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Research Article

Analysis of the *Glycine max* role of Syntaxin (SYP22) in resistance to *Rotylenchulus reniformis*

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Abstract
Syntaxin proteins are involved in the process of membrane fusion. *G. max* syntaxin genes (Gm-SYP22-3, and Gm-SYP22-4) that were similar in amino acid composition have been found to contribute to the ability of *Glycine max* to defend itselffrom infection by the plant- parasitic nematode *Rotylenchulus reniformis*. The Gm-SYP22-3and Gm-SYP22-4 genes were expressed in root cells (syncytia) undergoing a resistant reaction while not being expressed in control cells. The Gm-SYP22-3 and Gm-SYP22-4 genes have been isolated from genetically engineered in *G. max* [Williams 82/PI 518671], a genotype typically susceptible to *R. reniformis* parasitism. Genetically engineered plants in *G. max* [Williams 82/PI 518671] that lack the overexpression of Gm-SYP22-3 or Gm-SYP22-4 genes have also been produced to serve as a control. The transgenic Gm-SYP22-3 or Gm-SYP22-4 overexpression lines with their pRAP15 control have then been infected with *R. reniformis*. Infection was allowed to proceed for 30 days. At the end of the 30-day life span, *R. reniformis*stages were extracted from the soil and eggs from the roots, enumerated and compared to control plants. Plants overexpressing Gm-SYP22-3 or Gm-SYP22-4 had suppressed *R. reniformis*. In contrast, the gene expression levels of Gm-SYP22-3 and Gm-SYP22-4 were reduced in transgenic lines engineered for their RNA interference (RNAi) in *G. max* [Peking/PI 548402], a genotype normally resistant to *R. reniformis*. In comparison to genetically engineered control *G. max* [Peking/PI 548402] lines, RNAi of Gm-SYP22-3 or Gm-SYP22-4 resulted in an increase in parasitism in the normally *R. reniformis* resistant *G. max* [Peking/PI 548402].

Keywords: Glycine max. Syntaxin

INTRODUCTION

Glycine max (soybean) is a multifunctional legume that is incorporated in animal feed, human food, and biofuels (Barrett 2006). Furthermore, the health benefits of soybeans include providing essential amino acids, fiber, unsaturated fat, vitamins and minerals (Barrett 2006). In the United States, soybeans are processed to make animal food and domestic products including oils (Dwevedi 2011). In 2014, 315.1 million metric tons of soybeans were produced in the United States, the world leader of soybean production. In the past, half of soybeans produced in the U.S. have been exported to other countries with China accounting for most of these exports. Other countries include India (Barrett 2006; Dwevedi 2011). The reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira) is an economically important nematode parasite of cotton and soybean. In the United States, reniform nematode occur extensively in the southeastern region and is now considered the dominant plant-parasitic nematode species in this region. Currently, the primary means to manage this nematode includes resistant varieties, crop rotation and nematicide applications. With few resistant cultivars to the reniform nematode most of our producers rely on crop rotation and primarily nematicide applications.

The lipid bilayer membrane is a unifying component of cells. In eukaryotes, genes whose protein products function in membrane fusion have been originally identified genetically in *Saccharomyces cerevisiae*(Novick et al. 1980, 1981). The protein apparatus that mediates membrane fusion between vesicle and target is found in all eukaryotes and has a number of functions. In plants, one of the functions is plant defense to pathogens (Collins et al. 2003). In the plant genetic model *Arabidopsis thaliana*, one of these proteins called syntaxin 22 is a component of the endosome or prevacuolar compartment (PVC) (Sanderfoot et al. 2000). The endosome is a membrane delimited structure that forms from materials that are endocytized from the plasma membrane. Materials captured in the endosome may then become targeted for degradation or become targeted back to the *trans*-Golgi network. In *A. thaliana*, syntaxin 22 (SYP22) was first identified in a mutagenic screen and was called AtVAM3. SYP22 is closely related to another syntaxin called SYP23. SYP23 was first identified in a mutant screen and was called AtPLP.

The process of membrane fusion is facilitated by a structure called the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE). During membrane fusion, vesicle and target membrane

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proteins bind while other cytoplasmic proteins provide the energy for membrane fusion to occur (Jahn and Fasshauer 2012). The various cellular compartments contain different SNARE receptor gene family members each having either a very specific function While the genome of *A. thaliana* has 24 syntaxins, in comparison, *G. max* has 54 syntaxins.

Rationale for proposed work

Prior published work has shown the involvement of □-SNAP and SYP31 during the defense process of *G. max* to plant-parasitic nematode parasitism (Matsye et al. 2011, 2012; Pant et al. 2014, 2015). The effective nature of the overexpression of these genes opened questions as to whether other SYP22-3 and SYP22-4 genes also functioned in defense. As is shown, the expression of SYP22 during the resistant reaction made it a reasonable candidate for molecular analyses in examining the process of resistance. In the analysis, *G. max* homologs of SYP22 are analyzed to determine if they perform a role in defense to *R. reniformis*.

MATERIALS AND METHODS

Gene Cloning

BR signaling genes were identified by candidate gene analyses. To accomplish this, *A. thaliana* protein sequences of selected BR signaling genes were identified from Genbank and blasted against the genome of soybean which is housed at phytozome.net. The list of candidate soybean genes then was cross examined against an in-house gene expression database. The in-house database contains gene expression data obtained from Illumina deep sequencing studies of gene expression that happens in soybean as it resists SCN infection. That analysis allowed us to narrow down the number of candidate paralogs (duplicated genes). The cDNA nucleotide sequences were then extracted from Phytozome.net and used to design PCR primers for gene cloning experiments (Table 1).

Selection of candidate genes

PCR generated amplicons using Accuprime® from synthesized cDNA (SuperScript® First Strand Synthesis kit) and ran on a 1% agarose gel. Bands were excised from gel and purified according to protocol (Promega). Amplicon was cloned using pENTR™/D-TOPO® Cloning Kit with TOP10 Chemically competent cells (Life Technologies). Transformed cells were grown overnight (12-16 hours) on kanamycin plates (50ug/ml). Selected colonies were then grown at 37℃ overnight (14-16 hours) in LB-Kanamycin broth. Plasmid extraction was performed according to QIAprep Spin Miniprep Kit protocol. Prior to sequencing, Taq polymerase (Life Technologies) was used to confirm presence of insert in vector. Sequenced results are confirmed of correct insert and void of any mutations. Correct sequencing is then cloned into either the overexpression vector pRAP15 or transcriptional suppression vector pRAP17 (Figure 1). Cloning into these expression vectors is done through recombination with LR clonase II (Invitrogen). Insertion is done at recombination specific sites, attR1 and attR2. Recombination of insert at these sites replaces ccdb gene from the vector. This gene is toxic to the cell and is a mode of selection of insert. The cloning reaction was then transformed into One Shot® TOP10 Chemically Competent *E. coli* and grown on tetracycline plates (5μg/mL) plates overnight. Picked colonies were then grown overnight in LB-tetracycline broth at 37℃ overnight to amplify plasmid. Plasmid purification was done using QIAprep Spin Miniprep Kit protocol. Purified plasmid was then sent for sequencing to confirm correct insertion and was void of any mutations. Expressions vector were then transformed into *Agrobacterium rhizogenes* K599. Transformations are then plated on tetracycline plates grown overnight at 37℃ followed by an overnight growth in LB-tetracycline broth at 28 C. Stock samples of 30% glycerol and 70% LB broth are stored at -80 degrees for future purposes. (Matsye et al. 2011, 2012; Pant et al. 2014, 2015).

Soybean root transformation

One-week old soybeans are used in *A. rhizogenes K599*-mediated transformation experiments. Soybean roots were trimmed and soaked in a beaker of *A. rhizogenes K599* culture followed by a vacuum seal to ensure transformation. The pRAP15 vector was used for overexpression and was transformed in *G. max*$_{[\text{Williams 82/PI 518671}]}$ (*rhg1*$^{-/-}$) which provided a susceptible response to *R. reniformis*. The pRAP17 vector provided an RNAi response by deleting the gene of interest. pRAP17 was transformed into *G.max*$_{[\text{Peking/PI 548402}]}$ (rgh+/+) which provided a resistant response to *R. reniformis*. Control vectors included empty pRAP15 and pRAP17 vectors that contained the cytotoxic gene *ccdb*. Each empty vector was transformed into the specified soybean as described above. Transformed plants grew for approximately 3 weeks to develop a root system prior to infection. Roots were examined using fluorescent light to examine the presence of eGFP (enhanced green fluorescent protein) which is found in the vectors transformed in *A. rhizogene* K599. Under fluorescent light, non-transformed plants appeared brown while transformed plants appeared green. Roots that transformed were trimmed to prevent infection of non-transformed roots. Processed plants were then planted and prepared for infection. (Matsye et al. 2011, 2012).

Juvenile Extraction and infection soybean plants

Soil and water contents of the bucket set singly as substantive over (bucket 1) were teeming through a 60-mesh sieve till bucket 2. The contents of bucket 2 were sieved over the sink used a 325-mesh sieve. (Sieve No. 60, USA standard test sieve. Fisher Scientific Company, 250 micrometers. Sieve No. 325, USA standard test sieve, 45 micrometers. Fisher Scientific Company, USA.) Renew rinsing was done through the 325-mesh sieve with a gentle flow of water till 20 ml soil or minus remained on under most of the 325-mesh sieve. A 30-40 ml juvenile egg extract was collected by washing the 325-mesh sieve extract into a 150-ml beaker. The beaker content was allowed to settle for 2 hours. After 2 hours, water was rejected. A timer was set to 10 minutes. A 1.3 M sucrose solution was added to the bottom layer contents of the beaker to give a 50-ml volume and gently

Table 1. Primers currently used in the analysis.

Gene name	Accession	Primer type	Primer 5` ----3`
GmSYP22-3	Glyma.01G015600	PCR-F-OE	CACCATGAGTTTTCAAGACATCCAAGGTG
		PCR-R-OE	TGTCATCTTACTTGTTGTACTCATTTTTTAG
		qPCR-F	CACAACGTTGAAGTTAATGCAAGTAAG
		qPCR-R	AAGAAGTGCTTGCGGAACAAA
		qPCR probe	CACAGCGTCTTTCAGCGGAGAGG
GmSYP22-4	Glyma16g05040	PCR-F-OE	CACCATGAGCTTTCAGGACATCGAGG
		PCR-R-OE	CTAAGCAGCAAGAACAATGATGACG
		qPCR-F	ATGAGTTTTCAAGACATCCAAGGTG
		qPCR-R	GACGAGTCGCCGGAAAGTAG
		qPCR probe	AACCCTCCCTCTCGCCGGAAC

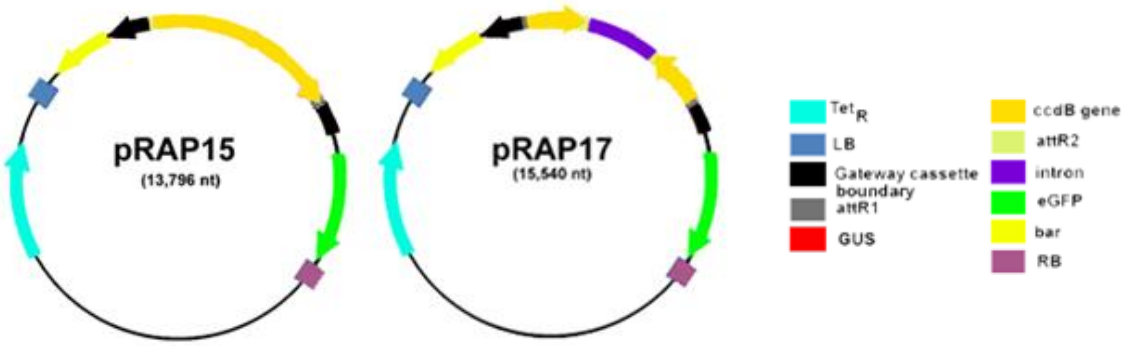

Figure 1.pRAP15, overexpression; pRAP17, RNAi; Right, legend of vector components. Functional cassette is between left and right border (LB, RB). Cyan, tetracycline resistance; blue, LB; black, Gateway cassette border; gray, *att*R1; red, GUS; orange, *ccd*B gene; olive, *att*R2; purple, intron; green, eGFP; yellow, *bar* gene; mauve, RB.

swirled. The sugar-nematode hang was leaving into 50 ml centrifuge tube and centrifuged for 1 min at 1500 rpm using a centrifuge from International Equipment Company (Model 120 Size 2 50/60 Hertz, 7.3 amps). After centrifugation, the supernatant was teeming off onto a 500mesh sieve grasped up the sink. The bead soil layer of the centrifuge tube was discarded. The extract was rinsed under a very gentle flow of working water to rinse off the 1.3 M sucrose solution and collected into a 150ml beaker. Water was added to bring the J2 egg extraction to 40 ml volume. Examination and count of eggs and juveniles on grated Petri dishes were done using the Olympus BH2 B071 microscope (Japan Model C35AD-4) at 40X magnification. (Aljaafri, Weasam A.R., 2017, Ayoub, S. M. 1980; Jenkins. W. R. 1964).

Life stages of R. reniformis Calculation

Determining the life stahes of *R. reniformis* represents the effect of overexpressing or suppressing gene expression in G. max when challenged with *R. reniformis*. The life stages including (Juveniles, eggs, and adult female) index is calculated with the equation Life stages =(Ns/Nx) *100, where Nx is the average of life stages on the test cultivar and Ns is the average number of life stages on the standard susceptible cultivar (Golden et al. 1970; Klink et al. 2009; Matthews et al. 2013). Life stages counted in transgenic plants transformed with pRAP15 and pRAP17 vectors represent Nx. Empty control vectors used represent Ns. The FI is calculated as a function of root mass, tested statistically using the Mann-Whitney-Wilcoxon (MWW) Rank-Sum Test, $p<0.05$ (Matsye et al. 2012). The effect of the overexpressed and suppressed gene on root growth was determined by using the Mann-Whitney-Wilcoxon (MWW) Rank-Sum Test, $p<0.05$ (Matsye et al. 2012). In this experimental study, each gene was replicated three times with 20 plants per replicate.*Rotylenchulus reniformis* was maintained in the greenhouse on cotton and corn respectively. The eggs for both nematodes were extracted from fresh roots by using NaOcl for 4 minutes with using 200 um pore sieves nested on 500 um pore sieves. (Mclean, K. S. 1993). Juveniles were extracted from the soil by sucrose centrifugal flotation. (Ayoub, S. M. 1980; Jenkins. W. R. 1964).Asuspension 2500 vermiform reniformnematode (*R. reniformis*) was pipetted into the pots at the time of planting.

Quantitative real-time PCR (qPCR)

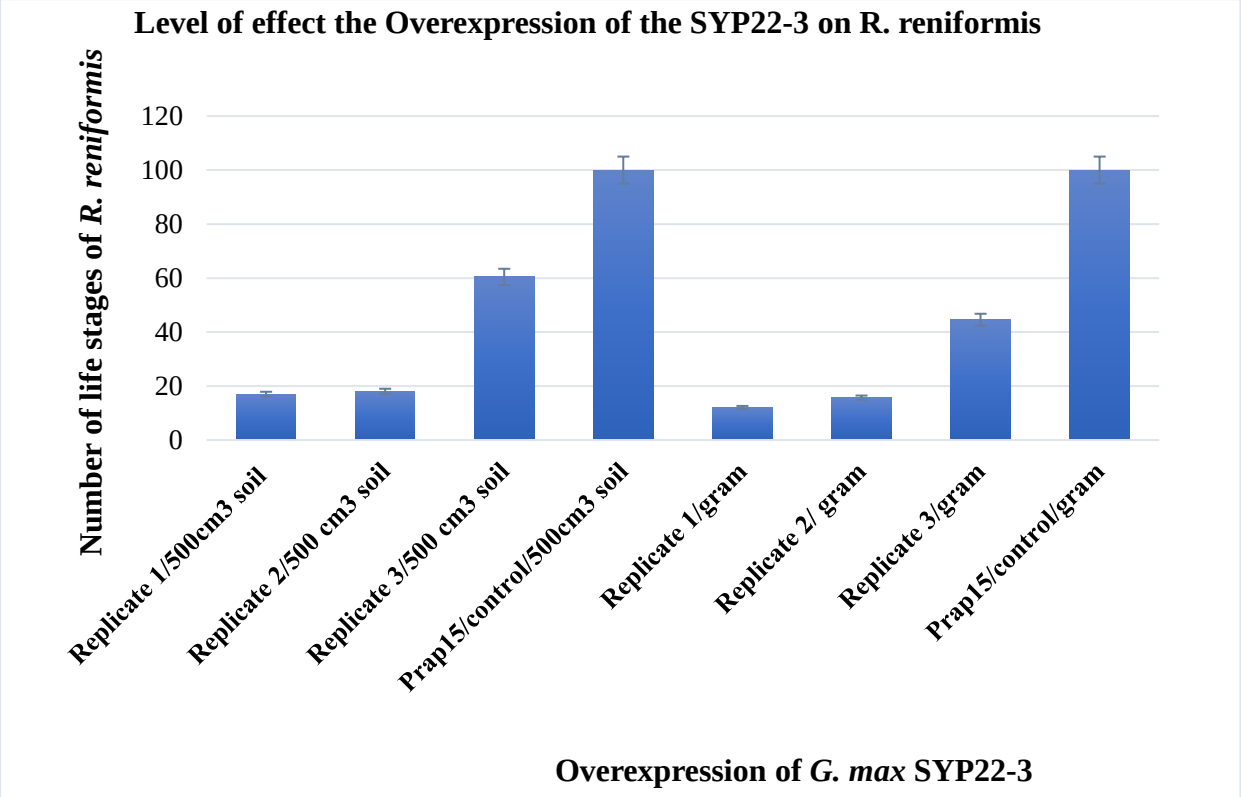

Figure 2: Level of effect the overexpression of *G. max* SYP22-3 has on *R. reniformis*parasitism in *G. max* as indicated by life stages of *R. reniformis*.
Statistically significant using Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, P <0.05 (Mann and Whitney 1947).

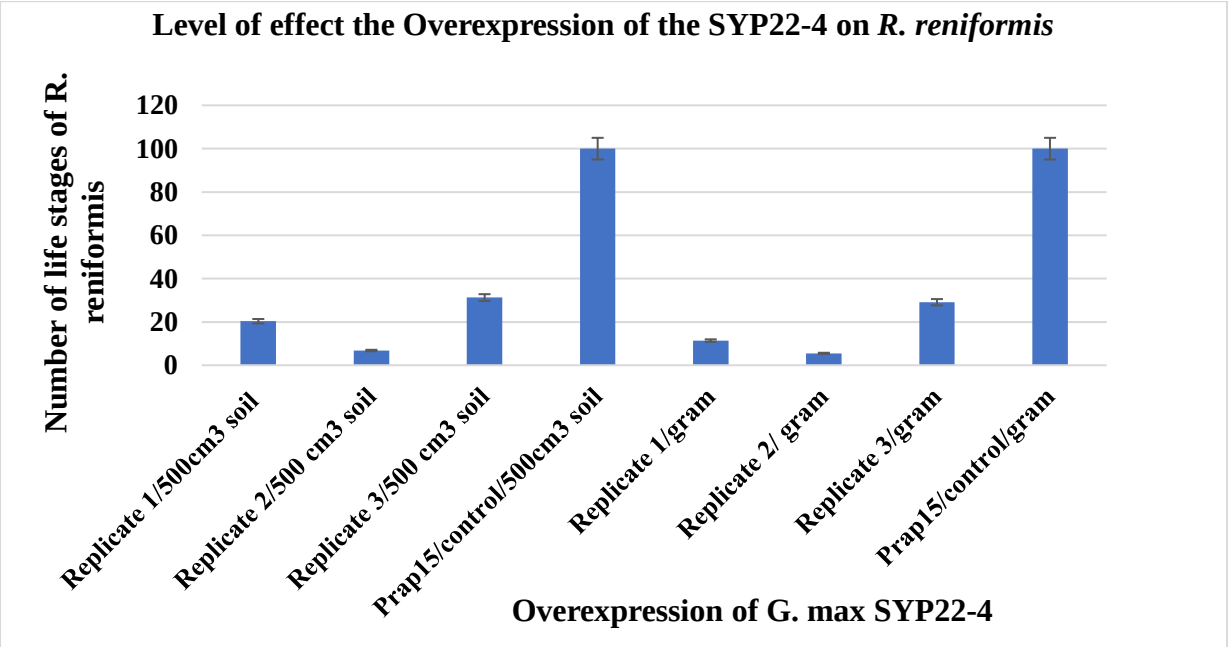

Figure 3: Level of effect the overexpression of *G. max* SYP22-4 has on *R. reniformis*parasitism in *G. max* as indicated by life stages of *R. reniformis*.
Statistically significant using Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, P <0.05 (Mann and Whitney 1947).

To determine fold change of gene expression, plant samples were collected 0, 3 and 6 days post infection (dpi). cDNA was synthesized from RNA samples collected and analyzed using qPCR. The ribosomal protein S21 served as a control for each reaction (Klink et al. 2005, Matsye et al. 2012). The experiments utilize the Taqman 6 carboxyfluorescein (6-FAM) probes with the Black Hole Quencher (BHQ1) (MWG Operon; Birmingham, AL). The qPCR reaction had 20µl Taqman Gene Expression Master Mix (Applied Biosystems;

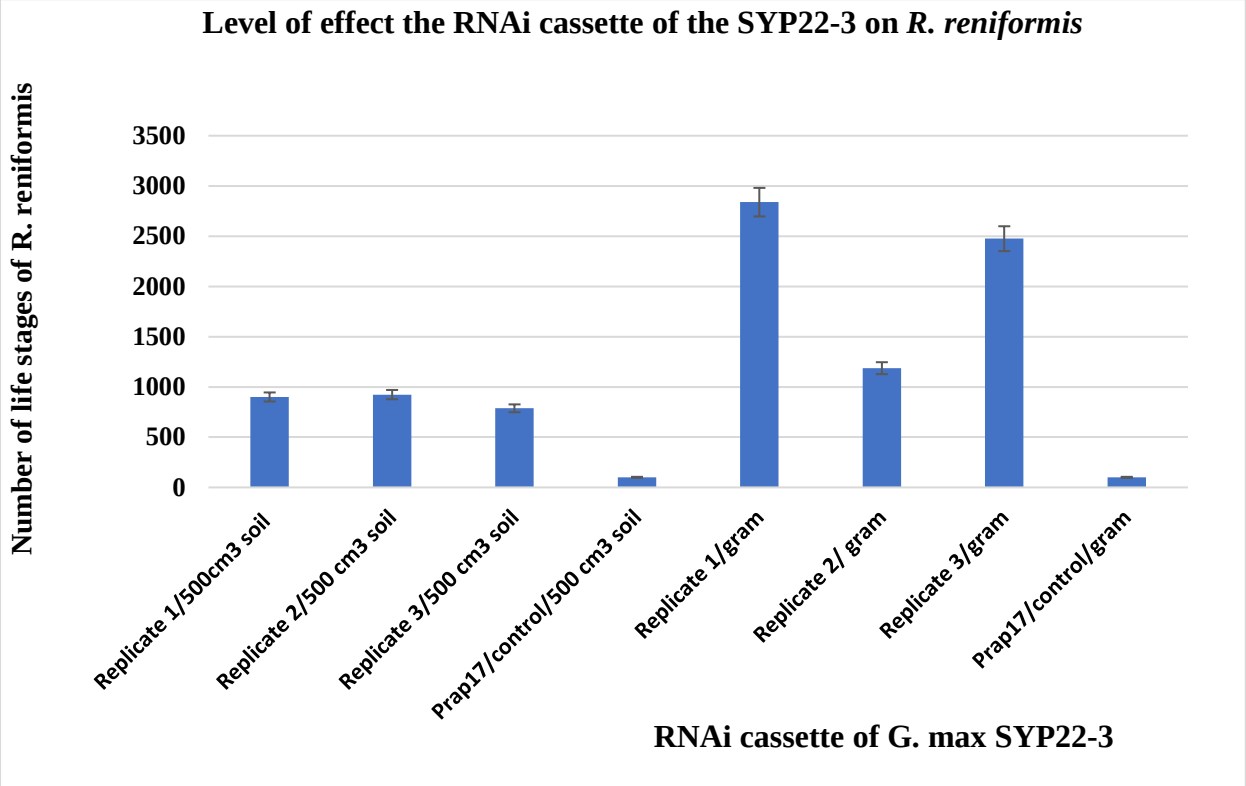

Figure 4: Level of effect the RNAi of *G. max* SYP22-3 has on *R. reniformis* parasitism in *G. max* as indicated by life stages of *R. reniformis*. P < 0.05., statistically significant using Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, P < 0.05 (Mann and Whitney 1947).

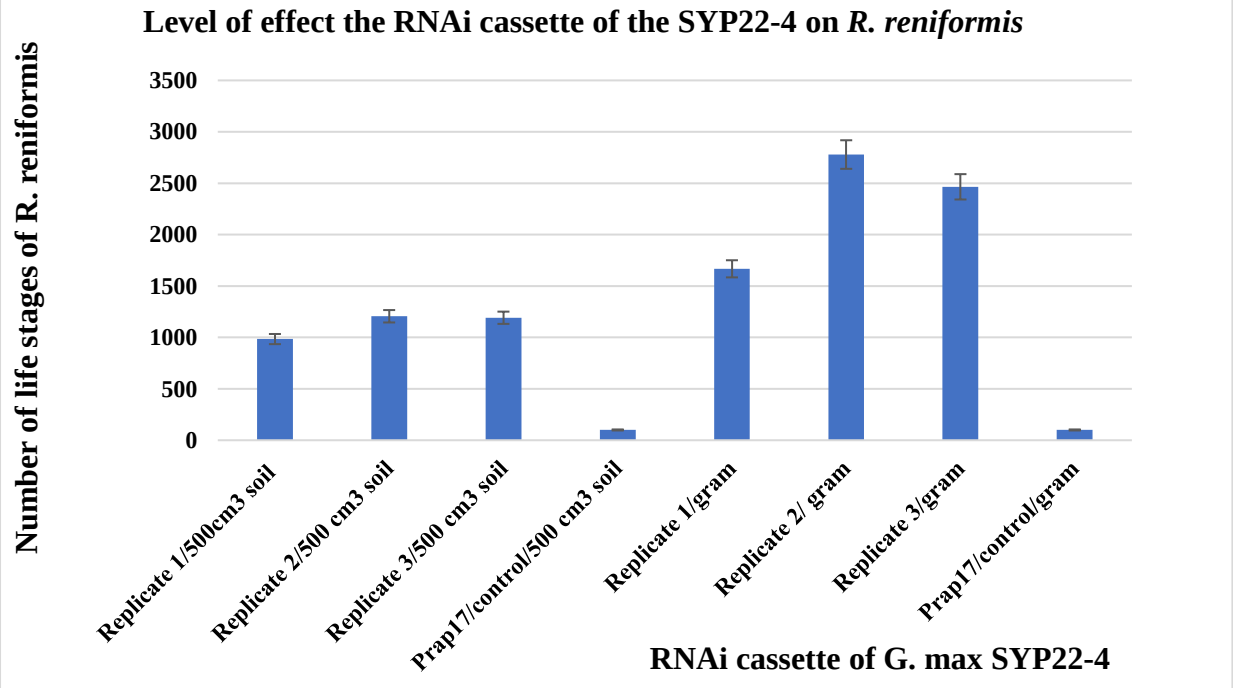

Figure 5: Level of effect the RNAi of *G. max* SYP22-4 has on *R. reniformis* parasitism in *G. max* as indicated by life stages of *R. reniformis*. P < 0.05., statistically significant using Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, P < 0.05 (Mann and Whitney 1947).

Foster City, CA), 0.9µl of µM forward primer, 0.9µl of 100µM reverse primer, 2 µl of 2.5 µM 6-FAM (MWG Operon®) probe and 9.0 µl of template of cDNA. The qPCR reaction is accomplished by preincubation of 50℃ for 2 minutes, followed by 95℃ for 10 minutes. Proceeding from this step is alternating 95℃ for 15

Table 2. Gene expression of candidate genes during a resistant reaction to *R. reniformis*.

Gene	Sample	Fold change
Glyma.01G015600 (OE)	0 dpi	3.84527
Glyma.01G015600 (OE)	3 dpi	6.103986
Glyma.01G015600 (OE)	6 dpi	2.422385
Glyma.01G015600 (RNAi)	0 dpi	-234.685
Glyma.01G015600 (RNAi)	3 dpi	-144.101
Glyma.01G015600 (RNAi)	6 dpi	-117.347
Glyma16g05040 (OE)	0 dpi	34.1835
Glyma16g05040 (OE)	3 dpi	2.748596
Glyma16g05040 (OE)	6 dpi	1.785616
Glyma16g05040 (RNAi)	0 dpi	-73.924
Glyma16g05040 (RNAi)	3 dpi	-288.196
Glyma16g05040 (RNAi)	6 dpi	-372.649

The relative levels of transcript abundance have been measured by qPCR in transgenic *G. max* overexpression and RNAi lines.

seconds then 60℃ for 1 minute for 40 cycles. The statistical analysis using $2^{-\Delta\Delta C_t}$ to calculate fold change was followed according to the derived formula presented in Livak and Schmittgen (2001).

RESULTS

Candidate gene cloning and gene testing

The identification of SYP22-3 and SYP22-4 functioning in the defense of soybean to *R. reniformis* implicated the Syntaxin proteins genes signaling cascade as also functioning in the process. This result led to the identification of other soybean genes that are homologous to A. thaliana genes that function in BR signaling.Through the examination of gene expression data of SYP22-3 and SYP22-4 genes, it was identified that a SYP22-3 and SYP22-4 homolog (Glyma.01G015600 and Glyma16g05040) were induced. Gm-SYP22-3 was cloned, sequenced and genetically engineered into susceptible *G. max* [Williams 82/PI 518671]. The genetically engineered plants then were infected with *R. reniformis*. *R. reniformis* infection was allowed to proceed for 30 days. At the conclusion of the 30day life cycle period, a life stages analysis was performed, demonstrating that the overexpression of Gm-SYP22-3 suppressed the life stages of *R. reniformis* parasitism (Figure 2,3). As shown in Figures 2,3 there was approximately an82.89% reduction in parasitism as measured by the life stages of *R. reniformis*.

The identification of Gm-SYP22-3 functioning in soybean defense to *R. reniformis* implicates its direct binding partners, Gm-SYP22-3 and Gm-SYP22-4. Downstream signaling genes include Gm-SYP22-3 and Gm-SYP22-4 that function to activate Gm-SYP22-3. Cloning experiments were performed as described, resulting in the isolation of soybean homologs of BR signaling genes, including Gm-SYP22-3 and Gm-SYP22-4 (Table1). These genes were currently being engineered into susceptible *G. max*[Williams 82/PI 518671]. In contrast, RNAi constructs are currently being engineered into *R. reniformis*-resistant *G. max*[Peking/PI 548402]. The results presented here indicate the Gm-SYP22-3 and Gm-SYP22-4 transcription factor functions in defense. The results are consistent with prior observations that demonstrated Gm-SYP22-3 and Gm-SYP22-4 functions in defense (Pant et al. 2014). These observations indicated that other members of the SYP cascade may also function in soybean defense to SCN parasitism.

Resultwas anticipated that the overexpression of Gm-SYP22-3 and Gm-SYP22-4 in the *R. reniformis* susceptible *G. max*[Williams 82/PI 518671]was result in engineered resistance like what has already been observed (Figure 2). In contrast, RNAi constructs are currently being engineered into *R. reniformis*-resistant *G. max*[Peking/PI 548402]. It was anticipated that the result of those experiments were be engineered susceptibility in the *R. reniformis* resistant *G. max*[Peking/PI 548402]. Similar results have been observed for SYP21 (Pant et al. 2014). To complement these studies, qPCR analyses wasbeing done to confirm that Gm-SYP22-3 and Gm-SYP22-4 were overexpressed. In contrast, it was be demonstrated that Gm-BES1-1 Gm-SYP22-3 and Gm-SYP22-4were suppressed in the resistant *G. max* [Peking/PI 548402] engineered with the Gm-SYP22-3 and Gm-SYP22-4 -RNAi cassette.(Table 2).The results of these experiments confirm transgenic roots of *G. max* containing SYP22-3 and SYP22-4 overexpression cassettes did exhibit higher relative transcript levels of each gene. In contrast, transgenic roots containing SYP22-3 and SYP22-4 RNAi cassettes exhibited lower relative transcript levels of each gene. These results demonstrate that the transgenic roots are behaving as they would be expected to function, based on the genetic cassette with which they have been engineered. With the transgenic roots made, each gene could be examined experimentally.Similar results have been observed for SYP21 (Pant et al. 2014). The same approachwas used with these candidate genes in this analysis. (Table 2). The outcome of the experiments was to identify whether other Gm-SYP22-3 and Gm-SYP22-4 genes function in resistance of soybean to *R. reniformis*.Prior analyses identified two SYP22-related genes in the *G. max* genome (Pant etal. 2014). The identified candidate *G. max* SYP22 genes were being studied to determineif they perform a role in defense to *R. reniformis*parasitism. Experiments were performed in *G. max* leading to the experimentally induced orsuppressed expression of *G. max* SYP22-3 and SYP22-4 (Figure 2,3).The transgenic SYP22-3 and SYP22-4 overexpression lines with their pRAP15control was then

being infected with *R. reniformis*. Infection was allowed to proceed for 30days. At the end of the 30-day life span, *R. reniformis* life stages were extracted from the soil, enumerated and compared to control plants. The experiments show that plantsoverexpressing SYP22-3 or SYP22-4 have impaired *R. reniformis*. (Figures2,3).The transgenic SYP22-3 and SYP22-3 RNAi lines with their pRAP17 controlwere infected with *R. reniformis*. Infection was allowed to proceed for 30 days. At theend of the 30-day life span, *R. reniformis*. Life stages were extracted from the soil, enumeratedand compared to control plants. The experiments show that the SYP22-3 or SYP22-4 RNAi lines had impaired resistance to *R. reniformis*. (Figures4,5). The data obtained from these complimentary approaches of gene overexpressionand RNAi in studying SYP22 resulted in combined opposite outcomes, respectfully. Theopposite outcomes were impaired susceptibility to *R. reniformis*. parasitism in the SYP22overexpression lines and impaired resistance to *R. reniformis*. in the SYP22 RNAi lines.These opposite outcomes were hallmarks of the involvement of the gene in the process ofresistance (Pant et al. 2014; Sharma et al. 2016, Aljaafri, W. A,2017).

DISCUSSION

A number of recent studies have pointed to the importance of components of the membrane fusion apparatus having a role during *G. max* resistance to plant-parasitic nematodes (Matsye et al. 2012; Pant et al. 2014; Sharma et al. 2016; Klink et al. 2017, Aljaafri, W.A,2017). Included in these analyses are observations of defense function of different members of the syntaxin gene family (Klink et al. 2017). The results presented here continue with the characterization of the *G. max* syntaxin gene family by functionally examining the syntaxin of plants two family members, SYP22. The result, along with a series of subsequent analyses have revealed the importance of vesicle transport, mediated by SYP121, to plant defense because the vesicles are responsible for the delivery of antimicrobials, enzymes and structural elements to the site of defense (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006; Johansson et al. 2014). The analyses resulted in the identification or both SYP22-3 and SYP22-4 being expressed specifically in syncytia undergoing the process of resistance in *G. max* [Peking/PI 548402] and *G. max* [PI 88788]. Important in the design of those studies is that each genotype can undergo a resistant reaction to *R. reniformis*. Prior studies performed in *G. max* that have tested gene function through genetic analyses demonstrate that genes expressed in the cells specifically undergoing the process of resistance have functional roles in resistance (Pant et al. 2014; Sharma et al. 2016; Klink et al. 2017). These observations indicate SYP22-3 and SYP22-4 had the highest probability of functioning during defense. These genes then were examined in functional studies including their experimentally induced overexpression and RNAi.

CONCLUSION

Recent studies have demonstrated the involvement of a cellular apparatus acting in the fusion of membranes functioning effectively in the resistance of *G. max* to *plant-parasitic nematode* (Matsye et al. 2012, Pant et al. 2014; Klink et al. 2017). In *A. thaliana*, the involvement of membrane fusion components during defense began with the genetic identification of *PEN1* (SYP121) (Collins et al. 2003).

The observation that *G. max* SYP22 functions in defense fills an important gap inour current understanding of resistance to *R. reniformis*and, perhaps, rootpathogens ingeneral. The results explain how materials can be delivered to thevacuole, a structurethat is central to cellular homeostasis while also having important roles in defense. Therole *G. max* SYP22-3 and SYP22-4 have in defense was explained by the vacuole serving as a site of storagefor enzymes andconjugate glucosides that can become activated during pathogeninvasion.

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