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Research Article

Transgenic Soybean Plants with Root-Expressing siRNAs Specific to *HgRPS23* Gene are Resistant to *Heterodera glycines*

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Abstract

The soybean cyst nematode (SCN, *Heterodera glycines*) poses a serious threat to soybean production worldwide. RNA interference approach was taken in this study to silence the essential SCN ribosomal protein gene *HgRPS23* in the roots of transgenic soybean plants. The treatment of SCN J2 worms with the *HgRPS23* dsRNA produced by *in vitro* transcription has been previously shown to lead to SCN lethality. Our analysis indicated that the conserved *HgRPS23* 225 bp shares high homology with 5'-terminal sequence of the *Caenorhabditis elegans CeRPL1* gene, and that the predicted small interfering RNA (siRNA) species from the *HgRPS23* 225 bp dsRNA finds its exact match in CePRL1. Our results showed that soaking *C. elegans* L2 worms in *HgRPS23* 225 bp dsRNA could result in *C. elegans* lethality. Transgenic soybean plants (cv. Williams 82) were produced with *Agrobacterium*-mediated transformation to express the hairpin structure of the *HgRPS23* in the roots with the *Arabidopsis* root-specific *pyk10* promoter. The TaqMan probe-based RT-qPCR analysis with a uniquely designed key-like RT primer demonstrated that the predicted *HgRPS23* siRNA was highly expressed only in the roots of transgenic soybean plants. Our data showed that the *HgRPS23* siRNA-expressing transgenic soybean plants were highly resistant to SCN infection.

Keywords: Heterodera glycines; *HgRPS23* siRNA; SCN Resistance; TaqMan RT-qPCR; Transgenic Soybean

Abbreviations

BAP: 6-Benzylaminopurine; Ce: *C. elegans*; ds: Double-Stranded; GA: Gibberellic Acid; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; RP: Ribosomal Protein; siRNA: Small Interfering RNA; RT-qPCR: Reverse Transcription Real-Time PCR; SCN: Soybean Cyst Nematode; wt: wild type

Introduction

The soybean cyst nematode (SCN, *Heterodera glycines*) poses a continual and serious threat to soybean production in the U.S. and around the globe [1]. Rotations of soybeans with non-host crops like corn can provide significant reductions in SCN population levels but they are not always practical or economical for soybean growers [2]. Resistance to SCN has been bred into available soybean cultivars and remains a mainstay in SCN management. Since the genetic base of resistance to SCN in cultivated soybean currently remains narrow, however, the relatively rapid emergence of HG-Types of SCN that break resistance continues to be a challenge to effective SCN management [2].

The production of transgenic soybean plants that are herbicide resistant through biotechnology has practically revolutionized soybean production in the world [3]. The potential to develop novel forms of resistance to SCN in genetically-engineered soybean also presents an opportunity to develop broad-spectrum and durable resistance to SCN. Over the last two decades, different transgenic soybean plants have been produced with improved resistance to different pathogens [4,5] pests [6] and altered or enhanced nutritional values [7]. The transgenic approach has also been used to confer resistance to SCN in transgenic soybeans. Lin., *et al*. showed that overexpressing a soybean salicylic acid (SA) methyltransferase gene, *GmSAMT1*, in the susceptible Williams 82 soybean hairy roots affected the expression of selected genes involved in SA biosynthesis and SA signal transduction, and resulted in significant reduction of SCN development [8]. Subsequently, transgenic soybean plants were produced to overexpress *GmSAMT1* and found to resist multiple SCN HG-Types [9]. Additionally, overexpression of the *Arabidopsis AtNPR1, AtTGA2* and *AtPR-5* genes that are involved in plant defense signaling in transgenic soybean roots decreased the number of SCN cysts by more than 50% compared to non-transformed roots [10].

RNA interference (RNAi) technology has also been explored to engineer SCN resistance in soybean plants. Potentially, transgenic soybean plants can be developed to express an RNAi construct that is specifically designed against SCN. If the RNAi construct is

designed against an SCN gene that is essential for SCN parasitism or survival, SCN feeding on transgenic soybean expressing the target double-stranded RNA (dsRNA) will trigger the RNAi pathway in SCN and disrupt the SCN life cycle. Using this technology, Steeves., *et al.* [11] have produced transgenic soybeans (cultivars Jack and Chapman) expressing small interfering RNAs (siRNAs) specific to a major sperm protein gene of SCN with the constitutive promoter of the *Arabidopsis ACT2* gene. Their results showed that SCN feeding on the T0 transgenic soybean plants resulted in up to 68% reduction in egg number per gram of root tissue [11]. Recently, Peng., *et al.* [12] showed that expressing the RNAi construct of *Hg-pel-6* encoding a nematode pectate lyase in the transgenic Williams 82 soybean hairy roots resulted in a 30.4-39.1% reduction in the number of SCN compared to the green fluorescence protein (GFP) control at 7 dpi (day post inoculation). Silencing of the *SCN 30C02* parasitism gene transcript by plant host-derived RNAi resulted in significant reduction in cyst female development on test plants [13]. Knocking down a soybean host factor has also been employed to engineer SCN resistance. It was shown that the expression of an RNAi construct targeting the putative soybean CLE (CLAVATA3/ENDOSPERM SURROUNDING REGION) receptor, CLAVATA2 by the soybean constitutive promoter p15 (promoter for Glyma15g06130) led to 32% reduction of SCN infection in transformed soybean hairy roots [14]. Using comparative bioinformatics approach, Alkharouf *et al.* identified nearly 8334 conserved genes between SCN and the model round worm *Caenorhabditis elegans* (*C. elegans*), of which 1508 have lethal phenotypes in *C. elegans* [15]. RNAi of one of the 1508 putative SCN lethal genes, *HgRPS23*, resulted in quantitative silencing of the critical ribosomal gene and subsequent lethality of the treated SCN J2 by soaking in dsRNA solution [15]. While plant host-derived RNAi targeted to nematode transcripts has generally used constitutive expression by CaMV 35S promoter leading to potential consumer concerns of genetically modified organism (GMO), the aim of this study was to engineer the expression of siRNAs against the *HgRPS23* gene only in the roots of transgenic soybean plants and assay for anti-SCN effects. Our results indicate that our T_1 and T_2 transgenic *HgRPS23*-RNAi soybean plants were resistant to SCN infection.

Materials and Methods

Construction of *HgRPS23* RNAi transformation vector

The reported 362 bp of *HgRPS23* gene (GenBank accession No. BF014259) [15] was compared to the *C. elegans CeRPL1* (Y71F9AL.13, GenBank accession No. NM_058660). The most conserved 225 bp within the 362 bp of *HgRPS23* was selected to make the hairpin construct for soybean RNAi vector. In order to express the siRNA only in soybean roots, the root-specific promoter of *Arabidopsis pyk10* gene [16,17] was chosen to drive the expression of the *HgRPS23* hairpin structure. The intron to separate the *HgRPS23* inverted repeats was from the pyruvate orthophosphate dikinase as in the pHANNIBAL vector (GenBank accession No. AJ311872). The terminator was from the octopine synthase as in pBI121 vector (GenBank accession No. AJ485783). The complete *HgRPS23* cassette was synthesized by GenScript (Piscataway, NJ), and digested by *Hind*III and *SacI* and then sub-cloned into the soybean expression vector pTF101.1 supplied by the Plant Transformation Facility, Iowa State University [18] resulting in pRD64 (Figure 1).

Evaluation of *HgRPS23* RNAi in *C. elegans*

The effectiveness of *HgRPS23* siRNA in silencing the worm's ribosomal protein gene was tested in *C. elegans,* following the previously published protocols [12,15] with modifications. A T7 promoter sequence (5'-TAATACGACTCACTATAG-3') was added to the 5' ends of both the forward and reverse primer to amplify the 225 bp of *HgRPS23* from pRD64 by PCR. The amplified PCR product was used to produce the double stranded *in vitro* transcript by the MEGAscript®T7 kit (Ambion/Invitrogen, Calsbad, CA, USA). Similarly, the T7 promoter sequences were added to the forward and reverse primers to amplify a 250 bp fragment of the enhanced green fluorescence protein (eGFP) gene (GenBank accession No. HQ423139) from a plasmid containing eGFP. eGFP dsRNA was also produced to serve as a control in the *C. elegans* soaking experiment.

The wild type (wt) N2 strain of *C. elegans* was obtained from the Caenorhabditis Genetics Center and maintained on Nematode Growth Medium (NGM) plates (60 mm Petri dishes) at room temperature. The worms were grown and synchronized using protocols from the WormBase (www.wormbase.org). The worms at L2 stage were soaked in 100 µl M9 buffer (22 mM KH_2PO_4, 4.7 mM NH4Cl, 43.6 mM Na_2HPO_4 and 2.1 mM NaCl) containing 50 mM octopamine (Sigma, St. Louis, MO, USA) to increase pharyngeal pumping and 2 mg/ml *HgRPS23* dsRNA or eGFP dsRNA. The negative control L2 worms were treated by soaking in M9 buffer containing 50 mM octopamine only. The positive control L2 worms were soaked in M9 buffer containing 50 mM octopamine and 10 µM cycloheximide (Sigma, St. Louis, MO, USA) as a translation inhibitor. *C. elegans* worms were treated in the wells of a 96-well

plate on a gentle shaker. The vitality of worms was observed under a stereo microscope. Treated worms were then collected by centrifugation at 300g for 2 min and stained with M9 buffer containing 1 µM SYTOX Green nucleic acid stain (Invitrogen, Calsbad, CA, USA). The stained dead worms were distinguished from the non-stained live worms by observation under the Olympus SZX16 stereomicroscope (Olympus, Tokyo, Japan).

Production of transgenic soybean plants expressing SCN-specific siRNAs

The *HgRPS23* siRNA vector pRD64 was provided to the Plant Transformation Facility, Iowa State University and was transformed into *Agrobacterium tumefaciens* strain EHA101 for soybean transformation [18]. Briefly, half-seed explants were excised from disinfected soybean (cv. Williams 82) mature seeds that had been soaked in sterile water overnight. The explants were incubated with A. *tumefaciens* containing pRD64 for 30 min and then co-cultivated for 5 days. Explants were incubated on shoot induction medium containing B5 medium and vitamins, 30 g/l sucrose, 1.11 mg/l 6-benzylaminopurine (BAP) and the antibiotic regime of 50 mg/l timentin, 200 mg/l cefotaxime and 50 mg/l vancomycin for 14 days. The explants were subsequently transferred to the shoot induction medium supplemented with 6 mg/l glufosinate for 14 days under 18:6 photo-period. After another 14 days on shoot induction medium with 6 mg/l glufosinate, the explants were transferred to shoot elongation medium containing MS salts and B5 vitamins supplemented with MSIII iron stock, 30 g/l sucrose, 0.1 mg/l indole-3-acetic acid (IAA), 0.5 mg/l (gibberellic acid) GA3, 1 mg/l zeatin riboside, the antibiotic regime as above and 6 mg/l glufosinate to select transgenic shoots. Individual shoot was dipped in 1 mg/l indole-3-butyric acid (IBA) and transferred to rooting medium containing MS salts and B5 vitamins, MSIII iron stock and 20 g/l sucrose, without the glufosinate. Rooted plantlets were transferred to soil and acclimatized to greenhouse conditions. T1 transgenic soybean seeds were shipped back to Rutgers University with the USDA-APHIS transgenic plant movement permit.

Regenerated T_0, T_1 and T_2 transgenic soybean plants at two trifoliate leaf stage were tested by the herbicide paint assay to confirm their expression of the *bar* gene [18]. The FINALE herbicide was diluted with water to contain 150 mg/l of the active ingredient of glufosinate ammonium. A Q-tip was used to evenly paint the diluted herbicide over the trifoliate leaves. Plants were observed visually every day for the symptoms of yellowing and necrosis on the leaves.

Analysis of *HgRPS23* siRNA expression level in transgenic soybean by TaqMan-based RT-qPCR

The siRNA species that could be potentially produced from the *HgRPS23* 225-bp RNAi repeat in transgenic soybean plants were predicted by the online program SIRNA from EMBOSS (http://emboss.sourceforge.net/apps/release/6.0/emboss/apps/sirna.html) [19]. siRNA species (5' AAACUACGACCCACAGAAGGA 3') had the top score of 9.0. The expression level of this *HgRPS23* siRNA in pRD64-transgenic soybean plants was evaluated by RT-qPCR using the uniquely designed key-like RT primer and TaqMan probe [19] (Figure 4). The 5× key-like primer and 20× TaqMan assay containing the forward and reverse primers and the TaqMan probe for the predicted *HgRPS23* siRNA species were synthesized by Applied Biosystems (Thermo Fisher, Waltham, MA, USA). Total RNA was isolated from each soybean plant using the TRIzol® reagent (Invitrogen, Calsbad, CA, USA) following the manufacturer's instructions. The RNA concentration was measured by a Nanodrop spectrophotomer (Thermo Fisher, Waltham, MA, USA). Using 100 ng RNA per sample, 1× key-like primer, reverse transcription reaction was conducted with the High Capacity cDNA Synthesis Kit (Applied Biosystems, Thermo Fisher, Waltham, MA, USA) in a 10 µl reaction. After 1:1 dilution of the cDNA with sterile water, qPCR reaction was performed with 1 µl of the diluted cDNA, 1× TaqMan assay, and 1× TaqMan master mix (Applied Biosystems, Thermo Fisher, Waltham, MA, USA) in a 10 µl reaction with the StepOnePlus thermocycler (Applied Biosystems, Thermo Fisher, Waltham, MA, USA). The cycling condition was as follows: 50oC, 2 min, 1 cycle; 95oC, 10 min, 1 cycle; 95oC, 15 sec, 60oC, 1 min, 40 cycles. The $2^{-\Delta\Delta Ct}$ relative quantification method was used to analyze the siRNA (5' AAACUACGACCCACAGAAGGA 3') level, in the roots with soybean MIR156b (5' UGACAGAAGAGAGAGAGCACA 3') [20] as the reference, and in the leaves with MIR159 (5' UUUGGAUUGAAGGGAGCUCUA 3') as the reference [21].

Analysis of transgenic soybean for SCN resistance

The T_1 and T_2 transgenic soybean plants were inoculated with *H. glycines* OP50 eggs as described [13] to test their resistance to SCN. Briefly, *H. glycines* OP50 nematodes were maintained on roots of greenhouse-grown soybean plants. Cysts were collected by stacked 850 µm and then 250 µm sieving from infected soybean roots after 2 - 3 months of infection. SCN eggs were isolated from the cysts by 70% sucrose solution separation and 25 µm sieving and quantitated. Transgenic and wt soybean (Williams 82) seeds were sowed in 150 ml plastic cone containers and plants were maintained in a greenhouse. After seeds were germinated for 2 weeks, 5000 SCN eggs were inoculated into each cone containing a single plant. Fifty days post inoculation, plant height was measured, roots were separated from plants and weighed. The SCN cysts and eggs were collected and counted from roots of test plants. The Student *t*-tests were used to assess the significance of difference between samples (*$p < 0.05$, **$p < 0.01$).

Results and Discussion

Construction of *HgRPS23* RNAi plant transformation vector

As shown by Alkharouf., *et al.* [15] RNAi with dsRNA produced from a 362 bp-fragment of the SCN ribosomal *HgRPS23* gene (accession No. BF014259) led to lethality of SCN J2 nematodes. By using the NCBI (National Center for Biotechnology Information) BLAST online tool, we found that the N-terminal 75 amino acid sequence from this 362-bp fragment of *HgRPS23* shares 73.3% homology with the *C. elegans* N-terminus of the ribosomal protein large subunit (WP: CE25552 Y71F9AL.13, *CeRPL1*, accession No. NM_058660). The nucleotide sequences between *HgRPS23* and *CeRPL1* also share 76.4% homology. We selected the most conserved 225 bp within the *HgRPS23* 362-bp fragment to construct the RNAi plant expression vector. The BLAST online tool and the soybean genomic database in NCBI were used to search for similarities between the *HgRPS23* 225 bp and any of the soybean genes. The negative search result ensured that our RNAi plant expression vector would not produce any siRNA against the soybean host gene.

Since SCN attacks the roots of soybean, we took the approach of expressing siRNA against the SCN ribosomal protein gene *HgRPS23* only in soybean roots with the *Arabidopsis* root-specific *pyk10* promoter to reduce potential off-target effects and to alleviate consumer's GMO concerns. It has been shown that the *Arabidopsis pyk10* (myrosinase or thioglucoside glucohydrolase) promoter (accession No. AJ292756) shows virtually no activities in other parts of the mature plant except in roots [16,17] showed that this promoter could drive the expression of the *Aspergillus ficuum* phytase gene in transgenic soybean roots. Therefore, the 1443 bp-fragment of Arabidopsis *pyk10* promoter was chosen to drive *HgRPS23* siRNA expression. As shown in Figure 2, the 225-bp *HgRPS23* sense (S) and anti-sense (AS) sequences are flanked by an intron (742 bp) from the *pyruvate orthohphophate dikinase* gene as in the siRNA vector pHANNIBAL (accession No. AJ311872), and terminated by the terminator from the *octopine synthase (ocs)* gene. The cassette of $P_{Atpyk10}$::*HgRPS23*-S::intron::*HgRPS23*-AS::T_{OCS} was synthesized by GenScript (Piscataway, NJ) and sub-cloned into pTF101.1 plant expression vector provided by the Plant Transformation Facility, Iowa State University (ISU).

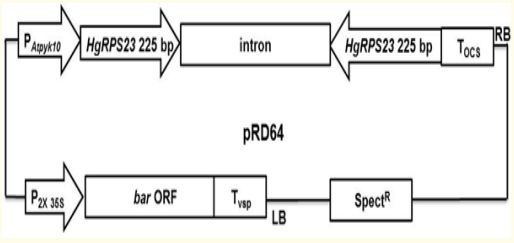

Figure 1: Schematic diagram of the *HgRPS23* siRNA plant transformation vector pRD64. The root-specific promoter of Arabidopsis *pyk10* gene, the 225 bp sense and anti-sense fragments from *HgRPS23* gene (GenBank accession No. BF014259), the pyruvate orthophosphate dikinase intron and the octopine synthase terminator were synthesized and sub-cloned into the pTF101.1 vector (Plant Transformation Facility, Iowa State University).

RNA interference in *C. elegans* by *HgRPS23* siRNAs

Using the online program SIRNA from EMBOSS (http://emboss.sourceforge.net/apps/release/6.0/emboss/apps/sirna.html) [19], the potential siRNA species were predicted from the *HgRPS23* 225 bp RNAi repeat. The siRNA species (5' AAACUACGACCCACAGAAGGA 3') with the top score of 9.0 matched to the 5' end of the *HgRPS23* 225-bp and the 5' end of the *C. elegans* ribosomal protein large subunit (accession No. NM_058660).

To test the RNAi phenotype of our *HgRPS23* siRNA on nematodes, we designed forward and reverse primers with T7 promoter sequence (5' TAATACGACTCACTATAG 3') at the 5' ends to amplify the 225 bp-fragment of the *HgRPS23* gene by PCR. The dsRNA was produced by T7 RNA polymerase and was used to soak and treat *C. elegans* (N2 wild type) at L2 stage [15]. It was observed that after only one day of treatment, the N2 worms treated with 2 mg/ml *HgRPS23* dsRNA were all dead likely due to the triggering of the RNAi pathway in this model nematode, evidenced by the non-movement with poking and the straightened phenotype of dead worms that stayed at L2 stage. The control N2 worms treated with M9 buffer containing 50 mM octopamine or M9 buffer containing 50 mM octopamine and 2 mg/ml eGFP dsRNA were 100% alive

and developed into L4 stage. The vitality rate of N2 worms treated with 10 µM cycloheximide, the translation inhibitor, was approximately 20% after one day of soaking. The treated worms were also stained with Sytox Green nucleic acid stain (Molecular Probes, Eugene, OR) to differentiate dead from living worms, confirming the phenotypic observation (data not shown). This experiment was repeated three times, with consistent result that *HgRPS23* dsRNA led to 100% lethality in *C. elegans* through RNAi after one day of soaking. It was reported that the *HgRPS23* dsRNA led to partial reduction in the vitality rate in SCN J2 worms after four days of soaking [15]. Thus, it seems *C. elegans* can serve as an efficient model to test the RNAi for SCN if their genes share homology. Differences in morphology, ingestion, metabolism, and gene expression between SCN J2 and C. elegans L2 worms may result in some quantitative differences in siRNA efficacy [22], but the trend is similar

HgRPS23 siRNAs are highly expressed in transgenic soybean roots

The *HgRPS23* siRNA plant expression vector pRD64 was provided to the Plant Transformation Facility at ISU. Five different lines of transgenic soybean plants were produced in three independent events via *Agrobacterium*-mediated transformation of the embryonic axis from mature Williams 82 seeds. The T_1 seeds were shipped to Rutgers for testing. Seeds from two lines were not tested due to low seed counts. The other three lines, RU3, RU6B and RU24 that were phenotypically indistinguishable from the wt plants, were tested for the integration of the bar cassette and the siRNA production by the TaqMan-based RT-qPCR assay.

Two weeks after the germination of transgenic soybean seeds in soil, the first trifoliate leaves were painted with 150 mg/l glufosinate ammonium with a Q-tip. Three days after the treatment, the herbicide sensitivity of the plants was recorded and transgenic plants were identified. Initially at least 10 T_1 seeds from each of RU3, RU6B and RU24 lines were tested. The 10 seeds from each of the T_2 plants were later similarly tested for the herbicide sensitivity. A T_2 line with all 10 plants resistant to the herbicide was identified as a homozygous line. A representative herbicide sensitivity test result is shown in Figure 2. The heights and seed yield of these transgenic soybean plants were measured and no significant difference was shown compared to wt plants (data not shown).

Figure 2: Representative herbicide sensitivity test to identify transgenic soybean plants. Untransformed wt displayed yellowing symptom on the painted trifoliate, indicating the sensitivity to glufosinate-ammonium. The trifoliate from transgenic line RU6B-3 remained green. All selected transgenic soybean plants were phenotypically indistinguishable from wt plants.

The total RNAs from transgenic soybean roots and leaves were isolated and used to conduct the TaqMan RT-qPCR for the expression levels of *HgRPS23* siRNA. The uniquely designed key-like RT primers modeled as previously described [19] extended the lengths of siRNAs in the cDNA products after reverse transcription reactions, which made it possible to quantitate the levels of siRNAs by qPCR analysis (Figure 3).

Figure 3: Assay design of TaqMan RT-qPCR analysis for HgRPS23 siRNA expression level. The key-like RT primer was uniquely designed to recognize the siRNA species that was predicted to be the most highly expressed in the RT reaction to extend the siRNA length. TaqMan probe and the primers were used in qPCR assays to assess the chosen siRNA expression level in the transgenic soybean plants.

The RT product was applied to qPCR with the forward, reverse primers and the TaqMan probe (as the TaqMan assay) for the predicted *HgRPS23* siRNA species (5' AAACUACGACCCACAGAAGGA 3'). With $2^{-\Delta\Delta Ct}$ analysis using MIR156b as the reference gene [20], our results showed that the T_1 lines RU6B-3, RU6B-6, RU6B-8, RU24-7 and RU24-9 had the predicted *HgRPS23* siRNA species expressed as high as 20.84-, 10.8-, 9.6-, 12.75- and 12.89-fold, compared to wt soybean roots. Plants from RU3 line showed relatively lower level (2-5-fold) of *HgRPS23* siRNA, so it was not used in further testing. The T2 plants RU6B-3-5, RU6B-3-8, RU6B-3-10, RU24-7-4, RU24-9-1 and RU24-9-6 were later shown to express the *HgRPS23* siRNA at levels of 18.37-, 21.93-, 20.62-, 15.96-, 16.78- and 15.68-fold by the RT-qPCR assay. The total RNAs isolated from the leaves of transgenic soybean plants were also analyzed by RT-qPCR with the TaqMan probe and the MIR159 as the reference gene. Our results demonstrated that the *HgRPS23* expression levels were negligible in leaves, indicating that the *HgRPS23* siRNA were indeed only expressed in the roots of transgenic soybean plants.

HgRPS23 siRNA-expressing transgenic soybean plants are resistant to SCN

The T_1 and T_2 transgenic soybean plants were tested in three batches (RU6B-3, RU24-7, RU24-9, wt; RU6B-6, RU6B-8, wt; RU6B-3-5, RU6B-3-8, RU6B-3-10, RU24-7-4, RU24-9-1, RU24-9-6, wt) for the SCN resistance. Five to ten plants were tested from each line. The number of SCN eggs per gram of root was averaged from two counts from each plant. The final egg number per gram of root was averaged from all the plants in each line and shown in Figure 4 with the student t-test statistical analysis. Our results showed that all *HgRPS23* siRNA transgenic lines had reduced SCN egg numbers per gram of root. The SCN egg numbers were highly significantly ($p < 0.01$) reduced in T_1 lines RU24-9, RU6B-6, RU6B-8, and significantly ($p < 0.05$) in T_1 line of RU6B-3. The SCN egg numbers were highly significantly ($p < 0.01$) reduced in all T2 lines (RU6B-3-5, RU6B-3-8, RU6B-3-10, RU24-7-4, RU24-9-1, RU24-9-6) tested. The percentage reduction of egg number ranged from 36.81% (RU24-9-6) to 79.68% (RU24-9) in transgenic lines compared to wt soybean (Figure 4). The correlation between the level of HgRPS23 siRNA expression and the egg number reduction in the two batches of T_1 plants tested was not clear, as the highest expresser RU6B-3 (20.84-fold) did not show the lowest SCN egg reduction (Figure 4). However, the T_2 lines RU6B-3-8 and 6B-3-10 that expressed the higher levels of *HgRPS23* siRNA at 21.93- and 20.62-fold supported the lowest number of SCN eggs/g root when compared to the other T_2 lines tested (Figure 4). Our data indicate that lines of these *HgRPS23* siRNA-expressing transgenic soybean plants were highly resistant to SCN infection.

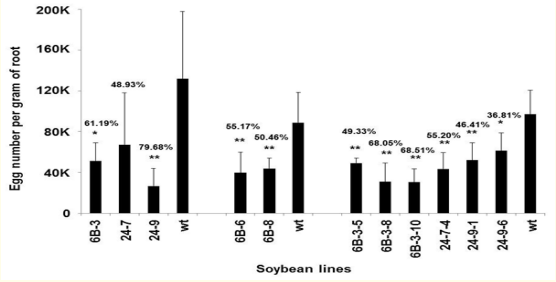

Figure 4: SCN resistance assay for *HgRPS23* siRNA-expressing transgenic soybean plants. SCN eggs were averaged from two counts of each plant, and then averaged from all plants tested in each line. Transgenic plants were compared to wt plant in each batch of testing. The percentage of reduction in egg number is shown above the bar for each transgenic soybean line. * $p < 0.05$, ** $p < 0.01$.

Conclusion

The worldwide presence of SCN and continued challenges of properly managing SCN in agricultural practice justify the development of resistant soybeans through transgenic approaches. Different from the previously reported transgenic soybean plants overexpressing soybean *GmSAMT1* [9], which was driven by the constitutive CaMV 35S promoter throughout the plants, our RNAi approach produced siRNA specific and lethal to SCN only in the soybean roots. Our data showed that our transgenic soybean plants were highly resistant to SCN infection, with egg number reduction ranging from 36.8% to 79.68% in the T_2 plants. It was reported that the SCN female index ranged from 43% to 79% in the GmSAMT1-transgenic soybean plants compared to the control plants [19]. Additionally, transgenic soybean plants overexpressing the *AtNPR1*, *AtTGA2* and *AtPR-5* transgenes in roots (by *A. rhizogenes* transformation) displayed less than 50% of the SCV cysts compared to the control plants [10]. Therefore, it seems that the SCN resistance level of our *HgRPS23* RNAi-transgenic soybean was comparable to these reported cases. This effective RNAi approach can be used to express siRNAs in soybean roots against other SCN effectors such as the pectate lyase genes (pel) [12] the SCN 30C02 effector transcript [13], and the genes of *HgBioB* encoding biotin synthase and *HgSLP-1* encoding a bacterial-like protein containing

a putative SNARE domain [23]. Strong resistance to cyst nematodes was observed in Arabidopsis plants that expressed dsRNA targeted to the SCN *30C02* effector gene, and may serve as a target in soybean for the SCN homologue of *30C02*. The RNAi approach to express siRNAs against these SCN genes specifically in soybean roots should confer SCN resistance in soybean plants and be more target-specific. As with all these technologies, the efficacy, specificity, and downstream field resistance to SCN would need to be confirmed.

This work does not have any relation with the enterprise Monsanto and their products such as transgenic soy that today are of high concern to the humanity.

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