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| Project Number:  | 1420-532-5640 |
| Project Title:  | Center for Soybean Genetic Engineering |
| Organization:  | University of Georgia |
| Principal Investigator Name: | Wayne Parrott |
| Project Overview - What key activities were undertaken and what were the key accomplishments during the life of this project?  |
| The USB-funded "Soybean Tissue Culture and Genetic Engineering Center" has been working for two decades to develop the basic tools needed to study soybean molecular genetics and genomics, and to incorporate the resulting discoveries into a form that can be used by breeders. These are technologies that lie at the intersection of plant breeding and genomics.  Today, transformation and genome editing technologies are indispensable for modern research and improvement, and the Center ensures the latest developments are available to the soybean breeding and research communities.Key activities and accomplishmentsGene Silencing for Nematode ResistanceNematodes secrete effector proteins into root cells to establish feeding sites that are essential for their survival. Gene silencing by RNA interference (RNAi) of the nematode gene making the effector was seen as a way by which resistance could be engineered in plants. Small-interfering RNA (siRNA) molecules targeting nematode genes and produced by transgenic plants could presumably ingested by the feeding nematodes, resulting in the silencing of the nematode genes. Gene targets and hairpin vectors previously tested to ensure expression in Arabidopsis were chosen for use in soybean. Twelve lines targeting five different SCN parasitism genes were tested to ensure expression and then subjected to a nematode bioassay. No significant differences in nematode counts, compared to wild-type controls, were observed. A second bioassay with 5 lines was performed and although siRNAs were readily detected in transgenic soybean lines, significant reductions in nematode infection were not observed. To determine if silencing of the targeted effector genes was occurring in the nematodes feeding on the transgenic lines, qRT-PCR was performed on transgenic and control roots infected with SCN. A non-segregating line that had consistently high siRNA counts was chosen. Unexpectedly, transcript levels of were 2-fold higher in nematodes feeding on the transgenic line than on control non-transgenic plants. It is unclear why transcript levels of the targeted gene would be upregulated in feeding nematodes. Bottom line is this gene is not a candidate to achieve nematode resistance. In addition, results from model systems such as *Arabidopsis* and hairy roots do not extrapolate to whole soybean plants in terms of obtaining nematode resistance.Soybean Promoter AnalysisPromoters are broadly defined as the regions of DNA immediately upstream of genes, which largely dictate when and how highly a gene is expressed. Providing a toolbox of soybean promoters and promoter elements remains critical to the development of future transgenics, and opens the door for the use of genome-editing applications. Since each soybean gene has a promoter and each promoter contains functional components that contribute to gene expression, this work will unquestionably lead to the discovery of new sequences that can be used to regulate transgenes, or that can be edited with the emerging genome editing techniques, such as CRISPR/cas9.Fourteen different soybean seed-specific promoters were identified from RNAseq data, PCR-amplified and cloned upstream of a green fluorescent protein (gfp) gene permitting them to be monitored. Seed specific or enhanced genes included serine protease inhibitor, kunitz trypsin inhibitor, cysteine protease, cupin, gamma thionin, subtilisin-like protease, and lectin. These 14 promoters were evaluated using a two tier lima bean cotyledon/soybean hairy root validation tool. Four of these soybean promoters (GmSeed3 - Protease inhibitor; GmSeed5 – cupin; GmSeed8 - subtilisin-like protease; GmSeed9 - lectin) were subsequently selected and stably introduced into embryogenic soybean tissue. Transgenic cell lines were recovered, validated through PCR, and plants were regenerated containing these 4 priority promoters. Although all promoters resulted in gfp expression, only the GmSeed5 (cupin) promoter-containing events showed low levels of GFP expression in proliferating somatic embryogenic tissues. No expression was visible in the embryogenic tissue of any other promoter construction, which was not too surprising as these promoters were supposed to primarily express later in seed development. Somatic embryos generated from events containing the GmSeed5 promoter also expressed during the later stages of embryo development (5+ weeks on maturation medium) or when undergoing desiccation. Expression was observed in the cotyledons of some transgenics, and surprisingly, in the hypocotyls of other plants containing the same promoter construction. No expression has yet been seen in somatic embryos of GmSeed3 (Protease inhibitor), GmSeed8” (subtilisin-like protease) and GmSeed9(lectin promoter). A previous introduction of a different lectin promoter construct did show cotyledon-specific expression of GFP in somatic embryos.Manipulation of flanking sequences of three G-boxes in a weak native GmScream5 (cupin) promoter revealed that the 4-bp upstream flanks was a major contributor to gene expression enhancement.Four RuBisCO small-subunit promoters from soybean were identified and cloned into our transient expression vector. RNAseq data for rbcS1-3 show high expression in green tissues, with rbcS1 displaying the highest expression in young leaf tissues. rbcS4 expressed only in the root and nodule, indicating that it was misidentified as a small-subunit promoter when the soybean genome was annotated. Transient gene expression in lima bean cotyledons closely match the levels shown with native transcript expression, and rbcS1 expressed transiently at over twice the level of the standard CaMV35S promoter. Each promoter:gfp construct was cloned into pCAMBIA for generation of soybean hairy roots, in order to rapidly assay for root-specific expression.. These promoters have recently been engineered into embryogenic tissue for generation of stable events and the first GFP expressing plants containing a rbcS promoter have been recovered. These events express GFP in the green proliferating embryogenic soybean tissue.Three different urease promoters were isolated and characterized.Two new constitutive promoters, GmubiXL and metalloprotease, were recently engineered into embryogenic tissue.Genomic EngineeringTargeted genomic engineering is an emerging technology with applications in both basic research and applied plant breeding. It can facilitate a diversity of activities, from removing anti-nutritional proteins/ metabolites from feed, to validating a candidate gene in a genomics program, to creating desirable alleles for a breeding program. First generation CRISPR/Cas9 vectors were created and validated in soybean. Two transgenes and six endogenous genes have been modified with this vector system.In addition to the original Cas9 from *Staphylococcus pyogenes,* three different Cas9-like genes were obtained, each with different targeting requirements. These come from *Staphylococcus* *aureus, Acidaminococcus spp.*, and *Streptococcus thermophilus*. Each one recognizes different DNA sequences in the genome. Thus using various different types of Cas9 (or the similar Cpf1) proteins gives researchers more flexibility to target different parts of the genome for editing. These new Cas9/Cpf1 genes have been placed in transformation vectors designed for the purpose. These vectors s use an expression cassette with sequences (promoter, terminator, and nuclear localization signal) from legumes. This cassette, termed a “clean cassette” as it is devoid of pathogen sequences, was cloned into a hygromycin selection vector for transformation. The use of such clean cassettes lessens the regulatory burden. In order to test the clean cassette and the efficiency of these Cas9 genes on plant cells, each construct has been entered into a binary vector to test on soybean hairy roots. In addition, these genes have been shot into cell lines, and plants recovered from the first set. Seed was harvested from 2 plants with Cas9 from *Staphylococcus aureus* and 1 with Cpf1 from *Acidaminococcus spp.*, while constructs with Cas9 from *Streptococcus pyogenes* and *thermophilus* were reshot and are currently under selection.  |
| Deliverables - List each deliverable and indicate whether or not it was supplied and if not supplied, please provide an explanation as to why. |
| **Gene-silencing**:* A set of silencing vectors for general purpose silencing in soybean. Supplied, and available to the public through AddGene
* Soybean plants with resistance to the soybean cyst nematode. The plants were obtained, but the strategy did not work as expected, and did not provide resistance.

**Promoters**:* ID of key regulatory elements from the best promoter. Supplied
* ID of 1-5 members of 1 new promoter family Supplied, and available – http://www.oardc.ohio-state.edu/SURE/.

**Genomic Engineering:*** Determine if CRISPR/Cas9 will provide a fast, economical and efficient system for soybean genomic engineering—Supplied, and first vectors available through Addgene
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| Did this project meet the intended Key Performance Indicators (KPIs)? List each KPI and describe progress made (or not made) toward addressing it, including metrics where appropriate.  |
| **Silencing for Nematode resistance:** * 5 genes, at 5 events per gene, will be characterized by small RNA deep sequencing and for cyst nematode resistance.

The goals to test the genes and characterize for soybean cyst resistance were met. While the genes were effectively expressed, they did not result in resistance.* A combinatorial strategy—which targets multiple genes at once- will be evaluated for nematode resistance.

The strategy was tried as planned, but did not work * **Silencing vectors:** One silencing vector system based on transacting silencing RNAs that can be used by soybean molecular biologists to modify traits in soybean

The vector system was developed, shown to be effective, and has been made available to the community. In addition, the “traditional” silencing vectors, based on hairpins rather than on transacting silencing RNAs, are also available.* **Promoters**: Requests for isolation, cloning and validation of additional soybean promoter families. Requests for existing promoter or element sequences by 1-2 different laboratories. These requests will suggest interest in soybean promoter cloning and characterization efforts.

Seven requests for promoters were received and filled. One request has been received to generate an additional promoter family. * **Promoters**: Key regulatory elements from the best promoter tested and validated. 1-5 members of a new family promoter family are identified.
* Identification and validation of 3 key promoter elements in the most useful promoter families, providing researchers with tools to precisely engineer gene expression.

Three different urease promoters were isolated and characterized. Elements within one of the GmERF promoters and some of the GmScream promoters were identified. Seven different seed specific soybean promoters (GmSeed family), a metalloprotease promoter, four RuBisCO small subunit, four root, and one elongation factor promoters were successfully isolated and cloned. Four publications in which USB was acknowledged described the 1) GmScream promoters, 2) GmERF promoters, 3) Soybean actin, heat shock protein and ribosomal promoters and 4) Intron and 5’ promoter regions of the GmUbi3 promoter.All the goals were met as planned and exceeded for the number of promoters characterized.**Genomic Engineering:*** Develop one vector that can target and cleave an endogenous gene target in soybean hairy roots, show genomic mutations at the DNA target site, Perform genomic re-sequencing of one knock-out and one control line and evaluate for off-target mutations.

All the goals were met as planned* Perform transformations of gene-targeting of an endogenous gene and generate at least five events with mutations in gene target and publish our results in a peer reviewed journal and make our sequences available to the public.

All the goals were met as planned* Build one set of CRISPR/Cas9 vectors devoid of pathogen sequences to facilitate shipment of transgenic seed and field trials

The goal of building the set has been met. As of yet, the verification that the vectors are functional is still pending.* Recover 1 soybean plant that had a gene added with CRISPR/Cas

Goal not met. |
| What, if any, follow-on steps are required to capture benefits for all US soybean farmers?Describe in a few sentences how the results of this project will be or should be used. |
| New tools are now available that expand the types of objectives that can be achieved with engineered soybeans. Specifically, simplified silencing vectors are available that can be used to remove traits, as are the first set of CRISPR/cas9 vectors for soybean genome-editing. Finally, a new set of seed-specific promoters will make it easier to engineer seed protein and oil quality. |
| **Describe any unforeseen events or circumstances that may have affected project timeline, costs, or deliverables.** |
| The genes selected for silencing in nematodes did not affect nematode survival as was originally expected, suggesting that nematodes have redundant gene function, such that the elimination of one can be covered by another. It will be necessary to devise alternative types of strategies to effectively control cyst nematodes in soybean. |
| **List any relevant performance metrics not captured in KPI’s.** |
| Cas9 plasmids deposited at Addgene have been requested by 54 researchers since 2014 (20 in 2016, 33 in 2015 and 1 in 2014), indicating that they are being widely used. |