RNA Interference of Effector Gene *Mc16D10L* Confers Resistance Against *Meloidogyne chitwoodi* in *Arabidopsis* and Potato

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ABSTRACT

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Meloidogyne chitwoodi, a quarantine pathogen, is a significant problem in potato-producing areas worldwide. In spite of considerable genetic diversity in wild potato species, no commercial potato cultivars with resistance to *M. chitwoodi* are available. Nematode effector genes are essential for the molecular interactions between root-knot nematodes and their hosts. Stable transgenic lines of *Arabidopsis* and potato (*Solanum tuberosum*) with resistance against *M. chitwoodi* were developed. RNA interference (RNAi) construct pART27(16D10i-2) was introduced into *Arabidopsis thaliana* and potato to express double-

stranded RNA complementary to the putative *M. chitwoodi* effector gene *Mc16D10L*. Plant-mediated RNAi led to a significant level of resistance against *M. chitwoodi* in *Arabidopsis* and potato. In transgenic *Arabidopsis* lines, the number of *M. chitwoodi* egg masses and eggs was reduced by up to 57 and 67% compared with empty vector controls, respectively. Similarly, in stable transgenic lines of potato, the number of *M. chitwoodi* egg masses and eggs was reduced by up to 71 and 63% compared with empty vector controls, respectively. The relative transcript level of *Mc16D10L* was reduced by up to 76% in *M. chitwoodi* eggs and infective second-stage juveniles that developed on transgenic pART27(16D10i-2) potato, suggesting that the RNAi effect is systemic and heritable in *M. chitwoodi*.

Potato (*Solanum tuberosum* L.) is the most important non-cereal food crop worldwide and makes up the staple diet of over 1 billion people (4,28). Plant-parasitic nematodes are a major threat to potato production worldwide, with root-knot (*Meloidogyne* spp.) and cyst nematodes (*Globodera* spp.) being the most widespread and causing most of the damage (6). In the Pacific Northwest of the United States, which is the leading potato growing area and accounts for more than half of the country's total production (2), the Columbia root-knot nematode (*Meloidogyne chitwoodi* Golden et al.) is the most common and significant nematode threat to sustainable potato cultivation. *M. chitwoodi* is not only a problem in the United States but has also been found in potato growing regions in Europe, Mexico, Argentina, and South Africa, making it a threat in some of the world's most important potato production areas (21). *M. chitwoodi* causes tuber quality defects and can render entire shipments unmarketable (24). It has been estimated that as little as one juvenile per 250 g of soil at the beginning of the growing season can lead to a total loss of marketability at harvest (42). To limit spread, *M. chitwoodi* has been designated as a quarantine pest, which has a significant impact on the international trade of potato tubers (1).

Meloidogyne spp. are obligate parasites that depend on their host plant for survival. The pathogenic part of the life cycle of root-knot nematodes begins with the second-stage juveniles (J2), which are the infective life stage and invade plant roots, rhizomes, and tubers. The J2 migrate intercellularly through host tissue until

they become sedentary and induce the formation of giant cells, which constitute the nematodes' feeding sites and sole source of nutrition. The exact mechanisms that lead to the formation and maintenance of giant cells are unknown, but generally it is thought that secretory proteins from the nematode esophageal gland cells, i.e., effectors, play a key role in the underlying processes (54). After a feeding site has been established, the J2 increase in size and undergo subsequent molts into third- and fourth-stage juveniles (J3 and J4). After a final molt they develop into adult females or males. Adult females deposit eggs into egg masses, a gelatinous matrix that protects eggs from desiccation. Inside the eggs, first-stage juveniles (J1) develop and molt into J2, which hatch under favorable conditions that are mostly dictated by soil moisture and temperature (58). Nematode control in most potato growing areas is based on routine applications of synthetic nematicides. This practice not only is costly but also potentially harmful to the environment because some products have been linked to negative effects on the atmosphere's ozone layer (60). Host resistance against root-knot nematodes would be an ideal control strategy, but is difficult to achieve.

In spite of the extremely rich genetic resources found in *Solanum* sect. *Petota*, which consists of wild and cultivated potatoes and includes up to 232 tuber-bearing and non-tuber-bearing species (25,27,64,65), exploiting this genetic diversity and introducing *M. chitwoodi* resistance into cultivated potato (*S. tuberosum* sp. *tuberosum*) has proven to be challenging. Germplasm evaluation identified resistance against *M. chitwoodi* in a number of potato species, including *S. brachistotrichum* (Bitter) Rydb., *S. bulbocastanum* Dunal, *S. cardiophyllum* Lindl., *S. chacoense* Bitter, *S. fendleri* Gray, *S. hougasii* Corr., and *S. stoloniferum* Schltdl. (7,9,10,44–46). Protoplast fusions and sexual hybridization were used to introgress *M. chitwoodi* resistance from *S. bulbocastanum*, *S. fendleri*, *S. hougasii*, and *S. stoloniferum* into cultivated potato (8,11,12,47) but recent analyses indicate that the same single dominant monogene, *R_{Mc1}*,

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might be responsible for the resistance found in *S. bulbocastanum*, *S. fendleri*, and *S. hougasii* (15). In spite of over two decades of breeding efforts no commercial potato cultivar with resistance to *M. chitwoodi* is available to date. The $R_{Mc1(blb)}$ gene from *S. bulbocastanum* has been used to develop advanced *S. tuberosum* breeding clones, but *M. chitwoodi* populations are very variable (34,35) and some isolates are able to overcome the $R_{Mc1(blb)}$ gene, presenting a significant problem for traditional resistance breeding (13,48). Furthermore, hairy nightshade (*S. sarrachoides* Sendtn.), a common weed in potato producing areas in the western United States and a host for *M. chitwoodi*, was found to undermine resistance under greenhouse and field conditions when it co-occurs with potato breeding lines carrying the $R_{Mc1(blb)}$ resistance gene (5).

Given these challenges, transgenic approaches, and in particular the use of RNA interference (RNAi), could be an attractive alternative for nematode control. Plant-parasitic nematodes are known to take up host cytoplasm during the infection process, making them vulnerable to host-derived compounds, including double-stranded RNA (dsRNA) and small interfering RNA (siRNA). This dependency has been exploited recently by engineering transgenic plant tissue that overexpresses dsRNA that is complementary to nematode genes (for recent reviews see 22, 49,51). However, plant-mediated RNAi technology to create nematode resistance has been restricted largely to the model plant *Arabidopsis thaliana* and *Agrobacterium rhizogenes*-induced transgenic hairy roots (31,40,63,71). Stable transformation of agriculturally important crops to implement host-mediated RNAi against nematodes has been achieved only in tobacco (*Nicotiana tabacum* L.) and soybean (*Glycine max* (L.) Merr.) to date, and with greatly varying levels of resistance (23,66,72). In some of these cases, nematode genes were targeted that are conserved in other animals, and could prove problematic when implemented in the field. Targeting genes that are specific to plant-parasitic nematodes to eliminate off-target effects might provide a better option if this technology is to be employed in crops. The effector gene *16D10* has shown great promise as an RNAi target in other *Meloidogyne* spp. (31), but it is unknown whether this gene exists in *M. chitwoodi* and whether it can be used to create stable resistance against this nematode, especially in agronomically important crops. Therefore, the objective of this study was to test whether creating stable transgenic lines of *Arabidopsis* and potato that overexpress dsRNA complementary to the *Meloidogyne*-specific effector gene *16D10* leads to resistance against *M. chitwoodi*.

MATERIALS AND METHODS

Nematode maintenance and extraction. *M. chitwoodi* isolate WAMC1 was maintained on tomato (*S. lycopersicum* L.) 'Rutgers' under greenhouse conditions (34). Tomato plants were grown in autoclaved sand. Three months after inoculation, *M. chitwoodi* eggs and parasitic life stages were harvested from tomato roots. To extract eggs, roots were cut into small pieces and agitated for 3 min in a 0.5% sodium hypochlorite solution (36). The root suspension was poured over a set of nested test sieves (850, 75, and 25 μ m pore size from top to bottom) and eggs were collected from the 25- μ m sieve. To purify samples, the *M. chitwoodi* egg suspension was divided into 50-ml tubes (20 ml per tube) and mixed with 20 ml of 70% sucrose. Ten milliliters of water was carefully placed on top of the suspension and the tubes were immediately centrifuged for 3 min at $375 \times g$ in a clinical centrifuge. After centrifuging, eggs were collected from the interface and rinsed on a 25- μ m test sieve. To hatch infective J2, purified eggs were incubated in a modified Baermann pan with sterile water at room temperature for 4 days; hatched infective J2 were collected by centrifugation (19). To extract parasitic nematode life stages, root pieces from which eggs were extracted were blended

in tap water and decanted over a set of four nested test sieves (250, 125, 45, and 25 μ m pore size from top to bottom). Each sieve was rinsed for 2 min with tap water. *M. chitwoodi* females were collected from the 125- μ m sieve, parasitic J3 and J4 from the 45- μ m sieve, and parasitic J2 from the 25- μ m sieve, respectively. To purify parasitic nematode stages, samples were transferred to 50-ml tubes and 10 ml of 25% $MgSO_4 \cdot 7H_2O$ was added to the bottom of each tube using a transfer pipet. Tubes were centrifuged as above and parasitic life stages were collected from the interface and rinsed on a 25- μ m sieve. To ensure purity of parasitic life stages and to remove remaining plant debris, samples were purified further by manually removing contaminants under a Stemi 2000C stereomicroscope (Zeiss, Jena, Germany). All developmental stages were rinsed either in DEPC-treated water before being stored at $-80^\circ C$ for use in quantitative real-time polymerase chain reaction (qRT-PCR) or resuspended in 4% formaldehyde in phosphate buffered saline for in situ hybridizations (see below).

Nematode RNA purification and cloning of *Mc16D10L* full-length cDNA. Total RNA was extracted from frozen tissue of all *M. chitwoodi* life stages using the PerfectPure RNA Fibrous Tissue kit (5Prime, Gaithersburg, MD) following the manufacturer's instructions. Approximately 500 ng of purified RNA of each life stage was used as template to synthesize cDNA using the Advantage RT-for-PCR kit (Clontech, Mountain View, CA) according to the manufacturer's protocol. Eighty microliters of DEPC-treated water was added to each 20- μ l reverse transcription product and samples were stored at $-80^\circ C$. To clone full-length cDNA of *Mc16D10L*, primers Mc16D10-F and Mc16D10-R were designed based on previously published *M. chitwoodi* expressed sequence tag (EST) sequence CD418743 (61). Using primers Mc16D10-F and Mc16D10-R and Phusion polymerase (New England Biolabs, Ipswich, MA), *Mc16D10L* was amplified from 5 μ l of cDNA of infective J2 in a Mastercycler pro thermal cycler (Eppendorf, Hamburg, Germany) with the following PCR program: $94^\circ C$ for 5 min; 40 cycles at $94^\circ C$ for 30 s, $52^\circ C$ for 45 s and $72^\circ C$ for 30 s, followed by $72^\circ C$ for 5 min. The PCR product was cloned into pGEM-T Easy (Promega, Madison, WI) and transformed into *E. coli* DH5- α (New England Biolabs). The plasmid was recovered using the GeneJET Plasmid Miniprep kit (Thermo Scientific, Rochester, NY) and sequenced (Elim Bio-pharm, Hayward, CA) to confirm the identity of the insert. Sequences were aligned using ClustalW and signal peptides were analyzed with SignalP 3.0 software.

In situ hybridization. To determine the spatial and temporal expression patterns of *Mc16D10L* in *M. chitwoodi*, in situ hybridizations were conducted for all life stages. After amplifying the *Mc16D10L* template with primers Mc16D10-F and Mc16D10-R using Phusion polymerase (New England Biolabs), the sense and antisense probes were synthesized with the PCR DIG Probe Synthesis kit (Roche, Indianapolis, IN) following the manufacturer's instructions. Eggs, infective J2, parasitic J2, J3, J4, and adult females that were fixed previously in 4% formaldehyde in phosphate buffered saline for 2 days at room temperature were cut at random with a razor blade, dehydrated, and hybridized in 1.5-ml microcentrifuge tubes with sense (for negative control) and antisense probes at $37^\circ C$ for 16 h (37). After incubation, residues were removed and probes were detected following Hussey et al. (37). Hybridization patterns were viewed and documented with an AxioObserver A1 inverted microscope equipped with differential interference contrast, AxioCam ICc1 digital camera, and ZEN imaging software (Zeiss). All experiments were replicated at least twice and showed similar results.

Generation of transgenic *Arabidopsis* and potato. *A. thaliana* ecotype Columbia (Col-0) was used as wild type in infection assays described below and as genetic background for generating transgenic RNAi lines following the floral dip method (17). Using *Agrobacterium tumefaciens* strain GV3101, the empty binary

vector pART27 and the silencing construct pART27(16D10i-2), which contained a pHANNIBAL RNAi cassette based on *Mi16D10* (31), were transformed into *A. thaliana*. For each construct, approximately 500 seeds from 24 individual plants were germinated on 1/2 strength Murashige and Skoog (MS) basal salts media (Caisson, North Logan, UT), supplemented with 3% D-sucrose, kanamycin sulfate at 50 µg/ml and timentin at 0.5 g/liter, and solidified with 0.6% Daishin agar at pH 5.7. Seedlings were maintained at 22°C in a growth chamber with a 12 h photoperiod. Selection-resistant seedlings were confirmed by PCR for presence of the transgene, and for each construct, six seedlings were carefully transferred to individual pots filled with potting mix and cultivated in the greenhouse (16 h daylight at 22°C and 8 h nighttime at 20°C). T₁ seeds were collected from five individual lines and about 50 T₁ seeds for each line were plated on selection media to ensure the presence of the transgene. One plant per line was grown in the greenhouse until maturity to collect T₂ seeds, later plated to confirm transgenic phenotypes by kanamycin resistance and PCR. For all experiments described here, T₃ lines were used; these were confirmed by PCR and Southern blots (Supplemental Figure 1). Three transgenic RNAi lines (D1, D2, and D4), one empty vector line (E2), and a wild type control line (Col-0) were used for infection assays.

The same constructs also were introduced into potato 'Désirée'. Internodal stem pieces were co-incubated with *Agrobacterium tumefaciens* strain GV3101, carrying the empty pART27 or pART27(16D10i-2) binary vector on sterile CIM media (MS basal salts, 0.25 ppm folic acid, 0.05 ppm D-biotin, 2 ppm glycine, 0.5 ppm nicotinic acid, 0.5 ppm pyridoxine HCl, 0.4 ppm thiamine HCl, 0.01% myo-inositol, 3% D-sucrose, 1 ppm 6-benzylaminopurine, 2 ppm 1-naphthaleneacetic acid, and 0.6% Daishin agar at pH 5.6) for 3 days in the dark at 19°C (14). For the empty pART27 lines, 200 internodal stem pieces were used for transformation, and 250 were used for pART27(16D10i-2), respectively. Stem segments were transferred every 2 weeks to sterile 3C5ZR media (MS basal salts, 0.5 ppm nicotinic acid, 0.5 ppm pyridoxine HCl, 1 ppm thiamine HCl, 0.01% myo-inositol, 3% D-sucrose, 0.5 ppm indole-3-acetic acid, 3 ppm zeatin ribose, timentin at 0.5 g/liter, kanamycin sulfate at 70 µg/ml, and 0.6% Daishin agar at pH 5.9) and maintained in a growth chamber at 22°C with a 12 h photoperiod (62). Newly formed plantlets that regenerated from callus tissue on 3C5ZR plates were transferred to propagation media (MS basal salts, 3% D-sucrose, kanamycin sulfate at 50 µg/ml, and 0.6% Daishin agar at pH 5.7) and maintained under the same growth conditions (55 plantlets for pART27 and 64 plantlets for pART27(16D10i-2)). Regenerated plantlets were analyzed by PCR and northern and Southern blots to ensure presence of the transgene. Three single-insertion pART27(16D10i-2) lines (D54, D56, and D57), one empty vector line (E29), and a wild type control line (DES) were chosen for infection assays in the greenhouse. BLAST searches between the gene-specific region of pART27(16D10i-2) and *Arabidopsis* and potato ESTs and genome sequences did not yield any significant hits, suggesting that no in planta off-target silencing effects were to be expected.

Infection assays. For *Arabidopsis*, Col-0 wild type and transgenic (E2, D1, D2, and D4) seeds were surface-sterilized and germinated in a growth chamber (22°C with a 12 h photoperiod) on 1/2 strength MS media supplemented with 3% D-sucrose and kanamycin sulfate at 50 µg/ml (for transgenic lines) and solidified with 0.6% Daishin agar at pH 5.7. After 9 days, seedlings were transferred to individual 500-ml pots filled with SunShine Mix #1 potting mix (Sun Gro Horticulture, Agawam, MA) and grown for 10 days under greenhouse conditions before being inoculated with 5,000 *M. chitwoodi* eggs per pot. For potato, single nodes of wild type 'Désirée' and transgenic (E29, D54, D56, and D57) lines were cut from plants maintained in tissue culture and propagated in a growth chamber (22°C with a 12 h photoperiod) on MS

media supplemented with 3% D-sucrose and kanamycin sulfate at 50 µg/ml (for transgenic lines) and solidified with 0.6% Daishin agar at pH 5.7 for 1 month. Following the 1-month period, plants were transferred to individual 500-ml pots filled with autoclaved sand, and cultivated under greenhouse conditions for 10 days before being inoculated with 5,000 *M. chitwoodi* eggs per pot. Sand was used to facilitate cleaning of potato roots at the conclusion of the infection assay. *Arabidopsis* did not grow well in pure sand, hence the use of potting mix. Both *Arabidopsis* and potato plants were maintained in the greenhouse for the duration of the experiment, and watered twice per day with 20-10-20 NPK liquid fertilizer. Infection assays were designed as randomized complete block designs with nine replications for each *Arabidopsis* line and timepoint (35 and 55 days after inoculation [DAI], respectively) and 10 replications for each potato line and timepoint. At 35 DAI, the roots were washed free of growth substrate and for potato, the root fresh weight was measured. Potting mix adhering to roots prevented reliable fresh root weight measurements in *Arabidopsis* and the weights were omitted. All root systems were stained with phloxine B at 0.15 g/liter for 15 min to visualize *M. chitwoodi* egg masses as well as facilitate counting under a Stemi 2000C stereomicroscope (Zeiss). At 55 DAI, root systems were washed free of substrate and for potato, root fresh weights were determined as before. For each root system, eggs were extracted as described above and eggs were counted under a stereomicroscope. Eggs were used to extract RNA or hatch infective J2 for qRT-PCR assays as detailed above. All infection assays were conducted twice and showed similar results. Representative results are shown.

Northern blots for transgenic *Arabidopsis* and potato lines.

Total RNA and small RNA were extracted from 0.5 g of leaf tissue of each line using the mirVana miRNA isolation kit (Ambion, Austin, TX). For each line, total RNA (20 µg for *Arabidopsis* and 4 µg for potato) was denatured in 50% deionized formamide (Amresco, Solon, OH), separated on a 1% agarose gel in 1× TBE buffer (0.9 M Tris base, 0.9 M boric acid, and 20 mM EDTA) and transferred by capillary action onto Nytran N nylon membrane (Sigma, St. Louis, MO) in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). In addition, 1 µg of denatured small RNA was separated on a 15% polyacrylamide gel with 8 M urea in 1× TBE buffer and transferred onto Nytran N membrane as described above. For northern blotting, [α -³²P] dATP probes (MP Biochemicals, Solon, OH) Mi16D10 and U6 were synthesized by PCR using primers 16D10F5, 16D10R5, U6F, and U6R, respectively (Table 1). Probes were labeled radioactively and purified with DECAprime II kit (Ambion) and Illustra NICK columns (GE Healthcare Life Sciences, Pittsburgh, PA). Membranes were UV-crosslinked and hybridized with radioactive probes overnight at 37°C in hybridization buffer (50% deionized formamide, 3× SSC, 0.1 mg/ml salmon sperm DNA, 1% sodium dodecyl sulfate, 0.05 M phosphate buffer, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, and 0.2% Ficoll) before being washed three times in 2× SSC and 0.2% SDS for 20 min at 46°C and exposed to X-ray films for 1 to 5 days at -80°C. All experiments were conducted twice and showed similar results.

qRT-PCR transcript levels of *Mc16D10L*. To analyze the transcript levels of *Mc16D10L* throughout the life cycle of *M. chitwoodi*, qRT-PCR was performed with cDNA from different nematode life stages harvested from greenhouse nematode stock cultures, as well as from eggs and infective J2 originating from plants used in infection assays (see above). Total RNA was extracted and cDNA generated as described above. qRT-PCR was performed in an iQ Real-Time PCR machine (Bio-Rad, Hercules, CA) using the iQ SYBR Green Supermix kit (Bio-Rad). Internal transcribed spacer 2 (ITS2) rRNA (JN241865) was chosen as internal control gene for qRT-PCR after comparing the expression pattern with that of other *M. chitwoodi* housekeeping genes, such as 18S rRNA (AY757835), actin 2 (CB930959), and glyceralde-

hyde-3-phosphate-dehydrogenase-1 (CB9332930). Primers McITS-RTF and McITS-RTR were designed based on the *M. chitwoodi* ITS2 gene and used for internal control qRT-PCR reactions (Table 1). *Mc16D10L* was amplified using primers Mc16D10-RTF and Mc16D10-RTR (Table 1). Each sample was run in triplicate using the following program: 94°C for 10 min; 45 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 30 s, followed by 91 cycles with a temperature increase of 0.5°C after each cycle from 50 to 95°C. The differences in the expression level of *Mc16D10L* in *M. chitwoodi* were analyzed using the $2^{-\Delta\Delta C_t}$ method (52) with cycle threshold (C_t) values exported from iQ5 Optical System Software (Bio-Rad). All experiments were conducted at least twice and showed similar results. Representative results are shown.

Data analysis. Number of egg masses, number of eggs, fold changes, and $\log_{10}$ fold changes of *Mc16D10L* expression were analyzed in Microsoft Excel to calculate means and standard errors. Statistically significant differences were estimated using a Student's *t* test with $\alpha = 0.05$ in SAS 9.2 (SAS Institute, Cary, NC).

RESULTS

***M. chitwoodi* effector gene *Mc16D10L* is a ortholog of *Mi16D10*.** In order to identify a putative *M. chitwoodi* ortholog of the *M. incognita* effector gene *Mi16D10*, *M. chitwoodi* ESTs were searched using BLASTN (32,61). *M. chitwoodi* EST CD418743 provided the best match and a full-length sequence spanning the complete open reading frame was cloned from infective J2 cDNA using the gene-specific primers Mc16D10-F and Mc16D10-R (Table 1). The coding sequence of this *Mi16D10*-like *M. chitwoodi* ortholog, which will be referred to as *Mc16D10L* henceforth, had a length of 153 bp. A pairwise sequence alignment showed that 70% of the sequences of *Mi16D10* and *Mc16D10L* were identical on the nucleotide level and 63% on the amino acid

level, respectively (Fig. 1). The N-terminal 32 amino acids of *Mc16D10L* represent a signal peptide, a characteristic of nematode effector gene products, indicating that this putative effector peptide is most likely secreted. Importantly, a region with similarity to the plant CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR) (CLE) motif (KRXXVPXGPNPLHNR) found in *Mi16D10* and other *Meloidogyne* spp. 16D10 orthologs (31,55) also was conserved in *Mc16D10L*. The nucleotide sequence of *Mc16D10L* was deposited in GenBank under accession number KF734590.

***Mc16D10L* expression changes during nematode development.** In situ hybridizations showed that *Mc16D10L* was expressed most strongly in the subventral gland cells of *M. chitwoodi* infective J2 (Fig. 2). Weaker expression was found in eggs and subventral gland cells of parasitic J2, whereas no gene activity could be detected in late parasitic life stages by in situ hybridizations. To corroborate the in situ data, qRT-PCR was performed with cDNA from all *M. chitwoodi* life stages except adult males (Fig. 3). Using the expression level of *Mc16D10L* in eggs as reference, *Mc16D10L* was upregulated 1.87-fold in infective J2 on a $\log_{10}$ scale, which represents a 78-fold upregulation in absolute terms. In parasitic J2, mixed J3/J4, and adult females, *Mc16D10L* was expressed at a lower level than in eggs, with a fold change of -0.72, -1, and -1.6 on a $\log_{10}$ scale (5-fold, 10-fold, and 33-fold downregulation in absolute terms), respectively.

Plant-mediated RNAi of *Mc16D10L* increases *M. chitwoodi* resistance. No overt phenotypical changes were observed in transgenic *Arabidopsis* compared with wild type controls. At 35 DAI, the average number of egg masses per plant was reduced ($P < 0.05$) by 36 to 45% in RNAi lines D1, D2, and D4 compared with the Col-0 wild type control, and by 50 to 57% compared with the empty vector control, respectively. At 55 DAI, the average number of eggs per plant in *Arabidopsis* RNAi lines was

TABLE 1. Primers and probes used for cloning, polymerase chain reaction, and northern and Southern blots

Gene	Primer name	Primer sequences (5'-3')
Mi16D10	16D10F5	GTTTACTAATTCAATTAAAAATTTAATT
(DQ087264)	16D10R5	CAATTATTTCCTCCAGGATTGGCCC
U6	U6F	GCGCAAGGATGACACGCA
(X60506)	U6R	GGCTGAGTTATTTTTTCTG
ITS2	McITS-RTF	GGGGTCAAACCTTTGGCAGCTCTGG
(JN241865)	McITS-RTR	GCGGGTGATCTCGACTGAGTTCAGG
Mc16D10L	Mc16D10-F	GATATTTAAATTAAATTATATTCTCTAA
(CD418743)	Mc16D10-R	GCTTTATTCAATTTATTTTATTATT
	Mc16D10-RTF	TTATTTTATCTGTTACTTTTGTGGATTGAGC
	Mc16D10-RTR	GCGACCATCATTTATTATCATTTCCACC
16D10dsRNA	35S-F	TTCGCAAGACCCTTCCTCTA
	OCS1	CTTCTCGTCTTACACATCACTTGTC

Fig. 1. Sequence alignment of 16D10 orthologs from *Meloidogyne incognita* (*Mi16D10*) and *M. chitwoodi* (*Mc16D10L*). A, Nucleotide level. B, Amino acid level.

Fig. 2. In situ hybridization of *Mc16D10L* in different *Meloidogyne chitwoodi* life stages. Spatial expression pattern of *Mc16D10L* in **A**, *M. chitwoodi* eggs, **B**, infective second-stage juveniles (J2), and **C**, parasitic J2. In infective and parasitic J2, *Mc16D10L* was expressed specifically in the subventral esophageal glands. **D**, Negative control using a sense probe. Scale bars = 20 μ m.

Fig. 3. Relative transcript abundance of *Mc16D10L* in different *Meloidogyne chitwoodi* life stages. Using the transcript level of *Mc16D10L* in eggs as a reference, *Mc16D10L* was upregulated significantly (1.87-fold on a log₁₀ scale) in infective second-stage juveniles (J2). In parasitic J2 (pJ2), mixed J3/J4 parasitic juveniles and adult females (F), *Mc16D10L* was downregulated significantly (–0.72, –1.0, and –1.6-fold on a log₁₀ scale, respectively). Each bar represents the log₁₀ transformed mean of qRT-PCR runs in triplicate with standard errors. Letters indicate statistically significant differences using a Student's *t* test ($P < 0.05$).

Fig. 4. Reproductive success of *Meloidogyne chitwoodi* on transgenic *Arabidopsis thaliana* expressing pART27(16D10i-2). **A**, Number of egg masses per plant at 35 days after inoculation (DAI), and **B**, number of eggs per plant at 55 DAI in Columbia-0 wild type (COL), transgenic empty pART27 vector control (E2), and transgenic pART27(16D10i-2) (D1, D2, and D4) plants. Each bar represents the mean of nine plants per independent line and timepoint with standard errors. Letters indicate statistically significant differences using a Student's *t* test ($P < 0.05$).

reduced by 68 to 74% compared with the wild type control, and by 59 to 67% relative to the empty vector control ($P < 0.05$) (Fig. 4). Since expression of dsRNA of *Mc16D10L* led to enhanced resistance against *M. chitwoodi* in the model plant *Arabidopsis*, the RNAi construct pART27(16D10i-2) was used to generate stable transgenic potato lines to test whether the same strategy could be applied to engineer *M. chitwoodi* resistance in potato. No overt phenotypical changes were observed in transgenic potato plants compared with wild type controls. At 35 DAI, the average number of egg masses per plant was reduced by 63 to 79% compared with the wild type control, and by 50 to 71% compared with the empty vector control ($P < 0.05$). Similarly, the average number of eggs per RNAi plant at 55 DAI was reduced by 65 to 74% compared with the wild type, and by 50 to 63% compared with the empty vector control lines ($P < 0.05$). Comparable results were obtained when nematode infection was measured as number of egg masses or number of eggs per gram of root fresh weight in potato, with reductions of 44 to 56% and 53 to 69% ($P < 0.05$) compared with the empty vector control, respectively (Fig. 5). Northern blots confirmed the expression of small RNAs ranging in size from about 50 to over 150 nt in all transgenic pART27(16D10i-2) *Arabidopsis* and potato lines when a *16D10* probe was used (Fig. 6).

Potato RNAi lines downregulate *Mc16D10L* in *M. chitwoodi*.

To investigate the effect of plant-mediated RNAi on the activity of the target gene in nematodes, qRT-PCR was used to analyze the relative transcript level of *Mc16D10L* in *M. chitwoodi* eggs and J2 harvested from potato plants at 55 DAI. Using the internal ribosomal spacer 2 (ITS2) of nuclear ribosomal DNA as reference (53), it was found that the expression level of *Mc16D10L* in *M. chitwoodi* eggs that developed on potato plants carrying pART27(16D10i-2) was reduced by an average of 27 to 76% compared with eggs harvested from wild type control plants ($P < 0.05$). Similarly, the expression level of *Mc16D10L* in infective J2 that hatched from eggs harvested from potato RNAi lines was reduced ($P < 0.05$) by 52 to 70% relative to J2 that developed from eggs collected from wild type control plants (Fig. 7).

DISCUSSION

Plant-mediated downregulation of the putative effector gene *Mc16D10L* provides resistance against *M. chitwoodi* in stable transgenic lines of *Arabidopsis* and potato. The effector gene *16D10* was identified originally in *M. incognita* but orthologs have since been found in *M. arenaria*, *M. hapla*, and *M. javanica* (31,33). Whereas *16D10* showed 95 to 98% nucleotide identity

and 100% amino acid identity between *M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica* (31), the *M. chitwoodi* ortholog cloned here was less conserved, with 70% nucleotide and 63% amino acid identity compared with *M. incognita*. This finding is in line with the more distant phylogenetic position of *M. chitwoodi* relative to other *Meloidogyne* spp. in which *16D10* orthologs have been found (30,69). In situ hybridizations and qRT-PCR indicated that *Mc16D10L* transcription was strongly upregulated in *M. chitwoodi* infective J2. Even though Huang et al. (33) failed to detect transcripts of *Mi16D10* in *M. incognita* infective J2 by in situ hybridizations, Huang and co-authors later reported that *Mi16D10* antiserum confirmed the presence of the *Mi16D10* peptide in the subventral glands of *M. incognita* J2 (32). This finding suggests that *Mi16D10* is in fact actively transcribed in *M. incognita* infective J2 and possibly escaped detection by Huang and colleagues' (33) earlier in situ hybridization assays. The function of *16D10* is unknown, but overexpression of the *M. incognita* ortholog *Mi16D10* increased root growth in tobacco hairy roots and in *A. thaliana* (32). All *Meloidogyne* *16D10* orthologs, including *Mc16D10L*, contain a region that is similar to the CLE domain of plant CLE signaling peptides, which could mean that this nematode effector has evolved to modulate root growth and vascular development, processes in which CLE peptides play a key role (31,55).

Stable transgenic *Arabidopsis* and potato lines overexpressing a 271-nt full-length *16D10* dsRNA construct showed strong resistance against *M. chitwoodi* in this study. No overt changes in plant morphology were observed in the *Arabidopsis* and potato

lines generated in the experiments described here and the level and range of resistance is comparable with what has been reported in other plant-mediated RNAi systems in which cyst or root-knot nematode genes have been targeted (49,51). Controlling *M. chitwoodi* under field conditions is challenging due to its wide host range, development at low temperatures and low damage threshold in potato (21). Standard control tactics heavily rely on the application of fumigant and nonfumigant nematicides, but not all compounds and application methods are equally effective at managing *M. chitwoodi* (41,42). Alternative control methods, such as green manure tend to reduce *M. chitwoodi*, but oftentimes the nematode population remains at economically damaging levels or recovers and quickly reaches previous densities once a susceptible crop is planted (56,70). Most likely, nematodes in deeper soil layers are not affected by synthetic nematicides and green manure applied at the soil surface, and enable a rapid infection of a following potato crop (41,70). It is unknown how transgenic *16D10i-2* potatoes would perform under field conditions, but the systemic nature of the resistance suggests that both roots and tubers would be protected at high levels, even in deeper soil layers. Most likely, an ideal *M. chitwoodi* management system would include diverse approaches, such as traditional nematicides, good crop rotation strategies, green manure, microbial antagonists, and germplasm that carries endogenous or transgenic resistance genes.

Importantly in this study, qRT-PCR indicated a significant reduction in *Mc16D10L* transcripts in the second-generation *M. chitwoodi* eggs and infective J2 that developed on the transgenic

Fig. 5. Reproductive success of *Meloidogyne chitwoodi* on transgenic potato expressing pART27(16D10i-2). **A**, Number of egg masses per plant at 35 days after inoculation (DAI), **B**, number of eggs per plant at 55 DAI, **C**, number of egg masses per gram of root fresh weight at 55 DAI, and **D**, number of eggs per gram of root fresh weight at 55 DAI in 'Desirée' wild type (DES), transgenic empty pART27 vector control (E29), and transgenic pART27(16D10i-2) (D54, D56, and D57) plants. Each bar represents the mean of 10 plants per independent line and timepoint with standard errors. Letters indicate statistically significant differences using a Student's *t* test ($P < 0.05$).

16D10i-2 potato lines. A recent report suggests that the RNAi effect of *Mc16D10L* in the nematode is systemic and proliferates upon the initial uptake of plant-derived dsRNAs or siRNAs from the esophagus through the entire body of the female, including the gonads and developing eggs, thereby transmitting the RNAi effect to the offspring (20). RNAi is known to be inheritable in

Caenorhabditis elegans, making it feasible that the underlying genetic mechanisms are conserved in other nematode taxa (26). In spite of repeated attempts it was not possible to verify the production of *16D10*-specific siRNAs in the *Arabidopsis* and potato lines created here, a problem that has been encountered previously for other genes, and most likely due to the limited sensitivity of northern blot assays (16,74). *Meloidogyne* spp. generate a feeding tube during each feeding cycle and it may act as a filter to prevent clogging of the nematode's mouth spear (38). Earlier studies have shown that root-knot nematodes are able to ingest molecules of 28 to 140 kDa, including the green fluorescent protein and crystal proteins formed by the biocontrol agent *Bacillus thuringiensis* (50,67,73). It is unknown if *M. chitwoodi* took up plant-derived dsRNAs and processed them into siRNAs or whether the nematode directly ingested plant-produced siRNAs in this study. Given that *Meloidogyne* spp. are able to ingest relatively large molecules, either possibility is conceivable. Previous experiments have demonstrated that a match of 21 nt or less between siRNAs and a target sequence is sufficient to trigger RNAi in animals and that several mismatched base pairs do not interfere with the silencing process (39,43,57). There are numerous conserved regions between *Mi16D10* (upon which pART27(16D10i-2) was designed) and *Mc16D10L* that fall within this size range and could thereby trigger RNAi in *M. chitwoodi*.

Almost all commercial potato cultivars are autotetraploid ($2n = 4x = 48$), which makes classic breeding schemes complicated and time-consuming, especially if the high level of heterozygosity related to tetrasomic inheritance is taken into consideration (4). Introgressing traits from wild *Solanum* spp., e.g., nematode resistance genes, compound already challenging breeding strategies and have to overcome additional complications, such as pre- and postzygotic incompatibility barriers (29). Root-knot nematode resistance in wild *Solanum* spp. is relatively poorly characterized and a largely untapped resource (59). Given the challenges associated with classic potato breeding, transgenic strategies as described here could present an attractive alternative to breed root-knot nematode-resistant potatoes. One of the advantages of creating transgenic plants with an RNAi-based resistance against nematodes is that in principle no foreign protein is expressed in planta, thereby making the end product potentially more desirable than transgenic crops that express nematocidal peptides or proteins, such as cystatins or neurotransmitter antagonists (3,22). Ideal candidate genes for RNAi-based approaches are specific and only present in the target organisms, even though it has been

Fig. 6. Production of small RNAs in transgenic *Arabidopsis* and potato plants. **A**, U6 small nuclear RNA (snRNA) loading control, **B**, small RNA, and **C**, total RNA in *Arabidopsis* Columbia-0 wild type (COL), transgenic empty pART27 vector control (E2), and transgenic pART27(16D10i-2) (D1, D2, and D4), and in potato 'Desirée' wild type (DES), transgenic empty pART27 vector control (E29), and transgenic pART27(16D10i-2) (D54, D56, and D57) plants.

Fig. 7. Relative fold change of *Mc16D10L* transcript level in second-generation *Meloidogyne chitwoodi* from transgenic potato lines. **A**, Relative transcript abundance of *Mc16D10L* in *M. chitwoodi* eggs. **B**, Relative transcript abundance of *Mc16D10L* in *M. chitwoodi* infective second-stage juveniles (J2). Eggs were harvested from transgenic potato lines expressing the silencing construct pART27(16D10i-2) and used either directly for qRT-PCR or allowed to hatch infective J2 in modified Baermann pans. Potato lines included 'Desirée' wild type (DES), transgenic empty pART27 vector control (E29), and transgenic pART27(16D10i-2) (D54, D56, and D57). Each bar represents the mean of qRT-PCR runs in triplicate with standard errors. Letters indicate statistically significant differences using a Student's *t* test ($P < 0.05$).

reported that silencing of host genes can also result in decreased susceptibility against cyst and root-knot nematodes in *Arabidopsis* (68). *Meloidogyne* effectors usually lack homology to genes in other taxa, which makes them a worthwhile group of target genes for safe and specific root-knot nematode control (18). Furthermore, RNAi constructs against more than one effector gene can be stacked to achieve additive effects and aid in creating a more durable nematode resistance (16). In summary, the experiments described here demonstrate that specific silencing of the putative effector gene *Mc16D10L* leads to *M. chitwoodi* resistance not only in *Arabidopsis* but also in stable transgenic lines of potato, thereby opening the door to improved molecular breeding strategies for nematode resistance in this extremely important food crop.

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