Iowa Soybean Association Contract Research Project Report
Half-yearly Report (November 15, 2024 – April 15, 2025)

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

Project Title: Bioengineering of an NLR gene for Creating Robust SDS resistance in Soybean

Research Needs: *Fusarium virguliforme* is one of the most damaging fungal pathogens. It causes sudden death syndrome (SDS) in soybean. In the U.S., the estimated soybean yield suppression from *F. virguliforme* is valued at up to $0.6 billion. More than 80 quantitative trait loci (QTL), each providing small SDS resistance effect, are reported. The SDS resistance governed by natural SDS resistance QTL provide soybean with only ***partial resistance***. The ***major genes*** conferring ***complete SDS resistance*** unlikely present in the nature. The major genes such as *Rps1*-k that confers race-specific *Phytophthora* resistance provides complete resistance against certain *Phytophthora sojae* isolates or races. The soybean *Rps1*-k locus contains two genes encoding coiled coil (CC) - nucleotide binding site (NBS) – leucine-rich repeat region (LRR) intracellular receptor proteins and this class of resistance proteins are abbreviated as NLR proteins (Gao et al. 2005).

The creation of a novel ***NLR*** gene conferring ***complete SDS resistance*** is an important research need. If we are successful, such a gene will complement the currently exploited SDS resistance QTL for SDS resistance and protect annual soybean yield losses valued over $300 millions across the soybean growing areas, where *F. virguliforme* is prevalent. The ***goal*** of this project is to generate a synthetic *NLR* gene that confers complete SDS resistance.

It has been demonstrated that the NLR receptor proteins Pikm-1 and Pikm-2 conferring resistance against the rice blast fungus, *Magnaporthe oryzae* can be modified to provide immunity of a wild tobacco species *Nicotiana benthamiana* against the *Potato Virus X* (PVX) (Kourelis et al. 2023).

We have applied the same system to generate an NLR receptor protein conferring complete resistance against *F. virguliforme* as follows (Figure 1).

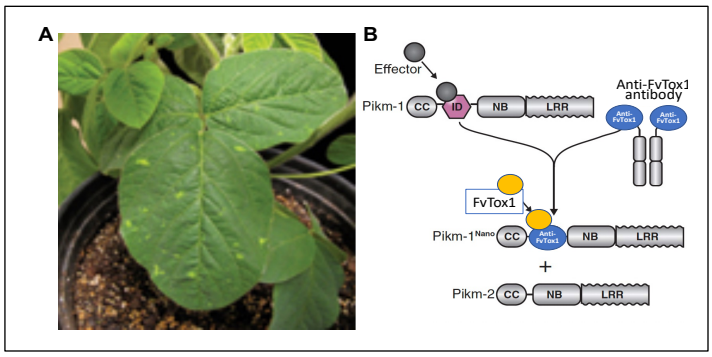

Figure 1. Bioengineering of *Pikm-1* for recognition of FvTox1**.** A, Expression of an anti-FvTox1 plant antibody enhances SDS resistance in soybean (Brar and Bhattacharyya 2012). B, Modification of Pikm-1. Pikm-1 has been engineered to carry any of the two anti-FvTox1 plant antibodies and nine FvTox1-interacting peptides (Wang et al. 2015), and 11 modified Pikm-1 receptor protein genes have been developed.

To determine if the proposed system can generate single *NLR* genes for providing complete SDS resistance in transgenic soybean plants, we developed a transient system in wild-type tobacco *N. benthamiana.* In this approach, we have transiently co-expressed each of the modified 11 Pikm-1 receptors with FvTox1 toxin encoded by the *FvTox1* gene (Brar et al. 2011).

The two vectors for this project were obtained from Sophien Kamoun, Sainsbury Laboratory, England. We have shown that at least three modified *Pickm-1* genes induced **h**ypersensitive cell death **r**esponse (HR) in presence of FvTox1 in a transient system in wild-type tobacco *N. benthamiana.* The three modified *Pickm-1* genes will be expressed in stable transgenic soybean lines. Our lab has recently established the

soybean transformation protocol, and we will initiate the generation of transgenic soybean plants in the second half of the Year 2 of the proposal.

The following are the deliverables were expected according to our proposed project.

Year 1

1. The 11 modified *Pikm-1* genes generated and co-expressed with *Pikm-2* in *N. benthamiana.*
2. The modified *Pikm-1* genes that do not produce **h**ypersensitive cell death **r**esponse (HR) caused by their self-activation in *N. benthamiana* in absence of FvTox1 will be identified.
3. The modified *Pikm-1* genes that initiate HR in *N. benthamiana* in presence of FvTox1 will be identified.

Year 2:

1. The modified *Pikm-1* gene(s) recognized by FvTox1 will be expressed along with the *Pikm-2* gene in stable transgenic soybean lines.
2. At least three independent transgenic soybean lines for each selected modified *Pikm-1* gene will be developed.
3. Transgenic T1 generation will be molecularly characterized for integration of the modified *Pikm-1* gene and the unmodified *Pikm-2* gene.

The project was started late due to delay in recruiting a graduate student and only from June 2024.

Our progress in the last six months is summarized under each of the above three deliverables of Year 1. We have recently completed the proposed research of Year 1 and research has started for the deliverables of Year 2. Here we describe the results gathered for the three deliverables of Year 1.

Deliverable 1. The 11 modified *Pikm-1* genes generated and co-expressed with *Pikm-2* in *N. benthamiana*

The pJK-B2-0529 vector provided by Dr. Kamoun carries the modified *Pikm-1* gene containing the anti-GFP pico-antibody (very small antibody raised against GFP) for binding to the GFP protein expressed from the pPVX-001. The pJK-B2-0529 vector is a large plasmid (16 kilo bases). Therefore, more than one site is found for most restriction endonuclease enzymes and engineering this plasmid is complex. The strategy to be followed for developing 11 modified pJK-B2-0529 vectors is described below.
The DNA sequence encoding the anti-GFP pico-antibody has been replaced by each of the 11 synthetic genes encoding nine FvTox1-interacting peptides and two anti-FvTox1 plant antibodies (Table 1).

The overall the cloning strategy applied is shown in Figure 2.

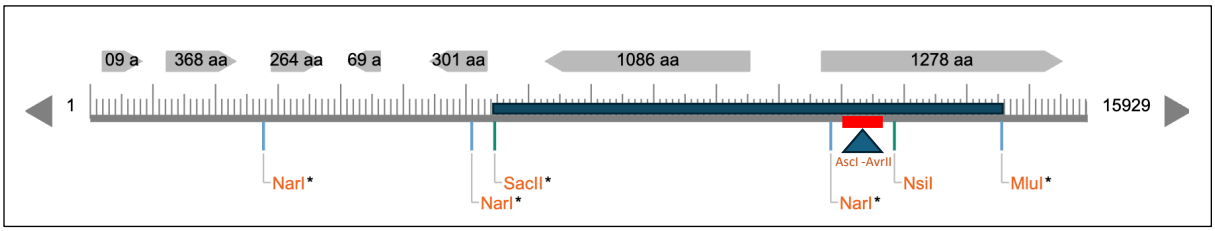

Figure 2. Linear restriction map of the pJK-B2-0529 vector containing the anti-GFP pico-antibody shown by red box (which is drawn not to the scale) in place integrated domain (ID) of the Pikm-1 NLR protein. The 1 kb *Nar*I-*Nsi*I fragment containing this pico-antibody sequence was replaced with a 689 bp synthetic DNA fragment containing *Asc*I-*Pac*I-*Avr*II sites for incorporation of each of the 11 DNA sequences encoding nine FvTox1-interacting peptides or two anti-FvTox1 plant antibodies.

In our earlier report we described the cloning steps and successfully cloning of 11 modified *Pikm-1* genes (Table 1). We replaced the DNA sequence encoding integrated domain of *Pikm*-1 with each of the 11 synthetic DNA sequences encoding nine FvTox1-interacting peptides (Peptide 1 to Peptide 9, Table 1) and two anti-FvTox1 plant antibodies (SCVF-1 and SCVF-2, Table 1) following the method described in previous report. For example, the complete Pikm-1 modified protein containing the Peptide 1 of Table 1 is presented in Figure 3. The yellow-highlighted 12 amino acids represent the Peptide 1 was identified by us ealier as an FvTox1 interacting peptide (Wang et al. 2015). The *A. tumefaciens* isolates carrying the modified pJK-B2-0529 constructs were identified by conducting PCR and were used to infect *N. benthamiana* for transient expression of the modified Pikm-1 protein. Results of the transient expression of the engineered genes in *N. benthamiana* are presented under Deliverables 2 and 3.

Table 1. The amino acid sequences of nine peptides and two single chain variable fragments that were used to modify the Pikm-1 protein (e.g., example Figure 3). The interacting peptide or variable fragment sequences are high-lighted with different colors.

Sl. No.	Name	Amino Acid Sequences
1	Peptide 1	MKETAAAKFERQHMDSPDLGTGGGSGDDDDKSPMGYRGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSLERLG
2	Peptide 2	MKETAAAKFERQHMDSPDLGTGGGSGDDDDKSPMGYRGSGGGGSGGGGSGGGGSVENKTRYHDREVGGGGSLERLG
3	Peptide 3	MKETAAAKFERQHMDSPDLGTGGGSGDDDDKSPMGYRGSGGGGSGGGGSGGGGSHEGAWHNYARSVGGGGSLERLG
4	Peptide 4	MKETAAAKFERQHMDSPDLGTGGGSGDDDDKSPMGYRGSGGGGSGGGGSGGGGSSNGRVADGGGGSLERLG
5	Peptide 5 (1+2+4 peptides)	MRGSHHHHHHMGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSELGGGGSGGGGSGGGGSVENKTRYHDREVGGGGSLEGGGGSGGGGSGGGGSSNGRVADGGGGSRLGSQV
6	Peptide 6 (1+2+3+4 peptides)	MRGSHHHHHHMGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSELGGGGSGGGGSGGGGSVENKTRYHDREVGGGGSLEGGGGSGGGGSGGGGSHEGAWHNYARSVGGGGSEGGGGSGGGGSGGGGSSNGRVADGGGGSRLGSQV
7	Peptide 7 (1+2 peptides)	MRGSGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSELGGGGSGGGGSGGGGSVENKTRYHDREVGGGGSRLGSLG
8	Peptide 8 (3X Peptide 1)	GAPGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSRLG
9	Peptide 9 (3X Pep 1 & 2)	GAPGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSGGGGSGGGGSGGGGSVENKTRYHDREVGGGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSGGGGSGGGGSVENKTRYHDREVGGGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSGGGGSGGGGSGGGGSVENKTRYHDREVGGGGSLG
10	SCVF-1	MKLGLNWVFLALILKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQTPEKRLEWVATISSGGSNYTYYGDGVKGRFTISRDNAKSTLYLQMSSLRSGDTAMYYCARHDRAIFDYWGQGTTLTVSSAKTTPPSVYPLAPVCGGLAPGGGGSGGGGSGGGGSGGGGSGISTMETDTLLLWVLLLWVPGSTGDIVLTQSPASLAVSLGQRATISYRASKSVSTSGYSYMHWNQQKPGQPPRLLIYLVSNLESGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHIRELTRSEGGPSWNLG
11	SCVF-2	MKLGLNWVFLALILKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQTPEKRLEWVATISSGGSNYTYYGDGVKGRFTISRDNAKSTLYLQMSSLRSGDTAMYYCARHDRAIFDYWGQGTTLTVSSAKTTPPSVYPLAPVCGGLAPGGGGSGGGGSGGGGSGGGGSGISTMAPAQLLVYNAKTLADGVPSRFSGSGSGTQYSLKINSLQPEDFGSYFCQHFWTTPWTFGGGTKLEIKRADAAPTVSIFPPSSVDPLPTIQCRPQACVNLG

MEAAAMAVTAATGALAPVLVKLAALLDDGECNLLEGSRSDAEFIRSELEAVHSLLTPNILGRMGDDDAACKDGLIAEVRELSYDLDDAVDDFL
ELNFEQRRSASPFGELKARVEERVSNRFSDWKLPAASLPPSSVHRRAGLPPPDAGLVGMHKRKEELIELLEQGSSDASRWRKRKPHVPLRGAP
GSTAIGMKETAAAKFERQHMDSPDLGTGGGSGDDDDKSPMGYRGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSLERLGSQVKEDVKEIT
AMLAPVKSICEFHEVKTICILGLPGGGKTTIARVLYHALGTQFQCRVFASISPSSSPSPNLTETLADIFAQAQLGVTDTLSTPYGGSGTGRAL
QQHLIDNISAFLLNKKYLIVIDDIWHWEEWEVIRKSIPKNDLGGRIIMTTRLNSIAEKCHTDDNDVFVYEVGDLDNNDAWSLSWGIATKSGAG
NRIGTGEDNSCYDIVNMCYGMPLALIWLSSALVGEIEELGGAEVKKCRDLRHIEDGILDIPSLQPLAESLCLGYNHLPLYLRTLLLYCSAYHW
SNRIERGRLVRRWIAEGFVSEEKEAEGYFGELINRGWITQHGDNNSYNYYEIHPVMLAFLRCKSKEYNFLTCLGLGSDTSTSASSPRLIRRLS
LQGGYPVDCLSSMSMDVSHTCSLVVLGDVARPKGIPFYMFKRLRVLDLEDNKDIQDSHLQGICEQLSLRVRYLGLKGTRIRKLPQEMRKLKHL
EILYVGSTRISELPQEIGELKHLRILDVRNTDITELPLQIRELQHLHTLDVRNTPISELPPQVGKLQNLKIMCVRSTGVRELPKEIGELNHLQ
TLDVRNTRVRELPWQAGQISQSLRVLAGDSGDGVRLPEGVCEALINGIPGATRAKCREVLSIAIIDRFGPPLVGIFKVPGSHMRIPKMIKDHF
RVLSCLDIRLCHKLEDDDQKFLAEMPNLQTLVLRFEALPRQPITINGTGFQMLESFRVDSRVPRIAFHEDAMPNLKLLEFKFYAGPASNDAIG
ITNLKSLQKVVFRCSPWYKSDAPGISATIDVVKKEAEEHPNRPITLLINAGYKEISTESHGSSENIAGSSGIDTEPAQAQHDNLPAVRDDYKG
KGILLDGRCPTCGRATKIEEETQDRVADIEIQTETTSGSGSGRGSHHHHHHDYDIPTTASENLYFQGELDYKDHDGDYKDHDIDYKDDDDK

Figure 3. The modified Pikm-1 protein containing Peptide 1 in place of integrated domain. Yellow-highlighted 12 amino acid sequence interacts with FvTox1 (Wang et al. 2015).

In the last six months we accomplished the following.

Deliverable 2. The modified *Pikm-1* genes that do not activate *Pikm-2* in *Nicotiana benthamiana* in absence of FvTox1 will be identified.

Each of the modified *Pikm-1* genes were transformed into *Agrobacterium tumefaciens;* and transformed *A. tumefaciens* strains carrying the 11 modified *Pikm-1* genes (Table 1) were infiltrated into the dorsal leaf surfaces of *N. benthamiana* for transient expression of each of the 11 genes. The modified *Pikm-1* genes, expressed alone transiently to determine if there is any self-activation of the engineered modified *Pikm-1* genes (responses shown by red broken circles in Figure 4). Some of the modified *Pikm-1* genes carrying Pep 1, Pep 2 and Pep 4 self-activated and caused HRs as showed in Figure 4A. Whereas, some of the modified *Pikm-1* genes containing Pep 5, Pep 6 and SCVF1 showed consistently lack or reduced levels of HR (Figure 4B). The HRs of all 11 modified *Pikm-1* genes when they were expressed individually are presented in Figure 4C. Note that some of the modified Pikm-1 genes will be ideal to explore if they are activated in presence of FvTox1.

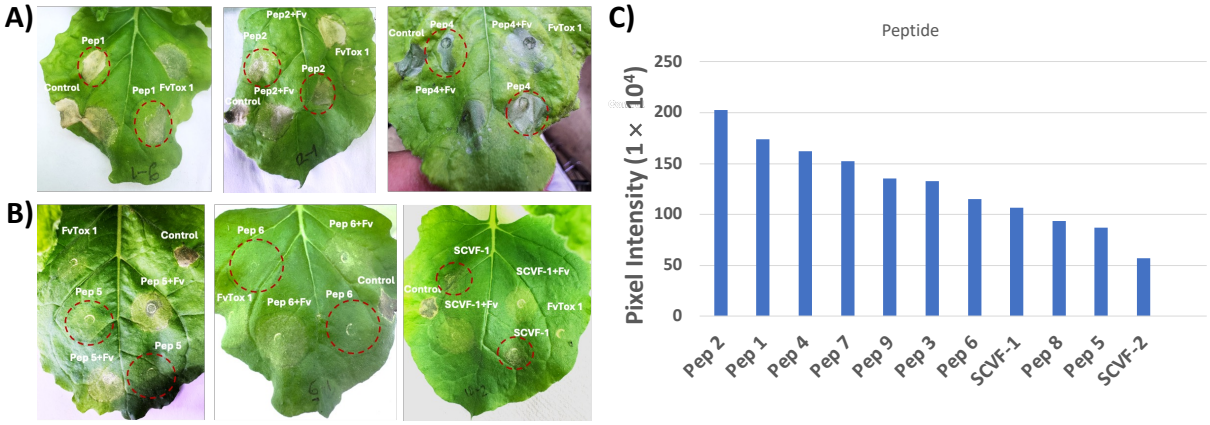

Figure 4. Identification of the modified Pikm-1 genes that self-activate and exhibit HR. A) The modified *Pikm-1* genes carrying Pep 1, Pep 2 and Pep 4 exhibited severe HRs (two red broken circles in each leaf)

following expression due to self-activation. B) The modified *Pikm-1* genes carrying Pep 5, Pep 6 and SCVF-1, exhibited negligible or reduced HR (two red broken circles in each leaf) following expression due to absence of self-activation. C) Levels of HRs induced due to self-activation of the 11 modified *Pikm-1* genes. All experiments were conducted six to 15 times and means are presented. The HRs were calculated from responses among 6-15 replications, each replication comprised of two infiltrations in each of six to 15 leaves.

The modified *Pikm-1* genes containing Pep 5, Pep 6, Pep 8, SCVF-1 and SCVF2 showing low levels of self-activation in some experiments are good candidates to determine if any of them are activated in presence of FvTox1.

Deliverable 3. The modified *Pikm-1* genes that initiate HR in *N. benthamiana* in presence of FvTox1 will be identified.

We co-expressed each of the 11 modified *Pikm-1* genes carrying 11 *F. virguliforme* interacting peptides (Table 1) with FvTox1 to determine if any of the genes are activated to induce the *Pikm-2* gene to cause severe HR. The *FvTox1* gene was cloned into the PVX vector and following expression in *N. benthamiana* leaves we conducted a western blot analysis to establish that the protein is expressed (Figure 5). Note that in one experiment the level of FvTox1 accumulation is much reduced presumably due to HR induced by a modified *Pikm-1* gene carrying Pep 5. In all four lanes of separated protein samples carrying expressed FvTox1showed the FvTox1 protein band (shown by an arrow) recognized by the anti-FvTox1 antibody.

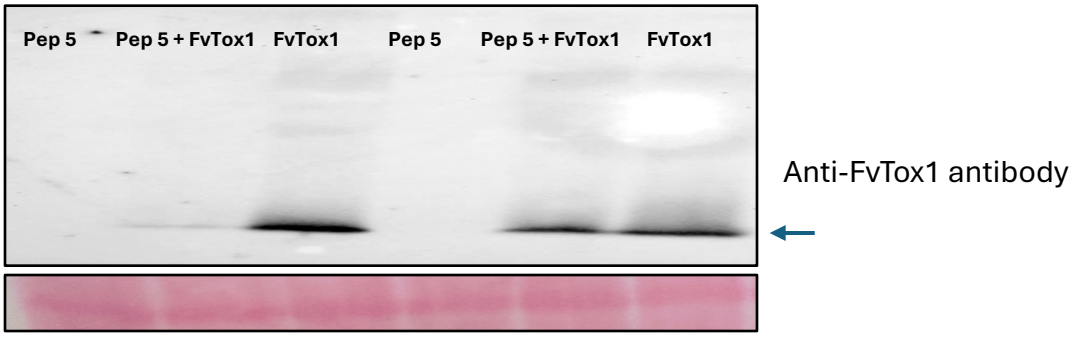

Figure 5: Expression of FvTox1 toxin in *N. benthamiana* 48 h following infiltration *with Agrobacterium tumefaciens* carrying the *FvTox1* in the PVX vector. Pep 5, the modified *Pikm-1* gene carrying Pep 5.

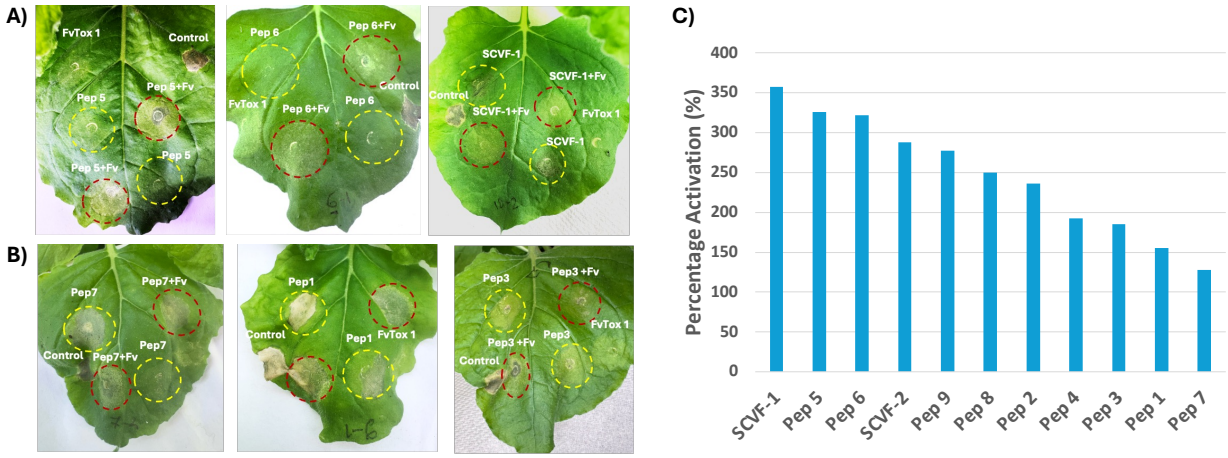

Figure 5. Identification of the modified *Pikm-1* genes that activat the *Pikm-2* gene and result in HR in presence of FvTox1. A) The modified *Pikm-1* genes carrying Pep 5, Pep 6 and SCVF-1 exhibited high levels

of HRs (two red broken circles in each leaf) due to their co-expression with the FvTox1 toxin. Note that the levels of self-activation of these three modified *Pikm-1* genes (shown by broken yellow circles) are negligible to very low. B) The modified *Pikm-1* genes carrying Pep 7, Pep 1 and Pep 3 exhibited some levels of HRs (two red broken circles in each leaf) in presence of FvTox1 but showed a high level of HRs (two yellow broken circles in each leaf) due to self-activation. C) Percentage activation of the modified *Pikm-1* genes as percentages of FvTox1-induced activation of HRs as compared to the corresponding levels of self-activation of individual modified *Pikm-1* genes. The percentage activations were calculated from responses among 6-15 replications, each replication comprised of two infiltrations in a leaf.

The modified *Pikm-1* genes containing Pep 5, Pep 6, and SCVF-1 with low levels of self-activation (Figure 4) and over 300% FvTox1-induced activation over the background levels of self-activation are selected for testing the technology in stable transgenic soybean plants.

Currently, we are studying the responses of the modified *Pikm-1* genes in *Agrobacterium rhizogenes*-induced hairy roots.

Self-evaluation:

Project milestones & deliveries: We have identified three modified *Pikm-1* genes that are strong candidates for testing their functions in stable transgenic roots.

Self-evaluation:

We have completed the proposed research for the three deliverables of Year 1 and research of for the Year 2 started. The graduate student started to work on this project from June 2024 and the proposed work for the Year 1 has been completed as expected.

The research of the Year 2 has been started.

References

Brar H.K., and Bhattacharyya, M.K. (2012) Expression of a single-chain variable-fragment antibody against a *Fusarium virguliforme* toxin peptide enhances tolerance to sudden death syndrome in transgenic soybean plants. *Mol. Plant-Microbe Interact*. 25:817-824.

Brar H.K., Swaminathan, S., and Bhattacharyya, M.K. (2011) The *Fusarium virguliforme* toxin FvTox1 causes foliar sudden death syndrome-like symptoms in soybean. *Mol. Plant-Microbe Interact.* 24:1179-1188.

Gao, H., Narayanan, N., Ellison, L., and Bhattacharyya, M.K. (2005) Two classes of highly similar coiled coil-nucleotide binding-leucine rich repeat genes isolated from the *Rps1*-k locus encode *Phytophthora* resistance in soybean. *Mol. Plant-Microbe Interact.* 18:1035-1045.

Kourelis J., Marchal C., Posbeyikian A., Harant A., Kamoun S. (2023) NLR immune receptor-nanobody fusions confer plant disease resistance. *Science*. 379:934-939.

Wang, B., Swaminathan, S., and Bhattacharyya, M.K. (2015) Identification of *Fusarium virguliforme* FvTox1-interacting synthetic peptides for enhancing foliar sudden death syndrome resistance in soybean. *PLoS ONE* 10: e0145156.