Iowa Soybean Association Contract Research Project Report

Half-yearly Report (April 1, 2024 – November 14, 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).

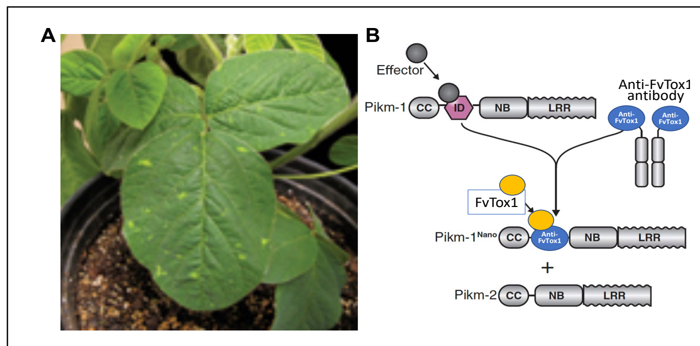

Figure 1. Bioengineering of *Pikm-1* for recognition of FvTox1**.** A, Expression of an anti-FvTox1 plant antibody enhances SDS resistance in soybean (Brar and Bhattacharyya 2012). B, Modification of Pikm-1. Pikm-1 will be engineered to carry any of the two anti-FvTox1 plant antibodies and nine FvTox1-interacting peptides (Wang et al. 2015), 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 (Brar et al. 2011).

To accomplish our goal, we proposed to modify the 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:

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 activate *Pikm-2* 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.

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 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 of the 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.

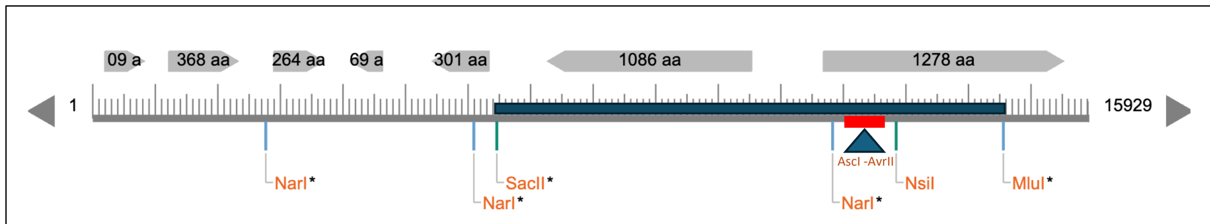

Figure 2. Linear restriction map of the pJK-B2-0529 vector containing the anti-GFP pico-antibody shown by red box (which is drawn not to the scale) in place integrated domain (ID) of the Pikm-1 NLR protein. The 1 kb *Nar*I-*Nsi*I fragment containing this pico-antibody sequence was replaced with a 689 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-FvToxI plant antibodies.

The following six steps were followed in developing the vectors carrying modified *Pikm-1* protein genes.

1. We 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.
2. The 689-base pair *Nar*I-*Nsi*I fragment was cloned the pBlueScript vector to sequence and confirm the identity of the fragment.
3. The 8 kb *Sac*II-*Mlu*I fragment of the pJK-B2-0529 vector was cloned into the modified pBlueScript vector developed by placing an *Mlu*I site in between *Bam*HI and *Eco*RI sites. The 689-base pair *Nar*I-*Nsi*I fragment was then cloned in the 8 kb *Sac*II-*Mlu*I fragment in the pBlueScript vector.
4. The 8 kb modified *Sac*II-*Mlu*I fragment was used to replace the 8 kb *Sac*II-*Mlu*I fragment of the pJK-B2-0529 vector.
5. Each of the 11 synthetic DNA fragments encoding each of the nine FvTox1-interacting peptide and two anti-FvTox1 plant antibodies were individually cloned into the *Asc*I -*Avr*II sites of the modified pJK-B2-0529 vector.

6. The resultant 11 modified pJK-B2-0529 vectors were transformed into *Agrobacterium tumefaciens* for transient expression of the fusion genes 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 # 3.

We have successfully cloned 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 in Table 1) and two anti-FvTox1 plant antibodies (SCVF-1 and SCVF-2 in Table 1). For example, the complete Pikm-1 modified protein containing the Peptide 1 of Table 1 Is presented in Figure 3. The green-highlighted 12 amino acids represent the Peptide 1 identified as an FvTox1 interactor (Wang et al. 2015).

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

Sl. No.	Name	Amino Acid Sequences
1	Peptide 1	MKETAAAKFERQHMDSPDLGTGGGSGDDDDKSPMGYRGSGGGGSGGGGSGGGGSSYLPE TIYEYRLGGGGSLERLG
2	Peptide 2	MKETAAAKFERQHMDSPDLGTGGGSGDDDDKSPMGYRGSGGGGSGGGGSGGGGSVENKT RYHDREVGGGGSLERLG
3	Peptide 3	MKETAAAKFERQHMDSPDLGTGGGSGDDDDKSPMGYRGSGGGGSGGGGSGGGGSHEGA WHNYARSVGGGGSLERLG
4	Peptide 4	MKETAAAKFERQHMDSPDLGTGGGSGDDDDKSPMGYRGSGGGGSGGGGSGGGGSSNGR VADGGGGSLERLG
5	Peptide 5 (1+2+4 peptides)	MRGSHHHHHHMGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSELGGGGSGGGGSG GGGSVENKTRYHDREVGGGGSLEGGGGSGGGGSGGGGSSNGRVADGGGGSRLGSQV
6	Peptide 6 (1+2+3+4 peptides)	MRGSHHHHHHMGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSELGGGGSGGGGSG GGGSVENKTRYHDREVGGGGSLEGGGGSGGGGSGGGGSHEGAWHNYARSVGGGGSEGG GGSGGGGSGGGGSSNGRVADGGGGSRLGSQV
7	Peptide 7 (1+2 peptides)	MRGSGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSELGGGGSGGGGSGGGGSVENK TRYHDREVGGGGSRLGSLG
8	Peptide 8 (3X Peptide 1)	GAPGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSGGGGSGGGGSGGGGSSYLPETIYEYRL GGGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSRLG
9	Peptide 9 (3X Pep 1 & 2)	GAPGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSGGGGSGGGGSGGGGSVENKTRYHDR EVGGGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSGGGGSGGGGSGGGGSVENKTR YHDREVGGGGSGGGGSGGGGSGGGGSSYLPETIYEYRLGGGGSGGGGSGGGGSGGGGSVE NKTRYHDREVGGGGSLG
10	SCVF-1	MKLGLNWVFLALILKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQTPEKR LEWVATISSGSNYTYYGDGVKGRFTISRDNAKSTLYLQMSSLRSGDTAMYYCARHDRAIFDYWG QGTTLTVSSAKTTPPSVYPLAPVCGGLAPGGGGSGGGGSGGGGSGISTMETDTLLLWVLLLW VPGSTGDIVLTQSPASLAVSLGQRATISYRASKSVSTSGYSYMHWNQQKPGQPPRLLIYLVSNLE SGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHIRELTRSEGGPSWNLG
11	SCVF-2	MKLGLNWVFLALILKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQTPEKR LEWVATISSGSNYTYYGDGVKGRFTISRDNAKSTLYLQMSSLRSGDTAMYYCARHDRAIFDYWG QGTTLTVSSAKTTPPSVYPLAPVCGGLAPGGGGSGGGGSGGGGSGISTMAPAQLLVYNAKTLA DGVPSRFSGSGSGTQYSLKINSLQPEDFGSYFCQHFWTTPWTFGGGTKLEIKRADAAPTVSIFP PSSVDPLPTIQCRPQACVNLG

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MEAAAMAVTAATGALAPVLVKLAALLDDGECNLLEGSRSDAEFIRSELEAVHSLLTPNILGRMGDDDAACKDGLIAEVRELSYDLDDAVDDFLELNF
EQRRSASPFGELKARVEERVSNRFSDWKLPAASLPPSSVHRRAGLPPPDAGLVGMHKRKEELIELLEQGSSDASRWRKRKPHVPLRGAPGSTAIGMK
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HEVKTICILGLPGGGKTTIARVLYHALGTQFQCRVFASISPSSSPSPNLTETLADIFAQAQLGVTDTLSTPYGGSGTGRALQQHLIDNISAFLLNKK
YLIVIDDIWHWEEWEVIRKSIPKNDLGGRIIMTTRLNSIAEKCHTDDNDVFVYEVGDLDNNDAWSLSWGIATKSGAGNRIGTGEDNSCYDIVNMCYG
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AEGYFGELINRGWITQHGDNNSYNYYEIHPVMLAFLRCKSKEYNFLTCLGLGSDTSTSASSPRLIRRLSLQGGYPVDCLSSMSMDVSHTCSLVVLGD
VARPKGIPFYMFKRLRVLDLEDNKDIQDSHLQGICEQLSLRVRYLGLKGTRIRKLPQEMRKLKHLEILYVGSTRISELPQEIGELKHLRILDVRNTD
ITELPLQIRELQHLHTLDVRNTPISELPPQVGKLQNLKIMCVRSTGVRELPKEIGELNHLQTLDVRNTRVRELPWQAGQISQSLRVLAGDSGDGVRL
PEGVCEALINGIPGATRAKCREVLSIAIIDRFGPPLVGIFKVPGSHMRIPKMIKDHFRVLSCLDIRLCHKLEDDDQKFLAEMPNLQTLVLRFEALPR
QPITINGTGFQMLESFRVDSRVPRIAFHEDAMPNLKLLEFKFYAGPASNDAIGITNLKSLQKVVFRCSPWYKSDAPGISATIDVVKKEAEEHPNRPI
TLLINAGYKEISTESHGSSENIAGSSGIDTEPAQAQHDNLPAVRDDYKGKGILLDGRCPTCGRATKIEEETQDRVADIEIQTETTSGSGSGRGSHHH
HHHDYDIPTTASENLYFQGELDYKDHDGDYKDHDIDYKDDDDK
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Figure 3. The modified Pikm-1 protein containing Peptide 1 in place of integrated domain. Green-highlighted 12 amino acid sequence interacts with FvTox1.

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 each of 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. None of the genes self-activated the *Pikm-2* gene since no hypersensitive cell death was recorded (Figure 4). We will investigate if each of the 11 fusion *Pikm-1* genes expressed in the infiltrated leaves by conducting western blot analysis.

3. **The modified *Pikm-1* genes that initiate HR in *N. benthamiana* in presence 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 two mature forms of the FvTox1 toxin, respectively (Brar et al. 2011).

Recently, we have cloned both *FvTox1-1* and *FvTox1-2* genes from RNA samples obtained from the *F. virguliforme* infected soybean roots and used to replace the *GFP* gene of the pPVX-001 vector plasmid, developed from *Potato Virus X* (PVX) as described in the previous report.

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. Neither of the FvTox1-1 and FvTox1-2 induced any hypersensitive response (HR) in *N. benthamiana* following transient expression in multiple experiments and therefore this transient system should be ideal in studying the 11 Pikm-1 fusion genes for inducing Pikm-2 following binding to FvTox1-1 and FvTox1-2.

We have started to study the interactions of FvTox1-1 and FvTox1-2 with each of the 11 fusion Pikm-1 proteins in *N. benthamiana* leaves following transient expression. Very preliminary results of the activation of Pikm-2 by the Pikm-1-Peptide 5 fusion protein is shown in Figure 4. There is no change in the chlorophyl color when the leaf was infiltrated with Agrobacterium strains carrying either Pikm-1-Peptide 7 (Peptide 7), FvTox1-1 or FvTox1-2. Interestingly, when Pikm-1-Peptide 5 (*Pep5*) was co-expressed with either FvTox1-1 or FvTox1-2, the entire infiltrated regions have started to show yellowing due to HR.

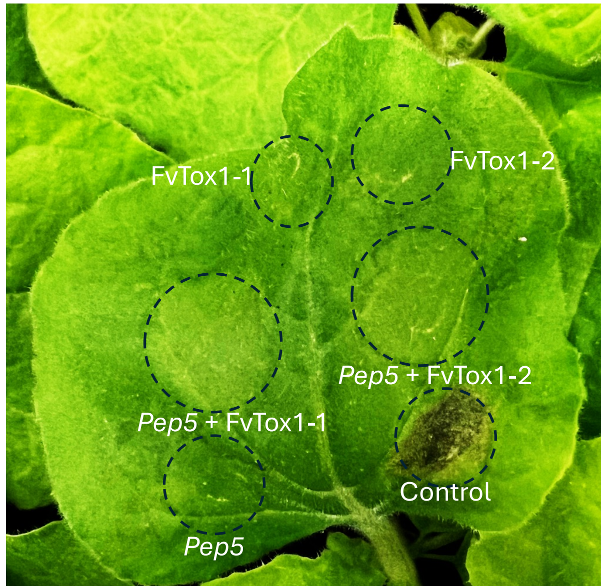

Figure 4. The transient expression genes in *N. benthamiana.* The dorsal side of the leaf was infiltrated with *Agrobacterium tumefaciens* containing (i) the pPVX-FvTox1-1, (2) pPVX-FvTox1-2 toxin and (3) *Pep5,* (Pikm-1-Peptide 5) genes. The observation was made four days following infection with *A. tumefaciens*.

Note that co-infiltration of *Pep5* (Pikm-1-Peptide 5) with either FvTox1-1 or FvTox1-2 exhibited yellowing of the infiltrated region shown with the dotted black circles.

Control, Co-expressed pJK-B2-0529 + pJK-PVX-001 showed HR because of interaction between Pikm-1-anti-GFP antibody and GFP from PVX1 vector.

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 have completed all cloning steps. We have cloned the two forms of the *FvTox1* gene and generated 11 modified *Pickm-1* genes.

We have demonstrated that none of the 11 modified *Pikm-1* genes self-activates Pikm-2.

We have observed that most likely the modified Pikm-1-peptide 5 protein activates Pikm-2 resulting in yellowing and HR.

References

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