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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: University of Nebraska-Lincoln; PIs: Ed Cahoon & Tom Clemente

Year 3 **of** 3 **research project**

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

The proposed research addresses the need for soybean germplasm with high-value oil quality traits for aquaculture feed. The current soybean-based aquaculture feedstocks lack EPA and DHA omega-3 fish oil fatty acids and other oil-based feed components. Because of these deficiencies, soybean-based aquaculture feed requires supplementation with fish oil and high-priced astaxanthin flesh pigments, particularly for farm-raised salmon. In addition, oils with enhanced omega-3 fatty acid content are prone to oxidation, which reduces the shelf life of fish due to the development of off-flavors and odors. The proposed research will address these limitations in oil quality for increased use of soybeans for aquaculture feed by:
1. Developing soybean germplasm with oils enriched in EPA and DHA omega-3 fatty acids.
2. Optimizing production of astaxanthin in soybean seeds.
3. Applying emerging synthetic biology techniques to stack EPA/DHA omega-3 fatty acid, astaxanthin, and high vitamin E antioxidant traits into Nebraska soybean germplasm.
4. Conducting physiological and field-evaluation of new aquaculture germplasm to optimize agronomic performance.

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

Summary of the major findings of FY21 research activities are:

• Seeds from field-grown soybeans in 2020 engineered for EPA/Astaxanthin/Vitamin E contained up to 30 µg/g dry weight of ketocarotenoids including astaxanthin. Plots of EPA/astaxanthin/vitamin E-producing lines were successfully grown in the ENREC biotech field in 2021 for evaluation of seed traits, which are currently underway.

• Seeds from DHA/vitamin E soybeans contained up to 6.9% EPA and 4.5% DHA. The seeds were sown in the green house and large-scale harvested for next field trials. We believe that these levels are at or approaching commercial relevance.

• Seeds (F3) from crosses between EPA/astaxanthin/vitamin E soybeans and high oil soybeans displayed restored seed shape relative to the EPA producing parent. EPA/astaxanthin/vitamin E analysis are underway. Crossed lines were advanced to the F4. This finding suggests that enhancing seed oil content may address some of the potential yield drag issues with EPA/DHA-enriched oil production in soybean seeds.

• Crosses between EPA/astaxanthin/vitamin E soybeans and a high C18 soybean line exhibited increased seed oil content (11%) were successfully germinated and their growth phenomenon was similar with wild type. Crossed lines were advanced to the F4.

• To improve production of EPA or DHA, new binary vectors containing EPA or DHA expression cassette along with Δ15 desaturase cassette were designed. Soybean transformations were initiated.

1. pPTN1331 soybean lines with EPA, astaxanthin and enhanced vitamin E in seeds

For our 2020 field trial, the pPTN1331 soybean lines were grown at Eastern Nebraska Research and Education Center (ENREC) in Mead, NE. and the seeds were harvested. Eight plants from four pPTN1331 events (1079-3, 1079-6, 1079-8 and 1079-9) were analyzed EPA level, seed oil level, astaxanthin level and vitamin E level.

Before analysis of their given-traits, we investigated transcripts level of eight transgenes in developing seeds (R5.5 developing stage and R6 developing stage) from pPTN1331 events. All transcripts of eight transgenes were detected in the R5.5 and R6 developing stage (Fig 1).

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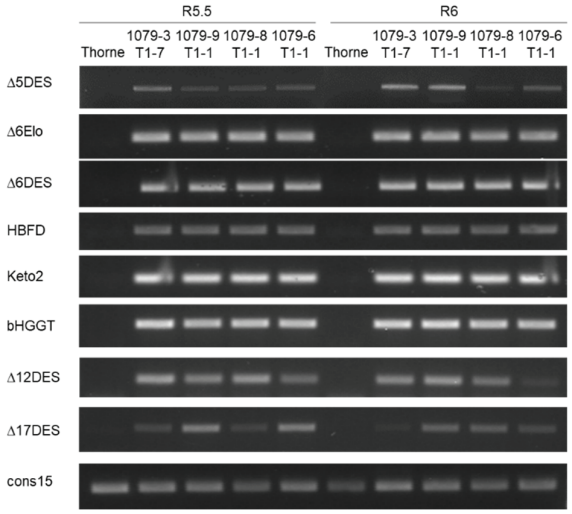

Figure 1. RT-PCR analysis of eight transgenes in R5.5 and R6 developing stages from Thorne and pPTN1331 events. The *cons15* gene used as a reference gene.

The EPA content in pPTN1331 T4 seeds ranged from 0.9 % to 2.5 % of total fatty acids (Fig 2A). Total seed oil level in pPTN1331 T4 seeds ranged from 162 µg/mg to 182 µg/mg, 69%-78% of the oil found in wild type Thorne seeds (234 µg/mg) (Fig 2B).

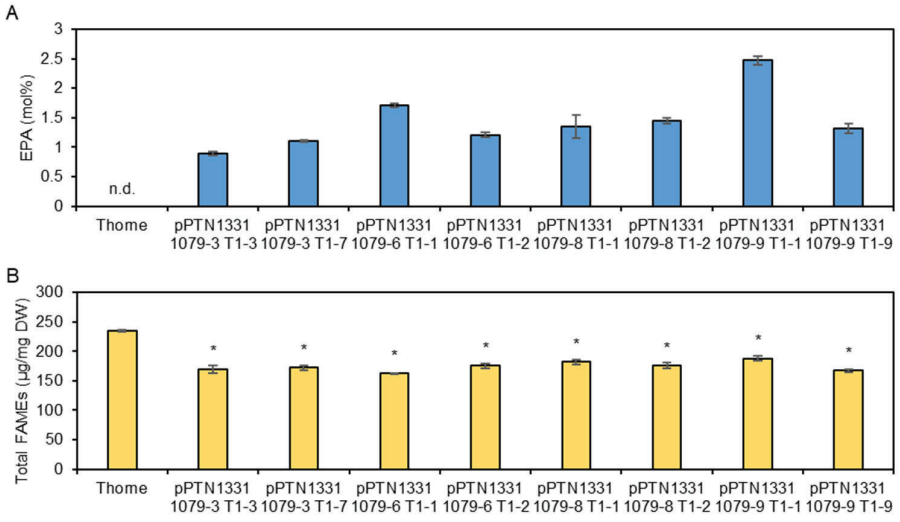

Figure 2. EPA levels (A) and total oil content measured as fatty acid methyl esters (FAMEs) (B) of dry seed from non-transgenic plant (Thorne) and pPTN1331 transgenic lines. Values are means ± SD of analyses of three independent samples. n.d., no detection. Student's t-test compared to Thorne. *, $p<0.005$.

Next, we investigated total vitamin E (tocochromanol) content in dry seeds of pPTN1331 transgenic line. Tocopherol content in seeds from pPTN1331 transgenic lines was average 190 µg/g, while that from Thorne was average 200 µg/g (Fig. 3). Vitamin E tocotrienols were not detected in seeds from Thorne, because wild type Thorne and content in seeds from pPTN1331 transgenic lines ranged from 616 µg/g to 998 µg/g (Fig 3).

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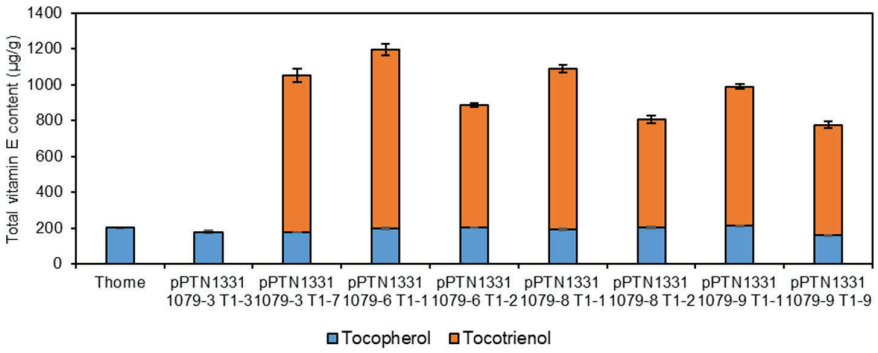

Figure 3. Total vitamin E content of dry seed from non-transgenic plants (Thorne) and pPTN1331 transgenic lines. Values are means ± SD of analyses of three independent samples. Student's t-test compared to Thorne. *, p<0.001.

The ketocarotenoids including astaxanthin and astaxanthin esters accumulate in red seeds from pPTN1331 transgenic lines. The best line of pPTN1331 event 1079-9 contained 30 µg/g ketocarotenoids (including astaxanthin) (Fig 4).

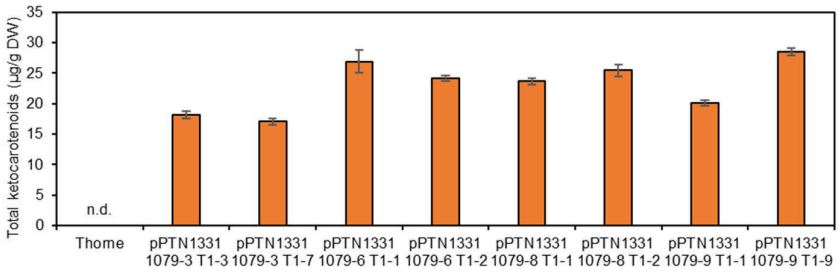

Figure 4. Total astaxanthin content of dry seed from non-transgenic plant (Thorne) and pPTN1331 transgenic lines. Values are means ± SD of analyses of three independent samples.

We conducted a field planting of events from pPTN331 during the summer of 2021 at ENREC in Mead, NE. Analysis of seed composition from these events are underway.

Figure 5. pPTN1331 soybean lines were harvested in the 2021 field at ENREC in Mead, NE.

2. pPTN1558 soybean lines with DHA and Vitamin E in seeds

T1 seeds from eight soybean pPTN1558 (DHA + vitamin E) events (1300-2, 1300-6, 1300-7, 1300-8, 1300-9, 300-11, 1300-38 and 1300-40) were harvested. T1 Seeds containing 6% EPA and 3% DHA from three pPTN1558 events (1300-6, 1300-7 and 1300-8) failed to germinate. The best T1 seed from pPTN1558 1300-11 event accumulated 0.8% EPA and 1.5 % DHA (1300-11 T1-8) (Fig. 6A). The top T1 seed from pPTN1558

1300-38 contains 9% EPA and 4% DHA (1300-38- T1-7) (Fig. 6B). In seeds from pPTN1558 1300-40 events, the maximum EPA and DHA level were 6% and 3%, respectively (Fig. 6C).

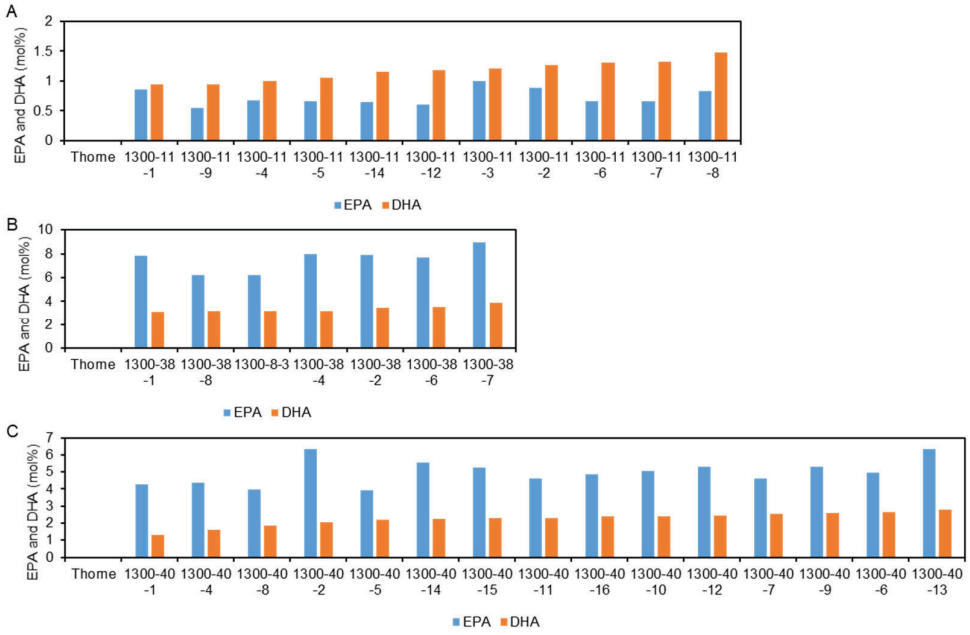

Figure 6. EPA and DHA level in the T1 seeds from three pPTN1558 events, 1300-11 (A), 1300-38 (B) and 1300-40 (C).

To investigate transcript level in developing seeds from three plant of pPTN1558 events, the seeds of R5.5 and R6 developing stage were collected and we performed RT-PCR using seed RNA and gene-specific primers. The transcripts of seven transgenes were detected in developing seeds (R5/R6) of T1 plants from two pPTN1558 events (1300-38 and 1300-40), indicating successful delivery and retention of the transgenes to produce EPA and DHA. However, the transcripts of Δ17DES gene were not detected in pPTN1558 1300-11 event (Fig 7).

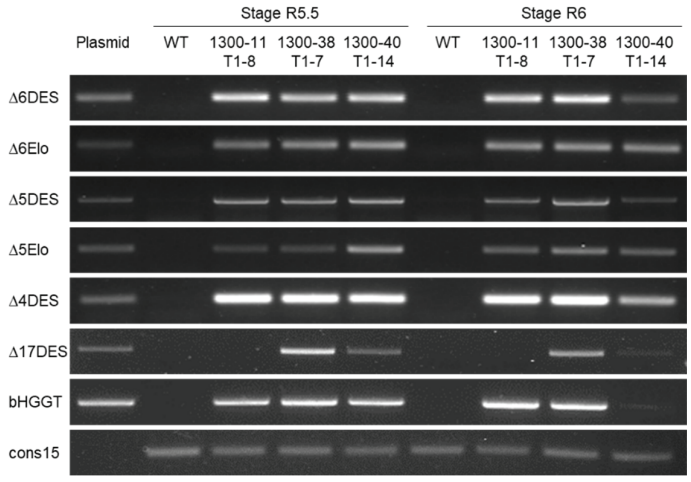

Figure 7. RT-PCR analysis of seven transgenes in R5.5 and R6 developing stages from Thorne and pPTN1558 events. *cons15* gene used as a reference gene.

EPA and DHA were analyzed in T2 dry seeds of two pPTN1558 events (1300-11 and 1300-38) grown in the

greenhouse accumulated EPA to ≤0.9% and DHA to ≤3.3% per dry weight, respectively, in the T2 dry seeds of pPTN1558 1300-11 event (Fig. 8A). EPA and DHA levels were ranged from 2.6% to 6.9% and 1.1% to 4.5% per dry weight, respectively, in the T2 seeds of pPTN1558 1300-38 event (Fig. 8B). T1 plants of the pPTN1558 1300-40 event are now growing in the greenhouse, and T2 seeds will be harvested.

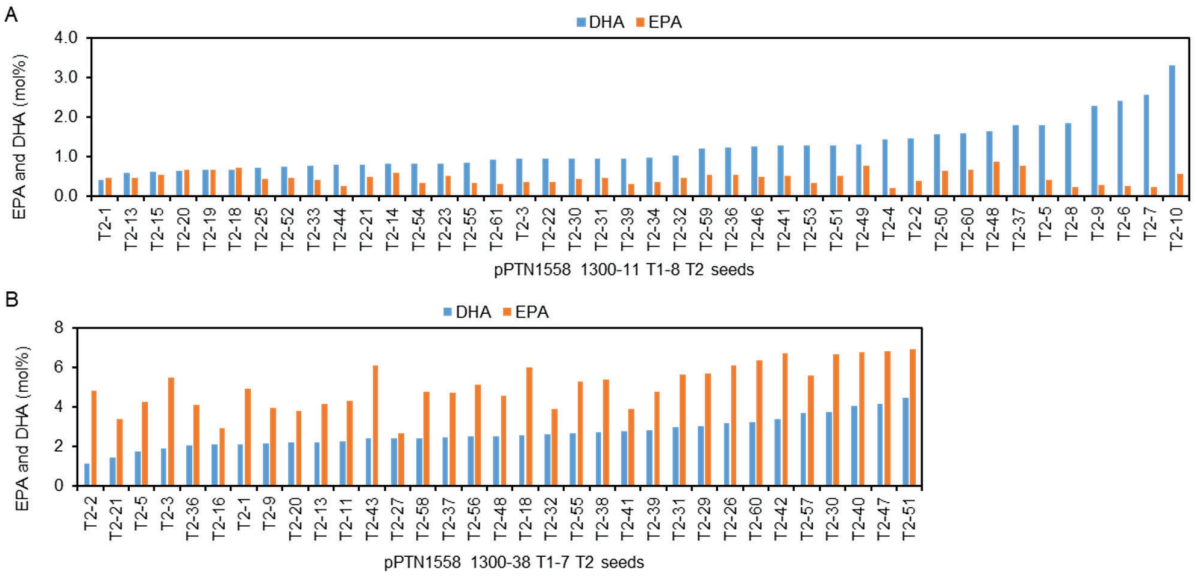

Figure 8. EPA and DHA level in the T2 seeds from three pPTN1558 events, 1300-11 (A) and 1300-38 (B).

For the 2021 field growing season, ~40 T2 seeds from two pPTN1558 events (1300-11 T1-5, T1-8 and 1300-38 T1-4 and T1-7) were sown in the field at ENREC, Mead, NE. To investigate T-DNA integration, we performed DNA-PCR using genomic DNA and *Δ6ELO* specific primers in the thirty-six pPTN1558 T2 plants. We obtained 22 pPTN1558 transgenic plants grown in the 2021 field: 9 plants from 1300-11 T1-5 generation, 5 plants from 1300-11-T1-8 generation, 2 plants from 1300-38-T1-5 generation and 6 plants from 1300-38-T1-7 generation) (Fig 9). The T3 seeds from pPTN1558 transgenic plants were harvested.

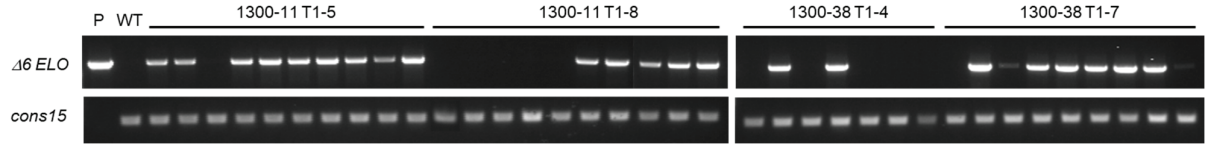

Figure 9. DNA-PCR analysis of pPTN1558 events. Genomic DNA were extracted from leaves of individual plant. P, plasmid.

We plan to harvest seeds and analyze fatty acid profiles and vitamin E level in the T2 seeds of pPTN1558 1300-40 and in the T3 seeds of pPTN1558 1300-11 and 1300-38 events.

3. pPTN1551 soybean lines with astaxanthin in seeds

Seeds from 58 greenhouse-grown T1 plants from nine pPTN1551 (Astaxanthin) events were analyzed for carotenoid content. Total orange seeds have been sown and T2 plants were grown in greenhouse. The integration of expression cassette of pPTN1551 was detected by DNA-based PCR in 34 plants. We selected two individual plants from four pPTN1551 event (1291-1, 1296-7, 1296-9 and 1296-18), and we investigated transcripts level in the R5.5 and R6 developing seeds of pPTN1551 lines. Except pPTN1551 1291-1 event, transcripts of three genes were detected in three pPTN1551 events (Fig. 10A). Finally, we obtained eight pPTN1551 transgenic lines with orange color (Fig 10).

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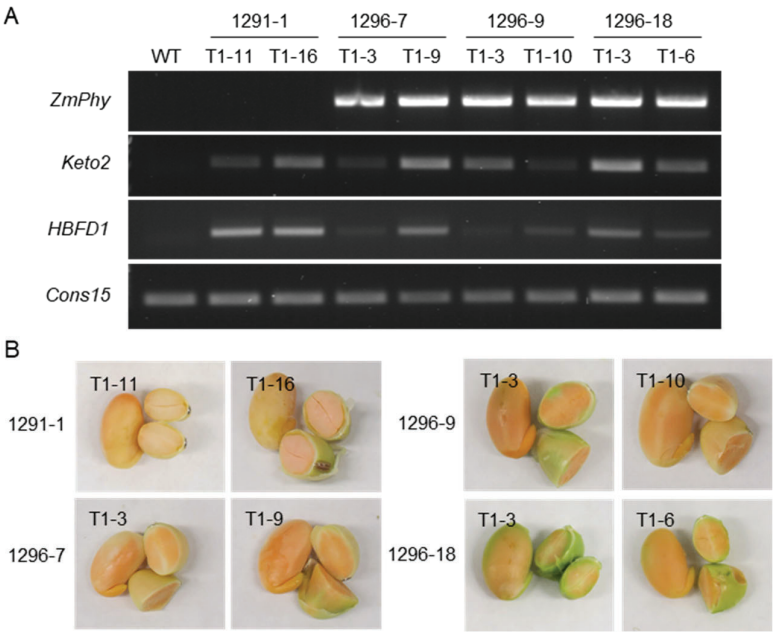

Figure 10. The RT-PCR analysis (A) and orange seeds image (B) of pPTN1551 events.

The T2 seeds from four pPTN1551 events (1291-1, 1296-7, 1296-9 and 1296-18) were harvested. Then, four transgenic seeds from two pPTN1551 events (1296-7 and 1296-9) which showed deep orange color in seeds analyzed the carotenoid component using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The seeds of pPTN1551 transgenic line showed yellowish orange rather than pink or red compared to the seeds of pPTN1331 transgenic line (Fig 11).

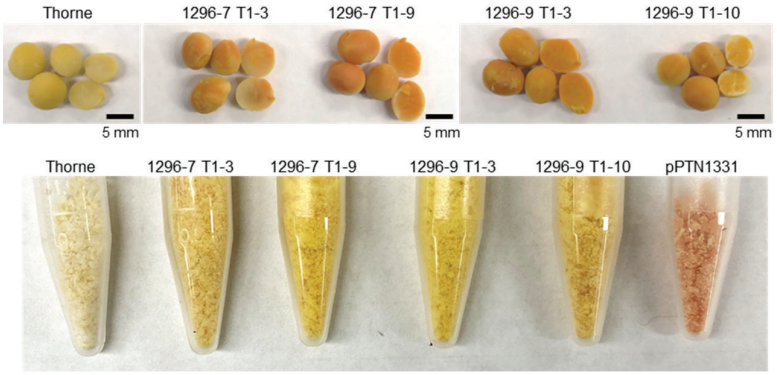

Figure 11. The seed color image of pPTN1551 transgenic lines and pPTN1331 transgenic line.

Carotenoids in transgenic seeds were primarily in the form of β-carotene (>90%), with little or no astaxanthin or other ketocarotenoids (Fig. 12). We suspect this is due to the inclusion of the phytoene synthase gene in these lines, which appears to not be effective for astaxanthin production.

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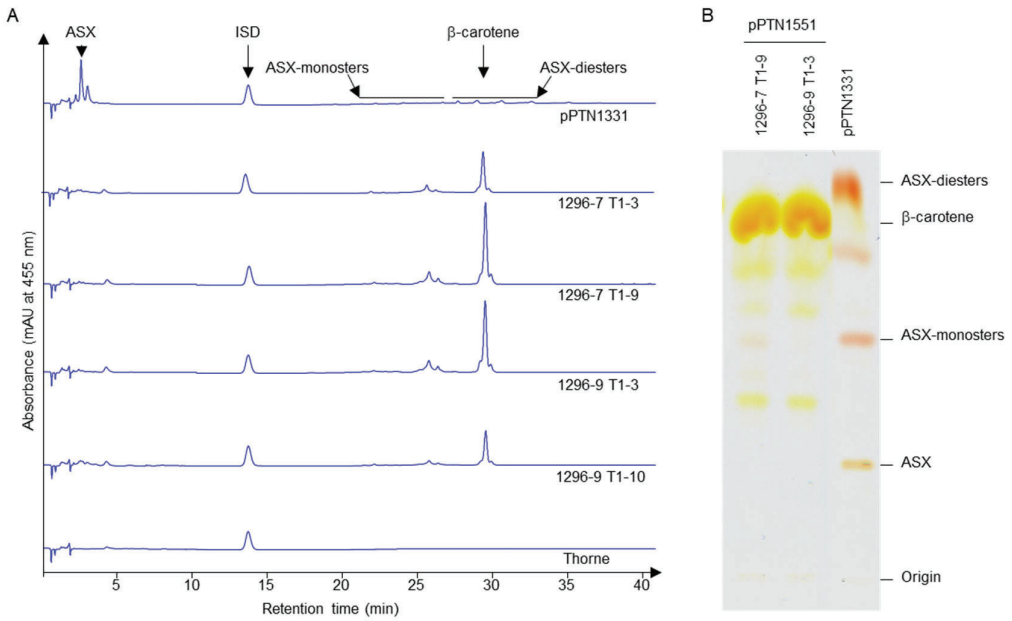

Figure 12. Chromatogram of HPLC analysis (A) and TLC analysis (B) in seeds from pPTN1551 transgenic lines (1296-7 and 1296-9) and pPTN1331 transgenic line.

To improve astaxanthin production, we modified binary vector to remove the phytoene synthase transgene. This modified vector contains the *Keto2* gene driven by the glycinin promoter and *HBFD* gene driven by the glycinin promoter (Figure 13). Soybean transformations have been initiated with this construct.

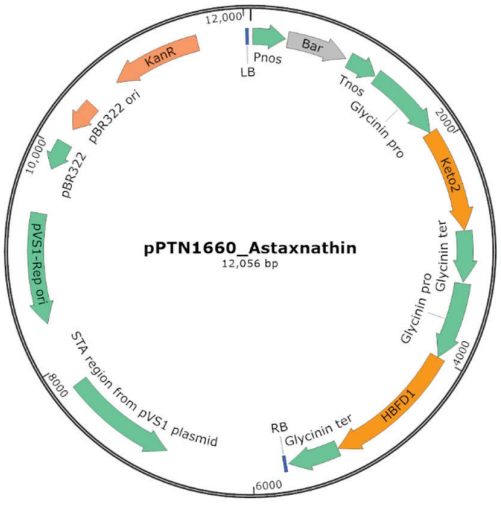

Figure 13. The pPTN1660 binary vector map.

5. Generation of high EPA content soybean line by crossing events.

1) Crossing events between pPTN1331 (EPA+Astaxanthin+Vitamin E) and pPTN1314 (KASII+AtWRI1)

Crosses (F3 plants) between pPTN1331 and pPTN1314 (KASII+WRII) for seed oil content enhancement were grown in the greenhouse. And the F4 seeds were harvested.

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Next, we plan to analysis fatty acid profiles and seed oil content in the F4 seeds of crosses grown in the greenhouse. F4 seeds will be bulked this winter in the greenhouse for large-scale field testing in 2022.

2) Crossing events between pPTN1331 (EPA + Astaxanthin + Vitamin E) and pPTN1248 (AtWRI1 + AtDGAT1)

Crosses (F3 plants) between pPTN1248 (DGAT1+WRII) for seed oil content enhancement were grown in the greenhouse and the F4 seeds from crosses (F3 plants) were harvested. In addition, five individual F3 plants of cross between pPTN1331 and pPTN1248 were grown in the 2021 field and the F4 seeds were harvested.

In the seeds from crosses grown in the green house and 2021 field, we found the seed shape from crosses were partially restored their seed shape while pPTN1331 parent seeds observed wrinkled shape (Fig 14).

Figure 14. The image of seeds from crosses between pPTN1331 and pPTN1248 grown in the green house (A) and 2021 field (B).

We plan to analysis fatty acid profiles and seed oil content in the F4 seeds of crosses grown in the greenhouse and 2021 field.

3) Crossing events between pPTN1331 and a high linolenic acid (ALA) line

The two individual crosses (ALA x 1331 F1-1 and ALA x 1331 F1-2) between pPTN1331 line and ALA line were harvested. The seed shape from individual ALA x 1331 F1-1 line was wrinkled and immature (still green); however, the seed shape from individual ALA x 1331 F1-2 line was similar with wild type (Thorne). The fatty acid composition in red seed from two individual F2 seeds was analyzed using gas chromatography. EPA levels in seeds of ALA x 1331 F1-1 were ranged from 11.7 % to 25.1 % of total fatty acids. EPA levels in seeds of ALA x 1331 F1-2 were ranged from 9.1 % to 17.6 % of total fatty acids. However, almost seeds were failed to germinate. Only three F2 plants were survived. The T-DNA integration of pPTN1331 and ALA and transcripts for 8 genes (*Keto2*, *HBFD*, *bHGGT*, *Δ17DES*, *Δ6Elo*, *Δ5DES*, *Δ6DES* and *Δ15DES*) were detected in three crosses (ALA x 1331 F1-2 F2-2, ALA x 1331 F1-2 F2-4 and ALA x 1331 F1-1 F2-3) (Fig 15).

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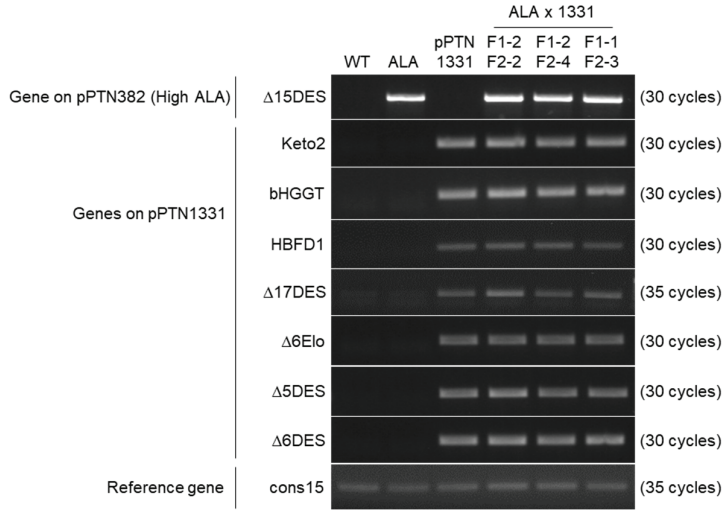

Figure 15. RT-PCR analysis of seven transgenes in R6 developing stages from Thorne, ALA line, pPTN1331 and crosses (ALA x pPTN1331). *cons15* gene used as a reference gene.

F3 seeds from three crosses (ALA x 1331 F1-1 F2-3, ALA x 1331 F1-2 F2-2 and ALA x 1331 F1-2 F2-4) were harvested and analyzed their fatty acid profiles. The EPA content showed range of 5.3% to 9.7% per dry weight, which is 9 to 16-fold change increase compared to pPTN1331 parent (1.6% of EPA). The EPA level in seeds of ALA x 1331 F1-2 F2-2 were ranged from 6.7% to 11.4% and that of ALA x 1331 F1-2 F2-4 were ranged from 5.2% to 11.0% (Fig. 16).

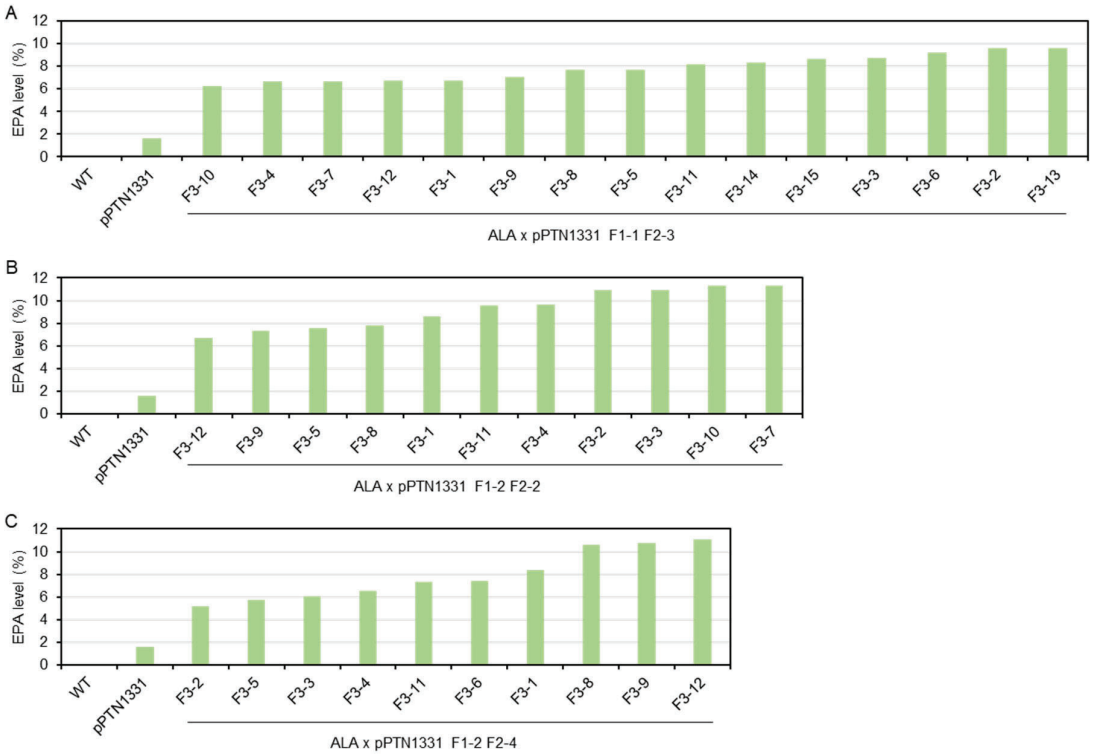

Figure 16. EPA level in the F3 seeds from three crosses between ALA and pPTN1331 line, ALA x pPTN1331 F1-1 F2-3 (A) and ALA x pPTN1331 F1-2 F2-2 (B) and ALA x pPTN1331 F1-2 F2-4 (C).

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Now, six F3 plants (one plant from ALA x 1331 F1-2 F2-2, three plants from ALA x 1331 F1-2 F2-4 and two plants from ALA x 1331 F1-1 F2-3) are growing in the green house. We plan to analyze fatty acid profile, vitamin E level, astaxanthin level in next gerneration.

6. New strategy for improvement of EPA/DHA production

To push the production of omega-3 fatty acids including EPA, we designed new vector including delta 15 fatty acid desaturase (Δ15DES) and delta 17 fatty acid desaturase (Δ17DES) genes driven by strong seed specific promoter (Fig. 17).

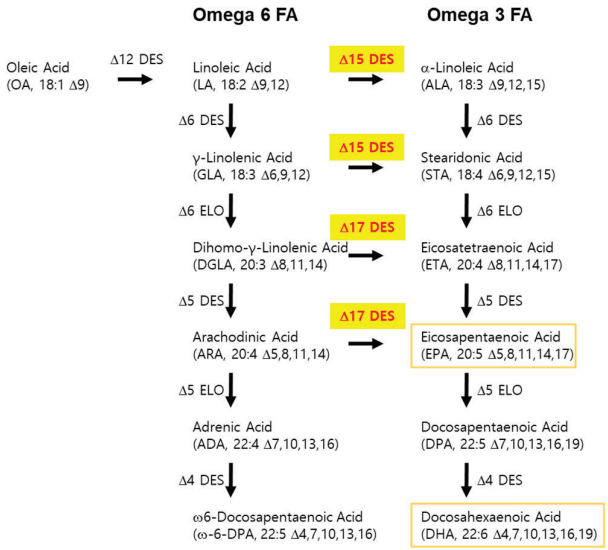

Figure 17. Metabolic engineering for the biosynthesis of omega 6 and omega 3 fatty acid.

Then, the construction of new binary vectors, pPTN1615 containing EPA expression cassette along with the Δ15DES and pPTN1642 containing DHA expression cassette along with the Δ15DES and vitamin E expression cassette were completed (Fig 18). The new binary vectors (pPTN1615 and pPTN1642) were stable in Agrobacterium cells.

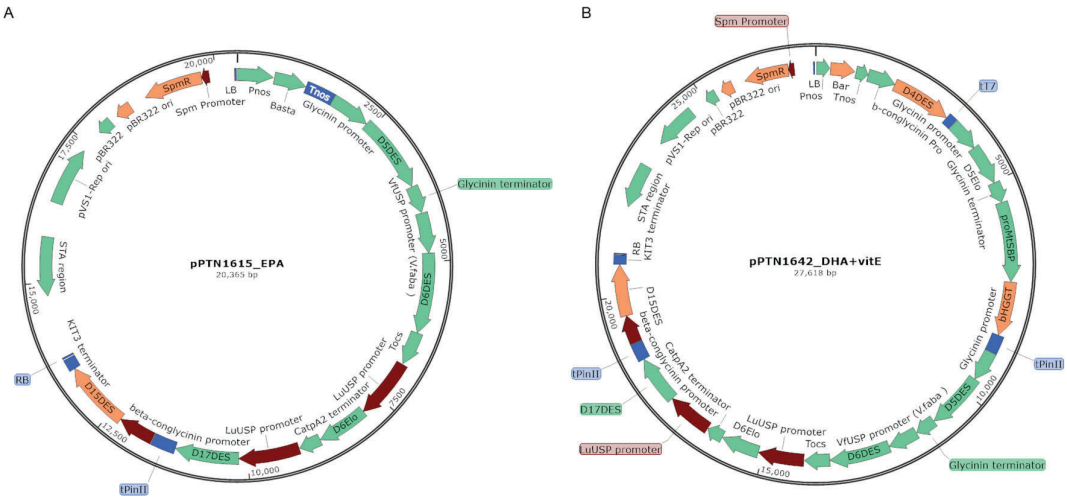

Figure 18. The pPTN1615 and pPTN1642 binary vector maps.

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Now, soybean transformation for pPTN1615 (EPA production) and pPTN1642 (vitamin E and EPA/DHA production) was initiated.

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. To date, these techniques have delivered soybean lines expressing eight transgenes that produce three high value aquaculture oil traits: fish oil EPA, astaxanthin, and high vitamin E antioxidants. These traits cannot be produced from conventional breeding. Also we have applied this technology to produce seeds with increased methionine content for improved meal quality. 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 has addressed the Nebraska Soybean Board focus area of germplasm improvement for composition and yield. The project has generated germplasm that produces seed oils with the key, high value traits: fish oil EPA, astaxanthin pigment for consumer-desired fish flesh color, and high vitamin E antioxidants to stabilize EPA from production of off-flavors. 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. Soybean is and will increasingly be a major sustainable source of aquaculture protein and oil feedstocks. Our research will increase the bushel price of soybeans and deliver high value oil traits that will increase the market share of Nebraska and US soybean for the aquaculture feed market.

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.

We presented project findings during the past year at two national conferences:

(a) Audience: farmers, public researchers

Oral presentation by Ed Cahoon at the Soybean Research Forum & Think Tank, August 25-26, 2021 Indianapolis, IN. Title: “Moving Soybean Beyond Commodity Markets”

(b) Audience: public researchers

Oral presentation by Ed Cahoon at the 2021 American Oil Chemists Society Annual Meeting & Expo, May 6, 2021. Title: “Synthetic Biology Application for Development of High-value Oil Traits”.

Publication in peer-reviewed journal:

Hagely, K., Konda, A. R., Kim, J. H., Cahoon, E. B., & Bilyeu, K. (2021). Molecular-assisted breeding for soybean with high oleic/low linolenic acid and elevated vitamin E in the seed oil. Molecular Breeding, 41(1), 1-13.

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.) Leveraged funding from NE Soybean Checkoff:

We were recently recommended for $150,000 of funding as Co-PI on a USDA-NIFA grant that is directed at strategies for seed oil enhancement. This is not directly related to this project but will generate strategies to enhance oil content, which will be useful for addressing oil yield drag with EPA/DHA production.

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