Project Title: Genetic engineering of soybean for production of DGLA-enriched oil

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Introduction

ω-6 polyunsaturated fatty acid (ω-6 PUFAs), including linoleic acid (LA), gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid (DGLA), and arachidonic acid (AA), play important roles in human health (Wang et al. 2012). LA is the main ω-6 fatty acid in the human diet. Upon taken into the human body, LA is converted to GLA by the enzyme delta-6-desaturase (D6D). GLA will then be converted into DGLA by the enzyme delta-6-elongase (D6E). DGLA is further metabolized to AA by delta-5-desaturase (D5D) (see Fig.1). Studies indicate that arachidonic acid (AA), the downstream of ω-6 PUFAs in the biochemical pathway, can generate deleterious metabolites through a series of reactions catalyzed by the enzyme cyclooxygenase (COX), thus promoting cancer invasion and growth (Gu et al. 2013; Xu et al. 2014). Qian and his coleagues at NDSU demonstrated that DGLA, an ω-6 PUFA in the upstream of arachidonic acid (AA), can produce a free radical byproduct (8-hydroxyoctanoic acid, 8-HOA) by the process catalyzed by the enzyme Cyclooxygenase-2 (COX-2), and thus inhibits cancer cell/tumor growth (Xu et al. 2016; 2018a). There are two forms of the enzyme COX: COX-1 and COX-2. COX-1 is produced by normal human tissues, while COX-2 is readily induced by all types of cancers (Liu et al. 2015). Previous studies showed that knocking down of D5D, an enzyme that mediates the conversion from DGLA to AA, promotes formation of 8-HOA (a cancer inhibitor) in cancer cells, leading to halting of cancer growth and spreading (Xu et al. 2016; Yang et al. 2016). More recently, Qian and his colleagues at NDSU discovered new anti-cancer drugs that can be used to effectively inhibit the enzyme D5D, leading to decrease in AA production while accumulating more DGLA to promote the formation of 8-HOA, consequently suppressing the growth and invasion of cancer cells (Xu 2018b; Yang et al. 2019). Therefore, a novel cancer therapy strategy can be developed by inhibiting D5D and dietary supplementation of DGLA in cancer patients (Qian et al. 2019).

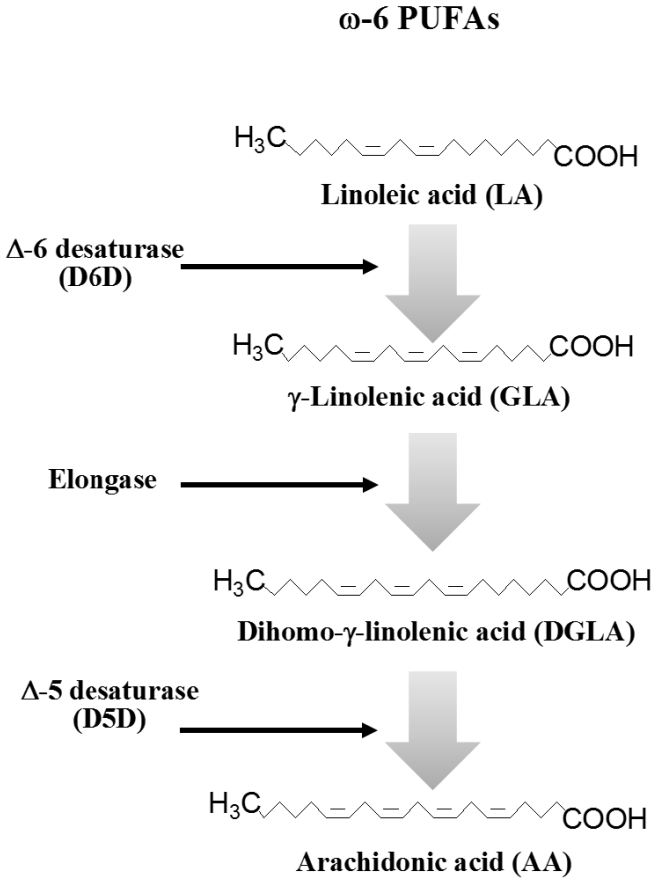

Fig. 1. Polyunsaturated fatty acid (PUFA) biosynthesis in humans

Due to the importance of DGLA in human health and cancer therapy, effective production of

large amount of DGLA is essential to meet the demand of pharmaceutical industry. A few plant species (such as borage, evening primrose, black currant, liverworts, mosses) are able to synthesize GLA or DGLA, but these plants are difficult to cultivate and with relatively low oil yields. On contrast, the major vegetable oil-producing crops (sunflower, safflower, corn and soybean) produce up to 57% LA in their oil, but they are unable to synthesize GLA and DGLA due to lack of the enzymes required for their biosynthesis. As GLA-containing oils are in high demand by the nutritional supplement market, much research in the past years has been focused on producing vegetable oils with significant amount of GLA (Haslam et al. 2013; Kim et al. 2016a; Nykiforuk et al. 2012). With the discovery of the role of DGLA in cancer therapy and other pharmaceutical uses, it is anticipated that the demand for DGLA will dramatically increase in near future. However, none studies have been done to produce vegetable oils with enriched DGLA. Soybean oil contains up to 51% LA and is the most widely used edible oil in the United States. Almost all margarine and shortenings contain soybean oil. Soybean oil is also frequently used in mayonnaise, salad dressings, frozen foods, imitation dairy and meat products and commercially baked goods. Our ultimate goal is to develop new soybean varieties that can produce DGLA-enriched oil for use in cancer therapy through biotechnology. In the first year of funding, we made a gene construct with the genes expressing the two enzymes (delta-6-desaturase and delta-6-elongase) for biosynthesis of DGLA, and introduced it into soybean cultivar William 82 through Agrobacterium-mediated transformation. Transgenic soybean plants were generated, which produced up to 22.7% GLA but only 1.98% DGLA in the soybean oil. We aimed to increase the DGLA productivity in transgenic soybean plants. Therefore, the specific objectives of the current research project were to:

1. Enhance expression of the two genes for delta-6-desaturase and delta-6-elongase) required for DGLA biosynthesis in transgenic soybean plants.
2. Generate more transgenic plants with high DGLA-enriched oil in other soybean varieties.
3. Evaluate transgenic soybean plants for DGLA and other oil contents.

Materials and Methods

Plant materials

Two soybean cultivars, William 82 and Thorne were used for transformation. These two soybean varieties were highly transformable and have been used in many gene transformation studies (Lee et al. 2012).

Gene constructs

We used the gene encoding the enzyme D6D in *Borago officinalis* and the gene encoding the enzyme D6E in *Physcomitrella patens* to prepare gene construct for plant transformation. The cDNA sequences containing the coding gene for the two enzymes were obtained from NCBI database (GenBank: EF495160.1 for D6D and AF428243.1 for D6E). To enhance the gene expression in soybean, we conducted codon-optimization for these two genes using the soybean genome sequence. To make the gene construct for soybean transformation, the soybean β-conglycinin alpha-subunit promoter was used for driving the expression of the gene for D6D and the gene D6E. Each gene cassette (promoter and the codon-optimized gene sequence) was

synthesized by Twist Bioscience (San Francisco, CA) and then cloned into the binary vector pBAtC (Kim et al. 2016b) to make the seed-specific expression construct pBAtC:Soybean-H1as shown in Fig. 2.

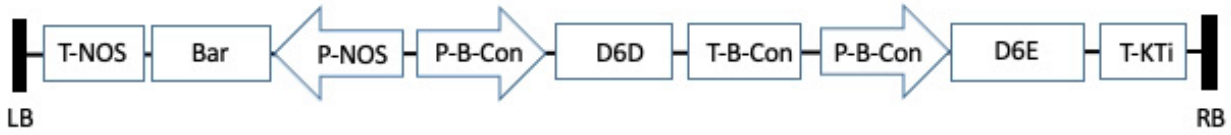

Fig. 2 pBAtC:Soybean-H1 represents the vector for expression of the Delta-6-desaturase gene (D6D) and the Delta-6-elongase gene (D6E) under the control of the soybean seed-specific promoter, β-conglycinin alpha-subunit promoter (P-B-Con). T-B-Con, β-conglycinin alpha-subunit terminator ; T-KTi, KTi gene terminater; P-NOS, nopaline synthase promoter; T-NOS, nopaline synthase terminator; Bar, resistance gene to the herbicide bialaphos under the control of the nopaline synthase promoter (P-NOS).

Plant transformation

The vector pBAtC:Soybean-H1was transformed into *Agrobacterium* strain EHA101. The *Agrobacterium* strain harboring the gene construct was used to transform the two soybean cultivars Williams 82 and Thorne using the "half seeds method" (Paz et al, 2006). The transformation was conducted in the Plant Transformation Facility at Iowa State University.

Molecular verification of transgenic soybean plants

To verify the transgenes in transgenic plants, DNA was extracted from seedlings of transgenic plants (T_0 and T_1). Primers designed from the coding genes for D6D and D6E were used for PCR to amplify the transgenes. DNA from non-transgenic plants was used as negative control in the PCR amplifications. The PCR products were separated in an agrose gel.

Fatty acid analysis

To determine the fatty acids compositions, the T_0 (initial generation) transgenic plants from William 82 and Thorne were grown in the greenhouse to produce T_1 (first generation) seeds. The T_1 seeds (15 g per sample) were sent to Creative Proteomics for fatty acids profiling. Fatty acid composition was analyzed by gas chromatography (GC) with flame ionization detection. The samples were weighed into a screw-cap glass vial which contained tritricosanoin as in internal standard (tri-C23:0 TG) (NuCheck Prep, Elysian, MN). The tissue samples were homogenized and then extracted with a modified Folch extraction. A portion of the organic layer was transferred to a screw-cap glass vial and dried in a speed vacuum. After samples were dried, BTM (methanol containing 14% boron trifluoride, toluene, methanol; 35:30:35 v/v/v; Sigma-Aldrich, St. Louis, MO) was added. The vial was briefly vortexed and heated in a hot bath at 100°C for 45 minutes. After cooling, hexane (EMD Chemicals, USA) and HPLC grade water was added, the tubes were recapped, vortexed and centrifuged help to separate layers. An aliquot of the hexane layer was transferred to a GC vial. Fatty acids were identified by comparison with a standard mixture of fatty acids (GLC OQ-A, NuCheck Prep, Elysian, MN) which was also used to determine individual fatty acid calibration curves.

Results and Discussion

In our previous study, we made a gene construct pBAtC:D6D::D6E using the promoter of beta-conglycinin alpha subunit gene from soybean for driving the expression of the D6D gene, and the KTI promoter for the D6E gene expression. However, transgenic soybean plants generated with pBAtC:D6D::D6E produced a high level of GLA, but low DGLA amount in soybean oil. This result indicated that the beta-conglycinin alpha subunit gene promoter was effective for D6D gene expression and LA was converted into GLA well, but the KTI promoter didn't function well since little GLA was converted into DGLA. To enhance D6E expression in transgenic soybean plants, we constructed a new vector (pBAtC:Soybean-H1), which used the beta-conglycinin alpha subunit promoter to drive the expression of both D6D and D6E genes. The coding gene sequences for D6D and D6E were obtained from the NCBI database (GenBank: EF495160.1 for D6D and AF428243.1 for D6E), and then were subjected to codon-optimization before gene synthesis and cloning into the vector.

The gene construct pBAtC:Soybean-H1 was first introduced into the Agrobacterium strain EHA105, which was used to transformed the two soybean cultivars Williams 82 and Thorne. A total of 24 T0 transgenic plants were obtained for Williams 82 and 21 of them were confirmed to contain the genes for D6D and D6E by PCR analysis. For Thorne, 22 T0 transgenic plants were obtained and 17 of them were confirmed to carry the genes for D6D and D6E based on PCR analysis.

T_1 seeds were from each T0 plant. Eight samples were sent to Creative Proteomics for fatty acids analysis. The non-transgenic soybean cultivar Barnes was included as control. The results are presented in Table 1. As expected, the control (Barnes) had a very low amount of GLA and no DGLA detected because it does not contain the enzymes D6D and D6E for GLA and DGLA production, respectively. Among the transgenic samples tested, several transgenic soybean samples were not significantly different from the control in GLA and DGLA content, indicating the two genes introduced might not be expressed. Some transgenic soybean plants produced a high level of GLA. For example, ST419-15 and ST441-53 contained 33.3% and 28.86% GLA in the soybean oil, suggesting that the introduced gene expressed D6D efficiently. GLA has been reported to have anti-inflammatory properties and may be used as supplemental to benefit some patients. The transgenic soybean varieties with high GLA production may be developed to produce GLA-enriched oil for pharmaceutical industry applications.

DGLA content was relatively low, which ranged from 0 to 10.25% among the samples tested. This result indicated that the expression level of the gene for D6E was different among the different transgenic plants. The transgenic plants with the beta-conglycinin alpha subunit gene promoter for driving the D6E gene produced a significantly higher amount of DGLA than those with the KTi promoter for expression of the D6E gene. This result indicated that D6E expression can be enhanced by a promoter change to produce more DGLA.

Table 1. Fatty acids profile of selected transgenic soybean plants

Fatty Acids		Barnes (control)	ST419-2	ST419-12	ST419-15	ST441-53	ST441-22	ST441-31
Myristic	C14:0	0.13%	0.12%	0.08%	0.08%	0.13%	0.13%	0.08%
Palmitic	C16:0	9.72%	11.43%	11.14%	11.11%	11.48%	11.65%	11.86%
Stearic	C18:0	3.54%	3.21%	2.92%	3.83%	4.12%	4.43%	4.57%
Oleic	C18:1n9	21.45%	17.99%	19.48%	15.96%	14.72%	14.90%	16.52%
Linoleic (LA)	C18:2n6	55.40%	40.75%	43.38%	36.95%	39.66%	44.37%	38.38%
Arachidic	C20:0	0.30%	0.26%	0.24%	0.30%	0.33%	0.33%	0.33%
gamma-Linolenic (GLA)	C18:3n6	0.13%	16.12%	13.74%	33.30%	28.86%	13.64%	18.23%
Eicosenoic	C20:1n9	0.18%	0.20%	0.20%	0.20%	0.20%	0.39%	0.50%
alpha-Linolenic (ALA)	C18:3n3	8.44%	5.81%	6.33%	5.16%	6.21%	9.00%	6.02%
Eicosadienoic	C20:2n6	0.05%	1.54%	1.49%	2.00%	0.20%	1.85%	2.20%
Behenic	C22:0	0.34%	0.28%	0.26%	0.28%	0.27%	0.26%	0.25%
Dihomo-g-linolenic (DGLA)	C20:3n6	0.00%	1.98%	0.43%	1.04%	5.44%	7.32%	10.25%
Lignoceric	C24:0	0.10%	0.08%	0.09%	0.10%	0.06%	0.06%	0.08%

In summary, we generated transgenic plants from two soybean cultivars (William 82 and Thorne) using the new gene vector with the same promoter (beta-conglycinin alpha subunit gene promoter) for expression of D6D and D6E, The transgenic plants produced up to 33.3% GLA and 10.25% DGLA in the soybean oil. We are generating more transgenic plants using different gene constructs in order to improve DGLA productivity.

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