Investigation of the contribution of oil biosynthetic enzymes to seed oil content in *Brassica napus* and *Arabidopsis thaliana*

Vesna Katavic, Lin Shi, Yuanyuan Yu, Lifang Zhao, George W. Haughn, and Ljerka Kunst[1]

Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4.
Received 17 May 2013, accepted 27 January 2014. Published on the web 5 February 2014.

Katavic, V., Shi, L., Yu, Y., Zhao, L., Haughn, G. W. and Kunst, L. 2014. **Investigation of the contribution of oil biosynthetic enzymes to seed oil content in *Brassica napus* and *Arabidopsis thaliana***. Can. J. Plant Sci. **94**: 1109–1112. One of the critical reactions in triacylglycerol (TAG) biosynthesis is activation of fatty acyl chains to fatty acyl CoAs, catalyzed by long-chain acyl CoA synthetases (LACS). In *Arabidopsis thaliana* there is a family of nine genes that encode LACSs. Studies to determine whether the products of two of these genes, LACS8 and LACS9, function together to contribute acyl-CoAs for storage oil biosynthesis in *A. thaliana* resulted in discovery that it is not LACS8 but LACS1 that functionally overlaps with LACS9 in TAG biosynthesis (published in Plant Journal). To elucidate regulatory mechanisms of seed oil synthesis, the potential roles of phospholipase D zeta (PLDZ) and rhamnose synthase 2 (RHM2/MUM4) in transcription factor GLABRA2 (GL2)-mediated regulation of seed oil biosynthesis and deposition were investigated. Results demonstrated that *PLDZ* genes are not involved in GL2-mediated seed oil accumulation and that GL2 regulates seed oil production, at least in part, through its influence on expression of the gene *RHM2/MUM4* required for the seed coat mucilage biosynthesis (published in Plant Journal). A novel *Arabidopsis* mutant with speckled seed coat and reduced seed oil phenotypes resulting from a mutation in a single unknown gene was identified, but attempts to isolate the gene by positional cloning have not been successful to date (unpublished results). Finally, seed oil content in near-isogenic double haploid *Brassica napus* lines was analyzed, ''low oil'' and ''high oil'' lines were identified, and developing seeds for expression profiling of target seed oil biosynthesis/bioassembly genes in selected double haploid lines were collected (unpublished results).

Key words: Seed oil, mucilage, long-chain acyl CoA synthetases, GL2, *mucilage modified 4*, phospholipase D zeta

Katavic, V., Shi, L., Yu, Y., Zhao, L., Haughn, G. W. et Kunst, L. 2014. **Étude sur l'apport des enzymes de la biosynthèse de l'huile à la teneur en huile des graines de *Brassica napus* et d'*Arabidopsis thaliana***. Can. J. Plant Sci. **94**: 1109–1112. Une des réactions cruciales dans la synthèse du triacylglycérol (TAG) est l'activation des chaînes d'acyles gras en acyl-CoA gras, que catalysent les acyl-CoA synthétases à chaîne longue (LACS). Chez *Arabidopsis thaliana*, une famille de neuf gènes code les LACS. Les études visant à établir si les produits de deux de ces gènes, LACS8 et LACS9, fonctionnent en tandem pour former les acyl-CoA servant à synthétiser l'huile de stockage chez *Arabidopsis thaliana* ont révélé que ce n'est pas le gène LACS8 mais bien le gène LACS1 qui intervient avec LACS9 dans la biosynthèse du TAG (article publié dans *Plant Journal*). Pour élucider les mécanismes qui régulent la synthèse de l'huile dans la graine, les auteurs se sont penchés sur le rôle éventuel de la phospholipase D zêta (PLDZ) et de la rhamnose synthase 2 (RHM2/MUM4) dans la régulation de la synthèse et du dépôt de l'huile dans la graine assistés par le facteur de transcription GLABRA2 (GL2). Les résultats de ces travaux montrent que les gènes *PLDZ* ne participent pas à l'accumulation d'huile assistée par GL2 et que le facteur GL2 régule en partie la production d'huile en influant sur l'expression du gène *RHM2/MUM4*, sans lequel il ne pourrait y avoir synthèse du mucilage des téguments de la graine (article publié dans *Plant Journal*). Les auteurs ont identifié un nouveau mutant d'*Arabidopsis* dont les graines présentent des téguments mouchetés et renferment moins d'huile, à la suite d'une mutation d'un gène inconnu, mais les tentatives pour isoler celui-ci par clonage positionnel ont toutes échoué jusqu'à présent (résultats non publiés). Enfin, les auteurs ont analysé la teneur en huile des graines de lignées à double haploïdie (DH) presque isogéniques de *Brassica napus* et identifié des lignées « pauvres » et « riches » en huile, puis recueilli des graines en développement, en vue du profilage de l'expression des gènes qui participent à la biosynthèse et à l'assemblage de l'huile dans les graines de quelques lignées DH (résultats non publiés).

Mots clés: Teneur en huile des graines, mucilage, acyl-CoA synthétases à chaîne longue, GL2, *mucilage modified 4*, phospholipase D zêta

[1]*Corresponding author (e-mail: kunst@mail.ubc.ca).*

Abbreviations: **GL2**, GLABRA2; **LACS**, long-chain acyl CoA synthetase; **PLDZ**, phospholipase D zeta; **RHM2**, rhamnose synthase 2; **TAG**, triacylglycerol

Efficient storage of carbon in seeds is crucial to plant fitness and to agricultural productivity. In oilseed plants, seed oils are major reserve material, and they also represent the largest resource of renewable reduced carbon chains available from nature (Schwender et al. 2004). To humans, seed oils are of great value in many food and non-food applications, including biodiesel, as an important source of renewable alternative energy. Because of growing demand and limited supply, genetic engineering has frequently been used in attempts to increase oil content in oilseed crops above levels normally present in the seed. Most of these attempts have been unsuccessful, due to incomplete understanding of lipid metabolic pathways and their regulation (Cahoon and Schmid 2008).

OBJECTIVES AND RELEVANCE TO GREENHOUSE GAS MANAGEMENT

The aim of our Green Crop Network research project was to utilize new molecular genetic tools available in *Arabidopsis thaliana* (complete sequence of *A. thaliana* genome, collection of T-DNA insertion mutant lines, collection of cDNA clones and microarray expression data) to identify the rate-limiting steps in lipid metabolic pathways, the factors that control carbon partitioning in the seed, and potential regulators of seed oil accumulation. Specifically, our objectives were: (1) to determine the physiological roles of long-chain fatty acid acyl-CoA synthases (LACS) in *A. thaliana* seed oil metabolism, (2) to investigate the role of the phospholipase D zeta (PLDZ) enzyme in *A. thaliana s*eed oil production, (3) to examine the mechanisms of regulation of seed oil accumulation by the GL2 transcription factor in *A. thaliana*, (4) to characterize the new *speckled testa* (*spt*) oil deficient mutant of *A. thaliana* and isolate the *SPT* gene by positional cloning, and (5) to analyze seed oil content in isogenic *Brassica napus* lines to identify those with low and high seed oil phenotypes in collaboration with Dr. McVetty (University of Manitoba).

The information obtained from this work is essential for successful genetic manipulation of the target *Brassica* crops to maximize carbon flow into storage lipid biosynthesis and enhance seed oil yield. Understanding limitations to carbon flow towards oil biosynthesis during seed development will allow us to increase overall oil yield in oilseeds for both food and fuel applications and result in reduced oil cost.

Consequently, this will result in greater use of renewable and biodegradable vegetable oil derived biodiesel (Weselake et al. 2009) and cause a significant reduction in greenhouse gas emission. According to Natural Resources Canada/ Office of Energy Efficiency (greenhouse gas emission derived from representative Canadian life-cycle assessment of agricultural based biofuels using Genius analyses), blended biodiesel fuel (a 20% vegetable oil/80% petrodiesel, blend offered at most biodiesel fueling stations) reduces carbon dioxide emissions by 13–14%.

GENERAL FINDINGS AND RELATIONSHIP WITH OTHER PUBLISHED WORKS

Long-chain Acyl CoA Synthetases Studies

De novo fatty acid synthesis in plants occurs in plastids. Fatty acids are activated to CoA thioesters during export from the plastid, and are used for stepwise acylation of glycerol-3-phosphate in the endoplasmic reticulum to generate molecules of oil [triacylglycerol (TAG)] (Kennedy 1961; Stymne and Stobart 1987). Given that acyl-CoA thioesters are key metabolites required for oil biosynthesis, and that activation of free fatty acids to acyl-CoAs is catalyzed by LACS, one of our research objectives was to identify LACS enzymes involved in TAG-related acyl-CoA formation, and assess their contribution to TAG accumulation in the seed. In *A. thaliana*, LACS enzymes are encoded by a family of nine genes (Shockey et al. 2002). Previous research revealed that LACS9 and one or more additional LACS isoforms are likely involved in the production of acyl-CoA for storage TAG biosynthesis (Schnurr et al. 2002). Based on its high sequence similarity to LACS9, and preliminary results from in vitro chloroplast assays, LACS8 was predicted to be the most likely candidate for this role. To test this hypothesis, and determine whether LACS8 and LACS9 jointly contribute acyl-CoAs for seed oil synthesis, we investigated the subcellular localization of the LACS8 protein, the LACS8 transcript level in the seed, and the TAG content in the *lacs8* single mutant and *lacs8 lacs9* double mutant. Unexpectedly, we found that it is not LACS8 but LACS1, which is also known to be the major isoform involved in cuticular lipid formation, that functionally overlaps with LACS9 in TAG biosynthesis (Zhao et al. 2010).

PLDZ and GL2 studies

The transcription factor GL2 has been shown to regulate seed oil accumulation, since a loss-of-function mutation in the *GL2* gene results in greater than wild type seed oil content (Shen et al. 2006). In addition, in vitro studies showed that GL2 can bind to the promoter of phospholipase D zeta1 (*PLDZ1*), suggesting that GL2 is a negative regulator of *PLDZ1* (Ohashi et al. 2003). Since PLDZ activity could contribute to seed oil synthesis, we tested the hypothesis that *PLDZ* is a target of GL2 in seeds by examining the seed expression of *PLDZ* genes in wild-type and the *gl2* mutant. To determine if PLDZ is involved in oil biosynthesis, we analyzed the seed oil content of *pldz* mutants. Collectively, our results demonstrated that the *PLDZ* genes are indeed targets of GL2 in seeds in the late stage of development, but that *PLDZ* genes are not involved in GL2-mediated seed oil accumulation.

Another target of GL2 in the seed coat is the gene *MUCILAGE MODIFIED 4* (*MUM4*) encoding a rhamnose synthase required for seed mucilage biosynthesis. We have shown that *mum4* mutants, just like *gl2* mutants, have an increased seed oil content in comparison with wild-type and that this effect is mediated through the seed coat tissue. These results indicate that GL2 regulates seed oil production, at least in part, through its influence on the expression of *RHM2*/*MUM4* in the seed coat. Thus, our results revealed a novel regulatory mechanism of carbon partitioning that occurs between the maternal seed coat and the filial embryo that can be exploited to increase the seed oil content in oil crops by blocking conversion of glucose to rhamnose in the seed coat (Shi et al. 2012).

Characterization of the *spt* Mutant and Positional Cloning of the SPT Gene

Our reverse genetic studies involved analyses of a collection of *A. thaliana* mutant lines with T-DNA insertions in genes with potential roles in carbon partitioning, lipid biosynthesis/deposition or regulation of lipid metabolism during seed development. Unexpectedly, while genotyping T-DNA lines with insertions in the *LACS4* gene, we identified a novel mutant with a "speckled" seed coat (testa) phenotype (*spt*), which exhibited a 30% reduction in its seed oil content. Co-segregation analysis demonstrated that both phenotypes were due to mutation in a single gene. Surprisingly, analyses of the seed coat phenotype in F_3 progeny homozygous for the T-DNA insert in the *LACS4* gene, as well as analyses of the seed oil content in the F_3 generation of reciprocal crosses between the *spt* mutant and wild type revealed that T-DNA insertion in the *LACS4* gene was not the cause of the seed coat and seed oil phenotypes. The *spt* mutant phenotype was due to mutation in an unknown (*SPT*) gene.

Microscopic analyses of seed coat outer integument, as well as staining with vanillin indicated that the speckled testa phenotype was a result of the reduced amount of tanins derived from proanthocyanidins in the pigmented layer of the seed coat (PA). These qualitative findings were confirmed by quantitative analyses of soluble and insoluble PA content, which was shown to be reduced by more than 50% in *spt* seed in comparison to wild type.

Positional cloning of the *SPT* gene was initiated by determining the location of the *spt* mutation on the upper arm of chromosome V. Fine mapping of the *spt* mutation to accurately pinpoint its location is in progress and should allow us to isolate the *SPT* gene in the near future. Subsequent molecular characterization and over-expression in a seed specific manner in *Arabidopsis* will then be carried out to evaluate the utility of this gene for increasing oil content in the target *B. napus* oilseed crop (Katavic et al., unpublished results).

Analyses of Seed Oil Content in Double Haploid *B. napus* Lines

Eighty-eight near-isogenic double haploid *B. napus* lines received from Dr. McVetty (University of Manitoba) were analyzed for seed oil content. The oil content was determined to be in the range 23–30% (wt/wt) for low oil lines and 42–44% (wt/wt) for high oil lines. The next generation of 20 selected near-isogenic double haploid *B. napus* lines was grown under controlled greenhouse conditions and developing seeds at the peak of oil accumulation were collected for 10 lines with low oil content, and 10 lines with high oil content. The collected developing seeds will be used for expression profiling of key genes involved in fatty acid biosynthesis and bioassembly/accumulation of seed oil to determine which genes are up-regulated in the "high oil" lines (Katavic et al., unpublished results).

CONCLUSIONS REGARDING RELEVANCE OF FINDINGS TO SHORT-TERM AND LONG-TERM GHG MANAGEMENT

Due to insights gained from our Green Crop Network project we moved a step closer to determining rate-limiting factors and regulation mechanisms governing plant seed oil metabolism. Cumulative knowledge of these complex mechanisms will eventually lead to significant increases in oil content in target oilseed crops, and contribute to a reduction in biodiesel price. In the long-term this will lead to higher biodiesel consumption and a concomitant drop in greenhouse gas emissions.

Cahoon, E. B. and Schmid, K. M. 2008. Metabolic engineering of the content and fatty acid composition of vegetable oils. Pages 161–200 *in* H. J. Bohnert, H. Nguyen, and N. G. Lewis, eds. Advances in plant biochemistry and molecular biology. Volume 1. Elsevier Ltd., Amsterdam, the Netherlands.

Kennedy, E. P. 1961. Biosynthesis of complex lipids. Fed. Proc. **20**: 934–940.

Ohashi, Y., Oka, A., Rodrigues-Pousada, R., Possenti, M., Ruberti, I., Morelli, G. and Aoyama, T. 2003. Modulation of phospholipid signaling by GLABRA2 in root-hair pattern formation. Science **300**: 1427–1430.

Schnurr, J. A., Shockey, J. M., de Boer, G.-J. and Browse, J. 2002. Fatty acid export from the chloroplast. Molecular characterization of a major plastidial acyl-coenzyme A synthetase from Arabidopsis. Plant Physiol. **129**: 1700–1709.

Schwender, J., Goffman, F., Ohlrogge, J. B. and Shachar-Hill, Y. 2004. Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds. Nature **432**: 779–782.

Shen, B., Sinkevicius, K. W., Selinger, D. A. and Tarczynski, M. C. 2006. The homeobox gene *GLABRA2* affects seed oil content in Arabidopsis. Plant Mol. Biol. **60**: 377–387.

Shi, L., Katavic, V., Yu, Y., Kunst, L. and Haughn, G. 2012. Arabidopsis *glabra2* mutant seeds deficient in mucilage biosynthesis produce more oil. Plant J. **69**: 37–46.

Shockey, J. M., Fulda, M. S. and Browse, J. A. 2002. Arabidopsis contains nine long-chain acyl-coenzyme A synthetase genes that participate in fatty acid and glycerolipid metabolism. Plant Physiol. **129**: 1710–1722.

Stymne, S. and Stobart, A. K. 1987. Triacylglycerol biosynthesis. Pages 175–214 *in* P. K. Stumpf, ed. The biochemistry of plants: A comprehensive treatise. Volume 9. Academic Press, Orlando, FL.

Weselake, R. J., Taylor, D. C., Shah, S., Laroche, A. and Harwood, J. H. 2009. Molecular strategies for increasing seed oil content. Pages 3–19 *in* C. H. Hou and J.-F. Shaw, eds. Biocatalysis and agricultural biotechnology. CRC Press, Boca Raton, FL.

Zhao, L., Katavic, V., Li, F., Haughn, G. W. and Kunst, L. 2010. Insertional mutant analysis reveals that long-chain acyl-CoA synthetase 1 (LACS1), but not LACS8, functionally overlaps with LACS9 in Arabidopsis seed oil biosynthesis. Plant J. **64**: 1048–1058.