Review

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Advances in the use of genetically modified plant biomass for biodiesel generation

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Abstract: Biodiesel is a low-carbon-intensity renewable fuel with up to 99% lower greenhouse gas emissions than petroleum-based diesel. The use of oil crops for biodiesel is under critical examination. It is expensive and suffers from the food versus fuel risk/benefit problem. Consequently, many countries (e.g. Malaysia and countries in the EU) are scaling back the use of oil crops as feedstock for biofuel production. The limitations of these traditional crops are leading the renewable fuels industry to consider innovative, sustainable, and profitable biomass-based platforms. Plant genetic engineering and other new breeding technologies are essential for developing such biomass-based platforms because they enhance plant tolerance to abiotic and biotic stresses, resulting in higher feedstock yields, greater net energy gain, and the generation of high-value co-products. We review and summarize the recent improvements of oil crops through plant genetic engineering that may increase widespread and cost-effective production of biodiesel and value-added co-products for green chemistry applications. © 2017 Society of Chemical Industry and John Wiley & Sons, Ltd

Keywords: biodiesel; fatty acid (FA); feedstock; plant genetic engineering; triacylglycerol (TAG); bioproduct gene technology

Introduction

Mitigating climate change and meeting increasing energy needs are two of the most challenging problems facing humanity in the twenty-first century.[1] Drop-in fuels such as renewable biodiesel appear to be sustainable alternatives to fossil fuel. These biomass-based options are expected to grow in the near future. Biodiesel production capacity is constrained by two related factors – high production costs and feedstock

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limitations – which hinder adoption of biodiesel fuel in the marketplace. Specifically, the cost of biodiesel production is strongly related to the price of the input triacylglycerol (TAG).[2] Since this raw material represents 70–95% of the total biodiesel production cost, TAG must become more readily available at lower cost to expand the commercial use of biodiesel.[3]

Biodiesel represents 80% of biofuel production in Europe and continues to grow in popularity due to political, environmental and economic objectives.[4] Biodiesel's benefits power its growth: lower carbon monoxide and carbon dioxide (CO_2) production, less smoke and particulates upon combustion, and reduced emission of air pollutants.[5] As a non-toxic and biodegradable alternative fuel, biodiesel confers additional advantages for engine performance, including a higher flashpoint and greater lubricity.[6] Energy content and physico-chemical properties of biodiesel are similar to conventional diesel. It can be used alone or mixed with conventional diesel in any diesel engine, without modifying the ignition system or the fuel injector.[5] Biodiesel fuel properties depend on length and degree of unsaturation of fatty acids (FAs); an ideal biodiesel contains a high percentage of monounsaturated FAs and fewer polyunsaturated acids.

Biodiesel is traditionally derived from vegetable oils (edible and non-edible), animal fats, and waste cooking oils through the transesterification process.[7] Vegetable oils include soybean, rapeseed and canola, corn, peanut, sunflower, carinata, camelina, and oil palm, all of which are currently used for biodiesel production, and algal oils are an emerging feedstock (Table 1).[8,9] The average European biodiesel plant uses approximately 61.9% rapeseed oil, 12.7% soybean oil, 10.9% palm oil, 8.0% used cooking oil, 3.4% tallow, 1.9% sunflower oil, and 1.4% from other sources.[10] Feedstock for

Table 1. Feedstocks for biodiesel production.

Edible oil crop seeds	Oil content % (w/w)	Non-edible oil crop seeds	Oil content % (w/w)
Soybean (*Glycine max*)	15-20	Jatropha (*Jatropha curcas* L.)	35-40
Palm (*Arecaceae*)	30-60	Karanja (*Pongamia pinnata*)	27-39
Rapeseed (*Brassica napus* L.)	38-46	Carinata (*Brassica carinata*)	40
Coconut (*Cocos nucifera*)	63-65	Castor (*Ricinus communis*)	53
Canola (*Brassica napus*)	40-50	Neem (*Azadirachta indica*)	20-30
Sunflower (*Helianthus annuus*)	25-35	Mahua (*Madhuca longifolia*)	35
Cotton seed (*Gossypium hirsutum*)	18-25	Camelina (*Camelina sativa*)	30-40
Peanut (*Arachis hypogaea*)	45-55	Rubber tree (*Hevea brasiliensis*)	40-50
Safflower (*Carthamus tinctorius* L.)	20-45	Sea mango (*Cerbera odollam*)	54
Mustard (*Brassica alba*)	25-30	Jojoba (*Simmondsia chinensis*)	44-59
Rice bran (*Oryza sativa* L.)	12.1-25	Linseed (*Linum usitatissimum*)	38 -44
Corn (*Zea mays*)	3.3-15.9	Croton (*Croton megalocarpus*)	40–45
Sesame (*Sesamum indicum*)	52-63	Polanga (*Calophyllum inophyllum* L.)	65–75
Macadamia seed (*Macadamia integrifolia*)	70	Yellow oleander seed (*Thevetia peruviana*)	60–65
Tung fruit (*Aleurites fordii*)	14-22	Tobacco (*Nicotiana tabacum*)	33-40
Avocado (*Persea americana*)	11.23-18.8	Hemp (*Cannabis sativa* L.)	30-35
Sugar apple seed (*Annona squamosa*)	24.5	Chinese tallow (*Sapium sebiferum* L.)	45–60
Ramtil (*Guizotia abyssinica*)	40	Beauty leaf (*Calophyllum inophyllum*)	46-75
Egusi (*Citrullus colocynthis* L.)	53	Moringa (*Moringa oleifera*)	40
Olive (*Olea europaea*) (pericarp)	15-16 (65)	*Hodgsonia macrocarpa*	71.65
		Silk cotton oil tree (*Ceiba pentandra*)	28.7
		Paradise (*Simarouba glauca*)	22-26
		Nagchampa seed kernel (*Calophyllum inophyllum*)	43–73
		Soapnut (*Sapindus mukorossi*)	23
		Hochst (*Crambe abyssinica*)	35.6-42.8
		Guizotia abyssinica L.	30

US biodiesel is about 50% soybean oil with animal fats and yellow grease, inedible corn oil, and canola oil making up the remainder.[11] Larger biodiesel quotas may increase demand for vegetable oil, most of which are currently used by food industries (81%), such that world demand for oilseed production is predicted to increase from 413Mt in 2010/2011 to 507Mt by 2020/2021.[10]

There are two strategic opportunities for biotechnology to improve vegetable oil and increase the quantities available for biodiesel production. First, vegetable oils often lack important biodiesel properties, such as improved oxidative stability and cold-flow properties for northern climates, which are sought by the biodiesel industry. Since most oil crops do not naturally meet the specifications required by biorefineries, biotechnology may improve the chemical composition and quality of oilseeds for cost-effective, efficient biodiesel production. Second, biotechnologies have significant potential for increasing the amount of plant-based feedstock per hectare of cultivable land, at a lower cost than other sources of TAG. More plant-based feedstock must be produced to avoid a food vs. fuel conflict, lower the cost of biodiesel production and thus increase the profit margins for biodiesel. In the short-term, it is imperative to invest in agronomic and biotechnological improvements that boost oil crop yields because biorefineries will continue to rely on these feedstocks for biodiesel generation in the near future.

Clearly, biotechnology offers solutions to overcome the bottlenecks to biodiesel production imposed by the high cost, low availability, and suboptimal chemical composition of vegetable oils. Forecasts of biodiesel production with traditional oil crops (status quo) indicate that converting world vegetable oil production completely to biodiesel may replace up to ten percent of current diesel needs.[12] Some agricultural economists have argued that by replacing more than 2% of world diesel with oil crops is problematic from several perspectives (i.e., negative impacts on food security, consumer prices, and land use). Diverting edible plant oils to produce biodiesel may have contributed to higher vegetable oil prices that increase the cost of biodiesel and related products, and have other economic impacts such as higher food prices.[13] Some forecasts have indicated that without subsidies (e.g. USA fuel tax exemption), biodiesel is not cost-competitive even with petroleum oil at $100/ barrel. Current expansion of crude petroleum stocks may last for years, with level or decreasing demand forecasted over a 20–30-year period stemming from incremental improvements in transport vehicle fuel efficiency. Concomitantly the global demand for vegetable oil as food is growing faster than population growth.[14] Regardless of the lead-up time for biodiesel costs to yield non-subsidized profits for this industry, biodiesel may play a future role in contributing to greenhouse gas reduction, and this could become a driving force for the development of biodiesel.[15] Economics and energy security are primary factors in the historical emergence of biodiesel but its continued evolution may profit from novel breakthroughs in plant science such as genome editing.[16]

The objective of this review is to describe and critically analyze how trait modifications achieved with biotechnology can enhance oil crop yield, chemistry and sustainable oilseed feedstock that is suitable for biodiesel production. As illustrated in Fig. 1, plant genetic biotechnologies present opportunities to (i) produce feedstock with higher oil content so a greater proportion of the energy/carbon in the oilseed can undergo FA esterification; (ii) increase oil accumulation in leaves and other vegetative tissue; (iii) develop feedstock with a lower environmental footprint/lower cost; (iv) produce an oilseed feedstock with characteristics required by biorefineries, reducing the processing time/energy; (v) produce high-value co-products from the feedstock such as enzymes and functional proteins; and (vi) improve the resistance of oilseed crops to biotic and abiotic stresses in the field. Together, these biotechnological advances are expected to improve the suitability of oilseed feedstock for biodiesel production, in that a modified oilseed could have greater energy density, lower cost, less environmental impact, and other favorable characteristics that are sought by biorefineries.

Metabolic engineering for increasing oil production

Increasing the supply and concentration of FAs and TAGs by novel means

Increasing seed or oil-kernel yield and/or oil yield per hectare allows oilseed crop farmers and plantation owners to produce a desired level of oil on existing farmland, thus avoiding competition with land allocated for food production and essential ecosystem services (e.g. wetlands). Oilseed, oil palm, and their oil content could be increased through genetic modification of growth, targeting pathways that allocate more carbohydrates to synthesize fatty acids and TAGs.[10, 17] Metabolic engineering should aim to maximize the flow of carbon into oil by:

- Increasing the supply of FA. Evidence suggests that oil synthesis may be limited by the production of FAs.[18] Thus, increasing flux of carbon to FA biosynthesis can significantly influence the amount of TAG produced in plant tissues.[19] The key rate-determining step that

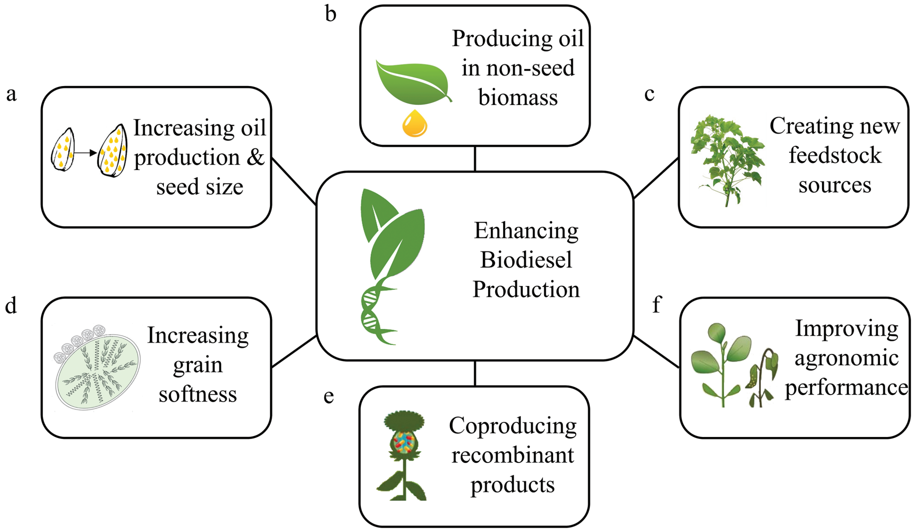

Figure 1. There are various strategies for modifying energy crop genetics and biomass processing, with the goal of optimizing biodiesel production. (a) For example, oil content in the seeds of energy crops can be increased by selecting for enlarged seed size (sink) and increasing oil production by molecular breeding or via genetic engineering. (b) Non-seed plant biomass that grows abundantly in a particular region can be modified to accumulate plant oils in leaves, stems roots and other non-seed organs. (c) Non-edible crops can be genetically modified to reach the desired concentration and composition of free fatty acids (FFAs), and reduce or eliminate any toxic compounds before refining their oils. (d) Crushability: Processing costs of oilseeds may be reduced by expression of grain softness genes such as puroindolines, pinA and pinB, that are known to influence oilseed milling properties. (e) Crops tailored for biodiesel oil production can co-host a parallel platform for the manufacture of high-value recombinant products such as biocatalysts for the emergent *Biodomain* industries (including Green Chemistry) as well as traditional biologics such as insulin production. (f) All candidate biodiesel plant species can benefit from the yield increase achieved through the application of high performance plant biotechnology, already proven in the field in over 30 countries and over 20 years to improve agronomic performance (biotic and abiotic stresses).

controls FA biosynthesis, acetyl-CoA carboxylase (ACCase) has been extensively studied in a few model plants. ACCase catalyzes the formation of malonyl-CoA to provide two carbon units for use by the FA synthase complex (Fig. 2). A study that over-expressed the ACCase gene from foxtail millet found the oil content of transgenic maize seeds was 24 to 65% higher than that of untransformed seeds.[20] If the rate determining role for ACCase were to be studied for major oilseeds and oil palms, significant yield increases may be achieved.

- Increasing the concentration of glycerol backbone. Glycerol-3-phosphate (G3P) is an important substrate that limits the amount of TAG formed and stored in plant reserve tissues. Glycerol-3-phosphate dehydrogenase (G3PDH) catalyzes the conversion of dihydroxyacetone phosphate (DHAP) to G3P (Fig. 2). Over-expression of yeast G3PDH in *Brassica napus* led to a three- to four-fold increase in G3P, resulting in a 40% relative increase in the lipid content of mature seeds.[21]
- Increasing the activity of TAG biosynthetic enzymes. In plants, the synthesis of glycerolipids in the endoplasmic reticulum (ER) involves the sequential acylation of G3P by the following enzymes: glycerol 3-phosphate acyltransferase (GPAT); lysophosphatidic acid acyltransferase (LPAAT); and diacyl-glycerol acyltransferase (DGAT, Fig. 2). Since these enzymes have a rate-limiting role in TAG synthesis, genetic modifications that would increase the GPAT, LPAAT, and DGAT activity in plants were evaluated in several model species. The result was increased seed oil content in *Arabidopsis* that was genetically modified to express plastidial safflower (*Carthamus tinctorius* L.)

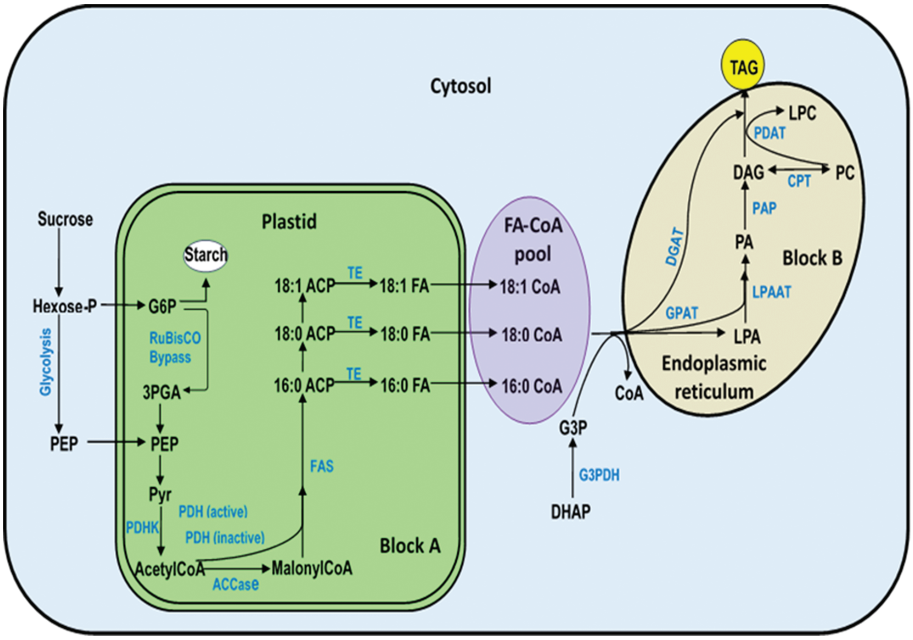

Figure 2. Overview of fatty acid and triacylglycerol production in plants. Oilseed species utilize different pathways for producing acetyl-CoA precursors and cofactors for fatty acid synthesis. For simplicity, not all possible reactions are shown and multiple reactions may be represented by single arrows. All pathways are not present in every species. The abbreviations used are: 3PGA, 3-phosphoglycerate; ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; CoA, coenzyme A; CPT, cholinephosphotransferase; DAG, diacylglycerol; DGAT, DAG acyltransferase; DHAP, dihydroxy acetone phosphate; FA, fatty acid; FA-CoA, fatty acyl-coenzyme A; FAS, FA synthase; G3P, glycerol 3-phosphate; G6P, glucose-6-phosphate; GPAT, G3P acyltransferase; G3PDH, sn-glycerol-3-phosphate dehydrogenase; Hexose-P, hexose-phosphate; LPA, lyso-phosphatidic acid; LPAAT, lyso-phosphatidic acid acyltransferase; LPC, lyso-phosphatidylcholine; PA, phosphatidic acid; PAP, PA phosphatase; PC, phosphatidylcholine; PDAT, phospholipid:DAG acyltransferase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; Pyr, pyruvate; Rubisco, ribulose-bisphosphate carboxylase/oxygenase; TAG, triacylglycerol; TE, thioesterase. Adapted from Durrett *et al.*[44] and Weselake *et al.*[111]

GPAT, and *Escherichia coli* GPAT during seed maturation.[22] Likewise, when a mutated yeast LPAAT gene (*SLC1-1*) was expressed in both *Arabidopsis* and high-erucic acid *B. napus*, under the control of CaMV35S promoter, the seed oil content increased, as much as 8 to 48% in the latter.[23] In subsequent field studies, high-erucic acid *B. napus* expressing *SLC1-1* increased seed oil content by as much as 13.5%.[24] In the downstream pathway of TAG synthesis, DGAT catalyzes the addition of acyl-CoA to the glycerol backbone with release of CoA; it is proposed to be a rate-limiting step because DGAT has lower enzyme activity than other enzymes in the pathway.[25] Over-expression of cDNAs encoding DGATs increased the oil content of canola, maize, and soybean. Transgenic *B. napus*, over-expressing *B. napus DGAT1* (*BnDGAT1*) had a four-fold increase in DGAT activity and up to 14% increase in seed oil content, as well as increased resistance to drought stress.[26] Zheng *et al.* ectopically expressed DGAT1-2 allele of maize, resulting in relative increases in seed oil content by up to 40%.[27] Likewise, the DGAT2A from the soil fungus (*Umbelopsis ramanniana*) was expressed in soybean throughout development, leading to an absolute increase in oil of 1.5% (by weight) in the mature seed.[28]

- Increasing lipid transfer proteins. An example of this novel approach to engineering carbon flow includes the expression of a lipid transfer protein relative, puroindoline. When expressed in maize, puroindoline caused a 25% increase in total seed oil content.[29, 30]

These examples provide compelling evidence that the glycerolipid synthesis pathway can be manipulated to increase oil yields (Table 2). Further advances can be achieved by conceptualizing the process of TAG biosynthesis in plants as occurring in two blocks of reactions - Block A (fatty acid synthesis) and Block B (lipid assembly). Metabolic control analysis (MCA) indicated that the control exerted by FA synthesis (Block A, Fig. 2) is greater than the glycerolipid synthesis reaction to produce oil in important crops, such as soybean, oil palm, and olive.[31] On the other hand, for oilseed rape, lipid assembly (Block B, Fig. 2) was more important. MCA offers a way of providing vital information that can be used to guide genetic manipulation.[32]

Redirecting carbon partitioning between lipid and other cellular metabolites

During seed development, oilseeds exhibit a transient accumulation of starch, which can be repressed to favor oil accumulation. In plants, the formation of acetyl-CoA from pyruvate and CoA is conducted by the mitochondrial pyruvate dehydrogenase (PDH) complex. This links glycolysis to the TCA cycle with concomitant release of CO_2 (Fig. 2). Pyruvate dehydrogenase kinase (PDHK), a negative regulator of the PDH complex, plays a pivotal role in controlling PDH complex activity. Antisense RNA technology has potential to improve oil accumulation by decreasing PDHK activity through seed-specific antisense repression of *Arabidopsis thaliana* PDHK cDNA during seed maturation.[33] This resulted in greater seed oil content and seed weight in transgenic plants, revealing PDHK as a suitable target for genetic improvement of seed oil content.[34]

Although more than 80% of vegetable oil production worldwide was derived from palm, soybean, rapeseed and sunflower oil, we cannot overlook the fact that cereal crops contain grain oil and this component could be manipulated through genetic technologies.[30] Cereal grains store carbon as starch in a large endosperm and store oil in an embryo of limited size, making the embryo the obvious target for increasing grain oil production. Increasing the embryo size and oil content of maize by ectopic over-expression of wheat puroindoline genes (*pinA*, *pinB*) resulted in 25% more kernel oil content in the transgenic lines.[29] A significant increase in kernel oil content was achieved via embryo-specific over-expression of maize gene *DGAT1-2* and/or fungal *DGAT2*.[27, 35] The ultimate goal for biodiesel feedstocks production (i.e., redirecting carbon from starch to oil in the endosperm), however, has not yet been achieved. More complex strategies aimed at modifying both the structure of the grain and the endosperm metabolism are required.[30] In the future, engineering of new ‘high-oil’ cereal varieties should take advantage of biodiversity previously noted in the “oiliest” genus among the common cereals, *Avena* (oat).[36] Biodiesel feedstocks generation may advance more rapidly with a renewed focus on the combination of efficient biotechnological solutions dedicated to the modification of the grain structure and stimulation of oil synthesis in grains.

Modifying transcription factors for seed oil accumulation

Modifying transcription factors (TFs) is an alternative strategy for altering complex traits such as accumulation of seed oil (Table 2). TFs are proteins that bind to DNA to induce the expression of a gene or suite of genes whose encoded product(s) (e.g. a protein) serve a particular purpose within the cell. Transcriptional regulator, WRINKLED1, is able to activate most of the genes involved in the FA biosynthetic pathway (Fig. 3).[37] WRI1 controls the expression of at least 15 enzymes, including ACCase, enzymes of FAS, and key enzymes and transporters that provide pyruvate and acetyl-CoA in the plastid.[38] Maize WRINKLED1 (Zm*WRI1*) controls transcription of the majority of known key enzymes that convert sucrose to FA. Thus, it is an ideal target for the design of biotechnological tools aimed at boosting FA production.[39] As a ‘master transcriptional regulator’, LEAFY COTYLEDON1 (LEC1) and LEC2, ABSISIC ACID INSENSITIVE3 (ABI3) are proposed to activate the expression of WRI1 (Fig. 3). Over-expression of transcription factors maize ZmLEC1 under the control of embryo-preferred promoters in transgenic maize lines significantly increased kernel oil content by 48%, but reduced seed germination and leaf growth in maize.[40] Overexpression of maize ZmWRI1 increased oil content to the same extent as the overexpression of maize ZmLEC1 without affecting germination, seedling growth, or grain yield.[41] Overexpression of ZmLEC1 increased seed oil by as much as 30% in field trials.[40] If these results can be extrapolated to field scale, plant oil production could be increased about 6 billion liters if ‘high-oil’ maize crops were grown worldwide.[42] There is evidence that WRI1 also up-regulated PDH in oil palm,[43] which may suggest a universal role of WRI1 in manipulating expression of transcription factors that regulates multiple enzymes in glycolysis and FA synthesis of both cereal grain and oil crops.

Table 2. Strategies to enhance oil production in seeds.

Strategies	Target gene	Source organism	GM plant	Increase in seed oil content	References
TA biosynthesis	ACCase	*Arabidopsis thaliana*	*Brassica napus*	5%	Roesler *et al.*[85]
	WRI	*Zea mays*	*Zea mays*	49%	Shen *et al.*[40]
	WRI	*Zea mays*	*Zea mays*	30% (field trials)	Shen *et al.*[40]
	WRI	*Arabidopsis thaliana*	*Camelina sativa*	14%	An *et al.*[86]
	WRI	*Brassica napus*	*Arabidopsis thaliana*	10–40%	Liu *et al.*[87]
	LEC1	*Zea mays*	*Zea mays*	48%	Shen *et al.*[40]
	LEC1	*Brassica napus*	*Brassica napus*	7-16%	Elahi *et al.*[88]
Glycerol backbone	G3PDH	*Saccharomyces cerevisiae*	*Brassica napus*	40%	Vigeolas *et al.*[21]
TAG biosynthesis	GPAT	*Carthamus tinctorius*	*Arabidopsis thaliana*	10-21%	Jain *et al.*[89]
	GPAT	*Escherichia coli*	*Arabidopsis thaliana*	15%	Jain *et al.*[89]
	LPAAT (SLC1-1)	*Saccharomyces cerevisiae*	*Brassica napus*	8-48%	Zou *et al.*[90]
	LPAAT (SLC1-1)	*Saccharomyces cerevisiae*	*Brassica napus*	13.5% (field study)	Taylor *et al.*[91]
	DGAT1	*Arabidopsis thaliana*	*Arabidopsis thaliana*	28%	Jako *et al.*[92]
	DGAT1	*Arabidopsis thaliana*	*Brassica juncea*	10%	Savadi *et al.*[93]
	DGAT1	*Zea mays*	*Zea mays*	41%	Zheng *et al.*[27]
	DGAT2	*Umbelopsis ramanniana*	*Glycine max*	1.5% (by weight)	Lardizabal *et al.*[28]
	MGAT	*Mus musculus*	*Arabidopsis thaliana*	10%	El Tahchy *et al.*[94]
Oil body stabilization/	SPD 1 RNAi	*Brassica napus*	*Brassica napus*	8%	Kelly *et al.*[95]
TAG degradation	SPD 1 RNAi	*Arabidopsis thaliana*	*Arabidopsis thaliana*	3.20%	van Erp *et al.*[96]
Combinatorial	WRI1 and DGAT1	*Zea mays*	*Arabidopsis thaliana*	18%	van Erp *et al.*[96]
approaches	WRI1, DGAT1 and SDP1RNAi	*Arabidopsis thaliana*	*Arabidopsis thaliana*	7.40%	van Erp *et al.*[96]
	GPDH, DGAT, GPAT and LPAAT	*Brassica napus, Saccharomyces cerevisiae*	*Brassica napus*	14.46%	Liu *et al.* [97]

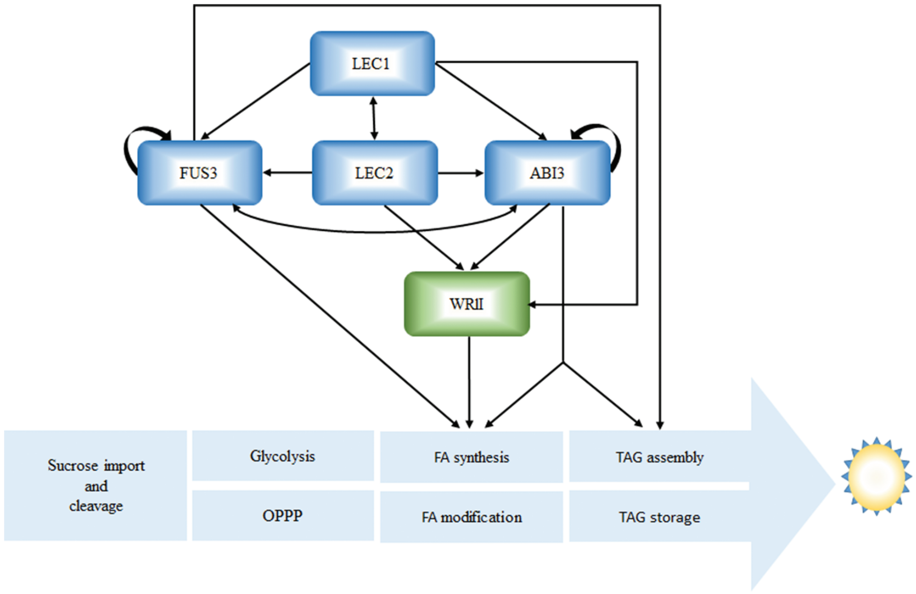

Figure 3. Interaction model of the transcription factors involved in seed oil synthesis. The precursors for de novo fatty acid synthesis in maturing embryos are derived from sucrose through the glycolytic pathway and/or the oxidative pentose phosphate pathway. Fatty acids produced in the plastids are exported towards the cytosol in the form of acyl CoAs and converted to triacylglycerols, ultimately stored in oil bodies. Transcription factors that induce lipogenesis are presented in colored boxes. Solid arrows represent transcriptional regulations. ABI3, ABSCISIC ACID INSENSITIVE3; FA, fatty acids; FUS3, FUSCA3; LEC1,2, LEAFY COTYLEDON1,2; OPPP, oxidative pentose phosphate pathway; TAG, triacylglycerols; WRI1, WRINKLED1*.* Adapted from Santos-Mendoza *et al.,*[112] Fatihi *et al.,*[113] Devic and Roscoe,[114] and Fobert.[115]

Producing oils in abundant vegetative tissues

The net energy yields from lipids are inherently high. Triacylglycerol has twice the energy content per carbon atom when compared to that of carbohydrates. Existing in nature usually in fluid form, plant oils can be extracted with low energy inputs and low costs.[42,44] Engineering crops to accumulate higher amounts of energy-dense oils in leaves and other vegetative parts is particularly attractive because many plants produce at least as much, if not more, biomass in leaves and stems as in the seed/grain components.[45] Several approaches have been explored thus far (Table 3):

- Increasing oil content in non-seed tissues by altering FA metabolism in leaves to favor oil production. When *A. thaliana* DGAT1 was expressed in *Nicotiana benthamiana*, the TAG levels in the leaf tissue exhibited about a 20-fold increase in TAG content, corresponding to 0.45% on a dry weight basis.[46]
- Producing the seed lipids in the leaf tissues by regulating TFs. TFs, such as LEC1, LEC2, WRI1, FUS3, and ABI3 were shown to control the expression of genes involved in oil synthesis in developing plant seeds. Induction of LEC2 in *Arabidopsis* and tobacco led to higher lipid contents in the leaf, with TAG profiles similar to those of *Arabidopsis* seed.[47,48] The constitutive expression of the *A. thaliana* WRI1 transcription factor in *N. benthamiana* yielded higher TAG levels in vegetative tissues. TAG levels increased 22-fold compared to the negative control and corresponded to 0.57% on a dry weight basis.[49] Unlike other master TFs (LEC1, LEC2), ectopic overexpression of WRI1 is not associated with negative effects on growth, germination, and somatic embryogenesis. Hence WRI1 is a target of significant interest to increase oil content in vegetative tissues.

Table 3. Genetic engineering approaches to increase lipid synthesis in vegetative feedstocks.

Strategies	Gene	Source organism	GM plant	TAG in leaf (% dry weight)	References
Upregulation of	WRI1	*Arabidopsis thaliana*	*Nicotiana benthamiana*	0.57%	Vanhercke *et al.*[49]
fatty acid biosynthesis	WRI1	*Avena sativa*	*Nicotiana benthamiana*	2.20%	Grimberg *et al.*[98]
(push)	LEC2	*Arabidopsis thaliana*	*Arabidopsis thaliana*	0.80%	Slocombe *et al.*[99]
	LEC2	*Arabidopsis thaliana*	*Nicotiana tabacum*	6.8% TFA	Andrianov *et al.*[48]
	MGD1 RNAi	*Nicotiana tabacum*	*Nicotiana tabacum*	8.3-fold increase	Wu *et al.*[100]
	AGPase RNAi	*Arabidopsis thaliana*	*Arabidopsis thaliana*	1.4-fold increase (seedling)	Sanjaya *et al.*[101]
	ACCase	*Arabidopsis thaliana*	*Solanum tuberosum*	0.03% (tuber)	Klaus *et al.*[102], Vanhercke *et al.*[103]
Increasing	MGAT1	*Mus musculus*	*Nicotiana benthamiana*	8.2-fold increase	Petrie *et al.*[46]
TAG assembly	MGAT2	*Mus musculus*	*Nicotiana benthamiana*	6.3-fold increase	Petrie *et al.*[46]
(pull)	DGAT1	*Arabidopsis thaliana*	*Nicotiana tabacum*	5.8-6%	Andrianov *et al.*[48]
	DGTT2	*Chlamydomonas reinhardtii*	*Arabidopsis thaliana*	0.36% (leaf); 1.0% (seedling)	Sanjaya *et al.*[104]
	PDAT1	*Arabidopsis thaliana*	*Arabidopsis thaliana*	2.60%	Fan *et al.*[105]
Preventing TAG and FA	cgi-58	*Arabidopsis thaliana*	*Arabidopsis thaliana*	0.20%	James *et al.*[106]
Turnover (protect)	pxa1	*Arabidopsis thaliana*	*Arabidopsis thaliana*	1.8% (senescing leaf)	Slocombe *et al.*[99]
Combinatorial	WRI1 and AGPase RNAi	*Arabidopsis thaliana*	*Arabidopsis thaliana*	5.8-fold increase (seedling)	Sanjaya *et al.*[101]
approaches	tgd1 with sdp1 or pxa1	*Arabidopsis thaliana*	*Arabidopsis thaliana*	9%	Fan *et al.*[107]
(push, pull and protect)	DGAT1 and OLEOSIN	*Arabidopsis thaliana*	*Arabidopsis thaliana*	2.1% (leaf); 6.5% (root)	Winichayakul *et al.*[108], Vanhercke *et al.*[103]
	WRI1, OLEOSIN and DGAT1	*Arabidopsis thaliana*	*Nicotiana tabacum*	15%	Vanhercke *et al.*[109]
	WRI1 and DGAT1	*Arabidopsis thaliana*	*Nicotiana benthamiana*	2.48%	Vanhercke *et al.*[49]
	WRI1 and DGAT2	*Arabidopsis thaliana*	*Arabidopsis thaliana*	2-3%	Kelly *et al.*[110]
	WRI1 and DGAT1, sdp1	*Arabidopsis thaliana*	*Arabidopsis thaliana*	5%	Kelly *et al.*[110]
	PDAT1 and OLEOSIN 1	*Arabidopsis thaliana*	*Arabidopsis thaliana*	6.4% (leaf); 1.5% (stem)	Fan *et al.*[105]
	PDAT1 and OLEOSIN 1, tgd1	*Arabidopsis thaliana*	*Arabidopsis thaliana*	8.6% (leaf); 3% (stem)	Fan *et al.*[105]

- Push Pull Protect: Optimizing the flux of carbon into TAG on multiple metabolic levels by upregulating FA synthesis ('Push'), increasing TAG assembly ('Pull') and preventing lipid turnover ('Protect').[50,51] With co-expression of *WRI1* and *DGAT1* (Push and Pull genes), a significant synergistic effect on leaf oil accumulation was observed with TAG levels increasing about 100-fold and representing 2.48% of the leaf dry weight.[49] Co-expression of three genes, *WRI1*, *DGAT1*, and an oleosin-encoding gene (*OLEOSIN*), led to the accumulation of more than 15% TAG by dry weight in tobacco leaves, while the total leaf starch content was reduced. This corresponded to a 75-fold increase compared to wild type TAG content. More importantly, the high level of storage TAG in senescing leaves did not have any detrimental effect on plant development and seed viability. These yields exceed the levels found in wild-type leaf tissue as well as previously reported engineered TAG yields in the vegetative tissues of *Nicotiana tabacum*.[50]

Recently, a combination of genetic strategies has achieved high levels of oil accumulation in plant biomass (Table 3). If TAG could accumulate to levels above ten percent on a dry weight basis in a high-yielding dedicated energy crop, the energy yield from the crop would be impressively increased by 30–40%, and oil yields per ha would exceed those of oilseed rape at least two-fold.[42] Caveat: In this research area of plant lipidology, any increases in TAG or fatty acid in vegetative tissues have sometimes been reported as 'fold increases'. While being a useful calculation, it does depend on measuring (often inaccurately) a very low concentration of TAG in the control samples; e.g. a 20-fold increase over 0.1% is not appreciable. When working with such novel plant lines, the absolute value (wt %) would be more indicative of the metabolomic plasticity.[52] If the 57.4 million ha of biotech maize now grown in the world were instead planted with such a crop, 114.8 billion liters of biodiesel could be produced from a non-food crop.[42, 53] Needless-to-say the assumptions that are made to come up with such estimates, for instance the conversion efficiency and bioconversion process, can be strengthened through additional detailed research studies. Thus, biomass crops engineered for high oil content in vegetative tissues could be a novel oil-based feedstock for meeting global production needs for low-cost, energy-dense oil sources.[54]

Producing biodiesel from non-edible crops

The widespread adoption and commercialization of biodiesel present several sustainability challenges. The biggest of these relates to land use regarding potential competition with food crop production and the limited supply of biodiesel feedstocks. Due to food security concerns, countries such as China and Japan prohibit the use of edible oils for biodiesel production.[55,56] Consequently, research on alternative non-edible oil sources is encouraged and supported by governments around the world.[55] Fortunately, many oil plants produce non-edible oils (Table 1). The most important non-edible oil plants are jatropha, *Brassica carinata*, camelina, karanja, tobacco, mahua, neem, rubber, sea mango, castor, cotton, and Chinese tallow.[3, 57-65] *Jatropha curcas*, *Madhuca indica*, and *Pongamia pinnata* are the feedstocks most frequently converted to biodiesel. For example, biodiesel extracted from the seeds of *B. carinata* and *Camelina sativa* was a successful jet-fuel that reduced net carbon emissions by about 80% during simulated flight.[66] These non-edible oil plants are cultivated in marginal lands that are unsuitable for growing edible crops, so they do not displace food crop production on prime agricultural lands. They also cost significantly less to grow and harvest than their edible oil counterparts. Some of the critical disadvantages include their high free fatty acids (FFAs) and toxic compounds. For example, curcin and purgative agents are expressed in jatropha, ricin in castor, glucoside cerberin in fruits of sea mango and toxic flavonoids pongamiin and karajiin in karanja oil.[3, 67] Genetic engineering has the potential to reduce these toxic compounds, as was demonstrated when RNAi was used to suppress curcin expression in the transgenic plants of *J. curcas*.[55] Biodiesel crops such as jatropha and other species may provide a small bioproduct contribution in some geographical areas, and this can be important to local rural populations. While these socio-political factors are critical as development goals, it should be borne in mind that low yields of carinata, jatropha, camelina, etc., are expected on marginal agricultural lands, so these crops contribute little to the socio-economic development in rural areas. Some elements in the 'protest industries' suggest that dedicated non-food oil crop benefits are overestimated, but producers on marginal lands will reap real benefits if plant breeding programs and/or biotechnology advances can improve crop traits and productivity of these oil crops.

Decreasing processing cost by increasing grain softness

Oil can be extracted from oilseed crops by mechanical, solvent, or enzymatic extraction. Mechanical pressing is the most commonly used method for commercial oil extraction. However, seed crushing requires energy and adds to the cost of biodiesel production. Thus, improving the softness of the seed will reduce the crushing costs. The molecular genetic basis of grain hardness provides strategies to soften the seed for better crushing. Soft endosperm texture is dominant in wild races of wheat, oats, rye, and barley. The puroindoline proteins (pinA and pinB) are believed to play critical roles in the hardness of wheat grain. Mutations or absence in either gene (*pinA* and *pinB*) will result in hard texture, as demonstrated in the harder textured cereal crops such as corn and rice that lack pinA and pinB. Pressure required to fracture kernels was reduced by 15.2% to 27.3% in soft textured rice seeds that expressed pinA or pinB, compared with control seeds.[68]

Puroindolines, formerly known as grain softness protein or friabilin, when expressed in transgenic rice softened grain texture of rice. The authors suggested that puroindolines might convey similar kernel properties to other cereals such as corn, sorghum, and wheat.[68] Modifying grain texture of seeds may yield numerous advantages. Softer texture could reduce crushing costs and lead to greater extraction efficiency of the oil. Furthermore, transgenic crops overexpressing pinA and/or pinB showed significantly increased resistance to plant diseases, which could be used as alternatives to conventional antibiotics to control plant and human diseases.[69,70] Thus, exploitation of variation in grain hardness may have numerous market niches.

Coproduction of high-value recombinant products in biodiesel crops

Feedstock accounts for up to 70–95% of biodiesel cost,[3] which affects the profitability of commercial biodiesel production. Just as the petroleum industry optimized profits by producing value-added co-products, the biorefinery industry strives to follow the same model.[71] Transgenic plants are already recognized as a bio-based platform that can produce high-value biopharmaceuticals and industrial products.[72,73] Snell *et al.* demonstrated co-production of poly-3-hydroxybutyrate (PHB), a renewable biodegradable plastic in *Camelina sativa*.[74] PHB accumulated at up to 15% of the mature seed weight in the plastids of seeds.[75] Scaled up via agricultural production of PHB-plants may provide a commercially attractive source of bio-polymer feedstock to the plastics, chemicals and feed supplements industries. Therefore, coproduction of recombinant biodegradable plastics in transgenic, non-edible bioenergy crops can add value to the crops. A similar co-product strategy could apply to feedstocks producing multiple co-products. For example, recombinant human insulin was successfully expressed and produced in oil bodies of *A. thaliana* seed, and insulin accumulated to high level in transgenic seed (0.13% of total seed protein).[76] The targeted expression of insulin in subcellular organelles allowed easy recovery of recombinant insulin during oil extraction. Designing genetically modified bioenergy feedstocks that produce high-value enzymes and proteins is expected to enhance the profitability of biorefineries.[77]

Furthermore, the enzymes used in the biotransformation of lipid into biodiesel could be biosynthesized by the biomass itself. The transesterification process is performed in the presence of base, acid, or enzyme catalysts. As for the downstream processing problems caused by chemical transesterification, enzyme-catalyzed (lipase) transesterification is a preferred method to achieve biodiesel production. In contrast to chemical catalysts, the advantages of using enzymatic catalysts include: the reactions are less energy intensive; can be carried out at mild conditions; do not promote side reactions; and enzymes are more environmentally friendly (soap is not formed; no problem of purification, washing, and neutralization).[78] However, biocatalysts are not very frequently used in industry, since higher costs are often associated with the production of catalyst. Currently, most commercial lipases, such as *Burkholderia cepacia* lipase (BCL), *Candida antarctica* lipase B (CALB), are mostly produced from microbial systems by fermentation. Recombinant protein manufacture costs have decreased somewhat with improved microbial fermentation strains and systems, but the capital investments of fermenter, media sterilization, pathogen control, downstream processing, media disposal, etc. are still limiting broader commercial applicability to the biodomain.[79] Using transgenic plants as a bioreactor for mass production of lipase could potentially reduce the cost of catalyst production significantly.[80] Yang *et al.* created transgenic rice, expressing the CALB gene under the control of the promoter of the rice seed storage protein GT1. The made-in-plant OsCALB protein accumulated to more than 40% of the total soluble protein in crude seed extracts (19.24 g rOsCALB per kilogram dry seeds), and was biochemically and functionally comparable to the protein issuing from conventional fermenter systems.[79]

Improving the agronomic performance of feedstocks via transgenic technology

Environmental stresses, such as drought, high salinity, and low temperature, have adverse effects on plant growth and seed productivity, and restrict land use. Diseases, insects, weeds, and other pests annually cause substantial losses in the yield and lower the quality of feedstocks produced. Most commercially available genetically modified crops possess incorporated yield traits, such as herbicide tolerance, insect resistance and fungicide resistance, which contribute to increased yields and agricultural growth.[10,81] Similarly, transgenic technology, already applied to food crops, could be used to enhance the biotic and abiotic stress tolerance in oil-producing crops. For example, the pathogenesis-related protein (JcPR-10a) showed RNase and antifungal activity against *Macrophomina*, indicating that genetic engineering JcPR-10a may be an advantageous solution to increase stress tolerance in jatropha as well as other plants susceptible to collar rot by *Macrophomina*.[82] Since ethylene responsive element binding protein (AP2/EREBP) participates in hormone signal transduction and in responses to biotic pathogens and abiotic environmental stresses, tolerance was observed to increase through overexpression of AP2/EREBP transcription factor.[83]

Summary and conclusions

Recent improvements in gene manipulation have enhanced biodiesel prospects significantly. Seed oil content has increased dramatically in greenhouse or field test plots, but this remains to be confirmed in multi-year field trials. Lab-scale results in growth chambers and in glasshouses may or may not be replicated under field conditions, a realistic environment with more variable conditions for plant growth, including abiotic and biotic stresses that can be eliminated in controlled environments: light intensity and quality are two of the many factors that can cause differences in lab-grown and field-grown plants. The seed industry has been targeting seed oil increases for over 25 years through major investments in plant molecular genetics. For example, Calgene (Monsanto) reported the transgenic expression of the DGAT2A gene from a soil fungus, *Umbelopsis ramanniana* in the seeds of soybean. This brilliant piece of lipidology resulted in an absolute increase in oil of 1.5% (by weight) in the mature seed. Such efforts surely continue, but to the authors' knowledge there are still no commercial crops on the market where oil has been increased by genetic engineering. Needless-to-say, conventional breeding has continued to spread forward, nudging oil content in *B. napus* crops up by one percent in some years.[84] Fortunately technical barriers and market dynamics continue to mutate faster, engendering prospects for commercial success viz. jet fuel emission analysts at NASA, NRC Canada and their colleagues studying camelina biofuel report that biofuel blending reduces mass emissions and particle number immediately behind a jet aircraft by 50 to 70 per cent.[116] However, there are still some outstanding questions that we might address, such as the following research gaps and promising avenues for future research:

- Stem/leaf oil production is feasible in field-grown crops to support sustainable food, high energy forage, and biofuel and biomaterial applications. However, this raises a complicated question. When oil accumulates in seeds, it seems to be more 'dense', yielding more oil per kg of oilseed than kg of vegetative tissue. Since foliage must be dried and ground prior to oil extraction, it cannot be transported as cheaply as oilseeds. Also, green parts are bulkier, costing more to transport and store than grain. Can harvesting equipment be modified, or a pre-processing step added to densify the oil contained in the leaf and stem components, to make it a cost-effective feedstock for biorefining?
- Most biotechnological approaches therefore have been directed at increasing the storage lipid content in either seed or vegetative tissues. Can high TAG levels be achieved simultaneously in seeds and vegetative tissues of the same plant? If entire biomass is used in renewable production of TAG, such 'dual depot' crops may yield oil at levels comparable to oil palm, currently the highest yielding oil crop.
- Transgenic seeds are used increasingly as bioreactors in plant molecular farming, producing recombinant biologics and biocatalysts on commercial scale, for example trypzean, avidin, aprotinin. Which co-products are compatible with biorefinery conversion pathways? An ideal strategy would preserve the biological activities of recombinant enzymes and other co-products, whilst allowing for maximum oil extraction from the crop. Such 'dual stream' crops may strengthen the financial feasibility of farms that decide to include oilseed crops destined for biorefining in their cropping systems.
- Plant seeds, especially cereal grains, are promising vehicles for co-producing biocatalysts. Can the high accumulation of recombinant protein, and high levels of protein stability, even during long storage time, favor controlled production scales? Since downstream processing costs are considerable, further research into efficient conversion technologies will allow biorefineries to capitalize on lower production costs of large-scale transgenic oil-based feedstocks.

References

1. Popp J, Lakner Z, Harangi-Rakos M and Fari, M, The effect of bioenergy expansion: Food, energy, and environment. *Renew Sustain Energy Rev* **32:** 559-578 (2014).
2. Naik SN, Goud VV, Rout PK and Dalai AK, Production of first and second generation biofuels: A comprehensive review. *Renew Sustain Energy Rev* **14:** 578-597 (2010).

3. Bankovic-Ilic IB, Starnenkovic OS and Veljkovic VB, Biodiesel production from non-edible plant oils. *Renew Sustain Energy Rev* **16:** 3621-3647 (2012).
4. Wisner R, Biofuels mandates outside the U.S. *AgMRC Renewable Energy and Climate Change Newsletter* (2013). Available at: http://www.agmrc.org/renewable-energy/biofuelsbiorefining-general/biofuels-mandates-outside-the-us/ [April 16, 2017].
5. Pragya N, Pandey KK and Sahoo PK, A review on harvesting, oil extraction and biofuels production technologies from microalgae. *Renew Sustain Energy Rev* **24:** 159-171 (2013).
6. Knothe G, Dependence of biodeisel fuel properties on the structure of fatty acid alkyl esters. *Fuel Process Technol* **86:**1059–1070 (2005).
7. Zhang Y, Dube MA, McLean DD and Kates M, Biodiesel production from waste cooking oil: 1. Process design and technological assessment. *Bioresour Technol* **89:**1–16 (2003).
8. Chisti Y, Biodiesel from microalgae. *Biotechnol Adv* **25:**294–306 (2007).
9. No SY, Inedible vegetable oils and their derivatives for alternative diesel fuels in CI engines: A review. *Renew Sustain Energy Rev* **15:**131–149 (2011).
10. Milazzo MF, Spina F, Cavallaro S and Bart JCJ, Sustainable soy biodiesel. *Renew Sustain Energy Rev* **27:**806–852 (2013).
11. Amarasekara AS, *Handbook of Cellulosic Ethanol*. John Wiley & Sons Inc., Hoboken, NJ pp. 1–27 (2013).
12. Maheshwari P and Kovalchuk I, *Industrial Oil Crops*. AOCS Press, Urbana, IL pp.379–412 (2016).
13. McKeon TA, Hayes DG, Hildebrand DF and Weselake RJ, *Industrial Oil Crops*. AOCS Press, Urbana, IL pp. 1–13 (2016).
14. OECD/FAO, *OECD-FAO Agricultural Outlook 2015.* [Online]. OECD Publishing. Available at: http://www.fao.org/3/a-i4738e.pdf [April 16, 2017].
15. Wan S, Ward TL and Altosaar I, Strategy and tactics of disarming GHG at the source: N_2O reductase crops. *Trends Biotechnol* **30:**410–415 (2012).
16. Lu CF, Napier JA, Clemente TE and Cahoon EB, New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. *Curr Opin Biotechnol* **22:**252–259 (2011).
17. Chapman KD, Dyer JM and Mullen RT, Commentary: Why don't plant leaves get fat? *Plant Sci* **207:**128–134 (2013).
18. Andre C, Haslam RP and Shanklin J, Feedback regulation of plastidic acetyl-CoA carboxylase by 18:1-acyl carrier protein in *Brassica napus*. *Proc Natl Acad Sci USA* **109:**10107–10112 (2012).
19. Chapman KD and Ohlrogge JB, Compartmentation of triacylglycerol accumulation in plants. *J Biol Chem* **287:**2288–2294 (2012).
20. Dong ZG, Zhao HJ, He JG, Huai JL, Lin H, Zheng J *et al*. Overexpression of a foxtail millet Acetyl-CoA carboxylase gene in maize increases sethoxydim resistance and oil content. *Afr J Biotechnol* **10:**3986–3995 (2011).
21. Vigeolas H, Waldeck P, Zank T and Geigenberger P, Increasing seed oil content in oil-seed rape (*Brassica napus L.*) by over-expression of a yeast glycerol-3-phosphate dehydrogenase under the control of a seed-specific promoter. *Plant Biotechnol J* **5:**431–441 (2007).
22. Jain RK, Coffey M, Lai K, Kumar A and MacKenzie SL, Enhancement of seed oil content by expression of glycerol-3-phosphate acyltransferase genes. *Biochem Soc Trans* **28:**958–961 (2000).
23. Zou J, Katavic V, Giblin EM, Barton DL, MacKenzie SL, Keller WA *et al*. Modification of seed oil content and acyl composition in the Brassicaceae by expression of a yeast *sn*-2 acyltransferase gene. *Plant Cell* **9:**909–923 (1997).
24. Taylor DC, Katavic V, Zou J, Mackenzie SL, Keller WA, An J *et al*. Field testing of transgenic rapeseed cv. Hero transformed with a yeast *sn*-2 acyltransferase results in increased oil content, erucic acid content and seed yield. *Mol Breed* **8:**317–322 (2002).
25. Yoon JM, Zhao L and Shanks JV, Metabolic engineering with plants for a sustainable biobased economy. *Annu Rev Chem Biomol Eng* **4:**211–237 (2013).
26. Weselake RJ, Shah S, Tang M, Quant PA, Snyder CL, Furukawa-Stoffer TL *et al*. Metabolic control analysis is helpful for informed genetic manipulation of oilseed rape (*Brassica napus*) to increase seed oil content. *J. Exp Bot* **59:**3543–3549 (2008).
27. Zheng P, Allen WB, Roesler K, Williams ME, Zhang S, Li J *et al*. A phenylalanine in DGAT is a key determinant of oil content and composition in maize. *Nat Genet* **40:**367–372 (2008).
28. Lardizabal K, Effertz R, Levering C, Mai J, Pedroso MC, Jury T *et al*. Expression of *Umbelopsis ramanniana* DGAT2A in seed increases oil in soybean. *Plant Physiol* **148:**89–96 (2008).
29. Zhang JR, Martin JM, Beecher B, Lu CF, Hannah LC, Wall ML *et al*. The ectopic expression of the wheat Puroindoline genes increase germ size and seed oil content in transgenic corn. *Plant Mol. Biol* **74:**353–365 (2010).
30. Barthole G, Lepiniec L, Rogowsky PM and Baud S, Controlling lipid accumulation in cereal grains. *Plant Sci* **185:**33–39 (2012).
31. Harwood JL and Guschina IA, Regulation of lipid synthesis in oil crops. *FEBS Lett* **587:**2079–2081 (2013).
32. Guschina IA, Everard JD, Kinney AJ, Quant PA and Harwood JL, Studies on the regulation of lipid biosynthesis in plants: application of control analysis to soybean. *Biochim Biophys Acta* **1838:**1488–1500 (2014).
33. Robert LS, Donaldson PA, Ladaique C, Altosaar I, Arnison PG and Fabijanski SF. Antisense RNA inhibition of beta-glucuronidase gene-expression in transgenic tobacco can be transiently overcome using a heat-inducible beta-glucuronidase gene construct. *Biotechnology* **8:**459–464 (1990).
34. Marillia EF, Micallef BJ, Micallef M, Weninger A, Pedersen KK, Zou J *et al*. Biochemical and physiological studies of *Arabidopsis thaliana* transgenic lines with repressed expression of the mitochondrial pyruvate dehydrogenase kinase. *J Exp Bot* **54:**259–270 (2003).
35. Oakes J, Brackenridge D, Colletti R, Daley M, Hawkins DJ, Xiong H *et al*. Expression of Fungal *diacylglycerol acyltransferase2* genes to increase kernel oil in maize. *Plant Physiol* **155:**1146–1157 (2011).
36. Matlashewski GJ, Urquhart AA, Sahasrabudhe MR and Altosaar I, Lipase activity in oat flour suspensions and soluble extracts. *Cereal Chem* **59:**418–422 (1982).
37. Baud S and Lepiniec L, Physiological and developmental regulation of seed oil production. *Prog. Lipid Res* **49:**235–249 (2010).
38. Bates PD, Stymne S and Ohlrogge J, Biochemical pathways in seed oil synthesis. *Curr Opin Plant Biol* **16:**358–364 (2013).
39. Marchive C, Nikovics K, To A, Lepiniec L and Baud S, Transcriptional regulation of fatty acid production in higher plants: Molecular bases and biotechnological outcomes. *Eur J Lipid Sci Technol* **116:**1332–1343 (2014).
40. Shen B, Allen WB, Zheng PZ, Li CJ, Glassman K, Ranch J *et al*. Expression of *ZmLEC1* and *ZmWRI1* increases seed oil production in maize. *Plant Physiol* **153:**980–987 (2010).

41. Pouvreau B, Baud S, Vernoud V, Morin V, Py C, Gendrot G *et al*. Duplicate maize *Wrinkled1* transcription factors activate target genes involved in seed oil biosynthesis. *Plant Physiol* **156:**674–686 (2011).
42. Ohlrogge J and Chapman K, The seeds of green energy: Expanding the contribution of plant oils as biofuels. *Biochemist* **33:**34–38 (2011).
43. Bourgis F, Kilaru A, Cao X, Ngando-Ebongue GF, Drira N, Ohlrogge JB *et al*. Comparative transcriptome and metabolite analysis of oil palm and date palm mesocarp that differ dramatically in carbon partitioning. *Proc Natl Acad Sci USA* **108:**12527–12532 (2011).
44. Durrett TP, Benning C and Ohlrogge J, Plant triacylglycerols as feedstocks for the production of biofuels. *Plant J* **54:** 593–607 (2008).
45. Vessey JK, Fei H, Burton DL, Bradley RL and Smith L, The bilateral influence of plant and rhizosphere characteristics in brassicas varying in seed oil productivity. *Can J Plant Sci* **94:**1113–1116 (2014).
46. Petrie JR, Vanhercke T, Shrestha P, El Tahchy A, White A, Zhou XR *et al*. Recruiting a new substrate for triacylglycerol synthesis in plants: The monoacylglycerol acyltransferase pathway. *PLoS ONE* **7**:e35214. doi:10.1371/journal.pone.0035214 (2012).
47. Mendoza MS, Dubreucq B, Miquel M, Caboche M and Lepiniec L, LEAFY COTYLEDON 2 activation is sufficient to trigger the accumulation of oil and seed specific mRNAs in *Arabidopsis* leaves. *FEBS Lett* **579:**4666–4670 (2005).
48. Andrianov V, Borisjuk N, Pogrebnyak N, Brinker A, Dixon J, Spitsin S *et al*. Tobacco as a production platform for biofuel: overexpression of *Arabidopsis DGAT* and *LEC2* genes increases accumulation and shifts the composition of lipids in green biomass. *Plant Biotechnol J* **8:**277–287 (2010).
49. Vanhercke T, El Tahchy A, Shrestha P, Zhou XR, Singh SP and Petrie JR *et al*. Synergistic effect of WRI1 and DGAT1 coexpression on triacylglycerol biosynthesis in plants. *FEBS Lett* **587:**364–369 (2013).
50. Vanhercke T, El Tahchy A, Liu Q, Zhou X-R, Shrestha P, Divi UK *et al*. Metabolic engineering of biomass for high energy density: oilseed-like triacylglycerol yields from plant leaves. *Plant Biotechnol J* **12:**231–239 (2014).
51. Napier JA, Haslam RP, Beaudoin F and Cahoon EB, Understanding and manipulating plant lipid composition: Metabolic engineering leads the way. *Curr Opin Plant Biol* **19:**68–75 (2014).
52. McKeon TA, *Industrial Oil Crops*. AOCS Press, Urbana, IL pp. 275–341 (2016).
53. ISAAA, A. biotech crop annual updates 2016 maize. [Online]. https://www.isaaa.org/resources/publications/biotech_crop_annual_update/download/biotech-crop-annual-update-maize-2016.pdf [April 16, 2017].
54. Troncoso-Ponce MA, Cao X, Yang ZL and Ohlrogge JB, Lipid turnover during senescence. *Plant Sci* **205:**13–19 (2013).
55. Yue GH, Sun F and Liu P, Status of molecular breeding for improving *Jatropha curcas* and biodiesel. *Renew Sustain Energy Rev* **26:**332–343 (2013).
56. Koizumi T, Biofuel and food security in China and Japan. *Renew Sustain Energy Rev* **21:**102–109 (2013).
57. Naik M, Meher LC, Naik SN and Das LM, Production of biodiesel from high free fatty acid Karanja (*Pongamia pinnata*) oil. *Biomass Bioenerg* **32:**354–357 (2008).
58. Jena PC, Raheman H, Prasanna Kumar GV and Machavaram R, Biodiesel production from mixture of mahua and simarouba oils with high free fatty acids. *Biomass Bioenerg* **34:**1108–1116 (2010).
59. Ramadhas AS, Jayaraj S and Muraleedharan C, Biodiesel production from high FFA rubber seed oil. *Fuel* **84:**335–340 (2005).
60. Kansedo J, Lee KT and Bhatia S, *Cerbera odollam* (sea mango) oil as a promising non-edible feedstock for biodiesel production. *Fuel* **88:**1148–1150 (2009).
61. De Oliveira D, Di Luccio M, Faccio C, Dalla Rosa C, Bender JP, Lipke N *et al*. Optimization of enzymatic production of biodiesel from castor oil in organic solvent medium. *Appl Biochem Biotechnol* **115:**771–780 (2004).
62. Georgogianni KG, Kontominas MG, Pomonis PJ, Avlonitis D and Gergis V, Alkaline conventional and in situ transesterification of cottonseed oil for the production of biodiesel. *Energy Fuel* **22:**2110–2115 (2008).
63. Ciubota-Rosie C, Ruiz JR, Ramos MJ and Perez A, Biodiesel from *Camelina sativa*: A comprehensive characterisation. *Fuel* **105:**572–577 (2013).
64. Picou L and Boldor D, Thermophysical characterization of the seeds of invasive chinese tallow tree: importance for biofuel production. *Environ Sci Technol* **46:**11435–11442 (2012).
65. Martínez G, Sánchez N, Encinar JM and González JF, Fuel properties of biodiesel from vegetable oils and oil mixtures. Influence of methyl esters distribution. *Biomass Bioenerg* **63:**22–32 (2014).
66. Fabijanski S, Lindenbaum M, Fu P and Marillia EF, Production of modified fatty acids in plants through rDNA targeted integration of heterologous genes. US Patent No. 8546645B2 (2013).
67. Leung DYC, Wu X and Leung MKH, A review on biodiesel production using catalyzed transesterification. *Appl Energy* **87:**1083–1095 (2010).
68. Krishnamurthy K and Giroux MJ, Expression of wheat puroindoline genes in transgenic rice enhances grain softness. *Nature Biotechnol* **19:**162–166 (2001).
69. Miao YJ, Chen L, Wang C, Wang YJ, Zheng Q, Gao C *et al*. Expression, purification and antimicrobial activity of puroindoline A protein and its mutants. *Amino Acids* **43:**1689–1696 (2012).
70. Zhang JR, Martin JM, Balint-Kurti P, Huang L and Giroux MJ, The wheat puroindoline genes confer fungal resistance in transgenic corn. *J. Phytopathol* **159:**188–190 (2011).
71. Greenham T and Altosaar I, Molecular strategies to engineer transgenic rice seed compartments for large-scale production of plant-made pharmaceuticals. *Methods Mol Biol* **956:** 311–326 (2013).
72. Zhang X, Wang D, Zhao S and Shen Z, A double built-in containment strategy for production of recombinant proteins in transgenic rice. *PLoS ONE* **9**: e115459 DOI: 10.1371/journal.pone.0115459 (2014).
73. He Y, Ning T, Xie T, Qiu Q, Zhang L, Sun Y *et al*. Large-scale production of functional human serum albumin from transgenic rice seeds. *Proc Natl Acad Sci USA* **108:**19078–19083 (2011).
74. Snell KD, Singh V and Brumbley SM, Production of novel biopolymers in plants: recent technological advances and future prospects. *Curr Opin Biotechnol* **32:**68–75 (2015).
75. Malik MR, Yang W, Patterson N, Tang J, Wellinghoff RL, Preuss ML *et al*. Production of high levels of poly-3-hydroxybutyrate in plastids of *Camelina sativa* seeds. *Plant Biotechnol J* **13:**675–688 (2015).

76. Baeshen NA, Baeshen MN, Sheikh A, Bora RS, Ahmed MM, Ramadan HA *et al*. Cell factories for insulin production. *Microb Cell Fact* **13:**141–149 (2014).
77. Sticklen MB, Expediting the biofuels agenda via genetic manipulations of cellulosic bioenergy crops. *Biofuels Bioprod Bioref* **3:**448–455 (2009).
78. Shahid EM and Jamal Y, Production of biodiesel: A technical review. *Renew Sustain Energy Rev* **15:**4732–4745 (2011).
79. Yang Y, Wang D, Zhang X, Fang J, Shen Z and Lin C, Transgenic rice as bioreactor for production of the *Candida antarctica* lipase B. *Plant Biotechnol J* **12:**963–970 (2014).
80. Altosaar I, Giband M, Schernthaner JP, Tanchak MA, Sardana RK and Potier B, *Strategies and Tactics for Cloning Genes, Coding for Lipase, from Higher-Plants*. Kluwer, Springer Science+Business Media, Dordrecht, pp. 373–381 (1992).
81. Qaim M and Zilberman D, Yield effects of genetically modified crops in developing countries. *Science* **299:**900–902 (2003).
82. Agarwal P, Bhatt V, Singh R, Das M, Sopory SK and Chikara J, *et al*. Pathogenesis-Related Gene, JcPR-10a from *Jatropha curcas* exhibit RNase and antifungal activity. *Mol. Biotechnol* **54:**412–425 (2013).
83. Tang MJ, Liu XF, Deng HP and Shen SH, Over-expression of *JcDREB*, a putative AP2/EREBP domain-containing transcription factor gene in woody biodiesel plant *Jatropha curcas*, enhances salt and freezing tolerance in transgenic *Arabidopsis thaliana*. *Plant Sci* **181:**623–631 (2011).
84. McKeon TA, *Industrial Oil Crops.* AOCS Press, Urbana, IL, pp. 75–112 (2016).
85. Roesler K, Shintani D, Savage L, Boddupalli S and Ohlrogge J, Targeting of the *Arabidopsis* homomeric acetyl-coenzyme A carboxylase to plastids of rapeseeds. *Plant Physiol* **113:**75–81 (1997).
86. An D and Suh MC, Overexpression of *Arabidopsis WRI*1 enhanced seed mass and storage oil content in *Camelina sativa*. *Plant Biotechnol Rep* **9:**137–148 (2015).
87. Liu J, Hua W, Zhan G, Wei F, Wang X, Liu G *et al*. Increasing seed mass and oil content in transgenic Arabidopsis by the overexpression of *wri1*-like gene from *Brassica napus*. *Plant Physiol Biochem* **48:**9–15 (2010).
88. Elahi N, Duncan RW and Stasolla C, Modification of oil and glucosinolate content in canola seeds with altered expression of *Brassica napus LEAFY COTYLEDON1*. *Plant Physiol Biochem* **100:**52–63 (2016).
89. Jain RK, Coffey M, Lai K, Kumar A and MacKenzie SL, Enhancement of seed oil content by expression of glycerol-3-phosphate acyltransferase genes. *Biochem Soc Trans* **28:**958–961 (2000).
90. Zou JT, Katavic V, Giblin EM, Barton DL, MacKenzie SL, Keller WA *et al*. Modification of seed oil content and acyl composition in the brassicaceae by expression of a yeast *sn*-2 acyltransferase gene. *Plant Cell* **9:**909–923 (1997).
91. Taylor DC, Katavic V, Zou JT, MacKenzie, SL, Keller WA, An J *et al*. Field testing of transgenic rapeseed cv. Hero transformed with a yeast *sn*-2 acyltransferase results in increased oil content, erucic acid content and seed yield. *Mol Breed* **8:**317–322 (2002).
92. Jako C, Kumar A, Wei Y, Zou J, Barton DL, Giblin EM *et al*. Seed-specific over-expression of an *Arabidopsis* cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. *Plant Physiol* **126:**861–874 (2001).
93. Savadi S, Naresh V, Kumar V and Bhat SR, Seed-specific overexpression of *Arabidopsis DGAT1* in Indian mustard (*Brassica juncea*) increases seed oil content and seed weight. *Botany* **94:**177–184 (2016).
94. El Tahchy A, Petrie JR, Shrestha P, Vanhercke T and Singh SP, Expression of mouse MGAT in *Arabidopsis* results in increased lipid accumulation in seeds. *Front Plant Sci* **6**: DOI:10.3389/fpls.2015.01180 (2015).
95. Kelly AA, Shaw E, Powers SJ, Kurup S and Eastmond PJ, Suppression of the *SUGAR-DEPENDENT1* triacylglycerol lipase family during seed development enhances oil yield in oilseed rape (*Brassica napus* L.). *Plant Biotechnol J* **11:**355–361 (2013).
96. van Erp H, Kelly AA, Menard G and Eastmond PJ, Multigene engineering of triacylglycerol metabolism boosts seed oil content in *Arabidopsis*. *Plant Physiol* **165:**30–36 (2014).
97. Liu F, Xia Y, Wu L, Fu D, Hayward A, Luo J *et al*. Enhanced seed oil content by overexpressing genes related to triacylglyceride synthesis. *Gene* **557:**163–171 (2015).
98. Grimberg A, Carlsson AS, Marttila S, Bhalerao R and Hofvander P, Transcriptional transitions in *Nicotiana benthamiana* leaves upon induction of oil synthesis by WRINKLED1 homologs from diverse species and tissues. *BMC Plant Biol* **15** DOI: 10.1186/s12870-015-0579-1 (2015).
99. Slocombe SP, Cornah J, Pinfield-Wells H, Soady K, Zhang Q, Gilday A *et al*. Oil accumulation in leaves directed by modification of fatty acid breakdown and lipid synthesis pathways. *Plant Biotechnol J* **7:**694–703 (2009).
100. Wu HY, Liu C, Li MC, Zhao MM, Gu D and Xu YN, Effects of monogalactoglycerolipid deficiency and diacylglycerol acyltransferase overexpression on oil accumulation in transgenic tobacco. *Plant Mol Biol Rep* **31:**1077–1088 (2013).
101. Sanjaya, Durrett TP, Weise SE and Benning C, Increasing the energy density of vegetative tissues by diverting carbon from starch to oil biosynthesis in transgenic *Arabidopsis*. *Plant Biotechnol J* **9:**874–883 (2011).
102. Klaus D, Ohlrogge JB, Neuhaus HE and Dormann P, Increased fatty acid production in potato by engineering of acetyl-CoA carboxylase. *Planta* **219:**389–396 (2004).
103. Vanhercke T, Petrie JR and Singh SP, Energy densification in vegetative biomass through metabolic engineering. *Biocatal Agric Biotechnol* **3:**75–80 (2014).
104. Sanjaya, Miller R, Durrett TP, Kosma DK, Lydic TA, Muthan B *et al*. Altered lipid composition and enhanced nutritional value of *Arabidopsis* leaves following introduction of an algal diacylglycerol acyltransferase 2. *Plant Cell* **25:**677–693 (2013).
105. Fan J, Yan CS, Zhang XB and Xu CC, Dual role for phospholipid: diacylglycerol acyltransferase: enhancing fatty acid synthesis and diverting fatty acids from membrane lipids to triacylglycerol in *Arabidopsis* leaves. *Plant Cell* **25:**3506–3518 (2013).
106. James CN, Horn PJ, Case CR, Gidda SK, Zhang D, Mullen RT *et al*. Disruption of the *Arabidopsis* CGI-58 homologue produces Chanarin-Dorfman-like lipid droplet accumulation in plants. *Proc Natl Acad Sci USA* **107:** 17833-17838 (2010).
107. Fan J, Yan C, Roston R, Shanklin J and Xu C, *Arabidopsis* lipins, PDAT1 ACYLTRANSFERASE, and SDP1 triacylglycerol lipase synergistically direct fatty acids toward β-oxidation, thereby maintaining membrane lipid homeostasis. *Plant Cell* **26:**4119–4134 (2014).
108. Winichayakul S, Scott RW, Roldan M, Hatier JH, Livingston S, Cookson R *et al*. In vivo packaging of triacylglycerols enhances *Arabidopsis* leaf biomass and energy density. *Plant Physiol* **162:**626–639 (2013).

109. Vanhercke T, El Tahchy A, Liu Q, Zhou XR, Shrestha P, Divi UK *et al*. Metabolic engineering of biomass for high energy density: oilseed-like triacylglycerol yields from plant leaves. *Plant Biotechnol J* **12:**231–239 (2014).
110. Kelly AA, van Erp H, Quettier AL, Shaw E, Menard G, Kurup S *et al*. The sugar-dependent1 lipase limits triacylglycerol accumulation in vegetative tissues of *Arabidopsis*. *Plant Physiol* **162:**1282–1289 (2013).
111. Weselake RJ, Taylor DC, Rahman M, Shah S, Laroche A, McVetty PBE *et al*. Increasing the flow of carbon into seed oil. *Biotechnol Adv* **27:**866–878 (2009).
112. Santos-Mendoza M, Dubreucq B, Baud S, Parcy F, Caboche M and Lepiniec L, Deciphering gene regulatory networks that control seed development and maturation in *Arabidopsis*. *Plant J* **54:**608–620 (2008).
113. Fatihi A, Boulard C, Bouyer D, Baud S, Dubreucq B and Lepiniec L, Deciphering and modifying LAFL transcriptional regulatory network in seed for improving yield and quality of storage compounds. *Plant Sci* **250:**198–204 (2016).
114. Devic M and Roscoe T, Seed maturation: Simplification of control networks in plants. *Plant Sci* **252:**335–346 (2016).
115. Fatihi A, Boulard C, Bouyer D, Baud S, Dubreucq B and Lepiniec L, Role of transcription factors in storage lipid accumulation in plants. [Online]. Available at: http://lipidlibrary.aocs.org/Biochemistry/content.cfm?ItemNumber=40316 [May 1, 2017].
116. Moore RH, Thornhill KL, Weinzierl B, Sauer D, D'Ascoli E, Kim J *et al.* Biofuel blending reduces particle emissions from aircraft engines at cruise conditions. Nature **543:**411–415 (2017).

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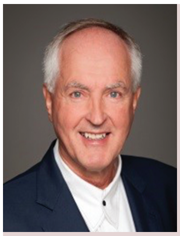

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