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BARD

FINAL REPORT

PROJECT NO. US-4158-08

Broad-Spectrum Resistance to Root-Knot Nematodes in Transgenic Cucurbits

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Cover Page**BARD Project Number:** US-4158-08**Date of Submission of the report:** June 24, 2013**Project Title:** Broad-spectrum resistance to root-knot nematodes in transgenic cucurbits**Investigators****Principal Investigator (PI):**
Eric L. Davis**Co-Principal Investigator (Co-PI):**
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ARO, IsraelARO, Israel
NC State University, U.S.**Keywords:** control, *Meloidogyne*, cucumber, RNA interference, parasitism gene, transgenic plant**Abbreviations commonly:**

CaMV – Cauliflower Mosaic Virus

RKN- Root-knot nematode

RNA – ribonucleic acid

RNAi – RNA interference

siRNA - small-interfering RNA

16D10, 8D05, 4D03 – parasitism genes of root-knot nematodes

Budget: IS: \$ 164,000

US: \$ 158,000

Total: \$ 322,000

 Signature
 Principal Investigator

 Signature
 Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	0	1	0	1
Submitted, in review, in preparation	0	0	0	0
Invited review papers	0	0	0	0
Book chapters	0	0	0	0
Books	0	0	0	0
Master theses	0	0	0	0
Ph.D. theses	0	0	0	0
Abstracts	0	0	0	0
Not refereed (proceedings, reports, etc.)	0	0	0	0

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

N/A

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	0	0	0	0
Longer Visits (Sabbaticals)	0	0	0	0

Description Cooperation:

The pHANNIBAL 8D05-RNAi constructs were provided by the U.S. scientists to our Israeli collaborators in Year 2 of the project to develop the first lines of transgenic cucurbits with these constructs. A construct that replaces the 35S promoter in pHANNIBAL with the RKN-inducible *NtCel7* plant gene promoter was provided by the U.S. scientists to our Israeli collaborators to produce an *NtCel7*-driven 16D10-RNAi and 8D05-RNAi constructs for cucurbit transformation. The pHANNIBAL 4D03-RNAi construct and the newly-modified 16D10-RNAi construct (for increased RNAi expression and efficacy) was provided by the U.S. scientists to our Israeli collaborators to develop the first lines of transgenic cucurbits with these constructs.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued (allowed)	0	0	0	0
Licensed	0	0	0	0

Abstract

Root-knot nematodes (RKN), *Meloidogyne* spp., are extremely destructive pathogens of cucurbit crops grown in the United States and Israel. The safety and environmental concerns of toxic nematicides, and limited sources of natural cucurbit resistance to the four major species of *Meloidogyne* that threaten these crops in Israel and the U.S., have emphasized the use of biotechnology to develop cucurbits with novel RKN resistance. The U.S. scientists have identified over 40 unique RKN parasitism genes that encode nematode secretions involved in successful plant root infection by RKN, and they have demonstrated that expression of a double-stranded RNA (dsRNA) complementary to a RKN parasitism gene (called 16D10) in *Arabidopsis thaliana* induced RNA interference (RNAi)-mediated silencing of the RKN16D10 gene and produced transgenic plants with strong resistance to all four major RKN species. The expression 8D05 parasitism gene was found to coincide with the timing of upregulation of *NtCel7* promoter (identified to be upregulated in giant-cells by US scientists). *NtCel7* promoter was used to express the genes at the right time (early stages of infection) and in the right place (giant-cells) in transgenic plants. US partners produced *NtCel7* (nematode-induced promoter)-driven 16D10-RNAi and 8D05-RNAi constructs, pHANNIBAL 4D03-RNAi construct and modified 16D10-RNAi construct (for increased RNAi expression and efficacy) for cucurbit transformation in Israel. In *Arabidopsis*, some 16D10-RNAi plant lines show greater levels of resistance to *M. incognita* than others, and within these lines resistance of greater than 90% reduction in infection is observed among almost all replicates in US. The level of observed nematode resistance is likely to be directly correlated with the level of RNAi expression in individual plants. In Israel, all the RKN parasitism genes-RNAi constructs were successfully transformed into cucumber and melon. The transgenic lines were evaluated for expression of the transgene siRNA in leaves and roots. Those displaying transgene siRNA accumulation were passed on for nematode resistance analysis. R1 seedlings from different lines were subjected to evaluation for resistance to *M. javanica*. None of the lines was resistant to the nematode in contrast with US partner's results in *Arabidopsis*. This could be for the following reasons: a) The level of transgene siRNA was insufficient in cucumber and tomato to cause resistance. b) The nematode species on cucumber may be different or act in a different manner. c) The assay was performed in soil with a high level of nematode inoculation, and not in petri dish, which may not permit the observation of a low level of resistance

Achievements in US

First year: RKN parasitism gene 8D05 that encodes a novel nematode secreted protein provided promising results as a potential target for disruption including 75% decrease in infection by host-derived RNAi and interaction of the 8D05 protein with major intrinsic proteins (MIPs) of plants. We determined that 8D05 interacts with a specific aquaporin member of the MIP family, tonoplast intrinsic protein 2 (TIP2), and that TIP2 is specifically upregulated only in the early stages of giant-cell formation by RKN in host roots. Quantitative analysis of 8D05 expression in parasitic stages of RKN showed upregulation only at similar times as TIP2 upregulation early in infection, suggesting a specifically timed interaction between the nematode and plant proteins. The timing of this expression exactly coincides with the timing of upregulation of *NtCel7* promoter (identified to be upregulated in giant-cells by our lab in previous research), suggesting that we can drive expression of RNAi targeted to 8D05 transcripts at the right time (early stages of infection) and in the right place (giant-cells) in transgenic plants as compared to constitutive expression throughout whole plants by the CaMV 35S promoter. We also developed an antibody to the 8D05 protein during Year 2 that specifically binds to 8D05 in RKN subventral esophageal gland secretory cells. We can now monitor both silencing of the 8D05 gene and protein in nematodes extracted from transgenic plants that express 8D05-RNAi to correlate the levels of target silencing observed with the amount of RKN resistance achieved.

Second year: The 4D03 RKN parasitism gene that also encodes a novel protein had showed significant reduction in RKN parasitism using RNAi in Arabidopsis and overexpression of the 4D03 protein induced accelerated Arabidopsis root growth. We determined that RKN infection increased in roots of Arabidopsis that expressed 4D03, suggesting that this nematode protein promotes the infection process. Different than 8D05, the 4D03 parasitism gene was found to be upregulated in nematodes from the early stages of plant infection through the adult life stages, with maximum expression in the fourth-stage juvenile females. Yeast two-hybrid analyses confirmed that the 4D03 protein specifically interacted with a plant chorismate synthase protein. Chorismate is a pivotal intermediate in the shikimic acid pathway in plant cells leading to the synthesis of plant defense compounds. Our new data suggest that RKN 4D03 protein may interact with the plant chorismate synthase protein to suppress plant defense response to the nematode. The expression of 4D03 throughout the nematode life cycle and increase in nematode parasitic success when 4D03 is overexpressed in plants are consistent with the potential role of 4D03 in suppression of plant defense.

Final year efforts of the project have focused on increasing the level and reproducibility of the 16D10 RNAi-based root-knot nematode resistance in transformed plants. Some 16D10-RNAi plant lines show greater levels of resistance than others, and within these lines resistance of greater than 90% reduction in infection is observed among almost all replicates (single outliers reduce the calculated mean). The level of observed nematode resistance is likely to be directly correlated with the level of RNAi expression in individual plants, so we are conducting quantitative small RNA analyses using next-generation (Illumina) RNA sequencing technology to identify the levels of expression and species of small interfering RNA (siRNA) among tplants within lines. We have also modified the design of the original RNAi transgene construct including a spliced (GUS) intron and (GmUbi) promoter to maximize 16D10-RNAi expression.

A part of the study has been published: Xue, B., Hamamouch, N., Li, C., Huang, G., Hussey, R. S., Baum, T. J., and Davis, E. L. 2013. The 8D05 parasitism gene of *Meloidogyne incognita* is required for successful infection of host roots. *Phytopathology* 103:175-181.

Achievements in ARO-Israel.

First year: We transformed cucumber and melon plants with binary vector pHANNIBAL 16D10 -RNAi and we started the molecular characterization of our T0 and T1 generations.

Second year: We continued our transformation with the same construct 16D10 in cucumber, melon, tobacco and tomato plants. We collected seeds from 24, 33, 10 and 7 independent transformed lines of cucumber, melon, tobacco and tomato, respectively. For molecular characterization of transgenic plants, we germinated transgenic seeds on kanamycin agar plates. T1 plants from each line (resistant to kanamycin) were tested by PCR for gene integration and then for accumulation of the transgene small-RNA by northern blot. We observed a variety of accumulation levels in different transgenic lines. In general the level of 16D10 siRNA in tomato and tobacco was higher than in cucumber and melon. Selected lines which exhibited transgene-siRNA accumulation were tested for biological resistance to nematodes. Evaluation of nematode resistance in transgenic lines of melon, tomato and tobacco plants was done by inoculating plants with second-stage juveniles and/or eggs of *Meloidogyne javanica*. Root galling index (0-5) and number of eggs per plant were recorded 6 weeks after inoculation. None of the plants showed significant resistance (lower galling index and number of eggs) to the nematode comparing with those of relevant control plants.

Third year: we transformed cucumber with 2 constructs. The first one is with a new gene 8D05 (contributed by the US partner) under control of the specific nematode promoter *NtCel7*. The second construct contains a chimeric gene with parts of 4 nematode genes (300 bp from each gene): 16D10, Splicing factor, 8D05 and Integrase in a hairpin configuration which is expressed under control of the nematode-induced promoter *NtCel7*. We generated 18 independent transformed cucumber lines and selfed to generate R1 plants. The transgenic lines were evaluated for expression of the transgene siRNA in leaves and roots. Those displaying transgene siRNA accumulation were passed on for nematode resistance analysis. R1 seedlings from different transgenic lines were subjected to evaluation for nematode resistance, in pots as in method 3 above. We do not observe resistance in any of the nematode parameters (eggs, galls).

Our results do not match the US partner's results with Arabidopsis. This could be for the following reasons:

- a. The level of transgene siRNA was insufficient in cucumber and tomato to cause resistance.
- b. The nematode species on cucumber may be different or act in a different manner.
- c. The assay was performed in soil with a high level of nematode inoculation, and not in petri dish, which may not permit the observation of a low level of resistance.

Unfortunately, no publication was achieved because no resistant transgenic line of cucurbits was obtained.