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| **Reporting Period** | 1211 Final Report Jan1, 2011 – Sept 30, 2013 |
| **Proposal** | [1211 Center for Soybean Tissue Culture and Genetic Engineering: Engineering Soybean for Effective Resistance to Soybean Cyst Nematode (Year 1 of 3)](http://moss.unitedsoybean.org/Lists/Proposals/DispForm.aspx?ID=2177&RootFolder=*) |
| **Committee** | Production |
| **Target Area** | Supply |
| **Project Start Date** | 1/1/2011 |
| **Project End Date** | 9/30/2011 |
| **Project Number** | 1211 |
| **Project Status** | The overall goal is to generate soybeans that will have effective race-independent resistance against soybean cyst nematode, by identifying the proper genes and engineering them into soybean.  Performance Measures:  1)         Determine the best way to place the nematode genes within the vector to ensure that RNAi is reliably formed.  Summary  A set of plants was engineered with 8 genes against SCN and 8 against RKN. The lack of positive results with the first transgenic plants indicated that the vector design for gene-silencing was not as straight forward as initially believed. The soybean vector produced small RNAs in soybean against reporter genes but not against nematode genes. To trouble-shoot the vector, we conducted a complete factorial experiment focusing on three high-priority SCN gene targets used by our collaborators. These three were selected due to promising results in Arabidopsis. However, the Arabidopsis vector employs a different promoter and intron than is present in our soybean vector. Therefore, we moved the target genes into our soybean vector and transformed them into Arabidopsis. If it is the vector limiting expression, we should see fewer small RNAs in the transformed Arabidopsis plants. In addition, we took the Arabidopsis vectors and used them to get hairy roots and stable transgenics in soybean. Again, if it is the vectors, we should see small RNAs produced in soybean at the levels seen for Arabidopsis.  Accordingly the SCN team reported that five SCN parasitism genes (4F01, 30C02, 10A06, 4G06 and 8H07) performed well in sugar beet cyst nematode challenges of Arabidopsis, and we showed that they also produced significant numbers of small RNAs. The same Arabidopsis vectors were used to generate soybean hairy roots and roots from three events of each of the five SCN gene targets were collected and small RNAs were detected from soybean.  Having established that the vectors are making small RNAs against the nematode target genes it was imperative to perform SCN bioassays with stably transformed soybean.  Therefore, the Arabidopsis gene silencing cassette was combined with a hygromycin selection vector to generate stably transformed soybean with a goal of ten homozygous events/gene target.  Overall, we harvested T1 seed from 106 PCR-verified events of the 5 high-priority SCN gene targets. Seed from 9 homozygous events of 10A06, 3 each of 4G06 and 8H07 have been collected.  We devised and verified a reverse transcription PCR protocol that correlated well with small RNA production. We identified 27 events that are expressing the silencing construct at high levels. We have 8 events of 10A06, 6 of 30C02, 3 of 4F01, 4 of 4G06 and 6 of 8H07.  We performed small RNA sequencing on heterozygous T1 soybean plants that were transformed with the Arabidopsis vectors. They were shown by RT-PCR to be expressing the nematode target genes. The longer gene targets (4F01 and 8H07) produced more small RNAs than the shorter targets (10A06 and 4G06).  We don’t know the level that is needed to be ingested by the nematode to reduce infection. Thus, the first obstacle – lack of RNAi production, was overcome.  We sent seed from five 10A06 events (3 expressing lines and 2 non-expressing lines) to our collaborator for a bioassay. Preliminary results showed there was no reduction in cyst number.  We then verified that the events were homozygous and had consistent expression. It may be that the 10A06 parasitism gene by itself is not a good target to confer resistance.  Three additional targets (4G06, 8H07 and 30C02) have been sent for bioassay. The experiment will be repeated, this time using better quality seed. Seed for the initial experiment were obtained when the growth conditions were less than ideal.  Targeting multiple nematode genes may increase resistance. While waiting for results from the single target constructs to guide us in determining the most promising combinations we performed a 5-way bombardment of the high-priority nematode gene targets. We identified 8 events having multiple targets and 3 events have all 5 gene targets. When these are ready they will be shipped to our collaborator for a bioassay.  To ascertain the combination of promoter, intron and terminator required to optimally make small RNAs, a vector swap experiment was designed that used the components of both the Arabidopsis and the soybean vectors. Nine different constructs were made. Hairy roots from three events per construct were PCR-verified and RNA sequencing was performed. The results showed approximately 50-fold lower counts for vectors containing the GmUbi promoter when compared to the 35S promoter that is present in the Arabidopsis vectors. The other components (intron and terminator) had a smaller effect on small RNA count number. This result was surprising as the Gmubi promoter has been shown to be much stronger in terms of GFP expression than several versions of the 35S promoter although the version of 35S present in the Arabidopsis vector was not compared.  An alternative silencing vector that uses an endogenous microRNA (miR1514) target has been used to suppress expression of three genes in soybean. The proof-of-concept experiments showed consistent silencing in all experiments. New miR1514 vectors targeting SCN genes 4G06, 4F01, 8H07, 10A06 and 30C02 were constructed and bombarded into soybean. We currently have 3 PCR-verified events of 1514:10A06, 16 of 1514:4F01 and 3 of 1514:30C02.  The SCN team has shown that members of the miR396 gene family play a major role during syncytium initiation/formation in the Arabidopsis/sugar beet cyst nematode interaction. Overexpression of the miRNA396 led to reduced nematode susceptibility as well as reduced root growth. The Baum lab has cloned seven miR396 genes from soybean. In addition, the Mitchum lab of the SCN team sent two nematode-inducible promoters that are turned on in the nematode feeding site. These have been combined into hairy root vectors and shipped to the Baum lab to determine the role that the miR396 regulatory system plays in soybean. Furthermore, vectors optimized for generating stably-transformed soybean were made and bombarded into soybean to obtain stably transformed events. We currently have 9 PCR-verified events of 396-b, 1 of 396-c, 4 of 396-d, 7 of 396-e and 6 of 396-f.  Assessment  The original goals of engineering soybean with genes from SCN were achieved, but the biology of the system was not what was anticipated, so resistance was not obtained.  A set of experiments was designed to dissect out the biology of the system. At this point, several variations and alternatives were tried, but more time is needed to test the results.    2)         Identify, clone and evaluate 1-2 root-specific and/or inducible promoters per year, using engineered soybean roots in culture as a model for quantification of promoter strength.  Summary  We identified and initiated characterization of members of three promoter families of soybean. They are the constituently expressed GmUbi, the wound-inducible GmERF and the GmScream which should be highly expressed based on RNAseq data.  We generated six constructs to evaluate the wound-inducible GmERF3 promoter. These constructs include the previously cloned 1.3 kb and a longer 2.8 kb promoter region in combination with either the native GmERF3 terminator or NOS terminator. We also built two GmERF3::GFP translational fusions. These constructs were characterized using transient expression and soybean hairy roots. This analysis was to identify additional regulatory regions involved with rapidity of wound induction mediated by the GmERF3 promoter. There are two "waves" of induction, one is a rapid induction (within 1 hr) in response to wounding and a secondary response that occurs after two days that seems to be associated with wound callus formation.  We analyzed *gfp* gene expression driven by GmScream8 and GmScream4 promoters in stably transformed soybean. Good levels of GFP expression were observed in newly growing embryogenic tissues as well as root tips of the germinated embryos in transgenic GmScream4-containing tissue while GmScream8-containing tissue only showed expression in embryos in early stages. Expression was expected to be quite high as RNAseq data indicated that the genes are expressed at high levels in most tissues. It is unclear why these promoters are not more active.  The GmScream promoters and our Gmubi promoter contain leading introns and we detected interesting interactions between intron-containing sequences (in synthetic introns) and proximal promoter element sequences (in synthetic promoters). One of the intronic sequences in one of our synthetic introns may contribute to tissue specific expression in hairy roots. As far as we know intron contribution to tissue specific expression hasn’t been previously reported. For promoter (and intron) analysis, we performed intron partial deletions from the leading intron of the GmScream8 promoter to see if the intron contains elements that interact with a synthetic promoter that contains a tetramer from the same promoter. The intensity of transient gene expression in lima bean cotyledons was reduced with one of the deletions while the other two had no significant decrease. This suggests a region within the intron, which interacts with sequence within the promoter. Stable expression in soybean hairy roots showed that expression intensity decreased greatly with some of the intron deletions, which indicate there may be additional intron/promoter proximity effects. However, no difference in gene expression pattern was observed within transformed hairy roots containing the three intron deletion constructs.  Assessment  A core set of novel promoters was obtained and made available to the community. These are now in use by public and industry researchers.    3)         Provide transformation assistance to other USB-funded research groups working on nematode resistance to enable them to meet their research goals and deliverables.  Summary  We assisted three USB-funded labs (Mitchum, Nyugen and Bent).  A biolistic vector was constructed by the Center and shipped to the labs. It contained a selectable marker cassette and a Gmubi promoter cassette for insertion of their gene of interest.  For the Mitchum lab we transformed with a vector containing a putative resistance gene into three SCN-susceptible soybean genotypes. We recovered 2 events from one genotype and have shipped the seed. We recovered 22 events in cv. Jack with seed harvested from 7 events. Seed from 1 event was shipped to the Mitchum lab and the others will soon follow.  Four constructs from the Nguyen lab designed for nematode resistance were bombarded into both JackX and Magellan and we have identified 19 PCR positive JackX events in total. They are at the tissue culture stage. The Bent lab sent us 4 different constructs for introduction into cv. Thorne. We recovered over a hundred putative events. Not all have been analyzed but of those tested to date only one is positive by PCR.  To increase the throughput of transformed soybean to other labs we investigated whether desiccated embryos could be shipped and regenerated into plants at their location. We can generate many of these quickly and most labs can make the media for embryo germination. This will alleviate constraints on our greenhouse facilities. We sent desiccated non-transformed embryos to the Mitchum lab and they reported a 77% germination rate and that the plants looked good.  We are very hopeful that this approach will work and we can deflect the growing and analysis of T0 plants to other programs.  Assessment  We have delivered transformed soybean to our collaborators however not all of their supplied genotypes have been optimized for success in tissue culture.  The SCN-susceptible genotypes received from the Mitchum lab were very poor at regenerating into plants therefore we also transformed the vector into cv. Jack. Jack is our standard for transformation and also is susceptible to specific races of SCN. The Thorne line is very fast growing and requires alternate selection and growth strategies. This is underway.  Shipping desiccated embryos will allow us to increase the throughput of transgenic soybean for other researchers. |
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