ASSESSING RNA INTERFERENCE GENE TARGETS OF ROOT-KNOT NEMATODES

by

BENJAMIN LIVINGSTON RAMBO-MARTIN

(Under the Direction of Wayne Parrott)

ABSTRACT

Root-knot nematodes (RKN) account for losses over $200 million in soybean yield year^{-1} in the United States. RKN establishes a feeding site within plant roots by altering gene expression in root cells, causing those cells to enlarge and adjacent cells to proliferate, netting visible galls. The identification of RKN genes involved in plant parasitism opened the possibility for developing RKN resistant soybean by disrupting these genes expression using RNA interference (RNAi). Accordingly, the effectiveness of targeting two RKN parasitism genes, 17H02 and 31H06, to confer resistance in soybean is evaluated. Composite soybean, 'Peking,' plants were created by inoculating newly emerging radicals with *A. rhizogenes* strain K599 harboring binary vectors designed to produce double-stranded RNA (dsRNA). Targeting two genes simultaneously is also tested. Finally, two promoters, a ubiquitin promoter from *Glycine max* (GmUbi) and a phosphate transporter promoter from *Medicago truncatula* (MtPt1) are compared for their effectiveness at obtaining RKN resistance.

INDEX WORDS: RNAi, root-knot nematodes, parasitism genes, composite soybeans, promoter analysis

ASSESSING RNA INTERFERENCE GENE TARGETS OF ROOT-KNOT NEMATODES

by

BENJAMIN LIVINGSTON RAMBO-MARTIN

B.S., The University of Georgia, 2006

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2009

ASSESSING RNA INTERFERENCE GENE TARGETS OF ROOT-KNOT NEMATODES

by

BENJAMIN LIVINGSTON RAMBO-MARTIN

Major Professor:	Wayne A. Parrott
Committee:	R. Kelly Dawe Richard S. Hussey

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2009

DEDICATION

This thesis is dedicated to the most important person in my life, my wife, Christine. As my best friend and life partner, she supported every aspect of me throughout this research and thesis writing. She kept me from going completely insane, consented to every long night I was in the lab and provided endless encouragement and inspiration to keep my eyes on the prize. I am so lucky to have a person who not only stood beside me during this work, but is ready to do it again during future studies. Thank you Christine, I Love You!

This work is also dedicated to my Mom and Dad. Thank you for sculpting me early into the person I have become. You both are an inspiration and I hope to live up to your expectations and make you proud. I have a great mirror in both of you to replicate my family by. Thank you for pushing me to achieve while also instilling in me values of the greatest importance.

Thank you to Granny and Papa, for endless Love, perpetual encouragement and a peaceful refuge. Thank you to Galen, Harrison, and Mackenzie for Love, maintenance, and the kind of friendship only brothers can provide. Thank you Molly for sisterly Love, and of course the competition, and also to Megan, for providing me shelter in my early graduate school days. This is dedicated also to the rest of my family, especially Betty and Billy Cooper, and Ronnie and Neal Livingston. Thanks also to the Rambo's for their Love, support, and for providing the most important person in my life.

Finally, this is dedicated to the only two who never displayed anything but overwhelming Love, comfort, and companionship towards me, Gita Paynts and Barley Hopperson.

ACKNOWLEDGEMENTS

Acknowledgements far above and beyond what is expressed here go to Wayne Parrott. His exuberance, incredible expectations, confidence, and above all faith in my abilities have truly created the person I am today. He is a precipice of ethics and an inspiration for what true drive in your life's work should be. I have nothing but limitless respect and admiration for him, but more importantly an implacable gratitude for his mentorship.

Secondly, the entire Parrott Lab is due enormous thanks! Specifically, thank you Donna Tucker, for patient instruction (and re-instruction) of tissue culture, donations of somatic embryos, adoption of transgenic lines, and along with Barbara Artelt, for keeping my ego in check. Also to Barbara, thank you for keeping the entire lab running smoothly, especially in tirelessly ordering supplies. Thanks also to Nathan Hancock, who was also willing to provide helpful insight, troubleshooting, and forethought to my defense presentation. Great thanks moreover to Pete LaFayette for teaching me the art of vector construction and RTFM and for the camaraderie both in the lab and during conference travel. Many thanks also to all the undergraduate and high school workers who have passed through the lab and helped my research along the way.

Acknowledgements are also due to all my fellow graduate students who have shared the woes and triumphs of classes and research. Particularly to Nathan Friday, thank you for being a great vent, roommate, and comfort-food provider. Becky Tashiro also deserves a special thank you for being a fantastic cubicle buddy, counselor, friend, and for her love of eating cheerios while she's watching her cartoons.

Thanks are also due to members of the Hussey Lab, specifically Penny Goodman for helping me with RKN DNA extraction, Guozhong Huang for information on *16D10* and Steve Finnerty for assisting in the RKN assay in the greenhouse.

Much appreciation is due to The United Soybean Board, for providing my assistantship and research funding. I also want to thank all the members of the Soybean Tissue Culture and Transformation Center, for making me feel welcomed in their meetings and granting me exposure into such a fantastic professional collaboration.

Finally, thanks are overdue to my committee members, Kelly Dawe and Richard Hussey, for their time, knowledge, and expertise.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

1. Introduction

Root-knot nematodes (RKN) are one of the most ubiquitous plant pathogens in the world, able to parasitize nearly every major crop species throughout various growing regions (Sasser and Carter, 1985). Belonging to the genus *Meloidogyne*, RKN are the most economically damaging plant-parasitic nematode worldwide, causing an average yield loss of 5% (Hussey and Janssen, 2002). In soybean, *Glycine max* (L.) Merr. yield loss estimates alone due to RKN have been over 354,000 metric tons worldwide (Wrather et al., 1997).

Soybean is the largest source of vegetable protein in the world, and the second largest source of vegetable oil, according to the American Soybean Association (SoyStats, 2009). It has tremendous value worldwide, and farmers produced 228.4 million metric tons of soybean in 2006 (USDA, 2006). Global demand for soy products has driven conventional breeding methods, whose focus has been primarily to increase yield, which has risen by 60% during the past 60 years. Breeding for disease resistance has been an important way to increase yield (Orf et al., 2004; Wilcox, 2001).

Conventional breeding for pest resistance has been facilitated by the development of molecular markers, which allow identification of quantitative traits during a breeding program (Fu et al., 2006; Gordon et al., 2007; Mienie et al., 2002; Wang and Roberts, 2006). Nematodes, in particular the Soybean Cyst Nematode (SCN), *Heterodera glycines*, have received much attention for breeding resistance to in soybean, as they are its greatest threat to yield worldwide (Wrather et al., 1997). After SCN, RKN are the

next most destructive nematodes on soybean, and the greatest nematode threat in the Southeastern United States (Ha et al., 2007; Wrather et al., 1997). There are four species in the genus *Meloidogyne* that are significant pests on plants; they are: ***M. incognita, M. javanica, M. arenaria,*** and ***M. hapla***. Respectively, each contributes 54%, 30%, 7%, and 7% to the total of plant RKN infestations (Sasser, 1980).

Genetically, resistance to RKNs is complex. Complete resistance to a single species, can require the presence of both a major and minor quantitative trait locus (QTL), two of which have been identified by restriction fragment length polymorphisms (RFLP) toward ***M. arenaria*** (Tamulonis et al., 1997b). Luzzi et al. (1995) demonstrated that soybean's resistance to ***M. javanica*** is also due to multiple QTLs, or possibly multiple alleles at a single loci. Following this multiple gene trend, single sequence repeat (SSR) markers validated earlier RFLP work, indicating two separate QTLs for conditioning ***M. incognita*** resistance in soybean (Li et al., 2001; Tamulonis et al., 1997a). While development of precise markers such as single nucleotide polymorphism (SNP) markers for ***M. incognita*** resistance, can hasten breeding efforts in broad spectrum resistance to the *Meloidogyne* genus, the complexity of incorporating numerous loci inhibits their breeding into evolving elite genotypes.

To date, only a single cultivar of soybean, 'Haskell,' has been developed containing resistance to the three major *Meloidogyne* species which infect soybean (Boerma et al., 1994). Resistance is thought to be obtained by incorporating six dominant 'R' genes (Roger Boerma, 2009, personal communication). It is therefore impractical to deploy this resistance in conventional breeding programs, even using

molecular markers. Hence, a transgenic approach is appealing to provide broad-spectrum RKN resistance for soybeans.

Transgenic approaches to nematode resistance have had limited success thus far. Transformation strategies that have been tried include engineering plants with cloned resistance genes (R-genes), expressing proteinase inhibitors or lectins, *Bacillus thurigiensis* (Bt) crystal (Cry) proteins, and transgenically produced antibodies complementary to nematode esophageal secretions. The *Mi-1.2* gene from tomato, while showing promising nematode resistance within the species as well as in transgenic eggplant (within tomato's genus, *Solanum*) (Goggin et al., 2006), was unable to provide comparable resistance in tobacco (Williamson, 1998). Similar interspecies inefficacies are found in transgenic expression of the tomato *Hero A* in tobacco (Sobczak et al., 2005). In general, R-genes possess additional negative attributes, including a lack of cross-species resistance. and specifically for the *Mi-1.2* gene, heat-labile function over 28°C.

Proteinase inhibitors have shown promising potential for nematode resistance when expressed transgenically in plants, but have not become a fail-safe approach due to the direct relationship between transgenic protein levels and resistance, which leads to infrequent high levels of resistance (Fuller et al., 2008). Plant lectins, such as the snowdrop lectin, have proven able to provide nematode resistance, but have serious limitations due to their inability to affect nematode progeny, lack of consistent levels of resistance, and most importantly, a possible increase in susceptibility to nematodes (Ripoll et al., 2003). A monoclonal antibody, 6D4, though able to complement an *M. incognita* secretory protein, provided no resistance when expressed in transgenic tobacco

(Baum et al., 1996). Finally, Bt Cry proteins have proven toxicity to nematodes (Marroquin et al., 2000), however they are effective against the entire phylum nematoda, which includes beneficial soil fauna, and have little effect on the immediate seasons galling, though do reduce the nematodes fecundity (Li et al., 2007; Wei et al., 2003) . The inherent limitations to the current transgenic approaches in achieving resistance to nematodes require an alternate method to be explored.

RNAi is a collective term for a series of natural processes for the down-regulation of gene expression (Della Vedova et al., 2005; Jorgensen et al., 1996; Kusaba, 2004; Tuteja et al., 2004). It involves generation and recognition of double stranded RNA molecules that, once processed by the enzyme Dicer into small interfering RNAs (siRNAs) or micro RNAs (miRNAs), incorporate into the RNA-induced silencing complex (RISC) which cuts the target mRNA via sequence homology, destroying its potential to be translated into a protein (Hannon, 2002). Since its discovery in 1998 by Fire and Mello, a tremendous amount of research has been built upon RNAi technology, ranging from gene function discovery in plants, animals, fungi, and bacteria to pest resistance in plants (Fire et al., 1998; Kaeberlein et al., 2007; Mao et al., 2007; Mery et al., 2008; Nakagawa et al., 2007; Nekhotiaeva et al., 2004).

The work done by Huang et al. (2006a) shows proof-of-concept for achieving transgenic nematode resistance in crops via RNAi. In their study, *Arabidopsis thaliana* plants were engineered to produce double-stranded RNA that went on to silence expression of an essential parasitism gene, 16D10 (GenBank DQ087264), within the subventral esophageal glands of RKNs (Huang et al., 2006a).

2. Root-knot Nematodes

Root-knot nematodes are microscopic roundworms parasitizing crops with a greater economic impact than any other nematode worldwide (Sasser and Freckman, 1987). RKN attacks an enormous variety of crop species including soybean, potato, sugar beet, rice, coconut palm, banana, pepper, tobacco, watermelon, tomato, peanut, and thousands of other plants (Mai, 1985; Sasser and Carter, 1985). RKN is a silent killer of profits. Often, it thrives as a parasite throughout a growing season in annuals, or over many years in perennial crops, without any above-ground sign or symptom. Only when harvest is over and yield has been quantified is the parasite's damage commonly seen (Mai, 1985). If undiagnosed, the infested field's lower yields may be misinterpreted by the farmer as a nutrient management problem, applying more fertilizers the subsequent year, and obtaining even lower profit margins due to the continued yield reduction. This is a world-wide problem, but one that is frequently undiagnosed, so proper estimates of total yield loss have been difficult to obtain, but likely amount to over $77 billion per year (Sasser and Freckman, 1987). Soybean yields alone lose over 350,000 metric tons $year^{-1}$ to the pest (Wrather et al., 1997).

Around the world, RKN control is typically by crop rotation, though it is not necessarily the most efficient means. RKN's ability to parasitize a large host diversity, prevalence of different, sometimes unknown races, and lack of proper sampling and identification in many areas, all contribute to the difficulty of control (Mai, 1985). Biological control is practiced in some cropping systems, with two organisms showing

moderate success, including *Bacillus penetrans* and *Verticillium chlamydosporium* (Mai, 1985). However, such control never has had the efficacy of chemical nematicides. Nematicides are often used in situations where other control techniques are not possible or not economical. Unfortunately, effective nematicides typically interact as acetyl-cholinesterase inhibitors between nerve cells (carbamates and organophosphates) or respiratory blockers through blockage of the electron transport chain (halogenated hydrocarbons), making them extremely dangerous to animals (Chitwood, 2003). Their toxicity is further generalized through their common ability to move through the soil rapidly, and they have a long persistence in the environment (Sasser and Carter, 1985; Schmitt, 1985). Clearly the most efficient method of control is by using resistant cultivars; however not all crops have resistance genes in their germplasm, or as is the case with soybean, broad resistance may require the incorporation of more genes than is feasible in a conventional breeding program (Boerma et al., 1994; Sasser and Carter, 1985).

A. Anatomy

Taxonomically, root-knot nematodes belong to the genus *Meloidogyne* under the family Meloidogynidae (Wouts 1979). They differ from the other two genera in the family in several key ways. Females in *Melododerella* have a cyst stage which is absent in *Meloidogyne* and *Meloinema* (Hirschmann, 1985). Juveniles in *Melodogyne* have a weakly developed cephalic framework and smaller body size than *Meloinema*; this trend in size is seen between each genera's respective male and females as well (Hirschmann, 1985).

Of key importance to all plant-parasitic nematodes, of which *Meloidogyne* is no exception, is the stylet. The stylet is the nematode's hypodermic needle-like structure which is embedded within its cephalic framework, but emerges from the head to pierce a host's cell wall to enable feeding. This hollow, rigid structure is connected to the lumen of the esophagus, which in turn is connected to three esophageal gland cells and the intestine.

The three esophageal glands, one dorsal and two subventral, are each an individual secretory cell (Hussey, 1989). While early studies noted that secretions from the dorsal gland were present during pathogenesis, the subventral glands were not thought to be involved in pathogenesis, and none of these cells were definitively linked to parasitism (Hussey, 1988; 1989). Each of the glands produces secretory proteins sequestered in secretory granules, which are released by exocytosis to be injected into plant cells via the stylet at regulated times (Hussey, 1988). During establishment of parasitism, the subventral glands predominate but degenerate as the dorsal gland enlarges, which seems to maintain the parasitic relationship (Bird, 1983; Davis et al., 2008; 2000). These glands synthesize secretory proteins which become the parasitome, encompassing the suite of nematode proteins necessary to parasitize a plant (Huang et al., 2006a).

B. RKN Infection

RKNs go through 5 life stages, separated by molting. They reach the second stage juvenile (J2) stage within the egg before being deposited, typically on the root surface, but sometimes within the gall (Hussey, 1985). After finding a host's root, likely

via chemotaxis, they enter behind the root cap, and travel intercellularly toward cells differentiating into vascular tissue (Hussey and Janssen, 2002; Perry, 1996). A feeding site for a single RKN can be 5-7 of these cells, which are modified into 'giant-cells' by the RKN (Hussey et al., 1994). The ability of RKN to create these giant-cells set them apart from other pathogens as one of the most complex host-pathogen relationships known (Hussey and Janssen, 2002).

The term, 'giant-cells' was coined in observation of their most noticeable feature, their size. These cells also contain other physical attributes, clearly setting them apart from an uninfected cell and facilitate the nematode's ability to parasitize. The first visible change within a cell in the process of becoming a giant-cell involves karyokinesis. However, while the nucleus divides, the cell does not proceed beyond what appears to be the beginning of a normal mitosis; cytokinesis never occurs, even though partial cell plates can begin to develop (Jones and Payne, 1978). Karyokinesis continues until ploidy levels reach 32x, or 64x, though over 100 nuclei have been observed in a single giant-cell (Huang and Maggenti, 1969). The nuclear envelope becomes an ameboid-like, increasing the surface area of the nuclear envelope, in contrast to the typical spherical shape (Yousif, 1979).

All of the changes in these giant-cells seem to serve the purpose of increasing the amount of nutrients available for the RKN. After the feeding site is established, the RKN's two subventral esophageal gland cells, which are responsible for the production of proteins necessary for host root penetration, intercellular migration and the creation of a giant-cell, contract, while the single dorsal esophageal gland cell enlarges and heightens activity in order to maintain the giant cell (Bird, 1968; Bird, 1969). The rapid and

constant amount of resources available to the nematode allows it to morph into a totally stationary lifestyle, as its muscles deteriorate. The RKN proceeds through its final three molts, ending with the adult stage in which eggs develop inside its body, and the process repeats. Over 200,000 eggs per gram of root can be hatched on susceptible plants, and the entire RKN lifecycle can be completed in a month, depending on temperature, thus leading to high growth rates of parasite populations on a single plant (Nyczepir et al., 1999; Ploeg and Maris, 1999). Each nematode parasitizing on cells, its created giant cells, and the accompanying hypertrophy and hyperplasia of adjacent cells results in the visible phenotype on the host's root, known as a gall.

As galls originate from vascular tissue, their growth could impede nutrient and water flow from the roots to the aerial sections of the plant. The suppressed root growth seen in RKN infestation likely contributes more heavily to yield reduction, though in general it is not well understood how RKNs affect crop yield (Hunter, 1958; Hussey, 1985). In minor infestations, an infection may result in yield loss, observed only at harvest time, from otherwise healthy looking plants. In major infestations, plant death can occur.

C. The Parasitome

The parasitome is the collective of proteins secreted from parasitic nematodes through their stylet in order to create a host suitable for sustaining the nematode's lifecycle (Gao et al., 2003). Williamson and Hussey (1996) hypothesized that these secreted proteins came from the subventral and dorsal esophageal gland cells and altered host gene expression, thus causing the creation of giant-cells. Cellulases (β-1,4-

endoglucanases) were the first proteins to be identified in *Heterodera* and *Globodera* and later in *Meloidogyne*, and are secreted during the nematode's root penetration and migration to its feeding site (Rosso et al., 1999; Smant et al., 1998; Yan et al., 1998).

The inefficacy of candidate parasitism gene discovery through expressed sequence tags was bypassed through the use of microaspiration techniques, whereby mRNA could be extracted directly from the esophageal gland cells (Gao et al., 2001; Wang et al., 2001). Development of cDNA libraries followed by suppression subtractive hybridization, determination of sequences with N-terminal secretion signal peptide, and large scale *in situ* mRNA hybridization, allowed for a more specific determination of potential parasitism genes (Huang et al., 2003). Twenty-seven of the 37 parasitism genes found in RKN have no homology to any known gene in the database. One of these, *16D10*, codes a small, 13-amino acid peptide, which was immediately thought to be involved in signaling due to its size (Huang et al., 2003). Over the 3 years which followed, *16D10* became the focus of several studies to determine its function and as the target of engineered pest resistance.

Huang et al. (2006c) ran *16D10* through a gauntlet of molecular tests aiming to determine its function in plant cells parasitized by RKN. Using 16D10 antiserum, immunolocalization confirmed the peptide's presence in the subventral esophageal gland cells and metacorpus of *M. incognita*, while ELISA and immunoblot assays established its occurrence in J2 stylet secretions (Huang et al., 2006c). Tobacco and *Arabidopsis* transgenics were produced to over-express post intronic *16D10*, creating startling results. In plants, expression *of 16D10* increased root growth by 65% in tobacco hairy roots and lengthened the primary root of *Arabidopsis* by nearly 85% without creating a significant

difference in shoot size (Huang et al., 2006c). Also of interest was the increase in lateral root growth and callus formation at the site of cut roots in the transgenic tobacco hairy roots. Using tomato root cDNAs in a yeast two hybrid assay, a 30-78% similarity was discovered between *16D10* and three scarecrow-like (SCL) transcription regulators, which manipulate development and growth (Huang et al., 2006c). A yeast two-hybrid system was used to confirm 16D10's interaction between the SAW domains of AtSCL6 and AtSCL21 while also using coimmunoprecipitation *in vitro*.

Proof of concept that broad resistance across RKN species is possible was published later in 2006 by the same group, by using transgenic *Arabidopsis* which was engineered to suppress the function of 16D10. Truncated and full-length *16D10* sequences were cloned into a pHANNIBAL vector in both a sense and antisense, head-to-head orientation, which when transcribed into mRNA, the transcripts fold to create a double stranded RNA molecule, triggering the RNAi mechanism (Huang et al., 2006a; Wesley et al., 2001). This vector was engineered into *Arabidopsis,* and when challenged with the four major RKN species, displayed broad RKN species resistance (Huang et al., 2006a). In a second experiment, which confirmed the RNAi vector's ability to silence *16D10* expression *in planta*, two *Arabidopsis* lines were crossed, one over-expressing *16D10* (giving rise to a phenotype of increased root growth) and the other producing double-stranded *16D10* RNA. The resulting F1 displayed a wild-type root phenotype, while an RNA-blot demonstrated the elimination of full-length *16D10* mRNA, with an increased presence of *16D10* siRNAs (Huang et al., 2006a). Thus, Huang et al. (2006a) fully demonstrated the potential to use RNAi targeting RKN parasitism genes to create broad species resistance in crop plants to this economically serious pathogen.

3. Soybean and Genetic Engineering

A. *Soybean's Importance*

The United States is the world's largest producer of soybean growing 32% of the total 219.8 million metric tons produced worldwide in 2007, creating a value over $26.8 billion for the US (USDA, 2008). Following the US in production is Brazil (28% of world gross), Argentina (21%), and China (7%) (USDA, 2008). Soybean derives its value from its ability to produce vegetable protein and oil (USDA, 2006). As the number two vegetable oil crop worldwide (second to palm oil), products derived from it include: margarine, cooking oil, ink, and biodiesel (USDA, 2008).

B. Conventional Improvement of Soybean

Soybean's versatility as a raw source for many products drives its demand, leading to increased value received by the soybean industry. This demand has bolstered breeding efforts, which, while focusing on improving yield, have also stressed breeding for pest resistance (Orf et al., 2004). Conventional breeding of soybean relies on the yearly selection of parents, accompanied with the selection of superior progeny from previous years' crosses, and using both single-seed descent and back-crossing for finished cultivar development (Orf et al., 2004; Pathan and Sleper, 2008). A major obstacle of conventional methods has been their imprecision in terms of introgression to obtain a single added benefit in an already superior genetic background. Many of the traits which breeders wish to introgress are found in wild soybeans or old varieties, which

have many undesirable characteristics. Historically, these undesirable traits were frequently incorporated in along with the desired trait. The advent of marker-assisted selection (MAS), based on SSRs and now SNPs, has greatly facilitated backcrossing for finished cultivar development (Orf et al., 2004; Pathan and Sleper, 2008).

While the use of MAS has lessened problems with introgression of undesirable traits, the development of genetic engineering in soybean is a powerful tool to bypass breeding limitations. It is particularly important when the trait of interest is otherwise unattainable through conventional breeding methods.

C. *Genetic Engineering in Agriculture*

Genetic engineering (GE), as a practice, defines the process by which sections of DNA are removed from one organism, or synthesized *de novo in vitro*, and incorporated into the genome of another. This exchange can allow for the transfer of genetic information between any levels of taxonomy. GE has provided the most value to soybeans, adding 6.05% to farm gate income, when compared to its conventional alternative (Brookes and Barfoot, 2006). Globally, GE soybean has benefited developing and developed countries similarly, with the increase in total value derived from GE soybean adoption at 55% and 45% respectively (Brookes and Barfoot, 2006). Developing countries have had greater benefits from GE soybean based on the allotment of total farm income going towards accessing the technology, with 10% for developing countries and 32% for developed (Brookes and Barfoot, 2006).

Aside from the overall gain seen by farmers of GE soybean, the environment too, has derived benefit. The volume of active ingredients in herbicides has fallen by 51.4

million kg which has reduced total environmental impact by equitable amounts in both developing and developed nations, with 47% and 53% respectively (Brookes and Barfoot, 2006).

The environment has also profited from GE soybean by slowing the release of CO_2 from agricultural lands. In 2005, an estimated 7,747 million kg of CO_2 was sequestered from the atmosphere in the U.S. from the use of GE herbicide-tolerant soybean, which is equivalent to removing 3,097,778 cars $year^{-1}$ from the road (Brookes and Barfoot, 2006).

D. Genetic Engineering of Soybean

Soybean was originally engineered using *Agrobacterium tumefaciens* harboring a binary plasmid containing neomycin phosphotransferase (*nptII*) from *Escherichia coli* for selection of subsequent developing shoots (Hinchee et al., 1988). While using *Agrobacterium tumefaciens* as a vehicle to deliver transgenes into soybean originally had a low transformation efficiency (0.3-2.2%), various modifications to the protocol, including the addition of acetosyringone (Stachel et al., 1985), tissue wounding prior to inoculation (cited in Parrott and Clemente (2004)), reducing the pH of the inoculation medium to 5.4 (Godwin et al., 1991), rooting in medium without herbicide selection pressure (cited in Parrott and Clemente (2004)) and the addition of L-cysteine, has increased transformation efficiency to as high as 16.4% (Olhoft et al., 2003).

Soybean transformation via particle bombardment was developed concurrently with *Agrobacterium*-mediated methods. The original microprojectile bombardment used tungsten particles coated with DNA and shot into onion cells (Klein et al., 1987). In

soybean, the embryonic apex was targeted with microprojectiles carrying the intended trans-DNA, and emerging shoots were selected based on the appropriate marker (β-glucuronidase (*gus*) expression or glyphosate, phosphinothricin, or imazapyr resistance) (Aragão et al., 2000; Christou et al., 1989; Martinell et al., 1999; McCabe et al., 1988; Russell et al., 1991). The use of apices as target tissue has too low a transformation efficiency to be widely accepted, due to the basic need of the second cell layer requiring transformation since only these cells become the germline (Parrott and Clemente, 2004).

The ability to genetically engineer soybean somatic embryos arose following the development of an efficient tissue culture system wherein immature soybean cotyledons could be induced to produce somatic embryos which could develop, mature and germinate into plants (Christianson et al., 1983; Parrott et al., 1988; Yin et al., 1982). A particle bombardment-mediated transfer system was developed for soybean, and has proven a reliable and consistent method for its engineering (Parrott and Clemente, 2004; Trick et al., 1997).

Particle-bombardment, with tungsten particles now largely replaced with gold, has proven to be highly efficient in soybean and other crops (Christou et al., 1988; 1989). Soybean has now been engineered with transgenes from many other organisms, a review of which can be found in Widholm et al. (2009)

From a commercial perspective, the most important application to date of genetic engineering in soybean is the development of transgenic herbicide tolerance (Brookes and Barfoot, 2006). In the US alone, herbicide tolerant soybean increased farm income over $1 billion in 2005 and globally over $2.2 billion, principally by lowering herbicide costs, and those of labor and machinery (Brookes and Barfoot, 2006). Monsanto

Company, St. Louis, MO, developed the first herbicide tolerant soybean, released in 1996, known as the Roundup Ready (RR) soybean. A second generation, RR2Y is available this year, as are Pioneer's TREUS™ low linolenic soybean (Pioneer Hi-Bred International, Inc., Johnston, IA) and Bayer's LibertyLink® soybean, which is resistant to Bayer's Ignite® herbicide (Bayer CropScience LP, Research Triangle Park, NC).

The over 54 million hectares of transgenic soybean planted around the world in 2005, accounting for the majority of soybean cultivation in major soybean growing countries such as the United States, Brazil and Argentina, represents the market's ability to accept genetically modified soybeans (Brookes and Barfoot, 2006).

4. Hairy Root Transformation Analysis

Stable genetic transformation of soybean, including particle bombardment-mediated transformation, has inherent limitations for the rapid assay of transgenes. Mainly, stable transformation requires enough time for the soybean to complete its life cycle. Regeneration from embryogenic tissue culture can take 12 months to recover T1 transgenic seed from the starting T0 seed, or a heavy labor demand in producing large numbers of independent transgenic events if targeting cotyledonary nodes (Parrott and Clemente, 2004; Trick et al., 1997). In contrast, the use of composite plants allows rapid evaluation of transgene function in those cases where the transgenic trait is meant to be expressed in the root system. Following inoculation with *Agrobacterium rhizogenes,* the resulting plants are considered to be composite because only their root system is transgenic, multiple events are present in the roots, and each root can represent one or more transformation events. With transgenes confined to the root system, seeds are not transgenic. The single greatest advantage of assaying transgenes using *A. rhizogenes* is the rapidity with which transgenic roots can be obtained and assayed—only a few weeks post inoculation.

A. Biology of Agrobacterium rhizogenes *Infection*

Agrobacterium rhizogenes, a gram-negative bacterium, acting in a similar manner to the popular genetic engineering tool, *Agrobacterium tumefaciens*, establishes an intimate parasitic relationship with various plant species to gain a carbohydrate and

nitrogen source provided by the plant in the form of opines (Dessaux et al., 1993). The primary difference between *A. rhizogenes* and *A. tumefaciens* is the disease phenotype. While *A. tumefaciens* causes an oncogenic display of heightened cell division, producing a visible gall, or tumor, on the plant's surface, *A. rhizogenes* produces a more precise phenotype of proliferative root growth and branching. The necessary genes for parasitism are on the Root-inducing (Ri) plasmid, some of which are transferred into plant cells and stably inserted into the host genome. These genes travel in a conglomerate of DNA and proteins called the transfer strand (T-strand). Gene-transfer is accomplished via the bacterium's type IV secretion system, the same protein network used in bacterial conjugation.

The host plant uses genes from *Agrobacterium* to produce a specific opine, i.e., nopaline or octapine (Chilton et al., 1982). The parasite is then able to catabolize its associated opine (Petit et al., 1983). Other genes inserted by the parasite cause plant production of auxins, and by *A. tumefaciens* cytokinins as well (Ove Nilsson, 1997). The production of these plant growth regulators, elicited by sequences transmitted from these bacteria, highlight the major difference between the two species. While both incorporate sequences to upregulate auxin production, only wild-type *A. tumefaciens* transfers genes for cytokinin biosynthesis in its host, giving rise to its hallmark gall phenotype; no galling occurs in *A. rhizogenes* infections (Ove Nilsson, 1997), which only synthesize auxins.

The first sequenced Ri plasmid, from *A. rhizogenes* strain MAFF301724, revealed striking evolutionary information in comparison to the Tumor-inducing (Ti) plasmid from *A. tumefaciens* and the Symbiosis (Sym) plasmid from *Rhizobium* spp. Moriguchi et al.

(2001) described the Ri plasmid as a chimeric hybrid of the Sym and Ti plasmids. Genes, such as the *tra*, *trb*, and *rep* families, involved respectively in conjugation and replication, are more closely related in sequence and synteny between the Sym and Ri plasmids, whereas *vir* (virulence) genes and opine catabolism genes have a greater similarity between the Ri and Ti plasmids (Moriguchi et al., 2001).

While work on the exact mechanism of DNA integration from *Agrobacterium* into its host has focused primarily on *A. tumefaciens*, the similarity in sequence of their *vir* regions suggests that *A. rhizogenes* gene transfer functions in a similar way to that of *A. tumefaciens* (Moriguchi et al., 2001). The transfer strand (T-strand) produced from the Ri or Ti plasmid is comprised of transfer-DNA (T-DNA) for host cell transformation, as well as associated proteins. Attachment of the bacterium to the host cell wall surface is required to initiate host infection. This is stimulated by host cell exudates of monosaccharides and phenolics from wounded tissue, which are perceived by bacterial membrane receptors, initiating membrane attachment proteins including ChvA, ChvB, PscA, and Att (Stachel et al., 1985; Tzfira and Citovsky, 2002). These signals allow the origin of transfer to be transcribed and translated into various DNA transfer (Dtr) and replication proteins, of which a relaxase enzyme knicks the DNA at the origin of transfer and remains covalently bound to the 5' end (Cascales and Christie, 2003). Other Vir proteins then bind to the nascent single-stranded DNA T-strand, super-coiling the strand and allowing for targeting of the mating-pore-formation (Mpf) proteins which make up the physical membrane spanning channel of the type IV secretion system (Cascales and Christie, 2003).

Once the T-strand is in the host's cytoplasm, plant chaperone proteins bind to the accompanying Vir proteins and probably help maintain correct Vir conformation and phosphorylation of the Vir proteins, particularly VirD2, enhancing overall virulence (Deng et al., 1998). Still other endogenous host proteins, such as *Arabidopsis*' VIP1 and VIP2 bind to the T-strand, acting as localization signals for membrane transport into the nucleus (Tzfira and Citovsky, 2002). Inside the host nucleus, VIP1 appears to play the integral role in DNA insertion into the plant chromosome, since it is able to interact with a TATA-box binding protein (TBP) and the H2A histone (Bakó et al., 2003; Mysore et al., 2000). The host DNA repair mechanism also plays a critical role in dsDNA synthesis of T-DNA, chromosomal double-stranded breaks, and final T-DNA insertion (Tzfira and Citovsky, 2006; Tzfira et al., 2003).

This foreign DNA, being completely incorporated into the host genome, persists for the life of the cell, and if said cell is totipotent and the *Agrobacterium* strain is disarmed, the foreign gene would persist in subsequent generations, segregating in Mendelian ratios. This is typically not the case with *A. rhizogenes* transformation, since transgenes are confined to root tissue. However, all root cells emerging from the engineered cell are transgenic, and entire root systems emerging from those transformed cells are transgenic (Veena and Taylor, 2007).

B. Hairy Root Culture Applications

Much research has been aimed at utilizing hairy root cultures of plants containing high-value secondary metabolites, providing a perpetual supply of plant tissue from which the products of interest can be extracted. A review of the multitude of metabolites

that can be produced using hairy root cultures can be found at Srivastava & Srivastava (2007). Hairy root cultures have also served as a medium themselves on which to cultivate or study obligate plant pathogens. Fungi such as *Polymyxa betae* have been cultured axenically on sugar beet hairy roots, as has the important symbiotic mycorrhizal fungus, *Glomus mosseae* on hedge bindweed hairy roots (Mugnier, 1987; Mugnier and Mosse, 1987). *Sinorhizobium meliloti*, a nitrogen-fixing endosymbiotic bacterium, was maintained and its interaction studied with hairy roots of *Medicago truncatula* (Boisson-Dernier et al., 2001). Nematodes too, such as *M. javanica,* have been maintained on potato and tomato hairy root cultures (Verdejo et al., 1988), *M. hapla* on onion, tomato, and dandelion hairy roots (Mitkowski and Abawi, 2002), *M. incognita* on melon hairy roots (Adachi, 1992), *Radapholus similis* on hairy roots of carrot (Elsen et al., 2000), *Heterodera schactii* on sugar beet hairy roots (Kifle et al., 1999) and *H. glycines* on soybean hairy roots (Cao et al., 2009; Narayanan et al., 1999).

A. rhizogenes use in plant transformation differs from *A. tumefaciens* in that *A. rhizogenes* strains typically are not "disarmed" by removal of all T-DNA sequences aside from trans-factors, such as the *vir* genes critical in DNA transport and integration (Mankin et al., 2007). Since the hairy root phenotype is often a desirable part of *A. rhizogenes* transformation, especially where strict root culture is needed, a completely disarmed strain is not desirable, as is the case for *A. tumefaciens*-mediated gene transfer systems. Strains that have been disarmed, such as A4RS, were done so primarily to study the host-parasite interaction, and showed a decreased level of virulence across host species, like soybean (Owens and Cress, 1985).

A. rhizogenes strain K599 is a cucumopine catabolizer, isolated from cucumber in the 1970's, and has become a popular biovar. Its main chromosome is similar to that of *A. tumefaciens* Biovar 1, however its Ri-plasmid is similar to that of *A. rhizogenes* Biovar 2 (Mankin et al., 2007; Sawada et al., 1993). The diversity between the genomic and plastid DNA may be the cause of its broad spectrum virulence across many species and cultivars. Strain K599 has been documented to induce a hairy-root phenotype on species including, cucumber, Russian olive, licorice, maize and tomato (Mankin et al., 2007; Mehrotra et al., 2008; Savka et al., 1990; Savka et al., 1992; Weller et al., 2004). Strain K599 has proven virulent across many genotypes of soybean too, including, 'Carter,' 'Fayette,' 'Hartwig,' 'Jack,' Lee 68,' 'Mandarin,' Maple Arrow,' 'Peking,' 'PI 437654,' and 'Williams 82' (Cho et al., 2000). Of these , Cho et al. (2000) determined 'Carter' and 'Peking' to be most susceptible to K599 transformation, with efficiencies of 95% and 90% respectively. Even the cultivar 'Hartwig,' with the lowest transformation efficiency obtained, had a relatively high efficiency at 54%. These findings prove *A. rhizogenes* K599 is a suitable candidate strain for soybean hairy-root transformation.

C. Composite Plants for Transgene Analysis

A. rhizogenes' ability to create composite plants which express transgenic DNA only in roots has had great use in transgene analyses, even if it has had little application for the recovery of stable transgenic plants. Even with the advent of disarmed strains, which avoid the phenotypic abnormalities seen in *A. rhizogenes*-mediated transgenic plants from early work, *A. rhizogenes* is not a mainstream technology used to create stable transgenic plants, likely due to the popularity of *A. tumefaciens* (Veena and Taylor,

2007). Transformation with armed strains of *A. rhizogenes* results in what is known as the T-phenotype. Aside from the hairy root phenotype of highly branching, agravitropic roots, the T-phenotype is also typified by shorter internodes, reduced male fertility, floral disfiguration, and wrinkled leaves (Tepfer, 1984), which are thought to result from cytokinin over-production.

The ease and speed of genetic transformation with *A. rhizogenes* across a wide array of plant species allows the system to be a rapid means of transgene analysis. Huang et al. (2006b) utilized a hairy root system in tobacco to further clarify the function of the essential parasitism gene from RKN, 16D10, which is discussed in chapter 2C. The *Mi* gene from tomato, which confers a high level of resistance to the three major RKN species, *M. incognita, M. javanica,* and *M. arenaria*, was examined using RKN-susceptible tomato hairy roots. The rapid rate of transformation using *A. rhizogenes* allowed for a faster elucidation of the various gene regions of *Mi,* including its nuclear-binding site (NBS) and leucine-rich repeat (LRR) domains of the final protein (Hwang et al., 2000).

Promoter efficiency has been compared in soybean hairy root systems. Narayanan et al. (1999), in preparation for proposed future nematode resistance gene assays, tested *Gus* expression being driven by: the cauliflower mosaic virus 35S (*CaMV 35S*), chalcone synthase-8 (*CHS*) from bean, or phenylalanine ammonia lyase (*PAL*) from *Arabidopsis thaliana*, promoters. Incorporating these constructs into 4 different soybean cultivars via *A. rhizogenes* showed that the *PAL* and *CHS* promoters provided significantly better *Gus* expression than did *CaMV35S* (Narayanan et al., 1999). This study also axenically challenged the *CHS*- and *PAL*-driven transgenic hairy roots of each

of the four soybean genotypes with SCN J2s. They determined that the two SCN-resistant genotypes (‘Bell’ and ‘Faribault’) averaged an 86% reduction in cyst numbers over the two susceptible genotypes (‘Agassiz’ and ‘Parker’). These results were congruent with the genotypes’ differences in resistance and susceptibility under both field and greenhouse conditions, providing assurance that the hairy-root system would not inherently augment soybean cyst nematode parasitism, and thus can be used as an accurate predictor of field performance (Narayanan et al., 1999).

5. RNA Interference

Silencing the expression of a gene has proven to be one of the more powerful tools in molecular genetics research. The most obvious application to gene knockouts is to infer its function based on its null phenotype. This approach is known as reverse genetics, whereby knowing the sequence of a gene can allow a researcher to stop its ability to give rise to its protein complement, therein deducing the gene's function. An older, but still commonly used method is by actual DNA mutation. Scrambling, replacing, or otherwise augmenting the present DNA sequence can result in a nonfunctional protein, leading to its hypothesized function. RNA interference (RNAi) brought forth another technique to silence protein production.

RNAi is an innate mechanism in animals, fungi, and plants for gene regulation and likely for virus resistance in plants (Bayne et al., 2005; Fagard et al., 2000; Fire et al., 1998; Hamilton and Baulcombe, 1999; Kusaba et al., 2003; Morel et al., 2002; Palatnik et al., 2003; Schwab et al., 2005). Specially designed transgenes resulting in an RNAi reaction *in vivo* to down-regulate expression of a specific mRNA target, rely on the modified organism's inherent RNAi machinery (Fagard et al., 2000). This ability not only creates a powerful reverse genetics tool, but provides crop genetic engineers a technology to reduce expression of specific detrimental products within a modified organism, or to reduce harmful parasitism products (Huang et al., 2006a; Mao et al., 2007; Plasterk, 2002).

A. RNAi History

Jacob and Monod (1961) correctly hypothesized two roles of genetic regulation by small noncoding RNA molecules, both pre- and post-transcriptionally. These ideas were dormant for nearly 40 years, overshadowed by the conventional wisdom that proteins were ubiquitously responsible for gene regulation (Bonnet et al., 2006). Curiosities were documented in petunia when transgenic over-expression of chalcone synthase resulted not in the hypothesized increase in pigment, but in some cases, a total lack of purple color (Napoli et al., 1990). This phenomenon, labeled "cosuppression," has now been attributed to RNAi both in the transgenic and naturally derived picotee pattern of variegated inflorescences (Metzlaff et al., 1997; Mol et al., 1983). While it had become understood that multiple copies of a gene could silence each other's expression, the molecular trigger was not defined until the 1998 publication by Fire et al. (1998) in a letter to *Nature*.

Caenorhabditis elegans, the nematode which serves as a model system in studying animal development, was utilized in this experiment out of Craig Mello's lab at the University of Massachusetts. In this work, sense, antisense, and a mixture of complementary sense and antisense molecules homologous to various *C. elegans* genes of described null phenotypes were injected into *C. elegans*. The effect on the target gene varied, depending on whether sense, antisense, or a mixture of the two, was injected. The injection of sense strands led from no to mild changes in phenotype. In contrast, the use of injections with mixed complementary sense and antisense RNA produced a phenotype similar to that of the corresponding null mutant. For example, the 742bp *unc-22*, a myofilament protein whose null mutants show drastic twitching motility, showed no

effect on phenotype when 724-bp of sense or antisense RNA strands were injected separately, but heavy twitching was obtained when both strands were injected simultaneously (Benian et al., 1993; Fire et al., 1998).

Fire et al., (1998) made several inferences from the study that have proven to be true, while others are still unknown. They determined regulation to be post transcriptional, due to the inability for molecules with intron homology to have any impact on protein expression. They saw that the silencing was systemic by injecting double stranded molecules into the head and finding silencing in the gonad, and vise versa. Progeny continued to show the null phenotype when high enough levels of molecules were present in the gonads. They predicted the power of this technology to be used in reverse genetics, but warned it was limited in complete gene product knockdown and that some cells may remain unaffected by silencing. Finally they also conceded its limitation in having a likelihood of down-regulating gene families which share large stretches of homology, producing phenotypes different than those obtained by the elimination of a single gene product. Limitations aside, their discovery of RNAi, triggered by double-stranded RNA molecules, led to Andrew Fire and Craig Mello being awarded the Nobel Prize in medicine in 2006.

B. The RNAi Mechanism

After the discovery that double-stranded RNA acted as a trigger for RNAi, much of the silencing pathway has been elucidated over the past 10 years (Figure 1). Double-stranded RNA is recognized by the enzyme DICER which binds to and digests the RNA

into small ~22nt RNA molecules, known as a small interfering RNA (siRNA) (Bernstein et al., 2001; Hamilton and Baulcombe, 1999). DICER is an RNase III nuclease which shares homology to the ARGONAUTE protein family which includes other RNAi linked enzymes, and is also conserved across animals, plants, and fungi (Bernstein et al., 2001). The small RNA molecule's next step in RNAi processing is its association with an RNA Induced Silencing Complex (RISC). RISC relies on the conglomeration of RNA and protein molecules, which when bound to a small noncoding RNA is guided to that homologous mRNA for mRNA destruction via primary endonuclease activity (Hammond et al., 2000).

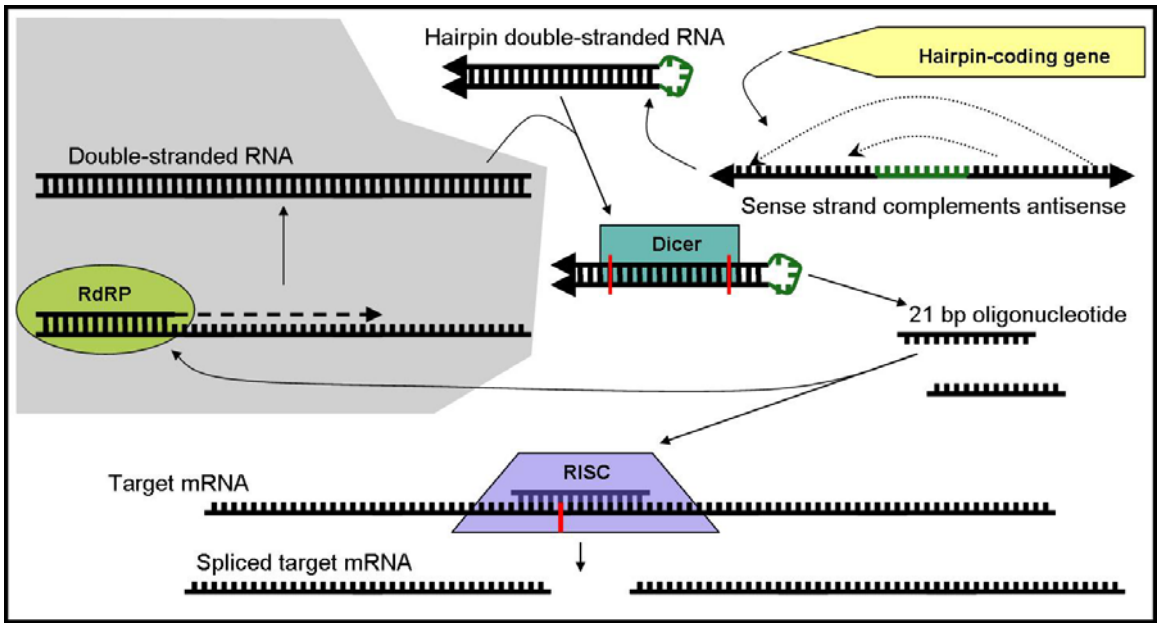

Figure 1. A hairpin-coding trans-gene creates a mRNA with complementary sense and antisense regions, separated by an intron. Double-stranded RNA is recognized by Dicer, and cleaved into short-interfering RNAs, ~22 nucleotides. This siRNA is associated with and guides an RNA-induced Silencing Complex of proteins to an mRNA complementary to the siRNA and cleaves the mRNA via endonuclease activity. An amplification step is highlighted in gray, whereby the siRNA, acting as a primer, can anneal to its target mRNA, but joined by an RNA-dependent RNA polymerase, synthesizes the mRNAs full length complementary strand creating a nascent dsRNA molecule, recognizable by Dicer. Modified from Kusaba et al., 2004.

While small RNAs can serve in RISC as guides for targeted destruction of homologous mRNA, resulting in gene silencing, they also can serve as a primer source in the creation of more double-stranded RNA molecules, leading to an amplification of RNAi. A small RNA bound to a complementary mRNA can drive an RNA-dependent RNA Polymerase (RDRP) to synthesize an increased length of dsRNA, 5'→3', being again recognized by DICER, stimulating an up-regulation of silencing potential via production of more siRNAs than a single dsRNA molecule could aptly produce (Sijen et al., 2001). This amplification ability increases the utility of RNAi technology for all of its potential applications, be it reverse genetic studies, or transgenic crop improvement.

A distinction was made early on between small noncoding RNAs that differ in their biogenesis. siRNAs, the originally discovered type of small RNA functioning in RNAi, are derived from double-stranded RNA created by intermolecular bonding of sense and antisense RNA complements, or via *de novo* synthesis by RDRP (Fire et al., 1998; Sijen et al., 2001). An important distinction is that siRNAs target the gene that produced them, along with homologous loci. In contrast micro RNAs (miRNAs) silence distant genes, separate from the miRNA gene (Bartel, 2004). Genes for miRNAs incorporate sequences of sense and antisense complementation in a single 5'→3' orientation, which are separated by various intronic or noncomplementary regions, thus creating a pre-miRNA hairpin molecule (Dykxhoorn et al., 2003).

Both siRNAs and miRNAs share some RNAi machinery, while also relying on different enzymes or enzyme constituent proteins. Members of the ARGONAUTE (AGO) protein family are the elemental pieces of the RISC, which can include different AGO proteins depending on the small RNA involved. *Ago1* and its closest paralog,

ago10, are used in both siRNAs and miRNAs. A lack of AGO1 causes hypersensitivity to viral infection, since virus resistance is an siRNA mediated RNAi pathway (Lynn et al., 1999; Morel et al., 2002). *Ago7* is critical in producing trans-acting siRNAs (ta-siRNAs), which relies on proper processing of guide miRNAs, though miRNAs do not require *Ago7* (Chapman and Carrington, 2007; Vazquez et al., 2004). *Ago4* and *ago6* work explicitly with heterochromatin modulation via siRNA production (Zheng et al., 2007).

Various DICER-Like (DCL) enzymes are also involved with different small RNAs. Twenty-one-nucleotide miRNAs rely on DCL1, though some variability in size has been found (Rajagopalan et al., 2006). DCL4 results in ta-siRNAs as well as in siRNAs produced via transgenes and viral siRNAs (Bouche et al., 2006; Deleris et al., 2006). RDRPs can also differ in their involvement with specific siRNA products, with RDR6 interacting with the 21-nt transgenic siRNAs and virus RNAs, and RDR2 interacting with 24-nt siRNAs created with DCL3 (Bouche et al., 2006; Deleris et al., 2006; Xie et al., 2004). Adding to the complexity of these regulation networks, these members often have the facility to compensate for each other, such as DCL2's ability to compensate when DCL3 or DCL4 are nonfunctional (Bouche et al., 2006).

C. Applications of RNAi in Crop Development

The first account of using RNAi for crop improvement was published by Waterhouse et al. (1998). The group successfully engineered tobacco to obtain resistance to Potato Virus Y (PVY). Cloning out a protease gene from the virus, the group experimented with sense, antisense, and sense-antisense formations of transgenic

tobacco, finding that transgenic tobacco producing double stranded RNA molecules homologous to PVY's protease gene conferred effective viral resistance (Waterhouse et al., 1998). This portrays perhaps the most obvious use of RNAi technology in crop improvement, pest resistance, though uses are certainly far-reaching and range from metabolic pathway manipulation to reverse genetic screening for gene function identification.

Proper vector creation was critical for researchers to properly engineer plants in producing siRNAs for RNAi. Smith et al. (2000) did the most influential work in this regard. They targeted *FAD3* in *Arabidopsis* using various permutations of sense:antisense vectors with and without assorted introns. The use of an intron to separate the sense and antisense DNA strands resulted in the most effective gene silencing, likely due to increased molecular flexibility for the formation of a hairpin structure (Smith et al., 2000). Currently many groups have contributed to the cloning of RNAi constructs by creating vectors for use in gene silencing transformation containing components required for different applications, including different selectable markers, *Agrobacterium*-transformation, different promoter:terminator combinations, chemically inducible silencing and multiple gene targets (Dafny-Yelin et al., 2007; Guo et al., 2003; Peretz et al., 2007; Wang, 2006; Wielopolska et al., 2005).

Critical to transgenic crop development is the stable transmission of the transgene to future progeny. Since RNAi is necessary for normal plant development, expression of novel siRNA molecules in a cell should not be a problem. In fact, a conventionally bred rice genotype, Low Glutelin Content-1 (LGC-1), created in the 1970's, was discovered to derive its low-glutelin phenotype from gene silencing of glutelin production via inverted

glutelin repeats, which were able to form double-stranded RNA via hairpin formation, producing siRNAs (Kusaba et al., 2003). Soybean too, has tissue-specific silencing of chalcone synthase in the seed coat of buff colored seeds, while wild-type black-coated seeds do not contain siRNAs homologous to *CHS* family members (Tuteja et al., 2004). This appears due to a large deletion, in the buff seed coat genotypes, between two *CHS* loci which are highly homologous, containing within themselves multiple inverted repeat copies (Tuteja et al., 2004).

Transgenic targeting of *FAD3* in *Arabidopsis* has also been shown to be stable for at least five generations (Stoutjesdijk et al., 2002). However, examples exist that show how gene silencing may not be stable, resulting from methylation of promoter regions (Fojtova et al., 2003; Mitsuhara et al., 2002). This type of epigenetic silencing is an aspect of all genomes, potentially silencing expression of any gene or transgene, and needs to be considered during the breeding process as transgenic products reach their final downstream applications.

Removing allergens via RNAi is another potential use of the technology. Apple's principle allergen, Mal d 1, was silenced via a hairpin producing transgene targeting *mal d 1*, and could potentially be grown in areas where widespread human reactions to Mal d 1 is present, i.e., in areas with high birch pollen counts (Gilissen et al., 2005). Ara h 2, one of the more potent allergens found in peanut, was successfully down regulated, resulting in a lower histological response according to ELISA tests, which demonstrates the possibility of a semi-hypoallergenic peanut (Dodo et al., 2008). Transgenic ryegrass has been created to silence the Lol p 5 allergen in pollen, though definitive results were unclear (Bhalla and Singh, 2004). Allergen Lyc e 3 in tomato also has been silenced with

transgenic RNAi to a level less than 0.5% that of its non transformed isogenic line (Le et al., 2006).

There are limits to the use of RNAi strategies for the attenuation of allergens. Transgenic RNAi approaches to achieve hypoallergenicity in foods could have serious consequences for consumers who are deathly allergic to some compounds, such as peanut. First, RNAi rarely achieves a complete knock-out of its targeted protein production. A consumer with a low threshold tolerance for the allergen could still be affected. Second, RNAi silencing suppressors exist in nature. One of these, a helper component-proteinase (HC-Pro) of potyviruses, is able to inhibit the accumulation of siRNAs due both to viral induced gene silencing (Anandalakshmi et al., 1998) and transgene-induced gene silencing (Brigneti et al., 1998; Mallory et al., 2001). If an allergen-susceptible consumer were to eat a transgenic food whereby the allergen production was silenced by RNAi, and that item had been infected by the potyvirus harboring HC-Pro, silencing could be reversed, leading to the allergen's production and potentially inflict substantial harm in the consumer.

Using RNAi to manipulate levels of certain innate plant products is also possible. Lysine content was increased in corn by gene silencing of lysine-ketoglutarate reductase/saccharopine dehydrogenase, a dual functioning lysine degradation enzyme, leading to an increase of over 600 ppm free lysine over wild-type levels in T3 kernels (Houmard et al., 2007). Also, siRNAs were found to only exist within kernels, and transport throughout other plant tissues was not detected (Houmard et al., 2007). Coffee plants were transformed to be intrinsically 70% decaffeinated by RNAi of theobromine synthase (Ogita et al., 2003). High oleic and high stearic acid cotton plants have also

been created by RNAi, in this case silencing expression of *ghFAD3-1* and *ghSAD-1* (Liu et al., 2002). Heavy metal bioaccumulation can occur at high concentrations in certain crops. Rice plants grown in cadmium-rich soils accumulate this toxic metal, unless it is rice engineered to silence a phytochelation synthase enzyme, which reduces cadmium concentrations by nearly 50% (Li et al., 2007).

Targeting of metabolic pathways can also have profound physical results important for commercial crop production. Plant height is influenced by multiple pathways controlled by numerous genes. The gibberellin phytohormones are key determinants of plant height. Popularly known as the "Green Revolution Gene," *sd-1* (*semi-dwarf 1*), was mutated, thus causing dwarfed rice. The *sd-1* allele, known formally in its wild-type as *OsGA20ox2*, was targeted by an RNAi -eliciting hairpin forming RNA homologous to 531 bases of *OsGA20ox2* and engineered into rice. The result was a height reduction of 54-58% from wild-type (Qiao et al., 2007). Transgenic rice had similar yields to that of the wild-type, while presenting a similar height reduction as the mutated *sd-1*, thus showing the potential for RNAi's use in metabolic pathways and laying the groundwork for drastic advances in crop production methods (Qiao et al., 2007).

Silencing gene expression in plant pests can have important applications. Since many plant pests establish intimate feeding relationships with their host, it becomes possible to disrupt the parasitism by destroying some functionality within the pest. The cotton bollworm, while currently controlled transgenically in cotton with various *Bt cry* genes, will likely become resistant to the crop at some point in time, making necessary a broadened resistance strategy (Pray et al., 2001). The use of RNAi-based strategies can

supplement Bt-derived resistance and help postpone the loss of effectiveness expected to affect Bt. Expressing hairpin-forming RNA identical to that of a cytochrome P450 monooxygenase, *CYP6AE14*, from cotton bollworm in both transgenic *Arabidopsis* and tobacco resulted in a lower growth rate of larvae exposed to the transgenic plants with gossypol as compared to growth on gossypol and wild type plants alone (Mao et al., 2007). By silencing the P450 monooxygenase, the corn earworms lost their ability to detoxify gossypol from cotton. Similarly, transgenic maize producing siRNAs targeting various western corn rootworm vascular ATPases: subunits A, D, and E, along with α-tubulin, experienced less feeding damage than its parental maize (Baum et al., 2007).

Nematodes have also been targeted for control using RNAi. Aside from Huang et al. (2006) who achieved effective RKN resistance by targeting *16D10*, other groups have shown potentially effective targets for control. Steeves et al., (2006) transformed soybean genotypes 'Jack' and 'Chapman' with a major sperm protein, *MSP*, which in females acts as a hormone for meiotic maturation of oocytes as well as ovarian muscle contractions (Kosinski et al., 2005; Miller et al., 2001), cloned from SCN, utilizing primer sequence determined from *C. elegans*. The vector contained a 231-base-pair fragment of *MSP* in a sense and antisense direction, separated by 103 bases of noncomplementary *MSP* DNA. Eight weeks after T_0 plants were challenged with SCN eggs (60 cm^{-3}), a 49% reduction in cysts g^{-1} root was found over control plants (Steeves et al., 2006). The group went on to quantify the transgenic plants' effect on SCN's reproduction in subsequent generations. They measured egg numbers produced by the offspring from eggs collected from transgenic roots and reapplied to the susceptible soybean, genotype 'Flyer.' The progeny showed 75% g^{-1} root reduction in egg numbers,

providing evidence that the gene suppression lasted at least one generation in nematodes (Steeves et al., 2006).

A separate team engineered tobacco to produce dsRNAs complementary to two different genes, a splicing factor and an integrase, which are present in both *Meloidogyne spp.* and *C. elegans*. They claimed a reduction of parasitism on transgenic roots, but showed no quantifiable results on gall or egg numbers, while claiming that 23 of the 25 transgenic plants inoculated were seemingly totally resistant to gall formation (Yadav et al., 2006). What they did show, unlike the other papers, was the absence of target mRNA in the feeding nematode populations, compared to those feeding on nontransgenic roots, proving the ability for host generated dsRNA to degrade homologous RKN genes via RNAi (Yadav et al., 2006).

Another group has looked for genes to potentially control SCN, using a functional genomics comparison between SCN and *C. elegans*. They isolated sequences from SCN that were conserved to those known to produce lethal results when mutated or absent in *C. elegans*. While the study presents a general strategy to identify potential target gene sequences, it also presents a dsRNA feeding study on SCN targeting *rps-23*, a ribosomal sequence, which effectively killed the nematodes (Alkharouf et al., 2007). Nevertheless, the use of genes homologous to a wider species array other than the target nematode increases the likelihood of inadvertently targeting other nematode species. Since free-living nematodes are beneficial and prevalent in soil fauna, interfering with their presence could produce undesirable outcomes. Furthermore, since dsRNA is taken up into *Meloidogyne* eggs after soaking them in a solution containing dsRNA, effectively silencing their target gene, this caution may prove more than rhetoric (Fanelli et al.,

2005). Hence, there are advantages to research targeting a single nematode species by fine-tuning the siRNAs to silence expression of genes found only in the intended pest.

Deriving nematode resistance by targeting essential SCN parasitism genes, was reported recently by Sindhu et al. (2009). Transgenic *Arabidopsis*, producing siRNAs created from soybean cyst nematode genes, but targeting homologous regions of the beet cyst nematode, decreased female nematode development over wild type plants by up to 64% (Sindhu et al., 2009). However, no transgenic lines singly targeting any of the four genes displayed complete beet-cyst nematode resistance.

6. Gmubi and MtPT1 Promoters

Gmubi is a soybean polyubiquitin promoter, hence Glycine max *polyubiquitin*. The promoter was isolated by John Finer and compared in two different forms to the *CaMV-35S* promoter. Since polyubiquitin promoters have often been found to require preintronic regions for proper/maximum functioning, the Finer group tested the promoter sequence with the intron (*Gmubi*) and without (*Gmupri*) (Plesse et al., 2001; Wang and Oard, 2003). Chiera et al. (2007) bombarded maturing lima bean cotyledons with a particle inflow gun to transiently express green fluorescence protein (GFP) driven by *Gmubi*, *Gmupri* or *CaMV-35S*. Using a robotic platform with a GFP-filter-enabled microscope and camera, images were gathered every hour for 95 h.

The use of Gmubi resulted in total expression that was 5x greater than that obtained by *CaMV-35S* (Chiera et al., 2007). Total expression levels were 2x greater using *Gmupri* compared to *CaMV-35S*. The ability for the two soybean ubiquitin promoters to have higher and longer expression levels than *CaMV-35S* over the 95 test hours was also noted (Figure 2) (Chiera et al., 2007). The strength, durability, and constitutive nature of *GmUbi* make it an ideal promoter choice to be used in soybean transformation to drive transgenes in which maximum, broad–tissue-type expression is desired.

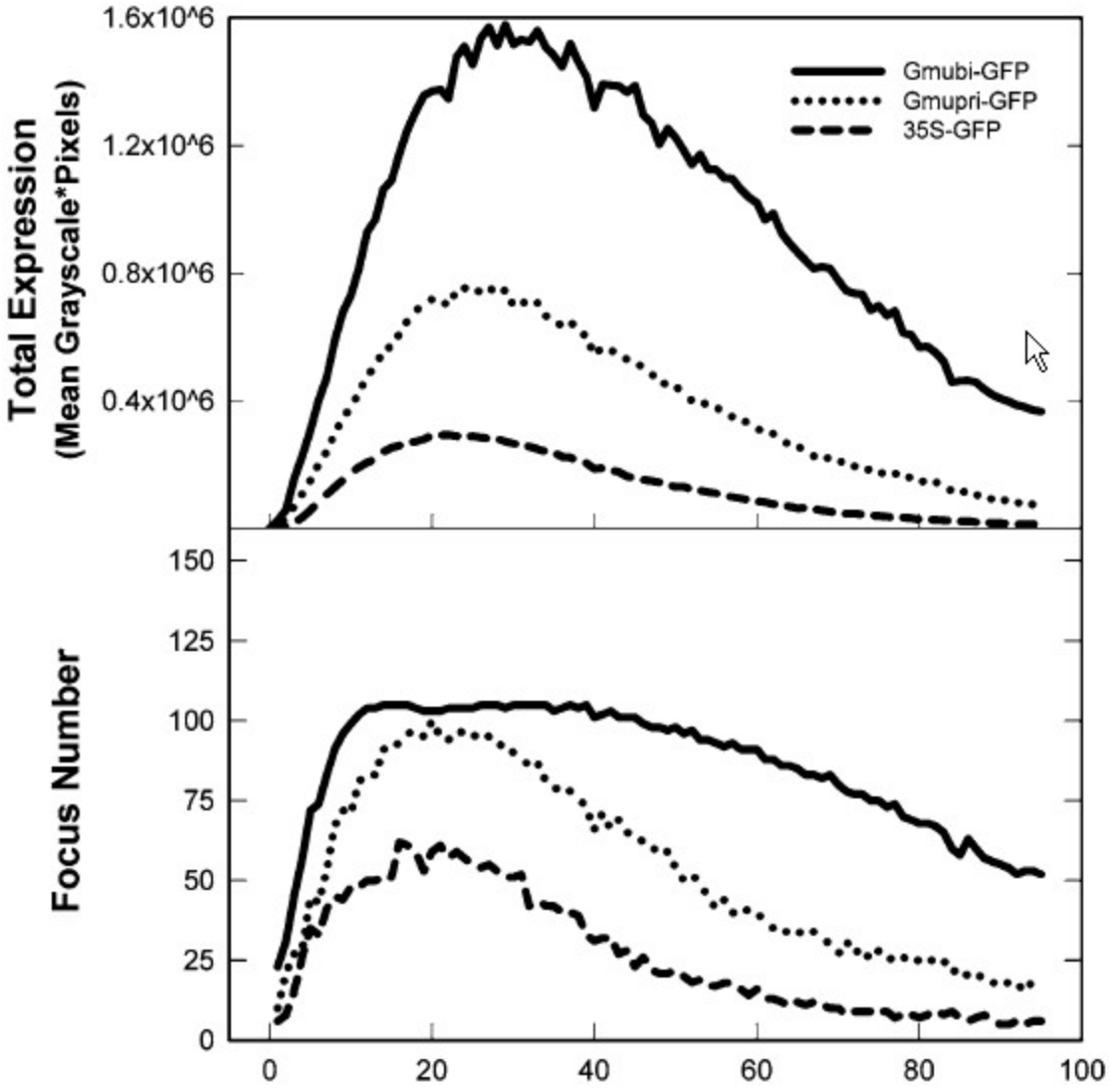

Figure 2. *Gmubi, Gmupri*, and *CaMV35S* expression profiles of 6 replications. "Total Expression" determination is described in "Materials and Methods" of Chiera et al., 2007.

MtPT1, Medicago truncatula *Phosphate Transporter 1*, was originally cloned from cDNA of a mycorrhizal *M. truncatula* root complex (*M. truncatula/Glomus versiforme*) (Liu et al., 1998). Phosphate transporters are required for uptake of orthophosphate available to plants, and for its transport to vascular elements (Chiou et al., 2001). This role in nutrient acquisition means the gene is preferentially expressed in root tissues, with transcription up-regulated in response to a decrease in available

orthophosphate (Chiou et al., 2001). Xiao et al. (2006) engineered *GFP* constructs driven by *MtPT1* and *MtPT2*, as well as the control *CaMV-35S* promoter into *M. truncatula*. Expression of *GFP* was confined to root tissues when driven by either of the *MtPT* promoters, while *CaMV-35S* showed root and apical tissue expression (Figure 3) (Xiao et al., 2006). Root cross sections have shown *MtPT2* having a greater expression over *MtPT1*, as determined by both intensity and extent in root tissues, including vascular elements and epidermal cells (Xiao et al., 2006). These results demonstrate *MtPT* promoters potential to be used in transgenic crop development to drive genes with preferential root expression.

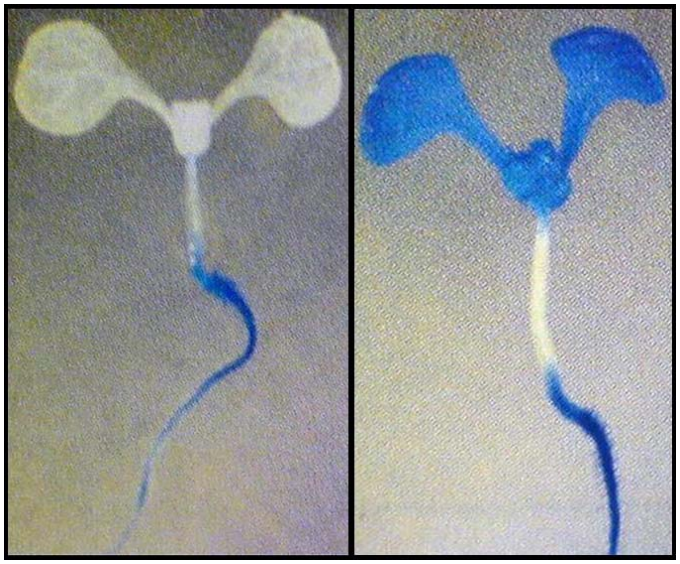

Figure 3. Gus expression profiles in Arabidopsis driven by MtPT1 (Left) and Cam35S (Right). Taken from Xiao et al., 2003.

7. Materials and methods

A. 17H02 *and* 31H06 *Identity*

Genes *17H02* (783 bp) and *31H06* (362 bp) were isolated and cloned into pGEM vectors by Huang et al. (2003) from M. incognita from the RKN dorsal and subventral esophageal gland cells, respectively. *31H06* is actively expressed at the onset of the host cell infection, likely making it a parasitism protein involved in the establishment of a feeding site. *17H02* is expressed after giant-cell initiation, after the dorsal gland has enlarged, likely making it involved in maintenance of the giant-cell.

B. *Conservation of 17H02i and 31H06i in Meloidogyne*

Since the objective is to obtain resistance against all species of RKN that parasitize soybean, it is important to know the extent to which the two targeted RKN nematode genes in this research are conserved across the four major RKN species of *M. incognita, M. javanica, M. arenaria,* and *M. hapla*. If target sequences are conserved, the likelihood of creating cross species control in soybean is high.

Before selecting the open reading frame (ORF) fragments of each gene to be cloned into the silencing vectors, the genes were examined by taking the largest region possible, up to 250 bp, and searching for those areas with the least amount of 18-25 bp matches to known plant sequences compiled in GenBank (Figure 4). Target regions were also determined to lack homology to the currently available soybean genome sequence.

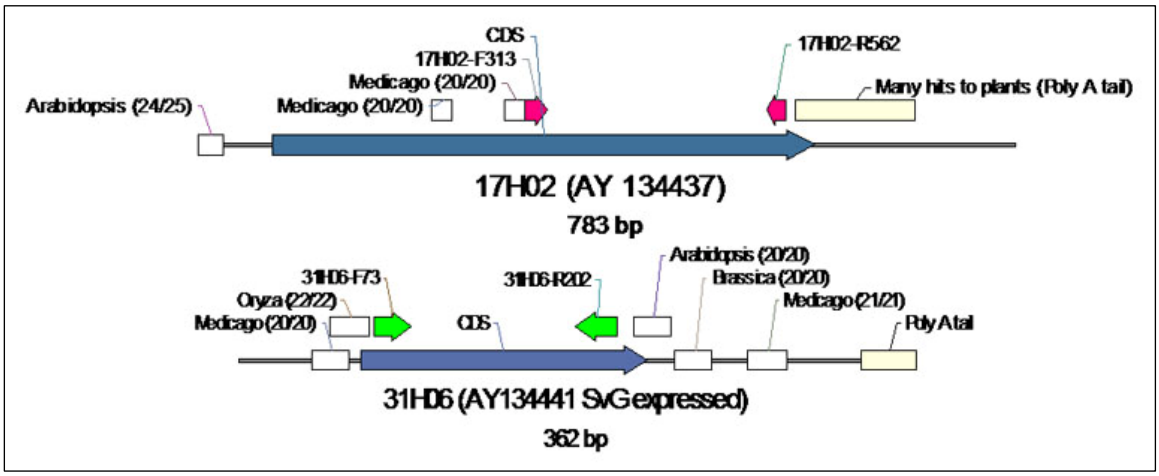

Figure 4. BlastN results yielded 20-24 bp homologous regions to known plant sequences which were avoided in choosing a target sequence for use in RNAi.

DNA isolation from Meloidogyne spp.

Frozen eggs, provided by Richard Hussey, from *M. incognita, M. javanica, M. arenaria,* and *M. hapla* were used as a source of genomic DNA to test for gene conservation according to the protocol developed by Ruihua Dong in Richard Hussey's laboratory. First, 500 µl of frozen RKN eggs were melted slowly on ice, followed by 15 min of grinding in liquid nitrogen in a pre-cooled rack. One ml of extraction buffer (0.2% 5M NaCl, 1% 1M Tris-HCl pH 8.0, 1% 0.5M EDTA, 0.1% SDS, 0.2% Proteinase K, 66% H_2O) was then added to lyophilized tissue and melted to room temperature (RT). Samples were incubated at 37° C for 30 min with inversion every 10 min followed by a 2-hr incubation in a 65° C water bath with inversion every 10 min. Homogenized liquid was taken into two aliquots and each was mixed with 750 µl phenol:chloroform (1:1) and then centrifuged at 16,060 x G for 15 min. The supernatant was transferred to a new tube and again mixed with 750 µl phenol:chloroform (1:1), centrifuged at 16,060 x G for 5', and moved to a new tube. Ten µl RNase (10 mg ml^{-1}) were added and incubated at 37° C

for 1 hr, before repeating the total chloroform extraction sequence. The supernatant was transferred to a new tube and combined with 0.8-1.0 volumes of isopropanol and mixed by inversion. Precipitated DNA was pulled out with a glass hook, washed twice with 80% EtOH,, and allowed to air-dry for 20 min. Dried DNA was dissolved in 200 µl of LTE buffer by incubating in a 70° C water bath before checking DNA concentration at 260 nm with an Ultrospec 200 spectrophotometer (Pharmacia Biotech, now GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

PCR amplification of fragment regions in RKN species and sequencing

RKN genomic DNA was diluted to 10 ng μl^{-1}. Target regions were amplified from genomic DNA by PCR using the following primers (sequences of all primers used in this research can be found in (Table 1): for *17H02*: 17H02-F313 + 17H02-R562 and for *31H06*: 31H06-F73 + 31H06-R202. GoTaq™ PCR (Promega Corporation, Madison, WI) was performed according to the manufacturer's guidelines at reaction conditions: 94° C – 4 min, (94° C – 30 s, 60° C – 30 s, 72° C – 20 s)X40, 72° C – 7 min, and run on a 1% agarose gel with TAE + cytidine and 0.005 % ethidium bromide. Original 17H02 and 31H06 cloned sequences in pGEM® (Promega Corporation, Madison, WI) vectors served as positive controls, being amplified from source DNA concentrations of 650 fg to be copy number equivalent to total RKN genomic DNA. DNA bands of correct size were excised and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., Orange, CA) according to the manufacturer's instructions. Purified DNA was sequenced by the Sequencing and Synthesis Facility at the University of Georgia using the respective primers used in amplifying the regions. Derived sequence data were aligned

and analyzed using MultiAlin Software by Corpet (1988) for complementation to each other.

Table 1. Primer sequences used throughout research. Underlined sequences represent the incorporation of restriction sites for cloning.

Primer	Sequence	Primer	Sequence
17H02-R562	TAGGATCCATTTAAAGGCATAGGTG GCGAAG	17H02-F313	TACCTAGGCGCGCCTTGAAGGCAAAG AATGTAGTGC
31H06-F73	TACCTAGGCGCGCCTCAGCCAATTA TTTGTTCTC	31H06-R202	TAGGATCCATTTAAAGACAAATACCAA ATATTCAGCG
17H02:GmUbi	CATTATATAGGCGCGCCTTGAAGGC AAAGAATGTAGTG	17H02:FAD	GTGAGATTACCATTTAAATGGCATAGG TGGCGAATCAG
FAD:17H02	CCACCTATGCCATTTAAATGGTAATC TCACTCTCACACT	FAD:17H02(anti)	CCACCTATGCCATTTAAATGGATCCAT CTACACATGTT
17H02(anti):FAD	TAGATGGATCCATTTAAATGGCATAG GTGGCGAATCAG	17H02(anti):RbcST	TCGAACCTAGGCGCGTTGAAGGCAAA GAATGTAGTG
31H06:GmUbi	CATTATATAGGCGCGCCAATTATTTG TTCTCTTAATA	31H06:FAD	GTGAGATTACCATTTAAATGACAAATA CCAAATATTCAGCG
FAD:31H06	TATTTGGTATTTGTCATTTAAATGGTA ATCTCACTCTCACACT	FAD:31H06(A)	TATTTGGTATTTGTCATTTAAATGGATC CATCTACACATGTT
31H06(A):FAD	TAGATGGATCCATTTAAATGACAAAT ACCAAATATTCAGCG	31H06(A):RbcST	TCGAACCTAGGCGCGCCAATTATTTGT TCTCTTAATA
17H02:31H06	AGAACAAATAATTGGGGCATAGGTG GCGAATCAG	MtPT1-Fnot	GAGAGAGCGGCCGCGTATGCATGGGC TGGAGTTCGAA-3
MtPT1-Rnot	GAGAGAGCGGCCGCCTAGGCTGAAT TTGTTACCTAGTTTTTCCCT	PZP303R	GATGTGCTGCAAGGCGATTAAG
RbcST_F	GTCCTAGGTTCGAGTATTATGGCATT	PZP303R	GATGTGCTGCAAGGCGATTAAG
RbcST_F	GTCCTAGGTTCGAGTATTATGGCATT	UbiqF	GCAGAGCTTACACTCTCATTC
Hyg504R	GTCGTCCATCACAGTTTGC	VirG73F	TTCAACCGGGTACTTGCATC
VirG472R	TTTCCAGGAAAGCGACGAG	PZPori4327F	AAAGATACCAGGCGTTTCC
PZPori4626R	TCGCTCTGCTAATCCTGTTAC	GmUbi842R	CGAGATTGCTTCAGATCCGTAC
RbcST_110R	CCATTTTCCATTTCACAGTTCG	MtPT1_1430F	CAGTTTATCCATTTCTTACCTC
Le-25F	CAGAATGTGGTTGTATCTCTCTCC	Le-593R	ATGAGAACCTTGGCTACTTTATTG

C. *Vector Construction*

p17H02i and p31H06i

Phusion™ High-Fidelity PCR (New England Biolabs, Inc., Ipswich, MA) was used to amplify both *17H02* and *31H06* from their original pGEM vectors according to

the manufacturer's protocol. Cloning of RNAi vectors utilized In-Fusion™ PCR Cloning (Clontech Laboratories, Inc., Mountain View, CA), which uses a one-step reaction to link together oligonucleotides that have 15 bp overlapping ends. To obtain these homologous ends, the following primers were used to clone target fragments of *17H02* and *31H06* into intermediary RNAi vectors to be used in a separate study utilizing particle bombardment: 17H02:GmUbi, 17H02:FAD, FAD:17H02, FAD:17H02(anti), 17H02(anti):FAD, 17H02(anti):RbcST 5', 31H06:GmUbi, 31H06:FAD, FAD:31H06, FAD:31H06(A), 31H06(A):FAD, 31H06(A):RbcST, 17H02:31H06. Reaction conditions for PCR were 98° C – 1 min, (98° C – 10 s, 63° C – 10 s, 72° C – 20 s) X 25, 72° C – 5 min, and then were isolated on a 1% agarose gel with TAE + cytidine and 0.005 % ethidium bromide, then excised and Zymo-purified before using In-Fusion™ cloning to build the particle bombardment vectors (Figure 5).

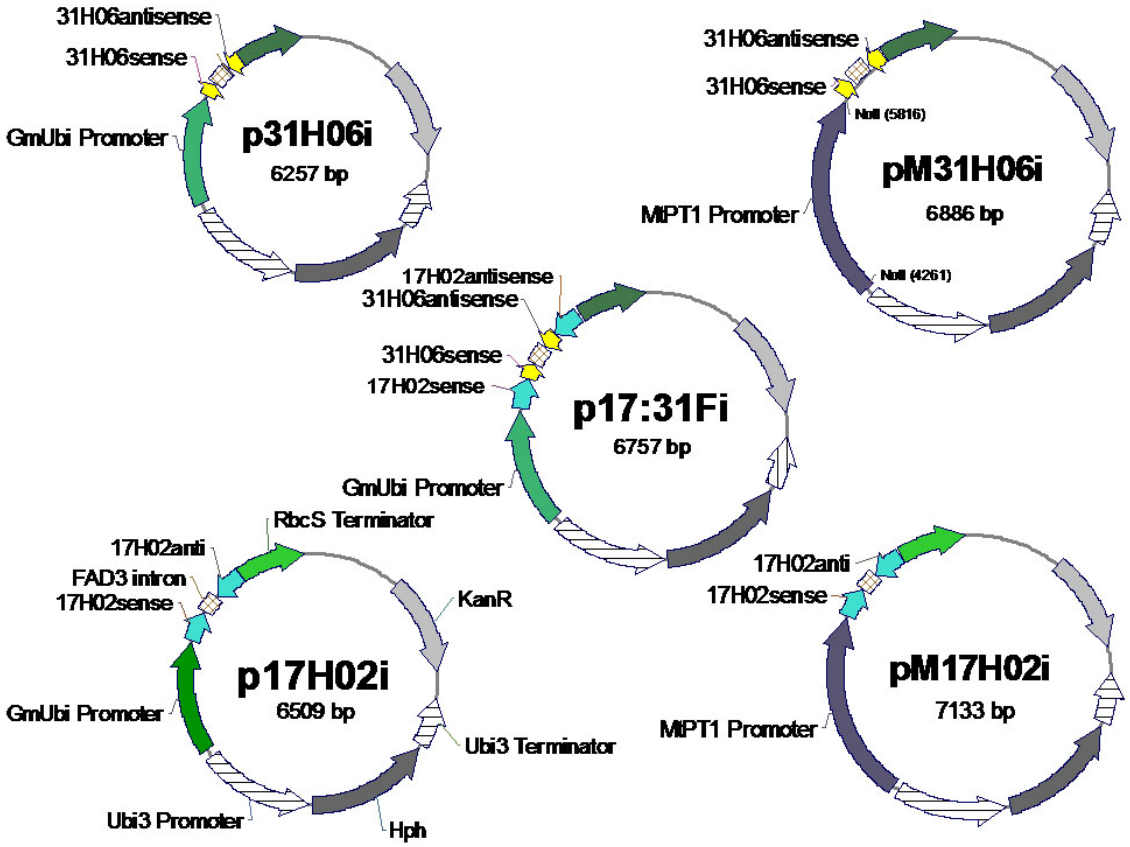

Figure 5. Fragments of RKN gland cell genes 17H02 and 31H06 in sense and antisense orientations, separated by a FAD3 intron, and followed by the RbCST terminator. The three vectors on the left are driven by the *Gmubi* promoter, while the two on the right are driven by MtPT1. The center vector is built to target 17H02 and 31H06 simultaneously. All vectors have resistance to kanamycin for cloning in *E. coli* and HPH regulated by the potato *Ubi3* promoter and terminator for selection with hygromycin in soybean.

pF17:31i

In order to target both *17H02* and *31H06* simultaneously, a single RNAi construct was built with both gene fragments, sense and antisense strands together separated by the *FAD3* intron (provided by Glenn Collins and used due to its proven ability to elicit RNAi (Siminszky et al., 2005)). Two oligonucleotides, one containing the target fragments *17H02* followed by *31H06* and then the *FAD3* intron, and another with just *17H02* followed by *31H06* were synthesized by SeqWright, Inc., Houston, TX. The synthetic

oligonucleotides contained 15-bp overlap homology to the adjacent strand in the final vector for correct orientation. They were cloned into the particle bombardment vector pGmuRNAi using In-Fusion™ cloning (Figure 5).

pM17H02i and pM31H06

The promoter *MtPt1* was amplified out of the vector pPZPH-MtPt1-DCPA1 using Phusion™ High-Fidelity PCR with the following primers: MtPT1-Fnot and MtPT1-Rnot which added a *Not*I restriction site. Reaction conditions were 98° C – 1 min, (98° C – 10 s, 64° C – 10 s, 72° C – 10' s) X 25, 72° C – 5 min, and then was separated on a 1% agarose gel with TAE + cytidine and 0.005 % ethidium bromide, then excised and Zymo-purified. Vectors p31H06i and p17H02i were digested along with amplified *MtPT1* fragments with *Not*I. Then *MtPT1* was cloned into the vectors p17H02i, p31H06i and pF17:31i, replacing *Gmubi*, with Fast-Link™ DNA Ligation kit (Epicentre Biotechnologies, Madison, WI) according to manufacturer's instructions, creating pM17H02i and pM31H06i (Figure 5). All vectors were then sequenced to verify their integrity.

Generation of stable transgenic soybean lines

Soybean genotype 'Jack' embryos were engineered using the method of initiation, somatic embryo proliferation, and particle bombardment per Trick et al. (1997). Plates containing embryos were bombarded at 7,584 kPa under 914 mbar of Hg vacuum. Selection took place using hygromycin-B (EMD Chemicals, Inc., Gibbstown, NJ) according to Samoylov et al., (1998) and regenerated as described by Schmidt et al. (2005). Stable embryonic lines were added to the pipeline of total research being done

by a United Soybean Board (USB) grant project encompassing multiple laboratories and numerous gene targets of RKN and SCN.

Transfer of RNAi cassettes to binary vectors for A. rhizogenes*-mediated transformation*

One µg each of p17H02i, p31H06i, pF17:31i, pM17H02i, and pM31H06i were first digested for 2 hr at 37°C in 15-µl reactions with 0.5 µl of *Sac*II (10 U μl^{-1}) (New England Biolabs, Inc., Ipswich, MA) (having 1 of its 2 restriction sites adjacent to the 3' RNAi cassette end) with 1.5 µl 10x Buffer 4 (New England Biolabs, Inc.), cutting the vectors in two pieces. Reactions were stopped by incubation at 65°C for 20 min and immediately treated with 1 U μg^{-1} T4 DNA polymerase 4 (New England Biolabs, Inc., Ipswich, MA) and 100 µM dNTPs at 12°C for 15 min to blunt ends by removing 3' overhangs and then run through Zymo spin columns to remove the polymerase. Entire reaction was digested for 1 hr at 37°C in 15 µl reactions with 0.5 µl *Spe*I (10 U μl^{-1}) , having a restriction site adjacent to the RNAi cassette 5' end, simultaneously with 0.5 µl *Xho*I (10 U μl^{-1}) (to allow for greater band separation by gel electrophoresis through increased band size differentials) and 2 µl BSA + 2 µl Buffer 2. Reactions were stopped by incubation at 65°C for 20 min and run on a 0.8% agarose TAE + cytidine gel until proper band separation was clear. Gel bands corresponding to the correct size of each respective RNAi cassette was excised and Zymo-purified.

Two µg binary vector pPZP201BK-UHU containing left and right borders required for *Agrobacterium*-mediated plant cell transformation, as well as the gene coding for hygromycin phosphotransferase (*hph*) driven by the potato ubiquitin-3 (*ubi3*), were digested for 1 hr at 37°C in a 30-µl reaction with 0.75 µl of *Stu*I (10 U μl^{-1}) (creating

a blunt end to fit the blunt end of the RNAi cassette) and 0.75 µl of *Spe*I (10 U μl^{-1}) and 3 µl Buffer 2. Each respective RNAi cassette was ligated into the opened pPZP201BK-UHU at a 2:1 ratio of insert:vector with T4 DNA Ligase according to manufacturer's instructions (New England Biolabs, Inc.) creating the binary vectors for *A. rhizogenes*-mediated transformation of soybean (Figure 6). Ligated vectors were transferred into chemically competent NEB 10-β cells according to manufacturer's instructions, plated on solid LB + kanamycin (50 µg ml^{-1}), and grown overnight at 37°C. Individual colonies were screened the following day by PCR using primers PZP303R and RbcST_F, which includes DNA from the vector and in the insert cassette, with GoTaq® polymerase according to manufacturer's instructions at cycle parameters: 94°C – 4 min, (94°C – 20 s, 55°C – 20 s, 72°C – 45 s)X 30, 72°C – 7 min.

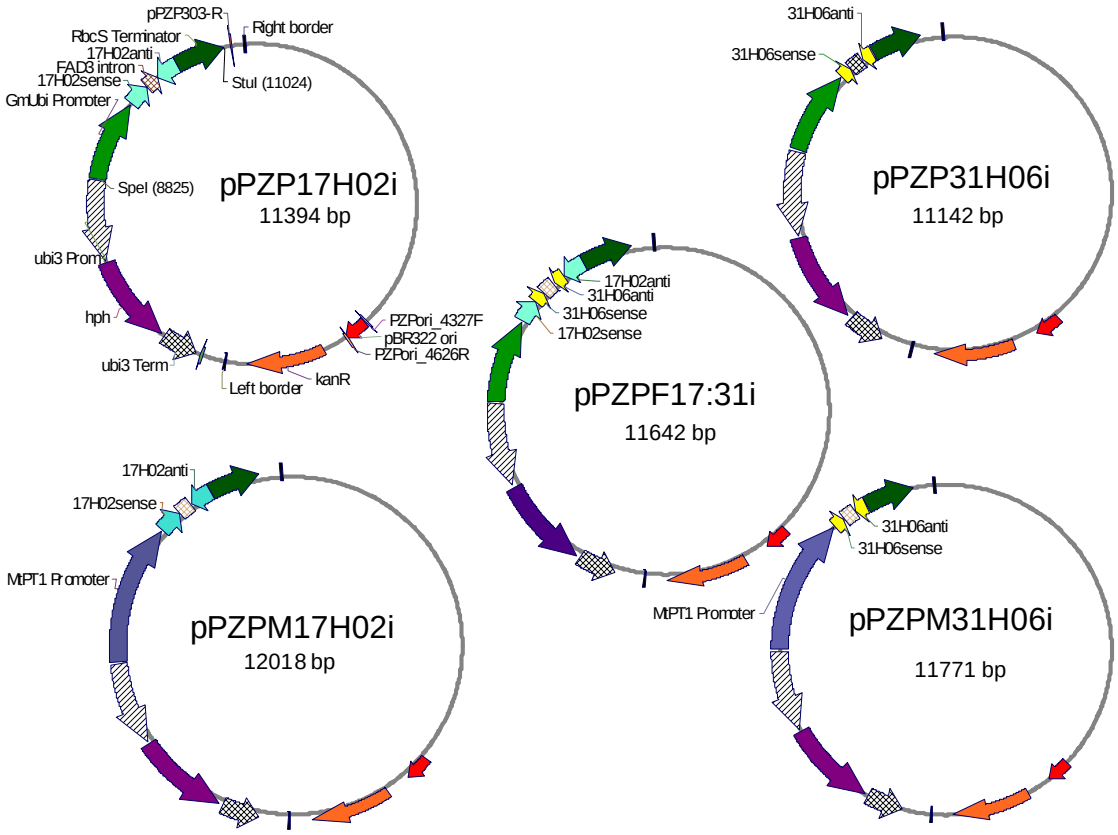

Figure 6. Binary vectors with RNAi cassettes from particle bombardment vectors containing left and right border sequences required for *A. rhizogenes*-mediated gene transfer

Agrobacterium rhizogenes strain K599 was provided by Harold Trick of Kansas State University. Electroporation transformation of *A. rhizogenes* was performed following a modified protocol by Lin (1995). K599 was cultured in 50 ml YM liquid broth (Vincent, 1985) at 28°C at 275 rpm to an OD_{600} of 0.4-0.5, pelleted by centrifugation at 3300g for 10 min at 4°C. The supernatant was decanted and cells were resuspended in ice-cold 10% glycerol and centrifuged at 3300g for 10 min at 4°C. This step was then repeated and the pellet was resuspended in 1.5-2.0 ml 10% glycerol.

For each binary vector, 30 µl of electrocompetent cells were gently mixed with 100 µg DNA. A Bio-Rad Micropulser (Bio-Rad Laboratories, Inc., Hercules, CA) electroporated samples in ice-cold 0.1 cm cuvettes at ~22 Kv cm^{-1} for ~5.5 ms. Samples

were instantly mixed with 500 µl YM and incubated at 28°C for 4 hr at 275 rpm. Then, 50 µl and 200 µl of each electroporated sample were each plated onto solid YM + kanamycin (50 µg ml^{-1}) and cultured for 2-3 days at 28°C. Individual colonies were screened by PCR using primers PZP303R and RbcST_F with GoTaq® polymerase according to manufacturer's instructions at cycle parameters: 94°C – 4 min, (94°C – 20 s, 55°C – 20 s, 72°C – 45 s) X 30, 72°C – 7 min.

D. *Composite Soybean Creation via* A. rhizogenes

Soybean genotype 'Peking' was used in this experiment due to its susceptibility to root-knot nematodes (Riggs et al., 1988) and to its susceptibility to *Agrobacterium rhizogenes* (Cho et al., 2000). The protocol followed for hairy-root induction was from personal correspondence with JiaRui Li in Harold Trick's laboratory at Kansas State University. Three-hundred seed were sterilized with chlorine gas and then germinated in Petri dishes on filter paper heavily soaked with ½ MSO liquid (salts: Murashige and Skoog (1962), vitamins: Gamborg et al. (1968)). Four to five days later, germinated seedlings were ready for inoculation. *A. rhizogenes* K599 harboring individual binary plasmids were plated from -80°C freeze stocks on YM + kanamycin (50 µg ml^{-1}) and cultured for 2-3 days at 28°C. Swabs of colonies were diluted in ~1 ml ½ PB Buffer to OD_{600} of 0.5-0.8.

Twenty seedlings were inoculated for each of the five vectors + a control using K599 after removing the radical tip from each seedling. The needle from a Hamilton syringe was used to inoculate the seedlings, administering three injections totaling 4 µl of prepared colony solution into the emerging radical at the root-stem interface, about 1 cm

below the cotyledons (Figure 7). Inoculated seedlings were placed into Petri dishes with filter and paper and liquid ½ MSO + 100 μM acetosyringone (97%) (Stachel et al., 1985) and grown for 3 days at 24°C with 16 hours of 1-6 μE m^{-2} s^{-1} light provided by of fluorescent tubes (>4200K Cool white bulbs, Sylvania Co,, Danvers, MA). After 4 days, seedlings were moved to solid MSO + MES (0.95g L^{-1}) + timentin (source, 500 μg ml^{-1}) in sterile GA-7 boxes (Magenta Co., Chicago, Illinois). Plantlets were grown in GA-7 boxes in a growth chamber for 15 days at 23° C with 16 hrs of light per day provided by mixed incandescent (60 W) and fluorescent lighting. Ten plants derived from each vector + control were moved to a sterile potting soil mix with a 2:1 ratio of Fafard 3B:sand, in GA-7 boxes and grown at the prior conditions. Two cm of three randomly selected roots per plant were harvested for further PCR analysis. Four random root sections ~1 cm in length were excised and plated on solid ½ MSO + 20 mg L^{-1} hygromycin-B + 500 μg ml^{-1} timentin to verify that the hairy roots contained *hph*. Plants in soil were acclimatized by gradually removing the GA-7 box lids and watering with non-sterile water.

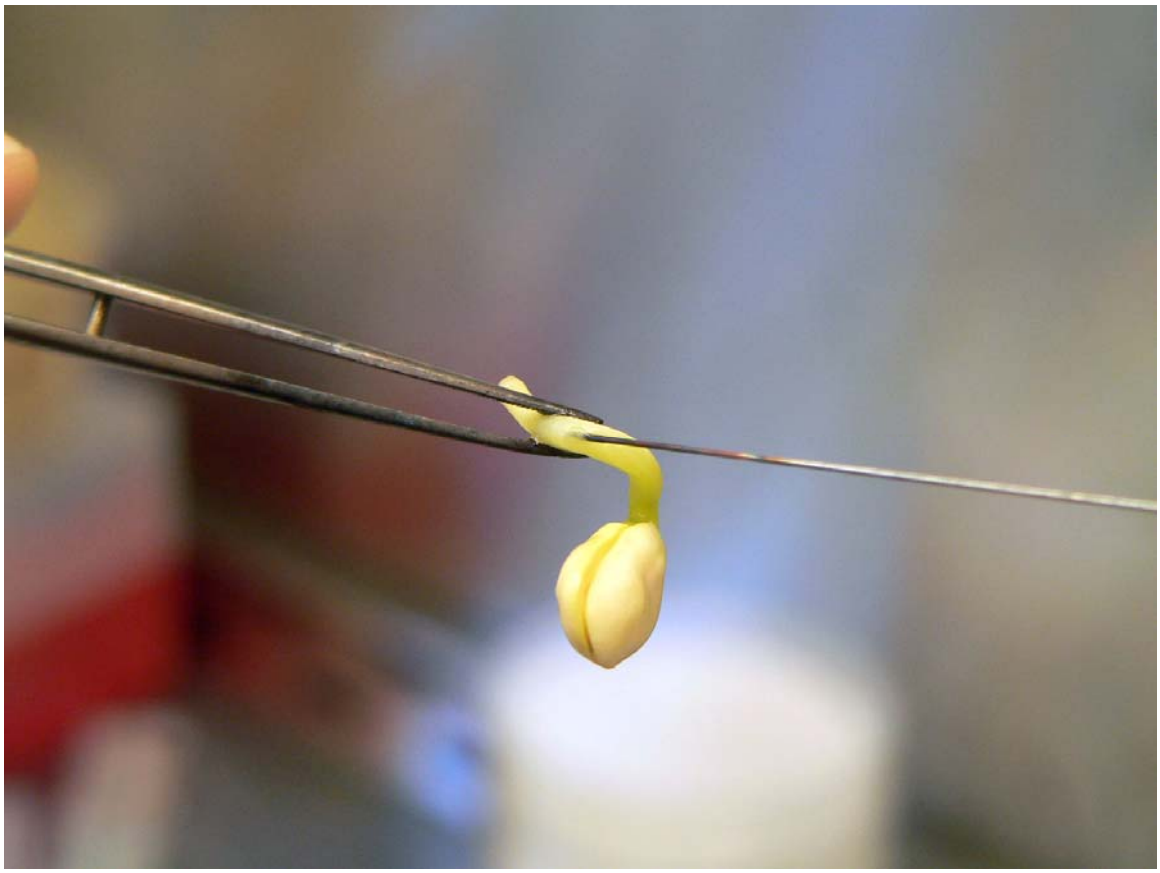

Figure 7. Four to five day old seedlings were inoculated with a *A. rhizogenes* with and without binary vectors. Radical tips were removed with a scalpel and ½ PB Buffer with *A. rhizogenes* OD600 of 0.5-0.8 was injected three times around the hypocotyl, 1 cm from the cotyledons, totaling 4 µl.

E. *PCR Analysis of Transgenes*

DNA was extracted from root tissues harvested during transfer of the composite plants to sterile soil according to Lassner et al. (1989). Four regions were amplified by PCR to check for transgene presence in roots and for contamination by *A. rhizogenes*. The presence of *hph* was checked with primer pair: UbiqF + Hyg504R. The presence of *VirG*, a part of *A. rhizogenes* endogenous Ri-plasmid, was checked with primer pair VirG73F + VirG472R. The presence of sense gene target fragments was tested with the sense primer pairs found in Table 1 To further check for *A. rhizogenes* contamination,

the origin or replication, *ori*, from the binary vector was amplified with primer pair PZPori4327F + PZPori4626R.

F. *RKN Screening Assay*

Ten plants of each vector were moved to a greenhouse and planted in a sandy loam in D40 Deepots™ (Hummert International, St. Louis, MO) arranged in a randomized complete block under supplemental 400-watt metal halide lamps 20 hr day^{-1}. They were hand-watered daily and fertilized with 20-20-20 (N = 20%, P = 8.7%, K = 16.6%) weekly. *M. incognita* eggs were collected from infected eggplant roots by cutting and shaking in 0.5% NaOCl + 10% Clorox, and collecting on a sieve. Eggs were counted in a 1-ml sample under a microscope. Five ml of *M. incognita* eggs (1000 eggs ml^{-1}) were applied to the root system of each plant with a digital dispensing pump. Seeded checks, consisting of cultivars known to be resistant or susceptible o *M. incognita*, including 'Peking,' were inoculated along with the trial plants. After 30 days, a subset of checks was harvested to gauge the advancement of galling. At 36 days, trial plants were taken out of soil which was washed from roots. Root tissue below 5 cm from the stem were removed,,frozen in liquid nitrogen, freeze-dried and stored as a reference. Individual roots emerging from the stem were removed and assayed separately. Each was given a rating of "one," "two," or "three," depending on a gall index whereby "one" = less than five galls 5 cm^{-1} emerging root system, "two" = five to 20 galls, and "three" = more than twenty galls. After indexing, the individual roots also were frozen in liquid nitrogen and freeze dried over night. One-way analysis of variance for gall number was conducted on the collected data, and p-values determined using SAS (SAS Institute Inc.,

Cary, NC). Ten random samples for each construct were selected, three that were assigned a gall rating of "one," and seven receiving a gall rating of "three," and checked for the presence of the sense strand utilizing primers in Table 1, as well as Le-25F + Le-593R which checked the quality of the DNA by amplifying an endogenous soybean sequence fragment of the *lectin* gene.

8. Results

A. *Conservation of 17H02i and 31H06i in* Meloidogyne

Identical primers developed from the *M. incognita* sequence were used to amplify genomic DNA extracted from all four major root-knot nematode species of *M. incognita, M. javanica, M. arenaria,* and *M. hapla*. The resulting sequence data were compared for homology using MultiAlin software from Corpet (1988). The target sequence of 17H02 is 98.75% identical across all four species (Figure 8). The target region in 31H06 however, contains a 50-bp insertion in the center of the *M. arenaria* and *M. javanica* sequences that is not present in the two other species (Figure 9). Each ~50 bp insert is different between the two species. Finally, *M. arenaria* also has 31 bp following the insert that is only 20% similar to the other species.

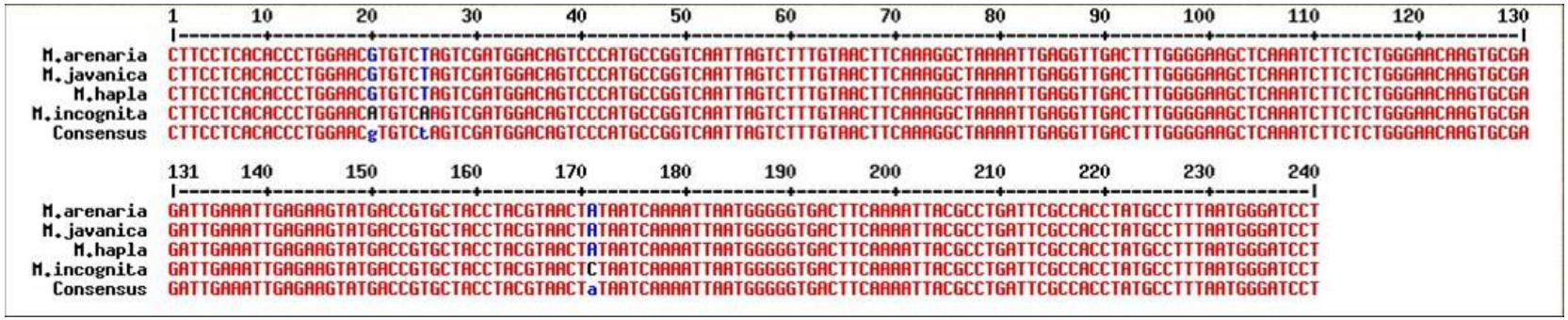

Figure 8. 17H02 target region is highly similar across RKN species sampled. Differences were all in M. incognita alone, compared against the other three *Meloidogyne* species.

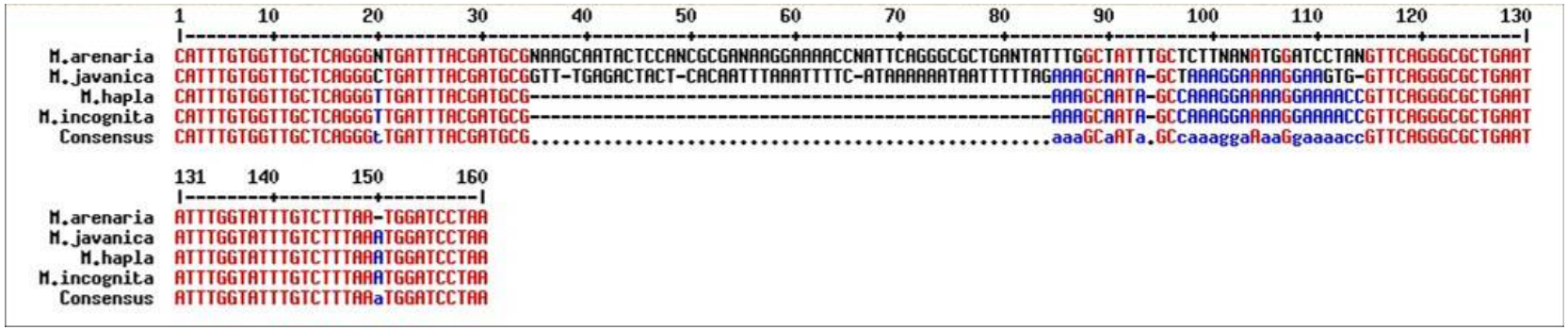

Figure 9. 31H06 target region is conserved between *M. hapla* and *M. incognita*, also at the first 34 bp and the last 45 bp over all 4 species. *M. arenaria* and *M. javanica* however have a 50-bp-insert not present in the other species.

B. *Root-Knot Nematode Resistance Assay*

Sixty composite soybean plants with hairy roots engineered to produce double-stranded RNA complementary to RKN esophageal gland secretory proteins were challenged with *M. incognita*. Two gene targets were compared, *17H02* and *31H06*, together and separately. Furthermore, two promoters, the *GmUbi* promoter and the *MtPT1* promoter were compared for their efficacy at driving the production of double-stranded fragments of *17H02* and *31H06*.

Assayed plants were harvested 36 days after inoculation, root tissue below 5 cm from the stem was removed, individual roots emerging from the stem were removed and separately indexed for gall numbers. The rating index was: 1 = less than five galls, 2 = five to 20 galls, and 3 = more than 20 galls 5 cm^{-1} emerging root system. Resulting data were analyzed using the GLM procedure of SAS software by building contrasts between gall data of control plants, inoculated with WT K599, and all RNAi plants, as well as the control versus individual constructs. No significant difference was seen between any of the factors and the control K599 plants (Table 2).

Table 2.

Comparison	P-value
K599 vs All RNAi	0.73
K599 vs 17H02i	0.64
K599 vs 31H06i	0.62
K599 vs F17:31i	0.49
K599 vs M17H02i	0.96
K599 vs M31H06i	0.42

To ensure the lack of resistance was not due to the lack of the transgene present in the hairy roots, seven random root samples given a gall rating of 3(>20 galls 5 cm^{-1}), and three random root samples given a gall rating of 1 (<5 galls 5 cm^{-1}) from each construct were checked for the presence of the sense strand of the transgene. All samples with quality DNA extractions (confirmed by PCR for the presence of an endogenous lectin sequence in soybean) were shown to be transgenic (Figure 10). Since all samples, showed presence of the sense transgene fragment, no correlation exists between gall rating and the presence of absence of the transgene.

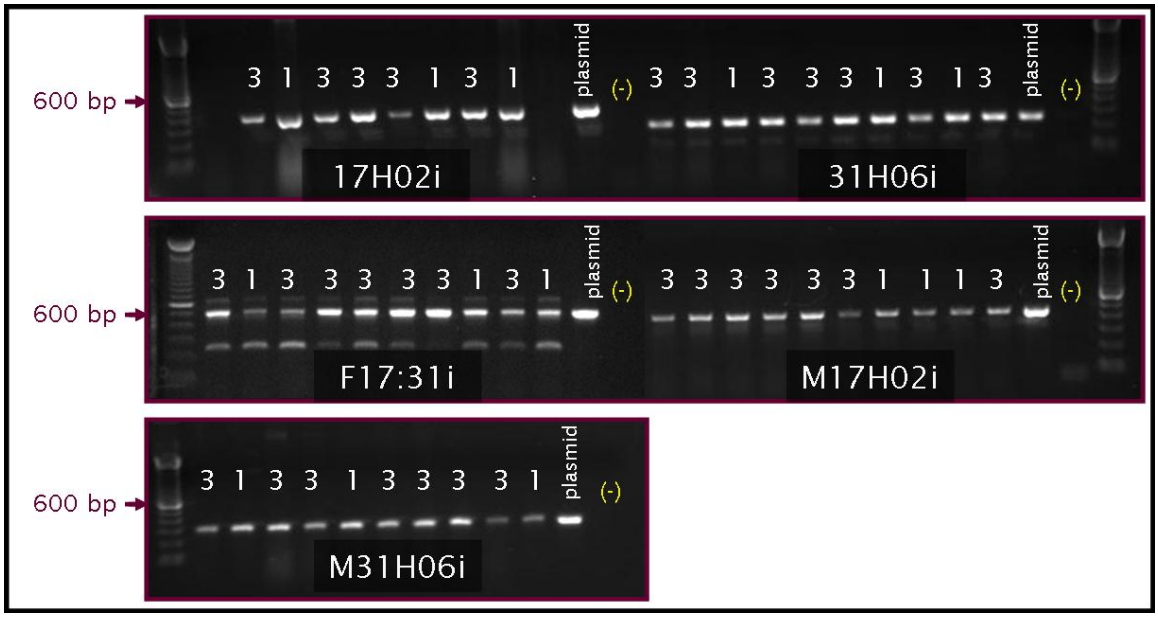

Figure 10. PCR validation for presence of sense fragment of RKN target gene in random sample of composite soybean roots. Ones represent samples which were given a gall rating of 1. Threes represent samples which were given a gall rating of 3.

9. Discussion

Root-knot nematodes cause devastating crop losses worldwide each year. The yield of soybean can also be reduced by RKNs. While nematicides are effective at decreasing nematode populations in soils, they are often cost-prohibitive and toxic to humans. The most desirable option in combating nematode infestations is through the use of resistant cultivars, but the development of such cultivars is challenging. Resistance to the three major RKN species parasitizing soybean requires the incorporation of six resistance QTLs, two for each species, making it impractical to routinely breed broad species resistance into current elite cultivars.

RKNs rely on an intimate molecular relationship with host cells for parasitism. Within their esophageal gland cells, proteins are synthesized which are secreted into host root cells, drastically altering the cell morphology and physiology for the benefit of the nematode. Phenotypic galling results, netting a reduction in plant yield in moderate to heavy infestations. Huang et al. (2006a) demonstrated the potential to engineer plants to produce siRNAs, homologous to RKN parasitism genes, to achieve RKN resistance via RNAi.

This work examined the ability to achieve RKN resistance in soybean by targeting two RKN esophageal gland cell parasitism genes, *17H02* and *31H06*. First, it was determined by sequencing complementary fragments of the four major RKN species: *M. incognita, M. javanica, M. arenaria* and *M. hapla*, that *17H02* is highly conserved. In contrast, *31H06* is conserved in *M. incognita* and *M. hapla*, while *M. javanica* and *M.*

arenaria have dissimilar ~50 bp inserts. Since *16D10* is conserved across these four species (data not shown), and Huang et al. (2006a) proved effective RKN resistance by targeting *16D10*, it can be inferred that if *17H02* proves a practical target for resistance to one RKN species, it will be effective against the three other major RKNs. However, *31H06* might not be an equally effective RNAi target across the genus. While it has some sequence conservation across species, the presence of sizeable inserts within two of the species precludes the presence of universal targets.

Originally, this gene target comparisons were to be assayed using only stable transgenic soybean produced via particle bombardment. Stable transgenic cell lines were produced for all five constructs. After some time it became apparent these lines would require another year to recover enough homozygous events per construct for proper RKN challenge analyses. These lines are currently in the pipeline to obtain homozygous plants as a part of the USB-funded multi-laboratory project evaluating RKN and SCN parasitism gene targets for creating respective nematode resistant transgenic soybean. Subsequently, bombardment vectors were retrofitted into binary vectors and utilized with *A. rhizogenes* to create composite soybean plants. Not only is the creation of hairy roots much faster than stable production, it also permits far greater numbers of transgenic events to be quickly and efficiently screened.

An attempt was made to compare the effectiveness of two different promoters, *GmUbi* and *MtPT1*, driving hairpin-forming dsRNA molecules homologous to these genes, as well as targeting these genes individually or simultaneously. Utilizing *A. rhizogenes* for gene transfer and formation of composite soybean plants containing chimeric transgenic root systems and challenging these plants with *M. incognita*, gall data

were obtained on a per-root basis. No significant difference was observed between roots inoculated with an empty strain of *A. rhizogenes* K599 versus roots obtained with any or all of the five RNAi cassettes targeting *17H02* or *31H06* or both. PCR of ten random root samples per construct, including seven highly galled roots (gall rating three) and three scarcely galled roots (gall rating one) proved there was no correlation between presence of the transgene and galling.

These results correlate with results found by the USB group in the stable soybean lines assayed thus far. In total, eight parsitism gene targets have been evaluated as targets for creating RKN resistance (two in composite plants and six in stable plants). Also, four different promoters have been utilized within these eight genes, producing no convincing RKN resistance. Moreover in this work, attempting to target two parasitism genes at once versus those independently failed to produce resistance.

The reason for soybean, engineered to form double-stranded RNA homologous to RKN parasitism genes, to show no RKN resistance remains uncertain. The current strategy's ineffectiveness could be attributed to several factors. First, the targeting of the parasitism genes *17H02* and *31H06* could be ineffective at deterring RKN parasitism, either because these genes are not essential for parasitism or they belong to a gene family with compensating members, however, this is unclear since there is no evidence that siRNAs are being made to any relevant extent. Three separate RNA blots (data not shown), including one produced during this work, have failed to detect small RNAs created from transgenes in stably engineered soybean. Thus the most likely explanation for the lack of RKN resistance in these composite soybean plants is the lack of siRNAs accumulating at a threshold level required for silencing within the feeding RKN. Mao et

al. (2007) engineered tobacco and *Arabidopsis* to produce siRNAs which when ingested by feeding cotton bollworms, reduced the insect's ability to produce a cytochrome P450 that is involved in detoxifying gossypol. They documented an inverse correlation between the amount of siRNAs being produced in the transgenic plants and the level of target mRNA in the cotton bollworm. An explanation must then be sought for the lack of siRNA production. One possible explanation is that the amplification step in the RNAi pathway, not being triggered in the plant due to lack of a homologous RNA target, could be required to amplify the level of siRNAs in the plant cell.

If too low a concentration of siRNAs targeting RKN genes are accumulating in transgenic soybean, manipulating the RNAi amplification processes may be critical in achieving RKN resistance in transgenic soybean. The amplification of siRNA occurs via an RNA-dependent RNA polymerase (RdRP). Vaistij et al. (2002) revealed that the ability for siRNAs targeting green fluorescent protein (*GFP*) to spread systemically in tobacco required not only RdRP, but also the production of a target *GFP* mRNA in the siRNA originating cell. Also made apparent was the inefficiency of certain gene sequences to allow for siRNA amplification or systemic effects, including ribulose-1,5-bisphosphate carboxylase/oxygenase genes and phytoene desaturase. Koscianska et al. (2005) engineered tobacco using *A. tumefaciens* with RNAi constructs targeting a viroid binding protein, *VirP1*, and found no difference between siRNA levels between plants engineered with hairpin-forming RNAi cassettes targeting *VirP1* and plants engineered with both the RNAi cassette and a cassette over-expressing *VirP1* mRNA. This further demonstrates the complexity of the RNAi machinery and potential hurdles in achieving RKN resistance in transgenic soybean.

Future work in achieving transgenic soybean resistance to RKN utilizing RNAi will need to focus on validation of siRNA production from the current vector design as well as investigating the incorporation of an overexpressed target sequence to which siRNAs can bind in the plant cell, possibly stimulating RdRP amplification, and providing the necessary concentration of siRNAs to confer RKN resistance.

This thesis research demonstrated the potential for rapid assay of RNAi vectors in composite soybean for achieving RKN resistance and has strengthened results found in stably transformed soybean with similar RNAi vectors (unpublished data). Previous success in the use of RNAi technology in transgenic *Arabidopsis* to achieve nematode resistance encourages further investigations in RNAi strategies for RKN resistance in soybean. Results produced in this thesis demonstrate the necessity for retooling of the transgenic molecules. Progress should be accelerated though the use of the hairy root system for rapid validation of siRNA production and RKN resistance, and after confirmation of their effectiveness, to be engineered into stable transgenic soybean.

REFERENCES

Adachi, H. 1992. Culture of Meloidogyne incognita on oriental-melon roots genetically transformed by Agrobacterium rhizogenes. Japanese Journal of Applied Entomology and Zoology 36:225-230.

Alkharouf, N.W., V.P. Klink, and B.F. Matthews. 2007. Identification of Heterodera glycines (soybean cyst nematode [SCN]) cDNA sequences with high identity to those of Caenorhabditis elegans having lethal mutant or RNAi phenotypes. Experimental Parasitology 115:247-258.

Anandalakshmi, R., G.J. Pruss, X. Ge, R. Marathe, A.C. Mallory, T.H. Smith, and V.B. Vance. 1998. A viral suppressor of gene silencing in plants, pp. 13079-13084, Vol. 95. National Acad Sciences.

Aragão, F.J.L., L. Sarokin, G.R. Vianna, and E.L. Rech. 2000. Selection of transgenic meristematic cells utilizing a herbicidal molecule results in the recovery of fertile transgenic soybean [Glycine max (L.) Merril] plants at a high frequency. Theoretical and Applied Genetics 101:1-6.

Bakó, L., M. Umeda, A.F. Tiburcio, J. Schell, and C. Koncz. 2003. The virD2 pilot protein of Agrobacterium-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants. Proceedings of the National Academy of Sciences, USA 100:10108-10113.

Bartel, D.P. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116:281-297.

Baum, J.A., T. Bogaert, W. Clinton, G.R. Heck, P. Feldmann, O. Ilagan, S. Johnson, G. Plaetinck, T. Munyikwa, and M. Pleau. 2007. Control of coleopteran insect pests through RNA interference. Nature Biotechnology 25:1322.

Baum, T.J., A. Hiatt, W.A. Parrott, L.H. Pratt, and R.S. Hussey. 1996. Expression in tobacco of a functional monoclonal antibody specific to stylet secretions of the root-knot nematode. Molecular Plant Microbe Interactions 9:382-387.

Bayne, E.H., D.V. Rakitina, S.Y. Morozov, and D.C. Baulcombe. 2005. Cell-to-cell movement of potato potexvirus x is dependent on suppression of RNA silencing. Plant Journal 44:471-482.

Benian, G.M., S.W. Lhernault, and M.E. Morris. 1993. Additional sequence complexity in the muscle gene, unc-22, and its encoded protein, twitchin, of Caenorhabditis elegans. Genetics 134:1097-1104.

Bernstein, E., A.A. Caudy, S.M. Hammond, and G.J. Hannon. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409:363-366.

Bhalla, P.L., and M.B. Singh. 2004. Knocking out expression of plant allergen genes. Methods 32:340-345.

Bird, A.F. 1968. Changes associated with parasitism in nematodes. Iv. Cytochemical studies on the ampulla of the dorsal esophageal gland of Meloidogyne javanica and on exudations from the buccal stylet. J. Parasitol 54:879-90.

Bird, A.F. 1969. Changes associated with parasitism in nematodes. V. Ultrastructure of the stylet exudation and dorsal esophageal gland contents of female Meloidogyne javanica. J. Parasitol 55:337-345.

Bird, A.F. 1983. Changes in the dimensions of the esophageal glands in root-knot nematodes during the onset of parasitism. International Journal of Parasitology 13:343-48.

Boerma, H.R., R.S. Hussey, D.V. Phillips, E.D. Wood, and S.L. Finnerty. 1994. Registration of Haskell soybean. Crop Science 34:541-541.

Boisson-Dernier, A., M. Chabaud, F. Garcia, G. Bécard, C. Rosenberg, and D.G. Barker. 2001. Agrobacterium rhizogenes-transformed roots of Medicago truncatula for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Molecular Plant-Microbe Interactions 14:695-700.

Bonnet, E., Y. Van de Peer, and P. Rouze. 2006. The small RNA world of plants. New Phytologist 171:451-468.

Bouche, N., D. Lauressergues, V. Gasciolli, and H. Vaucheret. 2006. An antagonistic function for Arabidopsis dcl2 in development and a new function for dcl4 in generating viral siRNAs. Embo Journal 25:3347-3356.

Brigneti, G., O. Voinnet, W.X. Li, L.H. Ji, S.W. Ding, and D.C. Baulcombe. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. The EMBO Journal 17:6739-6746.

Brookes, G., and P. Barfoot. 2006. GM crops: The first ten years - global socio-economic and environmental impacts. ISAAA, Ithaca, NY.

Cao, D., W.S. Hou, S.K. Song, H.B. Sun, C.X. Wu, Y.S. Gao, and T.F. Han. 2009. Assessment of conditions affecting Agrobacterium rhizogenes-mediated transformation of soybean. Plant Cell Tissue and Organ Culture 96:45-52.

Cascales, E., and P.J. Christie. 2003. The versatile bacterial type iv secretion systems.

Chapman, E.J., and J.C. Carrington. 2007. Specialization and evolution of endogenous small RNA pathways. Nature Reviews Genetics 8:884-896.

Chiera, J., R. Bouchard, S. Dorsey, E.H. Park, M. Buenrostro-Nava, P. Ling, and J. Finer. 2007. Isolation of two highly active soybean (Glycine max (L.) Merr.) promoters and their characterization using a new automated image collection and analysis system. Plant Cell Reports 26:1501-1509.

Chilton, M.D., D.A. Tepfer, A. Petit, C. David, F. Cassedelbart, and J. Tempe. 1982. Agrobacterium-rhizogenes inserts t-DNA into the genomes of the host plant-root cells. Nature 295:432-434.

Chiou, T.-J., H. Liu, and M.J. Harrison. 2001. The spatial expression patterns of a phosphate transporter (mtpt1) from Medicago truncatula indicate a role in phosphate transport at the root/soil interface. The Plant Journal 25:281-293.

Chitwood, D.J. 2003. Nematicides, *In* USDA-ARS, (ed.).

Cho, H.J., S.K. Farrand, G.R. Noel, and J.M. Widholm. 2000. High-efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. Planta 210:195-204.

Christianson, M.L., D.A. Warnick, and P.S. Carlson. 1983. A morphogenetically competent soybean suspension culture. Science 222:632-634.

Christou, P., D.E. McCabe, and W.F. Swain. 1988. Stable transformation of soybean callus by DNA-coated gold particles. Plant Physiology 87:671-674.

Christou, P., W.F. Swain, N.S. Yang, and D.E. McCabe. 1989. Inheritance and expression of foreign genes in transgenic soybean plants. Proceedings of the National Academy of Sciences, USA 86:7500-7504.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res 16 10881-10890.

Dafny-Yelin, M., S.M. Chung, E.L. Frankman, and T. Tzfira. 2007. Psat RNA interference vectors: A modular series for multiple gene down-regulation in plants. Plant Physiology 145:1272-1281.

Davis, E.L., R.S. Hussey, M.G. Mitchum, and T.J. Baum. 2008. Parasitism proteins in nematode-plant interactions. Current Opinion in Plant Biology 11:360-366.

Davis, E.L., R.S. Hussey, T.J. Baum, J. Bakker, and A. Schots. 2000. Nematode parasitism genes. Annual Review of Phytopathology 38:365-396.

Deleris, A., J. Gallego-Bartolome, J.S. Bao, K.D. Kasschau, J.C. Carrington, and O. Voinnet. 2006. Hierarchical action and inhibition of plant dicer-like proteins in antiviral defense. Science 313:68-71.

Della Vedova, C.B., R. Lorbiecke, H. Kirsch, M.B. Schulte, K. Scheets, L.M. Borchert, B.E. Scheffler, U. Wienand, K.C. Cone, and J.A. Birchler. 2005. The dominant inhibitory chalcone synthase allele c2-idf (inhibitor diffuse) from Zea mays (L.) acts via an endogenous RNA silencing mechanism. Genetics 170:1989-2002.

Deng, W.Y., L.S. Chen, D.W. Wood, T. Metcalfe, X.Y. Liang, M.P. Gordon, L. Comai, and E.W. Nester. 1998. Agrobacterium virda protein interacts with plant host cyclophilins. Proceedings of the National Academy of Sciences, USA 95:7040-7045.

Dessaux, Y., A. Petit, and J. Tempe. 1993. Chemistry and biochemistry of opines, chemical mediators of parasitism. Phytochemistry 34:31-38.

Dodo, H.W., K.N. Konan, F.C. Chen, M. Egnin, and O.M. Viquez. 2008. Alleviating peanut allergy using genetic engineering: The silencing of the immunodominant allergen Ara h 2 leads to its significant reduction and a decrease in peanut allergenicity. Plant Biotechnology Journal 6:135-145.

Dykxhoorn, D.M., C.D. Novina, and P.A. Sharp. 2003. Killing the messenger: Short RNAs that silence gene expression. Nature Reviews Molecular Cell Biology 4:457-467.

Elsen, A., S. Declerck, and D. De Waele. 2000. Reproduction of the burrowing nematode (Radopholus similis) on ri t-DNA transformed carrot roots. Nematology 2:247-249.

Fagard, M., S. Boutet, J.B. Morel, C. Bellini, and H. Vaucheret. 2000. Ago1, qde-2, and rde-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proceedings of the National Academy of Sciences, USA 97:11650-11654.

Fanelli, E., M. Di Vito, J.T. Jones, and C. De Giorgi. 2005. Analysis of chitin synthase function in a plant parasitic nematode, Meloidogyne artiellia, using RNAi. Gene 349:87-95.

Fire, A., S.Q. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806-811.

Fojtova, M., H. Van Houdt, A. Depicker, and A. Kovarik. 2003. Epigenetic switch from posttranscriptional to transcriptional silencing is correlated with promoter hypermethylation. Plant Physiol. 133:1240-1250.

Fu, S.X., Y. Zhan, H.J. Zhi, J.Y. Gai, and D.Y. Yu. 2006. Mapping of SMV resistance gene rsc-7 by SSR markers in soybean. Genetica 128:63-69.

Fuller, V.L., C.J. Lilley, and P.E. Urwin. 2008. Nematode resistance. New Phytologist 180:27-44.

Gamborg, O.L., R.A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Experimental cell research 50:151.

Gao, B., R. Allen, T. Maier, E.L. Davis, T.J. Baum, and R.S. Hussey. 2001. Identification of putative parasitism genes expressed in the esophageal gland cells of the soybean cyst nematode Heterodera glycines. Molecular Plant-Microbe Interactions 14:1247-1254.

Gao, B., R. Allen, T. Maier, E.L. Davis, T.J. Baum, and R.S. Hussey. 2003. The parasitome of the phytonematode Heterodera glycines. Molecular Plant-Microbe Interactions 16:720-726.

Gilissen, L.J.W.J., S.T.H.P. Bolhaar, C.I. Matos, G.J.A. Rouwendal, M.J. Boone, F.A. Krens, L. Zuidmeer, A. van Leeuwen, J. Akkerdaas, K. Hoffmann-Sommergruber, A.C. Knulst, D. Bosch, W.E. van de Weg, and R. van Ree. 2005. Silencing the major apple allergen mal d 1 by using the RNA interference approach. Journal of Allergy and Clinical Immunology 115:364-369.

Godwin, I., G. Todd, B. Ford-Lloyd, and H.J. Newbury. 1991. The effects of acetosyringone and pH on Agrobacterium-mediated transformation vary according to plant species. Plant Cell Reports 9:671-675.

Goggin, F.L., L. Jia, G. Shah, S. Hebert, V.M. Williamson, and D.E. Ullman. 2006. Heterologous expression of the Mi-1.2 gene from tomato confers resistance against nematodes but not aphids in eggplant. Molecular Plant-Microbe Interactions 19:383-388.

Gordon, S.G., K. Kowitwanich, W. Pipatpongpinyo, S.K.S. Martin, and A.E. Dorrance. 2007. Molecular marker analysis of soybean plant introductions with resistance to Phytophthora sojae. Phytopathology 97:113-118.

Guo, H.S., J.F. Fei, Q. Xie, and N.H. Chua. 2003. A chemical-regulated inducible RNAi system in plants. The Plant Journal 34:383-392.

Ha, B.K., R.S. Hussey, and H.R. Boerma. 2007. Development of SNP assays for marker-assisted selection of two southern root-knot nematode resistance QTL in soybean. Crop Science 47:S73-S82.

Hamilton, A.J., and D.C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950-952.

Hammond, S.M., E. Bernstein, D. Beach, and G.J. Hannon. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404:293-296.

Hannon, G.J. 2002. RNA interference. Nature 418:244-251.

Hinchee, M.A.W., D.V. Connorward, C.A. Newell, R.E. McDonnell, S.J. Sato, C.S. Gasser, D.A. Fischhoff, D.B. Re, R.T. Fraley, and R.B. Horsch. 1988. Production of transgenic soybean plants using Agrobacterium-mediated DNA transfer. Bio-Technology 6:915-921.

Hirschmann, H. 1985. The classification of the family Meloidogynidae, p. 35-45, *In* J. N. Sasser and C. C. Carter, eds. An advanced treatise on Meloidogyne, Vol. 1. Dept. of Plant Pathology, US Agency for International Development, Raleigh, N.C.

Houmard, N.M., J.L. Mainville, C.P. Bonin, S. Huang, M.H. Luethy, and T.M. Malvar. 2007. High-lysine corn generated by endosperm-specific suppression of lysine catabolism using RNAi. Plant Biotechnology Journal 5:605-614.

Huang, C.S., and A.R. Maggenti. 1969. Mitotic aberrations and nuclear changes of developing giant cells in Vicia faba caused by root-knot nematode, Meloidogyne javanica. Phytopathology 59:447-455.

Huang, G., R. Allen, E.L. Davis, T.J. Baum, and R.S. Hussey. 2006a. Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. PNAS 103:14302-14306.

Huang, G., R. Dong, R. Allen, E.L. Davis, T.J. Baum, and R.S. Hussey. 2006b. A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. Molecular Plant-Microbe Interactions 19:463-470.

Huang, G., B. Gao, T. Maier, R. Allen, E.L. Davis, T.J. Baum, and R.S. Hussey. 2003. A profile of putative parasitism genes expressed in the esophageal gland cells of the root-knot nematode Meloidogyne incognita. Molecular Plant-Microbe Interactions 16:376-381.

Huang, G.Z., R.H. Dong, R. Allen, E.L. Davis, T.J. Baum, and R.S. Hussey. 2006c. A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. Molecular Plant-Microbe Interactions 19:463-470.

Hunter, A.H. 1958. Nutrient absorption and translocation of phosphorus as influenced by the root knot nematode (Meloidogyne incognita acrita). Soil Science 86:245.

Hussey, R.S. 1985. Host-parasite relationship and associated physiological changes, p. 143-153, *In* J. N. Sasser and C. C. Carter, eds. An advanced treatise on Meloidogyne, Vol. 1. Department of Plant Pathology, NC State University, Raleigh, NC.

Hussey, R.S. 1988. Production of monoclonal-antibodies specific for secretory granules formed in esophageal glands of Meloidogyne species. Journal of Nematology 20:641-641.

Hussey, R.S. 1989. Disease-inducing secretions of plant-parasitic nematodes. Annual Review of Phytopathology 27:123-141.

Hussey, R.S., and G.J.W. Janssen. 2002. Root-knot nematodes: Meloidogyne species. Plant Resistance to Parasitic Nematodes:43–70.

Hussey, R.S., E.L. Davis, and C. Ray. 1994. Meloidogyne stylet secretions. Advances in Molecular Plant Nematology:233-249.

Hwang, C.-F., A.V. Bhakta, G.M. Truesdell, W.M. Pudlo, and V.M. Williamson. 2000. Evidence for a role of the n terminus and leucine-rich repeat region of the Mi gene product in regulation of localized cell death. Plant Cell 12:1319-1330.

Jacob, F., and J. Monod. 1961. Genetic regulatory mechanisms in synthesis of proteins. Journal of Molecular Biology 3:318-&.

Jones, M.G.K., and H.L. Payne. 1978. Early stages of nematode-induced giant-cell formation in roots of Impatiens balsamina. J. Nematol 10:70-84.

Jorgensen, R.A., P.D. Cluster, J. English, Q.D. Que, and C.A. Napoli. 1996. Chalcone synthase cosuppression phenotypes in petunia flowers: Comparison of sense vs antisense constructs and single-copy vs complex t-DNA sequences. Plant Molecular Biology 31:957-973.

Kaeberlein, M., C.R. Burtner, and B.K. Kennedy. 2007. Recent developments in yeast aging. Plos Genetics 3:655-660.

Kifle, S., M. Shao, C. Jung, and D. Cai. 1999. An improved transformation protocol for studying gene expression in hairy roots of sugar beet (Beta vulgaris L.). Plant Cell Reports 18:514-519.

Klein, T.M., E.D. Wolf, R. Wu, and J.C. Sanford. 1987. High-velocity microprojectiles for delivering nucleic-acids into living cells. Nature 327:70-73.

Koscianska, E., K. Kalantidis, K. Wypijewski, J. Sadowski, and M. Tabler. 2005. Analysis of RNA silencing in Agroinfiltrated leaves of Nicotiana benthamiana and Nicotiana tabacum. Plant Molecular Biology 59:647-661.

Kosinski, M., K. McDonald, J. Schwartz, I. Yamamoto, and D. Greenstein. 2005. C. Elegans sperm bud vesicles to deliver a meiotic maturation signal to distant oocytes. Development 132:3357-3369.

Kusaba, M. 2004. RNA interference in crop plants. Current Opinion in Biotechnology 15:139-143.

Kusaba, M., K. Miyahara, S. Iida, H. Fukuoka, T. Takano, H. Sassa, M. Nishimura, and T. Nishio. 2003. Low glutelin content1: A dominant mutation that suppresses the glutelin multigene family via RNA silencing in rice. Plant Cell 15:1455-1467.

Lassner, M.W., P. Peterson, and J.I. Yoder. 1989. Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. Plant molecular biology reporter 7:116-128.

Le, L.Q., Y. Lorenz, S. Scheurer, K. Fötisch, E. Enrique, J. Bartra, S. Biemelt, S. Vieths, and U. Sonnewald. 2006. Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-ltp (lyc e 3) expression. Plant Biotechnology Journal 4:231-242.

Li, X.Q., J.Z. Wei, A. Tan, and R.V. Aroian. 2007. Resistance to root-knot nematode in tomato roots expressing a nematicidal Bacillus thuringiensis crystal protein. Plant Biotechnology Journal 5:455-464.

Li, Z., L. Jakkula, R.S. Hussey, J.P. Tamulonis, and H.R. Boerma. 2001. SSR mapping and confirmation of the QTL from pi96354 conditioning soybean resistance to southern root-knot nematode. Theoretical and Applied Genetics 103:1167-1173.

Lin, J.-J. 1995. Electrotransformation of Agrobacterium, p. 171-178, *In* J. A. Nickoloff, ed. Electroporation protocols of microorganisms. Humana Press.

Liu, H., A.T. Trieu, L.A. Blaylock, and M.J. Harrison. 1998. Cloning and characterization of two phosphate transporters from Medicago truncatula roots: Regulation in response to phosphate and to colonization by arbuscular mycorrhizal (am) fungi. Molecular Plant-Microbe Interactions 11:14-22.

Liu, Q., S. Singh, and A. Green. 2002. High-oleic and high-stearic cottonseed oils: Nutritionally improved cooking oils developed using gene silencing. Journal of the American College of Nutrition 21:205-211.

Luzzi, B.M., J.P. Tamulonis, R.S. Hussey, and H.R. Boerma. 1995. Inheritance of resistance to the Javanese root-knot nematode in soybean. Crop Science 35:1372-1375.

Lynn, K., A. Fernandez, M. Aida, J. Sedbrook, M. Tasaka, P. Masson, and M.K. Barton. 1999. The pinhead/zwille gene acts pleiotropically in Arabidopsis development and has overlapping functions with the argonaute1 gene. Development 126:469-481.

Mai, W.F. 1985. Plant-parasitic nematodes: Their threat to agriculture, p. 11-17, *In* J. N. Sasser and C. C. Carter, eds. An advanced treatise on Meloidogyne, Vol. 1. Department of Plant Pathology, NC State University, Raleigh, N.C.

Mallory, A.C., L. Ely, T.H. Smith, R. Marathe, R. Anandalakshmi, M. Fagard, H. Vaucheret, G. Pruss, L. Bowman, and V.B. Vance. 2001. Hc-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. The Plant Cell Online 13:571-583.

Mankin, S.L., D.S. Hill, P.M. Olhoft, E. Toren, A.R. Wenck, L. Nea, L.Q. Xing, J.A. Brown, H.H. Fu, L. Ireland, H.M. Jia, H. Hillebrand, T. Jones, and H.S. Song. 2007. Disarming and sequencing of Agrobacterium rhizogenes strain K599 (ncppb2659) plasmid pri2659. In Vitro Cellular & Developmental Biology-Plant 43:521-535.

Mao, Y.-B., W.-J. Cai, J.-W. Wang, G.-J. Hong, X.-Y. Tao, L.-J. Wang, Y.-P. Huang, and X.-Y. Chen. 2007. Silencing a cotton bollworm p450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat Biotech 25:1307-1313.

Marroquin, L.D., D. Elyassnia, J.S. Griffitts, J.S. Feitelson, and R.V. Aroian. 2000. Bacillus thuringiensis (Bt) toxin susceptibility and isolation of resistance mutants in the nematode Caenorhabditis elegans. Genetics 155:1693-1699.

Martinell, B., L.A. Julson, M.A.W. Hinchee, D.C. Ward, D.E. McCabe, and C. Emler. 1999. Efficiency soybean transformation protocol. U.S. 1999.

McCabe, D.E., W.F. Swain, B.J. Martinell, and P. Christou. 1988. Stable transformation of soybean (Glycine max) by particle-acceleration. Bio-Technology 6:923-926.

Mehrotra, S., A.K. Kukreja, S.P.S. Khanuja, and B.N. Mishra. 2008. Genetic transformation studies and scale up of hairy root culture of Glycyrrhiza glabra in bioreactor. Electronic Journal of Biotechnology 11:15.

Mery, A., O. Taghli-Lamallem, K.A. Clark, M.C. Beckerle, X.S. Wu, K. Ocorr, and R. Bodmer. 2008. The Drosophila muscle lim protein, mlp84b, is essential for cardiac function. Journal of Experimental Biology 211:15-23.

Metzlaff, M., M. O'Dell, P.D. Cluster, and R.B. Flavell. 1997. RNA-mediated RNA degradation and chalcone synthase a silencing in petunia. Cell 88:845-854.

Mienie, C.M.S., H. Fourie, M.A. Smit, J. Van Staden, and F.C. Botha. 2002. Identification of AFLP markers in soybean linked to resistance to Meloidogyne javanica and conversion to sequence characterized amplified regions (scars). Plant Growth Regulation 37:157-166.

Miller, M.A., V.Q. Nguyen, M.H. Lee, M. Kosinski, T. Schedl, R.M. Caprioli, and D. Greenstein. 2001. A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation, pp. 2144-2147, Vol. 291.

Mitkowski, N.A., and G.S. Abawi. 2002. Monoxenic maintenance and reproduction of root-knot nematode (Meloidogyne hapla) on multiple-species in vitro root culture systems. Plant Cell Reports 21:14-23.

Mitsuhara, I., N. Shirasawa-Seo, T. Iwai, S. Nakamura, R. Honkura, and Y. Ohashi. 2002. Release from post-transcriptional gene silencing by cell proliferation in transgenic tobacco plants possible mechanism for noninheritance of the silencing. Genetics 160:343-352.

Mol, J.N.M., A.W. Schram, P. Devlaming, A.G.M. Gerats, F. Kreuzaler, K. Hahlbrock, H.J. Reif, and E. Veltkamp. 1983. Regulation of flavonoid gene-expression in petunia-hybrida - description and partial characterization of a conditional mutant in chalcone synthase gene-expression. Molecular & General Genetics 192:424-429.

Morel, J.B., C. Godon, P. Mourrain, C. Beclin, S. Boutet, F. Feuerbach, F. Proux, and H. Vaucheret. 2002. Fertile hypomorphic argonaute (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell 14:629-639.

Moriguchi, K., Y. Maeda, M. Satou, N.S.N. Hardayani, M. Kataoka, N. Tanaka, and K. Yoshida. 2001. The complete nucleotide sequence of a plant root-inducing (Ri) plasmid indicates its chimeric structure and evolutionary relationship between tumor-inducing (Ti) and symbiotic (Sym) plasmids in Rhizobiaceae. Journal of Molecular Biology 307:771-784.

Mugnier, J. 1987. Infection by Polymyxa betae and Plasmodiophora brassicae of roots containing root-inducing transferred DNA of Agrobacterium rhizogenes. Phytopathology 77:539-542.

Mugnier, J., and B. Mosse. 1987. Vesicular-arbuscular mycorrhizal infection in transformed root-inducing t-DNA roots grown axenically. Phytopathology 77:1045-1050.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant 15:473–497.

Mysore, K.S., J. Nam, and S.B. Gelvin. 2000. An Arabidopsis histone h2a mutant is deficient in Agrobacterium t-DNA integration, pp. 948-953, Vol. 97. National Acad Sciences.

Nakagawa, A., S. Sakamoto, M. Takahashi, H. Morikawa, and A. Sakamoto. 2007. The RNAi-mediated silencing of xanthine dehydrogenase impairs growth and fertility and accelerates leaf senescence in transgenic Arabidopsis plants. Plant and Cell Physiology 48:1484-1495.

Napoli, C., C. Lemieux, and R. Jorgensen. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 2:279-289.

Narayanan, R.A., R. Atz, R. Denny, N.D. Young, and D.A. Somers. 1999. Expression of soybean cyst nematode resistance in transgenic hairy roots of soybean. Crop Sci 39:1680-1686.

Nekhotiaeva, N., S.K. Awasthi, P.E. Nielsen, and L. Good. 2004. Inhibition of Staphylococcus aureus gene expression and growth using antisense peptide nucleic acids. Molecular Therapy 10:652-659.

Nyczepir, A.P., T.G. Beckman, and G.L. Reighard. 1999. Reproduction and development of Meloidogyne incognita and M. javanica on guardian peach rootstock. Journal of Nematology 31:334-340.

Ogita, S., H. Uefuji, Y. Yamaguchi, N. Koizumi, and H. Sano. 2003. RNA interference: Producing decaffeinated coffee plants. Nature 423:823-823.

Olhoft, P.M., L.E. Flagel, C.M. Donovan, and D.A. Somers. 2003. Efficient soybean transformation using hygromycin b selection in the cotyledonary-node method. Planta 216:723-735.

Orf, J.H., B.W. Diers, and H.R. Boerma. 2004. Soybeans: Improvement, production, and uses. Third ed. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI.

Ove Nilsson, O.O. 1997. Getting to the root: The role of the Agrobacterium rhizogenes rol genes in the formation of hairy roots. Physiologia Plantarum 100:463-473.

Owens, L.D., and D.E. Cress. 1985. Genotypic variability of soybean response to Agrobacterium strains harboring the Ti or ri plasmids. Plant Physiology 77:87-94.

Palatnik, J.F., E. Allen, W. Xuelin, C. Schommer, R. Schwab, J.C. Carrington, and D. Weigel. 2003. Control of leaf morphogenesis by micro RNAs. Nature 425:257.

Parrott, W.A., and T. Clemente. 2004. Transgenic soybean, p. 265-302, *In* H. R. Boerma and J. E. Specht, eds. Soybeans: Improvement, production, and uses, Third ed. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI.

Parrott, W.A., G. Dryden, S. Vogt, D.F. Hildebrand, G.B. Collins, and E.G. Williams. 1988. Optimization of somatic embryogenesis and embryo germination in soybean. In Vitro Cellular & Developmental Biology 24:817-820.

Pathan, M.S., and D.A. Sleper. 2008. Advances in soybean breeding. Genetics and Genomics of Soybean:113.

Peretz, Y., R. Mozes-Koch, F. Akad, E. Tanne, H. Czosnek, and I. Sela. 2007. A universal expression/silencing vector in plants. Plant Physiology 145:1251.

Perry, R.N. 1996. Chemoreception in plant parasitic nematodes. Annual Review of Phytopathology 34:181-199.

Petit, A., C. David, G.A. Dahl, J.G. Ellis, P. Guyon, F. Casse-Delbart, and J. Tempe. 1983. Further extension of the opine concept: Plasmids in Agrobacterium rhizogenes cooperate for opine degradation. Molecular Genetics and Genomics 190:204-214.

Plasterk, R.H.A. 2002. RNA silencing: The genome's immune system. Science 296:1263-1265.

Plesse, B., M.C. Criqui, A. Durr, Y. Parmentier, J. Fleck, and P. Genschik. 2001. Effects of the polyubiquitin gene ubi. U4 leader intron and first ubiquitin monomer on reporter gene expression in Nicotiana tabacum. Plant Molecular Biology 45:655-667.

Ploeg, A.T., and P.C. Maris. 1999. Effects of temperature on the duration of the life cycle of a Meloidogyne incognita population. Nematology 1:389-393.

Pray, C., D. Ma, J. Huang, and F. Qiao. 2001. Impact of Bt cotton in China. World Development 29:813-825.

Qiao, F., Q. Yang, C.-L. Wang, Y.-L. Fan, X.-F. Wu, and K.-J. Zhao. 2007. Modification of plant height via RNAi suppression of osga20ox2 gene in rice. Euphytica 158:35-45.

Rajagopalan, R., H. Vaucheret, J. Trejo, and D.P. Bartel. 2006. A diverse and evolutionarily fluid set of micro RNAs in Arabidopsis thaliana. Genes & Development 20:3407-3425.

Riggs, R.D., M.L. Hamblen, and L. Rakes. 1988. Resistance in commercial soybean cultivars to six races of Heterodera glycines and to Meloidogyne incognita. Ann. Appl. Nematol 2:70-76.

Ripoll, C., B. Favery, P. Lecomte, E. Van Damme, W. Peumans, P. Abad, and L. Jouanin. 2003. Evaluation of the ability of lectin from snowdrop (Galanthus nivalis) to protect plants against root-knot nematodes. Plant science (Limerick) 164:517-523.

Rosso, M.N., B. Favery, C. Piotte, L. Arthaud, J.M. De Boer, R.S. Hussey, J. Bakker, T.J. Baum, and P. Abad. 1999. Isolation of a cDNA encoding a ß-1, 4-endoglucanase in the root-knot nematode Meloidogyne incognita and expression analysis during plant parasitism. Molecular Plant-Microbe Interactions 12:585-591.

Russell, D.R., W.F. Swain, and J.T. Fuller. 1991. Soybean plants resistant to glutamine synthetase inhibitors. European 1991.

Samoylov, V.M., D.M. Tucker, F. Thibaud-Nissen, and W.A. Parrott. 1998. A liquid-medium-based protocol for rapid regeneration from embryogenic soybean cultures. Plant Cell Reports 18:49-54.

Sasser, J.N. 1980. Root-knot nematodes - a global menace to crop production. Plant Disease 64:36-41.

Sasser, J.N., and C.C. Carter. 1985. Overviewof the international Meloidogyne project 1975-1984, p. 20-24, *In* J. N. Sasser and C. C. Carter, eds. An advanced treatise on Meloidogyne, Vol. 1. Department of Plant Pathology, NC State University, Raleigh, NC.

Sasser, J.N., and D.W. Freckman. 1987. A world perspective on nematology: The role of the society, p. 7-14, *In* J. A. Veech and D. W. Dickson, eds. Vistas on nematology. Society of Nematologists, Hyattsville, Maryland.

Savka, M.A., B. Ravillion, G.R. Noel, and S.K. Farrand. 1990. Induction of hairy roots on cultivated soybean genotypes and their use to propagate the soybean cyst nematode. Phytopathology 80:503-508.

Savka, M.A., L. Liu, S.K. Farrand, R.H. Berg, and J.O. Dawson. 1992. Induction of hairy roots or pseudoactinorhizae on Alnus glutinosa, A. acuminata and Elaeagnus angustifolia by Agrobacterium rhizogenes. Acta Oecologica 13:423-431.

Sawada, H., H. Ieki, H. Oyaizu, and S. Matsumoto. 1993. Proposal for rejection of Agrobacterium tumefaciens and revised descriptions for the genus Agrobacterium and for Agrobacterium radiobacter and Agrobacterium rhizogenes. International Journal of Systematic and Evolutionary Microbiology 43:694-702.

Schmidt, M.A., D.M. Tucker, E.B. Cahoon, and W.A. Parrott. 2005. Towards normalization of soybean somatic embryo maturation. Plant cell reports(Print) 24:383-391.

Schmitt, D.P. 1985. Preliminary and advanced evaluation of nematicides, p. 241-248, *In* J. N. Sasser and C. C. Carter, eds. An advanced treatise on Meloidogyne, Vol. 1. Department of Plant Pathology, NC State University, Raleigh, N.C.

Schwab, R., J.F. Palatnik, M. Riester, C. Schommer, M. Schmid, and D. Weigel. 2005. Specific effects of micro RNAs on the plant transcriptome. Developmental Cell 8:517-527.

Sijen, T., J. Fleenor, F. Simmer, K.L. Thijssen, S. Parrish, L. Timmons, R.H.A. Plasterk, and A. Fire. 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107:465-476.

Siminszky, B., L. Gavilano, S.W. Bowen, and R.E. Dewey. 2005. Conversion of nicotine to nornicotine in Nicotiana tabacum is mediated by cyp82e4, a cytochrome p450 monooxygenase. Proceedings of the National Academy of Sciences, USA 102:14919-14924.

Sindhu, A.S., T.R. Maier, M.G. Mitchum, R.S. Hussey, E.L. Davis, and T.J. Baum. 2009. Effective and specific in planta RNAi in cyst nematodes: Expression interference of four parasitism genes reduces parasitic success. Journal of Experimental Botany 60:315.

Smant, G., J. Stokkermans, Y. Yan, J.M. de Boer, T.J. Baum, X. Wang, R.S. Hussey, F.J. Gommers, B. Henrissat, and E.L. Davis. 1998. Endogenous cellulases in animals: Isolation of beta-1, 4-endoglucanase genes from two species of plant-parasitic cyst nematodes. Proceedings of the National Academy of Sciences, USA 95:4906.

Smith, N.A., S.P. Singh, M.B. Wang, P.A. Stoutjesdijk, A.G. Green, and P.M. Waterhouse. 2000. Gene expression - total silencing by intron-spliced hairpin RNAs. Nature 407:319-320.

Sobczak, M., A. Avrova, J. Jupowicz, M.S. Phillips, K. Ernst, and A. Kumar. 2005. Characterization of susceptibility and resistance responses to potato cyst nematode (Globodera spp.) infection of tomato lines in the absence and presence of the broad-spectrum nematode resistance hero gene. Molecular Plant-Microbe Interactions 18:158-168.

SoyStats. 2009. Soybean . . . The miracle crop [Online]. Available by The American Soybean Association www.soystats.com.

Srivastava, S., and A. Srivastava. 2007. Hairy root culture for mass-production of high-value secondary metabolites. Critical Reviews in Biotechnology 27:29-43.

Stachel, S.E., E. Messens, M. Vanmontagu, and P. Zambryski. 1985. Identification of the signal molecules produced by wounded plant-cells that activate t-DNA transfer in Agrobacterium tumefaciens. Nature 318:624-629.

Steeves, R.M., T.C. Todd, J.S. Essig, and H.N. Trick. 2006. Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress Heterodera glycines reproduction. Functional Plant Biology 33:991-999.

Stoutjesdijk, P.A., S.P. Singh, Q. Liu, C.J. Hurlstone, P.A. Waterhouse, and A.G. Green. 2002. HpRNA-mediated targeting of the Arabidopsis fad2 gene gives highly efficient and stable silencing. Plant Physiology 129:1723-1731.

Tamulonis, J.P., B.M. Luzzi, R.S. Hussey, W.A. Parrott, and H.R. Boerma. 1997a. RFLP mapping of resistance to southern root-knot nematode in soybean. Crop Science 37:1903-1909.

Tamulonis, J.P., B.M. Luzzi, R.S. Hussey, W.A. Parrott, and H.R. Boerma. 1997b. DNA marker analysis of loci conferring resistance to peanut root-knot nematode in soybean. Theoretical and Applied Genetics 95:664-670.

Tepfer, D. 1984. Transformation of several species of higher plants by Agrobacterium rhizogenes: Sexual transmission of the transformed genotype and phenotype. Cell 37:959-967.

Trick, H.N., R.D. Dinkins, E.R. Santarem, R.D.V. Samoyolov, C. Meurer, D. Walker, W.A. Parrott, J.J. Finer, and G.B. Collins. 1997. Recent advances in soybean transformation. Plant Tissue Culture and Biotechnology 3:9-26.

Tuteja, J.H., S.J. Clough, W.-C. Chan, and L.O. Vodkin. 2004. Tissue-specific gene silencing mediated by a naturally occurring chalcone synthase gene cluster in Glycine max. Plant Cell 16:819-835.

Tzfira, T., and V. Citovsky. 2002. Partners-in-infection: Host proteins involved in the transformation of plant cells by Agrobacterium. Trends in Cell Biology 12:121-129.

Tzfira, T., and V. Citovsky. 2006. Agrobacterium-mediated genetic transformation of plants: Biology and biotechnology. Current Opinion in Biotechnology 17:147-154.

Tzfira, T., L.R. Frankman, M. Vaidya, and V. Citovsky. 2003. Site-specific integration of Agrobacterium tumefaciens t-DNA via double-stranded intermediates 1. Plant Physiology 133:1011-1023.

USDA. 2006. Soystats™ [Online] http://www.soystats.com.

USDA. 2008. Soystats™ [Online] www.soystats.com.

Vaistij, F.E., L. Jones, and D.C. Baulcombe. 2002. Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. The Plant Cell Online 14:857.

Vazquez, F., H. Vaucheret, R. Rajagopalan, C. Lepers, V. Gasciolli, A.C. Mallory, J.L. Hilbert, D.P. Bartel, and P. Crete. 2004. Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. Molecular Cell 16:69-79.

Veena, V., and C.G. Taylor. 2007. Agrobacterium rhizogenes: Recent developments and promising applications. In Vitro Cellular & Developmental Biology-Plant 43:383-403.

Verdejo, S., B.A. Jaffee, and R. Mankau. 1988. Reproduction of Meloidogyne javanica on plant roots genetically transformed by Agrobacterium rhizogenes. Journal of nematology 20:599-604.

Vincent, J. 1985. A manual for the practical study of root-nodule bacteria Ibp handbook, Vol. 15. Blackwell Scientific.

Wang, C.L., and P.A. Roberts. 2006. Development of AFLP and derived caps markers for root-knot nematode resistance in cotton. Euphytica 152:185-196.

Wang, J., and J.H. Oard. 2003. Rice ubiquitin promoters: Deletion analysis and potential usefulness in plant transformation systems. Plant Cell Rep 22:129-34.

Wang, S., Shi, Z., Liu, W., Jules, J., and Feng, X. 2006. Development and validation of vectors containing multiple siRNA expression cassettes for maximizing the efficiency of gene silencing. BMC Biotechnology 6.

Wang, X., R. Allen, X. Ding, M. Goellner, T. Maier, J.M. de Boer, T.J. Baum, R.S. Hussey, and E.L. Davis. 2001. Signal peptide-selection of cDNA cloned directly from the esophageal gland cells of the soybean cyst nematode Heterodera glycines. Molecular Plant-Microbe Interactions 14:536-544.

Waterhouse, P.M., H.W. Graham, and M.B. Wang. 1998. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proceedings of the National Academy of Sciences of the United States of America 95:13959-13964.

Wei, J.Z., K. Hale, L. Carta, E. Platzer, C. Wong, S.C. Fang, and R.V. Aroian. 2003. Bacillus thuringiensis crystal proteins that target nematodes. Proceedings of the National Academy of Sciences, USA 100:2760-2765.

Weller, S.A., D.E. Stead, and J.P.W. Young. 2004. Acquisition of an Agrobacterium Ri plasmid and pathogenicity by other a-proteobacteria in cucumber and tomato crops affected by root mat. Applied and Environmental Microbiology 70:2779-2785.

Wesley, S.V., C.A. Helliwell, N.A. Smith, M.B. Wang, D.T. Rouse, Q. Liu, P.S. Gooding, S.P. Singh, D. Abbott, and P.A. Stoutjesdijk. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. The Plant Journal 27:581-590.

Widholm, J.M., J.J. Finer, L.O. Vodkin, H.N. Trick, P. LaFayette, J. Li, and W.A. Parrott. 2009. Soybean transformation, *In* F. Kempken, et al., eds. Genetic modification of plants - agriculture, horticulture, and forestry. Springer - Verlag. In Press.

Wielopolska, A., H. Townley, I. Moore, P. Waterhouse, and C. Helliwell. 2005. A high-throughput inducible RNAi vector for plants. Plant Biotechnology Journal 3:583-590.

Wilcox, J.R. 2001. Sixty years of improvement in publicly developed elite soybean lines. Crop Science 41:1711-1716.

Williamson, V.M. 1998. Root-knot nematode resistance genes in tomato and their potential for future use. Annual Review of Phytopathology 36:277-293.

Williamson, V.M., and R.S. Hussey. 1996. Nematode pathogenesis and resistance in plants. Plant Cell 8:1735-1745.

Wouts , W.M. 1979. Characterization of the family Meloidogynidae with a discussion on its relationship to other families of the suborder tylenchina based on gonad morphology, p. 21-35, *In* F. L. a. C. E. Taylor, ed. Root-knot nematodes (Meloidogyne species) systematics, biology, and control. Academic Press, New York.

Wrather, J.A., T.R. Anderson, D.M. Arsyad, J. Gai, L.D. Ploper, A. PortaPuglia, H.A. Ram, and J.T. Yorinori. 1997. Soybean disease loss estimates for the top 10 soybean producing countries in 1994. Plant Disease 81:107-110.

Xiao, K., J. Liu, G. Dewbre, M. Harrison, and Z.Y. Wang. 2006. Isolation and characterization of root-specific phosphate transporter promoters from Medicago truncatula. Plant Biology 8:439-449.

Xie, Z.X., L.K. Johansen, A.M. Gustafson, K.D. Kasschau, A.D. Lellis, D. Zilberman, S.E. Jacobsen, and J.C. Carrington. 2004. Genetic and functional diversification of small RNA pathways in plants. Plos Biology 2:642-652.

Yadav, B.C., K. Veluthambi, and K. Subramaniam. 2006. Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. Molecular and Biochemical Parasitology 148:219-222.

Yan, Y., G. Smant, J. Stokkermans, L. Qin, J. Helder, T. Baum, A. Schots, and E. Davis. 1998. Genomic organization of four ß-1, 4-endoglucanase genes in plant-parasitic cyst nematodes and its evolutionary implications. Gene 220:61-70.

Yin, G.C., Z.Y. Zhu, Z. Xu, L. Chen, X.Z. Li, and F.Y. Bi. 1982. Studies on induction of pollen plant and their androgenesis in Glycine max (L.). Soybean Sci 1:69-76.

Yousif, G.M. 1979. Histological responses of four leguminous crops infected with Meloidogyne incognita. J. Nematol 11:395-401.

Zheng, X.W., J.H. Zhu, A. Kapoor, and J.K. Zhu. 2007. Role of Arabidopsis ago6 in siRNA accumulation, DNA methylation and transcriptional gene silencing. Embo Journal 26:1691-1701.