



11/1/2017

**Nebraska Soybean Board
Year-End Research Findings Report**

*Please use this form to summarize the practical benefits of your research project and what has been accomplished.
Your answers need to convey why the project is important and how the results impact soybean production.*

Project Title: Improvement of Soybean Germplasm for Aquaculture Feed (#1716)

Contractor & Principal Investigator: Ed Cahoon (PI), Tom Clemente (co-PI)

Please check/fill in appropriate box: Continuation research project
 Year 2 of 3 research project (for example: Year 1 of 2)

1. What was the focus of the research project or educational activity?

The proposed research addresses the need for development of a complete soybean-based aquaculture feed. The current soybean-based feed lacks sufficient levels of EPA omega-3 fatty acids and other oil-based feed components. Because of these deficiencies, soybean-based aquaculture feed currently requires supplementation with fish oil and high-priced astaxanthin pigments, particularly for farm-raised salmon. In addition, oils with enhanced omega-3 fatty acid content are prone to oxidation, which limits the shelf life of fish due to the development of off-flavors and odors. The proposed research will address these deficiencies by:

1. Applying emerging synthetic biology techniques to rapidly stack or combine omega-3 fatty acid, astaxanthin, and high vitamin E antioxidant traits into Nebraska soybean germplasm for optimized aquaculture feed.
2. Improvement of omega-3 EPA content in Nebraska soybean germplasm.
3. Improvement of astaxanthin pigment levels in Nebraska soybean germplasm.
4. Evaluation of new oil traits in aquaculture feeding studies.

2. What are the major findings of the research or impacts of the educational activity?

Summary of the major findings to date:

1. T₂ generation of soybean seeds that contain omega-3 EPA, astaxanthin, and enhanced vitamin E antioxidants were confirmed in the greenhouse and in field experiments at ARDC in Mead, NE.
2. The omega-3 EPA, astaxanthin, and enhanced vitamin E antioxidants were stably produced in T₂ soybean seeds.
3. Insertion of 8 genes in the soybean genome was confirmed by Southern blot.
4. Gene expression of 8 genes inserted in the soybean genome was confirmed by RT-PCR and northern blot. Large size of exogenous DNA is stable in T₂ generation.
5. Soybean T₂ lines engineered with bacterial genes for astaxanthin produce beta-carotene and astaxanthin.

Seeds from lines 1079-3, 1079-5, 1079-6, 1079-8, and 1079-9 that produce EPA, high vitamin E, and astaxanthin have been field planted at ARDC in Mead, NE (Fig. 1). Seed coatings were tested as a method to improve germination rates of these lines. The presence and copy number of transgenes in these lines were confirmed by Southern blot analysis (Fig. 2). The expression of each transgene integrated into the soybean genome was analyzed by RT-PCR, and all genes used for multi-gene stacking were expressed in soybean seeds from different lines (Fig. 3). Expression of the Keto2 gene for astaxanthin production was confirmed by Northern blot analysis of immature embryos (Fig. 4). All three traits, omega-3 EPA, vitamin E (tocotrienols) antioxidant, and astaxanthin, were stably transmitted to the T₂ generation in lines from

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the greenhouse and field. Astaxanthin-producing T₂ red seeds of lines 1079-3, 1079-5, 1079-6, 1079-8, 1079-9 produced EPA (2.5-5.8 mol%) and its precursors (27-33 mol%), tocotrienols (530-830 µg/g), and two astaxanthin forms astaxanthin 1 (52-154 µg/g) and astaxanthin 2 (29-75 µg/g) (Figs. 5, 6, 7). Total astaxanthin pigment concentrations as high as 210 µg/g were detected in T₂ seeds.

Soybean seeds from different T₁ lines, 1080-1, 1095-2, and 1099-2, were analyzed. 1080-1 and 1095-2 produced omega-3 EPA, astaxanthin, and vitamin E (tocotrienols). 1099-2 produced omega-3 EPA and astaxanthin but not vitamin E (tocotrienols).

To determine whether the vitamin E trait is able to increase astaxanthin content of seeds, our top lines for astaxanthin have been crossed with high vitamin E lines. Ten F₁ seeds were generated from crossing of 948-1 (19059, high astaxanthin line) and 488-3 (high vitamin E line). The F₁ seeds have been planted in soil to produce more seeds for analysis.

Soybean T₂ lines engineered with bacterial genes for astaxanthin production were also analyzed. Lines 18949, 18951, 20159, 20288, and 20293 had astaxanthin levels of 15 to 22 µg/g and high beta-carotene levels of 70 to 300 µg/g. Lines 19026, 19059, 19060, 19724, and 20278 had astaxanthin levels of 7 to 17 µg/g and very low beta-carotene levels of 2-5 µg/g. Lines 15251, 19782, 19783, 19784, and 26965 accumulated beta-carotene (100 to 300 µg/g) but had no detectable astaxanthin (Fig. 8).

Building on our success with stacking multi-transgenes for improved oil and astaxanthin compositions for aquaculture, we assembled four bacterial genes (MetA, MetB, MetC, and MetE) under control of seed-specific promoters to improve the methionine content of soybean meal. The gene expression construct was completed (Fig. 9) and have been introduced into soybean by the Clemente lab. This project is intended to optimize meal quality and oil and astaxanthin content of soybean seeds for aquaculture and other feed uses.

To produce a second fish oil component DHA (docosahexaenoic acid) in soybean, we have also been initiated to introduce two additional transgenes, *Ostreococcus tauri* elongase 5 (OtElo5) and *Emiliania huxleyi* desaturase 4 (EmiD4), into multi-gene genes stacking vector (pPTN1331).

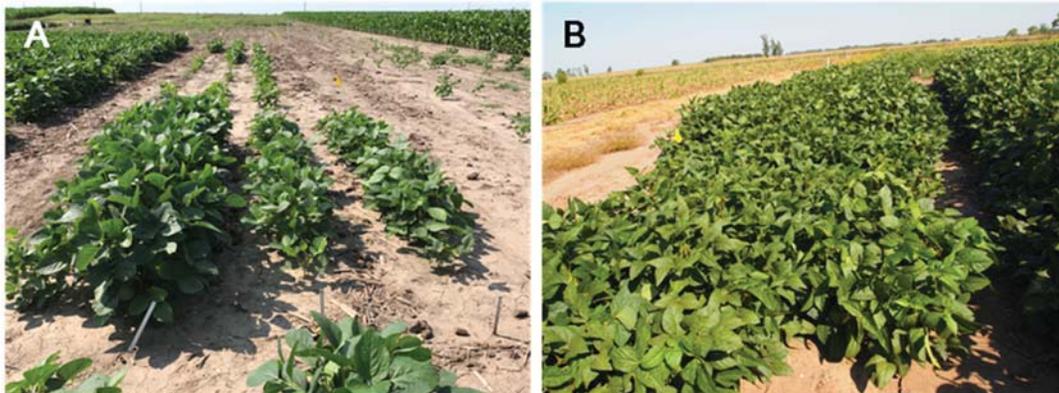


Figure 1. Soybean plants in field at ARDC in Mead, NE. (A) 50-day-old soybean plants. (B) 80-day-old soybean plants.

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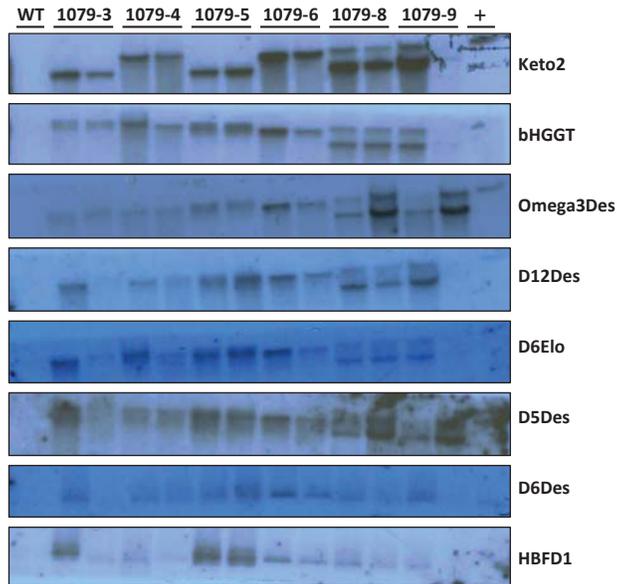


Figure 2. Transgenic allele integration analysis via southern blot of soybean plants carrying pPTN1331. Left to right: WT: Thorne, six soybean events (two plants each), and positive control. Each southern blot was hybridized with a fragment amplified via PCR from Keto2 (524 bp), bHGGT (538 bp), Omega3Des (548 bp), D12Des (553 bp), D6Elo (526 bp), D5Des (518 bp), D6Des (515 bp), or HBFD1 (593 bp). All plants were genotyped via PCR prior to southern blots.

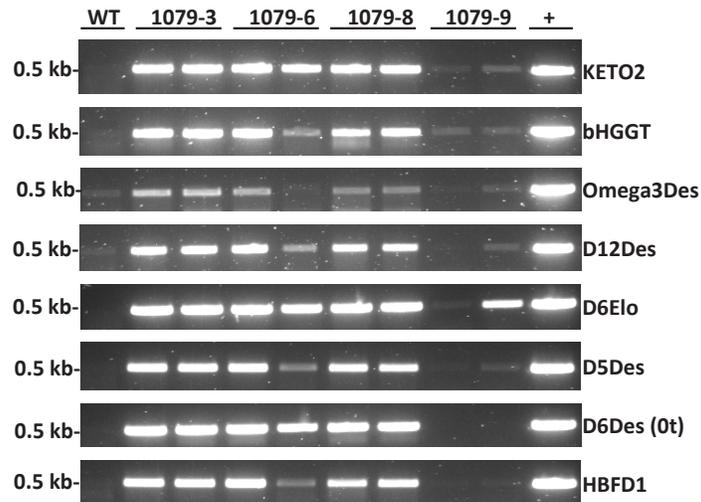


Figure 3. Gene expression analysis via RT-PCR of soybean seeds carrying pPTN1331. Left to right: WT: Thorne, and four independent events (two plants each), and positive control (pPTN1331). Keto2 (524 bp), bHGGT (538 bp), Omega3Des (548 bp), D12Des (553 bp), D6Elo (526 bp), D5Des (518 bp), D6Des (515 bp), and HBFD1 (593 bp).

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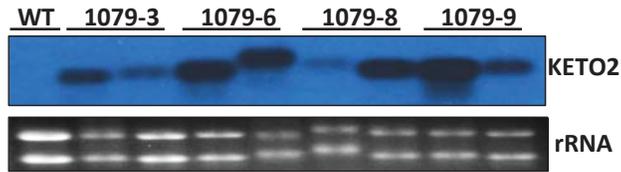


Figure 4. Keto2 expression analysis via northern blot of immature embryos derived from soybean plants carrying pPTN1331. Left to right: WT: Thorne and four independent events (two plants each). The northern blot was hybridized with a fragment amplified via PCR from Keto2 (524 bp).

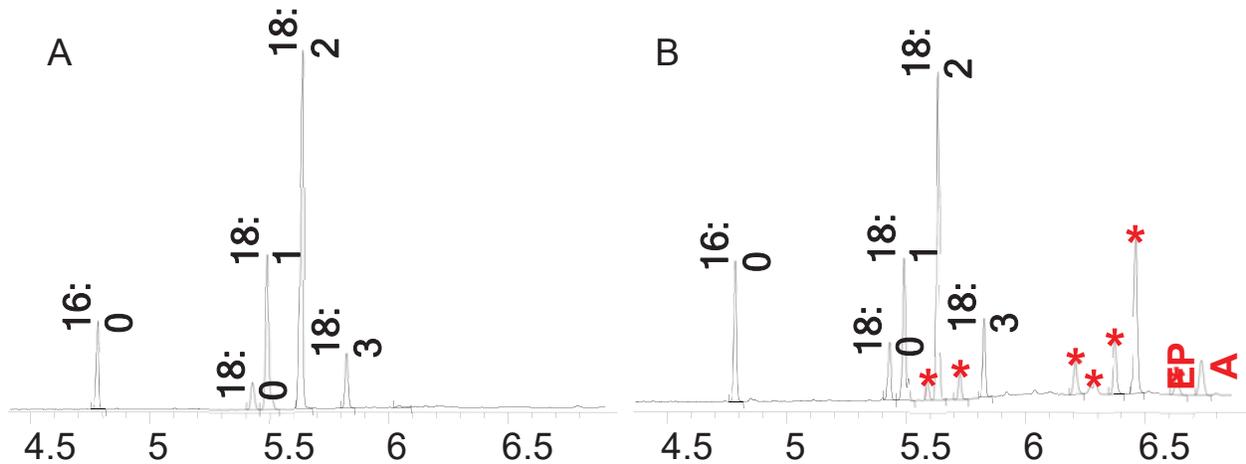


Figure 5. GC chromatogram of fatty acid analysis of transgenic soybean carrying pPTN1331. (A) Wild type soybean seed as negative control. (B) T₂ generation of soybean seed from field. Asterisks indicate the precursors of EPA.

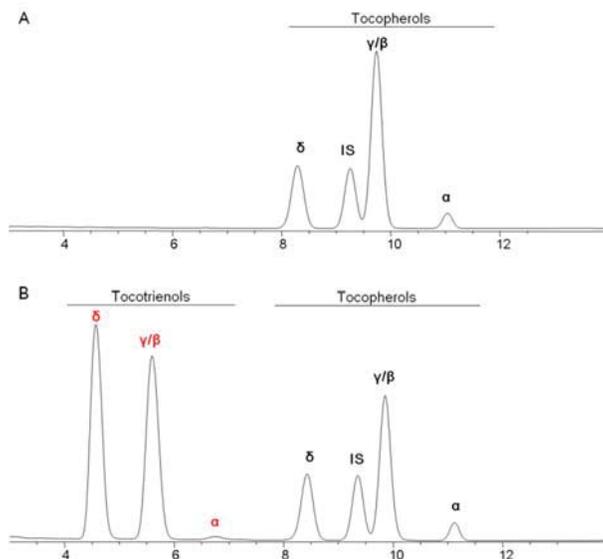


Figure 6. HPLC chromatogram of Vitamin E analysis of transgenic soybean carrying pPTN1331. (A) Wild type soybean seed as negative control. (B) T₂ generation of soybean seed from field. IS indicates tocol as an internal standard for quantification of vitamin E. Engineered soybeans in (B) accumulate high levels of the tocotrienol form of vitamin E.

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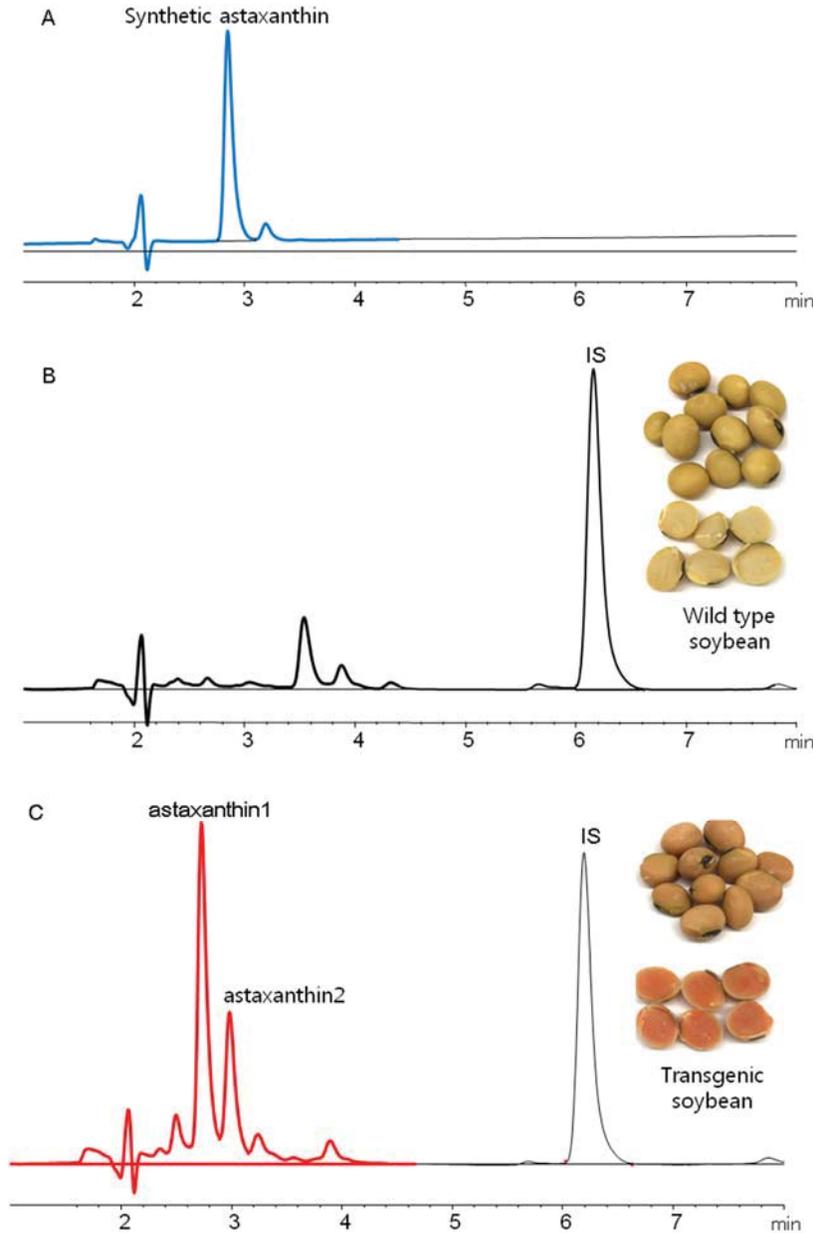


Figure 7. HPLC chromatogram of astaxanthin analysis of T₂ transgenic soybean carrying pPTN1331. (A) Synthetic astaxanthin as standard. (B) Wild type soybean seed as a negative control. (C) T₂ generation of soybean seed from field. IS indicates 8'-apo-beta-Carotenol as an internal standard for quantification of astaxanthin.

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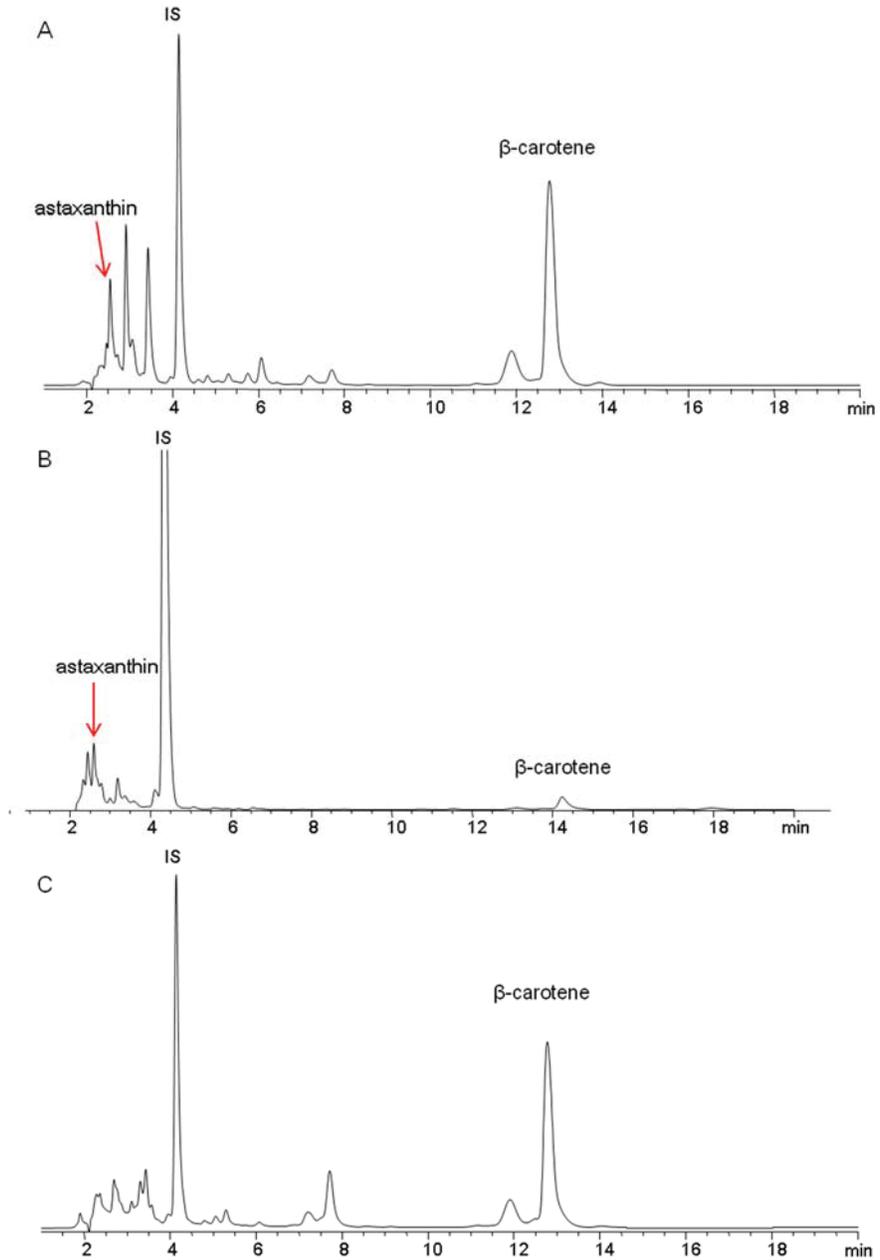


Figure 8. HPLC chromatogram of carotenoids analysis of T2 transgenic soybean with bacterial genes for astaxanthin production. (A) 18949. (B) 19724. (C) 15251. IS indicates 8'-apo-beta-Carotenol as an internal standard for quantification of carotenoids.

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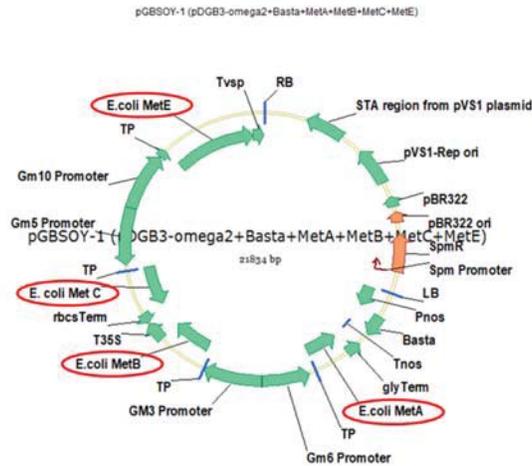


Figure 9. The map of binary vectors containing four genes to improve the methionine content of soybean meal.

Impacts

Our results to date demonstrate that new synthetic biology techniques are capable of delivering large numbers of transgenes to soybean to rapidly develop high-value seed quality traits. In one year, we have generated soybean germplasm with three aquaculture oil traits that could not be achieved by conventional breeding. **In this second year, we generated T2 soybean in field which produced omega-3 EPA, astaxanthin, and enhanced vitamin E antioxidants.** The findings to date are not only significant for soybean improvement for aquaculture feed but also pave the way for adopting synthetic biology approaches to target both output traits (e.g., increased yield) with seed quality traits for rapid improvement.

3. Briefly summarize, in lay terms, the impact your findings have had, or will have, on improving the productivity of soybeans in Nebraska and the U.S.

The project addresses the Nebraska Soybean Board FY17 focus area of germplasm improvement for composition and yield. The project is aimed at enhancing the value of Nebraska soybeans and increasing the use of soybeans for the expanding aquaculture feed market. Nearly 50% of fish that is consumed globally is farm-raised, and this production system is anticipated to expand as world population grows, ocean stocks of fish dwindle, and consumers place more emphasis on fish for healthy diets. Meeting this demand requires development of sustainable aquaculture feed sources, which can be met in large part by soybean-based feed. This research project will increase the amount of soybeans used in aquaculture feed by addressing deficiencies in the oil component of soybeans in current soy-based aquaculture feed rations. This is expected to translate into increased demand and expanded markets for Nebraska soybeans. The project builds on successes of the investigators in engineering improved compositional traits in soybean seeds, including enhancements in vitamin E antioxidant and omega-3 fatty acid content.

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The project also shows the power of synthetic biology technology to deliver large gene stacks to soybean that should provide a route for more rapid improvement of soybean germplasm for Nebraska and US farmers.

4. Describe how your findings have been (or soon will be) distributed to (a) farmers and (b) public researchers. List specific publications, websites, press releases. etc.

The findings have been distributed through research publications and a conference presentation, including:

Oral presentation, 2017 Phytochemical Society of North America, Columbia, MO, August 9, 2017.

Publications:

Park H, Weier S, Razvi F, Peña P, Sims N, Lowell J, Hungate C, Kissinger K, Key G, Fraser P, Napier JA, Cahoon EB, Clemente T (2017) Towards the development of a sustainable soya bean-based feedstock for aquaculture. *Plant Biotechnology Journal* 15:227-236.



5. Did the NE soybean checkoff funding support for your project leverage any additional state or Federal funding support? (Please list sources and dollars approved.)

Current leveraged funding from NE Soybean Checkoff:

USDA-NIFA grant, Cahoon (PI) Overcoming metabolic bottlenecks for enhanced vitamin E production in crop plants. 02/01/2015 - 01/31/2018 \$490,000

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