Evaluate the ability of Syntaxin genes to enhance resistance against *Fusarium virguliforme* and *Heterodera glycines*

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Abstract. Soybean sudden death syndrome (SDS) caused by *Fusarium virguliforme* is a common and widespread disease across soybean-growing regions worldwide. Experiments were conducted in temperature-controlled water baths in the greenhouse to test the effects of some genes from syntaxin group (SYP22-3 and SYP22-4) on *F. virguliforme* and the combination of SDS and soybean cyst nematode (*Heterodera glycines*). Both genes were tested in different combinations with currently two pathogens to examine their ability to manage studied pathogens. Syntaxin evaluated on soybean seedlings infected with *F. virguliforme* alone and both *F. virguliforme* and *H. glycines*. Results showed that there were significant effects of syntaxin genes to reduce the foliar disease severity on soybean plants. Furthermore, they reduced the number of *H. glycines* females cysts compared to control. Data analysis did not show any negative effect by syntaxin genes on plant growth.

1. Introduction

*Fusarium* species are varied pathogens, infecting all vegetative and reproductive parts of host plants [1]. They are economically important as pathogens attacking most agricultural and horticultural crops grown worldwide. *F. virguliforme* is an important member that causes the sudden death syndrome (SDS) disease on soybean in combining with soybean cyst nematode (*Heterodera glycines*) [2]. The disease is considered a real threat to soybean productivity annually, for instance, in 2010, SDS caused an estimated 4.7 million metric tons yield loss nationwide [3]. Sudden death syndrome infects soybean plants from mid- to late-season and its symptoms include root rot, vascular discoloration of roots and stems and crown necrosis [4]. Symptoms of SDS occur on leaves, beginning with chlorotic mottling and proceeding to interveinal chlorosis, necrosis and defoliation. The frequent of leaf symptoms become less in some growing seasons but roots may still be infected [5]. *F. virguliforme* pathogen has been isolated from lower stems and roots, but not from leaves [6]. To identify *F. virguliforme*, microscopic methods were used, however, it is difficult to detect the fungus in roots and soil due to other morphologically similar pathogens. Soybean cyst nematode (SCN) is the most harmful pathogen on soybean plants (*Glycine max* L.) worldwide [7; 8]. From 2006 to 2009, studies reported an estimated 34 million metric ton reduction in soybean production infected by
SCN in USA [8]. Several generations of SCN populations can be completed over one growing season [9]. Due to economic losses over time, it is important that new management strategies to be developed for successful management of these pathogens and decreasing their impact on soybean crop. Management options for SDS and SCN pathogens are the use of host plant resistance (resistant genes), chemicals and biological controls. Resistant genes are a new strategy for managing soybean disease. In plants, one function of genes is defense against pathogens [10]. Arabidopsis thaliana, as a plant genetic model contains some proteins including syntaxin 22 which is a component of the endosome or prevacuolar compartment (PVC) [11]. Syntaxin 22 (SYP22) was first identified in a mutagenic screen and was called AtVAM3. SYP22 is closely related to another syntaxin called SYP23 [12]. Genetic engineering has begun to take a considerable attention with potential as a practice to generate resistance against pathogens [18; 14]. Thus, the main objective of this study is to use syntaxin (SYP22-3 and SYP22-4) to promote resistance in soybean plant against F. virguliforme and H. glycines and to more fully understand of the process.

2. Materials and Methods
2.1. Genes selected and processing to transgenic plants
The candidate genes have been selected by mining data from published gene expression experiments [15; 13]. This procedure is an effective means to identify genes that function in Glycine max defense to H. glycines and F. virguliforme, proven further in independently-performed genetic mutational analyses [14; 16; 17]. In brief, A. thaliana protein sequences of selected signaling genes were identified from Genbank and blasted against the genome of soybean which is housed at phytozome.net. Candidate soybean genes were examined against an in-house gene expression database. The cDNA nucleotide sequences also were extracted from Phytozome.net and used to design PCR primers using Primer-BLAST program for cloning experiments (GmSYP22-3 Glyma.01G015600 PCR-F-OE CACCATGAGTTTTCAGACATCCAAGGTGPCROR0ETGTCTATCTTTGTTGACTCATTTTTTAG. GmSYP22-4.Glyma16g05040, PCR-F-OE CACCATGAGCTTTCAAGAACATCGAGG. PCR-ROE CTAAGCAGAAGACATGATGAGCG. The PCR amplicons generated using Accuprime® from synthesized cDNA (SuperScript® First Strand Synthesis kit) and run the gel with 1% agarose gel. PCR amplicons were excised from gel and purified according to instructions protocol provided by the Promega company. Amplicon was cloned using the pENTR™/D-TOPO® Cloning Kit with TOP10 and electroporated into chemically competent bacterial cells (Life Technologies). Transformed cells were grown overnight (12-16 hours) on kanamycin plates (50µg/ml). Selected colonies were then grown at 37̊C overnight (14-16 hours) broth. Plasmid extraction was performed according to protocol of QIAprep Spin Miniprep Kit. Prior to sequencing, Taq polymerase (Life Technologies) was used to confirm presence of gene inserted PCR product in the plasmid. Sequencing results were confirmed of correct insertion and to avoid any mutations. Correct sequencing was then cloned into either the overexpression vector pRAP15 or transcriptional suppression vector pRAP17. Subsequently, cloning into these expression vectors was done through recombination with LR clonase II (Invitrogen). Insertion was done at recombination specific sites, attR1 and attR2. Recombination of insertion at these sites replaced ccdB gene from the vector. This gene is toxic to the cell and is a mode of selection of insertion. The cloning reaction was then transformed into One Shot® TOP10 chemically competent E. coli and grown on tetracycline plates (5µg/mL) plates overnight. Selected colonies were grown overnight in LB-tetracycline broth at 37̊C overnight to replicate plasmid. Plasmid purification was done by using QIAprep Spin Miniprep Kit and sent for sequencing to confirm correct insertion and void of any mutations. Expressions vector was then transformed into Agrobacterium rhizogenes K599. Transformed A.rhizogenes were spread on tetracycline plates and grown overnight at 37̊C followed by an overnight growing in LB-tetracycline broth at 28̊C. Stock samples of 30% glycerol and 70% LB broth are stored at -80 degrees for future purposes. Soybean (Glycine max [Williams 82/PI 518671] and G. max[Peking/PI 548402] were employed for plant transformation of overexpression studies and RNAi studies, respectively. Soybean plants grew in the greenhouse for
one week and used for genetic transformation by cutting between the hypocotyls and the half-way point of the cotyledon according to Matsye et al. [14]. An Agrobacterium culture is prepared from stock by incubating at 28°C for two days. Culture is pelleted at 4000 rpm for 30 minutes, resuspended, OD-600 nm adjusted to approximately 0.2-0.3. After cutting the shoots in the culture, vacuum was drawn and held for 30 minutes. Overnight incubated plants were replanted in plastic pots and kept in culture room before transferred to the greenhouse environment. pRAP15 vector and pRAP17 vector with the gene of interest was transformed to G. max[Williams 82/PI 518671] and G. max[Williams 82/PI 518671] respectively. Controls for each experiment were carried out by transforming the applicable empty vector. The effect of overexpression and RNAi of the gene was analyzed compared with control through quantitative PCR [18; 14; 16]. Transformed roots exhibit green fluorescence when viewed under blue light due to the green fluorescent protein (GFP) reporter expression. Non-GFP roots were removed by trimming. Plants were replanted in vermiculite for 21 days. After a second trimming of non-GFP roots, plants were replanted in wet sand-soil mixture for 45 days.

2.2. Preparation of F. virguliforme and H. glycines inoculum

The source of F. virguliforme inoculum for all experiments was obtained from soybean roots showing SDS symptoms growing in a greenhouse at Mississippi State University, USA which already were confirmed to be infected by F. virguliforme. These diseased plants incubated at room temperature (estimated at 22°C) for one week, with briefly mixing every day by massaging the bag. Inoculum was then plated on potato dextrose agar (PDA), which was supplied with streptomycin and tetrachlorocycline to prevent any bacterial growth, to check that there was no fungal growth other than Fusarium virguliforme. A preliminary experiment was performed testing different substrates for the growth of F. virguliforme. The fungus then kept on autoclaved corn seeds until used. Plants were grown for 35 days and this duration was chosen to give SCN enough time to complete a single generation. Roots were washed in running tap water for three minutes then cut into 3-5 mm pieces with cortical and vascular tissues separated and placed on PDA then identified according to morphological characteristics of F. virguliforme. Severity was rated after 60 days using a 0-7 scale, where 0-no symptoms, 1-mosaicmottling, 2-chlorotic mottling, 3-intervenial chlorosis, 4-intervenial chlorosis with leaf edge necrosis, 5-intervenial necrosis, 6- defoliation with leaflets separating from thepetiole leaving the petiole attached to plant, 7- plant death [2]. H. glycines nematodes were obtained from infected plants in the greenhouse and maintained on Williams 82(PI 518671) that was used later as inoculum on the transgenic plant [16; 19]. Light brown to tan cysts dislodged from roots of plants with a strong water spray and collected on nested sieves with pore sizes of 20 and 100 um. Cyst were suspended in water then immediately poured through the 20 pore sieve nested on 100 pore sieve. [20]. Soybean Cyst nematode (H. glycines) counted on graded Petri dishes under an Olympus BH2 B071 stero-microscope (Japan Model C35AD-4) at 40X magnification. Eggs were released from the cysts using a modified seinhorst cyst crusher for 1 minute [20]. The resultant suspension was passed through a 200 um pore sieve nested on a 500 um pore sieve to remove broken cysts and debris. Each soybean plant was inoculated with 2500 eggs making the holes made near the base of the root system and for F. virguliforme used 1 gram of corn seeds that have the fungus. The holes were covered once inoculum soaked in to prevent expulsion from watering. Inoculum added to each pot by using calibrated micropipette adjusted to deliver 3ml total volume. Plants were maintained in the greenhouse for 45 days to allow the nematode to complete their lifecycle. To determine the effect of overexpression or suppression of specified genes, the female index (FI) was calculated by extracting cysts from roots. The female index represents the effect of overexpressing or suppressing gene expression in G. max when challenged with H. glycines. Transformed plants inoculated with the soybean nematode grown in the greenhouse for 45 days prior to harvest. The cyst life developmental stage, extracted and counted as previously described. The female index was calculated following the equation FI= (Ns/Nx)*100, where Nx is the average of females on the test cultivar and Ns is the average number of females on the standard susceptible cultivar [21; 22; 23; 18;
Cysts counted in transgenic plants transformed with pRAP15 and pRAP17 vectors represent Nx. Empty control vectors used represent Ns that means without genes only p15 or p17. The F1 was calculated as a function of root mass, tested statistically using the Mann-Whitney-Wilcoxon (MWW) Rank-Sum Test, p<0.05 [14]. The effect of the overexpressed and suppressed gene on root growth were determined by using the Mann-Whitney-Wilcoxon (MWW) Rank-Sum Test, p<0.05 [14]. In this experimental study, each gene replicated three times with 20 plants per replicate. The data acquired were analyzed using a Social Science Statistics existing the complete and the significant differences between means, the standard of significant was collected at 5% with two-tailed hypothesis.

3. Results and Discussions
For all experiment, there were significant effects of SYP22-3 and SYP22-4 genes on decreasing the severity of SDS disease. Results showed a clear band of PCR product of studied genes (Figure 1). Foliar disease index (DI) was decreased with both tested genes in overexpression lines. DI for SYP22-3 was 0.4 and 1.8%, respectively with *F. virguliforme* only and for *F. virguliforme* and *H. glycines* in comparison with 2.9 and 4.8 in the control treatment. Also, there was a significant effect of SYP22-4 that reduced the rating of the foliar disease index with overexpression experimental. The interaction between *F. virguliforme* and *H. glycines* increased the severity of foliar disease index compared to *F. virguliforme* alone(Figure2).

![PCR image](image-url)

**Figure 1.** PCR image conformed existing of both genes on the same location according to the number of pb paired of nucleotides of SYP22-3 and SYP22-4.
Figure 2. Severity of SDS disease with overexpression experimental. Data were means of the 3 replicates for each gene after 45 days. Means compared by using Fisher’s protected least significant difference test at $P<0.05$.

Further analysis of RNAi lines showed that for SYP22-3 and SYP22-4 genes, the transgenic SYP22-3 and SYP22-4 RNAi lines experimental had impaired resistance to *H. glycines* and *F. virguliforme*. Disease index (DI) for SYP22-3 gene was 2.4 and 4.8 compared to 0.4 and 1.2 in the control treatment for *F. virguliforme* only and both pathogens together *F. Virguliforme* and *H. glycines*, respectively. In addition, there was no negative effect on plant growth of soybean plants by SYP22-3 and SYP22-4 genes (Figure 3).
Figure 3. Severity of SDS disease with RNAi experimental recorded using a 0-7 scale. Means compared by using Fisher’s protected least significant difference test at $P<0.05$.

Present results showed the effect of SYP22-3 and SYP22-4 genes in reduction the number of cysts nematode that were decreased 13.3% and 21.1% in the first replicate respectively compared to 100% in control. There was no negative effect on plant growth by SYP22-3 and SYP22-4 genes in overexpression experiments (Figure 4).

Figure 4. Effect of Overexpression experimental of G. max SYP22-3 and SYP22-4 genes on H. glycines life stage including number of cysts per 500 cm$^3$ soil using Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, $P<0.05$. SYP22-3 and SYP22-4 genes with RNAi experiment were decreased resistance to H. glycines parasitism and F. virguliforme. Present results showed that in genes treatment there were 1130 and 571 cyst per 500cm$^3$ soil compared to 100 cysts in the p-17 control. Also, there were significant effect on plants growth with both genes and make soybean plants susceptible to both pathogens H. glycines and F. virguliforme (Figure 5 and 6).
Figure 5. Effect of RNAi in *G. max* SYP22-3 and SYP22-4 genes on *H. glycines* life stage including number of cysts per 500 cm³ soil using Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, P < 0.05.

Figure 6. Characteristics growth structures of soybean cyst nematode (*H. glycines*) identified from infected soybean plants under microscope showed A, Juvenile, B, cyst white and adults females.

The identified candidate GmSYP22 (SYP22-3 and SYP22-4) genes were being studied to determine if they perform a role in defense against *F. virguliforme* and Soybean Cyst Nematode (*H. glycines*). Results showed significant effect to reduce the rating of foliar disease index with *F. virguliforme* in both SYP22-3 and SYP22-4 genes. The genetically engineered plants challenged with *F. virguliforme* and Soybean Cyst Nematode (*H. glycines*) demonstrated the overexpression of SYP22-3 and SYP22-4 suppressed SCN parasitism. In addition, results of both genes were significantly affected RNAi. The damage caused by *H. glycines* variable based on population densities present at the beginning of a growing season. Francl and Dropkin [25] found that yield loss was observed with an egg count of 470 eggs per kilogram of soil at the beginning of the season. To reduce the yield loss exhibited by SCN, scouting and management are required. The use of a resistant cultivar is one form of management strategies that can further reduce nematode population densities [26]. Currently, most of SCN-resistant cultivars are derived from a single source of resistance (PI 88788) [27].
Since 1898, the USDA National Plant Germplasm System (http://www.ars-grin.gov/npgs/) has been collecting and preserving thousands of soybean accessions. Screening of 5,800 soybean accessions identified *H. glycines* resistance in the *G. max* accession, Peking. Other sources of resistance were identified later (reviewed in [28]). Genetic analyses identified the recessive resistance loci rhg1, rhg2 and rhg3 and dominant resistance loci Rhg4. The genes composing the rhg1 and Rhg4 loci have recently been identified [14; 29]. The metabolic networks that relate to these genes and resistance are not understood [29]. Thus, current study demonstrates the ability of syntaxin to enhance soybean resistance to SDS disease.

4. Conclusions
In current study, it has demonstrated the involvement of GmSYP22 genes (SYP22-3 and SYP22-4 provides an effective resistance against *F. virguliforme* and Soybean Cyst Nematode (*H. glycines*). The observation sowed that *G. max* SYP22-3 and SYP22-4 was functions in defense fills an important gap in our current understanding of resistance to *F. virguliforme* and *H. glycines*. The role of *G. max* SYP22-3 and SYP22-4 in defense was explained by these results in reducing the rating of foliar disease index and the number of cysts nematode.

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