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(54) Title: COMPOSITIONS AND METHODS FOR INDUCING RESISTANCE TO SOYBEAN CYST NEMATODE VIA RNAI

(57) Abstract: RNAi compositions and methods are provided which inhibit soybean nematode cyst infestation. Also disclosed are plants comprising said RNAi.

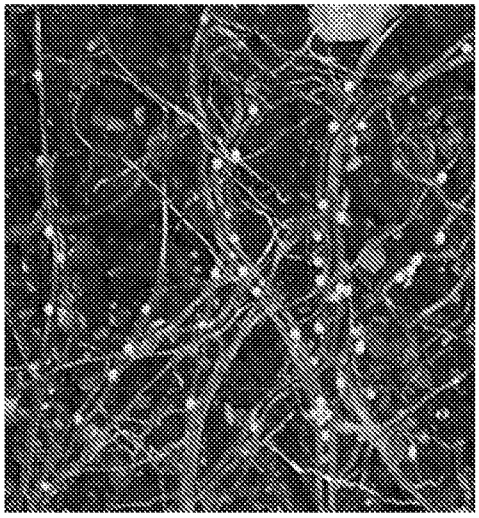

Fig. 1A

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Compositions and methods for inducing resistance to soybean cyst nematode via RNAi

This application claims priority to US Provisional Application No. 62/195,482 filed July 22, 2015, the entire contents being incorporated herein by reference, as though set forth in full.

Field of the Invention

This invention relates to the fields of transgenic plants and parasite resistance. More specifically, the invention provides RNAi molecules that effectively inhibit infection of transgenic soybean with cyst nematode.

Background of the Invention

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated by reference herein as though set forth in full.

Soybean cyst nematode (SCN, *Heterodera glycines*) poses a serious threat to the soybean production and causes approximately $1 billion yield loss per year in the U.S. (Koenning and Wrather, 2010). Agricultural practices such as crop rotation are normally employed to control SCN damage to soybean plants. However, SCN can survive in soil for many years in the form of tough cysts. Whenever soybean is planted, SCN infestation will start anew. Resistant soybean cultivars are also used to mitigate SCN infection, while compromising the yield of soybean crop. Additionally, the existence of at least fourteen different races of *H. glycines* further complicates the utilization of soybean cultivars with limited SCN resistance (Riggs and Schmitt, 1991).

The production of transgenic soybean plants that are herbicide resistant through biotechnology has practically revolutionized soybean production in the world (Duke, 2015). Since late last century, different transgenic soybean plants have been produced with improved resistance to different pathogens (Di *et al.*, 1996; Zhou *et al.*, 2014), pests (Yu *et al.*, 2014) and altered or enhanced nutritional values (Pandurangan *et al.*, 2015). Transgenic approach has 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 (Lin *et al.*, 2013). Subsequently, transgenic soybean plants were produced to over-express *GmSAMT1* and found to resist multiple SCN races (Lin *et al.*, 2016). Additionally, over-expression 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 (Matthews *et al.*, 2014).

RNAi (RNA interference) 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 survival (a lethal gene), feeding on transgenic soybean expressing the lethal gene by SCN will trigger the RNAi pathway in SCN and result in SCN death. Using this technology, Steeves *et al.* (Steeves *et al.*, 2006) has produced transgenic soybeans (cultivars "Jack" and "Chapman") expressing siRNAs (small interfering RNAs) 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 T_0 transgenic soybean plants resulted in up to 68% reduction in egg number per gram of root tissue (Steeves *et al.*, 2006). Recently, Peng *et al.* (Peng et al., 2016) 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 GFP (green fluorescence protein) control at 7 dpi (day post inoculation). Additionally, 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 (Guo *et al.*, 2015).

Clearly, there is a need in the art for more effective means to reduce and eradicate infestation of soybean crops with cyst nematodes.

Summary of the Invention

In accordance with the present invention, compositions and methods for controlling soybean cyst nematode infestation are provided. An exemplary method entails use of RNAi (RNA interference) technology to express small RNAs targeting *Hg-rps-23*, a critical SCN ribosomal gene under the root-specific promoter of Arabidopsis *pyk10* (myrosinase or thioglucosideglucohydrolase) gene. Homozygous transgenic soybean plants have been selected and tested against SCN. These transgenic plants demonstrate enhanced resistance to SCN relative to control plants lacking the RNAi molecules.

In one embodiment, a method for modulating soybean cyst nematode infestation in a plant comprising contacting the plant with an effective amount of a composition comprising an RNAi construct which is effective to down modulate expression of Hg-RPS23 in the root of said plant is disclosed. The present inventor has discovered that down modulation renders the plant resistant to soybean cyst nematode infestation. In a preferred embodiment, the method reduces the number of soybean cyst nematodes in the soil or plant, or on the plant, compared to a plant that has not been contacted with the composition. Use of the aforementioned method also promotes growth of the plant, compared to a plant that has not been contacted with the composition. In a particularly preferred embodiment, the RNAi construct comprises sense and antisense molecules targeting Hg-RPS23, said sense and antisense sequences being operably linked to an Arabidopsis Ppky10 root specific promoter. The RNAi construct can further comprising a selectable marker gene. Most preferably, the RNAi construct is contained within an expression vector, pRD64 (SEQ ID NO: 2).

In another aspect, an RNAi construct comprising an expression cassette encoding a selectable marker, a root specific promoter operably linked to sense and antisense molecules targeting Hg-RPS23 is provided. This construct is effective to increase resistance to soybean cyst nematode infestation upon expression in said soybean plant. In a preferred embodiment, the construct is contained within an expression vector, pRD64 (SEQ ID NO: 2).

Finally, the invention also provides soybean plant comprising the RNAi construct described above and nematode resistant progeny thereof.

Brief Description of the Drawings

Figures 1A -1C show micrographs of *Heterodera glycines* (Figs. 1A and 1B) and the sequence of *HgRPS23* (Fig. 1C; (SEQ ID NO: 1). Adult females and cysts of SCN are about 1/32 of an inch and appear long and visible to the unaided eye. The SCN life cycle is between 24-30 days and is divided into three phases, egg, juvenile and adult. One female SCN can produce 200-400 eggs in its tough sac. The pest is spread primarily through soil.

Figures 2A and 2B show a schematic diagram of the *HgRPS23* siRNA plant transformation vector pRD64 and the sequence of the vector (SEQ ID NO: 2).

Figure 3 shows a Herbicide sensitivity test. Untransformed wt displayed yellowing symptom on the painted trifoliate, indicating the sensitivity to glufosinate-ammonium. The trifoliate from transgenic line RU6B-3 remained green.

Figure 4 shows an assay design of TaqMan qRT-PCR for *HgRPS23* siRNA.

Figure 5 shows 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. * $p < 0.05$, ** $p < 0.01$.

Detailed Description of the Invention

There are no effective measures in the world to control SCN, as the nematodes produce tough cysts that can survive in the soil for many years. See Figures 1A and 1B. Rotation cropping is not effective. Chemical treatment is simply not economically feasible. Most production soybean plants are transgenic for herbicide resistance and the transgenes are expressed throughout the plants. However, the "transgene" of the present invention, a RNA-encoding DNA does not produce any protein, and is only expressed in the roots of soybean plants. This strategy is effective against SCN as it infects the soybean roots. Moreover, this strategy may reduce GMO concerns as the "transgene" is only expressed in the non-edible part of the soybean.

RNAi (RNA interference) technology has been exploited to express small RNAs targeting Hg-rps-23 (SEQ ID NO 1), a critical SCN ribosomal gene under the root-specific promoter of Arabidopsis pyk10 (myrosinase or thioglucosideglucohydrolase) gene. Homozygous transgenic soybean plants have been selected and tested against SCN. Our transgenic soybean plants displayed highly enhanced resistance to SCN.

In the description which follows, a number of terms are used. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided:

Resistant: As used herein, "resistant" or resistance means a soybean variety that is resistant to one or more races of Soybean Cyst Nematode (SCN). Where SCN attach to a resistant variety a majority of the nematodes die and/or do not reproduce.

Susceptible: As used herein, "susceptible" means a soybean variety susceptible to all races of Soybean Cyst Nematode. When SCN attach to a susceptible variety, the nematodes grow larger and reproduce.

Soybean Cyst Nematode (SCN): As used herein, "SCN" is a microscopic roundworm that attaches to soybean roots.

Cyst Nematode Race Spectrum: As used herein, "race spectrum" means a soybean variety having resistance to one or more races of SCN (i.e., spectrum of races).

Soybean Cyst Nematode Number (SCN #): As used herein, the "SCN #" is the number of SCN eggs per 100 cc of soil taken from the area adjacent to the soybean plant.

An “siRNA” refers to a molecule involved in the RNA interference process for a sequence-specific post-transcriptional gene silencing or gene knockdown by providing small interfering RNAs (siRNAs) that has homology with the sequence of the targeted gene. Small interfering RNAs (siRNAs) can be synthesized in vitro or generated by ribonuclease III cleavage from longer dsRNA and are the mediators of sequence-specific mRNA degradation. Preferably, the siRNA of the invention are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. The siRNA can be synthesized as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions. Commercial suppliers of synthetic RNA molecules or synthesis reagents include Applied Biosystems (Foster City, CA, USA), Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, Colo., USA), Pierce Chemical (part of Perbio Science,

Rockford, Ill., USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA) and Cruachem (Glasgow, UK). Specific siRNA constructs for inhibiting Hg-RPS23 mRNA may be between 15-35 nucleotides in length, and more typically about 21 nucleotides in length.

The term "vector" relates to a single or double stranded circular nucleic acid molecule that can be infected, transfected or transformed into cells and replicate independently or within the host cell genome. A circular double stranded nucleic acid molecule can be cut and thereby linearized upon treatment with restriction enzymes. An assortment of vectors, restriction enzymes, and the knowledge of the nucleotide sequences that are targeted by restriction enzymes are readily available to those skilled in the art, and include any replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element. A nucleic acid molecule of the invention can be inserted into a vector by cutting the vector with restriction enzymes and ligating the two pieces together.

A "chimeric gene" is a recombinant nucleic acid sequence in which a promoter or regulatory nucleic acid sequence is operatively linked to, or associated with, a nucleic acid sequence that codes for an mRNA or which is expressed as a protein, such that the regulator nucleic acid sequence is able to regulate transcription or expression of the associated nucleic acid sequence. The regulator nucleic acid sequence of the chimeric gene is not normally operatively linked to the associated nucleic acid sequence as found in nature.

A "coding sequence" is a nucleic acid sequence that is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Preferably the RNA is then translated in an organism to produce a protein.

As used herein the terms "to control" or "controlling" nematodes means to inhibit, through a toxic effect, the ability of nematode pests to survive, grow, feed, and/or reproduce, or to limit nematode-related damage or loss in crop plants. To "control" nematodes may or may not mean killing the nematodes.

A "nematode-controlling effective amount" as used herein refers to the concentration of an RNAi capable of inhibiting, through a toxic effect, the ability of nematodes to survive, grow, feed and/or reproduce, or of reducing or preventing nematode-related damage or loss in crop plants. "Nematode-controlling effective amount" may or may not mean killing the nematodes.

"Expression cassette" as used herein means a nucleic acid sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one that is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular nucleic acid sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter that initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, such as a plant, the promoter can also be specific to a particular tissue, or organ, or stage of development.

A "gene" is a defined region that is located within a genome and that, besides the aforementioned coding nucleic acid sequence, comprises other, primarily regulatory, nucleic acid sequences responsible for the control of the expression, that is to say the transcription and translation, of the coding portion. A gene may also comprise other 5' and 3' untranslated sequences and termination sequences. Further elements that may be present are, for example, introns.

A "heterologous" nucleic acid sequence is a nucleic acid sequence not naturally associated with a host cell into which it is introduced, including non-naturally occurring multiple copies of a naturally occurring nucleic acid sequence.

"Nematicidal" is defined as a toxic biological activity capable of controlling nematodes, preferably by killing them.

A "plant" is any plant at any stage of development, particularly a seed plant.

A "plant cell" is a structural and physiological unit of a plant, comprising a protoplast and a cell wall. The plant cell may be in the form of an isolated single cell or a cultured cell, or as a part of higher organized unit such as, for example, plant tissue, a plant organ, or a whole plant.

"Plant material" refers to leaves, stems, roots, flowers or flower parts, fruits, pollen, egg cells, zygotes, seeds, cuttings, cell or tissue cultures, or any other part or product of a plant.

A "plant organ" is a distinct and visibly structured and differentiated part of a plant such as a root, stem, leaf, flower bud, or embryo.

A "plant part" may be any part of a plant and include a plant cell, plant material, plant organ or plant tissue.

"Plant tissue" as used herein means a group of plant cells organized into a structural and functional unit. Any tissue of a plant in planta or in culture is included. This term includes, but is not limited to, whole plants, plant organs, plant seeds, tissue culture and any groups of plant cells organized into structural and/or functional units. The use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

A "promoter" is an untranslated DNA sequence upstream of the coding region that contains the binding site for RNA polymerase II and initiates transcription of the DNA. The promoter region may also include other elements that act as regulators of gene expression.

"Regulatory elements" refer to sequences involved in controlling the expression of a nucleotide sequence. Regulatory elements comprise a promoter operably linked to the nucleotide sequence of interest and termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

Many techniques are available to those skilled in the art to facilitate transformation, transfection, or transduction of the expression construct into a prokaryotic or eukaryotic organism. The terms "transformation", "transfection", and “transduction” refer to methods of inserting a nucleic acid and/or expression construct into a cell or host organism. These methods involve a variety of techniques, such as treating the cells with high concentrations of salt, an electric field, or detergent, to render the host cell outer membrane or wall permeable to nucleic acid molecules of interest, microinjection, peptide-tethering, PEG-fusion, and the like. Transformation of soybean can be can also be accomplished using Agrobacterium mediated transduction and biolistic delivery of RNAi coated microparticles.

The materials and methods set forth below are provided to facilitate the practice of the present invention.

Construction of *HgRPS23* RNAi transformation vector

The reported 362 bp of *HgRPS23* gene (GenBank accession No. BF014259; SEQ ID NO: 1) (Alkharouf et al., 2007) 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 (Li et al., 2009; Nitz et al., 2001) 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 *Sac*I and then sub-cloned into the soybean expression vector pTF101.1 supplied by the Plant Transformation Facility, Iowa State University (Paz *et al.*, 2006), resulting in pRD64 (SEQ ID NO: 2). Any RNAi between 18-23 nucleotides in length that hybridizes to SEQ ID NO: 1 which is effective to silence HgRPS23 may be used in the methods and constructs of the current invention.

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 (Alkharouf et al., 2007; Peng et al., 2016) with modifications. A T7 promoter sequence (5'-TAATACGACTCACTATAG-3'; SEQ ID NO: 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 (ds) *in vitro* transcript by the MEGAscript®T7 kit (Ambion/Invitrogen, Calsbad, CA, USA).

The wt N2 strain of *C. elegans* was obtained from the CGC (*Caenorhabditis* Genetics Center) and maintained on NGM (Nematode Growth Medium) plates (60 mm petri dishes) at room temperature. The worms were grown and synchronized using protocols from the WormBase (www.wormbase.org). The L2 worms were soaked in 100 µl M9 buffer (22 mM KH_2PO_4, 4.7 mM NH_4Cl, 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.

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 300 *g* 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 (Paz et al., 2006). 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 I containing B5 medium and vitamins, 30 g/l sucrose, 1.11 mg/l 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 I supplemented with 6 mg/l glufosinate for 14 days under 18:6 photoperiod. After another 14 days on shoot induction medium I 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 IAA, 0.5 mg/l GA_3, 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 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. T_1 transgenic soybean seeds were shipped back to Rutgers University with the USDA-APHIS transgenic plant movement permit. Notably, particle gun bombardment can also be used to introduce the siRNAi vector of the invention into recipient soybean plants.

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 (Paz et al., 2006). 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://bioweb.pasteur.fr/seqanal/interfaces/sirna.html#outseq) (Yang et al., 2009). siRNA species (5' AAACUACGACCCACAGAAGGA 3'; (SEQ ID NO: 4) 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 (Yang et al., 2009) (Figure 5). 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, the 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: 50 °C, 2 min, 1 cycle; 95 °C, 10 min, 1 cycle; 95 °C, 15 sec, 60 °C, 1 min, 40 cycles. The $2^{-\Delta\Delta Ct}$ relative quantification method was used to analyze the siRNA (5' AAACUACGACCCACAGAAGGA 3'; SEQ ID NO: 4) level, in the roots with soybean MIR156b (5' UGACAGAAGAGAGAGAGCACA 3'; (SEQ ID NO: 5) (Kulcheski et al., 2011)

as the reference, and in the leaves with MIR159 (5' UUUGGAUUGAAGGGAGCUCUA 3'; (SEQ ID NO: 6) as the reference (Itaya *et al.*, 2008).

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 (Hamamouch *et al.*, 2012) to test their resistance to SCN. Briefly, *H. glycines* OP50 was maintained on soybean plants. Cysts were collected by 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.

The following example is provided to illustrate certain embodiments of the invention. It is not intended to limit the invention in any way.

Example I

Construction of *HgRPS23* RNAi plant transformation vector

As shown by Alkharouf *et al.* (Alkharouf et al., 2007), RNA interference (RNAi) with dsRNA (double-stranded RNA) produced from a 362 bp-fragment of the SCN ribosomal *HgRPS23* gene (Accession No. BF014259) led to lethality of SCN J2 nematodes. Our bioinformatics analysis showed 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), confirming the author's claim in GenBank. 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.

Since SCN attacks the roots of soybean, we decided to express the *HgRPS23* siRNAs only in soybean roots. 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 (Nitz *et al.*, 2001). Li *et al.* (2009) (Li *et al.*, 2009) showed that this promoter could drive the expression of *Aspergillus ficuum* phytase gene in transgenic soybean roots. Therefore, the 1443 bp-fragment of *Arabidopsis pyk10* promoter was chosen to drive the *HgRPS23* siRNA expression. As shown in Fig. 2A, the 231-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 subcloned into pTF101.1 plant expression vector provided by the Plant Transformation Facility, Iowa State University (ISU).

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

Using the online program SIRNA from EMBOSS (http://bioweb.pasteur.fr/seqanal/interfaces/sirna.html#outseq) (Yang *et al.*, 2009), the potential siRNA species were predicted from the *HgRPS23* 231-bp RNAi repeat. The siRNA species (5' AAACUACGACCCACAGAAGGA 3'; SEQ ID NO: 4) with the top score of 9.0 matched to the 5' end of the *HgRPS23* 231-bp and the 5' end of the *C. elegans* ribosomal protein large subunit (Accession No. NM_058660).

To test the RNAi effectiveness of our *HgRPS23* siRNA, we designed forward and reverse primers with T7 promoter sequence (5' TAATACGACTCACTATAG 3'; (SEQ ID NO: 3) at the 5' ends to amplify the 231 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 (Alkharouf et al., 2007). It was observed that after only one day of treatment, the N2 worms treated with the *HgRPS23* dsRNA were all dead, 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 only M9 buffer containing 50 mM octopamine 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.

***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 then tested. Seeds from two lines were not tested due to low seed counts. The other three lines, RU3, RU6B and RU24, 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 3.

The total RNAs from transgenic soybean plants were isolated and used to conduct the TaqMan RT-qPCR for the expression levels of *HgRPS24* siRNA. The key-like oligo was used to carry out the RT reaction (Figure 4). 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 (Kulcheski *et al.*, 2011), 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 T_2 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* 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 5 with the student *t*-test statistical analysis. Our results showed that all transgenic lines had reduced 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 T_2 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 5). The heights of these transgenic soybean plants were also measured and no significant difference was shown compared to wt plants (data not shown). Our data indicate that our *HgRPS23* siRNA-expressing transgenic soybean plants were highly resistant to SCN infection.

Discussion

The worldwide presence of SCN and the difficulties of properly managing SCN by the agricultural practices warrant the development of resistant soybeans through the transgenic approach. Transgenic soybean plants over-expressing a soybean salicylic acid (SA) methyltransferase gene, *GmSAMT1*, has recently been shown to resist multiple races of SCN (Lin et al., 2016). The mechanism of enhanced resistance was attributed to the modulation of expression of genes involved in SA biosynthesis and SA signal transduction in the disease resistance pathways. The transgene was driven by the constitutive CaMV 35S promoter, leading

to the over-expression of *GmSAMT1* throughout the transgenic soybean plants. Transgenic soybean hairy roots over-expressing the *Arabidopsis AtNPR1, AtTGA2* and *AtPR-5* genes that are involved in plant defense signaling were also shown to produce decreased number of SCN cysts following infection (Matthews et al., 2014). However, transgenic soybean plants were not produced to show the effectiveness of heterologous *Arabidopsis* genes in conferring SCN resistance. The RNAi method taken by Steeves *et al.* (Steeves et al., 2006) to produce siRNA against the SCN major sperm protein gene seemed to be a better approach compared to the transgene expression to induce SCN resistance, as this RNAi was directly against SCN. However, the expression of siRNA was constitutively driven by the *Arabidopsis* ACT2 promoter, which means the siRNA against the SCN major sperm protein gene was expressed throughout the transgenic soybean plants, although it was only shown that siRNA was detected in the leaf tissues of transgenic soybean plants by Northern blot analysis (Steeves et al., 2006).

To circumvent potential GMO (genetically modified organism) issues, and to better target the pathogenic SCN at the soybean roots, 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. Our results showed that the *HgRPS23* siRNAs were indeed only expressed in the roots, but not the leaves, of our transgenic soybean plants. The expression level of *HgRPS23* siRNA assessed by the TaqMan probe-based RT-qPCR was shown to be 10- to 20-fold higher compared to the untransformed wt plants. The uniquely designed key-like RT primers modeled as previously described (Yang et al., 2009) 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, negating the utilization of radioactive materials in Northern blot analysis.

The *HgRPS23* RNAi has been shown to lead to lethality of SCN J2 worms (Alkharouf et al., 2007). Our bioinformatics analysis indicated that the 5' 225 bp of *HgRPS23* shares high homology with the *C. elegans CeRPL1* at both the nucleic acid and protein levels. The predicted *HgRPS23* siRNA (5' AAACUACGACCCACAGAAGGA 3'; SEQ ID NO: 4) shares the exact sequence found in *CeRPL1*. Our *in vitro* dsRNA produced from the 225 bp PCR product of *HgRPS23* resulted in 100% death rate in *C. elegans* after only one day of soaking and triggering the RNAi pathway in this model nematode. It was reported that the *HgRPS23* dsRNA led to partial reduction in the vitality rate in SCN J2 worms after four days of soaking (Alkharouf et al.,

2007). Our data suggest that *C. elegans* can serve as an efficient model to test the RNAi for SCN if their genes share homology.

Our SCN infection assay demonstrated that the high expression of *HgRPS23* siRNA in the T_1 and T_2 transgenic soybean roots led to 39-79% reduction in the SCN egg numbers, indicating the effectiveness of this RNAi approach. This approach can be used to express siRNAs in soybean roots against other SCN effectors such as the pectate lyase genes (*pel*) (Peng et al., 2016), and the genes of *HgBioB* encoding biotin synthase and *HgSLP-1* encoding a bacterial-like protein containing a putative SNARE domain (Bekal *et al.*, 2015). The knocking-down of *Hg-pel-6* by RNAi in transgenic soybean hairy roots using CaMV 35S promoter has shown to reduce the invading nematodes and suppress the development of SCN females (Peng et al., 2016), and the *HgBioB* and *HgSLP-1* genes have been shown to be involved in SCN virulence (Bekal et al., 2015). RNAi approach to express siRNAs against these SCN genes specifically in soybean roots should confer SCN resistance in soybean plants.

Furthermore, the recently advanced CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated)-gene editing technology (Jinek *et al.*, 2012) should be considered to knock-out soybean host factors to interrupt the interaction between soybean plants and *H. glycines*. CRISPR-gene editing has been used to knock-out the rice *OsMPK5* gene, which encodes a stress-responsive rice mitogen-activated protein kinase (a negative regulator of rice defense response) (Xie and Yang, 2013). The wheat Mildew-Resistance Locus A1 (*TaMLO-A1)* has also been mutated by CRISPR-editing (Wang *et al.*, 2014). It was recently shown that RNAi silencing of the putative soybean CLE (CLAVATA3/ENDOSPERM SURROUNDING REGION) receptors resulted in enhanced SCN resistance in transgenic soybean hairy roots (Guo et al., 2015). Employing the precise targeting and easy manipulating platform of CRISPR-gene editing, knocking-out soybean host factors such as the CLE receptors in soybean roots should potentially produce SCN-resistant soybean plants.

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While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. It will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the scope of the present invention, as set forth in the following claims.

What is claimed is:

1. A method for modulating soybean cyst nematode infestation in a plant comprising contacting the plant with an effective amount of a composition comprising an RNAi construct which is effective to down modulate expression of Hg-RPS23 in the root of said plant, said down modulation rendering the plant resistant to soybean cyst nematode infestation.

2. The method of claim 1, wherein the method reduces the number of soybean cyst nematodes in the soil or plant, or on the plant, compared to a plant that has not been contacted with the composition.

3. The method of claim 1, wherein the method promotes growth of the plant, compared to a plant that has not been contacted with the composition.

4. The method of claim 1, wherein the method increases the yield of seeds produced by the plant, compared to a plant that has not been contacted with the composition.

5. The method of claim 1, wherein said RNAi construct comprises sense and antisense molecules targeting Hg-RPS23, said sense and antisense sequences being operably linked to an Arabidopsis Ppky10 root specific promoter.

6. The method of claim 5, said RNAi construct further comprising a selectable marker gene.

7. The method of claim 5, wherein said RNAi construct is contained within an expression vector, pRD64 (SEQ ID NO: 2).

8. An RNAi construct comprising a expression cassette encoding a selectable marker, a root specific promoter operably linked to sense and antisense molecules targeting Hg-RPS23 having SEQ ID NO: 1, said construct being effective to increase resistance to soybean cyst nematode infestation upon expression in said soybean plant.

9. The RNAi construct of claim 8, being contained within an expression vector, pRD64 (SEQ ID NO: 2).

10. The RNAi construct of claim 8, wherein said root specific promoter is an Arabidopsis Ppky10 promoter.

11. A soybean plant comprising the RNAi construct of claim 8.

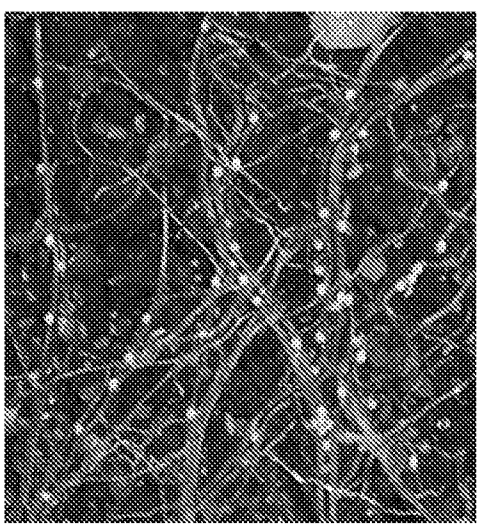

Fig. 1A

Fig. 1B

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Fig. 1C

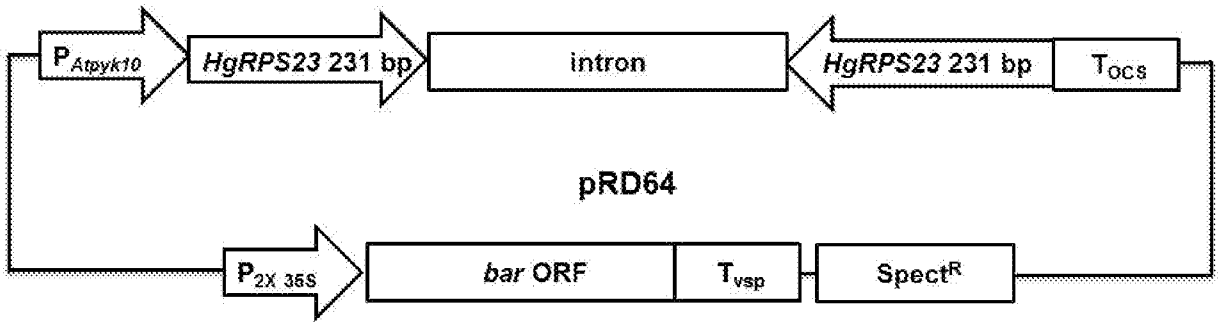
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Fig. 2A

Fig. 2B

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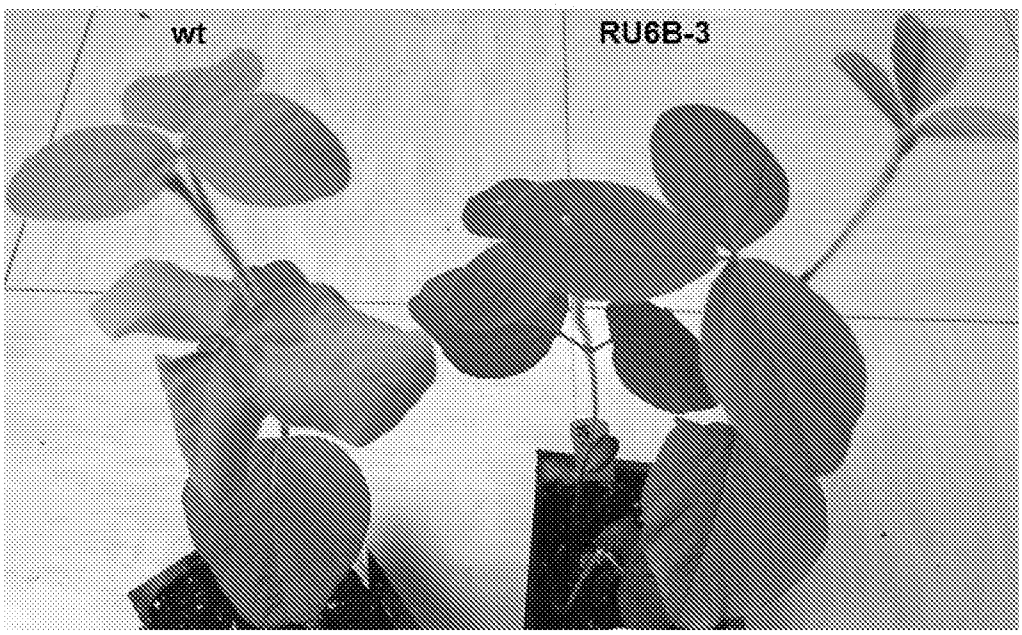

Figure 3

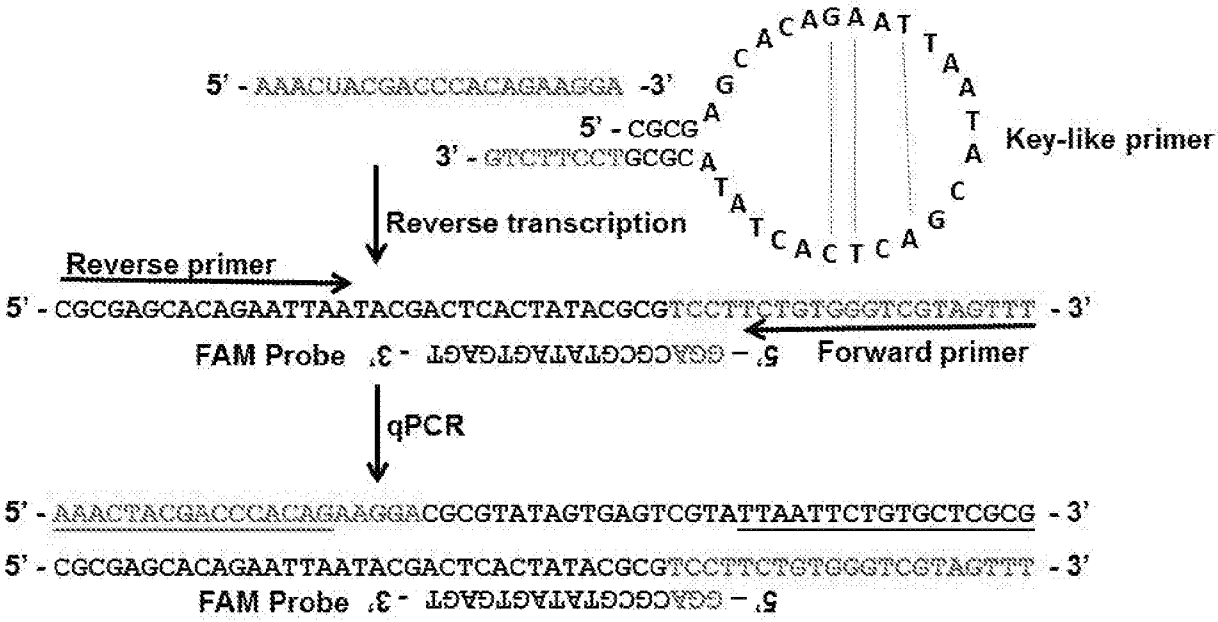

Figure 4

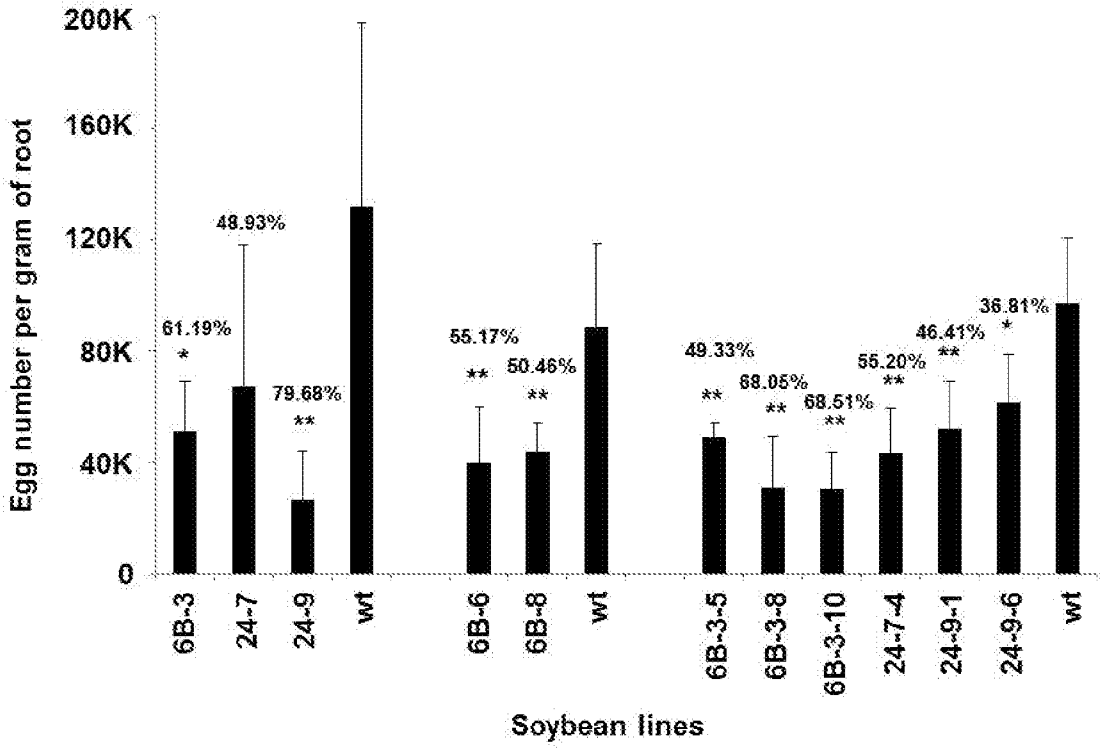
200K
160K
120K
80K
40K
0
Egg number per gram of root
61.19%
*
48.93%
79.68%
**
55.17%
**
50.46%
**
49.33%
**
68.05%
**
68.51%
**
55.20%
**
46.41%
**
36.81%
*
6B-3
24-7
24-9
wt
6B-6
6B-8
wt
6B-3-5
6B-3-8
6B-3-10
24-7-4
24-9-1
24-9-6
wt
Soybean lines

Figure 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/043706

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/11; C12N 15/113; C12N 15/82 (2016.01)
CPC - C12N 15/113; C12N 15/8218; C12N 15/8279; C12N 15/8285; C12N 2310/14 (2016.08)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC - C12N 15/11; C12N 15/113; C12N 15/82
CPC - C12N 15/113; C12N 15/8218; C12N 15/8279; C12N 15/8285; C12N 2310/14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/419; 435/468; 435/320.1; 536/23.1; 800/279; 800/301 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, PubMed
Search terms used: Soybean cyst nematode Hg rpo 33 pyk10 promoter

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2014/0007296 A1 (HUANG et al) 02 January 2014 (02.01.2014) entire document	1-4, 8, 11 --- 5, 6, 10
Y	VERCRUYSSEN et al. "Combining enhanced root and shoot growth reveals cross talk between pathways that control plant organ size in Arabidopsis," Plant Physiol. 03 January 2011 (03.01.2011), Vol. 155, Pgs. 1339-52. entire document	5, 6, 10
A	ALKHAROUF et al. "Identification of Heterodera glycines (soybean cyst nematode [SCN]) cDNA sequences with high identity to those of Caenorhabditis elegans having lethal mutant or RNAi phenotypes," Exp Parasitol, 18 October 2006 (18.10.2006), Vol. 115, Pgs. 247-258. entire document	1-11
A	US 2014/0325702 A1 (BOUKHAROV et al) 30 October 2014 (30.10.2014) entire document	1-11
A	GUO et al. "Enhanced resistance to soybean cyst nematode Heterodera glycines in transgenic soybean by silencing putative CLE receptors," Plant Biotechnol J, 12 January 2015 (12.01.2015), Vol. 13, Pgs. 801-10. entire document	1-11
A	US 2009/0012029 A1 (HUSSEY et al) 08 January 2009 (08.01.2009) entire document	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 2016	03 NOV 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/043706

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-10 were searched.