Enhancing Vegetative Oil Content through Optimized Lipogenic Factors

Sanket Anaokar[1]*(anaokars@bnl.gov), Yuanheng Cai[2], Yingqi Cai[1], John Shanklin[1, 2], and **Stephen P. Long[3]**

[1]Brookhaven National Laboratory, Upton, NY; [2]Stony Brook University, Stony Brook, NY; [3]University of Illinois at Urbana-Champaign, Urbana, IL

https://rogue.illinois.edu/

Project Goals

The main objective of ROGUE (Renewable Oil Generated with Ultra-productive Energycane) project is to engineer the two most productive American crops—energycane and *Miscanthus*—to produce a sustainable supply of biodiesel, biojet fuel, and bioproducts.

1. Increasing oil accumulation and targeting this to the mature stem
2. Increasing photosynthetic efficiency to power oil synthesis
3. Multi-gene construct transformation of energycane and Miscanthus
4. Field testing, processing and techno-economic analysis

Abstract

Most of the plant biomass is present in vegetative tissues and strategies to accumulate triacylglycerol (TAG, aka oil) in these tissues provides an attractive target for increasing oil yields relative to conventional oilseed crops. Generally, plants accumulate less than 0.1 % DW of oil in vegetative tissues, so, our goal is to use a multi-gene approach employing optimized components to increase oil biosynthesis and decrease oil degradation. Our strategies to enhance oil accumulation align with the proven 'push-pull-protect' model for fatty acid (FA) synthesis.

We targeted the Homomeric Acc1(HoAcc1) to the plastid to serve as a "push" factor. Accase represents the first committed step in FA synthesis. The native plastid-located heteromeric Accase is subject to multiple distinct forms of negative feedback regulation *in vivo*. Our previous transient expression results showed that targeting AtHoAcc1 to the plastids with an N-terminally fused plastid transit peptide and GFP tag can potentially circumvent this negative regulation, enhance FA synthesis, and promote TAG accumulation in tobacco leaves by increasing the malonyl-CoA supply. We stably transformed the AtHoAcc1 into Arabidopsis. Our preliminary analysis suggested that lines transformed with plastidially targeted AtHoAcc1 had larger seed size and higher seed weight without affecting the seed gemination and establishment. Moreover, they also showed higher vegetative TAG accumulation in 14-day old seedlings, demonstrating the effectiveness of using plastidial targeted HoAcc1 as a "push" factor in stably transformed plants to increase vegetative TAG accumulation.

The Pull factor used was diacylglycerol acyl transferase (DGAT), that catalyzes the final conversion of DAG to TAG in the ER. This can efficiently channel fatty acyl chains towards the synthesis of neutral lipid for storage. Overexpression of plant DGATs have been shown to increase TAG production. We identified a mammalian DGAT2 which was very efficient in producing TAG in tobacco transient overexpression assays. Tobacco leaves with DGAT2 overexpression driven by a senescence inducible promoter resulted in more than a 6-fold increase in TAG accumulation relative to controls.

The protect factor was an engineered Sesame oleosin (Ole1), that masks the oil bodies to protect the TAG from hydrolysis by lipases. Previous published research suggested that Ole1 could be degraded by ubiquitin conjugation to lysines, cysteines, serines, threonines, or the N-terminus of a target peptide. To inhibit ubiquitin ligation, we choose to replace all the lysine residues with arginine (KR) and delete the cysteine residues (CysDel) from a *Sesame* oleosin peptide to create an efficient Ole1 variant, Ole1_CysDel_KR. Transient expression of this variant in tobacco leaves indicated that CysDel_KR modifications resulted in an increase in TAG content compared to a Cys_Ole1 control. As the substitutions and deletions were applied to all lysine and cysteine residues, respectively, we predicted some of these changes could negatively impact the oil accumulation. Therefore, we performed a dropout experiment reverting each of these modifications to pinpoint those that contribute most to increased TAG accumulation. By combining the set of mutations that increased TAG accumulation we created Ole1_5 Mod. Our data from tobacco transient assays demonstrated that Ole1_5 Mod resulted in the most significant increase in TAG content. Based on these findings, we stably transformed the oleosin variants into Arabidopsis. Results from the stably transformed Arabidopsis lines are consistent with the data from transiently transformed tobacco in which Ole1_CysDel_KR and Ole1_5 Mod lines in combination with mammalian DGAT2 produced higher TAG and total FA content compared to CysOle1 with mammalian DGAT2.

In summary, our strategy was first to individually optimize the Push, Pull and Protect factors described above. We subsequently combined the optimized factors to test their ability to promote plant vegetative TAG accumulation. Our results show that the effects of each optimized factor are additive. When the three factors are transiently expressed in *N. benthamiana* leaves, TAG accumulation increased more than 18-fold relative to controls, reaching a final TAG content of ~4% (w/w) over a three-day period. The results from transient *N. benthamiana* expression were reproduced in stably transgenic Arabidopsis lines.

Funding Statement

The work of Sanket Anaokar, Yuanheng Cai and John Shanklin on Oleosin and ACCase was supported by the Office of Biological and Environmental Research (Award Number DE-SC0018254) and FWP 2021-BNL-BI-173. The work of Yingqi Cai and John Shanklin on the mammalian DGAT was supported by the Center for Advanced Bioenergy and Bioproducts Innovation (Award Number DE-SC0018420).