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Cover Page

Title: (no more than 15 words)	Enhancement of Soybean Through Genetic Engineering
Proposal to:	Kansas Soybean Commission 1000 SW Red Oaks Place Topeka, KS 66615
From:	Kansas State University 2 Fairchild Hall Manhattan, KS 66506-1103
Funding Period:	July 1, 2014 - June 30, 2015
Amount Requested:	$76,338
Project Status:	New X Continuing_____ If Continuing, Year ___ of ____ total years
Principal Investigators and Units:	Harold N. Trick,Tim C. Todd, Plant Pathology, William T. Schapaugh, Agronomy

By signature below, I certify that the investigator(s), department head(s), and appropriate Dean(s)/Director(s) associated with this proposal have indicated their agreement with the content, procedures, commitments, and obligations included in this submission. This submission is thus institutionally authorized.

10/14/2013

Paul R. Lowe, CRA
Assistant Vice President for Research
Kansas State University

Title: **Enhancement of Soybean through Genetic Engineering**

Principal Investigator(s) and Unit(s):

Harold N. Trick
Dept. of Plant Pathology
(785) 532-1426
Fax (785)532-5692
hnt@ksu.edu

William T. Schapaugh
Dept. of Agronomy
(785) 532-7242
Fax (785) 532-6094
scha0035@ksu.edu

Tim C. Todd
Dept. of Plant Pathology
(785) 532-1350
Fax (785)532-5692
nema@plantpath.ksu.edu

Objectives:

1. Enhance **Soybean Cyst Nematode (SCN)** resistance in transgenic soybean by modifying current silencing strategies.
2. **Test the effectiveness of gene silencing constructions for root knot nematode resistance** using RKN genes homologous to effective SCN genes.
3. Transgenic approaches for increased fungal resistance with **emphasis on SDS resistance**.

Procedures:

Objective 1: Enhance Soybean Cyst Nematode (SCN) resistance in transgenic soybean by modifying gene silencing strategies.

For the past few years we have been evaluating the effectiveness of traits to provide resistance to soybean cyst nematodes (SCN). Many of these traits have been designed to silence specific genes within the nematode and we have demonstrated a reduction in cyst numbers on these transgenic lines. We can further increase the resistance level by 1) using alternative gene sequences of these genes and 2) increasing the levels of siRNA produced by the plant. We have been targeting approximately 200-300 nucleotides of a given nematode gene with our current gene silencing approach. This is approximately 10 to 30% of the entire sequence of most target genes. Although we have demonstrated the effectiveness of method, targeting alternate sequences of a particular gene may improve the silencing effect. We propose to take two of the genes previously used (one high and one low cyst/egg reduction from the bioassay) and target alternative sequences of the genes for gene silencing. Such a study will provide us with critical data in regards to the selection of future target sequences.

In general, the RNAi mechanism for gene silencing is based on a large (exponential) amplification of small interfering RNA (siRNA) molecules that bind to a specific gene sequence. Many laboratories including our own use this approach to effectively silence the plant own genes. For endogenous plant genes, the RNAi mechanism will produce siRNA molecules that recognize the total gene sequence, even if only 10% of the entire gene sequence is targeted, which in turn will cause a very high degree (possibly complete) of gene silencing. Our current methodology produces only siRNAs that correspond to the specific sequence (200 to 300 bp) fragment found in our DNA construction. The quantity of siRNA species does not increase exponentially because the nematode gene target is not found in the plant. We propose to over-express the targeted nematode gene sequence (either in the sense or antisense orientation) together with the RNAi vector construction. This approach should allow the exponential accumulation of siRNA species in the transgenic soybean plants thereby allowing a greater number of siRNA molecules to be ingested by the feeding nematode. This increase in siRNA ingested by the nematode should translate into increased SCN resistance.

To assess the effectiveness of the above strategies greenhouse SCN bioassays on composite plants or transgenic soybean lines, as well as negative controls, will be performed. Lines will be planted into SCN infected soil (~6000 eggs/100 cm^3) and grown in the greenhouse

for five weeks. Soybean roots will then be washed free of soil and debris, SCN cysts removed from each plant and the number of cysts, eggs and root weight data will be collected for each replicate. Data collected from each bioassay will be examined by analysis of variance with the GLM procedure in SAS.

Transgenic lines generated from these research project will be incorporated into elite Kansas lines under the KSC funded project "Breeding and Management of Soybean for Improved Performance". Where intellectual property rights are involved, the Kansas State University Research Foundation will be advised and they will assist us in the transfer of technology to third parties.

Objective 2: Test the effectiveness of gene silencing constructions for root knot nematode resistance using RKN genes homologous to effective SCN genes.

Root-knot nematodes, particularly *Meloidogyne incognita*, pose an additional risk to soybean production in the United States, accounting for 127,000 tonnes in yield losses annually (Wrather et al., 2006). Although predominantly found in the southern soybean-producing states, *M. incognita* increasingly is recognized as a threat to soybean production in the Midwest (Allen at al., 2005; Kruger et al., 2008), and periodically is associated with stunted soybean plants in the Kansas River Valley. The nematode causes extensive galling of soybean roots, disrupting root function and resulting in seed yield losses up to and exceeding 50% in infested areas (Allen at al., 2005). Resistant varieties are used to manage *M. incognita* in the southern U.S., but availability of adapted resistant cultivars is limited for Kansas and the Midwest.

Table 1. Status of genes selected for RNAi bioassays.

Gene name/code	Process effected[1]	Reduction eggs/g root composite plants
cpn-1	embryonic lethal	**95%**
Y25C1A.5	embryonic lethal	**81%**
J15-001	overall fitness	84%
rnr-1	embryonic lethal	**54%**
prp-17	embryonic lethal	**79%**
J12-001	overall fitness	71%

[1]most genes have multiple processes effected

Target genes for RNA silencing will be selected based on research performed by our group evaluating this phenomenon in the soybean/SCN interaction. Genes showing a greater than 40% reduction in cyst or eggs in the soybean system will be our primary targets for the root knot nematode. In FY2014 we are continuing with greenhouse bioassays. **One of our stable lines demonstrating reduce SCN eggs (containing the *prp*-17 vector) have also shown a 64% reduction in RKN.** Composite plants made with other constructions and are also being screened for RKN resistance in the greenhouse. Plants will be grown for 1-2 months and the roots will be rated for the amount of galling using a standard gall index. The amount of nematode reproduction will be determined by extracting infective juveniles from the roots. In this next funding cycle we will continue produce transgenic lines and evaluate their effectiveness on RKN. Many of the transgenic lines made for SCN control have sequences similar enough to RKN genes so these will also be tested to see if they provide cross protection (i.e. resistance to both SCN and RKN).

Objective 3. Transgenic approaches for increased fungal resistance with emphasis on SDS.

Sudden Death Syndrome (SDS) is caused by *Fusarium virguliforme*, a soil-borne fungus. Disease symptoms have been attributed to specific toxins produced by the fungus. One study

indicated that when the fungal toxin gene FvTox1 was turned off in the pathogen by mutations, no symptoms developed on infected soybeans (Pudake et al., 2013). Our previous work using a gene silencing strategy targeting SCN genes are showing promising results and would serve as a model silencing the FvTox1 gene in *F. virguliforme*. We propose to create silencing vectors for the FvTox1 gene, create hairy roots expressing these silencing constructs, and challenge the transgenic material with the fungus. A positive result would be indicated by inhibition of fungal growth and absence of the disease.

Additionally, we will investigate separate approach to produce fungal resistance. Defensins and their relatives are peptides or small proteins that can inhibit antimicrobial growth (De Lucca and Walsh, 1999). These peptides are present in plants, insects, and vertebrates. Initially we have selected three peptides from various sources and created expression vectors. We will use bacterial expression systems to first characterize fungal inhibition in either *in vitro* or detached leaf assays. We will first evaluate growth inhibition on *F. virguliforme* (SDS) but will screen other pathogens such as *Macrophomina phaseolina* (charcoal rot). For bioassays we will cooperate with Dr. Chris Little, KSU's row crop pathologist. Genes from the effective peptides will be engineered into soybean cultures for *in planta* evaluations.

Justification:

Decreasing yield loss and increasing the value of soybeans is part of KSU's mission to improve Kansas' Agriculture. Our proposal is taking a genetic engineering approach to this mission allowing us to utilize traits outside the scope of conventional breeding.

Fungal pathogens and parasitic nematodes are important, persistent problems that cause large economic losses across the Midwest. For example, the total estimated loss for the US in 2010 due to SCN was 118 million bushels or $1.25 billion (Wrather, http://aes.missouri.edu/delta/research/soyloss.stm). Root Knot Nematodes is also a major factor soybean yield loss in the southern US and has the potential to become a problem for Kansas producers. Charcoal rot is the major fungal disease in the state of Kansas and losses in 2002 were estimated at 9%. *Phytophthora* root rot and *Fusarium virguliforme* (Sudden Death Syndrome, SDS) are other fungal pests that are beginning to make their presence in Kansas (SDS was at record levels in the 2004 growing season). It is timely to find methods to efficiently control to these pathogens as there is little or no natural sources of resistance found in our germplasm. Novel approaches such as using antimicrobial peptides have merit and should be explored. **Finding transgenic solutions to soybean diseases would complement the efforts of the conventional breeding program by adding additional sources of resistance.**

Objectives Addressed:

1A. & B. Breeding/Production/Environmental Programs: BMPs and Crop protection/pest management.

Project Location:

The project will be performed in Throckmorton Plant Sciences Center on the campus of Kansas State University and in selected field plots across the state. An application from APHIS will be required for the latter and will be submitted at least three months before planting.

Duration of the Project:

This project represents the 1st year of a four-year project. July 1, 2014 - June 30, 2018.

Budget:

Description	Budget Detail	Amount
A. Salaries	Research Associate(s)/Post Doctorate	$30,000
	(Under)Graduate Student(s)	$24,100
	Pre-baccalaureate Student(s)	$1,600
B. Fringe Benefits	34% RA; 5.9% GA; 1.0% Undergrads	$11,638
C. Expendable Materials and Supplies	Supplies for tissue culture and molecular analyses including greenhouse and computational/data supplies (approx. $500/month)	$6,000
D. Travel	PI and graduate student to present research at national meetings	$1,000
E. Subcontracts	None	$0
F. All Other Direct Costs	Sequencing, DNA primers, DNA synthesis, yearly service fee for one 600 sq. ft. greenhouse room.	$2,000
G. Total Amount of Request		**$76,338**

Justification: Funds are requested for 0.7 FTE research associate and a 0.5 FTE Graduate Student to perform molecular characterization, tissue culture and bioassays of transgenic soybean events. The pre-baccalaureate student will assist in the greenhouse care, nematode screening and routine lab maintenance.

Facilities and Equipment:

The Departments of Agronomy and Plant Pathology have state of the art equipment and facilities needed to perform the above projects. Dr. Trick's transformation facility has a biosafety containment level 1-P+ rating, which is at, or above CDC and NIH requirements for working with genetically engineered plants expressing the traits described above.

Cooperators:

Chris Little, KSU

Related work:

Dr. Trick's research interests pertain to the enhancement of Kansas crops by using biotechnological approaches to introduce novel traits. These traits include the protection against fungal pathogens and nematodes and the addition of value-added traits. Tim Todd's laboratory has significant experience with SCN both in the laboratory and field. Dr. Schapaugh's research focuses on soybean breeding. Specific aspects of his program includes developing high yielding, multiple pest resistant soybean varieties for full-season and double-crop production, developing soybean germplasm and cultivars resistant to soybean cyst nematode and to *M. phaseolina*, characterizing the influence of soybean cyst nematode resistance on nematode populations, developing procedures and strategies to improve the selection efficiency of important traits, and developing varieties for use in specialty markets, including: high protein, modified oil quality, and food uses.

Over the last three years KSC funding has allowed us to publish four manuscripts relating to our transgenic soybean research (Brady *et al.*, 2012; Lee *et al.*, 2011; Lee *et al.*, 2012; Li *et al.*, 2011). Funding from KSC has also helped leverage funds from both the United Soybean Board and the North Central Soybean Research Program. The USB grant (Trick and Todd) is evaluating a new approach called microRNAs to attempt to enhance silencing of the targeted genes discovered using KSC dollars. The NCSRP grant is in conjunction with University of Georgia, Ohio State University and the University of Illinois-Urbana upscale efforts in producing stable transgenic lines targeting a separate set of nematode genes.

Expected Outcome:

We anticipate the continued recovery of transgenic soybean plants expressing genes for fungal resistance proteins and nematode resistance. We expect to find some level of resistance to *F. virguliforme* (SDS), charcoal rot, and other fungal pathogens. However, the level of resistance of these plants will be unknown until these plants are challenged with the pathogens. Likewise we anticipate the recovery of nematode resistant lines of transgenic soybean. All projects above have the potential to reduce the negative impacts caused these pests and pathogens have on the soybean yield across the state and to increase the overall value of soybean.

The long-term outcome of this research will be the integration of these disease resistant traits into the soybean breeding program at KSU. Where intellectual property rights are involved, the Kansas State University Research Foundation will be advised and they will assist us in the transfer of technology to third parties.

References:

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