae resembled that of larvae fed a normal diet alone (**Fig. 1d**), indicating specific suppression of the

[1]Monsanto Company, 700 Chesterfield Parkway West, Chesterfield, Missouri 63017-1732, USA. [2]Devgen N.V., Technologiepark 30, B-9052 Ghent–Zwijnaarde, Belgium. [3]Correspondence should be addressed to J.R. (james.k.roberts@monsanto.com).

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Table 1 LC_{50} values determined from the WCR feeding assay

Plasmid	Nearest ortholog[a]	Annotation	LC_{50}[b]
pMON101053	F38E11_5	Putative COPI coatomer, β′ subunit[c]	0.57
pMON102873	CG6223	Putative COPI coatomer, β subunit[c]	0.73
pMON78428	CG8055	Putative ESCRT III_Snf7 ortholog[c]	1.20
pMON78416	CG11276	Putative ribosomal protein S4 ortholog[c]	1.30
pIC16005	CG2934-PA	Putative v-ATPase D subunit 1 ortholog[d]	1.72
pIC17504	CG3762	Putative v-ATPase A subunit 2 ortholog[d]	1.82
pMON78412	CG3416	Putative mov34 ortholog (proteosome)[c]	1.98
pMON98445	F37C12_9	Putative ribosomal protein rps-14 ortholog[c]	2.60
pMON101120	M03F4.2	Putative actin ortholog[c]	2.86
pMON96168	CG2331	Putative apple ATPase ortholog[c]	4.16
pMON102861	CG12770	Putative ESCRT I_Vps28 ortholog[c]	4.47
pMON78424	CG6141	Putative ribosomal protein L9 ortholog[c]	5.20
pMON78425	CG2746	Putative ribosomal protein L19 ortholog[c]	5.20
pIC17503	CG1913-PA	Putative alpha tubulin ortholog[d]	5.20
pMON78440	CG3180	Putative RNA polymerase II ortholog[c]	7.80
pMON102865	CG14542	Putative ESCRT III_vps2 ortholog[c]	11.96
pMON30694	CG9277	Putative beta tubulin ortholog[d]	51.98

[a]CG orthologs are from *D. melanogaster*, all others are from *C. elegans*. [b]LC_{50} values in ng dsRNA/cm^2. [c]Identified as part of the Monsanto Devgen collaborative research agreement. [d]Sequence identified and tested by Monsanto.

α-tubulin mRNA. Northern blot analyses for other gene targets confirmed the specificity of suppression of the targeted mRNAs by dsRNAs, including targets that do not cause mortality when silenced (**Supplementary Fig. 3** online).

Although suppression of targeted mRNAs could be observed by northern blot analysis within 1 d of exposure to dsRNAs included in an artificial diet, gross effects on larval growth and development were not immediately apparent. At applied concentrations of 52 ng/cm^2, even the best WCR dsRNAs had little visible effect on rootworm larvae after 7 d of exposure. Measurements of larval mass over time indicated significant mass reduction compared to the untreated control after only 3 d of feeding (data not shown). The small amounts of dsRNA required for gene silencing and larval mortality suggest an amplification pathway in which ingested dsRNAs are processed to siRNAs, presumably within insect gut epithelial cells, which may prime the synthesis of more abundant secondary siRNAs, as has been proposed for *C. elegans*[6]. Northern blot analysis of total RNA from whole WCR larvae revealed almost complete suppression of targeted transcripts from several housekeeping genes (**Fig. 1**), suggesting systemic spread of silencing beyond gut epithelial cells, the presumed initiation site of the RNAi response. The requirement for amplification and systemic spread could account in part for the time-lag between the gene silencing event and larval death. Although a search of the WCR-expressed sequence tag data set failed to identify an RNA-dependent RNA polymerase that would presumably be required for the amplification step as it is presently understood, this may be due to the incomplete nature of the data set. Orthologs of the *C. elegans sid-1* gene, responsible for systemic spread of dsRNAs in *C. elegans*[15], have been identified in insects such as *T. castaneum*, *Bombyx mori* and *Apis mellifera*[16]. A partial *sid-1*-like sequence was identified in the WCR data set but has not been demonstrated to function in RNAi.

Three other coleopteran species were tested for their sensitivity to dsRNAs in diet. WCR dsRNAs targeting putative β-tubulin, V-ATPase A and V-ATPase E orthologs (**Supplementary Table 1**) were observed to cause significant ($P < 0.05$) larval mortality when tested in a bioassay with the southern corn rootworm, *Diabrotica undecimpunctata howardii* (**Supplementary Fig. 4** online). Likewise, dsRNAs targeting the V-ATPase A and V-ATPase E orthologs each caused significant larval mortality in the Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) bioassay (**Supplementary Fig. 5** online). To extend these observations, we cloned the orthologous V-ATPase A and V-ATPase E sequences of CPB and used them as templates for dsRNA synthesis. The V-ATPase A target sequences from CPB and WCR share 83% nucleotide-sequence identity (**Supplementary Fig. 6** online) whereas the V-ATPase E target sequences from these organisms share 79% nucleotide-sequence identity (**Supplementary Fig. 7** online). As expected, the CPB dsRNAs appeared more active than the orthologous WCR dsRNAs in the CPB assay (**Supplementary Fig. 5**). More specifically, the CPB V-ATPase A dsRNA yielded an LC_{50} value of 5.2 ng/cm^2 in the CPB bioassay, whereas the orthologous WCR dsRNA yielded a LC_{50} value >52 ng/cm^2 a greater than tenfold difference in activity that can be attributed to divergence in target sequence. An appealing aspect of gene silencing for pest control is its potential for selectivity based entirely on nucleotide-sequence identity. However, other barriers to oral toxicity certainly exist. For instance, larvae of the cotton boll weevil, *Anthonomus grandis* boheman, showed no effects on mortality or mass upon ingestion of dsRNAs prepared using the orthologous boll weevil target gene sequences compared to control. This lack of response may result from the particular feeding physiology of the boll weevil, behavioral factors such as burrowing into the diet, or might indicate that some, but not all, coleopteran larvae are sensitive to orally delivered dsRNAs.

dsRNAs were prepared from contiguous sections of the full-length 1,842 base pair (bp) V-ATPase A coding region (**Supplementary Fig. 8** online) and tested for differences in activity in the WCR bioassay. Six dsRNAs of ~300 base pairs (bp) showed activity comparable to the full-length dsRNA, with no individual section yielding a superior target sequence. Additional sectioning experiments demonstrated that dsRNAs as small as 134 bp retained activity in the feeding assay. To test whether corn plants expressing a WCR-derived dsRNA were protected from rootworm feeding damage, we assembled a section of the putative V-ATPase A coding region from WCR into a corn transformation expression cassette designed to express dsRNA targeting a small section (246 bp) of the WCR gene (**Supplementary Fig. 9** online). The expression cassette (**Fig. 2a**) consisted of the CaMV e35S promoter[17], a maize heat shock protein (hsp)70 intron[18], the region of the V-ATPase A gene designed to assemble into a dsRNA upon expression, and the wheat hsp17 3′ untranslated region[18]. This expression cassette was combined with a plant selectable marker gene that confers tolerance to glyphosate and placed into an *Agrobacterium tumefaciens* plant transformation vector to give the

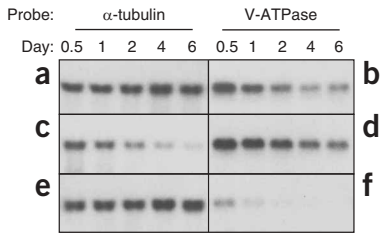

Figure 1 Suppression of target mRNA in WCR. (**a**–**f**) Neonates were allowed to feed for 0.5–6 d on an artificial diet (**a**,**b**) or the same diet containing 52 ng/cm^2 dsRNA derived from α-tubulin (**c**,**d**) or V-ATPase A (**e**,**f**) sequences, respectively. mRNA abundance in larvae was determined by northern blot analysis using α-tubulin or V-ATPase A probes.

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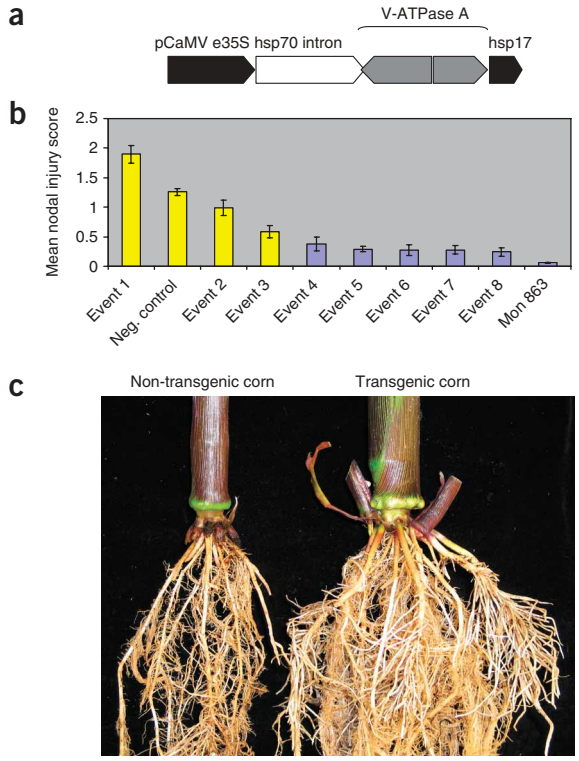

Figure 2 F1 plants expressing a V-ATPase A dsRNA are protected from WCR feeding damage. (**a**) Map of the expression cassette. (**b**) Mean root damage ratings for eight F_1 populations, the parental inbred line (negative control) and the corn rootworm–protected Cry3Bb event MON863; NIS, nodal injury score (Iowa State ranking system). (**c**) The plant on left is a non-transgenic control with average root damage, whereas the plant on the right shows the average root protection seen when the transgene is expressed.

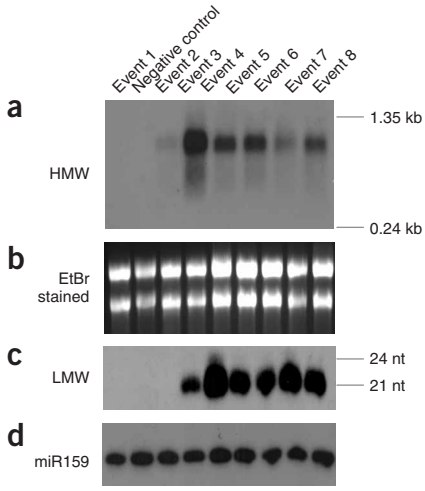

Figure 3 Northern blot analysis of large and small RNAs produced by F_1 plants expressing the V-ATPase A dsRNA transcript. (**a**–**d**) Individual plants from different transgenic events were infested with WCR and RNA was extracted from roots after scoring for damage. The full-length transcript from the WCR V-ATPase cassette accumulates in events 3–8 (**a**). Ethidium bromide–stained ribosomal RNA provided a loading control (**b**). Those events where the full-size transcript is present also contained small RNAs of about 21 base pairs (**c**). The small RNA blot was stripped and reprobed for maize miR159 to confirm equal loading (**d**). There was no large or small RNA from the WCR V-ATPase cassette in events 1 and 2, which had the most root damage.

plasmid pMON94805. pMON94805 maize transformants were transferred to soil[19] and backcrossed with an inbred line to generate F_1 hybrid progeny. F_1 hybrid plants from each cross containing one or two copies of the expression cassette, as checked by a quantitative PCR assay (data not shown), were evaluated using a growth chamber assay to assess rootworm feeding damage. Pots containing plants at the four-leaf stage were infested with ~1,000 WCR eggs in the root zone. Emerging larvae were allowed to feed on the roots for 3 weeks, after which the plants were removed from the soil and rated for larval feeding damage[20]. Similar hybrid plants expressing the Cry3Bb protein of *B. thuringiensis*[2] showed excellent root protection in the pot assay, providing a useful benchmark for efficacy. Progeny from several pMON94805 F_1 crosses showed obvious reductions in root damage when compared to the control hybrid line as evidenced by the low nodal injury scores (**Fig. 2b**) and healthy root masses (**Fig. 2c**). Progeny from one of the eight F_1 crosses scored at or lower than the accepted economic threshold nodal injury score of 0.25 (ref. 20). To further characterize the different transgenic F_1 hybrids used in this WCR feeding assay, we extracted RNA from individual plants for northern blot analysis. Transgenic events containing detectable RNA corresponding to the dsRNA construct expressed both the full-size transcript and small 21-bp siRNAs processed from the larger dsRNA (**Fig. 3a**,**c**). RNA corresponding to the dsRNA construct was not detected in plants with the highest nodal injury scores (event numbers 1 and 2). Similar results have been obtained with transgenic corn plants targeting other WCR genes, including feeding assays with isolated transgenic root tissue demonstrating severe larval stunting (**Supplementary Fig. 10** online).

The ability to trigger RNAi in coleopterans by oral delivery of dsRNAs has obvious commercial implications. RNAi provides a unique mode of action for the control of insect pests that could complement the current strategy of expressing *B. thuringiensis* (*Bt*) insecticidal proteins in crops such as corn, cotton and soybeans. As larvae of the WCR and related *Diabrotica* species are devastating pests of corn in the United States[21] and are refractory toward most *Bt* insecticidal proteins, it is especially noteworthy that WCR is sensitive to an oral RNAi approach. Accordingly, RNAi has the potential to increase both the efficacy and durability of insect-protected crops designed to control this important coleopteran pest.

METHODS

DNA and RNA manipulations. WCR cDNA libraries were prepared from whole larvae harvested at either the 1st instar stadium or the 2nd–3rd instar stadium. RNA was extracted using TRIzol reagent (Invitrogen) and Poly A+ RNA was isolated from the total RNA prep using Dynabeads Oligo dT (Dynal) following the manufacturers' instructions. A cDNA library was constructed from the Poly A+ RNA using the SuperScript Plasmid System (Invitrogen). cDNA was size-fractionated using chromatography. The fourth and fifth fractions were collected and ligated into the pSPORT1 vector (Life Technologies) between the *Sal*1 and *Not*1 restriction endonucleases recognition sites, and used to transform *E. coli* DH10B electro-competent cells by electroporation. Likewise, high molecular weight cDNA libraries from 1st instars and pooled 2nd-3rd instars were made from 20 micrograms of Poly A+ RNA using the SuperScript Plasmid System (Invitrogen). The cDNA was size fractionated on a 1% agarose gel in TAE, and cDNA between the range of 1 Kb to 10 Kb was collected and ligated into the pSPORT1 vector in between the *Sal*1 and *Not*1 restriction sites and transformed into *E. coli* DH10B electro-competent cells by electroporation. Sequencing was performed using Applied Biosystems capillary sequencers. Insect gene sequences were amplified from either genomic DNA or

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from cDNA using Taq DNA polymerase and the amplicons cloned into pCR2.1-TOPO or pCR4-TOPO (Invitrogen) for sequence confirmation. Amplified DNA framents flanked by T7 polymerase promoter sequences served as the template for dsRNA synthesis using the MEGAscript kit and recommended procedures (Ambion). Alternatively, dsRNAs were prepared using the T7 RiboMAX Express RNAi system and protocols (Promega). Purified dsRNAs were quantified by spectroscopy and examined by agarose gel electrophoresis to ensure their integrity. Target gene sequences from CPB and the cotton boll weevil were amplified from larval cDNA using *Taq* polymerase and degenerate primers designed from the alignment of the WCR target sequences with putative orthologs from several insect species. The putative V-ATPase A subunit 2 ortholog sequences were amplified using primers pr550 (5′-GGTGA CATGGCCACCATCCAGGT-3′) and pr552 (5′-ACCCCAGAACACCTGYACR ATACC-3′). The putative V-ATPase E ortholog sequences were amplified using primers pr591 (5′-ATCATGGART ACTAYGARAARAARGAG-3′) and pr592 (5′-GTTGCGWCCGAASAGMGCVT TWCGGATCTSSGG-3′). Amplified DNA fragments were cloned into pCR2.1-TOPO and confirmed by DNA sequencing. Primers pr568 (5′–TTAATACGACTCACTATAGGGAGACCAGT GTGCTGGAATTCGCC-3′) and pr569 (5′-TTAATACGACTCACTATAGGGAG AGGATATCTGCAGAATTCGCC-3′) were used as universal primers to amplify inserts cloned into pCR2.1, resulting in DNA templates containing flanking T7 RNA polymerase promoters suitable for *in vitro* synthesis of dsRNAs.

Insect bioassays. Varying doses of dsRNA were applied as an overlay to corn rootworm artificial diet according to the following procedure. *Diabrotica virgifera virgifera* (WCR) eggs were obtained from Crop Characteristics. The non-diapausing WCR eggs were incubated in soil for about 13 d at 25 °C, 60% relative humidity, in complete darkness. On day 13 the soil containing WCR eggs was placed between no. 30 and no. 60 mesh sieves and the eggs were washed out of the soil using a high-pressure garden hose. The eggs were surface disinfested, rinsed and dispensed onto sterile coffee filters and hatched overnight at 25 °C, 60% relative humidity, in complete darkness. Insect diet was prepared essentially according as described[22]. Twenty microliter volumes of test samples containing either control reagents or dsRNA in varying quantities were overlayed onto the surface of the insect diet. One WCR neonate larva was deposited to each well with a fine paintbrush. Plates were then sealed with mylar and ventilated using an insect pin. Twelve to 72 insect larvae were tested per dose, depending on the design of the assay. The bioassay plates were incubated at 27 °C, 60% relative humidity in complete darkness for 12–14 d. The number of surviving larvae per dose was recorded at the 12–14 d time point. Data were analyzed using JMP4 statistical software (SAS Institute, 1995) and a full factorial ANOVA was conducted with a Dunnet's test to look for treatment effects compared to the untreated control ($P < 0.05$). A Tukey-Kramer *post hoc* test was performed to compare all pairs of the treatments ($P < 0.05$).

Bioassays with the southern corn rootworm (SCR), *Diabrotica undecimpuctata howardii*, were conducted in a similar manner using insects purchased from Crop Characteristics. SCR eggs were incubated in soil for 6 d at 25 °C, 60% relative humidity, in complete darkness. On day 6, the soil containing SCR eggs was placed between no. 30 and no. 60 mesh sieves and the eggs were washed out of the soil using a high-pressure garden hose. The eggs were surface disinfested, rinsed and suspended in a 0.2% agar solution. SCR diet was prepared as previously desribed[23]. Twenty (20) microliter volumes of test samples containing either control reagents or dsRNA in varying quantities was overlayed onto the surface of the insect diet. Plates were allowed to dry before adding insect eggs suspended in 0.2% agar as described above, and were again allowed to dry to remove the water from the 0.2% agar. Plates were then sealed with mylar and ventilated using an insect pin. Twelve to 72 wells containing insects were tested per dose depending on the design of the assay. The bioassay plates were incubated at 27 °C, 60% relative humidity in complete darkness for 12–14 d. The number of surviving larvae per dose was recorded at the 12- to 14-d time point. Data were analyzed using JMP4 statistical software and a full factorial ANOVA was conducted with a Dunnet's test to look for treatment effects compared to the untreated control ($P < 0.05$). A Tukey-Kramer *post hoc* test was performed to compare all pairs of the treatments ($P < 0.05$).

Bioassays with the CPB were conducted using an artificial diet consisting of 13.2 g/l agar, 140.3 g/l Bio-Serve pre-mix, 5 ml/l KOH (18.3% wt/wt), and 1.25 ml/l formalin (37%). The diet was dispensed in 200-µl aliquots into 96-well plates and dried briefly before sample application. We applied 20 µl of test sample per well, with sterile water serving as the untreated check. Plates were allowed to dry before adding insect larvae. CPB larvae were obtained from the New Jersey Department of Agriculture. One neonate CPB larva was added per well with a fine paintbrush. Plates were sealed with mylar and ventilated using an insect pin. Forty larvae were tested per treatment. The bioassay plates were incubated at 27 °C, 60% relative humidity, in complete darkness for 10–12 d. The plates were scored for larval stunting and mortality. Data were analyzed using JMP4 statistical software.

Plant cloning vectors and transformation. Transgenic corn plants were generated by *Agrobacterium*-mediated transformation of corn embryos as previously described[19]. Initial transgenic (R0) plants were selected on medium containing glyphosate and were tested by PCR for the presence of the transgene. Plants containing a single copy of the transgene were selfed and pollen used to make hybrids (F_1) by crossing with a complementary genotype. The resulting hybrids were used in corn root feeding assays.

Northern blot analysis. Total RNA was extracted from rootworm larvae using the *mir*Vana miRNA isolation kit (Ambion) according to manufacturer's instructions. Ten micrograms of total RNA was loaded per lane, blotted and probed as described[24], except Dig Easy Hyb (Roche) was used as a hybridization buffer at 42 °C and final wash of 0.1× SS, 0.1% SDS was performed at 65 °C. Probe (section 4, **Supplementary Fig. 2**) for the detection of the V-ATPase A transcript was labeled with ^{32}P-dCTP using the RadPrime DNA Labeling System (Invitrogen). The entire α-tubulin cDNA (PIC17503, **Supplementary Fig. 1**) was used as probe after amplification with vector primers flanking the clone and labeling with ^{32}P-dCTP. Hybridization signals were visualized with BioMax MR film (Kodak). RNA from corn plants was extracted with Trizol (Invitrogen) and 10 µg of total RNA was loaded per lane. Small RNA gels were run as previously described[25] and the maize miR159 probe (CaGaGCTCCCTT CaaTCCaaa) was end-labeled[24] with ^{32}P ATP.

Whole plant feeding assays. Transgenic corn plants (F_1) generated using pMON94805 were planted into 10 inch pots containing Metromix soil after reaching an appropriate size. When plants reached the V4 growth stage, ~1,000 WCR eggs were infested into the root zone. Non-transgenic corn of the same genotype was infested at a similar growth stage to serve as a negative control. Eggs were pre-incubated so hatch would occur within 24 h of infestation. Larvae were allowed to feed on the root systems for 3 weeks. Plants were removed from the soil and washed so that the roots could be evaluated for larval feeding. Root damage was rated using the Iowa State nodal injury scale to score the level of damage where 0 indicates no damage, 1 indicates that one node of roots was pruned to within 1.5 inches, 2 indicates that two nodes were pruned, and 3 indicates that three nodes were pruned[20].

Corn root feeding assays. Transformed roots expressing α-tubulin dsRNA were fed to WCR larvae. Transgenic corn roots were maintained in Petri dishes with MSOD medium containing the antibiotics and glyphosate for *in vitro* selection. Two WCR larvae were infested per root in each dish with a fine tip paint brush. The dishes were sealed with Parafilm to prevent the larvae from escaping. The assays were placed in a 27 °C, 60% relative humidity Percival incubator in complete darkness. Contamination and larval quality were monitored. After 6 d of feeding on root tissue, the larvae were transferred to an artificial diet in a 96-well plate. The larvae were allowed to feed on the diet for an additional 8 d. Larval mass and survivorship were recorded for analysis. A one-way analysis was performed on the larval mass data and a Dunnett's test for statistical significance compared to LH244, an untransformed negative control.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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