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REVIEW – DOSSIER OPEN ACCESS

NEW PERSPECTIVES OF EUROPEAN OLEOCHEMISTRY
LES NOUVELLES PERSPECTIVES DE L'OLÉOCHIMIE EUROPÉENNE

DOSSIER

Camelina, a Swiss knife for plant lipid biotechnology

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Abstract – Camelina has emerged in the last decade as a multipurpose crop plant particularly suitable for engineering new lipids for diverse uses, including feed, biofuel and green chemistry. The rebirth of this ancient crop was based on several intrinsic favorable characteristics: robust agronomic qualities, attractive oil profile, genetic proximity with the model plant arabidopsis, ease of genetic transformation by floral dip. The need to increase both the production and diversity of plant oils, while improving the sustainability of agricultural systems, has been the driving forces behind the ever-increasing investment in camelina research. Worldwide interest in engineering camelina has led to the development of a remarkable pipeline that allows the rapid production and phenotyping of new lines; it includes specific tools, such as databases, collections of natural accessions, methods of genetic transformation and lipid analysis. Implementation of numerous metabolic pathways in camelina for the production of novel lipids has highlighted the potential as well as the versatility of this new "old" oilseed crop that is well on the way to becoming an ideal plant chassis for lipid synthetic biology.

Keywords: Lipid metabolism / oilseed / omega-3 / biofuels

Résumé – La cameline, véritable couteau-suisse pour la biotechnologie végétale des lipides. La caméline a émergé durant la dernière décennie comme une espèce agronomique polyvalente particulièrement adaptée à l'ingénierie des lipides pour divers usages comme les biocarburants, l'alimentation ou la chimie verte. La renaissance de cette espèce cultivée ancienne est fondée sur plusieurs caractéristiques : robustesse agronomique, profil en huile attractif, proximité génétique avec la plante modèle arabidopsis et très grande facilité de transformation génétique par infiltration des fleurs. Le besoin d'augmenter la production d'huile végétale tout en diversifiant la nature des lipides produits, associé à l'amélioration de la durabilité des systèmes de production, ont été les déterminants qui ont poussé le développement de la recherche autour de la caméline. L'intérêt de par le monde pour l'ingénierie des lipides chez la caméline a ainsi permis la mise en place d'un ensemble d'outils incluant des bases de données, des collections d'accessions naturelles, du matériel spécifique pour la transformation et le phénotypage, ainsi que des méthodes pour la production et la caractérisation de nouvelles variétés ou de lignées. Les nombreux exemples de mise en place de nouvelles voies métaboliques chez la caméline pour la production de lipides spécifiques illustrent le potentiel important de cette nouvelle « ancienne » espèce oleogineuse qui est en passe de devenir un chassis végétal idéal pour la biologie synthétique des lipides.

Mots clés : Métabolisme lipidique / oléagineux / oméga-3 / biocarburants

1 Introduction

Over the past decade, worldwide production and distribution of plant oilseeds and their products have undergone remarkable expansion; the area devoted to growing oilseeds has expanded by 19%, while production has increased by 34% since 2005 (source USDA, 2016). The plant oil market is driven by the demand for higher yield and more sustainable production of the main crops (palm, soybean, rapeseed and sunflower), but also by the need for increased crop diversification. This increased demand will require oilseed crops to be adapted to more diversified markets, in particular to provide sources of novel feedstock sources for the petroleum-based chemical industry. However, traditional oilseed crops suffer from several disadvantages that have limited their use in diversifying oil production. Each oilseed crop has relatively low genetic diversity, and the length of plant growth cycles impedes the potential to create new varieties by conventional breeding. Furthermore, for many oilseed crops, genetic engineering is a

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difficult and lengthy process. Finally, potential problems must be faced concerning how large-scale cultivation of oilseed crops for industrial purposes, such as the production of novel lipids, will coexist with the continued cultivation of the same oilseed crop for human consumption. The last decade has seen the emergence of camelina (*Camelina sativa* (L.) Crantz) as an alternative species for diversifying oilseed production. Combining camelina's very attractive agronomic traits with its unprecedented ease for genetic engineering, makes it an ideal plant chassis for biotechnology applications, in particular synthetic biology strategies (Napier *et al.*, 2014; Vollmann and Eynck, 2015; Bansal and Durrett, 2016; Haslam *et al.*, 2016). Also, since it remains a very minor crop in terms of human oil consumption, organizing co-existence should be easier than with the major oilseed food crops, such as rapeseed, soybean, or sunflower.

2 Camelina, the rebirth of an old crop

Camelina, also known as false flax, is an oilseed crop in the *Brassicaceae* family, and is closely related to well known wild species, including the intensively studied model species *Arabidopsis thaliana* and the widespread weed *Capsella bursa-pastoris*, shepherd's purse (Al-Shehbaz *et al.*, 2006). Under favorable conditions, camelina crops yield >2 t/ha, but lower yields (1.2 to 2.2 t/ha) are observed under conditions of limiting nutrients or water (Crowley and Frohlich, 1998; Gehringer *et al.*, 2006). The history of camelina as a crop is quite unusual, with an ancient history interrupted by a lengthy period of neglect, followed by a renaissance of interest over the past decade. Camelina is thought to have been first domesticated in the steppes of southeastern Europe or southwestern Asia, and the earliest archeological traces of camelina cultivation date to the Neolithic (Toulemonde, 2010). Within the presumed region of domestication, Ukraine and adjacent parts of Russia are still a major center of camelina genetic diversity (Ghamkhar *et al.*, 2010). Over the millennia following its domestication, camelina was widely grown in northern Europe, but starting at the end of the 19th century it was gradually replaced by higher yielding crops such as rapeseed. Nonetheless, during the 20th century, camelina continued to be cultivated on a small scale, essentially for production of oil for human consumption. Because of this century of neglect, camelina has undergone relatively little improvement by plant breeders, and thus the currently grown cultivars can be considered to be quite primitive, and should benefit greatly from the combined efforts of plant breeding and advanced techniques of modern biotechnology as described below.

Over the past decade, there has been a remarkable increase in scientific interest in camelina, as shown by the increase in the numbers of publications with "camelina" in the title (Fig. 1), but this increase was driven by several quite different potential end uses. The high levels of omega-3 lipids in camelina oil are perceived as beneficial for human health (Zubr, 1997; Eidhin *et al.*, 2003; Abramovič and Abram, 2005) and the high levels of tocopherols, including vitamin E, make camelina oil more stable to oxidation than other high omega-3 oils such as linseed oil (Abramovič *et al.*, 2007; Hrastar *et al.*, 2009). A further attractive feature is that the residual

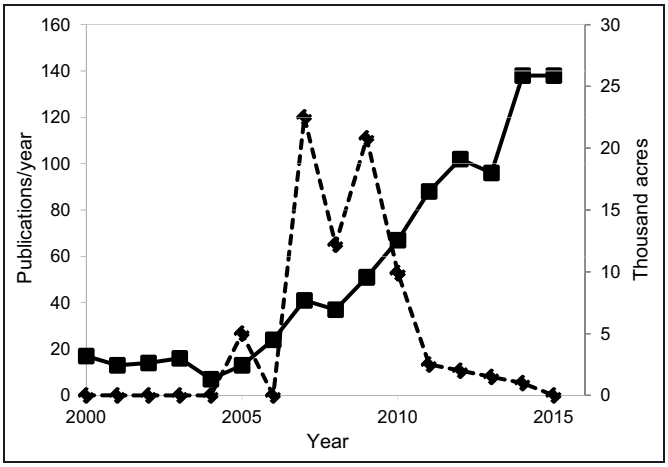

Fig. 1. Publications on camelina and acreage of camelina planted in Montana. Number of publications with camelina in the title referenced in Google Scholar (plain line) and the acreage of camelina grown in Montana (dashed line).

meal after pressing can be used for animal feed (Peiretti and Meineri, 2007; Pilgeram *et al.*, 2007; Aziza *et al.*, 2010; Bell *et al.*, 2010). In addition to current food uses, there are several micro-niches for camelina oil in cosmetics, soaps, lubricants, etc. (Bonjean and Le Goffic, 1999; Pilgeram *et al.*, 2007). Nevertheless, the intense recent activity in the USA and Canada regarding camelina has been driven primarily by camelina oil's potential as a low input source of biofuel (Fröhlich and Rice, 2005), with a particularly favorable greenhouse-gas life-cycle assessment (Shonnard *et al.*, 2010; Chen *et al.*, 2015; Keshavarz-Afshar *et al.*, 2015). As shown in Figure 1, this led to a sharp increase in the areas planted in camelina from 2005 to 2007 in Montana, but this was followed by a decline to levels even below the 2005 value, due to unexpectedly poor yields and the inability to compete with petroleum-based fuels (Mclaren and Sun, 2015). The current situation of low petroleum prices suggests that growing camelina for biofuel is unlikely to be economically viable in the near future, but that other uses, and particularly redesigning camelina oils to produce a variety of products including novel industrial feedstocks is a more realistic objective, and this will be the primary focus of this review.

3 Camelina is more than its oil

The increased interest in camelina oil occurred in parallel with increased interest in the crop because of its fundamental agronomic characteristics. Often cited as well adapted to growing on marginal soils, in fact camelina is remarkable adapted to a wide range of temperate climatic conditions, growing well in the semi-arid regions of western North America (Guy *et al.*, 2014) and also in the distinctly humid environment of Ireland (Crowley and Frölich, 1998). It has been described as a low-input crop, requiring little or no fertilization, and since it appears at present to be resistant to many pests and pathogens that affect other Brassicaceae neither insecticides nor fungicides are routinely used on camelina

(Seguin-Swartz *et al.*; Canadian Food Inspection Agency (CFIA), 2014; Vollmann and Eynck, 2015). Furthermore, the camelina life cycle is quite short; if planted in the spring, it can be harvested approximately three months later. Although spring-sown camelina is more widely grown, cultivars adapted to sowing in the fall to be harvested in the spring have also been developed (Bonjean and Le Goffic, 1999). Camelina's short lifecycle opens particularly interesting possibilities for double cropping. For instance, in the northern US corn belt, winter camelina harvested in the spring can be followed immediately by soybean (Gesch *et al.*, 2014), and winter wheat can be followed by spring camelina in the more arid Northern Great Plains. For northern Europe a fall-sown cereal crop could be followed by camelina sown in the spring (Groeneveld and Klein, 2014). These potential double-cropping systems are of great economic interest, but should also have a positive impact on protecting soils from erosion and increasing crop diversity. These strategies could be greatly facilitated if the camelina life cycle could be further shortened, although it could be of concern if life cycle shortening led to unacceptable loss of yield.

Although, as mentioned above, camelina cultivars have not benefitted from intensive plant breeding efforts, there is good evidence for potentially useful genetic variation in the camelina gene pool for important characters, such as plant height, flowering time, seed size, and seed oil composition (Vollmann *et al.*, 2005). Two collections in Germany and Canada are available with several hundred accessions (http://gbis.ipk-gatersleben.de/, http://pgrc3.agr.gc.ca/index_e.html). More recently, genetic maps based on RAPDs, SSRs AFLPs and SNPs have been created (Vollmann *et al.*, 2005; Gehringer *et al.*, 2006; Manca *et al.*, 2013; Singh *et al.*, 2015), and various transcriptome sequencing results have also been reported (Liang *et al.*, 2013; Nguyen *et al.*, 2013; Mudalkar *et al.*, 2014; Singh *et al.*, 2015). These efforts, as well as the draft complete genome sequence will be of great use in breeding improved camelina cultivars (Kagale *et al.*, 2014).

There had been previous indications of the hexaploid nature of the camelina genome from studies of genes encoding key steps in lipid biosynthesis (Hutcheon *et al.*, 2010), but this was fully demonstrated from the draft complete genome sequence (Kagale *et al.*, 2014). In essence, the camelina genome is composed of three equivalents of the genome of its close relative *Arabidopsis thaliana*. Two of the camelina sub-genomes are extremely similar to each other, and may be derived from an event of autopolyploidy, which then would have been followed by addition of the third, slightly more divergent genome. Sub-genome-specific transcriptomic studies showed a remarkably low degree of gene loss and gene functional differentiation among the three sub-genomes, and the homeologues of all three sub-genomes were most often expressed, with only a slight advantage in expression level for the last-added sub-genome (Kagale *et al.*, 2014). These features could make it difficult to create recessive mutants of interest, since in most cases all three homeologues would need to be mutated. The availability of new genome editing technologies like CRISPR/Cas9 should, however, eliminate these constraints, since CRISPR/Cas9 mutants have already obtained in polyploid species like wheat (Wang *et al.*, 2014).

4 Camelina: a model crop for genetic engineering

Since it was shown that camelina can be genetically transformed with ease by floral dip, using protocols similar to those used for arabidopsis (Lu and Kang, 2008), camelina has become an essential proving ground for seed oil modification (Fig. 2). A remarkably simple pipeline for testing oil modification strategies includes the following steps: (1) gene discovery; (2) construction of transgenes in a vector with a fluorescent protein marker gene (DsRed or GFP); (3) transformation by floral dip or vacuum infiltration; (4) screening T1 seeds for DsRed or GFP fluorescence, screening for changes in lipid profile and/or yield in T1 or T2 seeds, using a newly developed micropress. The overall process from transgene conception to preliminary screening of seed lipids in T2 seeds can be carried out in less than a year. Final validation of the introduced trait must, however, be carried out in the field.

An important issue in the choice of camelina as a model crop for genetic engineering is its ability to cross with wild relatives. Some understanding of the potential for gene flow from genetically modified (GM) crops to wild relatives is necessary for authorization for GM crop field releases, and the ability to use wild relatives as a source of potentially valuable genes is obviously of great interest for classic plant breeding. For both reasons, the resurgence of interest in camelina has included re-examination of its ability to cross with wild relatives. Among the wild *Camelina* species, only *C. microcarpa* and *C. alyssum* are widespread, and with both, fertile hybrids with cultivated camelina can be obtained (Séguin-Swartz *et al.*, 2013). These two species thus represent possible sources of genes of interest for future improvement of cultivated camelina. Since both are only occasionally observed in agricultural contexts, preventing pollen-mediated gene flow from GM camelina to *C. microcarpa* and *C. alyssum* in field trials should be easy to assure, as described by the Canadian Food Inspection Agency (CFIA 2012). In contrast to the relative rarity of the wild *Camelina* species, both *Arabidopsis thaliana* and *Capsella bursa-pastoris* are extremely abundant in agricultural environments, and occur in the margins of camelina fields (Tepfer, unpublished). Although both species flower primarily much earlier than camelina, they continue to flower throughout the summer, and are visited by the same potential pollinators (honeybees, bumblebees, syrphid flies (Groeneveld and Klein, 2014). In order to evaluate the potential for gene flow from camelina to these two wild relatives, crosses were made in the greenhouse (Julié-Galau *et al.*, 2014; Martin *et al.*, 2015). No F1 progeny seeds could be obtained with *Arabidopsis*, and with *Capsella*, very few F1 seeds were obtained, and the resulting F1 plants proved to be entirely male- and female-sterile (Julié-Galau *et al.*, 2014; Martin *et al.*, 2015). These results suggest that the likelihood of introgression of camelina transgenes in populations of *Arabidopsis thaliana* and *Capsella bursa-pastoris* is extremely low indeed.

5 Improving Camelina oil yield

Although, as described above, camelina has many attractive agronomic features, its relatively low oil yield compared

Fig. 2. Camelina pipeline for modification of lipid composition. (A) Genetic transformation can be carried out by floral dip, but as shown here is more efficient using vacuum infiltration of flowers. (B) Fluorescent protein markers, such as GFP and DsRed shown here, allow simple screening of transformants among seeds produced by infiltrated plants. (C) A micropress for extracting oil from small seed samples has been developed for preliminary screening of transformed lines. (D) A sample of camelina seeds and the oil extracted using the micropress. (E) Kilogram quantities of seed can be produced in the greenhouse. (F) For larger amounts of seed and for assessment of agronomic traits, field trials are necessary.

to rapeseed is a real limitation to its agroindustrial use. Improving camelina oil yield is thus a priority for development of the crop for any large-scale uses.

One strategy for increasing yield would be to improve the efficiency of photosynthetic carbon fixation. CO_2 fixation by Rubisco is limited by its oxygenase activity, initiating a photorespiration cycle leading to glycolate synthesis. Dalal *et al.* (2015) showed that expression in camelina of three *E. coli* enzymes that constitute a photorespiratory bypass led to a marked increase in vegetative biomass and and also increased seed yield by 57 to 73%. Although oil yield per seed was not changed, expression of the photorespiratory bypass should be reflected in important gains in yield/ha in the field (Dalal *et al.*, 2015). Similarly, the expression of arabidopsis purple acid phosphatase AtPAP2 modified carbon assimilation and distribution from photosynthetsis and led to higher seed yields (Zhang *et al.*, 2012). Increased photosynthesis efficiency associated with an increased seed mass and oil yield per plant was also achieved through the overexpression of the group III heterotrimeric Gγ-protein AGG3 (Roy Choudhury *et al.*, 2014).

A second strategy to improve oil yield is to globally enhance the levels of metabolic enzymes involved in triacylglycerol (TAG) synthesis by overexpression of specific transcription factors. WRINKLED1 (WRI1) was shown to be an essential transcription factor for TAG synthesis in many species, and its overexpression led to 10–30% increase in seed oil content in arabidopsis, rapeseed and even maize (Cernac and Benning, 2004; Baud *et al.*, 2007; Liu *et al.*, 2010; Pouvreau *et al.*, 2011; Wu *et al.*, 2014). Overexpression of arabidopsis AtWRI1 in camelina seeds led to an increase in seed weight and seed size, and as expected, to 8–31% increase in seed oil content (An and Suh, 2015). Although camelina already has three copies of endogenous *WRI1*, it is remarkable that significant yield gain could be achieved by overexpression of an orthologous gene.

A third strategy for improving seed oil content is to specifically target metabolic bottlenecks in TAG biosynthesis. Fatty acids produced by plastids are shuffled between phospholipid and TAG pools (Chapman and Ohlrogge, 2012). While phospholipids like phosphatidylcholine (PC) are essential for fatty acid desaturation, they represent an important pool of acyl chains not available for TAG production. Phospholipases A (PLA) are able to hydrolyse PC to lysophosphatidylcholine, releasing a free acyl chain available for TAG synthesis. Constitutive overexpression of several *PLA* genes in arabidopsis and camelina led to a significant increase in seed oil content, but at the expense of important developmental alterations (Li *et al.*, 2013, 2015). Similar effects on seed oil content, albeit more modest and variable, were obtained by seed-specific overexpression of arabidopsis PLAIIIΔ,Δ but without impacting overall plant growth, stressing the importance of targetting the desired metabolic modifications uniquely to the seed during

the maturation phase, while avoiding expression elsewhere and at other phases of the plant growth cycle (Li *et al.*, 2015).

Although this has not yet been described, combining strategies that will increase seed yield with those that increase the proportion of oil in the seeds should make a major contribution to enhancing the economic viability of growing camelina.

6 Improving camelina oil composition for food and feed

The high levels of alpha-linolenic acid (C18:3, ALA) in camelina oil provide an ideal plant chassis for the synthesis of omega-3 long chain polyunsaturated fatty acids (LC-PUFAs) like eicosapentaenoic acid (C20:5, EPA) or docosahexaenoic acid (C22:6, DHA). Omega-3 LC-PUFAs are central dietary recommendations for fetal development and adult cardiovascular and cognitive health. The main dietary source of LC-PUFAs is oil-rich fish, such as atlantic salmon. Since fish do not synthesize LC-PUFAs efficiently, farmed fish are fed with fish meals and fish oil enriched in LC-PUFA extracted from wild-caught fish. A more sustainable solution would be to replace fish oil by vegetable oil enriched in omega-3 LC-PUFA. Land plants do not synthesize polyunsaturated fatty acids longer than 18 carbons and with more than 3 double bonds. Metabolic transformation of ALA (C18:3) into EPA (C20:5) and DHA (C22:6) requires successive fatty acyl desaturation and elongation steps. The efficiency of this metabolic conversion is impeded by the substrate dichotomy paradigm. Indeed, fatty acid elongation in higher plants relies on the acyl-CoA pool, while desaturation takes place principally on phosphatidylcholine, implying that successful synthesis of LC-PUFA requires continuous shuffling of acyl chains between the two acyl pools, acyl-CoA and phospholipids. Substrate dichotomy was thus proposed to be one of the main metabolic bottlenecks in LC-PUFA synthesis (Domergue *et al.*, 2005a; Napier, 2007). A major breakthrough was the discovery that the acyl-CoA dependent Δ6 desaturase of the microalga *Otrococcus tauri* could convert ALA to stearidonic acid (C18:4, SDA) (Domergue *et al.*, 2005b; Sayanova *et al.*, 2012). The expression of the acyl-CoA dependent Δ6 desaturase in combination with Δ6 elongase and C20 Δ5 desaturase increased the accumulation of C20 intermediates of LC-PUFA biosynthesis in yeast and arabidopsis, demonstrating the potential for reducing substrate dichotomy (Sayanova *et al.*, 2012). Endogenous levels of the LC-PUFA substrate, ALA, has a clear effect on Δ6 desaturase activity, since camelina expressing Δ6 desaturase accumulated three times more SDA (C18:4) than arabidopsis, which synthesizes lower levels of ALA (Sayanova *et al.*, 2012). The complete expression of five enzymes of the LC-PUFA pathway including Δ12 desaturase, Δ15 desaturase, Δ9 elongase, Δ8 desaturase, Δ5 desaturase, for respectively the synthesis of linoleic acid (C18:2), ALA, eicosatrienoic acid (C20:3), eicosatetraenoic acid (ETA) and EPA, allowed efficient EPA synthesis in arabidopsis and camelina (Ruiz-Lopez *et al.*, 2015). Camelina again proved to be a better host, with about 8% EPA accumulated in seeds compared to 3.6% for arabidopsis. An iterative approach of LC-PUFA enzyme combinations allowed respectively a four- and a ten-fold improvement in EPA and DHA accumulation in arabidopsis seeds (Ruiz-Lopez *et al.*, 2013). Finally, the best constructs were validated in camelina, yielding LC-PUFA levels comparable to those found in fish, with 31% EPA and 14% DHA+12% EPA for the best EPA and DHA lines (Ruiz-Lopez *et al.*, 2014). Similar results were obtained by optimizing the expression of the Δ6 desaturase in multiple gene stacking combinations (Petrie *et al.*, 2014). The high potential of these lines to accumulate LC-PUFA in oil was confirmed in field trials (Usher, 2015). Three lessons can be learned from this success story. It is necessary to: (i) identify metabolic bottlenecks in the metabolic pathway (substrate dichotomy) (ii) systematically test all the possible enzyme combinations and (iii) choose an optimized plant host with the highest substrate availability (ALA). While arabidopsis and yeast have been valuable tools for identifying the appropriate enzymes, camelina was essential, not only for its oil profile, but also the ease of its genetic transformation, which facilitated the screening of a large number of transformants. Furthermore, EPA-enriched camelina oil was shown to be a suitable substitute for fish oil in aquaculture (Betancor *et al.*, 2015a, b). These results suggest that LC-PUFA-enriched camelina oil could also represent an interesting alternative source for LC-PUFA in human nutrition. This is reinforced by results obtained using mice fed an EPA-enriched camelina oil diet, which was found to be as efficient as fish oil for providing EPA, thus opening the way to human feeding trials (Tejera *et al.*, 2016).

Nervonic acid (C24:1Δ15) is a natural component of human breast milk fat, and is used in infant formula supplementation, but also in treatment of several neurological diseases, such as multiple sclerosis, adrenoleukodystrophy and Zellweger syndrome (Huai *et al.*, 2015). This fatty acid is found in oil from several *Brassicaceae*, such as *Lunaria annua,* but the production of this species is insufficient to meet the demand. Production of nervonic acid in camelina was developed by the overexpression of *L. annua* keto acyl synthase (KCS), the first enzyme of the cyclic elongation reaction that provides fatty acid specificity of the elongase complex. To improve fatty acid elongation efficiency in camelina seeds, combinations of two elongase enzymes from arabidopsis were associated with LaKCS (Huai *et al.*, 2015). Expression of LaKCS led to significant accumulation of nervonic acid in camelina seeds (up to 12% of lipids), but expression of the additional elongase genes did not improve the yield. Even if LaKCS expression allows nervonic acid synthesis, strategies could be implemented to improve its accumulation, such as: combine all four enzymes of the elongase complex rather than just two, use the elongase enzymes from camelina or *L. annua* directly, reduce the expression of endogenous KCS to minimize substrate competition by the different elongase complexes.

7 Developing new camelina oil profiles for industry

Due to its specific profile, camelina oil is already used in industrial applications. Highly unsaturated fatty acids are for instance more prone to epoxidation, which is of interest for adhesive properties (Kim *et al.*, 2015a). Epoxidation could be followed by partial acrylation and dihydroxylation, leading to acrylic polyols, which are the source of numerous polymers

(Li and Sun, 2015). Recent work demonstrated that alkyd resins used in the coating and paint industry could also be synthesized from camelina oil and polyglycerols (Nosal *et al.*, 2015).

Camelina oil metabolism could also be modified to enhance the accumulation of lipids of industrial value. Jet fuels require medium-chain fatty acid (MCFA) of 8 to 14 carbon length. Camelina plastids elongate acyl-ACP by fatty acid synthase until C16 and C18 carbon length, and the resulting acyl CoAs are then released in the cytosol by acyl thioesterases. C16:0-ACP is hydrolyzed by FatB thioesterases, while FatA is more specific to C18:0- and C18:1-ACP. *Cuphea* species that accumulate MCFA in their TAG have specific FatB genes (Kim *et al.*, 2015b). Expression of different *Cuphea* FatB genes associated with different MCFA profiles (C8, C10, C16) led to significant MCFA accumulation in camelina seeds. This effect was enhanced by the coexpression of coconut lysophosphatidic acid acyltransferase (LPAT) and the inhibition of the endogenous camelina plastidial beta-ketoacyl-ACP synthase CsKASII (Kim *et al.*, 2015b).

A similar strategy was used to enrich camelina oil in omega-7 fatty acids, like palmitoleic acid (C16:1Δ9) and cis-vaccenic acid (C18:1Δ11), which have neutraceutical and industrial value for polyethylene production (Nguyen *et al.*, 2015). Combined expression of six different transgenes allowed efficient redirection of fatty acid flux toward omega-7 lipids. First, plastidial C16:0-ACP was channelled toward C16:1Δ9-ACP by the combined inhibition of the 16:0-specific thioesterase (CsFatB) and the β-ketoacyl-ACP synthase (CsKASII) associated with the expression of C16:0-ACP Δ9 desaturase (COM25). To increase omega-7 fatty acid accumulation, the elongase FAE1 was also repressed by RNAi, and the cytosolic C16:0-CoA converted to omega-7 by the expression of C16:0-CoA Δ9 desaturase (Fat5). Altogether, this strategy successfully resulted in the accumulation of more than 60% palmitoleic acid and cis-vaccenic acid in camelina seeds.

Alternatively, the camelina oil profile could be modified simply by inhibiting the last steps of a metabolic pathway, such as for the synthesis of ALA. The down regulation of FAD2 desaturase by anti-sense or RNAi led to camelina oil with almost 50% oleic acid (Kang *et al.*, 2011; Nguyen *et al.*, 2013). Combined reduction of FAD2 with FAE1, the fatty acid elongase involved mainly in erucic acid synthesis in seeds, resulted in a further increase of oleic acid content up to 70% in camelina seeds (Nguyen *et al.*, 2013). Interestingly, spatial analysis of TAG and PC in camelina embryos revealed unexpected heterogeneity (Horn *et al.*, 2013). For instance, cotyledons were enriched in C20:1, while the embryonic axis accumulated more C18:2 in both lipid pools, suggesting tissue specificity in lipid metabolism within the embryo. The FAD2+FAE1 RNAi lines showed incomplete suppression of FAD2 in specific tissues, raising the question of either RNAi inefficiency or the presence of metabolic compartimentalization for oleic acid accumulation. These approaches, based on metabolic mapping by mass spectrometry, provide valuable information about the organization of lipid metabolism in the embryo that will help design further new strategies for modifying the camelina oil profile.

Camelina oil properties could also be profoundly changed by the accumulation of new lipids like acetyl-TAG. Acetyl-TAGs are unusual triacylglycerols in which the sn-3 position has an acetyl group instead of a fatty acyl group. This modification, which reduces viscosity and lowers the oil melting point compared to conventional TAG, is sought for lubricants, food emulsifiers and plasticizers. The main source of acetyl-TAG is the seeds of *Euonymus alatus* (Burning Bush) thanks to a specific acyltransferase (EaDAcT) that transfers the acetyl group of acetyl-CoA to the sn-3 position of DAG (Durrett *et al.*, 2010). Overexpression of EaDAct in camelina led to an average of 50% acetyl-TAG in seeds, a value that could be increased to 80% when combined with down-regulation of DAGT1 by RNAi (Liu *et al.*, 2015a). The effect of EaDAcT expression, combined or not with DGAT1 RNAi, was significantly higher in camelina transgenic lines compared to arabidopsis or soybean, confirming the particular value of camelina in oil engineering strategies. Interestingly, the high levels of acetyl-TAG accumulation in seeds did not impair seed yield, nor did it modify seed germination in several field studies (Liu *et al.*, 2015a, b). As expected, camelina oil enriched in acetyl-TAG showed lower viscosity, a higher crystallization temperature, and higher caloric content, providing a direct alternative for biodegradable lubricants, hydraulic fluids and transformer oils. The fact that camelina acetyl-TAGs are also rich in oleic acid, and are thus less susceptible to oxidation, could open new markets in the food industry, such as for water retention on food surfaces, emulsifiers, foam stabilizers and packaging plasticizers.

8 Camelina as an alternative producer of non-TAG high value products

Wax esters are neutral lipids that have higher energy density compared to TAGs, and their refinement does not produce glycerol as a side product. They could thus represent a valuable source of biodiesel. They are also used as lubricants, since they have low melting points, an excellent resistance to oxidation, and yet are biodegradable. The desert shrub jojoba is a natural source of wax esters, but since it is not adapted to high yield cultivation, particularly in temperate regions, a more efficient and sustainable approach is required. Only two enzymes are necessary for wax ester synthesis: a fatty acyl-CoA reductase (FAR) and a wax ester synthase (WS). The possibility to accumulate wax esters in seeds was validated in arabidopsis with different variants of FAR and WS from mouse or the bacterium *Marinobacter aquaolei* (Lardizabal *et al.*, 2000; Heilmann *et al.*, 2012). A total of seven different novel enzyme combinations were tested first in arabidopsis, after which the best three were introduced into camelina (Iven *et al.*, 2016). Similar types of wax esters were produced in arabidopsis and camelina, but the yield in camelina was half that in arabidopsis. Arabidopsis could accumulate 89–108 ng/seed (representing 43–59% neutral lipids) compared to 33–47 ng/seed (15–21%) for camelina. Since wax ester synthesis uses acyl-CoA as substrates, and thus competes directly with TAG synthesis, a possible improvement would be to favor substrate channelling toward wax ester biosynthesis instead of TAG. Recently, a relative 30% content in wax ester was achieved in camelina by the combined overexpression of jojoba FAR and WS with *L. annua* FAE1 and associated with FAD2 inhibition

(Zhu *et al.*, 2016). Nevertheless, camelina yields were lower than those of *Crambe abyssinica* transformed with the same constructs, perhaps because that the latter naturally accumulates high levels of very long chain fatty acids (Zhu *et al.*, 2016).

The high oil content of camelina seeds could also favor the accumulation of bioactive compounds such as terpenes, which are components of essential oils used in food additives, cosmetics, drugs, rubber and lubricants (Degenhardt *et al.*, 2009). Terpenes also increase oil caloric value, which is an important parameter for biofuel applications, in particular for kerosene. As a proof of concept, the synthesis of the monoterpene (4S)-limonene and the sesquiterpene (+)-delta-cadinene was investigated in camelina seed. Interestingly, these two terpenes are produced via two different pathways: the cytosolic mevalonate pathway and the plastidial 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, respectively. Combinatorial association of the different enzymes expressed either in the cytosol or in the plastids allowed direct comparison of the most efficient pathways (Augustin *et al.*, 2015). (4S)-limonene could only be accumulated in camelina seed via the expression of plastid-localized enzymes, while (+)-delta-cadinene was accumulated in camelina seeds expressing enzymes either in the cytosol or plastids. Interestingly, the ectopic localization of (+)-delta-cadinene biosynthetic enzymes in plastids was at least five times more efficient than that of the cytosol-localized enzymes. Globally, for both terpenes, the levels obtained were around 5–7 mg/g seed and about 22–29 mg/ml oil. The presence of terpenes in camelina oil increased its caloric value, but the ratio terpene/TAG was still too low for biofuel applications. The use of strong seed-specific promoters could improve terpene production in camelina seeds (Borghi and Xie, 2015). Nonetheless, the minimal loss of these volatile molecules during seed development, as well as their protection from possible degradation, makes camelina seed oil a potentially interesting alternative terpene source for cosmetics and pharmacological use.

There has been much interest in poly-3-hydroxybutyrate (PHB) for use as a biosourced biodegradable replacement for petroleum-derived plastics. Most plant-based PHB has been produced in leaves, but its accumulation in seeds of arabidopsis, rapeseed, tobacco or soybean has had limited success (7% PHB in rapeseed). Camelina was used in an attempt to alleviate this limitation, and the ectopic expression of the three biosynthetic enzymes with different combinations of seed-specific promoters allowed a modest increase to 15% for the best line (Malik *et al.*, 2015). PHB accumulation was however toxic to the embryo, with cotyledon chlorosis and weak seed vigor. The challenge to come will be to develop strategies to minimize PHB toxicity in the embryo cells in order to achieve yields compatible with industrial use.

9 Conclusions

In many studies, camelina has been shown to be an efficient host for bioengineering strategies, with higher oil yields compared to arabidopsis, rapeseed or soybean. The fact that camelina has potentially valuable agronomical characteristics should also increase its interest, not only as as a new crop, but also as a convenient translational tool for arabidopsis research results. In conclusion, current strategies for modifying oil yield or changing the endogenous lipid profile in camelina have had some real success, and can now provide market-compatible products derived from camelina seeds like omega-3 L-PUFA-enriched oil. Exciting challenges remain to improve production yield of new lipids in camelina to levels that are economically viable. New strategies are currently being implemented that couple knowledge of the intricated metabolic fluxes over time and space with synthetic biology tools in order to fine-tune lipid metabolism for specific needs.

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