**Executive summary:**

The ICON project has delivered 21 out of 29 scientific deliverables and achieved its overall aim, which was to demonstrate how plant gene technology can be used to develop renewable material with added value to replace fossil oil, in benefit for environment and rural economies. Wax ester oils have been produced in significant quantities in the three industrial oil crop platforms, Crambe abyssinica, Brassica carinata and Camelina sativa by the transfer of genes for fatty alcohol production (FAR) and wax ester synthesis (WS) from jojoba (Simmondsia chinensis). Crambe lines with near stable amount of wax esters amounting to 25% of the oil showed normal seed setting and only slightly delayed germination. The wax ester composition in Crambe and B carinata were very similar to the jojoba wax esters and dominated by very long chain (C20-C22) alcohols and fatty acids. When plants were transformed with gene constructs containing a fatty elongase gene in addition to the FAR and WS, they produced wax ester with high amount of 24:1 fatty alcohols and fatty acids. These lines had poor seed setting and poor seed germination even at rather low wax ester levels. Stable Crambe lines with up to 72% of erucic acid in the seed oil (from 59% in wild type) and Camelina and Crambe lines with over 80% of oleic acid were developed. Over 30 different FAR and WS enzymes were characterized in ICON. Enzymes with specificities towards very long chain, C10 to C18 fatty acids and both straight and branched fatty alcohols have been identified. However FARs with good activities towards fatty acids shorter than 14 carbon acids have not been identified. Artificial enzymes having both fatty acid reduction and wax ester synthase activities by fusion of FAR and WS genes were developed. A fatty alcohol reductase from Tetrahymena was shown to also have acyl transferase activity and is involved in the synthesis of ether lipids. Wax ester was tested for performance as lubricant in grease formulations. The wax esters of jojoba type were superior to triacylglycerol oils and the only major drawback was high melting point (around 10oC). Plastics were developed from Crambe seed cake mixed with gluten and was shown to have good mechanical properties and oxygen barrier. Field trials with GM Crambe with ultra-high erucic acid (70%) and 25% wax esters were performed in Sweden 2012. Due to regulation imposed by the Swedish authorities, we were forced to do the cultivation under tight insect net to prevent pollen to enter honey. This, combined with late planting and an unusual cold and rainy summer, led to that seeds were heavily infected by fungi and had to be harvest immature. Therefore, no estimation of the commercial viability of these lines could be made. However an ultra-high erucic acid line and one wax ester line with 2-gene construct (jojoba FAR+jojoba WS) showed the expected change in oil quality, although less pronounced than in the seeding seed, and close to the oil content of the control seeds. In scientific dissemination, 27 peer reviewed scientific papers have been published and 80 conference presentations have been performed. Extensive and positive media coverage was obtained in connection to our field trials with GM Crambe. Although scientifically successful, at least an of equal important impact coming from ICON is the increased public awareness of the potential benefits the GM technology can have on environment and rural economies.

**Project Context and Objectives:**

The main objective of the project was to demonstrate how plant gene technology can be used to develop renewable material with added value to replace fossil oil, in benefit for environment and rural economies. More specifically, the project focused on changing seed oil quality in oil crops to enhance the value of the oil and widen its industrial use. In order to achieve its overall objective, the main sub-objectives included in the project are listed below in the context of reaching the overall objective and how they have been dealt with in the project.

**Development of a 'safe' industrial oil crop platform.**
Background. Since the target oil qualities developed in ICON are for industrial purposes we considered it important to not use any food crop or any crop that could out cross with food or feed crops. The EU partners and China choosed the oil crop oil Crambe abyssinica. Crambe has many attractive features as a dedicated industrial oil crop. It does not cross out with any other agricultural crop and not with any wild relatives in northern part of Europe. Due to its high levels of erucic acid in the seed oil, it cannot be used for human consumption but this oil quality is an excellent background for producing the main ICON target oil qualities. ICON's Canadian partners chose Brassica carinata as their industrial oil crop platform. The reason being that they already had worked to develop this crop as an industrial crop for many years and had developed a GM high erucic line as a good background line for further transformation. Camelina sativa was introduced in the project when it was up and running. This plant was selected as a model plant instead of Arabidopsis since Camelina, like Arabidopsis, can readily be transformed by floral dipping and have much bigger seeds that facilitate seed analysis and breeding to homozygotes. It was also selected since it has been proposed as a potential industrial oil crop platform and since our US partners already worked with this plant. The drawback for the project was that it had less optimal oil quality for long chain wax ester synthesis than Crambe and B. carinata.

Research tasks and main results. Since no reproducible transformation protocol was developed for Crambe when the project started, this had to be developed. A protocol based on kanamycin selection was developed during the second reporting period where 40 transgenic lines with reporter genes were confirmed. Later this protocol was further optimized. Since there was a need to retransform the transgenic Crambe for further optimizing oil quality, a high efficiency transformation protocol was also developed based on hygromycin selection.

**Target oil qualities**
Background. Following target seed oil qualities was selected. 1) Very high erucic acid Crambe and B. carinata. 2) Long chain wax esters in Crambe, B. carinata and Camelina. 2) Oleoyl-oleatee wax esters in Crambe, B. carinata and Camelina. 3) Medium chain wax esters in Crambe, B. carinata and Camelina. 4) wax esters with mid hydroxy groups in Crambe, B. carinata and Camelina. 3) methyl branched wax esters in Arabidopsis. The target oil qualities were selected on the basis of following reasons: Erucic acid has wide industrial applications. Its main use is for production of erucamide, a common slipping agent in plastic films.

Today, high erucic rape (HEAR) and mustard provide most of the erucic acid oils and have erucic acid levels of 45-50% in the oil. The oil is priced on its level of erucic acid. Although Crambe has 55-60% erucic

acid and the GM B. carinata developed by our Candian partner had 56% erucic acid, they have hard to compete with rape and mustard since their seed cakes have virtually no value ´since it cannot be used for feed due to high levels of glucosinolates. A crop with 70% of erucic acid in its oil would have a double value compared to HEAR oil and would be a profitable crop for farmers as well as it would save both agriculture land and costs and energy used in the purification of the erucic acid from other fatty acids. Wax esters have excellent properties in lubrication since it withstands high pressure and temperatures in contrast to triacylglycerol plant oils. The only natural sources of wax esters are the spermaceti oil from the spermaceti whale and the jojoba oil. The spermaceti oil was widely used in lubricants until the whale was nearly extinct and a global ban on hunting was introduced in 1974.

The jojoba plant is a low yielding desert shrub and the production costs of the seed oil is very high, excluding its use in other applications than high price cosmetics. The Calgene company reported for over a decade ago wax ester production in Arabidopsis seeds by the transfer of genes from jojoba and thus this should also be technical feasible to achieve in an oil crops. The jojoba type of wax esters consists mainly of 20-24 carbon monounsaturated chains and have a melting point of around 10oC. This restrict the use of it in lubrication. Therefore we also had target wax ester qualities in the project with lower melting point and with modficiations in the carbon chains, such as hydroxy and methyl branch, that would further improve the lubrication properties.

Research tasks and main results. We were successful to increase erucic acid levels in Crambe from wild type 59% to 72% in stable lines by expressing three genes. These lines were field tested in Sweden 2012. Due to regulation imposed by the Swedish authorities, we were forced to do the cultivation under tight insect net to prevent pollen to enter honey. This, combined with late planting and an unusual cold and rainy summer, led to that seeds were heavily infected by fungi and had to be harvest immature. Therefore, no estimation of the commercial viability of these lines could be made. The attempts to increase erucic acid levels in B. carinata with the same genes were less successful, only increasing the levels from 56% to 59%. Codon optimized synthetic jojoba FAR and WS was transformed into Brassica juncea, Camelina and Arabidopsis. Although B. juncea and Arabidopsis produced some wax esters in their seeds, only small amounts were seen and it was not genetically stable. Camelina failed to give any wax esters. This was in sharp contrast to the published report from Calgene. The data indicated that the FAR gene gave an active enzyme but that the WS was inactive.

By an efficient collaboration between four participants, new gene constructs with cloned native genes were developed and transformed into Arabidopsis and Camelina. Now, both plants produced wax ester amounting to up to 60% of the oil. Subsequently Crambe and B. carinata were transformed and showed similar high amount of wax esters. These wax esters were of jojoba type dominated by C20 to C22 monounsaturated carbon chains if only the FAR or WS were expressed but also contained high amount of 24 carbon chains if a fatty elongase was included. Lines with higher amount of wax esters than 30% of oil had poor seed setting and poor seed germination frequency whereas Crambe lines with 25% wax esters in the oil and Camelina with 15% of wax esters showed near normal germination and seed yield. Crambe with about 20% of wax esters were field tested in Sweden 2012. Despite bad growing conditions of reasons mentioned above, one line gave harvest with about the same oil content

and just somewhat depressed seed yield compared control plants and had 16% of wax esters in their oil. Less progress was made in producing other types of wax esters than the jojoba type in seeds. It should be noted that there is no natural plant have been identified accumulating wax esters of these other types. As described below, a multitude of FAR and WS genes were cloned and characterized and 20 different gene constructs with combinations of these genes were transformed into Camelina having different fatty acid compositions. Camelina and Crambe lines with 70- 80% of oleic acid in the seed oils were developed to be used as background lines for further transformation for achieving high oleoyl-oleate wax esters. The target oleoyl-oleate wax esters were obtained in Arabidopsis seeds from a mutant line having high amount of oleic acid in the seed oil.

The best lines had about 16% of wax esters in their oil with 65% of the molecular species being oleoyl-oleate. However, so far no Camelina lines have shown to produce oleoyl-oleate, although lines with about 20% of wax esters of the oil and with significant amount of 16 and 18 carbon chains were obtained. Small amounts of medium chain fatty acids were found in wax esters in Camelina in a background producing high amount of 14:0 fatty acids in its seed oil. Many of the transformed Camelina plants are still under evaluation and new gene construct will be tested and this work will continue at least one year after the end of the project.

**Gene discover**
Background. At the start of the ICON project, only a couple of papers regarding fatty acid reductases and wax synthases, the two enzymes responsible for wax ester synthesis, had been published. Of them it was only the genes from jojoba and mouse that had been characterized in some detail. It was therefore important to clone additional FAR and WS genes encoding enzymes with novel specificities that could be used to produce other target wax ester qualities than the jojoba type.

Research tasks and main results. Over 30 different FAR and WS genes were cloned from various organisms from bacteria, protista, insects, mammals, birds and plants and their activity and specificity were investigated by expression in E. coli, yeast and in transient expression in Nicotiiana benthamiana leaf expression systems. Enzymes with specificities towards very long chain, C10 to C18 fatty acids and both straight and branched fatty alcohols was been identified. Out of many scientifically interesting results obtained we here mentioned a few.

i) Identification of the first bacterial enzyme able to produce fatty alcohol from fatty acids.
ii) Two chloroplast localized wax synthase/diacylgclycerol acyltransferase with a high preference for medium chain fatty acids was discovered. When co-expressed with a bacterial FAR in leaf assay system, high amount of wax esters with medium chain fatty acid were produced.
iii)A FAR from Tetrahymena having an acyl transferase activity and involved in ether lipid synthesis.
iv) Construction of fusion genes between FAR and WS giving rise to enzymes carrying out the complete synthesis of wax esters from fatty acids.

**The performance of wax esters in grease formulation and Crambe and B. carinata seed cake in plastic production.**
Background. Wax esters have previously been tested in lubrication oil and have been found to have excellent properties. In the project we further

investigated how wax esters performed as lubricants with the focus on grease manufacturing. When oil has been extracted from the seed, the so called seed cake will be left. In oil crops like rape this has a value in feed of 15-20% of the oil, adding significantly to the economy in oil rape production. However, Crambe seed cake cannot be used for this purpose because of its high levels of glucosinolate. Further, due to regulatory issues we do not want to use any part of our industrial oil crops in feed or food. Therefor research was performed to investigate if Crambe and Brassica carinata seed cakes could be used in plastic manufacturing.

Research tasks and results. In ICON, our industrial partner Axel Christiernsson tested the jojoba type of wax esters, similar in composition to what we have obtained in our GM industrial oil crop, in grease manufacture. The conclusion of the testing was that the wax esters could work as EP-additives in a wide range of products that already are using sulfur carrier additives and if the price was right they would definitely find a market niche. Further, greases based on wax esters have good lubricity and friction properties. The low heat emissions seen in the bearing test rig is very interesting from an energy conservation perspective. If the low temperature properties can be improved by lowering the melting point it should be possible to develop a high-performance biodegradable grease based on this oil. Such a product will be more or less unique on the market and if correctly priced it has the potential of attracting many users. Our industrial partner Innventia pursued the work on using seed cakes in manufacturing of plastics. Plastics with good mechanical properties and good oxygen barrier was achieved with a mixture of gluten and Crambe seed cake.

**Dissemination activities**

In scientific dissemination, 27 peer reviewed scientific papers have been published and 80 conference presentations have been performed. Extensive and positive media coverage was obtained in connection to our field trials with GM Crambe. Although scientifically successful, an at least of equal important impact coming from ICON was the increased public awareness of the potential benefits the GM technology can have on environment and rural economies. The details of dissemination and the societal implications are further elaborated in the last section of this final report.

**Project Results:**

The research tasks, its progress during the project and the main final results are described for each WP.

**WP1. Developing transformation protocols for Crambe**

It was realized in the start of the ICON project that the PCR positive putative Crambe transformants that had been developed by one participant were not stably transformed. Therefore the transformation work was much intensified by adding participant 1 (SLU) to this research task supported with resources from national grants. Transformation protocols was developed by all three partners, participant 1 (SLU), participant 4 HUBU and participant 12, (PRI), using kanamycin as a selectable marker during the second year of the project. The protocol developed by participant SLU was disseminated in a scientific journal1 and was further optimized to give near 100% regeneration and this work was published in another scientific article2. Further, participant SLU developed an efficient method for transformation with hygromycin selection and a scientific article on this work is now under revision3. A research task was allocated to one participant to transform microspore of Crambe in order to develop a rapid method for screening of changed oil quality in micropore derived embryos and raising transformed double haploids. However this work showed no progress during the first two years of the project and it was decided to delete this research task and the resources were re-allocated to other research tasks.

We found that Mendel Biotechnology, Inc. in US had filed a patent application covering a transformation method of Crambe. In order to see if this patent application could be a threat to ICON freedom to operate, we applied for patent of the method developed by SLU4. The PCT evaluators had no objection of any of our claims and it was clear that the Mendel application did not interfere with freedom to use the protocol developed by SLU. We therefore decide to not go further into national phase but the PCT evaluation could be used as a strong argument if Mendel would accuse any using the SLU protocol for patent infringement.

In summary, this WP achieved its main goal to develop efficient transformation protocol of Crambe using two different selection genes. It was considered important to develop another selectable marker for re-transformation of GM Crambe lines with additional genes to optimize the oil quality further.

**References:**

1. Li X, Ahlman A, Yan X, Lindgren H and Zhu L-H (2010) Genetic transformation of the oil crop Crambe abyssinica. Plant Cell, Tissue and Organ Culture 100, 149-156

2. Li X, Ahlman, A, Lindgren H, Zhu L-H. (2011) Highly efficient in vitro regeneration of the industrial oilseed crop Crambe abyssinica. Industrial Crops and Products 33, 170-175

3. Li X.Y., Fan J., Gruber J., Guan R., Frentzen M. and Zhu L.H. 2013. Efficient selection and evaluation of transgenic lines of Crambe abyssinica. Frontier in Plant Science (under revision).

4. Zhou L.H. Plant Protocol WO/2010/140961; PCT/SE2010/050595

**WP2. Work towards developing Crambe and B. carinata with ultra-high amounts of erucic acid**

The strategy employed was the same as reported successful in increasing oil content in high erucic rape1. This strategy involved the down regulation of the FAD2 gene, responsible for the desaturation of oleic acid to linoleic acid and thereby redrawing oleic acid from being elongated to erucic acid. Further, a gene encoding a limnanthes LPAAT should be expressed, yielding an enzyme that could effectively insert erucic acid in the middle position of the glycerol backbone, a capacity that is lacking in the endogenous LPAAT of Crambe and B. carinata. A third gene, encoding a fatty acid elongase should also be added to enhance the elongation capacity of oleic acid (a C18 carbon acids) to erucic acid (a 22 carbon fatty acids). Considerable technical problems were encountered to produce the three gene construct. Therefore initially separate transformations of Crambe were done with a one gene construct containing FAD2-RNAi for down regulation of oleate desaturation and a second gene construct containing Limnanthes LPAAT and a rape FAE. Many transformed lines from the FAD2RNAi construct had seeds with drastic reduction in polyusaturaed fatty acids but no increase in erucic acid whereas several lines with the 2-gene constructs showed increased erucic acid and synthesis of triacylglycerols with three erucic acid (which is not present in wild type seeds). Crossing were performed between these lines to achieve lines with all three genes expressed. During this time, also the three gene construct was achieved and transformed into Crambe.

Our Canadian participant19 (NRC-PBI) had already developed transgenic B. carinata lines expressing a Crambe FAE and a FAD2-RNAi so the best of these lines (having 56% of erucic acid) was re-transformed with a one-gene construct containing the Limnanthes LPAAT. The Crambe lines resulting from crossing as well as those with three gene constructs were taken to following generation through half seed analysis of its oil quality. Both the crossed lines and the three gene constructs showed seeds with over 70% of erucic acid in the oil, but the three gene constructs showed less variation between seeds in the best lines. Lines from the three gene constructs with single inserts were taken to T6 and a line was selected that showed little variation in erucic acid levels (72%) and good seed settings for taking further to following generation for selecting elite lines for field tests 2014. The work of developing these ultra-high erucic acid Crambe lines has now been published2 .

The transgenic B. carinata showed only moderate increase in erucic acid with the Limnantes gene expressed compared to the orginal transformants and the best T3 seeds showed maximum 59.9% erucic acid compared to 56% in the original line but was shown to contain tri-erucic acid triacylglycerols, demonstrating that the introduced LPAAT gene was functioning in the plants. Since the outcome of these transgenes was disappointing compared to the over 70% of erucic acid obtained in Crambe, new re-transformation of B. carinata XS more advanced lines with higher and more stable erucic acid have been done with the LPAAT gene and 27 hygromycin positive plants were taken to seed setting. However due to lack of funding as a consequence of re-prioritizing in-house research areas at NRC-PBI, no resources are currently present for any further analyses of these plants.

Biochemical studies have been performed on developing seeds of GM Crambe having 70% of erucic acid and compared to wild type Crambe, rape seed and safflower seeds. The conclusions that can be made is that Crambe has extremely low phospholipid:diacylglycerol cholinephosphotransferase

(PDCT) activity compared to the other oil seeds studied. It could further be concluded that the introduced genes confer high enzymatic activity even at early stages of seed development although the erucic acid content at that stage is not higher than in wild type Crambe. This indicate that the bottleneck in further increase in erucic acid is not due to inadequate activity of the introduced enzymes at this seed stage but might possibly be due to limiting amount of malonyl-CoA or in the reduction enzymes needed for elongation of erucic acid. Further, these work showed that in plants grown in green house, seed development continued up to 50 days compared to 40 days after flowering in the wild type and the oil content was reduced with about 10%. A manuscript on this work will be submitted to Plant Physiology.

In order to investigate if the activity of endogenous lipid metabolizing enzyme were limiting the amount erucic acid levels in Crambe, transformation of Crambe with a number RNAis targeted to the genes encoding these enzymes were performed. RNAis towards Crambe phospholipid:diacylglycerol acyltransferase (PDAT), phospholipid:diacylglycerol cholinephosphotransferase (PDCT) and lysophosphatidylcholine:acyltransferases (LPCATs) were transformed into wild type Crambe as single gene constructs as well as a three gene construct. Screening of T1 seeds by half seed analysis revealed some variation in fatty acid composition, of which none yielded more erucic acid than controls. The seeds showing most deviations in fatty acid profile were taken to next generation for further screening and biochemical characterization. This work will continue at least one year after the end of the ICON project with national funds.

Since the amount of erucic acid in the sn-2 position of the triacylglycerols was significant lower than the outer positions also with the Limnanthes LPAAT expressed, it indicates that endogenous Crambe LPAAT competes with the introduced LPAAT1. The Crambe LPAAT would transfer C18 carbon fatty acids to the sn-2 position and by this limiting the amount of erucic acid that could be acylated to the triacylglycerols. Therefore a fourth gene, encoding Crambe LPAAT-RNAi, was added to the three gene construct and the four gene construct were transformed into Crambe. Fifteen putative transformed lines were obtained and seeds will be analyzed after the end of the ICON project.

**Field tests**

T4 seeds from two transgenic lines, 3G7-6-7 and 3G7-6-13, having a mean value of 69% and 71% of erucic acid, respectively, were planted in plots of 100 m2 in the field in Sweden together with non-transformed Galactica seeds. The Swedish Agricultural Board, the authority approving GM field tests, imposed us to have a tight insect over our plots in order to prevent bees to come in contact with plants and thereby transfer pollen to their honey. If honey contains any pollen from non-approved GM events, it cannot be sold as food. This, combined with an unusually cold and rainy summer delayed plant development. The transgenes were germinating and establishing later than the control and had slower seed development. Thus, when harvest was done late September, many seeds had not matured and this was in particular the case for the transgenes, as reflected by their high chlorophyll levels. Further the seeds were heavily infected with fungi.

Thus no conclusions can be drawn on the basis of this field test. The lower yield of the transgene than the control and the low amount of erucic acid compared to the GM seeding seeds could mainly be attributed

to the fact that the majority of the seeds were not fully developed. Most of the oil and erucic acid are laid down late in seed development. The Swedish authorities has now allowed us to do the field tests without net provided that nearest bee hives is not closer than 3 km. We therefore intend to do another field trial this year (2013) without net with the some of the seeds collected from this field trial as seeding material.

Despite that the field trial failed to give us information about the performance of the GM lines it was a great public relation success. We invited the press to a meeting that included a visit to the field trial and this was reported in very positive manner in a number both national and local media. More details regarding this are reported under Impact section below.

**Reference:**

1. Nath, N.K., Wilmer, J.A., Wallington, E.J., Becker, H.C. and Möllers, C.
(2009) Increasing erucic acid content through combination of endogenous low polyunsaturated fatty acids alleles with Ld-LPAAT + Bn-fae1 transgenes
in rapeseed (Brassica napus L.). Theor. Appl. Genet. 118, 765–773.

2. Li X, van Loo EN, Gruber J, Fan J, Guan R, Frentzen M, Stymne S, Zhu LH. (2012) Development of ultra-high erucic acid oil in the industrial oil crop Crambe abyssinica.Plant Biotechnol J. 10:862-70.

**WP3. Gene discovery: Fatty acid reductase and wax synthase genes with different specificities**

Fatty acid reductase genes from Arabidopsis were cloned and characterized by heterologous expression in E. coli, yeast and transient in Nictoniana benthamiana leaf expression system. Their tissue specific expression in Arabidopsis and the effect of mutations in the genes were investigated. The work has resulted in four published articles1-4. Three enzymes under investigation more thoroughly were shown to be involved in suberin synthesis having defined specificities for either 18:0, 20:0 or 22:0 fatty acids and one enzyme (FAR6) was localized in the chloroplast and with unknown function.

Mouse FAR and WS had been reported to have good activity towards C18 unsaturated substrates5,6 and thus good candidate for producing oleoyl-oleate, a target wax ester in WP6. However, very little wax esters, but mainly free alcohols, were produced when the two genes were co-expressed in yeast. Similar results were obtained at University of Saskatchewan with the WS and FAR genes from Euglena expressed in yeast. Localization studies of the mouse enzymes in onion epidermis cells clearly show that mouse FAR1 was peroxisomal localized whereas the mouse WS was ER localized. In order to co-localize the enzymes, fusions genes with a oleosin gene were constructed. When both genes had oleosin fusion, the wax ester production in yeast was much increased. Genes construct with oleosin fusions were transformed into Arabiodospis fae/fad2 mutant, having much increased levels of oleic acid. Lines with wax esters levels up to 16% of oil and with 65% of the wax ester species being oleoyl-oleate were obtained (see also WP6). One patent application was filed on the method to co-localise the proteins7 and one scientific article was published8.

Methyl branched saturated wax esters would have superior function due to low melting point and excellent oxidation stability. Birds are known to

have secrete such wax esters from their preen glands. Five FAR and seven WS genes were clones from barn owl (Tyto alba), domestic chicken (Gallus gallus domesticus) and domestic goose (Anser anser domesticus) and characterized by expression in yeast. Some of the WS synthases were bifunctional and had also diacylglycerol acylating activity. WS with good activity towards medium chain alchols (10-12 carbon) and branched chain (farnesol and geranylgernaiol) as well as 2-methyl branched 16:0 and 18:0 fatty acids were identified. This work has been published 9. None of the WS in that study had good activity towards both methyl branched fatty acids and methyl branched fatty alcohols. However at the end of ICON a novel WS from chicken was identified being a variant of earlier characterized WS (GgWS1?1) that had much increased wax ester activity with branched chain alcohol and could also acylate branched chain acyl groups.

The bird FARs characterized were shown to have specificities ranging from 14:0 to 18:0 fatty acids and they also showed about the same specificity and activity with a 2-methyl branched substituted acyl-CoA (15- 19 carbon). This work has been published10.

Four WS genes were characterized from Tetrahymena termophila and were shown to some degree also having diacylglycerol acyltransferase activity. Specificities of the enzymes were characterized in membranes from yeast and howed generally good activity towards medium chain fatty alchols (10-12 carbons) but less activity with methyl branched acyl groups. The preferred acyl-CoAs has 14 to 18 carbon saturated acyl groups. This work has been published11.

A FAR (TtFARAT) from Tetrahymena termophila that contained an acyl transferase domain was characterized by expression in yeast. The enzyme had high specificity for 16:0-CoA in fatty alcohol production and was functional without the acyl transferase domain. By expressing the FARAT gene in yeast it could be shown that the enzyme is involved in ether lipid synthesis where the FAR domain supply the alkyl group and the acyltransferase domain encode a glyceronephosphate-O-acyltransferase. The bifunctional nature of the FARAT lead to the idea to fuse various WS and FAR using the TtFARAT as a model for these constructs. Most of these fusions were shown to be active in yeast, thus being novel enzymes that could carry out the entire wax ester synthesis from acyl-CoAs.

Two enzymes (PES1 and PES2) with homology to WS and predicted be chloroplast localized and were induced during leaf senescence in Arabidopsis were studied. Arabidopsis knockouts in the encoding genes showed drastic reduction of phytol esters in the senescent leaves, suggesting that the genes encoded a phytol ester synthase. The enzymes were studied by expression in yeast without plastid transit peptide and were showed to have broad acyl acceptor specificities, capable to acylate both diacylglcyerols and sterols. The enzymes was further shown to be able to well utilize both acyl-ACP and acyl-CoA and have a broad acyl group acceptance (from C8 to C20) and acylated both saturated and unsaturated acyl-CoAs. The work with the PES genes have been published12.The discovery of both FAR (FAR6) and a WS that were chloroplast localized prompted us to see whether we could produce wax esters in chloroplasts by co-expressing these genes in plants. Therefore, we transiently overexpressed FAR6 and PES2 in the Nicotiana benthamiana leaves. Substantial amount of wax esters were formed (up to 0.7% of leaf dry weight) with mainly 12:0 and 14:0 fatty acids and 16:0 alcohols. Although the levels of wax esters were low compared to what would be

required for being commercially interesting, it demonstrates that very interesting molecular species of wax esters can be produced in the chloroplasts and, if yield can be substantially improved, might be an alternative subcellular site beside the cytosol to accumulate these compounds.

Arabidopsis has eleven genes with homology to bifuntional wax synthase/diacylglycerol acyltransferases. Of the 11 WSD genes, 9 was expressed in yeast. One WSD1, was shown to be involved in wax ester synthesis in stems of Arabidopsis and had preference for acylation of very long chain alcohols. This work was published13. For two of the genes, no cDNAs are available in the datase (one might be a pseudogene, the other is of extremely low expression). When expressed in yeast H1246, the 9 genes give no production of TAG or wax esters. Only after feeding an alcohol and a fatty acid, then most of the clones produce wax esters, and some (WSD4) produce TAG. Work with this will continue after the end of the ICON project to further characterize these enzymes.

A protocol for expression and purification of FAR was developed. The FAR genes fused with a maltose binding protein were expressed in E. coli and further affinity purified. The enzymes rapidly lost activity after removing the maltos binding domain but the fusion protein was stable and showed the same specificity as the native enzyme as judged from the alcohols produced in-vivo in yeast and plants. The protocol was used to purify and characterize Arabidopsis FAR6 and the results have been published 2. The protocol was also applied to purify and characterize a FAR from Marinobacter aquaeolei VT8 that show gene sequence homologies with eukaryotic FARs. Fatty alcohol production from fatty acids in bacteria has been shown to be catalyzed by two separate enzymes, first an aldehyde forming enzyme and then an aldehyde reductase, but no enzyme that catalyzing both reaction, which is the case in eukaryotes, had been identified bacteria. However, for the first time we could show that also bacteria can have such a FAR. The enzyme showed good activities for 16 to 20 carbon acyl groups, including 18:1. These results have been published14. When the gene was expressed with a chloroplast transit peptide from FAR6 together with PES2 in N. benthamiana leaves it produced wax esters with similar fatty acids as with PES2 and FAR6 but with much higher proportion of 18:0 fatty alcohols. A fusion protein between a Marinobacter hydrocarbonoclasticus WS and the Marinobacter FAR was shown to be as effective as separately expressed enzyme in producing wax esters in chloroplast of N. benthamiana leaves. The combination of these enzymes resulted in wax esters mainly composed of 16:0 and 18:0 carbon chains both in fatty acid and alcohol parts.

A number of insect FAR with activity towards 14 and 16 carbon acyl groups was expressed transiently in N. benthamiana leaves together with insect desaturases acting on these acyl groups. Substantial amount of 14:1 and 16:1 alcohols were formed. These genes are of potential great interest in order to produce wax ester with 14 and 16 monounsaturated carbon chains. Such wax ester would have a low melting point and reasonable oxidation stability.

A comprehensive study was done on the specificities of jojoba WS and Marinobacter WS in microsomal preparations from yeast expressing the encoding genes. Different acyl-CoAs and fatty alcohols were tested in 211 different combinations to give guidelines for selecting genes for transformation work in plants to achieve a particular wax ester quality. Parts of these studies has been published15.

For the detailed analysis of the wax ester species composition a semiquantitative nanoESI-MS² method was developed that is based on multiple reaction monitoring (MRM) detection of the intact wax esters with acyl chain combinations from C16 to C24 and 0 to 3 double bonds at either the OH or the FA moiety. The quantification is achieved by calibration with an internal 17:0/17:0 wax ester standard. Detailed calibration of the method were performed on wax ester composition in the transgenic Arabidopsis plants applying updated calibration response factors . The method was further expanded to measure acyl chains up to 32:1 to analyse wax ester compositions of transgenic approaches aiming at the accumulation of very long chain wax esters. The developed method monitors intensity profiles of 785 wax ester species that were divided into 14 prototype groups based on their acyl chain structure. These prototype groups result from extensive study of ionisation and fragmentation behaviour of representative standards of the different wax ester classes and enable the calculation of response factors for each group to achieve a semiquantitative analysis of all 785 wax esters. Commercially not available standards of very long chained unsaturated wax esters were synthesised form respective fatty alcohols and acyl chlorides. In summary the developed method can now be offered to ICON partners providing a method for wax ester profiling of the various transgenic approaches. A manuscript describing the method has been submitted16.

**References**

1. Rowland O, Domergue F. (2012) Plant fatty acyl reductases: enzymes generating fatty alcohols for protective layers with potential for industrial applications. Plant Sci. 193-194:28-38

2. Doan T, Domergue F, Fournier A E, Vishwanath S J, Rowland O, Moreau P, Wood C C, Carlsson A S, Hofvander P. (2012) Biochemical characterization of a chloroplast localized fatty acid reductase from Arabidopsis thaliana. Biochim. Biophys Acta 1821:1244-55

3. Domergue F, Vishwanath SJ, Joubès J, Ono J, Lee JA, Bourdon M, Alhattab R, Lowe C, Pascal S, Lessire R, Rowland O. (2010) Three Arabidopsis fatty acyl-coenzyme A reductases, FAR1, FAR4, and FAR5, generate primary fatty alcohols associated with suberin deposition. Plant Physiol. 153:1539-54.

4. Doan TT, Carlsson AS, Hamberg M, Bülow L, Stymne S, Olsson P. (2009) Functional expression of five Arabidopsis fatty acyl-CoA reductase genes in Escherichia coli. J. Plant Physiol. 166: 787-96.

5. Cheng JB, Russell DW. (2004) Mammalian wax biosynthesis. I. Identification of two fatty acyl-Coenzyme A reductases with different substrate specificities and tissue distributions. J Biol Chem. 279:37789-97

6. Cheng JB, Russell DW (2004) Mammalian wax biosynthesis. II. Expression cloning of wax synthase cDNAs encoding a member of the acyltransferase enzyme family. J Biol Chem. 279:37798-807

7. Heilmann, M., Feussner I. 2011. Methods and means to alter lipid biosynthesis by targeting multiple enzymes to subcellular domains. Patent application: PCT WO2011048119A3

8. Heilmann M, Iven T, Ahmann K, Hornung E, Stymne S, Feussner I. (2012) Production of wax esters in plant seed oils by oleosomal co-targeting of biosynthetic enzymes. J Lipid Res. 53:2153-61
9. E.-M. Biester, J. Hellenbrand, J. Gruber, M. Hamberg and M. Frentzen (2012) Identification of avian wax synthases, BMC Biochemistry 13:4

10. J. Hellenbrand, E.-M. Biester, J. Gruber, M. Hamberg and M. Frentzen (2012) Fatty acyl-CoA reductases of birds, BMC Biochemistry 12:64

11. E.-M. Biester, J. Hellenbrand and M. Frentzen, Multifunctional acyltransferases from Tetrahymena thermophile (2012) Lipids 47:371-81

12. Li F, Wu X, Lam P, Bird D, Zheng H, Samuels L, Jetter R, and Kunst L .(2008). Identification of the wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase WSD1 required for stem wax ester biosynthesis in Arabidopsis. Plant Physiol. 148, 97-107

13. Lippold F, Vom Dorp K, Abraham M, Hölzl G, Wewer V, Yilmaz JL, Lager I, Montandon C, Besagni C, Kessler F, Stymne S, Dörmann P. (2012) Fatty Acid phytyl ester synthesis in chloroplasts of Arabidopsis. Plant Cell. 24:2001-14

14. Hofvander P, Doan T T P, Hamberg, M (2011) A prokaryotic Acyl-CoA reductase performing reduction of Fatty Acyl-CoA to fatty alcohol. FEBS Letters 585: 3538-3543

15. Miklaszewska, M., Kawinski, A., Banas, A. 2013. Detailed characterisation of mouse wax synthase substrate specificity. Acta Biochimica Polonica, in press.

16. Iven, T. Herrfurt, C., Hornung, E., Hofvander, P., Stymne, S., Zhu, L-H, Feussner I. (2013) Wax ester profiling of seed oil by nano-electrospray ionization tandem mass spectrometry. Submitted to BMC Plant Methods

**WP4. Work towards production of long chain wax esters in Crambe and B. carinata**
**WP5. Work towards production of monounsaturated long chain wax esters in Crambe, B. carinata and Camelina.**

Successful outputs of WP4 and WP5 were crucial for the success of the whole ICON project. The strategy was to produce wax ester of jojoba type using the method that was reported to work in Arbidopsis by the Calgene company1. Initially synthetic jojoba FAR, jojoba WS and Lunnaria annua FAE were ordered with codon optimization for expression in Brassicaceae species on the basis of sequences in the Calgene patents. A construct with these tree genes was transformed into B. juncea and Arabidopsis. However, transformed seeds were shown to contain only trace amounts of wax esters, in sharp contrast to the up to 60% of oil reported by Calgene. The results obtained were very similar to the results in Arabidopsis reported by Calgene when only jojoba FAR was expressed2. Further, no increase in 24:1 fatty acids were seen, which was expected to be produced by the introduced FAE. We therefore expressed the L. annua FAE in yeast and found no activity. In the meantime our Canadian partner (NPRC-PBI) had cloned the L. annua FAE and could show that the sequence that we used, published in a Calgene patent, contained errors. We suspected that also the published jojoba FAR and WS contained errors. Since the failure to repeat the Calgene results on wax production in

seeds jeopardized large part of the ICON project, we decided to clone and use the native jojoba gene and do this as rapidly as possible. At that time developing jojoba seeds were available in Australia and our participant CSIRO collected seeds and prepared RNA. This RNA was sent to participant UGOE who prepared cDNA and sent that further to participant UNL who cloned the jojoba WS, FAR and FAE genes. Sequencing the genes showed that the sequences were identical to published sequences. Nevertheless, ds Red selection gene constructs with the jojoba native FAR+WS and FAR+WS+FAE (FAE either from jojoba or from L. annua with correct sequence) were done and the three-gene constructs were transformed into Camelina and Arabidopsis. Transgenic seeds from both species now showed high amount of wax esters.

The plausible explanation of the failure in using the synthetic genes is that codon optimizing the jojoba WS gene of some reason gives an inactive (or no) protein. As soon as wax ester production was demonstrated in Camelina and Arabidopsis, constructs with the same genes but with kanamycin selection were transformed into Crambe and with hygromycin selection into B. carinata. The B. carinata line transformed was a GM line expressing a FAD2-RNAi and a Crambe FAE, having decreased amount of polyunsaturated fatty acids and increased erucic acid (56%). All three industrial oil crop platforms produced significant amounts of wax esters in their seeds.

Camelina lines contained up to 30% of wax esters of oil and these were dominated by 24:1 carbon fatty acids and alcohols. Gene constructs were also done where the L. annua FAE was exchanged with either Cardamine graeca or jojoba FAE, but gave similar wax ester profile and content. Introduction of a FAD2RNAi together with FAR,WS and FAE led to decreased levels of polyunsaturated carbon chains in the wax esters (mainly 18:2, 24:2 and 24:3) and large decrease in 24:0 fatty acid and much increased 22:1 alcohols. The oil content was depressed in all lines with about 50% but was partially restored in lines expressing the FAD2-RNAi. Germination was severely affected but was also partially restored in the FAD-RNAi lines. Germination and seed setting in lines with lower wax ester content (about 15% of oil) was close to normal under greenhouse conditions.

Crambe and B. carinata lines showed very similar wax ester profiles in their seeds. With two gene constructs, the wax esters had 22:1 as the nearly only fatty alcohol and 22:1 and 20:1 as the dominating fatty acids. Gene constructs also harboring the jojoba FAR had in addition to these carbon chains significant amount of 24:1 fatty alcohols and fatty acids. The amount of polyunsaturated carbon chains were very low in the wax esters of B. carinata whereas it amounted to about 7% of the wax ester species in Crambe, composed primarily of 18:2 and 18:3 fatty acids and 22:2 alcohols.

Crambe seeds with up to 60% of wax esters of oil were identified but these seeds did not germinate. Crambe seeds having the FAE had poor germination and poor seed setting even at low amount of wax ester whereas lines with 2-gene construct and about 30% of wax esters showed normal seed setting and only slight delay in germination. Lines with two gene and average three gene constructs with both an average of 25% wax esters were pooled and planted in field tests on 100 m2 in Sweden 2012. The Swedish Agricultural Board, the authority approving GM field tests, imposed to us to have a tight insect over our plots in order to prevent bees to come in contact with plants and thereby transfer pollen to their honey. If honey contains any pollen from non-approved GM events, it

cannot be sold as food. This, combined with an unusually cold and rainy summer delayed plant development. The transgenes were germinating and establishing later than the control and had a slower seed development. Thus, when harvest was done late September, many seeds had not matured and this was in particular the case for the transgenes, as reflected by their high chlorophyll levels.

The Crambe lines with three-gene constructs did extremely poor with only about 10% of the seeds developing into plants, which had very poor seed setting and no significant amounts of wax esters. It appears that only escapes made it to seeds. The lines with the two gene construct on the other hand made it surprisingly well considering the bad growing conditions and had a seed yield per acreage of 75% of control and with the same oil content as control and had 16.5% of wax esters of the seed oil. Harvest from lines with 2-gene constructs from this field trial will be used as seeding material in the field trials in Sweden 2013. Now authorities have given us permission to grow without insect net providing that it is 3 km to nearest bee hive.

Crambe wax ester lines with 2-gene construct were taken further through the generations to achieve stable wax ester lines with good seed setting. Lines with T5 seeds with 24% of wax ester with little variation between seeds and normal seed setting are now taken further to elite lines that will be tested in fields in Sweden 2014.

This WP also included tests of wax ester in grease formulations by our industrial participant Axel. Since the field trials were performed one year later than planned, Axel could not do the tests with wax esters from transgenic plants. However, the wax esters produced by B. carinata and Crambe were very similar in composition to the jojoba wax esters and results obtained with jojoba wax esters could be more or less be directly translated to the wax ester produced in these transgenic plants. Sulfurized oils have been used commercially in lubricants as extreme pressure (EP) additives for almost a hundred years. Sulfurized sperm whale oil was one of the most efficient sulfur carriers used until the bans on whale oil use were instituted in 1972. Since then new alternatives, at least as efficient, has been developed. The sulfurized jojoba oil tested in the project, and by many others before this study, will not bring anything revolutionary to the market if they were to be commercialized. They could work as EP-additives in the wide range of products that already are using sulfur carrier additives and if the price was right they would definitely find a market niche. The study shows that the use of wax esters as base fluid in lubricating grease improves the wear protection performance and the oxidation stability compared to existing environmentally adapted alternatives. In the two types of greases studies, anhydrous calcium soap and lithium soap, the effect is much more pronounced in the case of the calcium soap. It is known that esters generally reduce wear compared to mineral oils.

This is probably due to the strong dipole moment of the carboxylic bond that makes them adhere stronger to the metal surface. Why liquid wax esters should provide even better wear protection compared to triglycerides is unknown. Greases based on jojoba oil runs with very low heat formation in the R2F bearing test rig. This indicates that the greases based on wax esters have good lubricity and friction properties. The low heat emissions seen in the bearing test rig are very interesting from an energy conservation perspective. The drawbacks of using the currently available liquid wax esters is only the performance in low

temperature environment. Since the jojoba type of wax esters solidifies around 9°C and this will prevent use in central lubrication systems, increase the starting torque of lubricated equipment and stop oil bleeding and thereby starve the lubricating film. This limits the use of a grease based on this oil to areas with a warm climate. If the low temperature properties can be improved by tweaking the molecular structure of the wax esters, maybe in combination with using pour point depressants, it should be possible to develop a high-performance biodegradable grease based on this oil. Such a product will be more or less unique on the market and if correctly priced it has the potential of attracting many users.

1. Lardizabal KD, Metz JG, Sakamoto T, Hutton WC, Pollard MR, Lassner MW.(200) Purification of a jojoba embryo wax synthase, cloning of its cDNA, and production of high levels of wax in seeds of transgenic arabidopsis. Plant Physiol. 122:645-55.

2. Metz JG, Pollard MR, Anderson L, Hayes TR, Lassner MW. (2000) Purification of a jojoba embryo fatty acyl-coenzyme A reductase and expression of its cDNA in high erucic acid rapeseed. Plant Physiol. 122:635-44.

**WP.6. Production of wax ester with only C18 monounsaturated carbon chains in Crambe and B. carinata.**

As stated above in lubricatioin tests with wax esters of jojoba type, these have too high melting point for a wider use in lubricants, despite other excellent properties. The ICON project therefore included to develop other target wax ester levels with lower melting points and with the same and better oxidation stability than the very long chain wax esters. Such wax esters have not been reported to occur in any plants and thus it was not known if this actually could be achieved. Oleoyl-oleate (18:1-18:1) wax esters have a melting point of -4OC, considerably lower than the 9oC for the jojoba type and should have the same oxidation stability. We regarded oleoyl-oleate wax esters as the most easy other type of wax ester that could be obtained in transgenic plants.

In order to tailor suit the wax ester quality, the first step would be to alter the composition of fatty acids produced in the seeds. In case oleoyloleaste production it would mean to increase the amount of oleic acid as much possible. Therefore Crambe and Camelina were transformed with gene constructs with FAD2-RNAi + FAE-RNAi to prevent conversion of oleic acid to linoleic or very long chain fatty acids. Oleic acid levels were increased to over 70% in Camelina (Wt has 12%) and over 80% in Crambe (Wt has 16%). Next step was to identify FAR and WS enzymes/genes that have high activity towards C18 carbon chains. As mentioned above under WP3, mouse WS and FAR genes with desired properties could perhaps serve this function. However, they are not co-localised in the cell and therefore modification of the genes were done to co-localise the enzymes as described in WP3. Genes constructs with oleosin fusions were transformed into Arabiodospis fae/fad2, a mutant having much increased levels of oleic acid. Lines with wax esters levels up to 16% of oil and with 65% of the wax ester species being oleoyl-oleate were obtained (see also WP3). Less success have so far been achieved in crop plants. A number of combinations of candidate FAR and WS was introduced in a high oleic Camelina background or with an added FAD2-RNAi gene. The best candidates genes judged from their characterization in WP3 would be the mouse genes and Marinobacter FAR and Marinobacter WS and these have been transformed either as single genes or as fusion proteins (shown to be

active in yeast). The evaluation of these plants is still going on and will continue at least one year after the end of the ICON project. So far, the highest amount of C18 carbon chains has been achieved by combining the Marinobacter FAR with the jojoba WS. Camelina ines with up to 20% of wax esters composed mainly of C18 carbons were obtained, although a high proportion of these were polyunsaturated and the wax esters also contained significant amount of 20:1 carbon chains. The same gene construct is likely to give mainly C18 monounsaturated wax esters when transformed into a high oleic background. This work will be carried out after the end of ICON.

**WP7. Production of wax esters with medium chain fatty acids in model plants.**

If wax esters with saturated carbon chains shorter than C16 could be obtained, these would be very resistant to oxidation and have reasonable low melting point for certain applications. In the project description of ICON in Annex I it was originally suggested that we should demonstrate medium chain wax ester production in Arabidopsis as a model plant. However later in the project, Camelina was introduced as a model plant, due to its ease to transform and the much bigger seeds, but it was also introduced as a possible industrial oil crop platform by our US participants. By transferring different acyl-ACP thioesterase genes from Cupeha species, Camelina lines with seed oils composed of various amount of 8:0, 10:0, 12:0 and 14:0 fatty acids were developed as background for further transformation. However, no FAR with good activity towards medium chain acyl groups were identified in WP3 although some of the FARs showed activity towards 14:0 fatty acid. Therefore a number of combinations of these FARs were transformed into 14:0 producing Camelina lines together with selected WS. Evaluation of these plants is still ongoing and will continue at least one year after the end of the ICON project. Among the so far analysed Camelina lines, none has shown any substantial amount of medium chain carbon groups in wax esters. The highest amount of 14 carbon chain was achieved in transformation with fusion gene between Marinobacter and Mouse WS. Only trace amounts of 14:0 alcohols and about 10% of 14:0 acyl chains were found when the fusion gene was expressed in a 14:0 thioesterase expressing background with a total wax ester content of about 10% of the oil.

**WP8. Production of wax esters with hydroxy groups in model plants.**
Ricinoleic acid (12-OH-18:1?9) is appreciated for its good lubrication properties due to its in-chain hydroxy group. Could such in-chain hydroxy groups be introduced in the carbon chains of wax esters, it is predicted to significantly improve the lubrication properties of the wax esters. Ricinoleic acid is produced by hydroxylation of oleate by a ?12 hydroxylase while oleate is esterified to phosphatidylcholine1. The castor bean ?12 hydroxylase have been expressed in Arabidopsis and, together with other genes from castor bean, shown to give up to 30% of hydroxylated fatty acids in the seed oil2. The hydroxy fatty acid can be transferred from phosphatidylcholine to triacylglycerol oil by acyl-CoA independent acyltransferases such as PDCT and PDAT. However for synthesis of wax esters with hydroxy groups, these acyl groups have to be present in the acyl-CoA pool to be available for FAR and WS. The main effort in this WP was therefore allocated to search for genes/enzymes that could transfer such hydroxy fatty acid from PC to the acyl-CoA pool. Of special interest were enzymes from Lesquerella fendleri since these plants accumulate about 55% of lesquerolic acid but just a few percentage of ricinoleic acid in its oil. Lesquerolic acid is an elongation product of

ricinoleic acid produced on phosphatidylcholine3. Thus it can be predicted that virtually all ricinoleic acid produced in Lesquerella seeds are transferred to the acyl-CoA pool for elongation. Two enzyme types have been suggested to catalyse such transfer, ricinoleic acid specific phospholipases3 and possibly also lysophosphatidycholine acyltransferases (LPCATs). Work was carried out regarding the involvement of LPCAT in the removal of ricinoleic acid including biochemical characterisation of seven LPCAT genes from five different species.

The results (manuscript under revision) together with recently published in-vivo work4 strongly indicated that LPCAT is very efficiently transferring ricinoleoyl groups from PC to the acyl-CoA pool, at least in Arabidopsis and Lesquerella. The bottleneck in accumulation of ricinoleoyl groups in triacylglycerols in Arabidopsis was seen in the utilisation of diacylglycerols with ricinoleoyl groups4, which should not be a concern regarding accumulating of wax esters with hydroxy groups. Marinobacter FAR and Marinobacter WS were shown to have reasonable activities towards ricinoleoyl fatty acids and ricinoleol fatty alchols. Therefore a Marinobacter FAR -Mouse FAR fusion gene was transformed into Camelina lines expressing Claviceps pururea delta12 hydroxylase (having about 15% of hydroxy fatty acids in the seed oil). This fusion gene construct gave about 10% wax esters with substantial amounts of 18:1 and 20:1 fatty acids and alcohols when transferred to Wt Camelina (see WP6). The main hydroxylated species found in transgenic Camelina is ?12 hydroxy-18:1 (ricinoleic acid) and ?14hydroxy-20:1 (lesquerolic acid). Seeds will be harvest and analyzed after the end of ICON project. We have also identified a novel PLA enzyme from Arabidopsis, belonging to the so called LCAT enzyme family. The enzyme fulfill many criteria of the properties of enzymes removing unusual fatty acids, such as ricinoleic acids, from phosphatidylcholine. However such specificity could not be demonstrated by the Arabidopsis enzyme. The work has resulted in one publication5. Characterizing the homolog gene in Lesquerella has so far not yielded any information that indicates that this gene should have any different properties compared to the Arabidopsis gene.

Since participant USDA can transform Lesquerella, an additional work task was added to WP8 to increase the chances of progress in this WP: Transformation of Lesquerella fendleri with genes for wax ester synthesis. However no transformed plant with jojoba FAR and WS genes survived to seed setting.

**References:**

1. Bafor, M., Smith, M.A., Jonsson, L., Stobart, K & Stymne, S. (1991) Ricinoleic acid biosynthesis and triiacylglycerol assembly in microsomal preparations from developing castor-bean endosperm. Biochem. J. 280, 507-514.

2. Engeseth, N. & Stymne, S. (1996) Desaturation of oxygenated fatty acids in Lesquerella and other oil seeds. Planta 198, 238-245

3. Bates, P.D. and Browse J. (2011). The pathway of triacylglycerol synthesis through phosphatidylcholine in Arabidopsis produces a bottleneck for the accumulation of unusual fatty acids in transgenic seeds. Plant J. 68:387-99.

4. Ulf Ståhl, Bo Ek and Sten Stymne (1998) Purification and characterization of a low molecular weight phospholipase A2 from developing seeds of elm (Ulmus glabra) Plant Physiology 117, 197-205.

5. G. Chen, M. Greer, I. Larger, J. Yilmaz, E. Mietkiewaks, A.S. Carlsson, S. Stymne, R. J. Weselake. (2012) Identification and characterization of an LCAT-like Arabidopsis thaliana gene encoding a novel phospholipase A. FEBS Letters 586:373-377

**WP9. Production of branched chain wax esters in model plants.**
If methyl branched groups, preferentially with multiple methyl groups, could be introduced in the carbon chains of wax esters, this would drastically lower the melting point and improve lubrication properties without decreasing oxidation stability. To achieve such wax esters in seeds was the greatest challenge of all the research tasks in ICON. Birds are known to secret methyl branched wax esters from their preen glands. Although the de novo fatty acid biosynthesis of methyl branched fatty acids cannot be copied into plants the FAR and WS that act on these branched chain acyl groups in the birds would be of value to identify. This work in reported in WP3 and resulted in identification of FAR that could act on methyl branched acyl groups and WS that could esterify branched chain fatty acids and use branched chain fatty alcohol. In order to produce branched chain acyl groups in seeds following strategy was proposed: Seeds can be engineered to produce medium chain fatty acids (C10 to C14) by introduction of Cuphea acyl-ACP thioesterases with preferences for these acyl groups. These medium chain fatty acids are produced in the plastid and exported out in the cytosol of the cell. By introduction of enzymes in the cytosol producing methyl-malonyl-CoA and beta-keto synthases that could use methyl-malonyl-CoA and able to elongate these medium chain fatty acids, longer acyl groups with methyl groups inserted towards the carboxylic end would be synthesized. We wer building on the progress of the EC project REFLAX by participant CNRS but as mentioned above, avoided the bottleneck experienced in the export of the branched chain fatty acids out from the plastids and instead localize the synthesis in the cytosol of the plant cell. The first step in this WP was to introduce methyl branched fatty acid synthesis in yeast. In order to produce methyl-malonyl-CoA in the yeast cell, the methyl-malonyl-CoA synthase gene RtMAT from Rhizobium trifoli was expressed. Upon feeding the transgenic cells with methyl-malonate and analyzing extracts on HPLC, substantial amounts of methyl-malonyl-CoA were found. In order to introduce a condensing enzyme that could use this methyl-malonyl-CoA in elongation we expressed the genes encoding condensing and acyl transferase domains of the mycolic acid synthase (MAS) from Mycobacterium tuberculosis.

The encoded proteins were predicted to work together with endogenous yeast condensing enzymes to confer the ability of the yeast cell to condense methyl-malonate. By feeding 12:0-CoA, 14:0-CoA and 16:0-CoA and radioactive methyl-malonyl-CoA to membranes from the transformed cells, 2-methyl branched elongation products up to C18 was formed. Transformed cells co-expressing RtMAT , condensing and acyltransferase domain of MAS was shown to contain up to 2% of their fatty acids as methyl branched fatty acids when the cells were fed methyl-malonate. However , the production of branched chain fatty acids still rely on the feeding the cells methyl-malonate. Work will proceed after ICON to introduce the whole branched chain pathway into yeast including the methyl-malonate production. The first step will be to improve the connection between the domains from MAS and the endogenous yeast elongase domains.

**WP10:. Cellbiology of wax ester accumulation and mobilization in plants.**

In order to utilise the wax esters as energy source in a germinating seed, they first have to be cleaved by a lipase to fatty acids and fatty alcohols. The fatty alcohols have then to be oxidized to fatty aldehydes by a fatty alcohol oxidase (FAO) and further converted to fatty acids by a fatty aldehyde dehydrogenase (FADH) before they can be shunted into beta-oxidation and thus be used for energy for the germinating seed. It was anticipated that this mobilization of wax esters could be a bottleneck for seed viability in transgenic plants accumulating wax esters. Lipases, FAO and FADH genes highly expressed in germinating jojoba seeds were cloned. Proteomic analysis indicated that the FAO and FADH proteins can be detected on wax bodies, but they localized to the endoplasmic reticulum when they were expressed as amino-terminal green fluorescent protein fusions in tobacco (Nicotiana tabacum) leaves. The FAO and FADH genes were expressed in E. coli, purified and biochemically characterized. The recombinant proteins were active on very-long-chain fatty alcohol and fatty aldehyde substrates, respectively, and have biochemical properties consistent with those previously reported in jojoba cotyledons. Co-expression of jojoba FAO and FADH in Arabidopsis enhanced the in-vivo rate of fatty alcohol oxidation more than 4-fold. Taken together, the data suggest that jojoba FAO and FADH constitute the very-long-chain fatty alcohol oxidation pathway that is likely to be necessary for efficient WE mobilization following seed germination. This work has been published in Plant Physiology1.

A homolog to Arabidopsis triacylglycerol lipase SDP1 expressed in germinating jojoba seeds were cloned. Arabidopsis was transformed with constructs containing a synthetic and a native version of the jojoba SDP1 homologue under the control of the 35S promoter. The transformants was crossed into the sdp1-5 mutant and homozygous sdp1 lines containing the transgenes expressing native jojoba SDP1 was recovered. These lines are rescued for the sugar-dependent seedling growth phenotype of sdp1 (determined as hypocotyl growth in the dark after 5 days) and at least partially rescued for TAG breakdown (as determined by % of 20:1 FA remaining after 5 ways). By comparison Arabidopsis SDP1 under the 35S promoter fully complements the sdp1 mutant. Thus it appears that the jojoba enzyme is less effient than the Arabidopsis homolog in hydrolyzing triacylglycerols. The characterization of this enzyme will continue after the end of the ICON project.

In order to detrmine structure function relationships of FAR enzymes, attempts to crystalize FAR proteins were undertaken. A method was develop to purify large amount of active FAR proteins by expressing them as fusion proteins with a maltose binding domain (see WP3). However no protein crystals could be obtained from heterologous produced, MBP-tagged FAR from Marinobacter aquaeolei VT8 (MaFAR). Since separation of MaFAR and MBP-tag after cleavage does not work satisfactory, the MaFAR coding sequence was cloned into the pCOLD expression-vector. This vector encodes for the "trigger factor" chaperon-tag. Hopefully this will make it possible to separate MaFAR from the tag more efficiently, in order to get pure MarFAR protein for crystallization screens. Work with this will continue after the end of the ICON project.

Studies regarding mobilisation of wax esters in transgenic Camelina was carried out. One probem in these studies was that the trait had not been stabilized and thus individual wax ester content could differ substantially beteen the seeds from the same plant. Therefore the results obtained so far are inconclusive. It is clear that Camelina seeds with high amount of wax esters germinate poor, but this might be due to other

factors than inefficient wax ester mobilisation. The results show that triacylglycerols and wax esters are mobilized at about the same rate in the transgenic Camelina and that no free fatty alchols could be detected. Camelina wild type and two lines expressing Lunaria FAE/jojobaFAR/WS were grown in soil in the glasshouse under standard growth conditions (in a random block design exp.). A significant reduction in germination frequency and seedling establishment was observed in both transgenic lines and mature plants were on average smaller (less dry mass), producing less seed. However, the seed produced by the transgenic lines was slightly (but significantly) larger on average. Wax-ester content of the seed was measured by TLC - FAME and was in the range of 14 to 19% of the oil.

The harvest seeds were stored at 15oC / 15% relative humidity. Seed from batches with the highest wax-ester content were then grown for a further generation with the same experimental design. This time there was no significant reduction in germination frequency, seedling establishment, mature plant dry weight, seed number per plant or seed size. Wax-ester content of the seed produced remained at 16-18% of the oil. If the explanations for the difference is that growing the transgenic lines in optimal conditions yields seed that perform better in the subsequent generation or if seed quality deterioate upon storage is not yet established. Developing seed and leaf material was harvested for lipidomic profiling that will be carries out after the end of the ICON project.

Previously, we demonstrated that surface lipids, such as waxes, are much more resistant than internal lipids to degradation at plant senescence2. Therefore, accumulation of oils on the surface of plants is one strategy to prevent metabolic losses during senescence or to produce lipids that might be detrimental if accumulated inside plant cells. All plants accumulate waxes on their surface, and in some cases at very high levels. Bayberry (Myrica pensylvanica) is a perennial shrub that accumulates wax at 20-25% of its fruit mass, the highest amount of surface wax known in the plant kingdom. Unlike the wax of most other species Bayberry fruit surface wax is composed almost entirely of saturated (16:0 and 14:0) acylglycerides (DAG, TAG and MAG). Through collaboration with Joint Genome Institute (JGI), we have sequenced the transciptome of the tissue producing the wax. Analysis of the RNASeq data indicates very high expression of genes involved in surface lipid metabolism, as opposed to genes known to be involved in the biosynthesis of acylglycerides in seeds. Also very highly expressed were ABC transporters that are members of the ABCG family, known to secrete wax and cutin to surface of plants. We will test the ability of these to transport lipids outside of cells in heterologous systems. One other family of lipid genes that are highly expressed in Bayberry are sn-2 glycerol-3-phosphate acyltransferases (sn2 GPATs). In Arabidopsis, these genes catalyse the synthesis of sn-2 monoacyglycerol (sn-2 MAG), which is a precursor for the surface lipid polyester cutin. To determine if sn-2 MAG could also be a precursor for TAG in Bayberry, 14C acetate and 14C glycerol labelling was done. Indeed we observed that sn-2 MAG is the 1st labelled lipid product, with DAG and TAG appearing later. Taken together, we believe that Bayberry uses a previously unidentified pathway to produce TAG. We are currently cloning highly expressed acyltransferases expressed in Bayberry, to determine their activity and whether its pathway can be reconstituted in other plant species. This work will continue after the end of the ICON project.

**References**

1. Rajangam AS, Gidda SK, Craddock C, Mullen RT, Dyer JM, Eastmond PJ. (2013) Molecular characterization of the fatty alcohol oxidation pathway for wax-ester mobilization in germinated jojoba seeds. Plant Physiol. 161:72-80

2. Yang Z, Ohlrogge JB. (2009) Turnover of fatty acids during natural senescence of Arabidopsis, Brachypodium, and switchgrass and in Arabidopsis beta-oxidation mutants. Plant Physiol. 150:1981-9.

WP11. Improving agronomic value of Crambe with non-GM technology

Crambe is generally very susceptible to Altenaria infection and usually needs to be sprayed with fungicides to avoid yield losses. ICON suppported the ongoing breeding program on Crambe by praticipant PRI and to put the focus on improved germ plasm for Altenaria resistance. A large variation in seed yield potential was found among the 20 Crambe lines tested also without Alternaria attack, from below 1000 kg/ha to more than 2200 kg/ha. The relative yield loss due to Alternaria ranged from 0 % to about 50 %, showing a wide genetic variation for Alternaria resistance. The best yielding varieties still suffered a 15 % yield reduction from Alternaria. While some lower yielding varieties did not differ in yield with and without fungicide, which indicates that these varieties have a high relative level of resistance to Alternaria. Crossing these more resistant lines with the best yielding lines may lead to varieties that combine a high seed yield with a higher Alternaria resistance and will continue after the ICON project ended. An article regarding components determining Crambe seed yield has been published1. In order to improve Alternaria resistance interspecies crossing were done between C. hispanica X C. abyssinica and C. kralikii X C. abyssinica and hybrids obtained. A manuscript of this work has been submitted and is under review. Field tests of these plants for testing Altenaria resistance are planned after the end of the ICON project.

When erucic acid is purified from the Crambe oil, the remaining fatty acids have also a value. This value could be increased if the polyunsaturated fatty acids could be decreased and oleic acid increased. The conversion of oleate to linoleate in seeds are catalyzed by FAD2 enzymes. It has been shown that in many oil crops substantial reduction of polyunsaturated fatty acids could be obtained by mutating the FAD2 gene that is most highly expressed in seeds. Therefore a project was initiated to identify mutations in FAD2 genes in Crmabe by Tilling, using massive parallel sequencing. A saturated mutation population of Crambe lines was developed. Two mutants in the most strongly seed expressed FAD2 (FAD2C3) were detected by 454 amplICON sequencing. For one mutation (918, from Leucine to Phenylalanine), the homozygotes were identified from M4 family (8 homozygotes, 15 heterzygotes, 6 wildtype plants). For the other mutation (1190, from Proline to Leucine), one M3 line was identified as homozygote. The oil composition of these two mutants was analyzed but showed no significant difference between the mutants and the wild type. Rescreening of the mutation population was done by Illumina Hi-seq 2000 and one mutant producing a stop codon in FADC3 was identified in a pool. Individual will be identified and taken to homozygosity after the end of the ICON project. It should be emphasized that considerable efforts both in labor and costs were allocated to this research task and yet no mutant with desired phenotype was obtained. A FAD2RNAi was transformed into Crambe and efficiently reduced the polyunsaturated fatty acids in the seed oil from about 14% to 3% (see WP2), which is probably more drastic decrease than could be achieved by mutation. Due to the huge regulatory costs to take that GM line to market, this cannot be economic justified. Crops traits developed by mutation breeding are exempt from regulation

and thus even if the costs to develop such crops are a magnitude higher than with GM technology, it is still a more economically feasible alternative. This illustrates the problems with the present GM legislation that is based on a technique and not the consequences of the technique, in this case the high oleic trait that is introduced in the plant.

Crambe seed cake cannot be used for feed due to very high levels of glucosinolates and have virtually no present value. Also, in order to avoid expensive animal feed studies in the regulatory approval, we do not want to use any parts of our industrial oil crops to feed or food. Therefore we initiated studies to investigate if Crambe seed cake could be used for making plastics and thereby increasing its value. Despite that a multitude of different processes and additives were tested, plastics produced from Crambe seed cake as the only protein source showed inferior properties for commercial use. However, mixing Crambe seed cake with gluten showed very promising results. The materials were first hot pressed at 100 °C and 130 °C with glycerol used as plasticizer. Tensile properties, water absorption, oil absorption and moisture content were determined for the pressed films. Films pressed at 100 °C were slightly more susceptible towards water, moisture and grease. A longer milling time and higher wheat gluten content improved the tensile properties significantly. The most promising formulations were extruded at 130 °C. Urea was added to slow down the cross-linking effect thus enlarging the processing window of wheat gluten/crambe mixtures. Tensile properties, SEM and HPLC analysis were determined for the extrudates. Lowering the extrusion temperature to 105 °C improved the quality of the extrudate significantly. Tensile testing showed that a glycerol content of 30 % was found to be the best. Milling time had an influence as extrudates from ball milled material showed consistently better results. Up to a certain amount, urea improved the tensile properties significantly. An amount of 15 % urea was found to be the best, as it lead to the most elastic extrudates, while at 20 % urea the elasticity decreased. Scanning electron micrographs were taken from the extrudates, which were considered the most representative, to gain further knowledge about the homogeneity. A formulation was developed, which showed potential to be extruded, which is the most common processing method for plastics today. Work was done to investigate if said formulation could be extruded in a pilot extruder, a LTE 20-48 twin screw extruder from LabTech Engineering Company Ltd, Thailand.

The formulation could be extruded into strands that were pelletized. Bands with good mechanical properties could be extruded from pellets and from "dough" material. The bands exhibit slight melt fractures but otherwise appear homogenous. To mimic the injection moulding process extruded samples were compression moulded into translucent films. These were compared to compression moulded films from "dough" material. The compression moulded samples were analysed for oxygen barrier properties at 23° C and 50% RH according to ASTM F 1927-07 using Mocon Ox-Tran 2/21 equipment from Mocon, Inc., Minneapolis, USA. Results show that Crambe based bioplastics could be processed into materials with good oxygen barrier properties, homogeneity and great cohesion.

**Reference**

1. Banglian Huang, Yiming Yang, Tingting Luo, Shu Wu, Xuezhu Du, Detian Cai, Eibertus N. van Loo, Bangquan Huang. (2013) Correlation, Regression and Path Analyses of Seed Yield Components in Crambe abyssinica, a

Promising Industrial Oil Crop American Journal of Plant Sciences, 4: 42-47.

**Potential Impact:**

ICON have had an impact on the scientific community as well as on media and the general public. Its impact on the competitiveness of European industry warrants special comments and considerations since the commercial applications of plant biotechnology are presently blocked within EU. The impact on these levels are discussed below. In a separate attachment there are pictures and links showing some of the disseminations to media and public. There is also a presentation done by the Coordinator Sten Stymne at a meeting at the Royal Swedish Academy for Agriculture and Forestry entitled: 'Where is Plant Biotech Research in Europe Heading?'.

**Impact on the scientific community**

The obvious scientific impact of ICON is of course its external scientific disseminations in the form of 27 peer reviewed published articles and 80 conference presentations. It should also be noted that many more publications are expected as a result of the ICON research that has finished recently or still is going on. To our satisfaction, nearly all the ICON participants have declared that they will have other funds to continue their ICON research task, at least for the coming year and several have funding for more than two years after the end of ICON. We use to depict ICON as an icebreaker for GM crops. A journey on an icebreaker is not a holiday cruising. Occasionally the icebreaker got stuck, had to reverse and find new routes for the way forward. For being able to find the best way forward, the whole crew has to work together and this has created a loyalty and cooperative spirit between the participants that for sure will survive and live beyond ICON. Even if the icebreaker ICON has done its work and the crew has disembarked, the path created by ICON is closing with new ice drifting in and new ships have to clear the way. When ICON started, only three fatty acid reductase genes and three wax synthase genes had been reported in literature.

Only in ICON, over thirty such genes have been characterized and wax ester production in plants have become a 'hot topic' in plant biotechnology. ICON has re-vitalized European plant lipid research. ICON have had its General assembly meetings in connection to either the biannual European Plant Lipid meeting or the International Plant lipid meeting. This has led to a substantial increase in the number of participants at these meetings. As much as 30% of the participants in the European Plant Lipid Meetings have been involved in the ICON project. ICON also involves many scientifically outstanding participants outside Europe who now have participated and improved the scientific quality of these European meetings in a way that would not have happened without ICON. ICON had more participants outside EU than inside and this has led to a much closer contacts between scientists in US, Canada and Australia and has led to many transatlantic collaboration projects between these participants that will long survive the ICON project.

Since ICON research tasks have been a mixture of basic science and applied science, the potential application of their plant lipid research have captured the interest also by participating research groups that had previously been purely basic science orientated. Not the least have the emotional, political and legislative aspects on plant biotech been between different regions around the world been lively debated. Thus, ICON has improved the understanding among the participating scientists the factors that form public perception and political decisions around plant biotechnology. It is as important that scientist understand society

as that society understand science, if scientists want their research to have societal impacts.

**Dissemination to media and general public.**
Substantial efforts by the ICON coordinating team has been devoted to dissemination to media. Without doubt, it has influenced how media is portraying plant biotechnology and GM plants and thereby significantly increased both knowledge and acceptance of plant gene technology among the public in Sweden. Thus, ICON demonstrates how public attitudes can be changed from uncritically accepting charlatanism and political populism to a science based judgments of risk and benefits of new technologies. It also points out the importance of the involvement of scientists in this process and having good science communicator in this process.

In connection to the field tests with Crambe in Sweden 2012, media was invited to press conference and after that site visit at the field trials. The response was very good with both local and national TV and radio and a large number of daily newspapers as well as more specialized journals reporting about our research. All the reports were very positive to the research aims of ICON even if anti-GMO activists also were interviewed in some of the articles. The fact that we were forced by the Swedish authorities to grow the Crambe under insect net evoke a lot of interest and allowed us to explain the EU legislative reasons for this and also convey the message that the legislation around GM crops is not primarily a risk based legislation but a legislation based on the technology as such. It also gave us an opportunity to explain that a gene inserted gene technology is not any different from any other gene and the zero tolerance for pollen from GM field trials has nothing to do with risks. This was also reported accurately in all media. It should be noted that these field trials were done without any fence or other measurements to prohibit any destruction of the field tests. Yet, or perhaps because of that, no vandalism of the field trial occurs.

Sweden is probably the only country now in EU where GM field trials can be done without risks of destruction. The Governmental body, The Swedish Gene Technology Advisory Board (Gentekniknämnden, http://www.genteknik.se/sv/in-english ) also visited the field trials and was informed by the Coordinator (who is also a member of this board) about the aim of the ICON project. This Board consists of one member from each political party represented in the Swedish parliament as well as experts and have the Government's directive to assure that gene technology will used in a way that environment and health is protected as well as research in the area is stimulated. The Board should also follow the development of gene technology and inform the public about the progress and consequences of the technology. It is also considering proposed EU and national legislation and regulations in the area of gene technology, such as market approval or field tests of GM plants. The experience of having such a Governmental body comprised both of politicians and experts and having a mission to inform the public about GM technology might have significantly contributed to that the debate on GM plants in Sweden is more science based than in most other EU countries.

Not only were there many media reports about out field trials. It also evokes several debate articles in various Swedish media, questioning the validity of the arguments against GM technology expressed by many NGOs. Although scientifically successful, the most important impact of ICON might not be in the science but in the increased public awareness of the

potential benefits the GM technology can have on environment and rural economies that the project have conveyed.

**Impact of ICON on European competiveness and development of the Knowledge Based Bioeconomy.**

Twelve percentage of agriculture land in the world is now planted with biotech (GM) crops but in Europe it is close to nil. From only having a few traits (herbicide and insect resistance) introduced by genetic engineering in commercial crops, the number and sophistication of the traits introduced are now rapidly expanding. Genetic engineering is on the verge to be an integrated technology in plant breeding in many parts of the world. However, due to political and legislative hurdles, the application of gene technology in agriculture is in practice blocked in EU. This has resulted in that no biotechnology company is any longer developing GM crops for cultivation in EU. Also, the major parts of their research have moved outside EU. Thus, if any further research in this area should continue in EU, it has to be supported with public resources. It is of major concern that EC in their last FP7 call for proposal did not have any topic that fitted with the development of GM crops.

The demand for industrial support, expressed in the outlines for Horizon 2020 research, will effectively exclude any project aiming at using GM plants. Thus the present situation for plant biotechnology in Europe is alarming. If not plant biotech research is maintained and developed within EU it will be catastrophic for future EU agriculture competitiveness and for the attempts to develop a Biobased Economy. Due to the present above described situation, the achievements in ICON is not possible to exploit in EU. We have therefore initiated contacts with Brazil. Crambe is a potentially very promising crop in Brazil since it has shown to be an excellent break winter crop instead of maize after soybean cultivation during the summer. Further, Crambe grows well even without use of fertilizers after soybean harvest and can be cultivated using the same land and management tools as used with existing grain crops. Since it is a short rotation crop, it can be used as a second crop also in areas where it is not possible to grow both maize and soybean during the same year. Unlike other main grain crops cultivated in Brazil, Crambe reduces nematode attacks on following crops and can be a good rotation crop to these crops. We intend to sign a contract with Brazilian partners for field testing of our GM Crambe lines and, if these tests looks promising, taking Crambe with ultra-high erucic acand wax esters through to market approval and commercial cultivation. It is sad that we have to turn outside EU to exploit innovations developed with member state tax money but it is anyhow a better alternative than let these plant be left on the shelf. In this way they will anyhow benefit the global society and some revenues might float back to EU in form of license fees.

The coordination team of ICON realize that EC cannot change political decisions made by European council and European parliament, but we are somewhat disappointed that the The lenient attitude towards scientific charlatanism and pure lies regarding the GM technology put forward by lobbying groups have resulted in  a legislative nightmare regarding GM plants in EU. The only way forwards is to aggressively oppose such populist pressures and advocate for a science based biosafety legislation that is technology neutral to replace the present legislation. Unfortunately,  we have not seen any steps taken in this direction. Presently, it appears that the EC has surrendered and giving up the

aspiration that EU should use gene technology in their own agriculture but will increasingly be dependent on import of such products. We also fear that this will be reflected in the lack of funding of plant biotech research in Horizon 2020 and thus the loss of scientific competence in this area, a research area that without doubt sooner or later will be implemented also in EU, for the sake of the environment and the economic competitiveness in the agriculture sector.

The ICON participants have been driven of a mission that their research would benefit the European society and environment and the progress achieved have been very good. However the traits developed in ICON fade in comparison to what now can be achieved in plant biotech that was impossible to even think of when ICON application was written. The advancements in metabolic profiling and gene sequencing have just been breath taking the last five years. For example, our participant CSIRO has already demonstrated how metabolic engineering can be used to produce up to 17% oil content per dry weight in tobacco leaves, which translated to field corresponds to 3.4 ton oil per ha, i.e. approaching that of oil palm of 5 ton/ha. Plant biotechnology is a truly game changing technology that will radically change how agriculture products are produced, contributing to food security, maintaining wild habitats and deceasing environmental impact. EU has to be part of this development!

**List of Websites:**