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FUNCTIONAL ANALYSIS OF A GLUTATHIONE S-TRANSFERASE GENE FROM RENIFORM NEMATODE ON SOYBEAN

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FUNCTIONAL ANALYSIS OF A GLUTATHIONE S-TRANSFERASE GENE FROM RENIFORM NEMATODE ON SOYBEAN

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ABSTRACT

Nematode glutathione S-transferases (GSTs) have been implicated in plant-nematode interactions as effector proteins with an important role in the establishment of feeding sites. Studies with root-knot nematode in *Arabidopsis thaliana* suggest that GSTs may protect the nematode against oxidative plant defenses and modulate plant responses to parasitism. Our objective was to study the function of a GST from reniform nematode (*Rotylenchulus reniformis*) in soybean. We used a virus-induced gene silencing (VIGS) system, utilizing a Bean pod mottle virus (BPMV)-based vector and a partial sequence of *gsts-1* from *Meloidogyne incognita* to silence the putative reniform nematode homolog. The effect of silencing this gene on reniform nematode infection was evaluated by inoculating treated soybean plants with 3,000 nematodes per plant. The reproduction factor was calculated thirty-five days after inoculation, and the experiment was conducted three times. Gene silencing was assessed by qRT-PCR at 0, 2, and 4 days after nematode inoculation (dai), using pecific primers for the reniform nematode *gsts* gene and for reniform nematode 18S ribosome. Hydrogen peroxide concentration in the roots was measured at 0 and 2 dai, using a fluorometric assay. Roots from the treated and untreated plants were fixed and sectioned for observations on the histopathology of infection. Reproduction on the plants inoculated with the silencing virus construct was significantly lower than in controls inoculated plants, suggesting this gene of reniform nematode plays an important role in the infection of soybean. Hydrogen peroxide concentration 2 dai in

nematode-infected roots with the silenced gene was two times higher than that in roots without the silenced gene. We suggest that plant cells response to reniform nematode infection by producing superoxide and its dismutation product, hydrogen peroxide, both of which are toxic to plant-parasitic nematodes. The observed behavior of reactive oxygen species (ROS), cell wall thickening, and callose deposition support the possibility of this nematode-secreted protein potentially acting as a microbe-associated molecular pattern.

DEDICATION

This thesis is dedicated to everyone who has loved and supported me while I was working on my Master degree at Clemson University, especially my parents. Their unconditional love and support gave me the spirit to accomplish all this.

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I would like to thank Dr. Saara DeWalt from the Department of Biological Sciences for the opportunity to be a Teaching Assistant for the course Biology of Plants. It was an excellent experience and I really enjoyed it.

I want to thank all the past and current members of Clemson University Nematology laboratory. I will always be proud to be associated with Clemson University and a lab directed by Dr. Paula Agudelo.

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Finally, I offer my regards and blessings to all of those who supported me during the completion of the Master degree.

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INTRODUCTION

Fundamental research on plant-nematode interactions and plant breeding efforts constitute a two-pronged approach to increasing the arsenal of weapons to fight damage to soybeans from parasitic nematodes. The overall goal is to identify vulnerable points in nematode interactions with soybeans or within basic nematode biology to disrupt the nematode life cycle and protect soybean plants.

Considerable progress has been made in identifying potential molecular targets for disruption in the interactions between soybean and root-knot nematode (RKN) and soybean and soybean cyst nematode (SCN). In contrast, very little is known about the molecular basis for the parasitic relationship between reniform nematode and soybean. Some of the targets identified for RKN and SCN can be applied to addressing reniform nematodes. The candidate genes in either the plant or the nematode that are suspected to be essential for successful parasitism can be silenced through virus-induced gene silencing (VIGS). This system provides a way to study critical candidate genes that disrupt the nematode or its interaction with the plant.

To the best of my knowledge, this thesis research is the first work to use VIGS for functional analysis of a gene from reniform nematode. Chapter 1 describes the preliminary work undertaken to evaluate soybean responses to reniform and root-knot nematode infection after silencing of selected plant and nematode target genes. This preliminary work led to the selection of a glutathione S-transferase gene (*gsts*-1) and the

focus of this thesis. Chapter 2 describes the functional analysis of the selected glutathione transferase.

Reniform nematode. *Rotylenchulus reniformis* Linford and Oliveira 1940, the reniform nematode, was first found parasitizing cowpea (*Vigna unguiculata* (L.)) roots in Hawaii (Linford and Oliveira, 1940). The reniform nematode is a sedentary, semi-endoparasitic pest of a wide range of crops and is widely distributed in tropical, subtropical and warm temperate zones in South America, North America, the Caribbean, Africa, southern Europe, the Middle East, Asia, Australia, and the Pacific (Gaur and Perry, 1991; Robinson *et al*., 1997).

The life history of reniform nematode has been studied in detail by Linford and Oliveira (1940), Nakasono (1983), and Sivakumar and Seshadri (1971), among others. Eggs hatch one to two weeks after being laid. The first-stage juvenile molts within the egg, producing the second-stage juvenile (J2), which emerges from the egg. Following one molt in the egg, three superimposed molts without feeding give rise to an infective, vermiform adult with a slight decrease in size. Similar numbers of females and males are usually present, but males may be rare or absent in some populations. The vermiform female penetrates the root perpendicular to the stele and does not migrate longitudinally within the root tissue. The nematode usually stops to feed on a pericycle or endodermal cell, where it induces a multicellular syncytium of cells that are largely differentiated before infection. During the next two weeks, the female enlarges into a saccate form protruding from the root, is fertilized by males, and lays eggs in a gelatinous matrix. The

nematode completes its life cycle in 20 to 29 days. Temperature affects the life cycle duration and degree of damage.

A short life cycle and high density of feeding sites along roots contribute to rapid population development and high population densities. More than 300 plant species in 77 families are hosts (Gaur and Perry, 1991; Robinson *et al*., 1997). Extensive literature exists for pineapple in Hawaii, cotton and soybean in North America, and various legumes in India. Other noteworthy crops damaged or infected by *Rotylenchulus* spp. are cantaloupe (*Cucumis melo* L.), olive (*Olea europaea* L.), papaya (*Carica papaya* L.), pineapple (*Ananas comosus* Merr.), potato (*Solanum tuberosum* L.), sweet potato (*Ipomaea batata* L.), tobacco (*Nicotiana tabacum* L.), and tomato (*Solanum lycopersium*). The reader is referred to a review of the species by Gaur and Perry (1991) and a review of the genus by Robinson *et al*. (1997).

Worldwide, the reniform nematode appears most frequently associated with deep silty soils of volcanic or alluvial origin in river floodplains. Reproduction and development of *R. reniformis* are favored by fine textured soils with a relatively high content of silt and/or clay (Barker *et al*., 1994; Robinson *et al*., 1987). However, the nematode also occurs at high population densities in soils of high sand or clay content. Field symptoms in cotton, soybean, and pineapple tend to be uniform and include stunting and suppressed yield. In cotton, plants show potassium deficiency symptoms.

The greatest challenges to managing *R. reniformis* are its wide host range and ability to survive long periods without a host in dry soil. In cotton fields in the United States,

the nematode occurs at high population densities as deep as 1 meter, hampering fumigant efficacy (Westphal and Smart, 2003).

Virus-induced gene silencing using BPMV. This research used a previously described Bean pod mottle virus (BPMV)-based vector for functional analyses of genes in soybean (Zhang and Ghabrial, 2006). BPMV, a member of the genus *Comovirus* in the family Comoviridae (Lomonossoff and Ghabrial, 2001), infects nearly all known cultivars of soybean. The BPMV genome consists of two separately encapsulated positive-strand RNA molecules (RNA1 and RNA2) that are expressed via the synthesis and subsequent proteolytic processing of polyprotein precursors. RNA1 encodes for five mature proteins required for replication whereas RNA2 codes for a putative cell-to-cell movement protein (MP) and two coat proteins (L-CP and S-CP). A stable BPMV-based vector was generated by introducing restriction enzyme sites (BamHI and MscI) to enable insertion of target sequence into the BPMV RNA2. Proteinase cleavage sites flanking the restriction enzyme sites were added to ensure proper processing of the polyprotein (Zhang and Ghabrial, 2006).

Recombinant vectors containing target sequences, when used to co-infect soybean plants along with RNA1, can efficiently express these sequences in the infected plants. Thus, the vector (pGG7R2V) can be used for both overexpression of sequences as well as for virus-induced gene silencing (VIGS) in soybean. Overexpression is achieved when full-length target sequences are expressed via this vector. The pGG7R2V vector is able to stably express inserts up to 2.0 Kb in size (personal communication Ghabrial SA). Gene silencing is achieved when partial (~100-200 bp) target sequences are expressed via the

vectors. Soybean plants infected with such recombinant vectors induce their RNA silencing machinery to silence all virus-derived sequences, including the inserted partial sequence, also resulting in silencing of the endogenous gene.

The lab of Dr. Aardra Kachroo at University of Kentucky, where I spent time completing portions of this thesis work, routinely uses this BPMV-based vector for overexpression and gene silencing in soybean. For silencing members of multigene families, specificity is achieved by targeting unique sequences in the coding or 5'/3' non-translated regions. Kachroo and collaborators have silenced the related GmSGT1-1 and GmSGT1-2 (Fu *et al*., 2009) or GmRIN4a, GmRIN4b, GmRIN4c and GmRIN4d (Selote *et al*., 2013) individually, using this strategy. The gene silencing approach is advantageous because it enables the functional analysis of essential genes, complete loss (knock-out) of which could result in lethality.

CHAPTER ONE

PRELIMINARY WORK: SOYBEAN RESPONSE TO NEMATODE INFECTION AFTER VIRUS-INDUCED SILENCING OF SELECT CANDIDATE GENES

The sedentary endoparasitic life habits of root-knot and reniform nematodes require close biotrophic interactions with their host plants. Successful parasitism requires the formation of specialized feeding cells induced by the nematode. The feeding sites formed by reniform nematode (termed syncytia) and by root-knot nematodes (termed giant cells) are formed through modification of root cells and involve the participation of effector proteins. The term "effector" has been recently redefined to include pathogen proteins that alter host cell structure and functions regardless of whether these alterations facilitate infection or trigger defense responses (Hogenhout *et al*., 2009). The proteins that ensure infection success are often the same that elicit massive plant defense responses, which have to be overcome by the pathogen to mediate susceptibility (Hewezi and Baum, 2013).

Plants react to conserved pathogen molecules that initiate a series of basal defense responses, sometimes called microbe-associated molecular patterns (MAMPs). Although no nematode MAMPs have been identified, gene expression analysis and symptoms associated with nematode infection, such as the generation of reactive oxygen species (ROS), cell wall thickening, and callose deposition support the involvement of nematode MAMPs and suggest that certain nematode proteins could act as MAMPs (Hewezi and

Baum, 2013). Therefore, in addition to effectors that function in changing the host to allow parasitism (i.e. induce the formation of the feeding site), a number of effectors deal with the suppression of defense responses triggered by parasitism.

It has been demonstrated that RNAi-mediated silencing of genes encoding nematode effector proteins can reduce pathogenicity (Rosso *et al*., 2005; Shingles *et al*., 2007). Thus, strategies involving the use of effector protein-responsive genes in the plant and effector protein producing genes in the parasitic nematodes are likely to yield successful means for preventing nematode infection. The objective of this preliminary work is to evaluate the responses of soybean to infection with reniform and root-knot nematodes after silencing of each of eight selected candidate genes through a virus-induced system. The eight candidate genes have been previously implicated in the suppression of defense responses triggered by parasitism. Three genes are of plant origin and five are of nematode origin (Fig. 1.1.)

MATERIALS AND METHODS

Candidate genes. Candidate genes were selected based on previous work done in the Agudelo (Clemson University) and the Kachroo (Kentucky University) labs, and the choices were supplemented by evidence found in the literature for other plant-nematode pathosystems. The three candidate genes/proteins of plant origin chosen included:
- GmPAL, soybean phenylalanine ammonia lyase, it is the first and committed step in the phenyl propanoid pathway and is therefore involved in the biosynthesis of the polyphenol compounds.

- GmICS, soybean isochorishmate synthase, it participates in 2 metabolic pathways: ubiquinone biosynthesis and biosynthesis of siderophore group. In *Arabidopsis*, it is also required for salicylic acid synthesis (Wildermuth *et al*., 2006).
- GmA+D, soybean Acr1 (sterol-acyl carrier protein) plus desaturase, which contributes to synthesis of oleic acid (18:1), a fatty acid involved in *Arabidopsis* plant defense.

The five candidate genes/proteins of nematode origin chosen included:
- *cbp-1*: encodes a secretory cellulose-binding protein that could be secreted through the stylet.
- *gsts-1*: encodes *Meloidogyne incognita* glutathione S-transferase, may protect against reactive oxygen species.
- *map-1*: encodes putative map-1.2 avirulence secreted protein that may be involved in the early steps of recognition between (resistant) plants and (avirulent) nematodes.
- PK: pyruvate kinase, final enzyme in glycolysis, also can regulate gluconeogenesis. The gene is derived from reniform nematode sedentary parasitic female library.
- PREP: prolyl oligopeptidase protein. The protein's activity is confined to action on oligopeptides of less than 10 kD and it has an absolute requirement for the trans-configuration of the peptide bond preceding proline. This gene is derived from reniform nematode sedentary parasitic female library.

The candidate genes were silenced using a recently developed virus-induced gene silencing (VIGS) system that uses Bean pod mottle virus (BPMV) as vector. BPMV has a bipartite positive-strand RNA genome consisting of RNA1 and RNA2. RNA2 codes for a putative cell-to-cell movement protein (MP) and two coat proteins (L-CP and S-CP).

A stable BPMV-based vector can be generated by inserting a partial sequence of the target gene into the RNA2-encoded polyprotein open reading frame, between the MP and L-CP coding regions (Zhang and Ghabrial, 2006).

Soybean plants. Soybean (*Glycine max*) plants of cvs. Hutcheson, Perrin and Forrest were grown in the greenhouse at 25±5°C and 16 h photoperiod. 'Hutcheson' was used as a susceptible host for both root-knot and reniform nematode, 'Perrin' was used as a resistant host for root-knot nematode, and 'Forrest' was used as a resistant host for reniform nematode. The plants were grown in individual 150 cc PVC conetainers® filled with fine sand.

Virus constructs for candidate genes. A VIGS vector (pGG7R2V) derived from BPMV RNA2 was used (Zhang and Ghabrial, 2006). A partial sequence of each target gene was selected and cloned into pGG7R2V for building the silencing vector for each candidate. Transcripts for recombinant viral clones were mixed with transcripts of BPMV RNA1 and rub-inoculated on to soybean leaves. After BPMV symptoms were systemically developed (Fig. 1.2.), infected leaf tissues was collected and dried for use as inoculum. Inoculum was prepared by grinding 50 -100 μg of infected leaf tissue in 5 ml of potassium phosphate buffer (136.086 g/mol, pH 7.0). Virus constructs were rub-inoculated on fully expanded unifoliolate leaves at the VC stage. A small amount of fine carborundum was dusted on the leaves prior to applying the inoculum with a cheesecloth.

Nematode materials. Reniform nematodes were obtained from naturally infested cotton fields in St. Matthews, SC. Juveniles and vermiform adults were extracted from the soil by a modified gravity screening and centrifugation-flotation method (Jenkins,

1964). Plants were inoculated with nematode ten days after inoculation with the inoculation of virus construct. Root-knot nematode inoculum was maintained on susceptible tomato (*Solanum lycopersium* cv. Rutgers) in the greenhouse. Infected roots were harvested and egg masses on the root surface were then collected by dissolving in Clorox (0.6% NaClO). Calibrated aqueous suspensions were used to inoculate individual plants in 150 cc PVC Conetainers® with 1,000 root-knot eggs per plant or 1,000 reniform vermiform stages per plant (Fig. 1.3.). Forty-five days after inoculation with nematodes, roots were stained to count the number of egg masses and root galls per plant for root-knot nematode test. Sixty days after inoculation, the final population (Pf) in the soil was assessed for reniform nematode test.

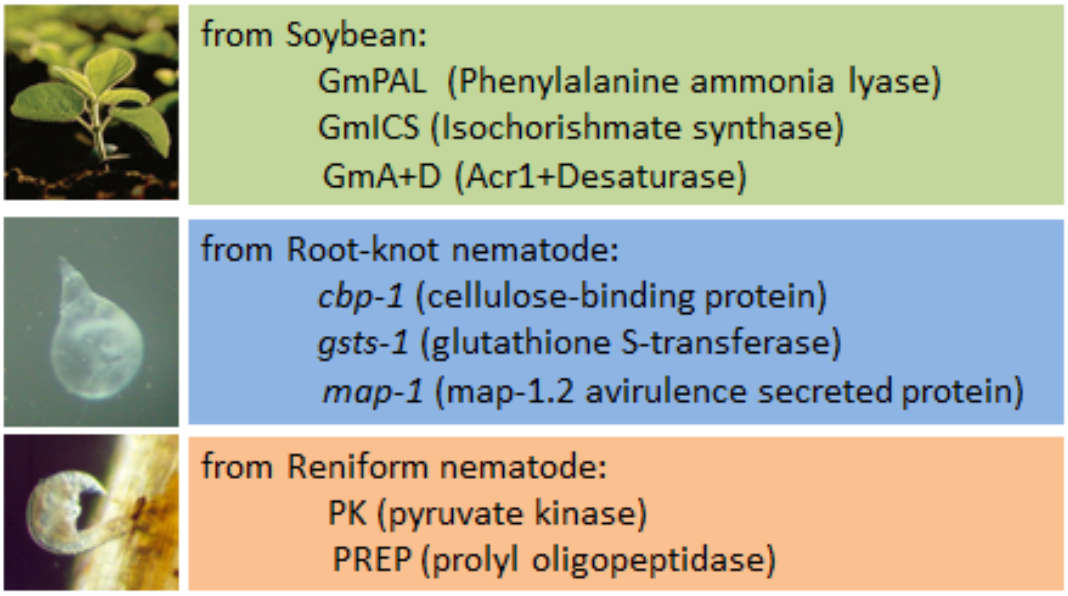

Figure 1.1. List of selected candidate genes and their origin, including candidate effector protein-responsive genes from the plant and candidate effector protein producing genes in the parasitic nematodes. A partial sequence of these genes (~150 bp) was cloned into the vector pGG7R2V for silencing.

Figure 1.2. Symptoms of Bean pod mosaic virus (BPMV) on soybean, crinkled leaves with a mosaic of light and dark green regions.

Figure 1.3. Soybean plants in the greenhouse ready for inoculation with reniform or root-knot nematode in calibrated aqueous suspensions and pipetted into the soil near the root.

RESULTS

GmPAL and GmICS. An effect of treatment was observed on the susceptible variety, but not on the resistant with both nematodes (Tables 1.1-1.4). In experiment 1 (Table 1.1.) with root-knot nematode on Hutcheson, higher nematode reproduction and galling were observed on the plants infected with the virus alone and the controls. At the time we hypothesized that BPMV infection increased SA accumulation in the plant, so empty vector-inoculated plants had more SA content than untreated plants. We also hypothesized that the tested constructs silenced key enzymes and reduced SA levels may shut down one pathway for SA biosynthesis and high SA was correlated with increased susceptibility to the nematode in the susceptible host. However, we didn't test if the target genes were silenced successfully, and we didn't measure SA content. Unfortunately, the subsequent three experiments did not validate our hypothesis.

GMA+D. Two experiments were done to test GmA+D virus construct on root-knot nematode. In the first experiment, GmA+D virus construct and BPMV control had similar egg masses and galls number which are significantly lower than control treated with buffer alone. In the second experiment there was no effect of treatments observed (Table 1.5). Similar inconsistent results were obtained for reniform nematode (Table 1.6.).

Nematode candidate genes. For most nematode candidate genes, results were either inconsistent or there was an effect of the virus vector alone that confounded the effect of the gene silencing (Tables 1.7 – 1.11). The effect of the virus vector seems to be present

for root-knot, but not for reniform nematode. For these reasons, *gsts-1* on reniform was selected for more detailed studies described in Chapter 2.

Based on the summary of candidate plant effector responsive genes tests on both root-knot nematode test and reniform nematode test (Table 1.12), we observed inconsistent of results among experiments. For root-knot nematode test, there was an effect of BPMV vector existed, even though GmICS showed consistent results in all four experiments. For reniform nematode test, the results were inconsistent. Based on the summary of candidate nematode effector genes tests on both root-knot nematode and reniform nematode test (Table 1.13), inconsistent of results was still observed. For root-knot nematode test, the effect of BPMV vector also existed. For reniform nematode test, two candidate genes, including PK and PREP, showed inconsistent result. However, we found *gsts-1* gene test on reniform nematode showed consistent result and there was no effect of BPMV vector exists.

DISCUSSION

BPMV-based VIGS system seems to have different influence on root-knot nematode and reniform nematode. The effect of BPMV vector was more frequently showed on root-knot nematode test. If there is an effect of vector, our reproduction results of the virus construct treated plants will not be reliable. This system may not be a perfect way to do functional analysis on root-knot nematode test. However, on reniform nematode test, especially, for *gsts-1* gene test, it seems to work really well. Further test is also

needed to identify if it is the gene's function causing the differences in reniform reproduction.

Table 1.1. Average galls and egg masses per plant 45 days after inoculation with *Meloidogyne incognita* on soybean cv. Hutcheson (susceptible host) and Perrin (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; GmPAL = *Glycine max* phenylalanine ammonia lyase virus construct. The experiment was run four times. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	25.2 a	28.4 a	1.9 a	2.4 a
BPMV control	6.5 b	9.2 b	0.7 a	0.7 a
GmPAL	30.4 a	33.8 a	1.4 a	1.4 a

EXPERIMENT 2

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	54 a	58.3 a	1.8 a	1.8 a
BPMV control	10.5 b	10.5 b	0.3 a	0.3 a
GmPAL	16.3 b	17.0 b	0.0 a	0.0 a

EXPERIMENT 3

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	62.3 a	67.0 a	1.8 a	1.8 a
BPMV control	12.7 b	15.0 b	0.3 a	0.3 a
GmPAL	6.3 b	9.3 b	0.0 a	0.0 a

EXPERIMENT 4

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	94.5 a	98 a	0.0 a	0.0 a
BPMV control	34.3 b	37 b	0.0 a	0.0 a
GmPAL	4 b	4.3 c	0.3 a	0.3 a

Table 1.2. Final population (Pf) per plant 60 days after inoculation with *Rotylenchulus reniformis* on soybean cv. Hutcheson (susceptible host) and Forrest (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; GmPAL = *Glycine max* phenylalanine ammonia lyase virus construct. The experiment was run twice. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	484 a	48 a
BPMV control	404 a	20 b
GmPAL	306 a	6 b

EXPERIMENT 2

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	704 a	20 a
BPMV control	600 ab	15 a
GmPAL	310 bc	44 a

Table 1.3. Average galls and egg masses per plant 45 days after inoculation with *Meloidogyne incognita* on soybean cv. Hutcheson (susceptible host) and Perrin (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; GmICS = *Glycine max* isochorishmate synthase virus construct. The experiment was run four times. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	25.2 a	28.4 a	1.9 a	2.4 a
BPMV control	6.5 b	9.2 b	0.7 a	0.7 a
GmICS	13.2 b	14 b	2.2 a	4.4 a

EXPERIMENT 2

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	54 a	58.3 a	1.8 a	1.8 a
BPMV control	10.5 b	10.5 b	0.3 a	0.3 a
GmICS	5.8 b	6.3 b	0.0 a	0.0 a

EXPERIMENT 3

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	62.3 a	67 a	1.8 a	1.8 a
BPMV control	12.7 b	15 b	0.3 a	0.3 a
GmICS	4.3 b	6 b	0.0 a	0.0 a

EXPERIMENT 4

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	94.5 a	98 a	0.0 a	0.0 a
BPMV control	34.3 b	37 b	0.0 a	0.0 a
GmICS	10 b	12.3 b	0.0 a	0.0 a

Table 1.4. Final population (Pf) per plant 60 days after inoculation with *Rotylenchulus reniformis* on soybean cv. Hutcheson (susceptible host) and Forrest (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; GmICS = *Glycine max* isochorishmate synthase virus construct. The experiment was run twice. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	484 a	48 a
BPMV control	404 a	20 b
GmICS	310 a	16 b

EXPERIMENT 2

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	704 a	20 a
BPMV control	600 a	15 a
GmICS	160 b	18 a

Table 1.5. Average galls and egg masses per plant 45 days after inoculation with *Meloidogyne incognita* on Hutcheson (susceptible host) and Perrin (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; GmA+D = *Glycine max* sterol-acyl carrier protein-desaturase virus construct. The experiment was run twice. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Galls	Egg masses	Galls	Egg masses
Control	15.8 a	10.8 a	4.8 a	1.8 a
BPMV control	2.8 b	1.8 b	0 b	0 b
GmA+D	0 b	0 b	0.6 b	0.6 ab

EXPERIMENT 2

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Galls	Egg masses	Galls	Egg masses
Control	6.6 a	5.8 a	1.2 a	0.6 a
BPMV control	3.0 a	3.0 a	1.4 a	1.2 a
GmA+D	2.0 a	2.0 a	0.2 a	0.2 a

Table 1.6. Average galls and egg masses per plant 45 days after inoculation with *Meloidogyne incognita* on soybean cv. Hutcheson (susceptible host) and Perrin (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; *cbp-1* = encoding cellulose-binding protein virus construct. The experiment was run twice. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Galls	Egg masses	Galls	Egg masses
Control	42.4 a	38.7 a	0.6 a	0.6 a
BPMV control	1.5 b	1.5 b	0.3 a	0.4 a
cbp-1	7.8 b	6.5 b	0 a	0 a

EXPERIMENT 2

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Galls	Egg masses	Galls	Egg masses
Control	54.7 a	52.7 a	2.3 a	2.3 a
BPMV control	22.6 b	22 b	3.5 a	3.2 a
cbp-1	0.4 c	0.4 c	0.4 a	0.2 a

Table 1.7. Average galls and egg masses per plant 45 days after inoculation with *Meloidogyne incognita* on soybean cv. Hutcheson (susceptible host) and Perrin (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; *map-1* = encoding putative map-1.2 protein virus construct. The experiment was run twice. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Galls	Egg masses	Galls	Egg masses
Control	42.4 a	38.7 a	0.6 a	0.6 a
BPMV control	1.5 b	1.5 b	0.3 a	0.4 a
map-1	1.8 b	1.8 b	0 a	0 a

EXPERIMENT 2

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Galls	Egg masses	Galls	Egg masses
Control	54.7 a	52.7 a	2.3 a	2.3 a
BPMV control	22.6 b	22 b	3.5 a	3.2 a
map-1	22.4 b	22.4 b	0.4 a	0.4 a

Table 1.8. Final population (Pf) per plant 60 days after inoculation with *Rotylenchulus reniformis* on soybean cv. Hutcheson (susceptible host) and Forrest (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; PK = encoding pyruvate kinase virus construct. The experiment was run twice. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	383.8 a	93 a
BPMV control	376.3 a	59 a
PK	387.5 a	66 a

EXPERIMENT 2

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	214a	55a
BPMV control	85b	140a
PK	114b	75a

Table 1.9. Final population (Pf) per plant 60 days after inoculation with *Rotylenchulus reniformis* on soybean cv. Hutcheson (susceptible host) and Forrest (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; PREP = prolyl oligopeptidase virus construct. The experiment was run twice. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	383.8 a	93 a
BPMV control	376.3 a	59 a
PREP	272.5 a	34 a

EXPERIMENT 2

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	214a	55a
BPMV control	302a	85a
PREP	120a	17.5a

Table 1.10. Average galls and egg masses per plant 45 days after inoculation with *Meloidogyne incognita* on soybean cv. Hutcheson (susceptible host) and Perrin (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; *gsts-1* = encoding *M. incognita* glutathione S-transferase virus construct. The experiment was run three times. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	54 a	58.3 a	0.8 b	0.8 b
BPMV control	1.8 b	1.8 b	0.0 b	0.0 b
gsts-1	43.3 a	47 a	3.3 a	3.3 a

EXPERIMENT 2

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	62.3 a	67 a	0.0 a	0.0 a
BPMV control	12.7 b	15 b	0.0 a	0.0 a
gsts-1	15.7 b	20 b	0.0 a	0.0 a

EXPERIMENT 3

	Hutcheson (n = 5)		Perrin (n = 5)	
Treatment	Egg masses	Galls	Egg masses	Galls
Control	94.5 a	98 a	3.8 a	5.3 a
BPMV control	20.7 c	23.7 c	1.8 a	1.8 a
gsts-1	39.5 b	47 b	1.3 a	1.8 a

Table 1.11. Final population (Pf) per plant 60 days after inoculation with *Rotylenchulus reniformis* on soybean cv. Hutcheson (susceptible host) and Forrest (resistant host). Control = buffer alone; BPMV control = Bean pod mottle virus alone; *gsts-1* = encoding *M. incognita* glutathione S-transferase virus construct. The experiment was run twice. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

EXPERIMENT 1

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	484 a	48 a
BPMV control	404 a	20 a
gsts-1	222 a	16 a

EXPERIMENT 2

	Hutcheson (n = 5)	Forrest (n = 5)
Treatment	Pf	Pf
Control	704 a	20 a
BPMV control	600 a	15 a
gsts-1	140 b	15 a

Table 1.12. Summary of candidate plant effector responsive genes tests on both *M. incognita* and *R. reniformis*.

Construct	Nematode	Exp. 1	Exp. 2	Exp. 3	Exp. 4
GmPAL	*M. incognita*	✗	✓*	✓*	✓*
GmICS	*M. incognita*	✓*	✓*	✓*	✓*
GmA+D	*M. incognita*	✓*	✗	-	-
GmPAL	*R. reniformis*	✗	✓*	-	-
GmICS	*R. reniformis*	✗	✓	-	-

✓: different from control

✗: not different from control

*: not different from BPMV control

Table 1.13 Summary of candidate nematode effector genes tests on *M. incognita* and *R. reniformis*.

Construct	Nematode	Exp. 1	Exp. 2	Exp. 3
cbp-1	*M. incognita*	✓*	✓	-
gsts-1	*M. incognita*	×	✓*	✓
map-1	*M. incognita*	✓*	✓*	-
PK	*R. reniformis*	×	✓*	-
PREP	*R. reniformis*	×	×	-
gsts-1	*R. reniformis*	✓	✓	✓

✓: different from control

×: not different from control

*: not different from BPMV control

CHAPTER 2

FUNCTIONAL ANALYSIS OF A GLUTATHIONE S-TRANSFERASE GENE FROM RENIFORM NEMATODE ON SOYBEAN

Nematode glutathione S-transferases (GSTs) have been implicated in plant-nematode interactions as effector proteins with an important role in the establishment of feeding sites. Studies with root-knot nematode in *Arabidopsis thaliana* suggest that GSTs may protect the nematode against oxidative plant defenses and modulate plant responses to parasitism. Our objective was to study the function of a GST from reniform nematode (*Rotylenchulus reniformis*) in soybean. We used a virus-induced gene silencing (VIGS) system, utilizing a Bean pod mottle virus (BPMV)-based vector and a partial sequence of *gsts-1* from *Meloidogyne incognita* to silence the putative reniform nematode homolog. The effect of silencing this gene on reniform nematode infection was evaluated by inoculating treated soybean plants with 3,000 nematodes per plant. The reproduction factor was calculated thirty-five days after inoculation, and the experiment was conducted three times. Gene silencing was assessed by qRT-PCR at 0, 2, and 4 days after nematode inoculation (dai), using specific primers for the reniform nematode *gsts* gene and for reniform nematode 18S ribosome. Hydrogen peroxide concentration in the roots was measured at 0 and 2 dai, using a fluorometric assay. Roots from the treated and untreated plants were fixed and sectioned for observations on the histopathology of infection. Reproduction on the plants inoculated with the silencing virus construct was significantly

lower than in controls inoculated plants, suggesting this gene of reniform nematode plays an important role in the infection of soybean. Hydrogen peroxide concentration 2 dai in nematode-infected roots with the silenced gene was two times higher than that in roots without the silenced gene. We suggest that one way plant cells responded to reniform nematode infection was by producing superoxide and its dismutation product, hydrogen peroxide, which are both toxic to plant-parasitic nematodes. The observed behavior of reactive oxygen species (ROS), cell wall thickening, and callose deposition support the possibility of this nematode-secreted protein potentially acting as a microbe-associated molecular pattern.

Reniform nematode (*Rotylenchulus reniformis*) is a sedentary semi-endoparasite that infects over 300 plant species, including important field crops like soybean and cotton (Robinson *et al*., 1997), in which it can cause economically important yield suppression (Koenning *et al*., 1996; Lawrence and McLean, 1996; Lawrence *et al*., 1990; Rebois *et al*., 1970). Parasitism by reniform nematode involves significant physiological changes in plant root cells leading to the formation of specialized feeding structures called syncytia (Gaur & Perry, 1991; Agudelo *et al*., 2005a). Events that occur at these feeding sites are thought to determine the degree of susceptibility of plants to reniform nematode.

In addition to inducing its permanent feeding site, reniform nematode needs effective mechanisms for combating host defense responses. In many plants, the first reaction in response to the attack by a pathogen involves an oxidative burst which generates toxic reactive oxygen species (ROS). Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are two kinds of ROS produced in the defense response of plants to nematode infection (Waetzig

et al., 1999). Symptoms of hypersensitive reaction and production of H_2O_2 have been documented in the incompatible interaction between *Arabidopsis thaliana* and the cyst nematode *Heterodera glycines* (Waetzig *et al*., 1999). A burst of H_2O_2 occurs during compatible interactions between tomato and *Meloidogyne incognita* (Melillo *et al*., 2006). It has been confirmed that the timing of H_2O_2 generation is an important determinant in blocking successful nematode development (Melillo *et al*., 2006).

Ascorbate-glutathione cycle is one of the main hydrogen peroxide detoxification systems in plant chloroplasts, in which ascorbate peroxidase is a key enzyme. Ascorbate peroxidase utilizes ascorbate as specific eletron donor to reduce H_2O_2 to water. Other than chloroplasts, ascorbate peroxidase and the ascorbate-glutathione cycle also play a role in ROS scavenging in plant cytosol, mitochondria and peroxisomes (Asada, 1999; Mittler *et al*., 2004; Noctor and Foyer, 1998; Shigeoka *et al*., 2002). Superoxide dismutase and ascorbate peroxidase have been detected in root-knot and cyst nematodes as enzymes for removing ROS and further protecting the parasite from host plant ROS damage (Molinari and Miacola, 1997; Robertson *et al*., 2000; Jones *et al*., 2004). However, little is known about the roles of these proteins in the host-parasite interaction and none of these proteins have been well characterized in reniform nematode. Glutathione S-transferases (GSTs) are a highly diverse family of enzymes catalyzing the addition of reduced glutathione (GSH) to electrophiles or the GSH-dependent reduction of hydroperoxides (Wilce and Parker, 1994; Sheehan *et al*., 2001; Jasmer *et al*., 2003). It has been reported that animal parasitic nematodes use GSTs to detoxify a wide range of endogenous and exogenous compounds (Campbell *et al*., 2001). GSTs are also involved

in the protection of *Caenorhabditis elegans* against oxidative stress (Leiers *et al*., 2003). GSTs are major cellular detoxification enzymes which are one kind of secretion expressed in the nematode subventral glands (Dubreuil *et al*., 2007).

One *Mi-gsts-1* gene, encoding a predicted GST of the sigma class, has been isolated and reported to be specifically expressed in *M. incogntia* subventral esophageal glands (Dubreuil *et al*., 2007). RNA interference (RNAi) was used to knock down the *Mi-gsts-1* gene, and results demonstrated that it may be required for successful nematode development after gall formation (Dubreuil *et al*., 2007). Studies on identification and characterization of proteins with RS scavenging functions in reniform nematode are non-existant. Our objective was to study the function of a GST from reniform nematode in soybean, using a virus-induced gene silencing (VIGS) system, consisting of a Bean pod mottle virus (BPMV)-based vector and a partial sequence of *gsts-1* from *Meloidogyne incognita*.

MATERIALS AND METHODS

Plant and nematode materials. Soybean (*Glycine max*) plants of the susceptible cultivar Hutcheson were grown in a walk-in-growth room at 28 ± 2°C, 40% relative humidity, and 16-h photoperiod. Reniform nematodes were obtained from naturally infested cotton fields in St. Matthews, SC. Juveniles and vermiform adults were extracted from the soil by a modified gravity screening and centrifugation-flotation method (Jenkins, 1964). Calibrated aqueous suspensions were used to inoculate individual plants in 150 cc PVC conetainers® filled with fine sand.

Viral vector and *gsts*-silencing construct. A virus-induced gene silencing (VIGS) vector (pGG7R2V) derived from bean pod mottle virus (BPMV) RNA2 was used to accomplish silencing (Zhang and Ghabrial, 2006). A partial sequence (~150-300 bp) of the *gsts* gene was generated based on a *Meloidogyne incognita* genomic sequence and cloned into pGG7R2V to build the silencing vector. Transcripts for recombinant viral clones were mixed with transcripts of BPMV RNA1 and rub-inoculated on soybean leaves. Leaf tissue was collected and dried for further inoculation after BPMV symptoms were systemically developed. This portion of the work was completed in the lab of Dr. Said Ghabrial at the University of Kentucky.

Effect of silencing of *gsts* on reniform nematode reproduction. Virus inoculum was prepared by grinding 100 μg infected leaf tissue in 5 ml potassium phosphate (pH 7.0) buffer. Inoculation of constructs was done by dusting carborundum powder GRIT 320 on fully expanded unifoliolate leaves at the VC stage (approximately 10 days after planting) and then rubbing the macerate onto the leaves with a piece of cheesecloth. Controls included plants inoculated with potassium phosphate buffer alone and plants inoculated with BPMV empty vector alone. Ten days after virus inoculation, plants were inoculated with 3,000 vermiform reniform nematodes per plant by dispensing 1 ml of a calibrated aqueous suspension of the nematodes into two small holes made in the soil near plant roots. Thirty-five days after inoculation, reproduction of reniform nematode was evaluated by extracting the nematodes present in the soil using a centrifugation-flotation method. The experiment was run three times.

Quantitative reverse-transcription PCR. Total RNA was extracted from nematode-infected roots at 10 dai using an RNeasy maxi kit (Qiagen). Total RNAs were treated with RNase-free DNase set (Qiagen). Quantitative reverse-transcription PCR (qRT-PCR) reactions were set up in 25 µl following the instructions of the QuantiTect SYBR Green RT-PCR kit (Qiagen) on a Stratagene Mx3000P qRT-PCR system, using 1 µl of the total RNA from soybean roots as templates. qRT-PCR parameters included initial reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; followed by 45 cycles of 94°C for 15 s, 52°C for 30 s, and 72°C for 30 s (at the end of this step, fluorescence data were collected). A portion of the 18S ribosomal RNA gene (150 bp) was included for all samples as reference for normalization of products. The primers used in the qRT-PCR were: gsts-F: 5′-AGGGGCGACCTTTTGAAGAT-3′ gsts-R: 5′-CCCATTCATTTTTGCCTGCCA-3′, 18S-F: 5′-ACCGTGGCCAGACAAACTAC-3′ 18S-R: 5′-GATCGCTAGTTGGCATCGTT-3′. The standard curve method was used to quantify differences in expression level. A dilution series for total RNA was used to create a standard curve for both *gsts* gene and 18S gene. Standard curves of *gsts* gene and 18S gene had r^2 of 0.989 and 0.99, respectively. Normalized fold expression of *gsts* was calculated using the $\Delta\Delta C_t$ method as part of the Bio-Rad CFX^{TM} Manager Software package (Bio-Rad, CA). Amplicons of *gsts* gene were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA) and sequenced at Clemson University Genomics Institute.

Northern blot analysis. Total RNA was extracted from nematode soybean leaves and infected roots 0, 2 and 4 dai using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. For Northern blot analysis, 7 µg of total

RNA was separated in 1.5% agarose gel containing 3% formaldehyde and blotted to the nylon membrane. Membranes were transferred with 20× SSC (1×SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2). The membranes were dried, UV cross-linked, and used for the hybridization. For probe synthesis, ascorbate peroxidase DNA fragement was amplified from soybean cv. Essex cDNA with gene specific primer: L10292-F: 5′-TCTCCTTCAGCTACCTTCTG-3′, L10292-R: 5′-ACGTCCAGCAAAATACATTC-3′. The probe was then labeled with α-^{32}P-dCTP (Perkin Elmer, USA). The membranes were pre-hybridized at 65°C for 1 h in a solution of 0.25 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA and 1% bovine serum albumin. Heat-denatured probes were mixed with fresh buffer, and hybridizations were carried out over night at 65°C. After hybridization, membranes were washed twice with 2× SSC, 0.5% SDS and once with 1× SSC, 0.1% SDS solution. The washed membranes were exposed overnight using a Storage Phosphor Screen (Amersham Biosciences) and scanned on a Typhoon 9400 Variable Mode Imager (GE Healthcare). The signal intensity was quantified by ImageQuant TL V2005 software.

Hydrogen peroxide (H_2O_2) production. For determination of H_2O_2 production in 0 and 2 dai soybean roots, 30 mg of root tissue was frozen in liquid nitrogen and ground, 200 µl of phosphate buffer (20 mM K_2HPO_4, pH 6.5) was added after the tissue had been thoroughly ground. After centrifugation, 50 µl of the supernantant was incubated with100 µM Amplex Red Reagent (10-acethyl-3, 7-dihydrophenoxazine) (Molecular probes, Eugene, OR, USA) and 0.2 U/ml horseradish peroxidase at room temperature for

30 min in the dark. The fluorescence absorption was measured at 590 nm (excitation) and 540 nm (emission) using a black 96-well plants in a microplate-flourometer .

Histopathology. Infected root segments from soybean plants treated with *gsts* virus construct and inoculated with 3,000 vermiform nematode per plant were collected 10 dai. Root segments were fixed in Formaldehyde/Acetic Acid (FAA) overnight, then dehydrated through a graded ethanol series (50, 70, 95, 100, and 100% ethanol), infiltrated, and embedded in ImmunoBed resin (Polysciences Inc., Warrington, PA) as per the manufacturer's instructions. Sections (2 μm) were counterstained with sequential immersions in Azure II (1% in 1% sodium borate) and Basic Fuchsin (0.05% in 2.5% ethanol). Observations of syncytium characteristics were made under an Olympus BH-2 light microscope.

RESULTS

Nematode reproduction on plants treated with the *gsts* virus construct. Reniform nematode reproduction (Rf = Final population/Initial population) was significantly lower ($p<0.01$) in the *gsts*-silenced treated plants than in the controls. Absolute final populations were different for each of the three experiments, but relative Rf (Rf treatment/Rf control) was consistently lower, ranging from 20 to 67% (Figure 2.1).

H_2O_2 level and histpathology. H_2O_2 production was significantly ($p<0.05$) higher in all treatments 2 dai (Table 2.1 and Figure 2.2) compared to 0 days. Hydrogen peroxide levels of *gsts* virus construct treated plants at 2 dai were significantly higher ($p<0.05$) than those observed in the controls for the same time. Similarly, ten days after

inoculation with the nematode, syncytia in the *gsts*-silenced treated plants appeared less developed, with more frequent presence of darkened cell walls (Figure 2.3).

qRT-PCR and Northern blot. For qRT-PCR, relative expression was calculated using the ΔΔCt method based on the standard curves (18S, y=-0.212lnx+30.215 and *gst*, y=-0.159lnx+27.9). Relative expression of *gsts* gene for "*gsts*-silenced" was 81% lower than Control treated with buffer alone (Figure 2.4). Northern blot analysis showed that ascorbate peroxidase transcription was higher 2 dai in *gsts*-virus construct treated plant leaves and roots compared to the controls (Figure 2.5).

DISCUSSION

We employed a reverse genetics approach to characterize the reniform *gsts* gene and study its parasitism-related functionalities. The *gsts* gene was silenced using a VIGS system and we were able to detect reduced *gsts* expression that resulted in decreased success in reniform parasitism and induced a defense-related phenotype. The importance of the *gsts* gene in reniform nematode parasitism was assessed by analyzing the ability of the nematode to complete its life cycle and reproduce.

It has been shown that plant cells respond to pathogen attacks by producing superoxide (O_2^-) and its dismutation product H_2O_2, both of which are toxic to the parasite (Dubreuil *et al*., 2007). A burst of H_2O_2 occurs during compatible interaction between tomato and root-knot nematode (Melillo *et al*., 2006). Our measurement of H_2O_2 production showed that H_2O_2 level was higher in all treatment 2 dai, which confirmed the former results that upon pathogen attack plants did generated more H_2O_2 to block

nematode attack. On the other hand, only 2 dai "*gsts*-silenced" plants ended up with significantly higher H_2O_2 level implying that *gsts* may play an essential role in reniform nematode parasitism. Reduced *gsts* expression impaired the nematode's ability to scavenge extra H_2O_2 which had been produced by plant's natural defense system. At the same time, our northern blot analysis of ascorbate peroxidase, which is a key enzyme of plant antioxidant system and playing a central role in H_2O_2 scavenging, showed that ascorbate peroxidase expression in both soybean leaves and roots was higher in 2 dai "*gsts*-silenced" treated plants than the other two controls.

In this study, soybean roots were inoculated with 3000 vermiform and cross sections of 10 dai roots were examined. For each treatment, the nematodes were able to establish healthy feeding sites. However, the syncytia cells in "*gsts*-silenced" treated roots were much less developed than the other two treatments. This result, confirmed our observations from the nematode reproduction that "gsts-silenced" treated plants' pathogenicity has been weakened. Our qRT-PCR showed that *gsts* expression in "*gsts*-silenced" treated plants was 80% reduced compared to plants treated with buffer alone. This provided us a direct evidence that the other changes in phenotypes were due to differential of *gsts* expression. Meanwhile, it proved that BPMV-based VIGS system is an effective and efficient way for gene silencing and other functional analysis for other genes and other research.

In summary, there is evidence of *gsts* gene acting as parasitism gene during reniform nematode infection process. Transcripts of glutathione S-transferase (GSTs) gene *Mi-gsts-1* were 27 times more abundant in *M. incognita* J3 than in J2 (J2 is the infective

stage), indicating that the gene products are secreted during infection (Dubreuil *et al*., 2007). Functional analysis also identified GSTs as effectors of parasitism required for the full establishment of the nematode in plant tissue. The secretion of GSTs may protect the parasite against oxidative plant defenses or modulate plant response to parasitism (Dubreuil *et al*., 2007). In the case of reniform nematode, we have found that *gsts* expression levels are relevant to nematode infection success. Its effect on syncytium development is unclear, but it does seem implicated in the nematode's ability to fight the plant's oxidative burst defense system, especially during scavenging H_2O_2, and further impact nematode reproduction.

GSTs play an important role in host-parasite interfaces of reniform nematode and plants. Our studies suggest that the particular *gsts* reniform homolog may be significant effector. Further studies will be necessary to identify the specific involvement of the gene and the corresponding protein in regulation of this host-nematode interaction.

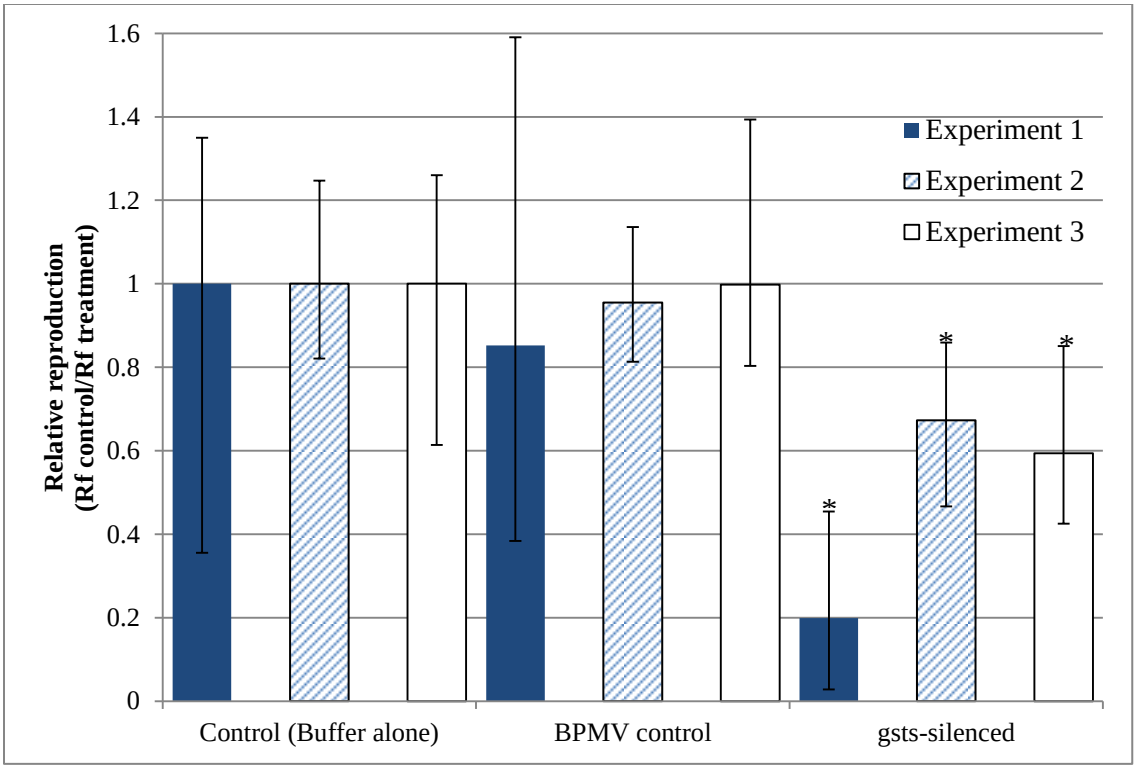

Figure 2.1. Relative reproduction (reproduction factor control/reproduction factor treatment) of reniform nematode on soybean plants (cv. Hutcheson) treated with *gsts*-virus construct. Experiment was performed three times, and the evaluation was done thirty-five days after nematode inoculation with seven replicates for each treatment. The data were analyzed separately by experiment by analysis of variance (ANOVA) and means of *gsts*-silenced plants were significantly (*) lower ($P \leq 0.05$) in all three experiments.

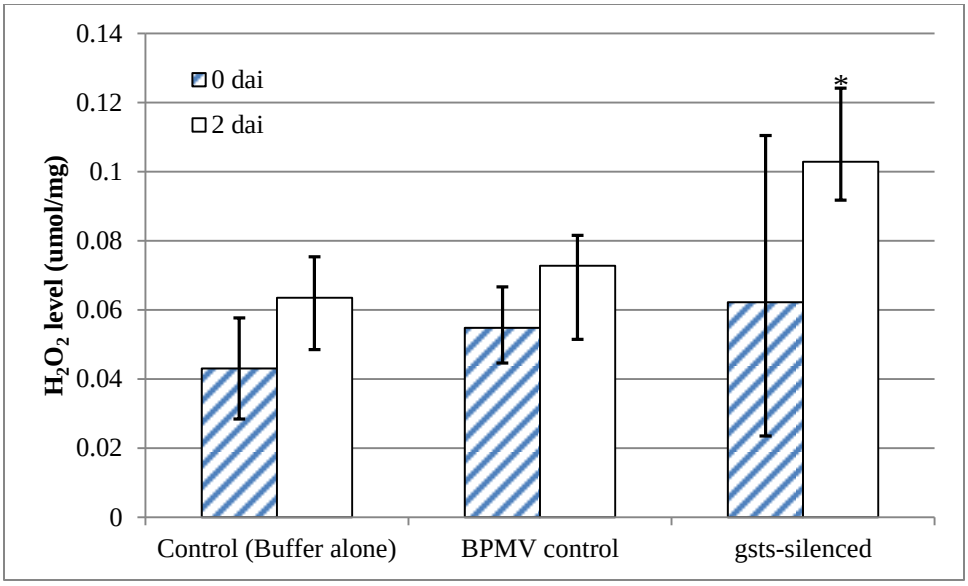

Figure 2.2. Soybean root H_2O_2 level 0 and 2 days after nematode inoculation (dai). Means are a result of three biological replicates and six technical replicates within each biological replicate. The data were analyzed by analysis of variance (ANOVA) and means of H_2O_2 production in 2 dai *gsts*-silenced plants were significantly higher (*) than the other means ($P \leq 0.05$). Error bars represent the standard error of the means.

Table 2.1. Soybean root H_2O_2 level 0 and 2 days after nematode inoculation (dai). Means are a result of three biological replicates and six technical replicates within each biological replicate. The data were analyzed by analysis of variance (ANOVA) and different letters after means within a column in each experiment indicate significant differences ($P \leqslant 0.05$) according to Student's t test.

Treatment	0 dai (umol/mg)	2 dai (umol/mg)
Control Buffer alone	0.0431 c	0.0635 bc
Control BPMV	0.0548 bc	0.0727 b
gsts-silenced	0.0622 bc	0.103 a

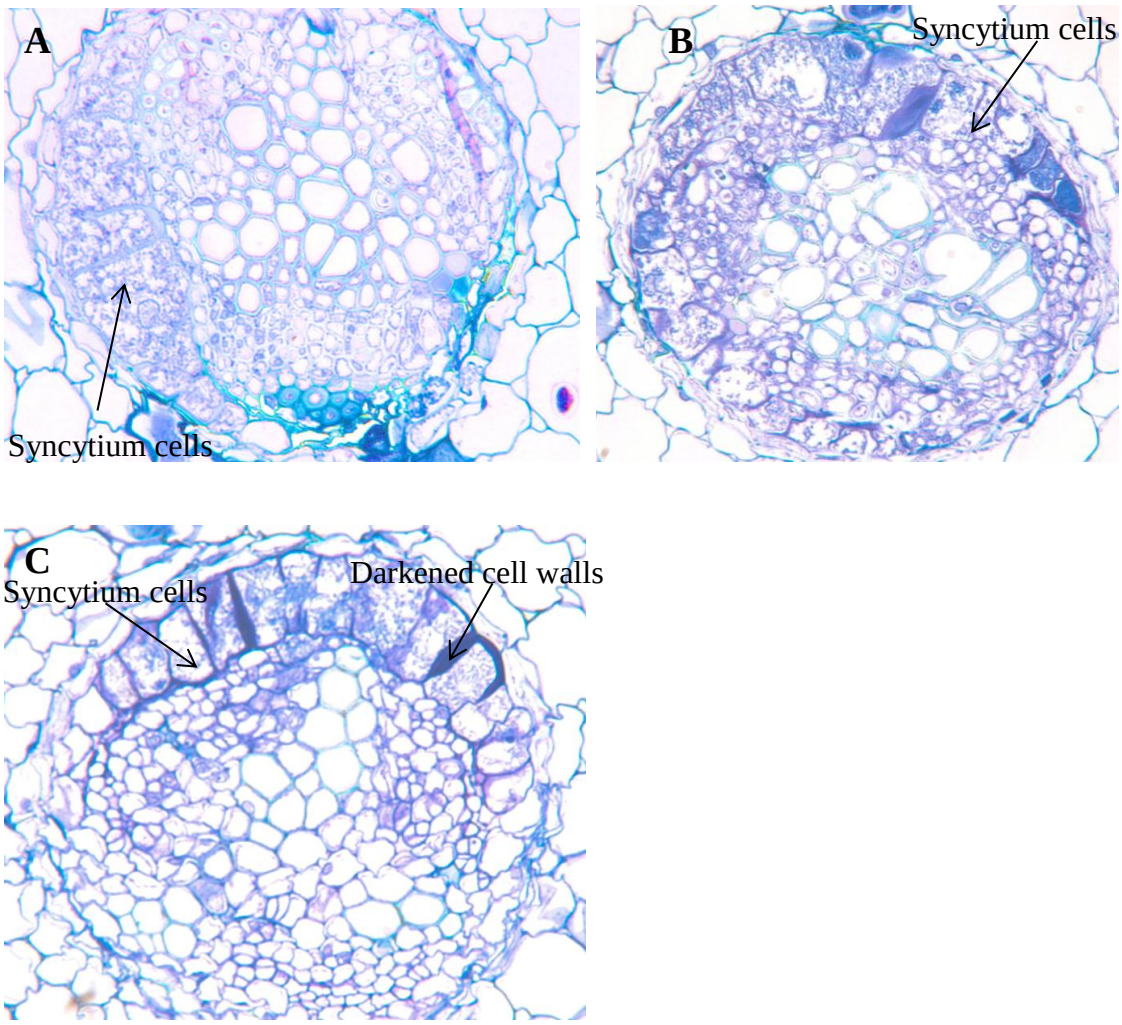

Figure 2.3. Cross sections (2 µm) of soybean roots treated 10 days after inoculation and stained with Azure II and Basic fuchsin. A and B. Normal syncytia in Control (buffer alone) and Virus control (empty BPMV vector) treated plant root segments; C. syncytia in *gsts* virus construct treated plant root segments showed more darkened cell walls.

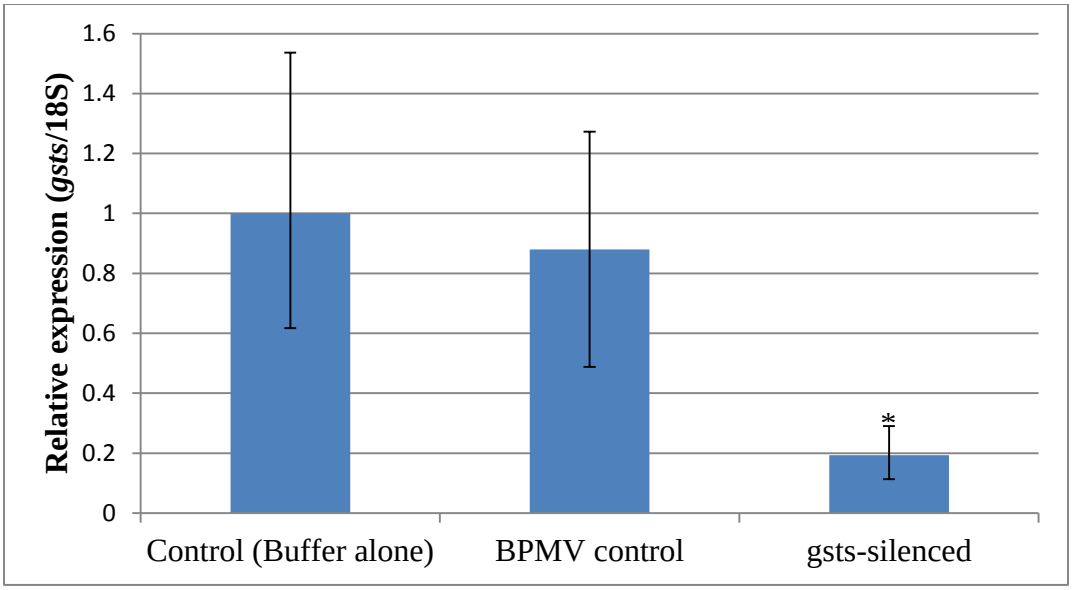

Figure 2.4. Relative expression (*gsts*/18S) of a glutathione S-transferase gene from *Rotylenchulus reniformis* in plants treated with *gsts*-silenced virus construct. The differential gene expression was detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Expression levels of *gsts* gene were normalized by 18S ribosomal gene. The relative expression level was separately computed for each treatment. Three biological replicates and four technical replicates per biological replicate were included per treatment. The data were analyzed by analysis of variance (ANOVA) and relative expression in *gsts*-silenced plants were significantly (*) lower than the other treatments ($P \leq 0.05$). Error bars indicate the standard deviation.

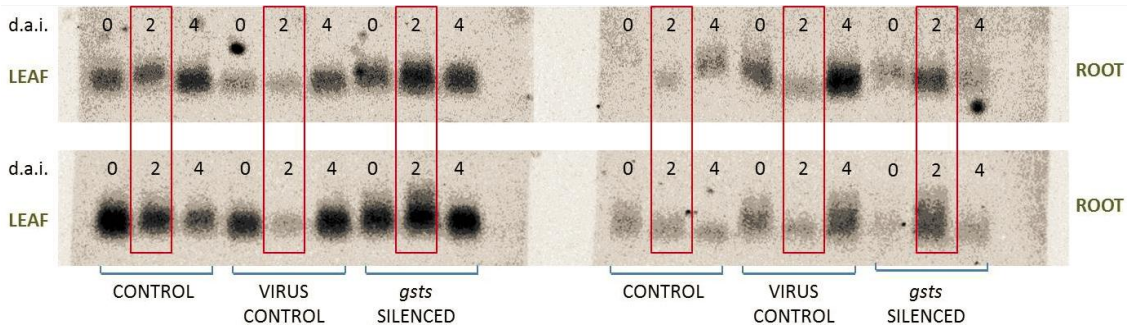

Figure 2.5. Northern blot analysis of ascorbate peroxidase expression in *gsts* virus construct treated plants. RNA was extracted from 0, 2, and 4 days after inoculation (dai) soybean leaves and roots. Ascorbate peroxidase signal in roots and leaves 2 dai *gsts* virus construct treated plants was much stronger than in the controls.

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