Summary of quarterly reports and Final Report for 2011
Virginia Peanut Growers Association grant 457338
Protocol Development and Validation for Detecting Genetically Modified Peanuts
Elizabeth A. Grabau

1st quarter activities report

1. Acceptance of manuscript
   Sclerotinia Blight Resistance in Virginia-Type Peanut Transformed with a Barley Oxalate Oxidase Gene by D. E. Partridge-Telenko, J. Hu, D. M. Livingstone, B. B. Shew, P. M. Phipps, and E. A. Grabau in Phytopathology
2. Continuation of data collection for resubmission of petition for deregulated status of transgenic Blight Blocker peanuts to the Animal and Plant Health Inspection Service Biotechnology Regulatory Services (APHIS BRS)
3. Invited presentation at the annual meetings of the American Society for the Advancement of Science:
   "A view from the trenches: Challenges bringing GM crops to the market place." Invited speaker at Symposium entitled "Genetically modified crop regulations: safety net or insurmountable obstacle?" at the AAAS annual meetings in Washington, D.C., February 18, 2011.

2nd quarter activities report

1. We continued collecting data to revise our petition to the Animal and Plant Health Inspection Service (APHIS) Biotechnology Regulatory Service (BRS) for deregulation of transgenic Blight Blocker peanuts.
2. Appearance of field testing data in press. The article was chosen as the's Editor's Pick (Phytopathology Editor's Pick,Dr. Niklaus Grunwald, Editor-in-Chief)
   "Sclerotinia blight is a major disease of peanut in the United States. Partridge-Telenko and colleagues effectively demonstrate that the barley derived oxalate oxidase gene can provide resistance to Sclerotinia blight in field trials. This approach shows promise for eliminating fungicide for control of Sclerotinia blight, increasing profits, and promoting more environmentally sound approaches to disease management.
   http://www.apsnet.org/publications/Emails/APSupdate42.htm

3rd quarter activities report

1. Maintenance and monitoring of transgenic plots at the Tidewater AREC (Phipps)
2. Continued editing the petition to USDA for resubmission. Data added to petition includes the items requested by the USDA APHIS Biotechnology Regulatory Services (Phipps and Grabau)
3. Performed work outlined in the objectives of the 2011 grant. (Grabau)
   a. We designed oligonucleotide primers for PCR reactions to test for oxalate oxidase transgene and hygromycin resistance gene as the selectable marker. We have identified a number of primer sequence pairs and have used several primer sets to demonstrate amplification of the target sequence with the primers chosen.
   b. We have continued to optimize DNA purification procedures from peanut material. We have identified best procedures for DNA purification from leaves and germinating hypocotyls. We are still working on testing a procedure to isolate DNA from dry peanut seeds.

c. We have developed PCR conditions that confirm presence of the transgene from both peanut genomic DNA of transgenics and plasmids containing the Oxalate oxidase gene. We are still working on determination of the sensitivity of the procedure.
d. Once sensitivity levels are determined, we will test mixtures of milled peanuts in various for detection of transgenics in bulked seeds.

4th quarter activities report

1. Invited to participate in a Specialty Crop Regulatory Assistance workshop entitled "Nuts and Bolts of US Regulatory Dossiers for Genetically Engineered Crops" as a case study in the regulatory process. The Blight Blocker project was presented along with commentary by USDA APHIS, EPA and FDA, helping to clarify the required elements of petitions through the approval process.
2. Prepared genomic DNA for sensitivity testing from transgenic and non-transgenic lines.
3. Germinated new high folate transgenic peanut lines and planted in pots in Blacksburg environmental chambers for eventual transfer to AREC for seed increase. (Plants were transferred Friday, Jan. 6).
4. Submitted pre-proposal to Virginia Agricultural Council for research support of transgenic peanut project.
5. Planned for proposal submission to the Biotechnology Risk Assessment Grants (BRAG) program.

2011 Final Report

Research on the transgenic peanut project in 2011 consisted of four major activities: 1) continued collection of data for submission to regulatory agencies, 2) field increase and testing of Blight Blocker peanuts, 3) optimization of protocols for transgene detection, and 4) increase of high folate peanuts for future biochemical testing (the latter continues into 2012).

1) The three federal regulatory agencies involved in deregulation of transgenic crops include USDA APHIS Biotechnology Regulatory Services (BRS), Environmental Protection Agency (EPA) and Food and Drug Administration (FDA). The Blight Blocker project and obstacles to deregulation have been the topic of several presentations in efforts to clarify and streamline the process. Dr. Grabau was part of a panel presentation at the national meetings of the American Association for the Advancement of Science (AAAS) in February and at a workshop of the Specialty Crops Research Assistance group in December (details reported above in quarterly reports). The petition to BRS is under revision. One remaining data item being sought before submission is to sequence the region of the peanut genome directly flanking the oxalate oxidase insert. Data collection for EPA is the subject of a research proposal to be submitted February 1, 2012 to the Biotechnology Risk Assessment Program (BRAG) program.

2) Blight Blocker peanuts lines were advanced another generation in the field at the Tidewater AREC by Dr. Pat Phipps. Plots were monitored for disease resistance, yields, and agronomic and market traits. Research results were published in Phytopathology in 2011.

Complete citation: Partridge-Telenko, D.E., Hu, J., Livingstone, D.M., Shew, B.B., Phipps, P.M. and Grabau, E.A. 2011. Sclerotinia blight resistance in Virginia-type peanut transformed with a barley oxalate oxidase. Phytopathology 101: 786-793.

3) As of the end of 2011, procedures had been developed for transgene detection but testing to determine the dilution factor at which the transgene detected continued into early 2012 (see data below).

4) Transgenic peanuts engineered for high folate have been recovered and transgene presence and expression evaluated. Positives are being grown in the greenhouse at Tidewater for seed increase and planting in the field in summer 2012.

Activities since December 2011

1) Wrote and submitted a grant for $500,000 to the Biotechnology Risk Assessment Grants (BRAG) Program through National Institute for Agriculture (NIFA). The grant proposes to examine the possible ecological impacts of Blight Blocker peanuts on the potential for weediness, on insect populations and on soil microbial communities. Granting decisions will be made by the agency by the end of July 2012.

2) Conducted a series of detection tests using dilutions of peanut genomic and transgene DNA to discover the lower limit at which the Blight Blocker transgene (oxalate oxidase) could be detected in a batch of non-transgenic peanuts.

Experimental design: Conduct PCR amplification of different mixtures of peanut genomic DNA from transgenic (Blight Blocker, also called P39) and non-transgenic parent (Perry) peanuts.

Plasmid PCR:

First objective was to design and test primers for the oxalate oxidase (OxOx) gene. The final primers chosen showed good amplification of the target OxOx sequence. Listed below are the two primer sequences and an illustration of the alignment of the primers with the OxOx sequence (Figure 1). Forward OxOx primer: CCCTCTACAGGACTTCTGCG; Reverse OxOx primer: CTGGCTGTTGAAGGAACACAA.

Figure 1. Barley oxalate oxidase sequence (675 bp) with the primer locations highlighted in yellow.

ATGGGTTACTCTAAAAACCTAGGGGCTGGCCTGTTCACCATGCTGCTCCTTGCTCCGGCCATCATGGCTACCGACC

Forward primer---------->

CTGA**CCCTCTACAGGACTTCTGCG**TCGCGGACCTCGATGGCAAGGCGGTCTCGGTGAACGGGCATACGTGTAAGC

CCATGTCGGAGGCCGGCGACGACTTCCTCTTCTCGTCCAAGCTGACCAAGGCCGGCAACACGTCCACCCCGAACG

GCTCGGCCGTGACGGAGCTCGACGTGGCCGAGTGGCCCGGTACGAACACGCTGGGCGTGTCCATGAACCGTGTG

GACTTCGCGCCGGGCGGCACCAACCCGCCGCACATCCACCCGCGTGCAACCGAGATCGGCATGGTGATGAAAGG

TGAGCTCCTCGTTGGAATCCTCGGCAGCTTTGACTCCGGAAACAAGCTCTACTCCAGGGTGGTGCGTGCCGGAGA

←----------

GACTTTCGTCATCCCGCGCGGCCTCATGCACTTCCAGTTCAACGTTGGTAAGACGGAAGCCTACATGG**TTGTGTCC**

Reverse primer

TTCAACAGCCAGAACCCTGGCATCGTCTTCGTGCCGCTCACACTCTTCGGTTCCAACCCGCCCATCCCCACACCGGT

GCTCACCAAGGCTCTTCGGGTGGAGGCCGGGGTCGTGGAACTTCTCAAGTCCAAGTTCGCCGGTGGGTCTTAA

Once primers were tested, they were used in PCR reactions containing a mixture of peanut DNA with different concentrations of the pOxOx plasmid to demonstrate the sensitivity of the amplification and to verify that the plasmid copy of OxOx could be detected and amplified in the presence of peanut DNA. Peanut genomic DNA amount per each reaction = 1 µg. The amount of pOxOx plasmid DNA varied in each reaction, from 10 ng to 0.0001 ng in 10-fold dilutions. The OxOx gene was detected by PCR amplication down to a level of 0.001 ng = 1 pg. This indicates that the PCR reaction can amplify at very low levels of the OxOx gene (on a plasmid).

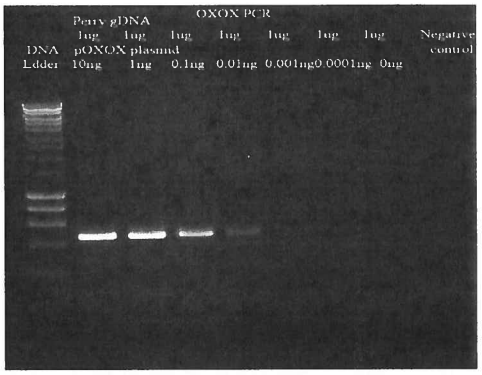

Figure 2. Amplification of OxOx DNA in the presence of peanut genomic DNA. OxOx gene is on a 7 kb pOxOx plasmid. Genomic DNA is from cultivar Perry.

To confirm the amplification results, we also tested for the presence of the selectable marker gene for hygromycin resistance (Hyg) also present on the plasmid using the same series of dilutions. The results (Figure 3) showed the same level of detection for hyg as for OxOx (0.001 ng) as expected.

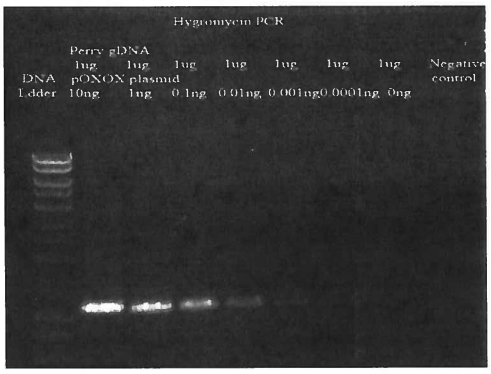

Figure 3. PCR amplification of hygromycin phosphotransferase gene in the presence of peanut genomic DNA. Hyg gene is on a 7 kb plasmid. Genomic DNA is from cultivar Perry.

The OxOx gene is located on a 7 kb plasmid which was introduced in into the peanut genome of 2,800 Mb during the transformation of Perry to yield the Blight Blocker line. (That translates to a peanut genome that is approximately 400,000 times the size of the introduced plasmid.) Another way to express this is that to simulate detection of a single copy of the OxOx gene integrated in a peanut genome, it would require a plasmid dilution factor of 400,000-fold. The amount of plasmid DNA in Figure 2 represents dilutions from 100-fold (10 ng) through 10-million fold (0.0001 ng) relative to 1 µg genomic DNA used in PCR reactions. The gene was detected down to the 1-million fold dilution, indicating that detection sensitivity is greater than a simulated single copy gene in the genome.

Genomic PCR:

To test levels of detection of OxOx in peanut DNA mixtures, genomic DNA from transgenic P39 (Blight Blocker) and parent cultivar Perry was mixed at different ratios. The total DNA amount per amplification reaction was kept constant at 1 µg. Detection of the OxOx gene was seen at a ratio of 1:10 transgenic to non-transgenic but was undetectable at 1:100 ratio (Figure 4). This result indicates that under the amplification conditions used, contamination of a non-transgenic batch of seeds with Blight Blocker could only be detected at a level of 1 seed in 10.

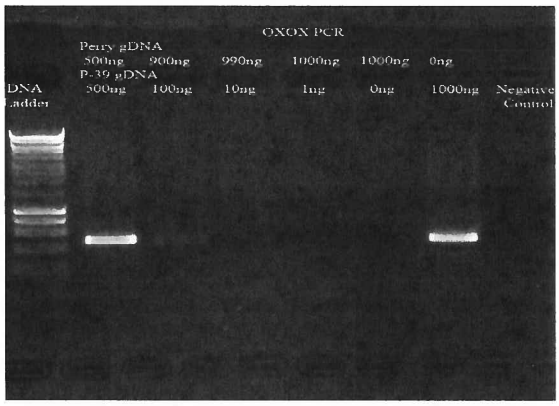

Figure 4. PCR amplication of OxOx gene from genomic DNA mixtures. Mixtures ranged from 1:1, 1:10, 1:100, 1:1000 of transgenic Blight Blocker P39 to Perry genomic DNA. Total amount of DNA remained constant per reaction (1 µg).

We predict that the relatively low detection ratio using genomic DNA mixtures was as a result of the relatively high concentration of DNA used (due to viscosity). We will repeat the experiments using a lower total concentration of DNA with the same ratios. We will also use reactions conditions with a greater number of amplification cycles (40 rather than 35).

Components and conditions for the OxOx PCR reactions:
Total reaction volume of 50 µl
2X immoMix red, PCR polymerase
DNA template 1 µg
Primer concentration is 0.2 pmol for both primers
Annealing temperature, 58°C, 35 cycles