Progress report 04012023

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.
Two 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 two systems, which are named PE1 and PE2 below, were used separately to make prime editing constructs targeting soybean genes encoding CDPK47, CDPK48, CDPK49 and CDPK50. The PE1 and PE2 systems will be compared to determine which one is best for creation of precise genetic changes for improved traits in soybean. Table 1 summarizes the progress on 10 prime editing constructs in hairy root or stable soybean transformation.

Table 1. Status of ten prime editing constructs

PE1	PE2
For hairy root transformation 1. pH-AtePPE-CDPK49-50 (Editing detected in hairy roots, continued for stable transformation) 2. pH-AtePPE-CDPK47 (No editing detected in hairy roots) 3. pH-AtePPE-CDPK48 (No editing detected in hairy roots)	**For hairy root transformation** 1. pG3H-Atubip-PE3mx-GFP-CDPK49-50* (Efficiency is not yet tested in hairy root system) 2. pG3H-Atubip-PE3mx-GFP-CDPK47* (Efficiency is not yet tested in hairy root system) 3. pG3H-Atubip-PE3mx-GFP-CDPK48* (Efficiency is not yet tested in the hairy root system)
For stable transformation 1. pSoy2-AtEC-ePE1-CDPK49-50: From this construct, we have 24 T0 plants in the green house. Among them, four have grown big and started giving pods. Others have recently transferred into the soil. If all of them survive, there will be enough plants for this construct.	**For stable transformation** 1. pSoy2-AtEC-ePE2(PE3mx)-CDPK49-50: Stable transformation for this construct already started. Right now, it is in the regeneration medium. 2. pSoy2-AtEC-ePE2(PE3mx)-CDPK48: Stable transformation for this construct already started. Right now, it is in the regeneration medium. 3. pSoy2-AtEC-ePE2(PE3mx)-CDPK47: Stable transformation for this construct has not started yet, and will start transformation for this construct right after spring break.

During this work, we found that the hairy root system that we previously used was not very efficient. Mostly the roots produced were not successfully transformed and were lacking the GFP marker gene that we use to report on the success of the transformation. Currently, we are trying to establish a new hairy root transformation system that is simpler and more effective.

We expect to have data from hairy roots for the PE2 system within 1-1.5 months and will know then if the new hairy root transformation system performs better.

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. So far, we have obtained three transgene positive plants for the NK44 construct with a few more awaiting genotyping. We have obtained seven transgene positive plants for the NK46 construct. Transgenic plants derived from CRISPR knockout constructs have started setting seeds, some of which have been harvested. The genotyping will be initiated soon in the T1 generation to determine if the desired edits have occurred in the target genes and if the edits were passed on to the next generation.

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 2): 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 2. 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 2. 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 3).

Table 3. 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:

Earlier we reported that the six constructs 1 to 6 (Table 3) were cloned into two binary vectors, (i) P1300-2X35S-Cas9-ccdB (vector A) for the generation of soybean hairy roots to check the success of gene knockout; and (ii) P1300-AtEC-Cas9-GFP-ccdB (vector B) for the generation of stable soybean transgenic lines. Because stable plant transformation using vector B in *Agrobacterium tumefaciens EH105* is a long process and CRISPR-Cas9-mediated gene knockout is well-established in soybean, we prioritized the stable soybean transformation using vector B. After transformation using vector B is underway, we plan to use vector A to generate hair roots for validation of the performance of the constructs.

Stable soybean transformation for five constructs is ongoing (Table 4). Construct #7 containing designed to edit four NLR receptors and two LRR receptor kinases genes is yet to be transformed into *Agrobacterium* strains. The details of our progress on stable soybean transformation are presented below in Table 4. Our goal is to inoculate at least 240 explants (cotyledonary nodes) for each construct. The transformation efficiency is expected to be 2%, so approximately five independent transformants are expected from the 240 explants, which should be sufficient for this study. In Table 4, the number of explants transformed for each of the five constructs and selected in subsequent selection steps are presented in parentheses. We have used two types of *Agrobacterium* strains: (1) thymidine auxotroph (shown with T at the end of the strain names, e.g., EHA105-T) and (ii) wild-type strain (with no T, e.g., EH105)). The thymidine auxotroph *Agrobacterium* strains are being eliminated from the transformed tissues following transformation just by growing the explants in medium containing no antibiotics. Note that some of our early attempts at soybean transformation had to be terminated due to contamination issues (highlighted in grey in Table 4). We have overcome that problem and some of the explants are now in the second round of shoot induction medium. We have observed that over 60% of the explants that were selected continued to grow in the second round of shoot induction medium.

Table 4. Stable soybean transformation for five CRISPR-Cas9 constructs.

Constructs #	# of units	Co-cultivation media	Soot Induction I	Soot Induction II	Soot Elongation (SE)
1	4	16-Feb			
		3/22/2023 (180)	3/27/2023 (144)	10-Apr	24-Apr
		3/30/2023 (180)	3-Apr	17-Apr	1-May
		(AGL-1T)			
2	2				
3	2	13-Feb	3-Mar	17-Mar	
		3/1/2023 (100)			
		3/7/2023 (100)	3/10/2023 (90)	3/24/2023 (64)	7-Apr
		(EH105)			
4	2	16-Feb	20-Feb	6-Mar	
		24-Feb	28-Feb		
		3/16/2023 (100)	3/21/2023 (80)	4-Apr	18-Apr
		(EH105)			
5	2	1-Mar	6-Mar	20-Mar	3-Apr
		3/8/2023 (180)	3/13/2023 (150)	3/27/2023 (112)	10-Apr
		3/10/2023 (180)	15-Mar	3/29/2023 (152)	12-Apr
		3/15/2023 (90)	3/20/2023 (64)	3-Apr	17-Apr
		(EH105-T)			
6	6	10-Feb	14-Feb	28-Feb	13-Apr
		3/15/2023 (180)	3/20/2023 (144)	3-Apr	17-Apr
		3/23/2023 (90)	3/28/2023 (56)	11-Apr	25-Apr
		3/29/2023 (200)	2-Apr	16-Apr	30-Apr
		(EH105-T)			

Failed experiments due to contamination are highlighted in grey.