Resistance to *Ditylenchus destructor* Infection in Sweet Potato by the Expression of Small Interfering RNAs Targeting *unc-15*, a Movement-Related Gene

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ABSTRACT

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Stem nematode (*Ditylenchus destructor*) is one of most serious diseases that limit the productivity and quality of sweet potato (*Ipomoea batatas*), a root crop with worldwide importance for food security and nutrition improvement. Hence, there is a global demand for developing sweet potato varieties that are resistant to the disease. In this study, we have investigated the interference of stem nematode infectivity by the expression of small interfering RNAs (siRNAs) in transgenic sweet potato

that are homologous to the *unc-15* gene, which affects the muscle protein paramyosin of the pathogen. The production of double-stranded RNAs and siRNAs in transgenic lines with a single transgene integration event was verified by Northern blot analysis. The expression of *unc-15* was reduced dramatically in stem nematodes collected from the inoculated storage roots of transgenic plants, and the infection areas of their storage roots were dramatically smaller than that of wild-type (WT). Compared with the WT, the transgenic plants showed increased yield in the stem nematode-infested field. Our results demonstrate that the expression of siRNAs targeting the *unc-15* gene of *D. destructor* is an effective approach in improving stem nematode resistance in sweet potato, in adjunct with the global integrated pest management programs.

Plant-parasitic nematodes are major agricultural pathogens that cause severe damage and huge agricultural losses annually on a global scale (Atkinson et al. 2012; Jones et al. 2013; Nicol et al. 2011; Oerke 2006). *Ditylenchus destructor* Thorne, commonly known as stem nematode, is one of the major pathogens of sweet potato (*Ipomoea batatas*) and causes significant yield loss of 20 to 50%; in endemic regions, there is a 100% yield loss (Xie et al. 2004; Xu et al. 2015; Zhang et al. 2006). In China, sweet potato is the fourth largest crop, and the parasitic nematode causes severe losses in the major growing regions, especially in North and East China (Xu et al. 2015). Because the situation is worsening with each passing year, an effective technique for the management of the disease is necessary (Xie et al. 2004; Xu et al. 2015). Currently, nematicides, crop rotation, and the use of resistant varieties are practical techniques for controlling plant-parasitic nematode diseases (Overstreet 2009). However, nematicides cause environmental pollution and have a deleterious effect on human health; crop rotation has some intrinsic limitations; and the narrow genetic background of cultivated sweet potato for nematode resistance makes it difficult to breed resistant cultivars by traditional breeding (Xie et al. 2004). Genetic engineering represents a promising approach for generating novel germplasm that is resistant to the parasitic nematode, and might additionally lead to a sustainable and effective control of the disease (Atkinson et al. 2012).

The main symptoms of stem nematode infection in sweet potato are stem necrosis and storage root rot; hence, severe infection often results in a dramatic decrease in yield or no yield (Scurrah et al. 2005). The above-ground part of infected sweet potato plants generally displays a weak disease phenotype, or none at all during the entire planting period, until the rot storage roots are finally harvested. Hence, the nature of the stem nematode infection also increases the difficulty of early diagnosis and control of the disease (Dutta et al. 2015; Wan et al. 2008). Study of stem nematode has been focusing on the analysis of population development, infestation development, and pathogenicity (Jones et al. 2013; Subbotin et al. 2005). Only recently, improved host resistance to stem nematode has been reported by the introduction of protein inhibitors (Gao et al. 2011) or molecular markers (Zhou et al. 2005), in sweet potato. In contrast to the stem nematode, most researches have been focused on cyst and root-knot nematodes for their broad host range and devastating impact on global agriculture (Charlton et al. 2010; Fairbairn et al. 2007; Fanelli et al. 2005; Huang et al. 2006; Yadav et al. 2006). Consequently, this knowledge has served as a useful guide in stem nematode studies.

RNAi technology has been useful for studying the interaction between plant host and parasitic nematode (Dutta et al. 2015). RNAi effects on *Caenorhabditis elegans* genes provided useful references and predictions for potential RNAi effects of homologous genes in plant-parasitic nematode (<http://www.wormbase.org>). For example, screening of candidate genes of *Heterodera glycines*, whose homologous genes in *C. elegans* have lethal phenotypes or phenocopies, revealed that the silencing of some genes, e.g., *Hg-rps-23*, by feeding on the double-stranded RNAs (dsRNAs) of the target genes led to loss of viability in the worm or development

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of fewer females (Alkharouf et al. 2007; Klink et al. 2007). The expression of hair-pin dsRNAs of nematode target genes in the host plants, following the dsRNA degradation into small interference RNAs (siRNAs) that silence the target gene of the nematode, has been proved to be effective in improving host resistance to nematode (Charlton et al. 2010; Dutta et al. 2015; Yadav et al. 2006). Successful RNAi target genes include a large number of parasite genes that expressed in the esophageal gland cells of nematodes and related to specific functions of the nematode such as penetration, migration, and maintenance (Bakhetia et al. 2008; Davis et al. 2008; Dinh et al. 2014; Huang et al. 2006; Souza Ddos et al. 2011).

In addition to the parasitic genes in nematodes, research was also focused on host plant genes, such as those expressing cellulose binding protein, 1,4- β -glucanase gene family, and pathogenesis-related genes (Hamamouch et al. 2011; Hewezi et al. 2008; Wiecezorek et al. 2008). Extensive genetic research on the host and parasite enabled a deeper insight into their interaction (Abad and Williamson 2010).

In order to effectively control stem nematode growth and propagation in sweet potato, it was opined that it would be suitable to interfere with the function of movement. Since movement is essential for its life cycle, interfering with muscle function might greatly affect nematode infectivity and propagation. The *unc-15* gene codes for paramyosin, which is a major thick filament component, playing an important role in muscle assembly (Benian et al. 2006). In nematodes, most muscle cells are located in the body wall, which aids in locomotion. RNAi phenotypes of the *unc-15* gene in *C. elegans* show severe symptoms, such as, being lethal to the larva, abnormal egg, abnormal locomotion, etc. (Fraser et al. 2000). This study investigated the interference effect of siRNAs homologous to the *unc-15* gene in transgenic sweet potato.

MATERIALS AND METHODS

Stem nematode cDNA library construction and sequence analysis. Stem nematodes (*D. destructor*) were collected from infected storage roots of cultivar Xushu22 after 30 days inoculation at room temperature. The cDNA library of stem nematode was constructed by Shanghai OE Biotech Co. Ltd. (Shanghai, China). Briefly, mRNAs were isolated from all stages of stem nematode reproduction and used to synthesize cDNAs by mRNA reverse transcription. These cDNA fragments were then inserted into a pMD-18T vector (TakaRa Dalian Co. Ltd., China) and transformed into *E.coli* DH5 α . About 2,000 clones were randomly selected and used for plasmid DNA extraction; sequencing was done using ABI 3730 DNA analyzer. Sequence analysis was performed using the blastx program in the blastall package (NCBI, version 2.2.9).

Identification of target genes and construction of RNAi binary vector. After sequencing, *D. destructor unc-15* clones were identified from the cDNA library. A 350-bp fragment encoding 116 amino acid C-terminal UNC-15 (Fig. 1A) was selected as the target region for RNAi. The sense and antisense fragments of *unc-15* were inserted into a modified cloning vector pBlueScript (PBS) that harbors a synthetic intron-containing hair-pin expression cassette. The synthetic intron is an AU-rich intron (Syn7) that can be processed efficiently in plant cells (Goodall and Filipowicz 1991). The hair-pin dsRNA-expressing cassette from the PBS was subcloned into the binary vector pCAMBIA1301 to generate the final binary vector pC-RNAi-unc15 (Fig. 1B). Primers used for vector construction are listed in Table 1.

Genetic transformation of sweet potato and transgenic plant regeneration. Sweet potato cultivar Xushu22, a popular starch-rich variety in North and East China, was used for the production of transgenic plants. The *Agrobacterium tumefaciens* strain LBA4404 harboring pC-RNAi-unc15 was used for sweet potato transformation as described by Yang et al. (2011). Briefly, embryogenic suspension of Xushu22 was used as explant for cocultivation with the LBA4404 strain harboring pC-RNAi-unc15, and transformed suspension clusters were selected from the SBM medium

(MS plus VB₁ at 0.3 mg/liter plus sucrose at 30 g/liter) containing hygromycin at 10 mg/liter, for 1 month. Then, hygromycin-resistant calli or somatic embryos were isolated and regenerated in the fresh SBM-cf200 medium (SBM plus cefotaxime at 200 mg/liter) containing hygromycin at 10 mg/liter. Regenerated plantlets were propagated and rooted on fresh regeneration medium. Independent transgenic plants were transferred to the greenhouse and fields for subsequent analysis.

Molecular analysis of transgenic plants by Southern and Northern blots. Total genomic DNA was isolated from the leaves of in vitro plants by the method described by Kim and Hamada (2005). DNA (20 μ g) was digested with *Hind*III, which is a unique restriction enzyme site in the T-DNA region of pC-RNAi-unc15 (Fig. 1B). A 500-bp DIG-labeled DNA fragment of *hpt* was amplified using the following specific primers: 5'-TTCTACACAGC CATCGGTCC-3' and 5'-CCCATGTGTATCACTGGCAA-3' with PCR DIG Probe Synthesis Kit (Roche Applied Science, Mannheim, Germany). Gel electrophoresis, DNA blot, hybridization, and detection were conducted using Roche hybridization and detection kit according to manufacturer's instruction (Roche Applied Science).

Northern blot was performed by the method described by Huang et al. (2006) with minor modification. Small RNAs were extracted from the mature leaves of greenhouse plants by using the mirVana miRNA isolation kit (Ambion, Austin, TX). Aliquots of 15 μ g of RNAs were separated on 15% acrylamide, 7 M urea, and 1 $\times$ Tris-borate-EDTA gels, and electro-transferred onto Hybond-XL nylon membranes (Amersham Biosciences, Piscataway, NJ) in 0.5 $\times$ Tris-borate-EDTA buffer. Probes of the target gene were labeled from the gel-purified restriction fragments of *unc-15* with biotin-dUTP using the North&South Biotin Random Prime DNA Labeling Kit (Thermo Scientific, Rockford), respectively. Hybridization was performed at 41°C for 16 h using North&South Chemiluminescent Hybridization and Detection Kit (Thermo Scientific). Stringency wash was performed at room temperature, and the membranes were exposed to X-ray films for 2 h before taking photos.

Plant growth evaluation in greenhouse and fields. The shoots of transgenic plants in bottles were transferred to round plastic pots (diameter, 35 cm; height, 25 cm) containing a mixture of vermiculite, turf and humus (1:1:1, vol/vol/vol) at a greenhouse in SIPPE, Shanghai for further growth. When transgenic plants grew for 2 to 3 months in pots, stems with apical part were cut (20 cm length) from the plants for field propagation. For infection trial, wild-type (WT) and transgenic plants were transferred to the field at the same time in the experimental station of Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu province. Thirty stems per line were randomly planted in the nursery field infested with stem nematodes (>100/g of soil). Simultaneously, five stems per line were planted in a normal field. Field conditions were the same in the nursery field and normal field. After 5 months of growth, the storage roots of WT and transgenic plants were harvested and their yields were recorded. Plant phenotype was recorded by photography during the growth period.

Storage root infection assessment by artificial inoculation. Storage roots harvested from normal field after 5 months of growth were used for assessing the extent of infection. Nematode *D. destructor* strain was provided by M.-S. Lin, Nanjing Agricultural University, and maintained by infected storage roots of sweet potato. Stem nematode was inoculated into storage roots of WT sweet potato for propagating controls. Second-stage juveniles (J2) of stem nematode were hatched from the egg mass collected from the infected sweet potato storage roots and used as inoculum (in 30% glycerol) for the subsequent infection assay (Southey 1986). Nematode inoculation assay was performed using the method described by Lin (1989). Each well-developed and healthy storage root was inoculated with approximately 1,000 J2 nematodes and the inoculated storage roots were maintained in an incubator in the dark at 22°C. Five storage roots per line were chosen for the nematode inoculation assay. All infection assays were conducted in triplicate.

To evaluate resistance of nematode infection by sweet potato, infection areas of the inoculated storage roots from the WT and transgenic plants were recorded 45 days after infection as described with modifications (Xie et al. 2009; Xie et al. 2010). The severity of disease symptoms in mature storage roots was evaluated on a scale of 0 to 4 (0, no symptom; 1, faint rotten, less than 5% rotten; 2, middle rotten, 5 to 10% rotten; 3, severe rotten, up to 50% rotten; and 4, severe rotten, with 50 to 100% rotten). The severity was evaluated using imageJ software (Schneider et al. 2012).

Gene expression analysis of nematodes cultivated by transgenic plants. Storage roots were infected by stem nematodes at different inoculation times and infected samples used for RNA preparation were harvested from the storage roots around the inoculation regions. For quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), total RNAs were extracted from the samples mixed by at least three storage roots per line using RNAlplant Plus Reagent (Tiangen Biotech, Beijing, China) as per the protocol provided by the manufacturer. Aliquots (2 µg) of total RNAs were reverse transcribed using 20 µl of ReverTra Ace (Toyobo, Osaka, Japan). The resulting cDNA was subsequently

diluted 10-fold with buffer. Gene-specific primers of the *unc-15* gene were designed as shown in Table 1. In order to avoid the amplification of regions from RNAi construct in the host plant, the primers for qRT-PCR were designed to amplify the N-terminal sequence from 49 to 200 bp of UNC-15. PCR (50 µl) was performed in SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), along with 1 µl of cDNA template and 5 µM each of forward and reverse primers. The PCR cycling parameters were set at 95°C for 10 min, followed by 40 cycles for 95°C for 15 s and 60°C for 1 min in CFX96 thermocycler (Bio-Rad, CA). qRT-PCRs were performed in triplicate with WT as negative control. The *D. destructor actin* gene (GenBank accession no. JN248784) was used as an internal control. The relative fold change was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All experiments were repeated three times, and the Student *t* tests were performed for significance analysis.

RESULTS

Identification of *unc-15* gene of *D. destructor* from a cDNA library. In *C. elegans*, muscles are characterized by

Fig. 1. Sequence analysis of *unc-15* and schematic diagram of the double-stranded RNA expression cassette targeting *unc-15* in the T-DNA region of the binary plasmid. **A**, Amino acid sequence alignment of the target region of *Ditylenchus destructor unc-15* in comparison with that of other parasites. The alignment was performed using ClustalX 2.0 and DNAMAN software. Black background indicates conserved residues among all the proteins selected. Nonconserved residues are indicated by shaded boxes. **B**, Two 350-bp fragments obtained from the C-terminal *unc-15* cDNA sequence were inserted into the cassette region in an opposite direction and were separated by the plant synthetic intron Syn7. **C**, Phylogenetic relationship between the proteins of *unc-15* and other parasites. The phylogenetic tree was generated by the neighbor-joining method using MEGA 5.0.

a dense body and M-line. Thick filaments are composed of myosin (UNC-54), paramyosin (UNC-15), and myosin heavy chain A (MYO-3), and anchored to the M-line on one side and bound to the dense body on the other side by the protein titin (Klink et al. 2007). UNC-15 of nematodes is an important component of muscle constructs. Two thousand clones from a cDNA-library of *D. destructor* were randomly selected for sequencing, in order to identify the *unc-15* gene. Several clones of the candidate *unc-15* gene and their full cDNA length were assembled from derived sequences (GenBank accession no. JN216830.1). The phylogenetic tree analysis (Fig. 1C) of *unc-15* shows that *unc-15* in *D. destructor* has 91% identity with homologous genes in *C. elegans*, *C. briggsae*, and *C. remanei*, has 90% identity with that in *Dictyocaulus viviparus* (AAT36324), and 89% with that in *Dictyocaulus viviparus* (ABW05396), and identity values with other nematode species were $\leq 88\%$. This gene was used as the target gene for interfering *D. destructor* development and growth by RNA silencing. Since their C-terminal showed relatively less sequence homology among aligned sequences (Fig. 1A), the mRNA sequence of this region was selected as target for constructing RNAi expressing cassette (Fig. 1B).

Production and Southern blot analysis of *unc-15* RNAi transgenic plants. The embryogenic suspensions of Xushu22 induced from shoot meristems were used for *Agrobacterium*-mediated transformation of sweet potato (Yang et al. 2011). In total, 21 independent transgenic lines transformed with pC-RNAi-unc15 were produced and propagated in vitro by shoot culturing. The success of T-DNA integration was verified by Southern blot analysis, wherein more than half of transgenic plant lines had undergone single insertion events (14 single copy lines). Of these, five *unc-15* RNAi plant lines having a single integration event (Fig. 2A) were transferred into pots for further growth, and eventually planted into field for phenotype analysis.

DsRNA and siRNA detection in *unc-15* RNAi transgenic plants. To confirm the proper expression of hair-pin dsRNA,

Northern analyses were performed using the small RNAs extracted from the leaves of greenhouse-grown transgenic plants. Two ~20- to 24-nt siRNA bands and the 300-bp dsRNA fragment of *unc-15* (Fig. 2B) were detectable in all the RNAi transgenic plants, after hybridization with biotin-labeled *unc-15* probe, indicating that the dsRNAs expressed from the RNAi cassette of transgenic plants could be successfully processed to form siRNAs. Among the five test lines, *unc-15-4* detected the weakest signal of siRNA, although its dsRNA signal was the strongest.

Phenotype and yield evaluation of *unc-15* RNAi transgenic plants. Under the normal greenhouse condition, all the plant lines showed a normal development and growth was indistinguishable between WT and transgenic plants (Fig. 3A). Once the plants were transferred to the field, all transgenic lines showed a healthy growth, and produced storage roots without any changes in yield, when compared with WT after 5 months (Fig. 3B). In order to study the effects of stem nematode infection on storage root production, the WT and transgenic plants were also grown in the nematode-infested field for 5 months, simultaneously. After harvesting, four of five tested transgenic plants produced significantly more storage root biomass than WT plants in the nematode-infested field (Fig. 3C). The line *unc-15-4* showed no difference with WT control, possibly due to its low siRNA level as shown in Figure 2B. These results imply that the transgenic plants developed a high resistance to stem nematode infection and performed better under the biotic stress.

Stem nematode infection assay of harvested storage roots by artificial inoculation. As sweet potato resistance assessment toward stem nematode in the field is time-consuming and unreliable (Gao et al. 2011), early screening of nematode resistance in sweet potato was unsuitable. In laboratory, artificial inoculation is often used as an alternative approach for sweet potato resistance evaluation. After inoculation, the brown infection region around the inoculation spot in sweet potato storage root demonstrated the extent of infection (Fig. 4). The nematodes usually develop

TABLE 1. Primers used for amplifying gene fragments of double-stranded RNA expression cassette and target and nontarget genes in polymerase chain reaction (PCR) analyses

Primer name	Primer sequence (5'-3')	Product size (bp)	Gene name and accession number
PCR detection			
unc-15-F	ACCGCGATTTGGAAACAGCT	350	<i>Ditylenchus destructor unc-15</i> JN216830
unc-15-R	GATGGGAGTTGCTCTATTGGCG		
Quantitative RT-PCR detection			
qRT-act-F	TGGTTACTCGTTCACCACCA	210	<i>D. destructor actin</i> JN248784
qRT-act-R	AAGGAAGGCTGGAAGAGAGC		
qRT-acdh-1-F	TCTCGAAAGTTGACCTTGCT	214	<i>D. destructor acdh-1</i>
qRT-acdh-1-R	TAGTCGTCCGCGTCTTTCTT		
qRT-fum-1-F	TCCGAGTGAACGTGATACA	188	<i>D. destructor fum-1</i>
qRT-fum-1-R	GAGCCCGTATTCTTGTTCA		
qRT-sp1-F	CGGAAAAGTTGGTGTCAC	234	<i>D. destructor sp1</i>
qRT-sp1-R	TACTCCGCGATTGGTATCC		
qRT-sp1-4-F	AGCTCAACTCGCAGGTCAAT	169	<i>D. destructor sp1-4</i>
qRT-sp1-4-R	TTGAGGGGCCATGTAAGAAG		
qRT-unc-15-F	CCGAAGTTGAACCTCGAGGAG	152	<i>D. destructor unc-15</i> JN216830
qRT-unc-15-R	ATCAGCAGCGTGGAGTTCTT		
qRT-unc-22-F	GCGATGTGTTTCAGCTTCAAA	163	<i>D. destructor cts-1</i>
qRT-unc-22-R	TGCATCGGTATGCACAATTT		
qRT-unc-29-F	TTTGATTGATTTCGCAACG	228	<i>D. destructor cts-1</i>
qRT-unc-29-R	AGAGGTTTGGTGTGGTCTCTG		
qRT-unc-31-F	GATTAGCCCGGTACGATGAA	163	<i>D. destructor cts-1</i>
qRT-unc-31-R	CCTCCGGTCGTGATAGTTGT		
qRT-unc-60B-F	CGGCCAAAATCAATCAAGTT	246	<i>D. destructor unc-60B</i>
qRT-unc-60B-R	AAGTGATCATCGAGCCATCC		
qRT-unc-87-F	CAAGAGGGCATGACAGGATT	192	<i>D. destructor unc-87</i>
qRT-unc-87-R	GTGCCTTGAGGACATTCGTT		
qRT-unc-101-F	GAGAGATGCGGACACTCCTC	224	<i>D. destructor unc-101</i>
qRT-unc-101-R	CCTGGATTCCGGACGTAGTA		
qRT-unc-105-F	CTGCTGGAGGCGAAAATAAG	181	<i>D. destructor unc-105</i>
qRT-unc-105-R	ACAACATCTCCGGTGTGTGA		
qRT-vars-1-F	CGCGGTAAGAAGGTATTGGA	244	<i>D. destructor vars-1</i>
qRT-vars-1-R	GCGAACCGAACAAAAACAAT		

a concentric circle infection area that is seen in transverse sections of the sweet potato storage root from the inoculation site. The resistance evaluation method of Xie et al. (2009, 2010) was adopted, with some modifications. Infection areas observed in the transverse sections of storage root were evaluated 45 days postinfection (Fig. 4). The results showed a significant difference in storage roots between WT and transgenic plants. The WT storage roots showed the typical severe symptom of storage decay due to the infection by stem nematodes. Some roots showed completely rotted with white eggs of stem nematodes while others were partially infected (Fig. 4A). The infection area of transgenic storage root was considerably lower than that of WT storage roots. Compared with WT's, the infection areas in transgenic lines were lower by at least 50% (Fig. 4B). Some inoculated storage roots of transgenic lines showed no significant spreading of nematodes in the inoculation site (Fig. 4A). Weak rot around their outer layer was detected in some plants. These observations confirmed that the transgenic lines were able to develop resistance against stem nematode infestation. These results indicated that the RNAi of target gene *unc-15* satisfactorily interrupted the nematode development, which led to less damage to sweet potato storage roots.

qRT-PCR analysis of genes related to stem nematode locomotion. To assess whether the expression of target gene *unc-15* in stem nematode was interfered, the mRNA transcript levels after inoculation of storage roots were performed by qRT-PCR. There was no significant change in the expression of *unc-15* in stem nematodes that fed on WT plants, at all time points. This was

consistent with the stem nematode life style, which migrates in sweet potato storage root throughout its life, with no sedentary stage. In contrast, mRNA transcript of *unc-15* was significantly down regulated by two to three folds in the transgenic storage roots at all magnifications (Fig. 5). The results showed that the targets gene was silenced after stem nematode infection of transgenic storage roots.

Fig. 2. Molecular characterization of *unc-15* RNAi transgenic sweet potato by Southern and Northern blot analyses. **A**, Integration pattern of T-DNA in the *unc-15* RNAi plant lines detected by DIG-labeled hygromycin transferase gene (*hpt*) probe in Southern analysis. Genomic DNA (20 µg) extracted from transgenic plants and wild-type (WT) plants were digested with *Hind*III. M, DNA molecular marker; P, plasmid control digested by *Hind*III; WT, wild-type plant of cultivar Xushu22; Line 1 to 5, different *unc-15* RNAi transgenic plant lines. **B**, Double-stranded RNA and siRNA detection in *unc-15* RNAi transgenic lines (1 to 5) with 300-bp biotin-labeled *unc-15* probe in Northern analysis. Small RNA (15 µg) extracted from leaves of WT and *unc-15* RNAi transgenic plants was used.

Fig. 3. Phenotypic evaluation of wild-type (WT) and *unc-15* RNAi transgenic plant lines in greenhouse and field conditions under the normal and stem nematode-infested fields after 5-month growth. **A**, Plant growth status in pots under greenhouse condition. **B**, Storage root biomass of WT and the transgenic plants in a normal field. Each bar represents the mean + standard error (SE) of five plants. **C**, Storage root biomass of the transgenic and WT plants in stem nematode-infested field. Each bar represents the mean + SE of 30 plants. Asterisks indicate a significant difference from that of WT at * $P < 0.05$ or ** $P < 0.01$ by the Student's *t* test.

To further check the impact of silencing of *unc-15* on the expression of other genes that were related to movement, 12 genes were further analyzed for their expression in storage roots at 30 days postinfection. Three of the twelve genes, *unc-105*, *unc-29*, and *unc-31*, were found to have altered expression levels, especially *unc-29* (Fig. 5B). These changes indicate that the silencing of *unc-15* expression directly affect the function of the close-knit UNC family genes.

DISCUSSION

Improvement in resistance toward stem nematode infection has become an important objective in sweet potato breeding and field management. In this study, transgenic sweet potato plants that expressed siRNA targeting to *unc-15* were obtained. The results of this study reveal that transgenic plants have normal phenotype and high resistance toward stem nematode infection, both in field

evaluation and artificial inoculation assay, than that of WT plants. This indicates that interference in stem nematode movement by the expression of siRNAs targeting to *unc-15* in sweet potato is effective.

It is widely accepted that the expression of hair-pin dsRNA in plant hosts could have an interfering effect on its pathogen, in which the dsRNA of the host could be degraded to siRNAs for silencing the target gene. This technique has been explored successfully in cyst and root-knot nematodes (Charlton et al. 2010; Fairbairn et al. 2007; Fanelli et al. 2005; Huang et al. 2006; Ibrahim et al. 2011; Li et al. 2010; Urwin et al. 2002; Yadav et al. 2006). To select the target genes, one strategy was based on RNAi with evident phenotypes of corresponding genes in *C. elegans*. The lethal phenotype was preferred for selecting homologous genes of the pathogen. For example, Alkharouf et al. (2007) obtained many candidates genes of *H. glycines* that were homologous with *C. elegans* genes with a lethal phenotype and one of these genes, *Hg-rps-23*, was silenced successfully leading to death of the nematode. The expression pattern of the movement-related genes that belong to the uncoordinated (*unc*) family was also investigated in *H. glycines* (Klink et al. 2007). The UNC family members perform many important functions in *C. elegans*, including focal adhesion, architecture, and stimulation, thus rendering a lethal phenotype by RNAi (Zengel and Epstein 1980). UNC-15 plays an important role in nematode movement, and an RNAi phenotype in the larva is lethal in *C. elegans*.

Fig. 4. Comparison of stem nematode resistance in storage roots of wild-type (WT) and *unc-15* RNAi transgenic plants after 45 days post inoculation (dpi). **A**, Extent of rot in the storage roots. At least three storage roots per line were used for the assay. **B**, Evaluation of infected areas in storage roots according to the symptom scales. Each bar represents the mean + standard error (SE) of three storage roots per line. All transgenic lines showed significantly less infection than that of WT plants ($P < 0.05$ and 0.01 , Student t test).

Fig. 5. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis of *unc-15* and other 12 movement-related genes in stem nematodes collected from inoculated storage roots. **A**, Transcript changes of *unc-15* gene in infected wild-type (WT) and five transgenic lines at 0, 10, 20, and 30 days postinfection (dpi). **B**, Transcript changes of 12 movement-related genes at 30 days postinfection in the *unc15-1* transgenic line. The sample at 0 dpi served as a control. *spn-1*, serpin 1; *unc-105*, degenerin; *spn-4*, SPiNdle orientation defective 4; *acd-1*, acyl-CoA dehydrogenase 1; *fum-1*, fumarase 1; *unc-101*, adaptin; *vars-1*, valyl-tRNA synthetase; *unc-22*, twitchin; *unc-29*, nonalpha subunit of the nicotinic acetylcholine receptor (nAChR) superfamily; *unc-31*, pleckstrin; *unc-60B*, cofilin; and *unc-87*, actin-bundling protein. Each bar represents the mean + standard error of qRT-PCR reactions performed in triplicate.

In our study, interferences in the *unc-15* expression in *D. destructor* by expressing siRNAs of *unc-15* reduced its infectivity and rendered the transgenic plant resistant to stem nematode infection.

RNAi mechanism is well known in plant, but reports on simultaneous detection of dsRNA and siRNA in transgenic plants (Huang et al. 2006) were limited. In our study with transgenic sweet potato, both dsRNA and siRNA were detected by Northern blot analysis, indicating that the simultaneous detection of dsRNA by hair-pin dsRNA-expressing cassette and siRNAs in sweet potato cells was feasible. Transgenic sweet potato plants expressing with *unc-15* siRNAs developed resistance to stem nematode in their storage roots, thus proving the feasibility of studying the interaction between plant host and pathogen. The results of our study confirmed a change in the expression of other native genes in stem nematodes collected from inoculated storage roots of transgenic plants.

Differences in the expression pattern of *unc-15* in *D. destructor* and *H. glycines* in nontransgenic plants might be due to differences in their life cycle. As a major component of muscles, thick filaments are composed by myosin (UNC-54), paramyosin (UNC-15), and myosin heavy chain A (MYO-3) in *C. elegans* (Klink et al. 2007). The *unc-15* is expressed at all stages of the lifecycle in *C. elegans*. This result was in agreement with *D. destructor* parasite life cycle that does not have sedentary stage and syncytium formation, and they are in constant locomotion in sweet potato storage root throughout their entire life cycle. In *H. glycines*, *unc-15* expression level was the lowest at 15 days postinfection and the highest at the J2 stage, which is suitable for its sedentary stage with less movement (Klink et al. 2007).

There are two different strategies in sweet potato to avoid the nematode infection namely, anti-invading and anti-extending. Proper assessment of the resistance is necessary. Currently, the two different approaches for evaluating sweet potato resistance toward stem nematodes is either by field evaluation in stem nematode-infested field or by artificial inoculation of stem nematode in harvest storage roots (Xie et al. 2010). In a field, stem nematode can cause dramatic reduction in sweet potato yield, when their population is high; and because this duration lasts several months, it is the most reliable method for screening resistant cultivars. In our study, the yield of sweet potato in the infested field was significantly lower than the *unc-15* RNAi transgenic plant lines, which had a better yield. This suggests that the transgenic plants were truly resistant to stem nematodes. The resistance capacity in transgenic plants was further confirmed by the artificial inoculation of mature storage roots. One major difference in artificial inoculation was the evasion of the epidermis barrier by the stem nematodes during invasion, thereby facilitating a direct entry of the nematodes into the inner tissues of sweet potato storage roots. Our results on artificial inoculation clearly demonstrate that the passage of stem nematodes inside the transgenic storage roots was repressed, as evidenced by a reduction in the rotten area around the inoculation sites.

In summary, the RNAi approach is an efficient tool for improving resistance to stem nematode by sweet potato or for studying the host–pathogen interaction. The siRNA generated in the storage roots of transgenic sweet potato silenced the *unc-15* gene expression and changed the expression of other nontarget genes in stem nematode, thus rendering the transgenic plants resistant to stem nematode infections. This study showed that targeting the interference of stem nematode movement is feasible in sweet potato for achieving resistance against stem nematode.

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