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.
Three prime editing systems have been made for soybean hairy root and stable transformation and genome editing based on two different variants of SpCas9 nickase and reverse transcriptase of M-MLV. The three systems, which are named PE1, PE2, and PE3 were used separately to make prime editing constructs targeting soybean genes encoding CDPK47, CDPK48, CDPK49 and CDPK50. The PE1 and PE2 systems were compared to determine which one is best for creation of precise genetic changes for improved traits in soybean. Unfortunately, these two systems were not effective in creating mutations in the four CDPK genes in hairy roots. Therefore, we decided to test additional genes, FAD2 and EPSPS, using the PE2 system, and again, did not find evidence that the target genes were modified. A third prime editing version, named PE3, was also tested for ability to edit the FAD2 and EPSPS genes in hairy roots and this was also unsuccessful.

The PE1, PE2, and PE3 prime editing constructs do not appear to be functional in soybean, so we are taking alternative approaches to modify the vectors to produce the prime editing guide RNAs using different strategies. These constructs will be tested during the next reporting period. In summary, the application of prime editing in soybean is not efficient using strategies that have worked in other plants. We continue to work to identify a prime editing strategy that will be efficient in soybean.

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

1. We have designed two different CRISPR-Cas9 constructs to knockout the function of CDPK genes that are predicted to affect soybean responses to drought.

CRISPR-Cas9 based gene knockout of the soybean CDPK family genes (CDPK47, 48, 49, and 50) Two CRISPR constructs (NK44, and NK46) have been built to knockout two combinations of CDPK genes.

a. NK44: pAtEC-Incas9-gCDPK49-50 (Targeting CDPK49 and CDPK50)

b. NK46: pAtEC-Incas9-gCDPK47-50 (Targeting CDPK47, CDPk48, CDPK49 and CDPK50)

Soybean transformation was performed with these two constructs and regenerated plants were genotyped for the presence of the transgenes. We have obtained a total of four transgene positive plants for the NK44 construct. We obtained a total of seven transgene positive plants for the NK46 construct. Seeds were harvested from these 11 transgenic plants, and we refer to these seed as the T1 generation. At least 24 T1 seedlings were germinated for each line, and we conducted PCR to first establish that the NK44 or NK46 constructs were inherited, and we

tested if any of the plants carried mutations in the target genes. Unfortunately, we found that the constructs were either not inherited or if they were inherited they carried deletions that rendered them ineffective. In line with these observations, no mutations were detected in the target genes. We hypothesize that soybean may not tolerate loss of function of these genes, and we are investigating alternative approaches to test roles of these genes in soybean defense and stress responses.

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	no
7	6	no	no	no	no	no

Progress on Objective 3 since last report:

We were able to develop a transgenic soybean plant carrying the construct #3 designed to knockout two *GmWRKY* genes (Table 1). This plant is now growing in the growth chamber and being analyzed for the presence of any possible mutations in the *GmWRKY* factor gene. Genomic DNA was extracted from the leaves of the candidate transgenic line and analyzed by conducting PCR. The forward primer from the gene and reverse pCR8-R primer from the pAtgRNA 1 and 2T vector were used in PCR (Table 3). The presence of PCR products demonstrated the presence of the intact construct #3 and thus successful transformation. We also conducted the restriction digestion of the PCR products of the target regions of two *GmWRKY* genes amplified by gene-specific primers. Digestion was done either with *Alu*I or with *Msp*JI. The observed bands indicated that there might be a mutation in gene 1 (*Glyma.04G223200*) because there is a faint un-digested larger band for one of the WRKY genes. The promoter fused to Cas9 is egg-cell specific and therefore we expect to have mutations in the WRKY genes in the seeds that are being developed in the transgenic plant.

Table 3. Primers used to characterize the transgenic plant.

Name	**Sequence**	**Origin**
1. 3200-Fw1	GGACAAGGACAGCATAGCTGG	Soybean Glyma.04G223200, CRISPR guide RNA spacer sequence
2. 3200-Rev1	CCAGCTATGCTGTCCTTGTCC	Soybean Glyma.04G223200, CRISPR guide RNA spacer sequence
3. 3200-Fw2	GCTAGTCTAATGCAGGAATGG	Soybean Glyma.04G223200, CRISPR guide RNA spacer sequence
4. 3200-Rev2	CCATTCCTGCATTAGACTAGC	Soybean Glyma.04G223200, CRISPR guide RNA spacer sequence
5. pCR8-R	5'-TGTTGTGGTGTGTAGGGACAG-3'	from the pAtgRNA vector

We have developed additional gene editing constructs, which are summarized below in Table 4. We created 5 constructs in total (N1, N2, N3, TF, and DR1) (Table 4). These constructs are being used in creating transgenic soybean lines (Table 5) using recently harvested soybean seeds. We have observed that the new seeds are highly responsive, and we already have several explants in the "Shoot Elongation 3" medium (Table 5). In summary, we have developed a transgenic soybean line using old seeds that were not very responsive to the transformation process and were frequently contaminated. Recently harvested soybean seeds are showing good responses to the transformation process, and we expect to obtain mutants for most of the target genes selected for this study.

Table 4. Five constructs created for mutating Kinase, LRR genes and transcription factors.

Gene name	Annotation	Constructs name (number of units)
LRR-receptor kinases		
Glyma.18G254000	LRR Receptor-Like Protein Kinase	1 construct **N3** (4 units)
Glyma.16G186600	LRR Receptor-Like Protein Kinase	
Glyma.16G184200	LRR Receptor-Like Protein Kinase	
Glyma.16G182866	LRR receptor-like Ser/Thr- Protein kinase GSO1	
Glyma.14G118800	LRR receptor-like Ser/Thr- Protein Kinase	
NBS-LRR-type Disease Resistance Receptors		
Glyma.16G137600	TIR-NBS-LRR class	1 construct **N2** (6 units)
Glyma.16G137000	TIR-NBS-LRR class	
Glyma.16G136600	TIR-NBS-LRR class	
Glyma.12G135600	TIR-NBS-LRR class	
Glyma.11G153000	TIR-NBS-LRR class	
Glyma.03G053602	TIR-NBS-LRR	
Glyma.03G053500	TIR-NBS-LRR	
Glyma.03G048200	TIR-NBS-LRR	
Transcription factors		
Glyma.03G042700	WRKY DNA-binding protein	1 construct **T** (6 units)
Glyma.18G256500	WRKY DNA-binding protein	
Glyma.04G238300	WRKY DNA-binding protein	
Glyma.04G223300	WRKY DNA-binding protein	
Glyma.04G223200	WRKY DNA-binding protein	
Glyma.04G057700	integrase-type DNA-binding superfamily protein	
Kinases + LRRs		1 construct **N1** (6 units)
Glyma.10g094800	Infection inducible gene	A construct **DR1** (4 units)

Table 5. Ongoing transformation status (number of transformants for each construct)

Construct	Co-cultivation	Shoot Induction 1 &2	Shoot elongation 1	Shoot elongation 2	Shoot Elongation 3
N1		60	60		
N2			50	35	8
N3	0	0	0	0	0
TF	0	58	42	0	0
DR1	160	92	0	6	0