Final Project Report

Iowa Soybean Association

May 2, 2022

Project Title: "Stacking four plant genes to provide durable and enhanced SCN and SDS resistance in soybean"

Investigator: Madan K. Bhattacharyya
Agronomy Hall G303
Iowa State University
Ames, IA 50011
515-294-2505
mbhattac@iastate.edu

Overview: Soybean is the most important legume crop that provides both protein and oil. Soybean seeds contain approximately 40% protein and 20% oil. It is an important source of animal and fish feed in addition to its major role in human nutrition. In the United States, the average annual soybean yield is valued at around $40 billion. Unfortunately, 12-15% of its yield potential is suppressed annually by pathogen attacks. Among the soybean pathogens, *Heterodera glycines*, commonly known as soybean cyst nematode (SCN), and *Fusarium virguliforme* are two of the most serious soybean pathogens. *F. virguliforme* causes sudden death syndrome (SDS). Soybean suffers average annual yield suppression valued close to $2 billions from SCN and SDS diseases. Our ***long-term goal*** is to alleviate soybean yield suppression from these two most serious pathogens, SCN and *F. virguliforme*, by breeding novel SCN and SDS resistant soybean cultivars.

In this project, we proposed to evaluate the joint or combined effect of four transgenes in improving the SCN and SDS resistance of a soybean line. The four transgenes considered were identified in previous transgenic studies conducted in our laboratory with the support from Iowa Soybean Association, Consortium of Plant Biotechnology Research, and United States Department of Agriculture - National Institute of Food and Agriculture (USDA-NIFA). The four genes use distinct mechanisms to confer both SCN and SDS resistance, when overexpressed in transgenic soybean plants (e.g., Ngaki et al. 2021: Plant Biotechnology Journal 19: 502–516 https://onlinelibrary.wiley.com/doi/pdf/10.1111/pbi.13479; Kambakam et al. 2021: Plant Journal 107:1432-1446 https://onlinelibrary.wiley.com/doi/10.1111/tpj.15392). Of the four genes, two are from soybean and two are from *Arabidopsis thaliana*. The two soybean genes, *GmDR1* and *GmSAMT2,* encode a receptor-like protein and a salicylic acid methyl transferase, respectively. The two *Arabidopsis thaliana* genes, *PSS30* and *PSS25,* encode a folate transporter and a putative transcription factor, respectively.

We ***hypothesize*** that since the resistance mechanisms encoded by *PSS25, PSS30, GmDR1* and *GmSAMT2* are distinct, the functions of the four genes are complementary to each other and together they are expected to provide soybean with stable and robust resistances against both SCN and *F. virguiforme*.

The four transgenes selected for this study govern novel mechanisms for SCN and SDS resistances. Therefore, ***outcome*** of this proposed research is expected to be ***highly significant*** because the SCN and SDS resistances governed by these genes will complement the resistance mechanisms currently available in soybean cultivars. The project will therefore lead to development of soybean lines with robust resistance against two most serious soybean pathogens, SCN and *F. virguliforme*, that together suppress soybean yield valued at close to $2 billions. Furthermore, *GmDR1* also provides tolerances to soybean aphids and spider mites, which are serious pests of soybean (Ngaki et al. 2021: Plant Biotechnology Journal 19: 502–516 https://onlinelibrary.wiley.com/doi/pdf/10.1111/pbi.13479). Therefore, this project is expected to significantly improve the soybean growers' farm economy.

In this project, ***our goal*** was to stack all four transgenes into single soybean plants and then determine if the SCN and SDS resistances of the resultant soybean lines with all four transgenes are further improved.

We proposed to conduct following five objectives to accomplish the goal of this 3-year project.

1. **Objective 1.** Map the four fusion genes, *PSS25, PSS30, GmSAMT2* and *GmDR1,* among the transgenic soybean lines.

2. **Objective 2.** Identify Williams 82 lines that carry combinations of three fusion genes: (i) *PSS25, PSS30* and *GmDR1* and (ii) *PSS30, GmDR1* and *GmSAMT2.*
3. **Objective 3.** Identify Williams 82 lines that carry all four transgenes: *PSS25, PSS30, GmDR1* and *GmSAMT2.*
4. **Objective 4.** Evaluate Williams 82 lines carrying *PSS25, PSS30, GmDR1* and *GmSAMT2* fusion genes for resistance to *F. virguliforme.*
5. **Objective 5.** Evaluate Williams 82 lines carrying *PSS25, PSS30, GmDR1* and *GmSAMT2* fusion genes for resistance to *H. glycines.*

Here we report the progress made under each project during the entire project period starting October 1, 2019.

Objective 1. Map the four fusion genes, *PSS25, PSS30, GmSAMT2* and *GmDR1,* among the transgenic soybean lines.

In this objective, we have successfully mapped all four transgenes to be stacked into single plants in Year 1 of the project. Results confirmed that all four transgenes are unlinked and stacking them into a single plant is feasible.

Objective 2. Identify Williams 82 lines that carry combinations of three fusion genes: (i) *PSS25, PSS30* and *GmDR1* and (ii) *PSS30, GmDR1* and *GmSAMT2*.

In 2020, we reported the generation of the segregating lines for various combinations of transgenes. To identify the segregating lines carrying expected combinations of transgenes, we conducted polymerase chain termination reactions (PCR) using synthesized oligo-nucleotide primers, specific to the transgenes. In the previous reports, we mentioned the identification of 335 plants carrying various combinations of transgenes. We also reported that among these plants, 37 contained four transgenes and 74 carried combinations of two or three genes. We harvested seeds from these plants in the greenhouse between April and July 2021. Seeds of 40 transgenic lines, harvested before the first week of June, 2021, were planted along with control nontransgenic cultivars in the field this summer, 2021 to determine the responses of the transgenic soybean lines carrying combinations of transgenes to *F. virguliforme* (Table 1).

Table 1. The transgenic and nontransgenic lines planted in three blocks of a field located at the ISU Horticulture Research Station, Ames, IA during the summer of 2021. *F. virguliforme* inoculum was added to the rows during planting to determine the responses of the transgenic soybean lines against the infection with the SDS pathogen. The numbers under three blocks are plot numbers, in which seeds of individual lines were sown.

Serial No.	Fusion Gene(s)	Line Name	Block 1	Block 2	Block 3
Transgenic Lines					
1	Prom1-PSS25	ST306-3-2-186	13	125	221
2	Prom1-PSS25	ST306-14-6-131	16	111	216
3	Prom2-PSS25	ST307-12-1-312	75	146	223
4	Prom2-PSS25	ST307-17-6-308	32	149	182
5	Prom2-PSS25	ST307-14-2-35	39	116	195
6	Prom2-PSS25	ST307-12-6-9	41	83	151
7	Prom2-DR1	Prom2-DR1-24	53	89	225
8	Prom3-DR1	Prom3-DR1-4	51	97	160
9	Prom3-DR1	Prom3-DR1-12	61	101	163
10	Prom1-SAMT2	Prom1-SAMT2-5	28	88	220
11	Prom3-SAMT2	Prom3-SAMT2-19	27	147	171
12	35S-Pss30	35S-Pss30-16-1	74	82	152
13	Prom2-Pss30	Prom2-Pss30-5-7	70	93	174
14	Prom2-Pss30	Prom2-Pss30-5-480	47	102	207
15	Prom2-Pss25	Prom2-Pss25-33	73	119	164
16	Prom3-DR1/Prom2-Pss30	Prom3-DR1/Prom2-Pss30-HM37	65	144	167

17	Prom3-DR1/Prom2-Pss30	Prom3-DR1/Prom2-Pss30-HM47	58	132	183
18	Prom3-DR1/Prom2-Pss30	Prom3-DR1/Prom2-Pss30-HT54	15	108	198
19	Prom3-SAT2/Prom2-Pss25	Prom3-SAMT2/Prom2-Pss25-84	72	122	179
20	Prom3-SAMT2/Prom2-Pss25	Prom3-SAMT2/Prom2-Pss25-86	33	76	217
21	Prom3-SAMT2/Prom2-Pss25	Prom3-SAMT2/Prom2-Pss25-87	4	143	181
22	Prom3-SAMT2/Prom2-Pss25	Prom3-SAMT2/Prom2-Pss25-89	36	86	165
23	Prom1-SAMT2/Prom2-Pss25	Prom1-SAMT2/Prom2-Pss25-93	21	98	209
24	Prom1-SAMT2/Prom2-Pss25	Prom1-SAMT2/Prom2-Pss25-94	1	85	224
25	Prom3-DR1/Prom2-Pss30/Prom2-Pss25	Prom3-DR1/Prom2-Pss30/Prom2-Pss25-12	56	131	212
26	Prom3-DR1/Prom2-Pss30/Prom2-Pss25	Prom3-DR1/Prom2-Pss30/Prom2-Pss25-115	24	145	204
27	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2-41	20	126	190
28	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2-118	67	80	154
29	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2-106	9	118	210
30	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2-221	57	81	173
31	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2-39	35	112	153
32	Prom2-Pss30/Prom2-Pss25/Prom3-SAMT2	Prom2-Pss30/Prom2-Pss25/Prom3-SAMT2-150	52	103	178
33	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -109	60	95	187
34	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -34	8	130	194
35	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -36	44	84	155
36	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -40	25	148	176
37	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -42	29	113	168
38	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -108	71	78	189
39	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2-120	54	133	175
40	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -128	17	120	192
Non-transgenic Lines					

41	None	Williams 82-1	26	137	172
42	None	Williams 82-2	30	134	205
43	None	Williams 82-3	62	127	177
44	None	Williams 82-4	31	96	170
45	None	Williams 82-5	63	100	200
46	None	Spencer-1	14	90	188
47	None	Spencer-2	69	92	166
48	None	Spencer-3	38	104	186
49	None	MN1606-1	10	114	197
50	None	MN1606-2	43	150	191
51	None	MN1606-3	45	105	203

Williams 82 and Spencer are SDS susceptible cultivars; MN1606 is an SDS resistant cultivar.

Stacked Lines Carrying Two Transgenes:

We harvested seeds from at least eight individual F_3 plants from each of the F_3 homozygous families, viz., 84, 86, 87, and 89 carrying *Prom2-PSS25* and *Prom3-GmSAMT2*, and 93 and 94 carrying *Prom2-PSS25* x *Prom1-GmSAMT2*. The F_4 plants homozygous for both *PSS25* and *GmSAMT2* genes were grown during this summer for SDS resistance, along with the three F_4 lines carrying the two transgenes *Prom3-DS1* and *Prom2-Pss30* identified and analyzed in the summer of 2020 (Table 2). The plot numbers for these lines are presented in Table 1.

Table 2. Nine F_4 lines carrying a combination of two transgenes, *Prom3-DS1*/*Prom2-Pss30*, *Prom2-PSS25/Prom3-SAMT2,* and *Prom2-PSS25/Prom1-SAMT2* transgenes, grown in the field during summer 2021.

Serial No.	Stacked Fusion Genes	Line Name
1	Prom3-DS1/Prom2-Pss30	Prom3-DS1/Prom2-Pss30-HM37
2	Prom3-DS1/Prom2-Pss30	Prom3-DS1/Prom2-Pss30-HM47
3	Prom3-DS1/Prom2-Pss30	Prom3-DS1/Prom2-Pss30-HT54
4	Prom3-SAMT2/Prom2-Pss25	Prom3-SAMT2/Prom2-Pss25-84
5	Prom3-SAMT2/Prom2-Pss25	Prom3-SAMT2/Prom2-Pss25-86
6	Prom3-SAMT2/Prom2-Pss25	Prom3-SAMT2/Prom2-Pss25-87
7	Prom3-SAMT2/Prom2-Pss25	Prom3-SAMT2/Prom2-Pss25-89
8	Prom1-SAMT2/Prom2-Pss25	Prom1-SAMT2/Prom2-Pss25-93
9	Prom1-SAMT2/Prom2-Pss25	Prom1-SAMT2/Prom2-Pss25-94

Stacked Lines Carrying Three Transgenes:

In 2020, we identified F_3 plants carrying a combination of three transgenes. Eighteen of those lines including five carrying *Prom3-GmDR1, Prom2-PSS30* and *Prom2-PSS25* transgenes; 11 carrying *Prom3-GmDR1, Prom2-PSS30* and *Prom3-GmSAMT2*; 19 plants harboring *Prom2-PSS25, Prom3-GmDR1* and *Prom3-GmSAMT2*; and two carrying *Prom2-PSS30, Prom2-PSS25* and *Prom3-GmSAMT2* transgenes were planted in the field to evaluate their responses to *F. virguliforme* (Table 3). Thirteen F_3 plants carrying a combination of three transgenes from *Prom2-PSS30, Prom3-GmDR1*, *Prom3-SAMT2*, and *Prom2-PSS25* were also grown in the field for SDS infection and/or seed increase (Table 4).

Table 3. Eighteen F_4 plants carrying combinations of three transgenes were planted in the field to determine their responses to *F. virguliforme* infection SDS infection.

Serial No.	Sacked Fusion Genes	Line Name
1	Prom3-DR1/Prom2-Pss30/Prom2-Pss25	Prom3-DR1/Prom2-Pss30/Prom2-Pss25-115
2	Prom3-DR1/Prom2-Pss30/Prom2-Pss25	Prom3-DR1/Prom2-Pss30/Prom2-Pss25-124

3	Prom3-DR1/Prom2-Pss30/Prom2-Pss25	Prom3-DR1/Prom2-Pss30/Prom2-Pss25-126
4	Prom3-DR1/Prom2-Pss30/Prom2-Pss25	Prom3-DR1/Prom2-Pss30/Prom2-Pss25-132
5	Prom3-DR1/Prom2-Pss30/Prom2-Pss25	Prom3-DR1/Prom2-Pss30/Prom2-Pss25-37
6	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2-176
7	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2-214
8	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2-221
9	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2-221
10	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2-223
11	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2-238
12	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2	Prom3-DR1/Prom2-Pss30/Prom3-SAMT2-39
13	Prom3-DR1/Prom2-PSS25/Prom3-SAMT2	Prom3-DR1/Prom2-PSS25/Prom3-SAMT2 -100
14	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2-106
15	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2-118
16	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2	Prom3-DR1/Prom2-Pss25/Prom3-SAMT2-41
17	Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -149
18	Prom2-Pss30/Prom2-Pss25/Prom3-SAMT2	Prom2-Pss30/Prom2-Pss25/Prom3-SAMT2-150

Table 4. Thirteen F_3 plants carrying a combination of three transgenes from *Prom2-PSS30, Prom3-GmDR1*, *Prom3-SAMT2*, and *Prom2-PSS25* were grown in the field for SDS infection and/or seed increase.

Serial No.	Fusion Gene	Line
1	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -102
2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -108
3	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -109
4	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -128
5	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -15
6	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -28
7	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -30
8	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -31
9	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -34
10	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -36
11	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -40
12	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2 -42
13	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2	Prom3-GmDR1/Prom2-PSS30/Prom2-PSS25/Prom3-SAMT2-120

Objective 3. Identify Williams 82 lines that carry all four fusion transgenes: Prom2-*PSS25, Prom2-PSS30, GmDR1* and *GmSAMT2.*

In our original proposal, we proposed to stack two combinations of four genes; viz., (i) *PSS25, PSS30* and *GmDR1* and (ii) *PSS30, GmDR1* and *GmSAMT2*. In Year 2 of the project, we added this objective to stack all four transgenes in single plants. To obtain all four transgenes in one transgenic plant, we generated two F_1 plants carrying either (i) *Prom3-DS1* and *Prom2-Pss30* or (ii) *Prom2*-PSS25 and *Prom3-SAMT2* genes.

In 2020, we reported three F_1 plants that carry all four transgenes: *PSS25, PSS30, GmDR1* and *GmSAMT2* developed by crossing the two F_1s. F_2 seeds of these plants were planted in two greenhouses to raise the F_2s. The F_2 plants were evaluated by conducting PCR to identify plants carrying all four transgenes. Thirty-eight plants carrying all four transgenes were identified. From April to July 2021, seeds from these lines were harvested. Seeds of eight lines were randomly planted in three blocks of the field (Table 1). At least 30 seeds of each line were planted in individual rows of each block. For the rest five lines, the number of seeds were low and were only grown with other eight lines in the fourth block just for seed increase.

To identify the individual plants carrying all four transgenes in homozygous condition, leaf samples were collected from randomly selected over 800 single plants. The pods of these selected plants were then harvested at maturity.

In November and December of 2021, the seeds of the harvested pods were threshed and stored in cold room in individual seed envelopes.

In January and February, DNA was prepared from 700 of the 800 leaf samples harvested earlier in the summer of 2021. We conducted molecular analysis for identifying the plants containing all four fusion transgenes. In four-gene segregation with independent assortment (genes are not linked, segregate independently), screening of a large segregating population of 256 individuals is required for identifying a single individual with all four genes in homozygous condition with a low probability. From screening 800 F_2 plants, we expect to obtain only three homozygous F_2 plants with an approximately 95% certainty of finding

a single homozygous plant for all four transgenes. In February, we analyzed DNA of the first batch of 96 plants (Plate # 1 in Table 5) individually for each of the four transgenes, *Prom2-PSS30, Prom3-GmDR1*, *Prom3-SAMT2*, and *Prom2-PSS25,* and results are presented in Table 5.

Table 5. Lines carrying all four transgenes, *PSS25, PSS30, GmDR1* and *GmSAMT2* identified by PCR. Y, indicates the presence of the specific transgene. Plate 1, the first group of 96 genotypes.

Plate 1	TRANSGENES				Plate 1	TRANSGENES			
Plant #	GmDR1	PSS30	PSS25	SAMT2	Plant #	GmDR1	PSS30	PSS25	SAMT2
1		y	y		49	y	y	y	y
2	y	y	y	y	50	y	y		y
3	y	y	y	y	51	y	y	y	y
4		y		y	52	y	y	y	y
5		y		y	53	y	y	y	y
6		y	y	y	54	y	y	y	y
7	y	y	y	y	55	y	y		y
8	y	y	y	y	56	y	y	y	y
9	y	y	y	y	57	y	y	y	y
10	y	y	y	y	58		y		y
11		y	y		59	y	y	y	y
12					60		y	y	y
13	y	y		y	61		y		y
14					62	y	y	y	y
15	y	y	y	y	63	y	y	y	y
16	y	y	y	y	64	y	y		y
17	y	y		y	65	y	y		y
18	y	y	y	y	66	y	y	y	y
19	y	y	y	y	67	y	y		y
20		y		y	68		y		y
21	y	y		y	69		y		y
22	y	y		y	70	y	y		y
23					71	y	y		y
24					72		y	y	y
25		y	y	y	73		y	y	
26	y	y	y	y	74		y		y
27	y	y	y	y	75	y	y	y	y
28	y	y	y	y	76	y	y		y
29	y	y	y	y	77	y	y	y	y
30	y	y		y	78				
31	y	y	y	y	79	y	y		y
32	y	y	y	y	80	y	y	y	y
33		y	y	y	81	y	y	y	y
34		y	y	y	82	y	y	y	y
35	y	y	y	y	83	y	y		y
36		y		y	84				y
37		y	y	y	85				
38	y	y	y	y	86	y		y	y
39	y	y		y	87	y	y	y	y
40	y	y	y	y	88	y	y	y	y
41		y	y	y	89	y	y		y
42		y	y	y	90	y	y		y
43		y	y	y	91	y	y	y	y
44	y	y		y	92		y	y	y
45	y	y		y	93	y	y	y	y
46	y	y			94	y	y	y	y
47	y	y	y	y	95	y	y		y
48		y	y	y	96				

Nearly half (40 plants) of the 96 plants showed PCR amplification for all four transgenes. Of the 40 plants positive for all four transgenes, 32 showing strong PCR amplification for all four transgenes were selected for further investigation. The selected 32 plants are shown with red font in Table 5.

Objective 4. Evaluate Williams 82 lines carrying *PSS25, PSS30, GmDR1* and *GmSAMT2* fusion genes for resistance to *F. virguliforme.*

In our original proposal, we planned to generate lines containing only three fusion genes. We expanded the scope of the project and generated lines that carry all four fusion transgenes. Lines carrying two or three fusion genes along with population segregating all four fusion genes were planted the summer of 2021 in the field to evaluate their responses against *F. virguliforme* infection (Table 1). Unfortunately, acceptable SDS symptoms were not developed in the experimental plot.

This year, the soybean growing season was very dry. We continuously irrigated the plot from the 4th week of August when the soybean lines just reached the R3 stage (starting to form pods). We irrigated twice a day, ¼ inch water in the mid-day and 1/4th inch water at dusk. The soil was muddy and moist with high humidity. Despite our effort of maintaining the ideal condition for SDS foliar disease development, the disease appeared only in a limited number of plants of some of the rows. The scored data are not meaningful to report. This is the first time we failed to observe any meaningful SDS foliar disease development since 2013, the year we started to conduct field trial for SDS resistance screening.

Objective 5. Evaluate Williams 82 lines carrying *PSS25, PSS30, GmDR1* and *GmSAMT2* fusion genes for resistance to *H. glycines*

We have evaluated transgenic soybean lines carrying both *PSS30* and *GmDR1* for responses to *H. glycines* infection. The lines carrying both genes were more tolerant to the pathogen than lines carrying either of the transgenes.

Of the selected 32 plants with strong PCR amplifications for all four transgenes in Objective 3 (Table 5), 19 were selected randomly for responses to SCN infection. For each genotype, six seedlings were individually infected with SCN. Thirty days following sowing soybean seeds in soil containing SCN cysts, the numbers of the newly developed cysts on soybean roots of individual plants were counted under a microscope by Madeline Thomson, an undergraduate student. A total of 79 plants representing the selected 19 random genotypes (Table 5), containing all four transgenes along with four plants of A95-684043 (A95), an SCN resistant control line, and six plants of transgenes recipient nontransgenic Williams 82 line were investigated for responses to SCN infection and results are presented in Figure 1.

The important take-home message from this study is that we were able to identify 17 individual segregants (Figure 2) from nine lines carrying all four transgenes (Figure 2) that are highly SCN resistant. We identified five plants (Plant # 29, 63, 66, 80, and 94) that exhibited two or three progenies with FI below 20. Progenies of these five plants will be grown to develop the next generation for identifying lines homozygous for all four genes. These lines will also be tested for possible SDS resistance in the field this summer; and in 2023 through 2025, if we can secure a grant.

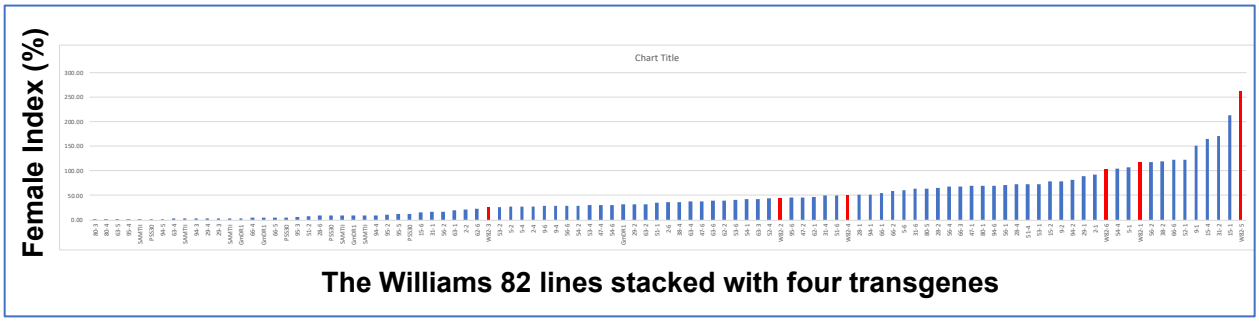

Figure 1. Responses of 79 progenies from 19 plants positive for all four transgenes to SCN 30 days following sowing of soybean seeds in SCN HG Type 2.5.7-infected soil. The bars indicate the extent of SCN cysts presented in female indices (FI) (female index is expressed in percentage cyst number of a genotype over the average cyst number of six Williams 82 nontransgenic plants). The red bars represent

FI of Williams 82 plants. Note that many progenies showed very small FI values suitable for protecting soybean against SCN. These lines are presented in Figure 2.

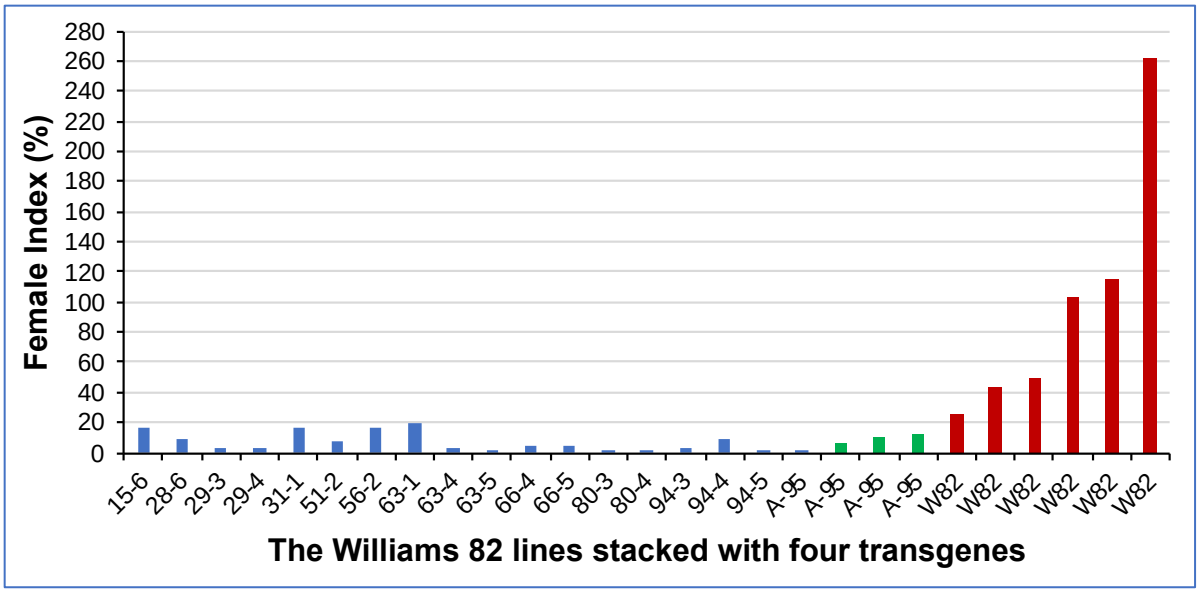

Figure 2. Responses of 17 progenies from nine plants carrying all transgenes. Female indices of soybean plants infected with the SCN HG Type 2.5.7 were calculated 30 days following sowing the seeds in SCN infected soil. Individual plants of transgenic lines carrying all four transgenes are shown with blue bars, those of the resistant check A95-684043 (A95) with green bars, and that of the SCN susceptible transgenes recipient line Williams 82 are shown with red bars. Each bar represents response of one plant.

Key Performance Indicators/Performance Metrics: We expect to accomplish in Year 3 of the project the following.

1. The F_1 and F_2 populations will be evaluated and lines with either two, three or four transgenes will be generated.
   Self-evaluation: We have identified soybean genotypes carrying either two, three or four fusion gene combinations. Seeds of these plants were harvested.

2. Levels of SCN and SDS resistances of lines carrying 2, 3 or 4 fusion gene combinations will be known.
   Self-evaluation: Responses of lines carrying two fusion genes to *F. virguliforme* and SCN have been reported earlier. We failed to observe the foliar SDS symptoms among the transgenic lines in the summer of 2021. We however plan to identify the homozygous lines for 2, 3 and 4 gene combinations and then evaluated them for their responses against *F. virguliforme* and SCN under the support of a possible support of renewal proposal.

Economic Impact/Significance

In the U.S., the total annual soybean yield suppression from SDS and SCN is approximately $1.8 billion. Even if we can reduce the SDS and SCN incidence by 20% through cultivation of novel SDS and SCN resistant cultivars to be generated from the outcomes of this project, we can expect to have significant increase in the annual soybean yield values close to $360 million in U.S. and approximately $50 million in Iowa.

Timelines and Milestone for Deliverables: Timelines and milestones for deliverables are:

1. We will deliver the seeds of genotypes carrying all four transgenes by May, 2021.
   Self-evaluation: Seeds of thirteen genotypes carrying all four transgenes or fusion genes were generated in greenhouse and planted in the field in the summer of 2021.

2. SCN and SDS resistances of lines carrying 2, 3 or 4 fusion gene combinations will be known by September, 2021.
   Self-evaluation: We have shown earlier that two genes further enhance SDS and SCN resistance due to complementary effects between the two transgenes. We failed to get the foliar SDS symptoms in 2021 despite we irrigated routinely during the reproductive phase starting R3 stage. The season was very dry and we observed only sporadic foliar symptoms among the transgenic lines. We have shown that 17 progenies of nine lines carrying all four transgenes exhibited enhanced SCN resistance to level, which is comparable to that of an SCN resistant soybean cultivar. Progenies of three of the nine lines showed very few SCN cysts.