The heterologous expression of a Glycine max homolog of NONEXPRESSOR OF PR1 (NPR1) and α-hydroxynitrile glucosidase suppresses parasitism by the root pathogen Meloidogyne incognita in Gossypium hirsutum

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**ABSTRACT**

Experiments in Glycine max (soybean) identified the expression of the salicylic acid signaling and defense gene NONEXPRESSOR OF PR1 (NPR1) in root cells (i.e., syncytium) parasitized by the plant parasitic nematode Heterodera glycines undergoing the process of resistance. Gm-NPR1-2 overexpression in G. max effectively suppresses parasitism by H. glycines. The heterologous expression of Gm-NPR1-2 in Gossypium hirsutum impairs the ability of the parasitic nematode Meloidogyne incognita to form root galls, egg sacs, eggs and second-stage juvenile (J2) nematodes. In related experiments, a G. max β-glycosidase (Gm-BG-4) related to Lotus japonicus secreted defense gene α-hydroxynitrile glucosidase LjBGD7 suppresses M. incognita parasitism. The results identify a cumulative negative effect that the transgenes have on M. incognita parasitism and demonstrate that the G. max–H. glycines pathosystem is a useful tool to identify defense genes that function in other agriculturally relevant plant species to plant parasitic nematodes with different strategies of parasitism.

**KEYWORDS**

α-Hydroxynitrile glucosidase; glucoside; NONEXPRESSOR OF PR1; nematode; soybean; cotton; cytochrome P450

**INTRODUCTION**

Salicylic acid (SA) is an important hormone that is sensitive to increased environmental temperature, but functions effectively in plant defense to pathogens (Malamy et al. 1992; Cao et al. 1994). The genetic pathway for SA signaling has been determined in the plant genetic model Arabidopsis thaliana. In this genetic pathway, the lipase ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), which functions upstream of events directly involved in SA synthesis, heterodimerizes with the lipase PHYTOALEXIN DEFICIENT 4 (PAD4) (Zhou et al. 1998; Falk et al. 1999; Feys et al. 2001). SALICYLIC-ACID-INDUCTION DEFICIENT2, a putative chloroplast-localized isochorismate synthase, and its allelic ENHANCED DISEASE SUSCEPTIBILITY16 (EDS16) in conjunction with the multidrug and toxin extrusion efflux transporter EDS5, function downstream to activate SA biosynthesis. These interactions lead to the expression of NONEXPRESSOR OF PR1 (NPR1) (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997; Nawrath & Métraux 1999; Nawrath et al. 2000; Wildermuth et al. 2001). In NPR1-dependent SA signaling, SA binds to NPR1, stimulating its movement to the nucleus where it dimerizes with the TGA2 transcription factor in the presence of copper ions (Kinkema et al. 2000; Fan & Dong 2002). The complex then binds to the as-1 promoter sequence 5′TGACGT3′, driving target gene expression (Fan & Dong 2002). An identified downstream transcriptional target gene is the secreted protein PR-1 (Niggegew et al. 2000). Ultimately, gene expression that results in a successful defense response occurs.

Microarray and RNAseq experiments have identified the expression of Glycine max homologs of EDS1 and NPR1 in RNA samples isolated from parasitized root cells called syncytia produced by its major pathogen, the parasitic nematode Heterodera glycines, undergoing the natural process of resistance (Klink, Hosseini et al. 2010; Klink, Overall et al. 2010; Matsye et al. 2011). The Gm-EDS1 transcript has been observed to represent 0.03675 of the RNAseq tags identified in parasitized cells undergoing the process of resistance at 9 days post infection (dpi) (Matsye et al. 2011). In other works, both Gm-EDS1 and Gm-NPR1 have been observed to be expressed at statistically significant levels in syncytia undergoing the process of resistance at 3 and 6 dpi (Klink, Hosseini et al. 2010; Klink, Overall et al. 2010). To examine...
their potential role in defense, functional experiments have then been performed, demonstrating Gm-EDS1 and Gm-NPR1 function during resistance in G. max to H. glycines parasitism (Pant et al. 2014). These results have been reinforced further by related experiments showing the heterologous expression of A. thaliana TGA2, PAD4 and NPR1 in G. max results in defense to H. glycines and the root knot parasitic nematode Meloidogyne incognita (Youssf et al. 2013; Matthews et al. 2014). Reciprocal experiments examining whether the G. max SA signaling genes Gm-TGA2, PAD4 and NPR1 function to suppress nematode parasitism in other plant systems or whether Gm-TGA2 and Gm-PAD4 suppress nematode parasitism in G. max were not examined (Youssf et al. 2013; Matthews et al. 2014).

The knowledge that A. thaliana NPR1 functions to drive the expression of the secreted protein gene PRI indicates that a functional secretion system is important to the defense of plants to parasitic nematodes. Such a role for the plant secretion system has been determined in the G. max – H. glycines pathosystem with the identification of alpha-soluble N-ethylmaleimide-sensitive factor attachment protein (α-SNAP) composing part of the major H. glycines resistance locus rhg1 in G. max (Matsye et al. 2011, 2012). These results were confirmed by the observation that overexpression of the α-SNAP interacting protein, syntaxin 31 (Gm-SYP38), known to function at the cis face of the Golgi apparatus, functions effectively to impair H. glycines parasitism (Banfield et al. 1995; Lupashin et al. 1997; Leyman et al. 1999; Collins et al. 2003; Peng & Gallwitz 2004; Bubeck et al. 2008; Pant et al. 2014; Pant, Krishnavajhal et al. 2015). In contrast, suppressed Gm-SYP38 expression in the H. glycines-resistant genotype G. max[Peking/PI 548402] results in the normally resistant genotype becoming susceptible to the parasite (Pant et al. 2014).

Secreted proteins that function in plant defense to various shoot pathogens include β-glycosidases. β-Glycosidases activate β-glycosides, secondary metabolites that perform a wide array of cellular functions including the induction of symbiosis, breakdown of endosperm cell wall during germination, phytohormone activation, lignification, the production of aromatic compounds and defense (Nisius 1988; Poulton 1990; Dharmawardhana et al. 1995; Leah et al. 1995; Hungria & Stacey 1997; Kristoffersen et al. 2000; Mizutani et al. 2002; Morant et al. 2003; Fia et al. 2005; Maicas & Mateo 2005; Escamilla-Trevino et al. 2006; Halkier & Gershonson 2006; Lee et al. 2006; Suzuki et al. 2006; Naoumkina et al. 2007; Stauber et al. 2012). Some of the defense β-glycosides include α-hydroxyconitriile glucosides, benzoazinoid glycosides, avenacosides, isoflavonoid glycosides and glucosinolates (Niemeyer 1988; Nisius 1988; Poulton 1990; Cairns et al. 2000; Halkier & Gershonson, 2006). Among these, the α-hydroxyconitriile glucosides are the best characterized, found in phylogenetically diverse taxa including ferns, gymnosperms, monocots, dicots and many agriculturally relevant plants and spanning more than 3000 plant species (Seigler & Brinker 1993; Jones 1998; Bak et al. 2006; reviewed in Gledow & Moller 2014). α-Hydroxyconitriile glucosides accumulate in the vacuole while their hydrolyzing α-hydroxyconitriile glucosidases contain signal peptides allowing them to enter the vesicle transport system (Saunders et al. 1977; Saunders & Conn, 1978). This process ensures that α-hydroxyconitriile glucosides are physically separated from their cognate α-hydroxyconitriile glucoside until they are activated during pathogen attack.

In the analysis presented here, roots that have been shown to express a G. max NPR1 homolog (Gm-NPR1-2) in Gossypium hirsutum (Pant, McNeece 2015) have been infected by M. incognita. The expression of Gm-NPR1-2 in G. hirsutum results in the suppression of the formation of galls, the production of egg sacs, the production of eggs and the development of J2 nematodes as compared to controls lacking the transgene. During the course of these experiments, a G. max β-glycosidase (Gm-β-g) has been identified as being expressed in G. max roots overexpressing its Gm-NPR1-2 gene, indicating that it performs a defense function during parasitism by H. glycines. The heterologous expression of Gm-β-g in G. hirsutum roots is shown to suppress gall formation, egg sac production, egg production and the development of J2 nematodes as compared to controls lacking the transgene. The experiments show that genes functioning in defense in the G. max – H. glycines pathosystem function effectively to control M. incognita parasitism in G. hirsutum. Furthermore, there appears to be a cumulative negative effect on the different stages of the M. incognita life cycle which is a characteristic of β-glycosidases that function in defense in other plant–pathogen systems (Zagrobelny et al. 2008).

**Materials and methods**

**Selection of candidate genes**

The selection of candidate genes to be expressed heterologously in G. hirsutum to affect the defense response has been aided by mining data from published gene expression experiments performed in G. max that resulted in its resistance to H. glycines (Klink, Hosseini et al. 2010; Klink, Overall et al. 2010; Matsye et al. 2011). These experiments prove that the method works to identify candidate genes that function in G. max defense to H. glycines parasitism (Matsye et al. 2011; Matthews et al. 2013, 2014; Pant et al. 2014, Pant, Krishnavajhal et al. 2015). In brief, G. max[Peking/PI 548402] and G. max[PI 887788] are infected with H. glycines[Peking/PI 548402] results in a resistant reaction as proven histologically in unengineered roots which is the natural resistance response found in these G. max genotypes (Ross 1958; Endo 1965, 1991; Klink et al. 2007, 2009, 2011; Klink, Hosseini et al. 2010; Klink, Overall et al. 2010). Roots are processed for histology and laser microdissection (LM), a procedure that has been used to collect syncytia undergoing the defense response (Klink et al. 2005, 2007, 2009, 2011; Klink, Hosseini et al. 2010; Klink, Overall et al. 2010). These procedures allow for the isolation of mRNA that then is converted to probe for hybridization onto the Affymetrix® Soybean GeneChip® (Klink et al. 2007, 2009, 2011; Klink, Hosseini et al. 2010; Klink, Overall et al. 2010; Matsye et al. 2011). The hybridizations are run in triplicate (arrays 1–3) using a probe derived from RNA isolated from LM-collected syncytia obtained from three independent replicate experiments each run independently in the two different H. glycines-resistant genotypes (Klink et al. 2007, 2009, 2011; Klink, Hosseini et al. 2010; Klink, Overall et al. 2010). For the gene to be considered expressed at a given time point in the analysis presented here, probe signal is measurable above threshold on at least half of the arrays (three of six total arrays), combining both G. max[Peking/PI 548402] and G. max[PI 887788] (six total arrays), p < .05 (Klink et al. 2007, 2009, 2011; Klink, Hosseini et al. 2010; Klink, Overall et al. 2010). The original analysis
procedure is performed as follows: the measurement for a particular probe set (gene) transcript on a single array is determined using the Bioconductor implementation of the standard Affymetrix® detection call methodology (DCM) (Klink et al. 2007, 2009, 2011; Klink, Hosseini et al. 2010; Klink, Overall et al. 2010). DCM consists of four steps, including (1) removal of saturated probes, (2) calculation of discrimination scores, (3) p-value calculation using Wilcoxon’s rank test and (4) making the detection call (present [P]/marginal [M]/absent [A]). Ultimately, the algorithm determines if the presence of a gene transcript is provably different from zero (P), uncertain or marginal (M), or not provably different from zero or absent (A) (Klink et al. 2007, 2009, 2011; Klink, Hosseini et al. 2010; Klink, Overall et al. 2010; Matsye et al. 2011).

**RNA sequencing**

These procedures have been described in Pant, Krishnavajhala et al. (2015). In brief, *G. max* RNA is extracted from roots using the UltraClean® Plant RNA Isolation Kit (Mo Bio Laboratories®, Inc.; Carlsbad, CA) and treated with DNase I to remove genomic DNA (Matsye et al. 2012; Pant et al. 2014). The RNAseq procedures (Illumina® HighSeq 2500® platform; Eurofins MWG Operon; Huntsville, AL) identify transcript (tag) counts and chromosomal coordinates of the *G. max* genome (Schmutz et al. 2010) along with the associated gene ontology annotations (Harris et al. 2004) and are outlined here. The qualities of raw reads are checked using the program FASTQC. The genome sequence and associated gene ontology annotations (Harris et al. 2004) are used unless specified. Briefly, the raw reads for each sample are mapped on *G. max* genome using TopHat v2.0.6 (Trapnell et al. 2009), followed by Cufflinks v2.0.2 (Trapnell et al. 2010) to assemble the mapped reads into transcripts. The fragments per kilobase of exon per million fragments mapped (FPKM) values are calculated for all genes in all samples (Trapnell et al. 2010).

**Gene constructs and genetic transformations**

The cloning of Gm-NPR1-2 and Gm-ßg-4 has been described (Pant et al. 2014). Overexpression studies are performed using the pRAP15 vector transformation system (Matsye et al. 2012; Pant et al. 2014; Pant, McNeece et al. 2015). The transgenic roots are produced by using Agrobacterium rhizogenes strain 15834 (Pant, McNeece et al. 2015). The transgenic roots are produced by using the pRAP15 vector transformation system (Pant et al. 2014). Amplicons are ligated into the pRAP15 destination vector using LR Clonase® (Invitrogen®). The pRAP15 control and engineered vector are used to transform chemically competent *A. rhizogenes* (Hofgen & Willmitzer 1988; Haas et al. 1995) on tetracycline (5 µg/ml) according to Matsye et al. (2012). Genetic transformation experiments resulting in heterologous gene expression in *G. hirsutum* are performed according to Pant, McNeece et al. (2015). RNA is extracted from *G. hirsutum* roots using the UltraClean® Plant RNA Isolation Kit (Mo Bio Laboratories®, Inc.; Carlsbad, CA) and treated with DNase I according to the manufacturer’s instructions (Invitrogen®). The cDNA is synthesized from RNA using the SuperScript First Strand Synthesis System for real time-polymerase chain reaction (RT-PCR) (Invitrogen®) with oligo d(T) as the primer (Invitrogen®) according to the manufacturer’s instructions. Transformation of *G. hirsutum* is carried out by using the chemically competent *A. rhizogenes* (Pant, McNeece et al. 2015). Chemical selection of *A. rhizogenes* is carried out on LB-tetracycline (5 µg/ml) plates (Pant, McNeece et al. 2015). A PCR reaction using pRAP15 primers that amplify the 717 bp eGFP and the 690 bp *A. rhizogenes* root-inducing (Ri) plasmid (EU186381) VirG gene (VirG) confirms that *A. rhizogenes* contains both plasmids prior to *G. hirsutum* transformation (Supplemental Table 1). The presence of the engineered amplicons in pRAP15 is confirmed by PCR using primers for the respective genes and DNA sequencing (Pant et al. 2014).

**A. rhizogenes-mediated transformation of *G. hirsutum***

The procedure and plants generated in Pant, McNeece et al. (2015) for the non-axenic transformation of *G. hirsutum* has been used in the analysis presented here. Seeds of *G. hirsutum* are planted in pre-wetted sterilized sand, germinated and grown for 14 days at ambient greenhouse temperatures (∼26–29°C). The plants are cut at the hypocotyl with a freshly unwrapped, clean and sterile scalpel in a Petri plate containing a 10-ml pool of the pRAP15-transformed *A. rhizogenes*. The procedure ensures that infection by *A. rhizogenes* occurs at the exact moment the plant root is cut. The rootless plants (∼25 plants per beaker) are placed in 400 ml beakers containing *A. rhizogenes* cultured in Murashige and Skoog (MS) media (1962), including vitamins (Duchefa Biochemie, The Netherlands) and 3.0% sucrose, pH 5.7 (MS media). Only the bottom 0.5 cm of the hypocotyl end of the plantlet is submerged in the MS. No chemical selection is performed during the cocultivation. *G. hirsutum* undergoes vacuum infiltration for 30 min. The vacuum then is released slowly, allowing the *A. rhizogenes* suspension to infiltrate the tissue. Cocultivation is performed overnight in MS media in the 400-ml beaker on a rotary shaker at 28°C without chemical selection. After an overnight cocultivation, the cut ends of
G. hirsutum are placed individually 2–4 cm deep into fresh, coarse, non-sterilized, vermiculite (Palmetto Vermiculite Co., Woodruff, SC) in 50-cell flats. The 50-cell flat containing the G. hirsutum plantlets are placed in a covered 32 quart Sterlite® Clearview Latch Box®. The plants then are covered and grown at a distance of 20 cm from standard fluorescent cool white 4100 K, 32-watt bulbs emitting 2800 lumens (Sylvania*, Danvers, MA) for 14 days at ambient lab temperatures (~22°C). The plants are subsequently uncovered and transferred to the greenhouse. Genetically engineered roots are identified by carefully dislodging the plant and root ball from its pot and inspecting them for the expression of EGFP using the Dark Reader Spot Lamp (Clare Chemical Research, Dolores, CO). The remaining vermiculite is removed from these plants by washing the root ball in distilled, deionized water in a 1000-ml plastic beaker. The easily identified untransformed roots, evident by lacking fluorescence, are excised from the plants. The resulting chimeric plants are genetic mosaics (having transformed roots and untransformed aerial stocks). The chimeras are planted in a sterilized 50–50 mixture of a Freestone fine sandy loam (46.25% sand, 46.50% silt and 7.25% clay) and a sandy (93.00% sand, 5.75% silt and 1.25% clay) soil and allowed to recover for two weeks prior to experiments.

The infection of G. hirsutum by M. incognita

Procedures involving M. incognita infection of G. hirsutum are performed according to Diez et al. (2003). The M. incognita race 3 population originally growing at Humphrey County, Mississippi, on G. hirsutum has been isolated from egg masses. M. incognita are bulked up under ambient greenhouse conditions. M. incognita race 3 population is confirmed by the North Carolina differential host test (Myers 1990). M. incognita are extracted from greenhouse cultures by gravity screening and centrifugal flotation (sucrose sp gr = 1.13) (Jenkins 1964). M. incognita eggs and J2 are extracted from Lycopersicum esculentum (tomato) by a 4-min root immersion in 0.525% NaOCl (Hussey & Barker 1973). The solution is subsequently poured through a 75-μm pore sieve nested over a 28-μm-pore sieve. The collected eggs, present on the 28-μm-pore sieve, are placed in water maintained at 28 ± 1°C for 3 days. The J2s hatch and are collected in a 28-μm-pore sieve at 24-h interval over a period of 3 days. M. incognita J2s are maintained in water at 4 ± 1°C until inoculation (Tang et al. 1994). The quantity of inoculum is calculated in a Petri dish with an enumeration grid using a stereomicroscope. At the end of the experiment, M. incognita are extracted from the soil as described previously. M. incognita populations are determined, calculating number of galls, egg masses, eggs and J2. Further determination of life cycle stages is accomplished using a modified acid-fuchsin staining-destaining procedure (Byrd et al. 1983). M. incognita life-stage development has been described using a modified Christie’s method (Christie 1946; Christie & Cobb 1946; Tang et al. 1994). Parasitism has been assayed at four different points in their life cycle by calculating gall number, egg mass number, egg number and number of juveniles.

Quantitative PCR

Primers used in quantitative polymerase chain reaction (qPCR) gene expression experiments are provided in Supplemental Table 1. The G. hirsutum S21 primers have been designed from Gossypium raimondii S21 (Gorai.009G233700.1) and serve as a control (Pant, McNeece et al. 2015). The qPCR experiments use Taqman® 6-carboxyfluorescein (6-FAM) probes and Black Hole Quencher (BHQ1) (MWG Operon; Birmingham, AL). The qPCR differential expression tests are performed using RNA samples isolated from three independent replicates. The qPCR reaction conditions include a 20-μl Taqman Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 0.9 µl of 100 µM forward primer, 0.9 µl of 100 µM reverse primer, 2 µl of 2.5 µM 6-FAM (MWG Operon®) probe and 9.0 µl of template DNA. The qPCR reactions are performed on an ABI 7300 (Applied Biosystems®). The qPCR conditions include a pre-incubation of 50°C for 2 min, followed by 95°C for 10 min. This step is followed by alternating 95°C for 15 s followed by 60°C for 1 min for 40 cycles. The statistical analysis using 2-ΔΔCt to calculate fold change is followed according to the derived formula presented in Livak and Schmittgen (2001).

Microscopy

Stereoscope images are obtained on a Wild Heerbrugg stereoscope. The lenses are Wild Heerbrugg Makrozoom 1:5 having a 6.3–32× scale. Image capture is carried out using the IMT i-solution computer package (IMT i-Solution Inc., Ho Chi Minh City, Vietnam).

Results

Heterologously expressed Gm-NPR1-2 activates endogenous defense gene transcription

Gm-NPR1-2 and its upstream activator Gm-EDS1-2 are expressed in G. max roots undergoing the process of resistance (Supplemental Tables 2 and 3). G. max roots overexpressing Gm-NPR1-2 results in resistance to H. glycines parasitism, indicating that it could be expressed heterologously in G. hirsutum to affect the defense response to M. incognita (Pant et al. 2014). A candidate gene screen examining the expression of G. max defense genes occurring during its resistance to H. glycines has led to the identification of a 525 amino acid (aa) signal peptide-containing G. max β-glycosidase (Gm-βg-4 [Glyma11g13810]), which is also expressed in G. max roots undergoing the process of resistance (Supplemental Tables 2 and 3). The Gm-βg-4 gene is induced by 1.59-fold in G. max roots genetically engineered to overexpress NPR1-2 (Table 1). The demonstrated roles for β-glucosidases in plant defense have led to the experiments presented here in G. hirsutum (reviewed in Gleadow & Moller, 2014).

Table 1. Gm-βg-4 gene expression is induced in G. max roots overexpressing (OE) Gm-NPR1-2. qPCR primers

| Gene        | qPCR primers | Gm-NPR1-2-OE | βg-4 |
|-------------|--------------|--------------|------|
| NPR1-2      | 2.07*        | 1.59         |      |

*The expression of NPR1-2 in Gm-NPR1-2-OE roots was presented in Pant et al. (2014).
Confirming heterologous expression of analyzed transgenes in *G. hirsutum*

Prior gene expression experiments demonstrate that the heterologous expression of Gm-NPR1-2 in *G. hirsutum* can be achieved (Pant, McNeece et al. 2015). Those *G. hirsutum* roots heterologously expressing Gm-NPR1-2 are compared here with roots heterologously expressing Gm-βg-4 and control roots lacking the expression of either transgene (Figure 1). While the experimental procedure presented here in *G. hirsutum* is complicated by the heterologously expressed *G. max* gene not being present in the control roots, experiments are presented here confirming the heterologous expression of Gm-βg-4 in *G. hirsutum* (Figure 2). *G. hirsutum* plants heterologously expressing Gm-NPR1-2 or Gm-βg-4 have then been examined to determine their effect on *M. incognita* parasitism, analyzing gall formation, egg mass number, egg count and number of juveniles (Figure 2).

Analysis of the effect of heterologous expression of Gm-NPR1-2 on *M. incognita* parasitism

An examination of the effect of the heterologous expression of Gm-NPR1-2 in *G. hirsutum* on *M. incognita* parasitism has been performed (Figure 3). The impact that Gm-NPR1-2 expression has on *M. incognita* parasitism is presented in

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**Figure 1.** The qPCR procedure is used to determine the expression of the transgene in RNA extracted from transgenic *G. hirsutum* roots. Left panel, roots transformed with the empty pRAP15 vector. Right panel, roots transformed with Gm-βg-4.

**Figure 2.** Genetically engineered *G. hirsutum* roots. (a) pRAP15 overexpression (OE) control lacking the Gm-NPR1-2 or Gm-βg-4 genes. (b) Gm-NPR1-2-OE. (c) Gm-βg-4-OE. Bar = 1 cm.
two different ways. Firstly, these data are presented in relation to \textit{M. incognita} parasitism in the whole root mass. However, to account for potential variations caused by differences in the mass of the root, analyses on gall formation, egg mass number, egg count and number of J2s have also been calculated per gram of root tissue (Figure 4, Supplemental Table 4). The percent decrease for each of the three replicates is presented (Table 3). It is noted that there appears to be an accumulative negative effect occurring during the course of the \textit{M. incognita} life cycle as revealed in the low levels of J2s (Table 3).

An examination of the effect of Gm-βg-4 expression in \textit{G. hirsutum} on \textit{M. incognita} parasitism has been performed (Figure 5). The analysis examines its effect on gall formation, egg mass number, egg count and number of J2s in relation to the whole root mass and per gram of root tissue (Figure 6; Supplemental Table 5). The percent decrease for each of the three replicates is presented (Table 3). It is noted that there appears to be an accumulative negative effect occurring during the course of the \textit{M. incognita} life cycle as revealed in the low levels of J2s (Table 3).

Discussion

A number of plant pathogenic nematodes that infect \textit{G. max} are capable of parasitizing other plant species. Among them is \textit{M. incognita} which is able to successfully parasitize \textit{G. hirsutum}. The \textit{M. incognita} life cycle is composed of six stages including the egg that is encased within an egg mass, four juvenile stages and the adult. During infection, the \textit{M. incognita} J2s burrow into roots and establish a nurse cell called a giant cell from which they feed. However, unlike syncytia formed by plant parasitic nematodes such as \textit{H. glycines}, the growth and development of the giant cell results in the formation of swollen root regions around the giant cells leading to the formation of galls. This characteristic of infection by \textit{M. incognita} presents advantages for studying their pathology since the parasitized root regions are easily identifiable without the aid of histological procedures. \textit{M. incognita} presents other advantages for the study of resistance to root parasitic nematodes. For example, plants parasitized by \textit{M. incognita} allow for an analysis of the effect that the expression of gene cassettes have at different stages of the nematode life cycle and their influence on giant cell development through the examination of gall formation. Therefore, any cumulative negative effect exerted by the plant on nematode fitness can be determined since multiple stages in its life cycle can be easily quantified. In the analysis described here, results are presented showing the effect that the heterologous expression of Gm-NPR1-2 or Gm-βg-4 has on four sequential processes associated with \textit{M. incognita} development including gall formation, egg mass formation, egg production and J2s that have hatched.

Heterologously expressed \textit{G. max} NPR1 in \textit{G. hirsutum} effectively impairs \textit{M. incognita} fitness

The effective defense response that has been obtained in \textit{G. hirsutum} by the heterologous expression of Gm-NPR1-2 is consistent with the observations, showing that its expression is important to the defense response of \textit{G. max} to \textit{H. glycines} (Pant et al. 2014). These results are consistent with observations showing that the heterologous expression of the \textit{A. thaliana} SA signaling genes \textit{PAD4}, \textit{TGA2} and \textit{NPR1} suppresses \textit{M. incognita} development in \textit{G. max}. Therefore, the results presented here confirm the
importance of the SA signaling pathway in defense to parasitic nematodes (Youssef et al. 2013; Pant et al. 2014; Matthews et al. 2014).

Gm-β-glucosidase is part of a conserved cellular process that functions in defense. The results presented here show the heterologous expression of Gm-β-g4, having characteristics of a secreted protein, functions effectively in G. hirsutum to suppress parasitism by M. incognita. The results confirm prior observations of the importance of the plant secretion system playing a major role in the defense of plants to parasitic nematodes in general (Matsye et al. 2011, 2012; Pant et al. 2014, Pant, Krishnavajhala et al. 2015). For example, earlier observations have been made in G. max in an examination of its syntaxin 31 homolog Gm-SYP38 (Pant et al. 2014) and earlier analyses of the alpha-soluble N-ethylmaleimide-sensitive fusion protein (α-SNAP) (Matsye et al. 2011, 2012).

The results indicate that the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) will

**Figure 4.** G. hirsutum roots genetically engineered to overexpress Gm-NPR1-2-OE affect M. incognita parasitism. (*) denotes statistically significant p < .05. Values used in the analysis are provided in Supplemental Table 4.

**Table 2.** The expression of Gm-NPR1 in G. hirsutum has a cumulative negative effect on M. incognita parasitism during successive stages of its life cycle.

|                     | Replicate 1 | Replicate 2 | Replicate 3 |
|---------------------|-------------|-------------|-------------|
| Control-wr          | 100         | 100         | 100         |
| Control-pg          | 100         | 100         | 100         |
| Galls-wr            | 29.2        | 30.43       | 39.8        |
| Galls-pg            | 23.25       | 47.17       | 31.54       |
| Egg masses-wr       | 20.46       | 47.15       | 30.53       |
| Egg masses-pg       | 15.55       | 43.96       | 35.95       |
| Eggs-wr             | 20.57       | 38.28       | 28.08       |
| Eggs-pg             | 15.7        | 23.62       | 28.02       |
| J2-wr               | 9.9         | 15.61       | 5.17        |
| J2-pg               | 10.62       | 21.12       | 2.51        |

Note: Data are presented as a percent, compared to the control from analyses of M. incognita in whole root (wr) and per gram (pg).

**Gm-β-glucosidase is part of a conserved cellular process that functions in defense**

The results presented here show the heterologous expression of Gm-β-g4, having characteristics of a secreted protein, functions effectively in G. hirsutum to suppress parasitism by M. incognita. The result confirms prior observations of the importance of the plant secretion system playing a major role in the defense of plants to parasitic nematodes in general (Matsye et al. 2011, 2012; Pant et al. 2014, Pant, Krishnavajhala et al. 2015). For example, earlier observations have been made in G. max in an examination of its syntaxin 31 homolog Gm-SYP38 (Pant et al. 2014) and earlier analyses of the alpha-soluble N-ethylmaleimide-sensitive fusion protein-associated protein (α-SNAP) (Matsye et al. 2011, 2012). The results indicate that the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) will
perform a prominent role in defense (reviewed in Jahn and Fasshauer 2012).

Bioinformatics analyses show that Gm-βg-4 is most closely related to the root-specific *Lotus japonicus* α-hydroxynitrile glucosidase LjBGD7, belonging to a small family of enzymes involved in the production of cyanogenic α-hydroxynitrile glucosides (Morant et al. 2008; Takos et al. 2010). In the α-hydroxynitrile glucoside metabolic pathway, active α-hydroxynitrile glucosides are produced through a pathway involving cytochrome p450 79 D4 (CYP79D4) which converts an amino acid to an oxime. A second enzyme, CYP71, converts the oxime to an α-hydroxynitrile. The α-hydroxynitrile is then converted to a cyanogenic monoglucoside by the activity of UDP-glucosyltransferase and can be stored in this form. Subsequent activity by α-hydroxynitrile glucosidase removes the glucose moiety, activating the α-hydroxynitrile. The α-hydroxynitrile is then metabolized by α-hydroxyynitril lyase or experiences a spontaneous event, resulting in the production of hydrogen cyanide (HCN) which is toxic to the pathogen. HCN is later detoxified by β-cyanoalanine synthase (Gleadow & Moller 2014). The effective and conserved function that Gm-βg-4 has on suppressing *M. incognita* parasitism indicates that the cognate α-hydroxynitrile glucoside is also present in *G. hirsutum*. While the *G. hirsutum* genome is not available at this point, comparative analysis of the *G. max* and *G. raimondii* genomes has identified closely related homologs of all components of the α-hydroxynitrile glucoside metabolism. Thus, it is evident that Gm-βg-4 is involved in synthesizing active α-hydroxynitrile glucosides in *G. hirsutum*. If so, our result is similar to those presented by Forslund et al. (2004), demonstrating that the heterologous expression of a *Manihot esculenta* (cassava) CYP79D2 in *L. japonicus* results in the accumulation of the cyanogenic α-hydroxynitrile glucosides lotaustralin and linamarin.

**Gm-NPR1-2 and Gm-βg-4 have a cumulative negative impact on *M. incognita* parasitism**

In the analysis presented here, the percent effect that the heterologous expression of the Gm-NPR1-2 and Gm-βg-4 transgenes has on *M. incognita* parasitism in *G. hirsutum* increases at subsequent stages of its parasitism. This observation indicates a cumulative negative effect caused by expression of these transgenes. This observation is very similar to those observed by Zagrobelny, Bak, Ekstrom et al. (2007), Zagrobelny, Bak, Olsen et al (2007), and Zagrobelny et al. (2008), showing the cumulative negative effect of β-glucosidases at different and subsequent stages of the life cycle in the Lepidopteran *Zygaena filipendulae* grown on *L. japonicus*. Thus, it appears that the heterologous expression of Gm-NPR1-2 and Gm-βg-4 functions in a similar manner producing an effective action that progressively and negatively impacts the *M. incognita* parasitism in *G. hirsutum*. Since the genes function effectively against *H. glycines* parasitism in *G. max*, it appears that they would have a broad spectrum action.

| Table 3. The expression of Gm-βg-4 in *G. hirsutum* has a cumulative negative effect on *M. incognita* parasitism during successive stages of its life cycle. |
|---------------------------------|----------------|---------------|----------------|
| **Galls-wr** | **Eggs-wr** | **Egg masses-wr** | **J2-wr** |
| Control-wr | 100 | 100 | 100 |
| Control-pg | 100 | 100 | 100 |
| Galls-wr | 44.15 | 72.12 | 100 |
| Galls-pg | 34.96 | 69.65 | 8.71 |
| Eggs-wr | 20.17 | 32.68 | 15.63 |
| Eggs-pg | 20.4 | 63.81 | 39.31 |
| Egg masses-wr | 27.8 | 70.78 | 32.68 |
| Egg masses-pg | 15.42 | 25.44 | 21.42 |
| J2-wr | 14.5 | 20.4 | 14.35 |
| J2-pg | 8.71 | 44.15 | 1.99 |

Note: Data are presented as a percent as compared to the control. Data presented as a percent from analyses of *M. incognita* in whole root (wr) and per gram (pg).
The use of chimeric plants in analyzing plant parasitic nematodes

The hairy root system used here has been employed in *G. hirsutum* to analyze plant parasitic nematode fitness in transgenic roots (Triplet et al. 2008; Pant, McNeece et al. 2015). Many plant parasitic nematodes including *H. glycines* and *M. incognita*, among others, are obligate root parasites. The infection of transgenic hairy roots of *G. max* and *G. hirsutum* by *H. glycines* and *M. incognita*, respectively, indicate that these organs have the identity of roots. This characteristic is clear when examining pathogenicity, revealing levels of parasitism in genetically engineered control plants lacking the transgene that are similar to unengineered control plants. Furthermore, histological observation demonstrates that the development of *H. glycines*-induced syncytia or *M. incognita*-induced giant cells is similar in genetically engineered control plants lacking the transgene and unengineered control plants. These observations are consistent with the presence of root hairs and other morphological and anatomical features of roots in the hairy root organs, indicating that these structures are roots and function as roots (Tepfer et al. 1984). The plants produced by this experimental procedure, however, are genetic chimeras that have transgenic roots and nontransgenic shoots (Collier et al. 2005; Klink et al. 2009; Matsye et al. 2012; Pant, McNeece et al. 2015). The hairy root method is rapid, allowing for the production of libraries of genes that can rapidly and inexpensively be tested (Matthews et al. 2013). Therefore, the method allows for the testing of genes in plants amenable to the procedure that are not tractable genetically. Genes that function effectively in defense then can be targeted in genetic screens or engineered in through homologous recombination or other screening procedures (Jinek et al. 2012).

**Figure 6.** *G. hirsutum* roots genetically engineered to overexpress Gm-βg-4-OE affect *M. incognita* parasitism. (*) denotes statistically significant *p* < .05. Values used in the analysis are provided in Supplemental Table 5.
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Disclosure statement

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References

Bak S, Paquette S, Morant M, Morant AV, Saito S, Bjarnholt N, Gronberg M, Jørgensen K, Osmanski S, Simonsen HT, et al. 2006. Cytocinogenses: a case study for evolution and application of cytochromes P450. Phytochem Rev. 5:309–329.

Banfield DK, Lewis MJ, Pelham HR. 1995. A SNARE-like protein required for traffic through the Golgi complex. Nature. 375:806–809.

Bubek J, Scheuring D, Hummel E, Langhans M, Viotti C, Foresti O, Denecke J, Banfield DK, Robinson DG. 2008. The syntaxins SYP31 and SYP18 control ER-Golgi trafficking in the plant secretory pathway. Traffic. 9:1629–1652.

Byrd DW Jr., Kirkpatrick T, Barker KR. 1983. An improved technique for clearing and staining plant tissue for detection of nematodes. J Nematol. 15:142–143.

Cairns JRK, Champattanachai V, Srisomsap C, Wittman-Liebold B, Thiede B, Svatij V. 2000. Sequence and expression of Thai rosewood beta-glucosidase/beta-fucosidase, a family 1 glycosyl hydrolase glycoprotein. J Biochem. 128:999–1008.

Cao H, Bowling SA, Gordon AS, Dong X. 1994. Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell. 6:1583–1592.

Christie JR. 1946. Host-parasite relationships of the root-knot nematode, Heteroder a marioni. II. Some effects of the host on the parasite. Phytopathology. 36:340–352.

Christie JR, Cobb GS. 1946. Notes on the life history of the root nematode, Heteroder a marioni. Proc Helminthol Soc Washington. 8:23–26.

Collier R, Fuchs B, Walter N, Luette KW, Taylor CG. 2005. Ex vitro composite plants: an inexpensive, rapid method for root biology. Plant J. 43:449–457.

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrik E, Qiu JL, Hückelhoven R, Stein M, Frei ldenhoven A, Somerville SC, Schulze-Lefert P. 2003. SNARE-protein mediated disease resistance at the plant cell wall. Nature. 425:973–977.

Delaney TP, Friedrich L, Rylas JA. 1995. Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc Natl Acad Sci USA. 92:6062–6066.

Dharmawardhana DP, Ellis BE, Carlso n JE. 1995. A beta-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferyl. Plant Physiol. 107:331–339.

Diez A, Lawrence GW, Lawrence KS. 2003. Competition of Meloidogyne incognit a and Rotylenchulus reniformis on cotton following separate and concomitant inoculations. J. Nematol. 35:422–429.

Endo BY. 1965. Histological responses of resistant and susceptible soybean varieties, and backcross progeny to entry development of Heterodera glycines. Phytopathology. 55:375–381.

Endo BY. 1991. Ultrastructure of initial responses of susceptible and resistant soybean roots to infection by Heterodera glycines. Revue Nematol. 14:73–84.

Ecsacki-Trevino LL, Chen W, Card ML, Shi h MC, Cheng CL, Poulton JE. 2008. Arabidopsis thaliana beta-glucosidases BGLU45 and BGLU46 hydrolyse monoglucosyl glycosides. Phytochemistry. 67:1651–1660.

Falk A, Feys BJ, Frost LN, Jones JDG, Daniels MJ, Parker JE. 1999. EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proc Natl Acad Sci USA. 96:3292–3297.

Fan W, Dong X. 2002. In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell. 14:1377–1389.

Feys BJ, Moisan LJ, Newman MA, Parker JE. 2001. Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO J. 20:5400–5411.

Fia G, Giovanni G, Rosti I. 2005. Study of beta-glucosidase production by wine-related yeasts during alcoholic fermentation: a new rapid fluorimetric method to determine enzymatic activity. J Appl Microbiol. 99:509–517.

Fordlud K, Morant M, Jørgensen B, Olsen CE, Asamizu E, Sato S, Tabata S, Bak S. 2004. Biosynthesis of the nitrile glucosides rhodocyan oside A and D and the cyanogenic glucosides lotaustralin and linamarin in Lotus japonicus. Plant Physiol. 135:71–84.

Glazebrook J, Rogers EE, Ausubel FM. 1996. Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics. 143:973–998.

Gleadow RM, Moller BL. 2014. Cyanogenic glucosides: synthesis, physiology, and phenotypic plasticity. Annu Rev Plant Biol. 65:155–185.

Haas JL, Moore LW, Beam W, Manulla S. 1995. Universal PCR primers for detection of phytopathogenic Agrobacterium strains. Appl Environ Microbiol. 61:2879–2884.

Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, Foulger R, Elbeck K, Lewis S, Marshall B, Mungall C, et al. 2004. Nucleic Acids Res. 32:D258–D261.

Halkier BA, Gershenzon J. 2006. Biology and biochemistry of glucosinolates. Annu Rev Plant Biol. 57:303–333.

Haus H, Willmitzer L. 1988. Storage of competent cells for Agrobacterium transformation. Nucleic Acids Res. 16:9877–9877.

Hofgen R, Willmitzer L. 1988. Storage of competent cells for Agrobacterium transformation. Nucleic Acids Res. 16:9877–9877.

Huston RS, Barker KR. 1973. A comparison of methods of collecting inocula of Meloidogyne eggs, including a new technique. Plant Dis Rep. 57:1025–1028.

Jahn R, Faaberg H. 1999. Molecular machines governing exocytosis of synaptic vesicles. Nature. 490:201–207.

Klink VP, Alkharouf NW, MacDonald M, Matthews BF. 2005. Laser capillary electrophoresis for the lignin precursor coniferin. Phytochemistry. 67:1651–1660.

Klink VP, Hosseini P, MacDonald M, Matthews BF. 2009. A nuclear localization of NPR1 is required for activation of PR gene expression. Plant Cell. 20:1025–1028.

Jenkins WR. 1964. A rapid centrifugal flotation technique for separating nematodes from soil. Plant Dis Rep. 48:692–693.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in eukaryotic cells. Science. 337:1896–1903.

Jones DA. 1998. Why are so many food plants cyrogenic? Phytochemistry. 47:155–162.

Klink VP, Alkharouf NW, Matthews BF. 2009. A gene expression analysis of syncytia laser microdissected from the roots of the Glycine max (soybean) genotype PI 584902 (Peking) undergoing a resistant reaction after infection by Heterodera glycines (soybean cyst nematode). Plant Mol. Biol. 71:525–567.

Klink VP, Hosseini P, MacDonald M. 2010. Shoot cell gene expression in Glycine max[PI 88788] roots

"
undergoing a resistant reaction to the parasitic nematode *Heterodera glycines*. Plant Physiol Bioch. 48:176–193.

Klink VP, Hosseini P, Matsye PD, Alikharouf NW, Matthews BF. 2011. Differences in gene expression amplitude over a conserved transcriptomic program occurring between the rapid and potent localized resistant reaction at the syncytium of the *Glycine max* genotype Peking (PI 548402) as compared to the prolonged and potent resistant reaction of PI 88788. Plant Mol. Biol. 75:141–165.

Klink VP, Overall CC, Alikharouf N, MacDonald MH, Matthews BF. 2007. Laser capture microdissection (LCM) and comparative microarray expression analysis of syncytial cells isolated from incompatible and compatible soybean roots infected by soybean cyst nematode (*Heterodera glycines*). Planta. 226:1389–1409.

Klink VP, Overall CC, Alikharouf NW, MacDonald MH, Matthews BF. 2010. Microarray detection calls as a means to compare transcripts expressed within syncytial cells isolated from incompatible and compatible soybean (*Glycine max*) roots infected by the soybean cyst nematode (*Heterodera glycines*). J Biomed Biotechnol. Article ID 491217. doi:10.1155/2010/491217.

Kristoffersen P, Brzobohaty B, Hohfeld I, Bako L, Mellkonian M, Palme K. 2000. Developmental regulation of the maize Zm-g601 gene encoding a beta-glucosidase located to plastids. Planta. 210:407–415.

Leah R, Kigel J, Svendsen I, Mundy J. 1995. Biochemical and molecular characterization of a barley seed beta-glucosidase. J Biol Chem. 270:15789–15797.

Lee KH, Piao HL, Kim HY, Choi SM, Jiang F, Hartung W, Hwang I, Kwik JM, Lee JH, Hwang I. 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. Cell. 126:1109–1120.

Leyman B, Geelen D, Quintero FJ, Blatt MR. 1999. A tobacco syntaxin gene suppresses plant parasitic nematode infection. Plant Mol Biol. 41:283–300.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 25:402–408.

Maicas S, Mateo JJ. 2005. Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. Appl Microbiol Biotechnol. 67:322–335.

Malamy J, Hennig J, Klessig DF. 1992. Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to Tobacco mosaic virus infection. Plant Cell. 4:359–366.

Matsye PD, Kumar R, Hosseini P, Jones CM, Tremblay A, Alikharouf NW, Matthews BF, Klink VP. 2011. Mapping cell fate decisions that occur during soybean defense responses. Plant Mol Biol. 77:513–528.

Matsye PD, Lawrence GW, Youssef RM, Kim K-H, Matthews BF, Lawrence KS, Klink VP. 2012. The expression of a naturally occurring, truncated allele of an α-SNAP gene suppresses plant parasitic nematode infection. Plant Mol Biol. 80:131–155.

Matthews BF, Beard H, Brewer E, Kabir S, MacDonald MH, Youssef RM. 2014. Arabidopsis genes, AtNPRI, AtTGA2 and AtT-P5, confer partial resistance to soybean cyst nematode (*Heterodera glycines*) when overexpressed in transgenic soybean roots. BMC Plant Biol. 14:96. doi:10.1186/1471-2229-14-96.

Matthews BF, Beard H, MacDonald MH, Kabir S, Youssef RM, Hosseini P. 2013. Expression of an α-SNAP gene suppresses plant parasitic nematode infection. Planta. 201:151–161.

Myers RF. 1990. Identification of species and races of Meloidogyne by differential host assay. In: Zuckerman, B., Mai, W. F. Krusberg, L. R., editors. Plant nematode laboratory manual. Amherst, MA: University of Massachusetts Agricultural Experiment Station. pp. 86–90.

Nawrath C, Métraux JP. 1999. Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell. 11:1393–1404.

Nawrath C, Heck S, Parinthawong M, Métraux J-P. 2000. EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. Plant Cell. 14:275–286.

Niemyer HM. 1988. Hydroxamic acids (4-hydroxy-1,4-benzoazin-3-ones), defense chemicals in the Gramineae. Phytochemistry. 27:3349–3358.

Niggeweg R, Thurov C, Kegler C, Gatz C. 2000. Tobacco transcription factor TGA2.2 is the main component of as-1-binding factor ASF1 and is involved in salicylic acid- and auxin-inducible expression of as-1-containing target promoters. J Biol Chem. 275:19897–19905.

Nirus A. 1988. The stomatometer in Avena plasstis: an aggregation of beta glucosidase responsible for the activation of oat leaf sapinins. Planta. 173:474–481.

Pant SR, Krishnavajhala A, Lawrence GW, Klink VP. 2015. A relationship exists between the cis-Golgi membrane fusion gene syntaxin 31, salicylic acid signal transduction and the GATA-like transcription factor, LESION SIMULATING DISEASE1 (LSD1) in plant defense. Plant Signal Behav. 10(1): e977737.

Pant SR, Matsye PD, McNece BT, Sharma K, Krishnavajhala A, Lawrence GW, Klink VP. 2014. Syntaxin 31 functions in *Glycine max* resistance to the plant parasitic nematode *Heterodera glycines*. Planta Mol Biol. 85:107–121.

Pant SR, McNece BT, Sharma K, Nirula PM, Jiang J, Harris JL, Lawrence GW, Klink VP. 2015. A plant transformation system designed for high throughput genomics in *Gossypium hirsutum* to study root-organism interactions. J Plant Interact. 10(1): 11–20.

Peng R, Gallwitz D. 2004. Multiple SNARE interactions of an SM protein: Sed5p/Sly1p binding is dispensable for transport. Eur Mol Biol Org J. 23:3939–3949.

Poulton JE. 1990. Cyanoangin in plants. Plant Physiol. 94:401–405.

Ross JP. 1958. Host-Parasite relationship of the soybean cyst nematode. Phytopathology. 48:578–579.

Saunders JA, Conn EE. 1978. Presence of cyanogetic glucoside dhurrin in isolated vacuoles from Sorghum. Plant Physiol. 61:154–157.

Saunders JA, Conn EE, Lin CH, Stocking CR. 1977. Subcellular localization of cyanogetic glucoside of Sorghum by autoradiography. Plant Physiol. 59:47–652.

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen J, Cheng J, et al. 2010. Genome sequence of the palaeopolyploid soybean. Nature. 463:178–183.

Seigler DS, Brinker AM. 1993. Characterisation of cyanogetic glucosides, cyanolipids, nitroglycosides, organic nitro compounds and nitrile glucosides from plants. Methods Plant Biochem. 85:131–157.

Shah J, Tsui F, Klessig DF. 1997. Characterization of a salicylic acid insensitive mutant (sai1) of *Arabidopsis thaliana*, identified in a selective screen utilizing the light inducible expression of the tms2 gene. Mol Plant Microbe Interact. 10:69–79.

Stauber EJ, Kuczka P, van Ohlen M, Vogt B, Janowitz T, Piotrowski M, Beurele T, Wittstock U. 2012. Turning the ‘mustard oil bomb’ into a ‘cyanide bomb’: aromatic glucosinolate metabolism in a specialist insect herbivore. PLoS One. 7:e33545. doi:10.1371/journal.pone.0033545.

Suzuki H, Takahashi S, Watanabe R, Fukushima Y, Fujita N, Noguchi A, Kato T, Uno S, Nakayama T, Miki H, Nakamura K. 2006. An isoflavo n conjugate-hydrolyzing beta-glucosidase from the roots of soybean (*Glycine max*) seedlings: purification, gene cloning, phylogenet ic, and cellular localization. J Biol Chem. 281:30251–30259.
Tang B, Lawrence GW, Creech RG, Jenkins JN, McCarty JC Jr. 1994. Post-infection development of Meloidogyne incognita in cotton roots. USDA and Mississippi Agricultural and Forestry Experimental Station Technical Bulletin. No. 195:43.

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.

Trapnell C, Pachter L, Salzberg SL. 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 25:1105–1111.

Trapnell C, Roberts A, Gott L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc. 7:562–578.

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010. Transcript assembly and quantification during cell differentiation. Nat Biotechnol. 28:511–515.

Triplet BA, Moss SC, Bland JM, Dowd MK. 2008. Induction of hairy root cultures from Gossypium hirsutum and Gossypium barbadense to produce gossypol and related compounds. In Vitro Cell Dev Biol Plant. 44:508–517.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defense. Nature. 414:562–565.

Youssef RM, MacDonald MH, Brewer EP, Bauchan GR, Kim K-H, Matthews BF. 2013. Ectopic expression of AtPAD4 broadens resistance of soybean to soybean cyst and root-knot nematodes. BMC Plant Biol. 13:67.

Zagrobelny M, Bak S, Ekstrom CT, Olsen CE, Moller BL. 2007. The cyanogenic glucoside composition of Zygaena filipendulae (Lepidoptera Zygaenidae) as effected by feeding on wild-type and transgenic Lotus populations with variable cyanogenic glucoside profiles. Insect Biochem Mol Biol. 37:10–18.

Zagrobelny M, Bak S, Moller BL. 2008. Cyanogenesis in plants and arthropods. Phytochemistry. 69:1457–1468.

Zagrobelny M, Bak S, Olsen CE, Moller BL. 2007. Intimate roles for cyanogenic glucosides in the life cycle of Zygaena filipendulae (Lepidoptera, Zygaenidae). Insect Biochem Mol Biol. 37:1189–1197.

Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J. 1998. PAD4 functions upstream from salicylic acid to control defense responses in Arabidopsis. Plant Cell. 10:1021–1030.