Iowa Soybean Association Contract Research Project Report

Half-yearly Report (October 1, 2023 – March 31, 2024)

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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 (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 conferring complete resistance against *F. virguliforme* as follows (Figure 1).

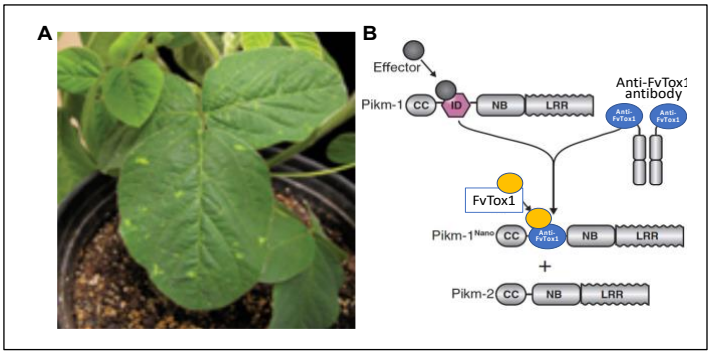

Figure 1. Bioengineering of *Pikm-1* for recognition of FvTox1**.** A, Expression of an anti-FvTox1 plant antibody enhances SDS resistance in soybean (Brar and Bhattacharyya 2012). B, Modification of Pikm-1. Pikm-1 will be engineered to carry any of the two anti-FvTox1 plant antibodies and nine FvTox1-interacting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

To determine if the proposed system can generate single *NLR* genes for providing complete SDS resistance in transgenic soybean plants, we are developing a transient system in wild-type tobacco *N. benthamiana.* In this approach, we will transiently express each of the modified 11 Pikm-1 receptors with each of the two FvTox1 proteins encoded by the *FvTox1* gene.

To accomplish our goal, we proposed to modify two vectors received from Sophien Kamoun, Sainsbury Laboratory, England. If we are successful in showing that one or more of the 11 modified Pikm-1 genes generate HR following co-expression with one or both FvTox1 proteins, we will express that modified Pickm-1 gene in stable transgenic soybean lines. Our lab has recently established the soybean transformation protocol and we will generate transgenic soybean plants in the Year 2 of this project as proposed in our funded proposal.

In Year 1, the proposed deliverables are:

- The 11 modified *Pikm-1* genes generated and co-expressed with *Pikm-2* in *N. benthamiana.*
- The modified *Pikm-1* genes that do not activate *Pikm-2* in *N. benthamiana* in absence of FvTox1 will be identified.
- The modified *Pikm-1* genes that initiate HR in *N. benthamiana* in presence of FvTox1 will be identified.

Our progress in the last six months is summarized under each of the above three deliverables:

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 carrying the modified *Pikm-1* gene that contains 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 of the restriction endonuclease enzymes and therefore 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 will be replaced by each of the 11 synthetic genes generated for expressing two anti-FvTox1 plant antibodies and nine FvTox1-interacting peptides.

   The overall the cloning strategy is shown in Figure 2.

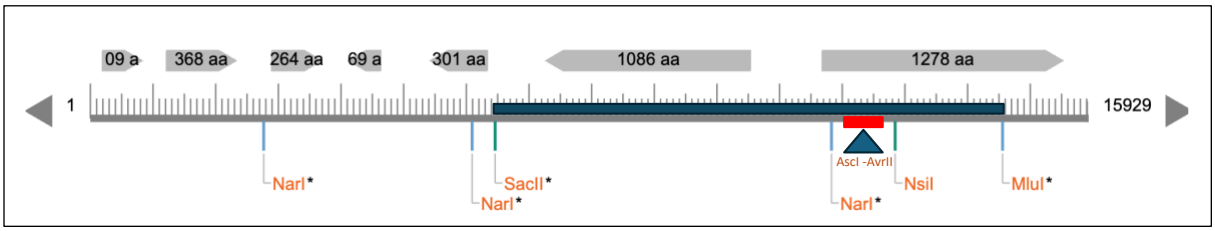

Figure 2. Linear restriction map of the pJK-B2-0529 vector containing the anti-GFP pico-antibody shown by red box (which is drawn not to the scale) in place integrated domain (ID) of the Pikm-1 NLR protein. The 1 kb *Nar*I-*Nsi*I fragment containing this pico-antibody sequence will be replaced with a 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-FvToxI plant antibodies.

Towards delivering this deliverable, we have accomplished the following:

I. We have synthesized the 689-base pair *Nar*I-*Nsi*I fragment containing the *AscI-PacI-AvrII* cloning sites to replace the 1 kb *Nar*I-*Nsi*I fragment containing the anti-GFP piko-antibody in the pJK-B2-0529 vector.

II. The 689-base pair *Nar*I-*Nsi*I fragment, to be cloned into the 8 kb *Sac*II-*Mlu*I fragment (Figure 2), is being cloned in the pBlueScript vector.

III. The 8 kb *Sac*II-*Mlu*I fragment has been being cloned into the modified pBlueScript vector which we developed by placing an *Mlu*I site in between *Bam*HI and *Eco*RI sites as shown below (Figure 3).

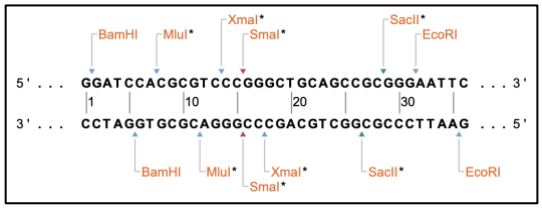

Figure 3. The generated modified pBlueScript vector showing the inserted *Mlu*I site in between *Bam*HI and *Eco*RI.

IV. Once the 689-base pair *Nar*I-*Nsi*I fragment containing the *Asc*I-*Pac*I-*Avr*II restriction sites is cloned into the 8 kb *Sac*II-*Mlu*I fragment in pBlueScript, the modified *Sac*II-*Mlu*I fragment will then be used to replace the 8 kb *Sac*II-*Mlu*I fragment of the pJK-B2-0529 vector.

V. We will then clone each of the 11 synthetic DNA fragments encoding each of the nine FvTox1-interacting peptide and two anti-FvToxI plant antibodies in the *Asc*I -*Avr*II sites of the modified pJK-B2-0529 vector.

VI. the resultant 11 modified pJK-B2-0529 vectors will then be transformed in *Agrobacterium tumefaciens* for transient expression in *N. benthamiana* along with each of the two modified pJK-PVX-001 constructs that have been engineered to carry each of two *FvTox1* DNA fragments described under the Deliverable # 2.

2. **The modified *Pikm-1* genes that do not activate *Pikm-2* in *N. benthamiana* in absence of FvTox1 will be identified.** To accomplish this deliverable, we have replaced the green fluorescence protein gene (GFP) from the pJK-PVX-001 construct, obtained from Dr. Kamoun, with either *FvTox1-1* or *FvTox1-2. FvTox1-1* and *FvTox1-2* encode the entire FvTox1 protein or processed matured FvTox1 toxin, respectively (Brar et al. 2011).

   We have already cloned both *FvTox1-1* and *FvTox1-2* genes and used in replacing the *GFP* gene of the pPVX-001 construct developed in the vector developed from *Potato Virus X* (PVX). The steps involved in cloning the *FvTox1-1* and *FvTox1-2* are described below.

   I. For cloning the *FvTox1* genes, we digested the pJK-PVX-001 construct with *Nhe*I and *Sac*I and the large vector fragment was purified and stored. The small fragment is the GFP fragment and was used in PCR cloning of the two *FvTox1* gene fragments.

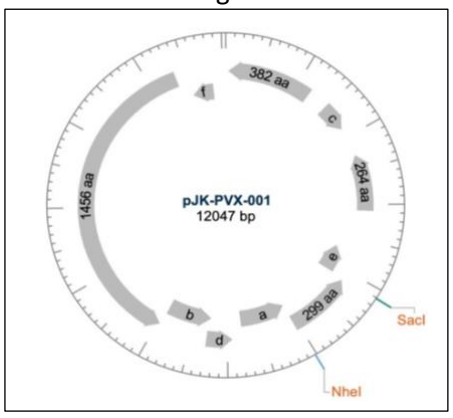

Figure 4. The 1 kb *Nhe*I and *Sac*I fragment of the 12 kb pJK-PVX-001 construct used for cloning the two *FvTox1* gene fragments.

   II. Two-step PCR was applied to fuse a section of the small *Nhe*I and *Sac*I fragment with the two *FvTox1* fragments. The two *Nhe*I and *Sac*I fragments containing FvTox1 sequences were sequenced to confirm that there was no mutation added during the PCR, and then cloned into the large *Nhe*I and *Sac*I fragment, i.e., the rest of the pJK-PVX-001 construct (Figure 4). The two modified pJK-PVX-001 constructs containing the *FvTox1-1* and *FvTox1-2* DNA fragments were named pPVX-FvTox1-1 and pPVX-FvTox1-2.

   III. The two modified pJK-PVX-001 constructs, pPVX-FvTox1-1 and pPVX-FvTox1-2 were transformed into *A. tumefaciens*. The *A. tumefaciens* isolates carrying either pPVX-FvTox1-1 or pPVX-FvTox1-2 were identified by conducting PCR and were used to infect *N. benthamiana* for transient expression of the pPVX-FvTox1-1 and pPVX-FvTox1-2 toxin. The very preliminary results suggest that neither the FvTox1-1 nor FvTox1-2 induced any hypersensitive response (HR) in *N. benthamiana* following transient expression (Figure 5). *N. benthamiana* is a nonhost for *F. virguliforme* and FvTox1 is expected not to induce in HR in this nonhost plant. If the observed

phenotype “absence of HR” is reproduced in two subsequent experiments, this model system will be ideal for investigating the interactions of FvTox1-1 and FvTox1-2 with the modified 11 Pikm-1 genes carrying either of the nine FvTox1-interacting peptides or two anti-FvTox1 plant antibody genes.

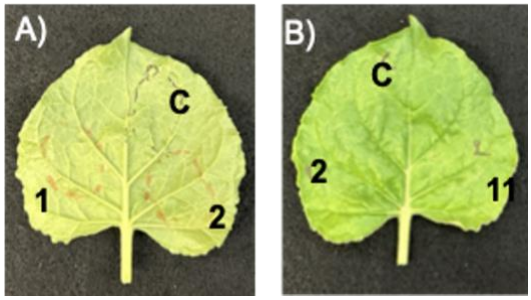

Figure 5. The *N. benthamiana* infected with *Agrobacterium tumefaciens* containing either the pPVX-FvTox1-1 or pPVX-FvTox1-2 toxin genes. The infected sites are shown with C, 1 and 2: C, empty vector control; 1, FvTox1-1 and 2, FvTox1-2. The observation was made five days following infection with *A. tumefaciens*. A, ventral side, and B) dorsal side.

3. **The modified *Pikm-1* genes that initiate HR in *N. benthamiana* in presence of FvTox1 will be identified.** The activities for this deliverable will be started as soon as we have a few of the 11 modified pJK-B2-0529 vectors are available and we expect to complete this task before the end of Year 1.

Self-evaluation:

Project milestones & deliveries:

By the end of Year 1, it will be known:

1. If any of the 11 modified *Pikm-1* genes that do not activate the Pickm-2 NLR protein in absence FvTox1.
2. If any of the modified *Pikm-1* genes that do not activate Pikm-2 do activate HR in presence of FvTox1.

Self-evaluation: Our progress is in the right track. We already cloned the two forms of the *FvTox1* gene and generated two modified vectors pPVX-FvTox1-1 and pPVX-FvTox1-2. We have started to study if any of these two vectors causes any hypersensitive response (HR) in *N. benthamiana.* Considering *N. benthamiana* is a nonhost to *F. virguliforme*, therefore we do not expect observe any HR responses following transient expression of the two genes in *N. benthamiana* leaves. The results of the first assay support this expectation.

We are also close to developing the 11 modified *Pikm-1* genes in the pJK-B2-0529 vector. Once the modified 11 *Pikm-1* genes are developed, we will determine if any of these genes can activate Pikm-2 receptor protein in presence of FvTox1 and produce HR. We should be able to complete all research activities and deliver all deliverables proposed for Year 1 by September 30, 2024.

Year 2: The modified *Pikm-1* genes that do not activate *Pikm-2* in *N. benthamiana* in absence of FvTox1 but activate in presence of FvTox1 will be expressed in stable transgenic soybean lines.

Year 3: The transgenic lines carrying the modified *Pikm-1* genes and *Pikm-2* will be tested for their responses to *F. virguliforme* infection under growth chamber and field conditions.

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.

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

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