11/10/2020, 10:12:48 am

Nebraska Soybean Board

Year-End Summary Research Report Form For Multi-Year Projects

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 will impact soybean production.

Note that this form must be submitted with the 4th Quarter Report in all multi-year projects.

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

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

Year of Multi Year: 2 ***of*** 3 ***(For example: Year 1 of 3, Year 2 of 2)***

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 FY20 research activities are:

- Seeds (F2) from crosses between EPA/astaxanthin/vitamin E soybeans and high oil soybeans displayed increased seed oil content to amounts up to levels approaching those in the Thorne wild-type control and EPA levels in these seeds was unchanged (4 to 6% of total fatty acids) relative to the EPA-producing parent. Astaxanthin levels in seeds of the cross ranged from 41-128 μg/g fresh weight and vitamin E levels in seeds of the crosses were up to 1218 μg/g fw or about twice that found in the parental line. Crossed lines were advanced to the F3.
- Crosses between EPA/astaxanthin/vitamin E soybeans and a high C18 soybean line exhibited increased seed oil content (35%) and increased levels of C18-C20 fatty acids compared to the parental line at the F2 stage. Astaxanthin levels in seeds of this cross ranged from 32 – 70 μg/g dry wt, and vitamin E ranged from 678-1034 μg/g dry wt. Crossed lines were advanced to the F3.
- Seeds from field-grown soybeans in 2019 engineered for EPA/Astaxanthin/Vitamin E contained up to 131 μg/g dry weight of astaxanthin. Seed wrinkling and reduced amounts of the phytohormone abscissic acid (ABA) in was observed the top astaxanthin-producing engineered seeds. ABA spraying of pods in 2020 field plots showed some effectiveness in reducing seed wrinkling.
- A new expression vector was successfully constructed with modules for DHA and vitamin E production and seven transgenic events were obtained.
- A new expression vector optimized for astaxanthin production was generated and 23 regenerated plants from 6 transformation events were obtained.
- Crosses between EPA/astaxanthin/vitamin E soybeans and a high linolenic acid soybean line for enhanced EPA production yielded two fertile F2 lines that were advanced to obtain F3 seeds.
- Plots of EPA/astaxanthin/vitamin E-producing lines were successfully grown in the ENREC biotech field in 2020 for evaluation of seed traits, which are currently underway.

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.

Our research was highlighted in the Soybean Information and Research Network and in the Fall 2020 issue of SoybeaNebraska magazines:
https://soybeanresearchinfo.com/research-highlight/thinking-big-soybean-germplasm-for-aquaculture-research/

The findings have been distributed through research publications in FY2020:

1. Konda, A.R., Nazarenus, T.J., Nguyen, H., Yang, J., Gelli, M., Swenson, S., Shipp, J.M., Schmidt, M.A., Cahoon, R.E., Ciftci, O.N., Zhang, C., Clemente, T.E., Cahoon, E.B., 2020. Metabolic engineering of soybean seeds for enhanced vitamin E tocochromanol content and effects on oil antioxidant properties in polyunsaturated fatty acid-rich germplasm. Metabolic Engineering 57, 63-73.
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5. Did the NE soybean checkoff funding of your project, leverage additional State or Federal funding support? Please list sources and dollars approved.

We have a grant proposal pending with USDA-NIFA on additional strategies for enhancing the asxtaxanthin trait and agronomic performance of engineered soybean lines. The proposed research extends and complements research funding from the Nebraska Soybean Board. The proposal is for $490,000 and was submitted in April 2020. A funding decision is expected in January 2021.

Please email this completed form to the Agriculture Research Division (jmcmahon10@unl.edu) based on the reporting schedule given to you. If you have any questions, please call Jen McMahon at the ARD 2-7082.

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 2 **of** 3 **research project**

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

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- Crosses between EPA/astaxanthin/vitamin E soybeans and a high C18 soybean line exhibited increased seed oil content (35%) and increased levels of C18-C20 fatty acids compared to the parental line at the F_2 stage. Astaxanthin levels in seeds of this cross ranged from 32 – 70 µg/g dry wt, and vitamin E ranged from 678-1034 µg/g dry wt. Crossed lines were advanced to the F_3.
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- Plots of EPA/astaxanthin/vitamin E-producing lines were successfully grown in the ENREC biotech field in 2020 for evaluation of seed traits, which are currently underway.

1. pPTN1398 soybean line with high methionine content in seeds.

The total RNA were isolated from developing seeds of pPTN1398 transgenic soybean line. Among 12 transgenic events, one transgenic event (event 1177-1) detected transcripts of *MetA*, *MetB*, *MetC* and *MetE* by RNA based PCR (RT-PCR), but with weak transcripts level of *MetE*. The T_2 seeds of transgenic events 1177-1, 1179-1 and 1179-2 were grown in the 2020 season at Eastern Nebraska Research and Education Center in Mead, NE. and T_3 seeds were harvested. To generate the best pPTN1398 soybean lines with high methionine content, two additional transformation events (T1; 1214-1 and 1225-1 events) were obtained and grown in the greenhouse. Transcript levels of *MetA*, *MetB*, *MetC* and *MetE* were investigated in developing seed from six transgenic events using RT-PCR analysis. The transgenic event 1214-1 showed high transcripts level of those genes compared to levels in other transgenic events (Fig. 1). The *MetB* and *MetE* transcript levels were markedly higher in 1214-1 event than in other events (Fig. 1B, D).

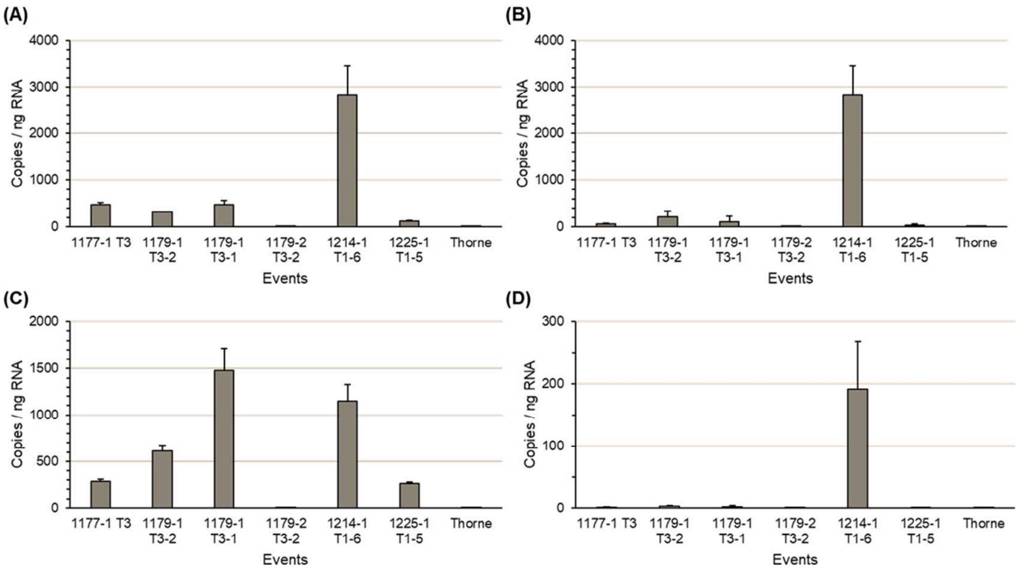

Figure 1. RT-PCR analysis of *MetA* (A), *MetB* (B), *MetC* (C) and *MetE* (D) genes in developing seeds of pPTN1398 transgenic events.

The developing T_3 seeds from four pPTN1398 events (1177-1, 1179-1, 1179-2 and 1225-1) grown in the 2020 field were analyzed free or total methionine content, but no differences compared to levels in seeds of the Thorne wild-type control (Figure 2). The T_3 seeds from 1177-1, 1179-1 and 1179-2 events and T_1 seeds from 1214-1 and 1225-1 events were harvested for analysis of methionine content in dry seeds of six transgenic events, including 1214-1 event.

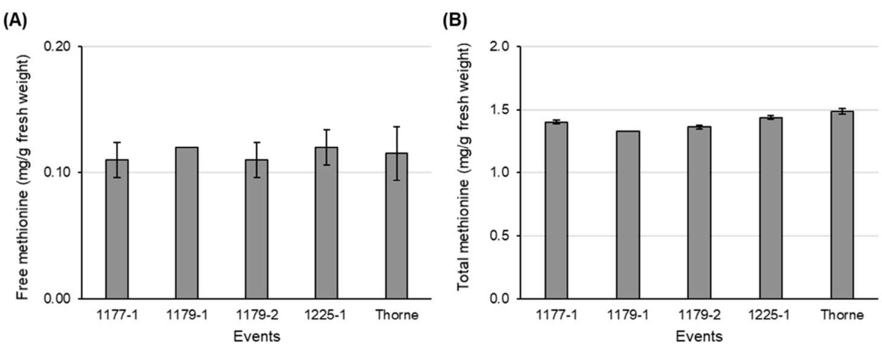

Figure 2. Free methionine (A) and total methionine (B) analysis in developing seeds from pPTN1398 transgenic events.

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

Red seeds from pPTN1331 soybean lines grown in the 2019 field were analyzed astaxanthin content. Astaxanthin content of red seeds ranged from 54 ng/g fresh wt (fw) to 131 ng/g fw (Figure 3A). After harvesting seeds of pPTN1331 soybean lines, we found wrinkled shape of red seeds (Figure 3B). β-carotene serves as a precursor for the ABA biosynthesis as well as the astaxanthin biosynthesis. The plant hormone ABA is important during seed maturation to regulate accumulation of seed reserves, maintenance of seed dormancy, and induction of desiccation tolerance. Next, we investigated ABA amount in developing seeds and dry seeds.

Nebraska Soybean Board
Year-End Research Findings Report

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During seed maturation, ABA content (8.5 µg/g fw) in developing seeds from Thorne (wild type) was decreased from 8.5 µg/g fw at R5 stage to 6.1 µg/g fw at R6-R7 stage. ABA content in seeds from pPTN1331 soybean lines was decreased from from 6~7.4 µg/g fw at R5 stage to 1.1~1.5 µg/g fw at R6-R7 stage. ABA content in seed from pPTN1331 soybean lines was decreased by 10~30% at R5 stage and 75~83% at R6-R7 stage compared to Thorne (Figure 3C). In the dry seeds, ABA content of seeds from pPTN1331 soybean lines ranged from 15~77 ng/g fw, which is less than 10% of Thorne content (327 ng/g fw) (Figure 3D). These results indicate that ABA content was significantly reduced due to the lack of precursors due to the biosynthesis of astaxanthin in the R5 stage. We are working to understand the relationship between reduced ABA and wrinkled seed shape.

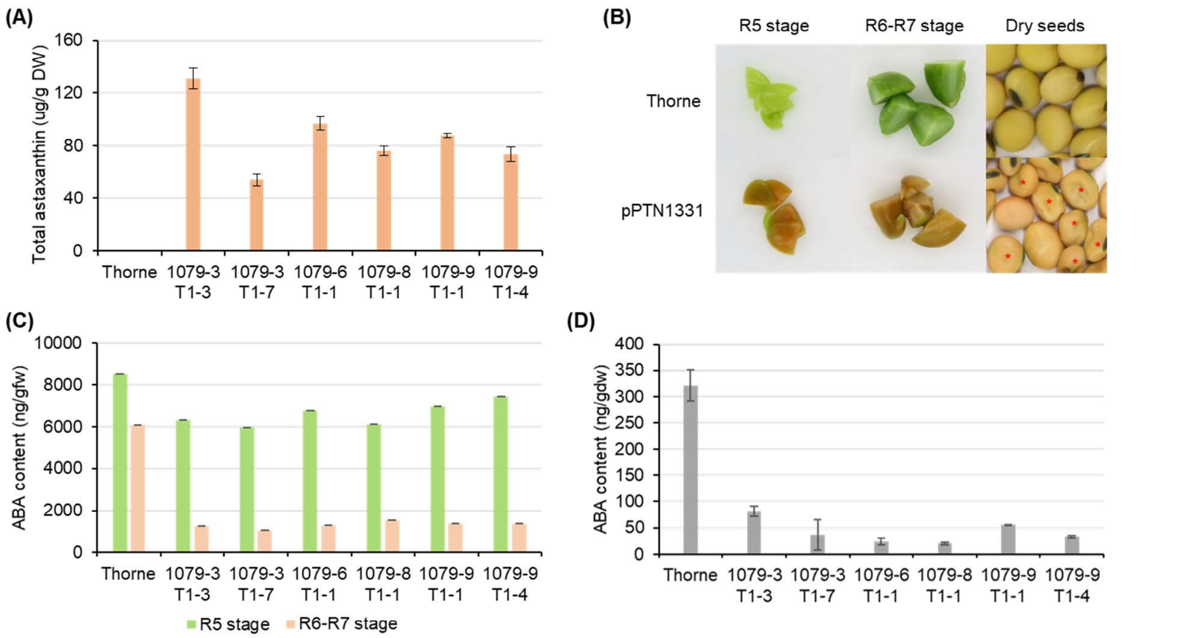

Figure 3. Astaxanthin and ABA analysis of seeds from pPTN1331 soybean grown in the 2019 field.
A, Astaxanthin content in dry seeds. B, developing seed image. Asterisks indicate wrinkled seeds. C and D, ABA amount in the developing seeds (C) and dry seeds (D).

For 2020 field trial, the pPTN1331 soybean lines were grown at Eastern Nebraska Research and Education Center in Mead, NE. and the T_4 seeds were harvested (Figure 4). We plan to analyze EPA, astaxanthin, vitamin E and ABA content.

Figure 4. T4 seeds of pPTN1331 soybean lines were harvested in the 2020 field at ENREC in Mead, NE.
A, Growth image at Sept. 2020. B, Dry seeds harvested from the 2020 field.

*****This form must be completed and submitted with the fourth quarter report.***

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

The construction of a new expression vector (pPTN1558) containing DHA vitamin E production modules was completed (Figure 5). Soybean transformations with pPTN1558 were initiated and seven transgenic events were obtained.

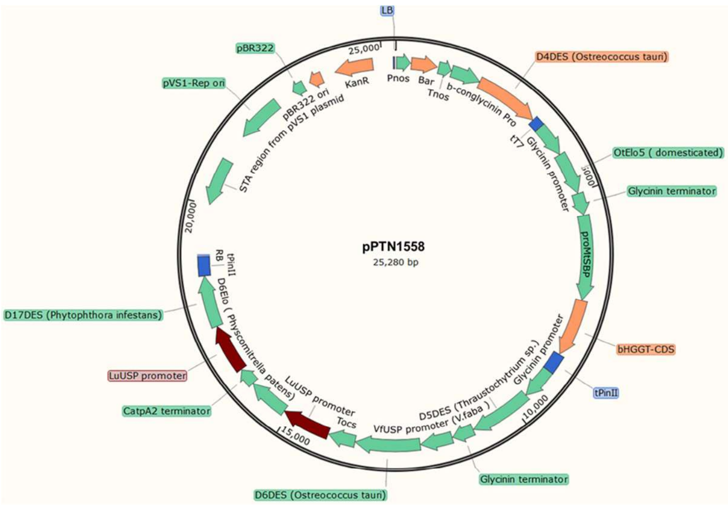

Figure 5. The pPTN1558 binary vector map. DHA module and vitamin E module are controlled under strong seed-specific promoter, LuUSP promoter, VfUSP promoter, Glycinin promoter, MtSBP promoter and β-conglycinin promoter.

4. pPTN1551 soybean lines with astaxanthin in seeds.

To avoid unstable transgenic soybean lines, a new expression vector (pPTN1551, high astaxanthin) that contains the herbicide resistance gene adjacent to the T-DNA Left Border (LB) was generated, and soybean transformations with this expression vector were started (Figure 6). We obtained 23 regenerated plants from six transformation events.

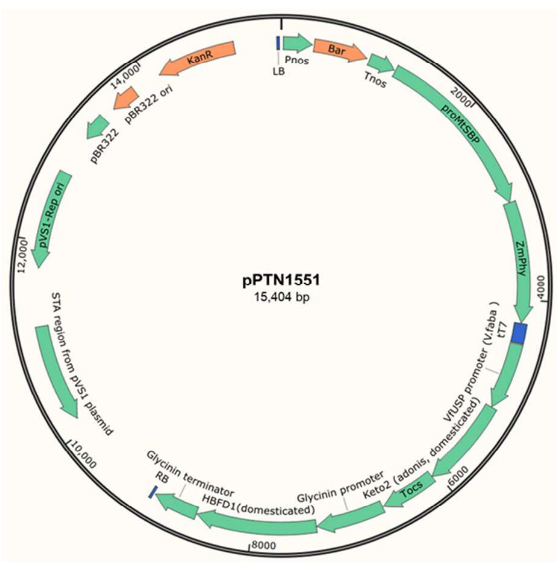

Figure 6. The pPTN1551 binary vector map.

5. Construction of new expression vector to produce high oil, EPA/DHA, Vitamin E, astaxanthin.

The new expression vector producing a high level of triacylglycerol, EPA/DHA, astaxanthin and vitamin E were designed and assembly of expression cassettes were initiated. And we checked the stability of binary vector in

Agrobacterium tumefaciens strain GV3101 every assembly step. Then we found that binary vector assembled with GmDGAT1 cassette was unstable in Agrobacterium cells (Figure 7). We are now testing the stability of other binary vector with GmDGAT1 cassette.

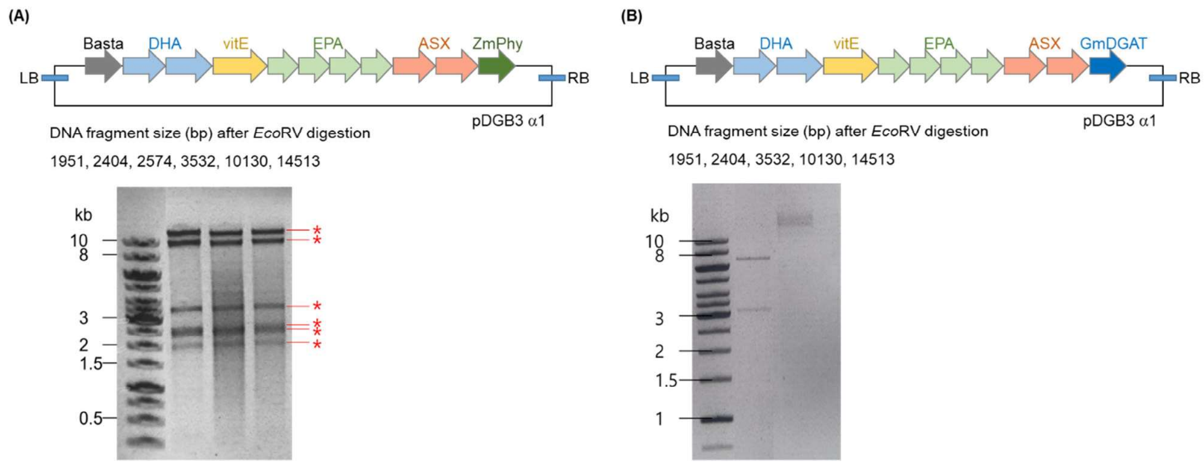

Figure 7. The binary vector diagram and DNA gel electrophoresis images after digestion of *Eco*RV restriction enzyme. The binary vector was transformed into the Agrobacterium and extracted from Agrobacterium.

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

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

The oil content and fatty acid composition of F_2 seeds from crosses between pPTN1331 (EPA+Astaxanthin+Vitamin E) and pPTN1314 (KASII+AtWRI1) were analyzed. The seed oil content of pPTN1331 lines was decreased by 35% compared to Thorne (140 µg/g dry wt vs. 214 µg/g dry wt), while those of crossed lines were partially restored to the level of Thorne (Figure 8A). Moreover, the ratio of C16 fatty acid amount to C18~C20 fatty acids amount in wild type ‘Thorne’ and pPTN1331 lines were 1:7.4 and 1:8.5, respectively, while that in cross lines were 1:11 (Figure 8B). These results indicate that the seeds of crossed lines have increased levels of EPA precursors, which may allow for enhanced production of fish oil-type fatty acids in optimized genetic backgrounds.

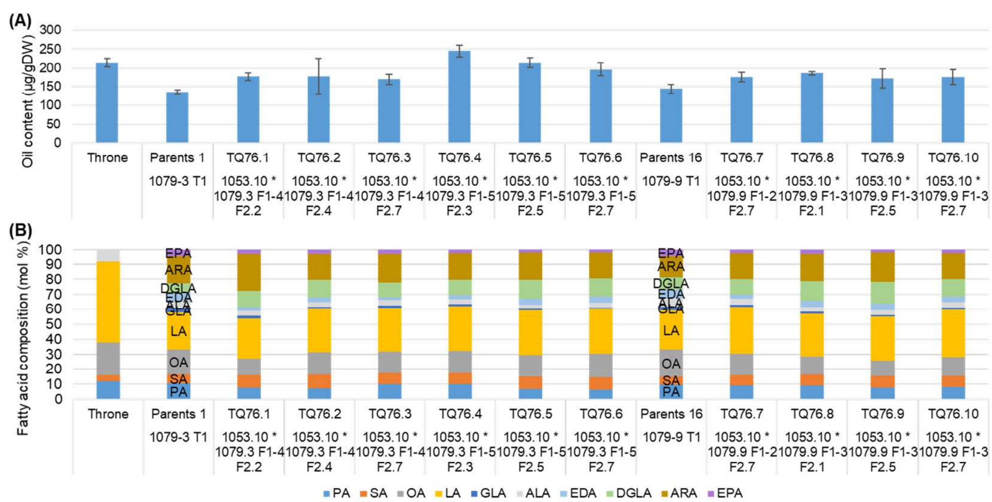

Figure 8. Oil content (A) and fatty acid composition (B) in F_2 seeds from crosses between pPTN1331 (EPA+Astaxanthin+Vitamin E) and pPTN1314 (KASII+AtWRI1).

We analyzed astaxanthin and vitamin E levels in the F_2 seeds of cross lines between pPTN1331 and pPTN1314. Astaxanthin was not detected in the Thorne seed and 2 cross lines (TQ76.8 and TQ76.9). Although the Astaxanthin content in the seeds of cross lines was lower than those in seeds of parent lines. However, vitamin

E content in seeds of cross lines was increased compared to levels in the parent lines (916~1,556 µg/g dry wt for seeds of crosses vs. 678 µg/g dry wt for parent lines) (Figure 9). The crossed lines were grown in the greenhouse to obtain sufficient amounts of F_3 seeds for field production.

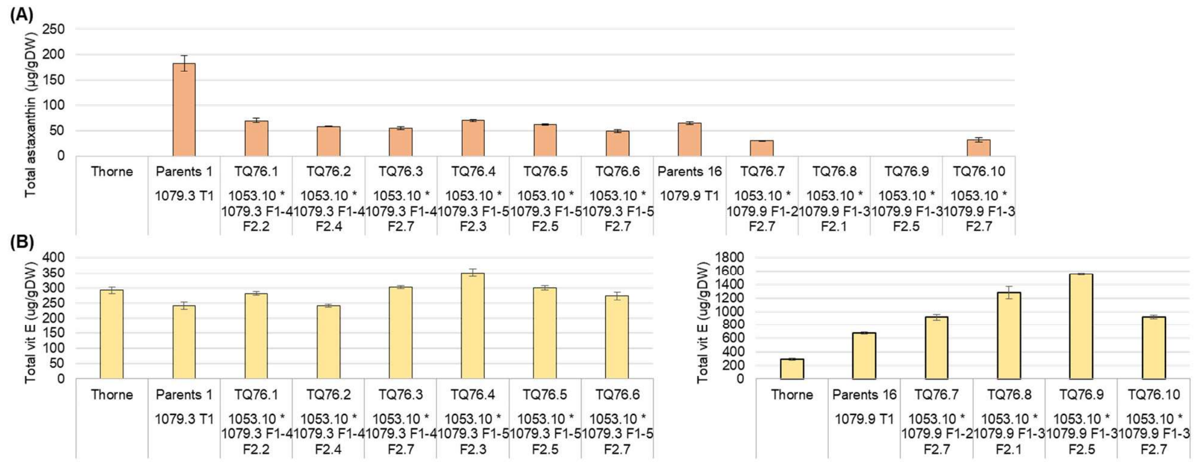

Figure 9. Astaxanthin (A) and vitamin E (B) content in F_2 seeds of cross lines between pPTN1331 and pPTN1314

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

The oil content and fatty acid composition of F_2 seeds from crosses between pPTN1331 (EPA + Astaxanthin + Vitamin E) and pPTN1248 (AtWRI1 + AtDGAT1) were analyzed. The EPA levels in seeds of crossed lines were similar to those of the parental lines (4% to 6% of total fatty acids). However, total oil content in seeds of crosses was partially restored to the level of wild type (165~211 µg/g fw for seeds of crosses vs. 214 µg/g fw for 'Thorne') (Figure 10).

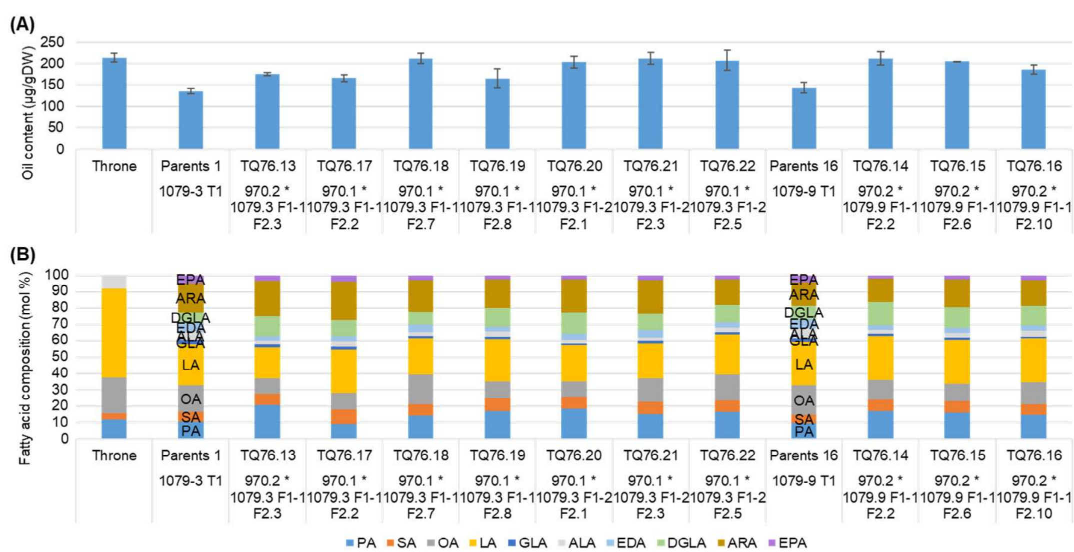

Figure 10. Oil content (A) and fatty acid composition (B) of F2 seeds from crosses between pPTN1331 (EPA + Astaxanthin + Vitamin E) and pPTN1248 (AtWRI1 + AtDGAT1).

Next, the astaxanthin and vitamin E content of seeds from crosses between pPTN1331 and pPTN1248 were analyzed. Astaxanthin levels in seeds of crossed lines was as high as 52 µg/g dry wt. Vitamin E content in seeds of crosses was increased compared to the level of pPTN1331 parents line (1,034~1,218 µg/g dry wt for seeds of crosses vs. 678 µg/g dry wt for parent lines) (Figure 11). The cross lines were grown in the greenhouse for the large-scale harvest of F_3 seeds for field testing.

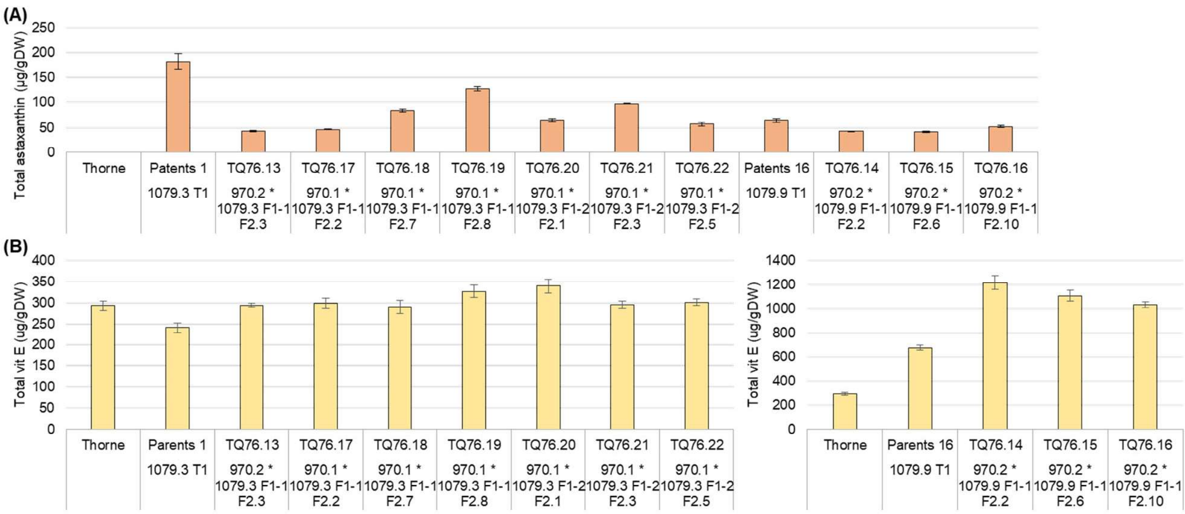

Figure 11. Astaxanthin (A) and vitamin E (B) content in F_2 seeds of cross lines between pPTN1331 and pPTN1248.

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

Nine individual F_2 crosses between pPTN1331 and a high linolenic acid line to increase EPA content were obtained and they were grown in the greenhouse. However, seven individual F_2 crosses showed delayed seed germination and did not survive when they were transferred to soil. Finally, two individual F_2 crosses were successfully grown in the greenhouse, and the F_3 seeds were harvested for trait analysis.

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.

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