Objective 1: Develop efficient PAMless Cas9 and Prime Editing platforms for soybean.
This is a gene editing tool development objective that builds upon the CRISPR-Cas9 gene editing platform that we previously developed.

Building a Prime Editing system for soybean.
As we previously reported, the application of prime editing has proved to be more challenging in soybean than anticipated. Recently, successful prime editing was reported for two dicotyledonous plant species for the first time. These species are tomato and Arabidopsis thaliana. We have revised our approach to prime editing in soybean based on this new report, and six new prime editing constructs were designed and built for testing efficacy of editing in soybean hairy roots:

1. AtUbiPro-nCas9-RT3(M-MLV)-MASpro-eGFP-pegFAD2A
2. AtUbiPro-nCas9-RT5(M-MLV)-MASpro-eGFP-pegFAD2A
3. AtUbiPro-nCas9-RT2(SPV)-MASpro-eGFP-pegFAD2A
4. AtUbiPro-nCas9-RT2(CaMV)-MASpro-eGFP-pegFAD2A
5. AtUbiPro-nCas9-RT5(CaMV)-MASpro-eGFP-pegFAD2A
6. AtUbiPro-nCas9-RT2(SCMV)-MASpro-eGFP-pegFAD2A

These constructs have different RT (reverse transcriptase) components as defined here:
RT3(M-MLV) – Moloney Murine Leukemia Virus Reverse Transcriptase (engineered version 3)
RT5(M-MLV) – Moloney Murine Leukemia Virus Reverse Transcriptase (engineered version 5)
RT2(SPV) – Soybean Putnam Virus Reverse Transcriptase (engineered version 2)
RT2(CaMV) – Cauliflower Mosaic Virus Reverse Transcriptase (engineered version 2)
RT5(CaMV) - Cauliflower Mosaic Virus Reverse Transcriptase (engineered version 2)
RT2(SCMV) - Soybean Chlorotic Mottle Virus Reverse Transcriptase (engineered version 2)

These constructs have been made and their sequences were confirmed. Each construct has been introduced into the *Agrobacterium rhizogenes* strain K599, and the hairy root transformation is in progress. The next plan is to collect the GFP positive hairy roots (those roots that carry the construct), and genotype them for prime editing efficiency by deep sequencing the PCR amplicons at the target site in the GmFAD2A gene.

Objective 2: Apply base editing and Prime Editing to modify genes affecting soybean responses to drought.

No progress to report this period, because we are revisiting the prime editing approaches needed to carry out this objective.

Objective 3: Application of CRISPR-Cas-based gene editing to identify genes that are critical for SDS resistance in soybean.
We have reported earlier that overexpression of *GmDR1* enhances broad-spectrum resistance against two soybean pathogens and two pests including *Fusarium virguliforme* that causes sudden death syndrome (SDS). Our results suggested that enhanced resistance against *F.*

virguliforme in plants overexpressing *GmDR1* is mediated by a number of genes including those that encode disease resistance-like receptors, receptor-like kinase, and WRKY transcription factor. The rationale of the proposed study is that once we establish that overexpressed *GmDR1* mediates defense functions by regulating the expression of genes encoding disease resistance-like receptor proteins, receptor kinases and a transcription factor, it will be feasible to utilize these genes in enhancing SDS resistance in soybean. At the end of the three-year project period, we expect to establish the defense functions for six signaling and regulatory genes. Once we establish the role of these genes in SDS resistance, one could use these as markers in breeding soybean for SDS resistance. We have generated CRISPR-Cas9 DNA constructs, using resources optimized for soybean, to knockout six target genes for determining their role in defense responses. The egg cell-specific promoter that we demonstrated to work well in expressing Cas9 in soybean has been used in generating the constructs. The constructs will be evaluated in hairy root assays prior to time consuming stable soybean transformation. It has been shown that multiple genes can be mutated simultaneously in one plant through CRISPR-Cas9 system. We will determine if all six genes can be knocked out in hairy root assays. If we are successful, then we will generate stable transgenic soybean lines to knock-out all six target genes. The stable transgenic mutant plants will be evaluated for responses to *F. virguliforme*, *P. sojae*, and SCN infections.

Selected genes and construction of CAS9 vectors

Based on our earlier RNA-seq and qRT-PCR results, nine genes were selected for being knocked out to investigate their involvement in soybean immunity against *F. virguliforme* (Table 1): four encode disease resistance-like receptors leucine-rich repeat (LRR), two encode the LRR receptor kinases, and 3 are encode regulatory genes. Next, primers were designed for the guide-RNA (gRNA) of each of the selected genes using the Iowa State University Crop Bioengineering Consortium's CRISPR Genome Analysis Tool http://cbc.gdcb.iastate.edu/cgat/ (Zheng et al., 2020).

Table 1. Constructs created for selected genes

Constructs #	Genes	# of units	pAtgRNA expression vector used (each gene in 1 vector)
1	4 LRR (all As)	4	pAtgRNA1, 2, 3, 4T
2	2 kinases (all Bs)	2	pAtgRNA1 and 2T
3	WRKY DNA -binding domain (C1)	2	pAtgRNA1 and 2T
4	NAD(P)-linked oxidoreductase (C2)	2	pAtgRNA1 and 2T
5	F-box family protein (C3)	2	pAtgRNA1 and 2T
6	3+4+5	6	pAtgRNA1, 2, 3, 4, 5, 6
7	1+2	6	pAtgRNA1, 2, 3, 4, 5, 6

We created seven constructs to knock out the selected genes in various combinations as shown in the Table 1. After cloning each individual CRISPR guide RNA spacer sequence into pAtgRNA expression vector, the constructs were assembled into pENTR4-ccdB vectors using the Golden Gate-cloning technology. Each of the constructs were sequenced to confirm the identity of each of the seven constructs. Each construct was transferred into two different binary vectors using the LR Gateway cloning system to obtain the following two plant expression vectors:

1. P1300-2X35S-Cas9-ccdB (vector A) for the generation of soybean hairy roots in order to check the success of knocking out the genes.
2. P1300-AtEC-Cas9-GFP-ccdB (vector B) for the generation of stable soybean transgenic lines.

Except for construct # 7, all the 6 other constructs have been cloned in both binary vectors and transferred to *Agrobacterium rhizogenes k599* for soybean hairy roots, and to *A. tumefaciens EH105* for production of stable soybean transgenic plants (Table 2).

Table 2. Status of the constructs

Constructs #	# of units	In pENTR4-ccdB	In vector A	In vector B	In *A. rhizogenes*	In *A. tumefaciens EH105*
1	4	yes	yes	yes	yes	yes
2	2	yes	yes	yes	yes	yes
3	2	yes	yes	yes	yes	yes
4	2	yes	yes	yes	yes	yes
5	2	yes	yes	yes	yes	yes
6	6	yes	yes	yes	yes	yes
7	6	no	no	no	no	no

Stable Transformation of Soybean

We have conducted stable soybean transformation for the six constructs (Table 3). Five constructs are at the stage of shoot induction and will go soon to shoot elongation. Our goal is to have at least 240 explants inoculated for each construct. We faced contamination at the beginning (rows in grey).

Table 3. Soybean transformation schedule in 2023.

Constructs #	# of units	Co-cultivation media	Shoot Induction I	Shoot Induction II	Shoot Elongation (SE)
1	4	16-Feb			
		22-Mar	27-Mar	10-Apr	24-Apr
		30-Mar	3-Apr	17-Apr	1-May
		(AGL-1T)			
2	2				
3	2	13-Feb	3-Mar	17-Mar	
		1-Mar	6-Mar	21-Mar	4-Apr
		7-Mar	10-Mar	24-Mar	7-Apr
		31-Mar			
4	2	(EH105)			
		16-Feb	20-Feb	6-Mar	
		24-Feb	28-Feb		
		16-Mar	21-Mar	4-Apr	18-Apr
5	2	(EH105)			
		1-Mar	6-Mar	20-Mar	3-Apr
		8-Mar	13-Mar	27-Mar	10-Apr
		10-Mar	15-Mar	29-Mar	12-Apr

		15-Mar	20-Mar	3-Apr	17-Apr
6	6	(EH105-T)			
		10-Feb	14-Feb	28-Feb	13-Apr
		15-Mar	20-Mar	3-Apr	17-Apr
		23-Mar	28-Mar	11-Apr	25-Apr
		29-Mar	2-Apr	16-Apr	30-Apr
		(EH105-T)			

Green color: done; white: yet to be done; grey: discarded.

Progress on Objective 3 since last report:

The progress made in the last two months after January 30, 2024, is presented below.

Previously, we reported that a T_0 transgenic plant carrying a construct designed to knockout two *GmWRKY* genes was generated. PCR analysis using forward primer from the gene-specific and reverse pCR8-R primer from the pAtgRNA 1 and 2T vector confirmed the presence of the transgene in the transformant. We harvested 58 seeds from this plant. The first five plants (from the first two pods) were analyzed by sequencing PCR products of the target regions of two *GmWRKY* genes. Unfortunately, none of the five T_1 plants carried any mutations at either of the two gRNA target sites. The remaining 53 seedlings are now at the unifoliate leaves and will be analyzed to determine if there is any mutation among these T_1 progenies. These seedlings will first be screened for resistance to basta herbicide and the PCR analysis for the basta resistance gene to confirm that they are not generated from a non-transgenic chimeric branch. Selected progenies carrying the transgene will then be evaluated for the presence of the mutation in the *Glyma.04G223200* gene.

We now have developed transformants for additional constructs, N1 (*TNLR* and *kinase* genes), N2 (eight *TNLR* genes), TF (six transcription factor genes) and DR1 (*GmDR1*) (Tables 1, 4 and Figure 1).

Figure 1. Putative T_0 transgenic plants carrying N1, N2, and DR1 constructs being grown in soil following root development.

More putative transgenic plants from additional constructs are being developed and are at different stages of transformation on plates. Table 4 below gives a summary of the transformation events in various stages. Examples of additional plants transformed with the N1, N2, and DR1 constructs that are forming roots in rooting medium are shown in Figure 2.

Table 4. The number of transformants at different stages of the transformation process.

Construct	Shoot elongation	Rooting	Soil
N1	7	3	2
N2	10	3	3
N3	5	0	0
TF	23	2	0
DR1	15	6	1

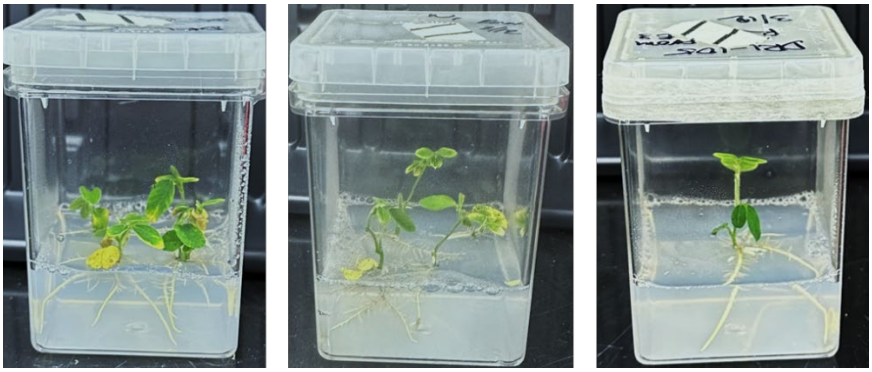

Figure 2. The eight of 14 transformants (Table 4) with developed roots in rooting medium. Plants in rooting media (photo taken on March 29, 2024) are from the N1, N2, and DR1 constructs (Table 1).