PRODUCTION OF WAX ESTERS IN *CRAMBE*

epobio

Realising the Economic Potential of Sustainable Resources
- Bioproducts from Non-Food Crops

PRODUCTION OF
WAX ESTERS IN *CRAMBE*

Outputs from the EPOBIO project
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EPOBIO: Realising the Economic Potential of Sustainable Resources - Bioproducts from Non-food Crops

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CONTENTS

EXECUTIVE SUMMARY

EPOBIO is an international project to realise the economic potential of plant-derived raw materials by designing new generations of bio-based products that will reach the marketplace 10-15 years from now. At a Workshop held in Wageningen in May 2006 a wide range of experts considered the Flagship theme of plant oils and identified a lubricant product developed in the non-food oilcrop *Crambe abyssinica* as the first target for EPOBIO to consider. This report sets out the conclusion of a detailed literature review and an analysis of environmental impacts and the economic case. It also takes account of inputs from international scientists and industrialists.

Bio-renewables, such as plant-derived oils, are a sustainable means of providing the essential products needed by society. In this context, plant oils are already major agricultural commodities with around 20% by value used for non-food applications. Two plant-derived fatty acids, erucic and lauric acid, have been competing with petroleum alternatives for many years. Historically, cost has been the major bottleneck limiting the development of new plant-derived oils. But, in the context of the escalating cost of crude oil and also the increasing concerns about both finite supply and security of supply, there is an emerging strategic need to develop additional renewable products from plant oils.

This report shows that the production of wax esters for the manufacture of lubricants, from the non-food oilcrop *Crambe abyssinica* can become viable in Europe. Viability would be further enhanced in scenarios where the hulls and meal remaining after oil extraction are used to produce heat and/or electricity for use in the production process. In addition to the economic benefit of using the co-products for bioenergy, this alternative to the use of fossil-derived energy would have the advantage of reducing carbon dioxide emissions and making a contribution to renewable energy targets. The reduced environmental impact of this renewable product demonstrates sustainable production.

Crambe is a low input crop when compared to many other oilcrops that could be cultivated in Europe. This offers potential to reduce the use and hence environmental burden of fertilisers and water. *Crambe* has been chosen as the candidate crop platform for industrial production of wax esters because its oil is not suitable for use in food applications. This is an essential requirement since the manufacture of wax esters in *Crambe* can only be achieved through genetic modification of the plant. The report recommends a gene discovery programme to identify the relevant enzymes for production of the relevant wax esters in high yields in parallel to optimisation of a routinely applicable plant transformation system for *Crambe* and an agronomy programme to achieve a robust, mainstream agricultural crop.

There is existing intellectual property (IP) in the area of wax ester production in oilcrops – this is typical of many applications involving the use of plant biotechnology. Analyses of the existing patent landscape and the opportunity for commercial development of wax esters in *Crambe* will be essential tasks needed to underpin future research and development of this application.

The implications for the use of a genetically modified plant, the impact of current GMO regulations in Europe and the associated substantial regulatory compliance costs have to be considered. Small and medium sized enterprises are unlikely to be able to bear the costs associated with these issues and so future exploitation is likely to be undertaken only by multinationals. Taken together, these constraints have the potential to limit development in Europe and lead to a continuing dependence on imported fossil oil and a continuing loss of competitive advantage to other countries and regions where the cultivation of genetically modified crops is not constrained.

The risks associated with the use of a genetically modified crop can be mitigated in a number of ways. First, the use of a crop which cannot be used for food or feed is important. This is considered essential from a regulatory perspective, given that the infrastructure in agriculture cannot ensure ‘fail-safe’ separation of different varieties/traits in the same crop species. However, the use of a non-food crop can have negative consequences since oilcrops such as *Crambe* have not been optimised for mainstream agriculture and their oil yield needs to be improved. Second, risks can be further mitigated by the choice of a crop for which inter-species crosses with the closest-related species give sterile offspring. It is not anticipated that *Crambe* will be able to cross easily with its related species. A third means of risk mitigation is the adoption of the same identity preservation practices for the cultivation of non-food GM crops as those already in place for the cultivation of GM foodcrops.

The Common Agricultural Policy (CAP) has decoupled subsidy from production and brought a new emphasis on market forces. *Crambe* for oil production could be grown on maincrop land and also on set-aside land, for as long as that cultivation option is retained in the CAP. The development of a new crop with clear market potential could help underpin commercially focussed farming in the future. Developments such as this will help to create and sustain employment both in the farming sector and in rural areas. In addition, the optimisation of processing an oilcrop for wax ester production will link into opportunities for integrated and zero waste, rural biorefineries further delivering economic benefit in rural areas.

Beyond Europe there is significant potential to develop alternative sources of wax esters. The report notes that current jojoba varieties have scope for improvement in collaboration with developing countries. Collaboration with the US on alternative crops such as soybean could benefit from information exchanged by research programmes on European oilcrops such as *Crambe*.

1 INTRODUCTION

This report targets a new bio-based wax ester product for lubricants. In particular, three classes of wax esters are identified as target oils to be used in lubricating formulations.

Previously, wax ester production in oil seed rape was developed pre-market during the 1980/90s. But a strategic decision by Monsanto was made to terminate the project. Nevertheless, there are considerable environmental and economic drivers to develop bio-lubricants. Of the various potential bio-lubricants, it is widely regarded that wax esters have excellent performance properties, but due to the high cost of obtaining waxes from existing sources, they are limited in use to specialised and high value product areas such as cosmetic and/or specialty lubricants.

European agriculture faces great challenges in the coming decades. Because of successively decreasing subsidies and import taxes, most of European agriculture will not be able to compete in bulk production in the free world-trade market. However, if agriculture can be re-directed for production of industrial products that replace mineral oil-based products, this will open new and huge potential markets. The steadily declining cost of agricultural products and the rapid increase in the price of the mineral oil favours an increased use of agricultural products in areas where traditionally only mineral oil-based products have been used. Mineral oil is a finite resource that provides essential fuels and raw materials for industry, and as this resource becomes limiting, oil prices will inevitably escalate to an even greater level than we currently experience. This will have a major negative impact on our economy and society unless alternative, sustainable sources of energy and industrial feedstocks are developed (Cambell and Laherrere, 1998). The rapid development of new plant molecular biology tools during the last two decades makes it now possible to produce novel chemicals tailor-made for technical purposes in our agricultural crops that can compete with mineral oil. Vegetable oils are the agriculture products that chemically are most similar to mineral oil and probably have the greatest potential to replace mineral oil in the chemical industry.

Wax esters are esters of long chain fatty alcohols and fatty acids. Wax esters have lubrication properties that are superior to ordinary vegetable oil, i.e. triacylglycerols (TAGs), due to their high oxidation stabilities and resistance to hydrolysis. High performance lubricating oils are often based on synthetic esters (such as wax esters) sometimes with the fatty acid part from plant sources. The only natural sources of wax esters have been the seeds of the desert shrub jojoba and sperm whale oil. The latter is now banned for hunting and the jojoba oil is too expensive for use in other products than in cosmetics.

This project is aimed at identifying the bottlenecks and underpinning research required to reach the target, that is, to produce wax esters cheaply in a non-food oil crop.

2 LUBRICANTS: PROPERTIES AND CHARACTERISTICS

2.1 General function of lubricants and examples of their uses

The movement of two surfaces against each other is restricted due to the force that is called friction. Depending on the situation the friction may be desirable or undesirable. For the sake of a safe ride, the driver of a car on an icy road is probably asking for as much friction as possible between the tyres of the car and the icy road. However, the same driver naturally asks for the lowest possible friction between all the parts of the car engine in order to avoid a premature failure that otherwise would follow due to wear and heat generation. A proper lubricant will help to minimise friction and is therefore a vital factor necessary for engines and machines to work properly. Its main function is to reduce wear and heat between two sliding surfaces. This is achieved by inserting the lower-viscosity material of the lubricant between the two surfaces having a relatively high-coefficient of friction. In addition, lubricants also conduct important tasks such as protecting the metal surfaces from corrosion, flush out contaminants, absorb shocks as well as seal foams and so forth.

2.2 Lubricating properties

The scientific studies of lubricants and lubrication technology are done in tribology, that is, the science of friction, wear and lubrication. These studies provide an understanding of the interplay between friction and lubrication and what factors are influencing this. The lubricating properties of a material are further determined from certain important characteristics. The most important are viscosity, viscosity index, compressibility, stability, pour point, cloud point, flash point and acid number (US Army Corps of Engineers, 1999).

Viscosity	Viscosity is a measure of the tendency of the lubricating oils to shear or more simply its resistance to flow. If an oil layer is imagined as several fluid layers superimposed on each other,

	viscosity is a measure of the resistance of flow between the layers. High viscosity implies a high resistance to flow and low viscosity a low resistance to flow. Viscosity varies inversely with temperature and is also affected by pressure. A higher pressure will cause the viscosity to increase, thereby increasing the load-carrying capacity of the oil.
Viscosity index (VI)	The viscosity index number shows the tendency of the viscosity of an oil to change as temperature changes. A lubricating oil with a high viscosity index will show only small changes in viscosity over temperature extremes and therefore is considered to have a stable viscosity. Knowing the viscosity index is specifically important in very hot or cold climates.
Compressibility	Compressibility is a measure of the amount of volume reduction due to pressure. It increases due to temperature and pressure and is an especially important characteristic for hydraulic oils.
Stability	The stability of a hydraulic fluid is an important property affecting service life. A number of physical and chemical changes to the oil induced by mechanical stress, cavitations, oxidations, hydrolysis and so forth, will each have an effect on the performance characteristics of the fluid. It is therefore important to choose an optimal additive(s) to stabilise the fluid in formulation for a specific application.
Pour point	The lowest temperature where the lubricating oil still flows is refereed to as the pour point.

Cloud point	Dissolved particles and substances can start to solidify and separate from the oil at certain temperatures. These temperatures are called cloud points. Depending on the composition of the oil some oils therefore need to operate above a certain temperature (cloud point) in order to avoid clogging filters.
Flash point and fire point	The flash point is the lowest temperature to which a lubricant needs to be heated in order for its vapour, when mixed with air, will ignite but not continue to burn. The temperature at which the lubricant vapour, after catching fire, will continue to be on fire is called fire point.
Acid number	Acids are formed as oils oxidise due to age and usage. The acid number is a measure of how much potassium hydroxide is needed to neutralise the acid in the oil. This number can be taken as an indication of the age of the oil and can be used to determine when it is time for changing the oil.

2.3 Lubricating base oils

A typical lubricant is comprised of more than 80% of a base-oil that together with various additives makes up the final product. The base oil predefines a whole range of property qualities such as high biodegradability, low volatility, high solvency for lubricant additives, miscibility with other types of system fluids, among others. It has also a major influence on other qualities like oxidative stability, low temperature solidification, hydrolytic stability and viscometric properties. On the other hand, additives and/or impurities are mainly responsible for lubricity, antiwear protection, load carrying capacity, corrosion prevention, acidity, ash content, colour, foaming, demulsibility, water rejection and so forth (Erhan and Asadauskas, 2000).

Base oils are mainly derived from distillation of crude petroleum and are classified as refined or synthetic. Paraffinic and naphthenic oils are refined from crude oil and synthetic oils are manufactured from chemically-derived precursors. Base oils derived from vegetable oil still only make up a small part of the total volume. However, for certain applications they do capture a distinct portion of the market and have a growing presence.

2.3.1 Mineral base oils

Paraffinic

The molecular structure of paraffinic oils consists of long linear chains of hydrogen and carbon atoms. They contain paraffinic wax and are the most widely used base stock for lubricating oils. Paraffinic oils are characterised by (US Army Corps of Engineers, 1999).

- Excellent stability (higher resistance to oxidation)
- Higher pour point
- Higher viscosity indexes
- Low volatility that gives a high flash point
- Low specific gravities

They are preferentially used for manufacturing lubricants such as engine oils, transmission fluids and gear oils.

Naphthenic

Naphthenic oils do not contain wax and behave differently from paraffinic oils. This is because they consist of a molecular structure with hydrocarbons rings. These oils are used for applications with narrow temperature ranges and where a low pour point is required. In comparison to paraffinic oils naphthenic oils have (US Army Corps of Engineers, 1999)

- Good stability
- Lower pour point due to the absence of wax
- Lower viscosity indexes
- High volatility (lower flash point)
- Higher specific gravities

The lower pour point and good solvency characteristics of naphthenic oils make them ideal for manufacturing of low temperature hydraulic oils, refrigeration oils, rubber process oils, metal working oils and greases.

2.3.2 Synthetic base oils

Synthetic oils are composed of oil components that have been synthesised rather than refined from petroleum or vegetable oils. Because their compositions are much more precise synthetic oils are generally superior to the refined oils. They perform better than the previous two in the following respects (US Army Corps of Engineers, 1999)

- Better oxidation stability or resistance
- Better viscosity indexes
- Much lower pour point, as low as -46°C
- Lower coefficient of friction

The qualities of synthetic oils are especially noticeable at either very low or very high temperatures. Their improved oxidative stability and lower coefficient of friction permit extended operation at high temperatures. The better viscosity index and lower pour point allows for smooth operation at low temperature.

The main disadvantage of synthetic oils is that they are approximately three times more expensive to purchase than mineral-based refined oils (US Army Corps of Engineers, 1999). However, this is somewhat balanced with their longer lifetime compared to conventional lubricants. But because of their high cost, synthetic oils should not be considered for high leakage or total loss applications.

There are several categories of synthetic oils of which some are listed below:

- Synthesised hydrocarbons – These synthetic oils have functions and qualities much like mineral oil-derived lubricants. Polyalfaolefins (PAO) and dialkylated bezenes are two examples and applications where synthesised hydrocarbons are used include engine and turbine oils, hydraulic fluids, gear and bearing oils, and compressor oils.
- Organic esters – Dibasic acid and polyol ester are the most common types and applications include crankcase oils and compressor oils.
- Phosphate ester – These are used in applications where fire-resistance is needed.
- Polyglycols – These are used for applications such as gear, bearings, and compressors for hydrocarbons gases.

2.3.3 Vegetable-based base oils

Vegetable oils have very good tribological properties. With few exceptions such as the waxes of jojoba oil, they consist of triacylglycerol esters containing 3 fatty acids with chain lengths of C8 through C24, with C16 and C18 as the dominating carbon chains. Due to their relatively inert hydrocarbon structures their positive properties are:

- Good boundary lubrication
- General wear protection
- High viscosity index
- High flash point
- Low volatility

Studies on the tribological properties of vegetable oils have concluded that (Bhuyan *et al*, 2006)

- Oils with longer carbon chains show lower friction and lower wear than shorter ones
- Oils containing saturated compounds show improved oxidative stability in high temperature/pressure applications in comparison to oils containing unsaturated ones
- Free fatty acids have a high degree of polarity and will react readily with metal surfaces to form a protective layer. They exhibit better tribological properties (improved boundary lubrication, lower friction and anti-wear abilities) compared to alcohols and methyl esters as well as triacylglycerol esters whose polarity is less. Free fatty acids and/or sulfurised fatty acid can therefore be used as additives to improve the lubricating properties of vegetable-based oils (Cao *et al*., 2000; Fox *et al*., 2004).

The main disadvantages with vegetable oils as lubricants are their poor low temperature fluidity behaviour and sensitivity to oxidation at high temperatures. For these reasons vegetable oils are limited in what they can be used for in applications up to 120°C (Maleque *et al*, 2003). The reason for the poor temperature properties is found in the molecular structure, i.e., the presence of double bonds on the alkyl side chains and the central β–CH group on the glycerol molecule. The β–hydrogen atom is easily eliminated from the molecular structure through oxidation. This leads to a weakening of the centrally positioned and usually very stable ester linkage, -C-O-R were R is a fatty acid. Heat alone will then break the ester linkage creating a carboxylic acid and a glycerol radical that leads to further degradation of the oil (Wilson, 1998; Wagner, 2001). Double bonds on the carbon chain are especially reactive and react with molecular oxygen to form radicals that lead to polymerisation and degradation. Polymerisation increases the viscosity of the oil, which reduces its lubrication functionality. Degradation results in breakdown products that are volatile, corrosive and diminish the structure and properties of the lubricants (Kodali, 2002).

The oxidative susceptibility of double bonds increases exponentially with addition of one and two double bonds, which is shown in Table 1.

Table 1 Fatty acids – rate of oxidation (Kodali, 2002).

	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)
Double bonds	0	1	2	3
Relative oxidation rate	1	10	100	200

A conventional method to improve the oxidative stability of the vegetable oil is by hydrogenation of the fatty acid. This eliminates double bonds and increases oxidative stability but at the same time destroys low temperature properties like pour point and viscosity index. Advanced plant breeding and genetic engineering are used to develop high oleic (HO) varieties with improved oxidative stability. HO varieties of rapeseed, sunflower and soybean with oleic acid levels of up to 90% have been developed in recent years (Cole *et al*., 1998, Stoutjesdijk *et al*., 2000). The chemical structure of such highly monounsaturated oils can be modified chemically to further improve the functional properties of the oil. An example is a family of bio-based polyols that Cargill successfully has introduced on the market and that has much improved low temperature fluidity, is oxidative stabile, has very high viscosity index, low volatility and is biodegradable (Kodali, 2002).

Additives can be used to improve the low temperature properties of a plant-based lubricating oil formulation. In addition, inclusion of functional groups such as hydroxy or branched fatty acids in the base oil itself could also be considered. For example, the irregularity imparted by hydroxy groups or branched-chain fatty acids would disrupt the lipid packing ability of the hydrocarbon chains, thereby reducing the melting temperature of the oil.

Wax esters constitute another target molecule by which the properties of a vegetable oil can be improved. The high linearity of wax esters enhances the viscosity index of the oil and imparts specific characteristics such as antirust, antifoam, antiwear and friction reduction properties to the lubricant (Bisht, *et al*., 1993; Kinway, 2004).

2.4 Classes of lubricants

Lubricating oils find applications in engines, industrial uses, grease and transmission fluids. The major oil uses (by volume) are for engine oils, transmission oils, gear oils, hydraulic oils and grease, where the first three make up about 70%, hydraulic about 10% and grease 3% of the world total use of lubricants.

2.4.1 Engine oils

Engine oils have to perform in different types of vehicles that run on either petrol or diesel, and also must operate in particularly harsh environments. Combustion gases, fuel and water in the oil as well as outside contaminants (dirt, sand, and other airborne materials) accelerate oil degradation, cause unique filtration problems, and result in much shorter change intervals compared to other typical automotive lubricants. The properties and composition of the engine oil are also important for a vehicles fuel economy and emissions. A low performance engine oil may risk contaminate the emission system of the vehicle as well as deteriorate the catalyst with increased emissions as a result (Tung & McMillan, 2004).

2.4.2 Transmission oils

Transmission oils lubricate an engine’s transmission. To provide the desired shift properties, one of the additives present in the oil is typically a long-chain hydrocarbon that has a polar group on one end. The long chain hydrocarbon portion permits the additive to dissolve in oil, and the polar group on the end is attracted to metal surfaces and provides the desired amount of friction (Tung & McMillan, 2004).

2.4.3 Gear oils

Gear oils typically operate at significantly lower temperatures that result in inherently less oxidation of the oil. Of importance is that the lubricating gear oil prevents premature wear of the gear tooth surfaces. The requirements of this type of lubricant are influenced from the type of gear application. Enclosed gears, where the oil is contained in an oil-tight housing, requires more of additives e.g. to prevent rust and oxidation, than open gears. For open gears it is vital that the lubricant does not disappear from the tooth surfaces due to the centrifugal force. These lubricants therefore are highly adhesive such like heavy oils, asphalt-based compounds or soft greases (US Army Corps of Engineers, 1999).

2.4.4 Hydraulic oils

The main purpose with any hydraulic fluid is to:

- Transmit power mechanically through hydraulic power systems. In this respect it is important that the fluid is incompressible and can flow easily.
- Provide lubrication to all parts of the hydraulic system and protect them from corrosion, rust, oxidation, wear and demulsibility (water release). These qualities are usually provided through additives.
- Provide the seal necessary between the low-pressure and high-pressure side of valve ports. The amount of leakage will depend on the viscosity and the tolerance between adjacent surfaces.
- Remove heat generated throughout the system.

In many applications where hydraulic fluids are used there is risk of leakage of the lubricant from the system to the environment. This has led to the development of environmental adapted lubricants (EALs) that is a growing segment for vegetable-based oils (Erhan and Asadauskas, 2000; Lea, 2002; Norrby, 2003).

2.4.5 Grease

Grease is composed of a fluid lubricant, a thickener and additives. The function of a thickener is to constitute a kind of three-dimensional structure that will hold the fluid in place in order for it to execute its lubricating properties. The fluid can be mineral, synthetic or vegetable oil. Thickeners can be soaps and organic or inorganic soap thickeners.

"The function of grease is to remain in contact with and lubricate moving surfaces without leaking out under gravity or centrifugal action, or be squeezed out under pressure. Its major practical requirement is that it retain its properties under shear at all temperatures that it is subjected to during use. At the same time, grease must be able to flow into the bearing through grease guns and from spot to spot in the lubricated machinery as needed, but must not add significantly to the power required to operate the machine, particularly at start-up". (Boehringer 1992).

Grease is generally used for machinery that is used intermittently or in storage for long periods. Further, grease provides good lubrication for machinery that is not easily accessible for frequent lubrication and for those that operate under extreme conditions such as high temperatures, high pressures or low speed. Grease can maintain thicker films in clearances enlarged by wear and thereby provide an opportunity to extend the life span of those equipments (US Army Corps of Engineers, 1999).

2.4.6 Process oil

Process oils are applications for which vegetable-based oils could expand into by replacing existing mineral-based oils. Process oil is used as ingredients in the manufacturing of diverse products like adhesives, explosives, fertilisers, industrial rubber, PVC and for making tyres for cars. For this production, manufacturers usually make use of the cheapest base oil that exists on the market. Recently the industry, for environmental reasons among others, shifted from aromatic base oils to using naphthenic base oils for process oils. However, if tyres e.g. were to be made from vegetable-based oils, this would result in even greater environmental gains due to the shift to a biobased renewable resource. The demand for process oils corresponds to about 10% of the total volume of all lubricating oils that corresponds to a substantial volume of 3.6 million tons.

2.5 Additives

Additives are important for improving the properties of the lubricating base oil up to a level where it can meet the specific requirements for different applications. In this respect they serve three main functions (U.S. Army Corps of Engineers, 1999):

- Surface additives - Protect the lubricated surface of the machinery
- Performance-Enhancing additives - Improve the performance of the lubricant
- Lubricant Protective additives - Protect the lubricant itself

2.5.1 Surface additives

- *Rust and Corrosion inhibitors* – These additives bind to the surface of metal parts in the machinery. In such a way they create a layer that prevents water from reaching the metal and rust from starting to develop. This layer also suppresses oxidation and the formation of acids.
- *Extreme pressure agents (EP)* – Certain points on the metal surfaces may experience increased friction, e.g. asperities of irregularities on wearing surfaces. EP agents react with these points, creating compounds with lower shear strength than the metal. This protective coating will reduce friction, wear and scoring. These agents are used in applications that experience heavy loading or shock such as turbines, gears, and ball and roller bearings. Sulphurised jojoba oil and sulphurised fatty acids (oleic and erucic) have shown excellent performance as added EP agents in vegetable-based oil (O'Lenick, 1999; Cao *et al.*, 2000).

- *Tackiness agents* – For applications where the oil needs to adhere to the surface extremely well. Examples are polymers of e.g. aluminium soaps of long-chain fatty acids.
- *Antiwear agents (AW)* – AW agents are polar molecules that bind or attach themselves to metal surfaces. In this way they form a layer that protects the metal surface from wear that can occur during boundary lubrication conditions e.g. direct contact between asperities. This reduces friction and wear up to 250°C. Anti-wear improvements can be achieved from adding sulphurised fatty acids to the base oil (Cao *et al*., 2000).
- *Detergents and dispersant* – Are added to a lubricant to keep compounds and substances dissolved in the liquid and avoiding a deposition onto metal surfaces.
- *Compounded oil* – A mineral oil that is blended with animal or vegetable oil in order to improve its performance as a lubricant.

2.5.2 Performance-Enhancing additives

- *Pour-point depressants* – The temperature where an oil does not pour or flow any more is referred to as the pour point. Molecules such as paraffin waxes tend to solidify and stiffen at low temperatures. Pour point depressants reduce the size and cohesiveness of these crystal structures, which reduces the pour point of the base oil and increases flow at lower temperatures.
- *Viscosity index (VI) improvers* – VI improvers are used to limit the rate of change of viscosity with temperature. Their effects are generally observed at higher temperatures where they allow the viscosity of the oil to increase within a limited range permitted by the oils and the concentration of the additive.
- *Emulsifiers* – Soluble oil requires that water can be emulsified rapidly and form stable emulsions. Emulsifiers are used in lubricants used for cutting, grinding, and drilling applications in machine shops.

- *Demulsifiers* – These additives help water and oil to stay separated from each other in lubricants exposed to water.

2.5.3 Lubricant Protective additives

- *Oxidation inhibitors* – During use and over time hydrocarbons will incorporate oxygen in their structure. This reaction will produce acids and other compounds that will damage and pollute the metal parts. They function by reducing the quantity of oxygens reacting with oil by forming inactive soluble compounds. These inhibitors are used up with time and it is therefore important to monitor the oil periodically for inhibitor level.
- *Foam inhibitors* – Air and other gases can get mixed up in the lubricating oil during action in an engine or machine and this will produce foam. Foam inhibitors function by reducing this foaming.

2.6 Lubricant Classification

Lubricants are formulated and produced for numerous types of applications, machines, equipment and so forth. When selecting the proper lubricant it is therefore important to have an accurate understanding of the situation where it is to be used, i.e. the lubricating regime, as well as an ability to interpret and apply the producer's product data specification. This has been facilitated by the work of professional societies and organisations that have established classifications for oil and grease. Some of the more widely encountered systems are listed below.

API	American Petroleum Institute (API Standard, 2002)
SAE	Society of Automotive Engineers (SAE, 2006)
ISO	International Standards Organisation (ISO Standard, 1992)
NLGI	National Lubricating Grease Institute (NLGI, 2006)

Oil classification can be based on (US Army Corps of Engineers, 1999):

- *Viscosity grade* – Common methods of describing the oil. The most used classification systems are those of SAE, AGMA and ISO.
- *Additives* – Classifies the oil on which additives have been used to improve the performance properties as follows: rust and oxidation inhibition, anti-wear (AW), extreme pressure (EP), compounded (mix-in of fatty acid or synthetic fatty oils) and residual.
- *Usage* – This classification is based on the usage of the lubricant as an engine oil (automotive, aircraft, marine, commercial), gear oil, cutting oil (coolants for metal cutting), insulating oil (transformers and circuit breakers), hydraulic oil and so forth.
- *Non-specialised industrial oils* – Some oils are not formulated for any specific application, and thus are referred to as either “general purpose oil” or EP gear oils. General purpose oils contain RO (rust and oxidation) additives, AW agents, anti-foamants, and demulsifiers. EP gear oils have generally higher viscosity and can be regarded as general purpose oils with EP additives.
- *Producers' brand name* - Oils are also referred to by the producers' brand names or their product data and specifications. These are highly varying and a user is usually left with studying the brochures, pamphlets, handbooks, or other information sources from the producer and trying to apply this to the lubricating regime where the lubricant is to be used.

Grease is classified by penetration number (established by NLGI) and by the type of soap or other thickener. Viscosity of the oil included in the grease also needs to be considered in selecting greases for certain applications.

3 CURRENT AND FUTURE POTENTIAL FOR BIO-LUBRICANTS

Major driving forces behind the growth of bio-lubricants are the efforts trying to reduce the number of pollutants released into nature and reducing the amount of CO_2 released to the atmosphere. Unlike petroleum-based fluids, vegetable oils can be used to prepare environmentally adapted and rapidly biodegradable lubricants and there is no net increase of CO_2 from them. Another consideration is that mineral-based lubricants use a limited and steadily decreasing resource (Goodstein, 2004) while the base for bio-lubricants is sustainable.

Table 2 Comparison of chemical and physical properties of biodegradable base fluids with mineral oil (average values) (Toscano, 2005).

Properties	**Mineral oil**	**Glycol**	**Vegetable oil**	**Jojoba oil**[a]	**Synthetic Ester**
Density at 20°C, kg/L	0.880	1.100	0.940	0.866	0.930
Viscosity index	100	100 to 200	100 to 200	210-233	120 to 220
Shear stability	Good	Good	Good	Very Good	Good
Pour point °C	-15	-40 to +20	-20 to +10	+9	-60 to –20
Cold flow properties	Good	Very good	Poor	Poor	Very Good
Miscibility with mineral oil	----	Not miscible	Good	Good	Good
Solubility in water	Not soluble	Very good to not soluble	Not soluble	Not soluble	Not soluble
Behaviour against paintings/coatings	Good	Poor	Good	Good	Good to poor
Biodegradability %	10 to 35	10 to 100	70 to 100	70 to 100	10 to 100
Oxidation stability	Good	Good	Fair	Good	Good
Hydrolytic stability	Good	-------	Poor	Good	Fair
Costs	1	2 to 4	2 to 3	10	4 to 20

[a] (Bisht *et al.*, 1993; Kinaway, 2004)

Over the years and due to the change in our environmental thinking the focus on lubricants has shifted from biodegradability to renewability. This has also influenced the way these kinds of products should be designated. Since any oily substance will tend to stain soil, fur or feathers, Norrby (2003) suggests that the most sensible description is environmental adapted lubricants (EAL) i.e. adapted to minimal harm and hazard to nature. According to him, alternative descriptions as environmentally friendly or compatible lubricants suggests either that there is an absolute quality that is unlikely to be attainable in practice or that there is no or low negative interaction with the natural surroundings. There are several aspects that make a lubricant EAL (Norrby, 2003; Pettersson, 2006)

- Biodegradability
- Toxicity
- Renewability
- Bioaccumulability and biomagnification
- Life cycle assessment (LCA)
- Energy saving and fuel economy

Since the first biodegradable lubricants were introduced in the 1980s (Lea, 2002), i.e. two-stroke engine oils for boating (1980) and chain saw lubricants (1985), there has been a strong growth in the area of biodegradable lubricants and there are now numerous applications on the market, as seen in Table 3.

Of the above application groups the hydraulic fluids, chain bar oils and two-stroke oils have seen a high conversion into bio-based lubrication formulas. The market driver has mainly been the efforts of removing mineral-based oils from the “total loss” applications where the lubrication oils are lost into the environment with all the negative consequences that follow. In applications where engine oils and gear and transmissions lubes are used the oils are contained in the engine of the vehicle and therefore do not constitute the same direct environmental problem. However, the major market potential for bio-lubricants lies in the conversion of automotive and industrial oils (italicised in Table 3 below) that make up more than 70% of the total world production of lubricants.

Table 3 Major applications for biodegradable lubricants (Lea, 2002).

Total loss lubricants	**Sealed/circulating systems**
Chain bar oils	Hydraulic fluids
Concrete mould release oils	Compressor oils
Two-stroke oils	Milking machine lubes
Chain and conveyor lubes	*Engine oils*
Greases	*Gear and transmission lubes*
Corrosion preventive oils	*Metalworking fluids*
Wire rope lubricants	*Machine tool lubricants*

3.1 Market considerations

Vegetable oils have already captured a significant portion of the lubricant market and have good potential to continue to grow, substantially due to several market drivers as described below.

3.1.1 Drivers for an increased use

Some drivers that are important in the development of the EAL market are (Norrby, 2003):

- Environmental interests from ordinary people create a situation where people are prepared to pay a surcharge on lubricants that meet criteria for EAL.
- Environmental policies and legislations that are exercised through governmental and international organisations. One such example is BMVEL (Germany), a government-sponsored market conversion programme for lubricants and fuel based on renewable resources.

- Environmental sustainability indexes that influence where investors make investments.
- NGOs such as Greenpeace, Bellona, Friends of the Earth etc. have a strong focus at influencing the society towards sustainable and environmentally friendly solutions.
- Increased use of Life Cycle Analysis (LCA) in the manufacturing processes of new products.

Further, USDA has proposed (2006) rules that set the minimum bio-based content for a number of products. These conditions state e.g. that 2-cycle engine oils should have a minimum of 7% bio-based content, stationary equipment hydraulic fluids should have 46% and fluid-filled transformers should have 66% (Tocci, 2006).

The real opportunity for a substantial growth of the volume of vegetable oil used for industrial applications lies in the continuous conversion of lubrication applications from petroleum-based to bio-based EAL applications. Examples are conversion of all types of hydraulic fluids, gear oils and automotive transmission fluids as well as metalworking applications to renewable and biodegradable fluids. The rising price of petroleum base oils can also be seen as a driver such that more of vegetable-based base oils will be used in lubrication formulas that will become more competitive in price. Further, the future EU chemicals policy, REACH, may also be a driver in more usage of bio-based EAL applications.

3.1.2 Barriers for an increased use

- Quality - if new and improved plant oils are not able to meet the specifications from manufacturers of lubrication formulations
- Price – production cost for vegetable oil-derived base oils is too high to be competitive with the mineral-based oils
- Opinion – development of new and novel non-food oilcrops will be delayed because a negative opinion among people in Europe does not allow the use of genetic engineering.

3.1.3 General sizes of market, world-wide and EU

The importance of well-suited lubricants can be exemplified by a calculation from the US Department of Energy that reducing friction and wear in engine and train components could save the US economy up to $120 billion per year (Tung & McMillan, 2004).

The annual consumption of oil-based lubricants in the world (2003) was 36 million metric tons (without the marine oils) with a value of about $28 billion. Europe (western and central/eastern Europe) consumes about 27%, the US share of the consumption is about 30% while Asia/Pacific makes up 31% of the world lubricant consumption (Lubricant, Industry Sourcebook, 2005-2006). More than 70% of total lubricant volume is used in motor oils for automotive engines and roughly 10% is used as hydraulic oils.

Table 4 Cost of various base oils in 2004.

Base oil		**Cost ($/Kg)**
Vegetable oils	Soybean oil	0,48-0,55
	Canola oil	0,638
	High-oleic sunflower oil	0,99-1,21
Synthetic oils	TMP[b] trioleate	~2,20
	TMP[b] trioleate (high-oleic)	~2,76
	PAO8[c]	~1,98
	Mineral base oil (Group I and II)	~0,55

[a] Price will vary based on quantity, customer supplier relationships, and market conditions; [b] TMP, trimethylolpropane; [c] PAO, polyalphaolefin.

In Western Europe, bio-lubricants were 3,1% of total lubricant consumption in 2002 (Whitby, 2004).

4 THE CASE FOR WAX ESTER PRODUCTION IN THE NON-FOOD OIL CROP *CRAMBE*

A case study was performed to evaluate the potential of producing a bio-based oil with outstanding lubrication properties that can be used in lubricant formulation and replace the present use of petroleum. Wax esters were chosen as the target oil because it is highly recognised for its performance as a lubricant but still too costly to be used in the bulk lubricant market.

4.1 Non-food oil crop platform *Crambe*

The reasons to choose *Crambe* as a platform for production of bio-based feedstocks such as wax esters are several:

- Today there is a consensus by all major biotech companies that there is a risk in developing industrial feedstock production in the same crop that is also used for the food market. This is due to the fact that infrastructure in agriculture today does not allow “fail-safe” separation of different qualities of the same crop e.g. the ‘StarLink’ maize incident is an example (Bucchini and Goldman, 2002; Kuiper and Kleter, 2003). The industrial oils to be produced in genetically engineered plants are not intended for food use (although not necessarily toxic) and must therefore not enter the food or feed chain. Dominating and high yielding oilcrops in Europe such as rapeseed, flax and sunflower are all more or less used for food production and are therefore not alternatives.
- In order to avoid the risk of unintentional events (due to seed mixing or out-crossing) it is important to consider specific non-food oil crop platforms for production of industrial raw materials. This report therefore considers the potential of using a new oil crop, *Crambe*, as a producer of wax esters. *Crambe* naturally contains a high amount of erucic fatty acid which disqualifies it for food production. In fact, *Crambe* is already used to produce an industrial feedstock (erucic acid).

- *Crambe* naturally contains up to 60% of erucic acid, a suitable long chain fatty acid for conversion into the fatty alcohols needed in the production of several of the wax ester types suggested in this report (see chapter 5).
- *Crambe* is already a high yielding oil crop (similar yield as spring rapeseed) and can grow wherever rapeseed is grown. It also has a high oil content of 35% (60% when excluding the pod).
- The risk of outcrossing to wild relatives is very high for oilcrops such as rapeseed and sunflower, while for *Crambe* this is an unlikely event. In addition, *Crambe* has a seed morphology distinct from established oilcrops which helps prevent admixtures of seed lots by simplifying identity preservation.
- Unlike major oilcrops, crambe is a self-pollinating crop which reduces the outcrossing of the different traits among varieties.
- For a typical oilcrop, major contributions to the environmental loads are the cultivation steps (Bernesson *et al.*, 2003). With regard to cultivation, *Crambe* is a low input crop compared to many others. This reduces the environmental burden in respect to use of fertilisers, water and so forth.
- A further and much deeper environmental analysis on *Crambe* will be made and presented in the next EPOBIO report on non-food crop platform.

4.2 GM-based production of wax ester

Jojoba is a plant species that is unusual in that it accumulates a high amount of wax esters in its seed oil rather than triacylglycerol. This wax oil has outstanding qualities for lubrication applications but is a very expensive commodity. Wax esters can also be produced enzymatically from alcohol and fatty acid (bio-based or mineral-based) but this production is also rather expensive. To establish a production of wax esters in *Crambe* as suggested in the report, the only choice available is to make use of genetic engineering.

4.2.1 Transformation method of *Crambe*

A transformation protocol is critical in the process of providing *Crambe* with the necessary genes for wax ester production. No public transformation protocol has so far been available for *Crambe*; however, recently a research laboratory in China established an *Agrobacterium*-based protocol for *Crambe* transformation (Banquan Huang, personal communication). Still this protocol will need to be developed further and optimised in order to transform other varieties. Therefore, this is an area where more research should be directed. In this respect it can be noted that like *Arabidopsis* and *B. napus*, *Crambe* is a member of the *Brassicaceae* family, where many members are routinely transformed.

Production of high levels of wax esters in *Crambe* will need efficient and tissue-specific promoters. Seed-specific promoters such as napin or FAE (Stålberg *et al*., 1993; Rossak, *et al.*, 2001) have a good track record for expression in *Arabidopsis* and *B. napus* and are therefore considered to be a good choice for *Crambe*.

There are several advantages with choosing a strategy involving marker-free selection i.e. where the marker is removed when stable insertion of the transgene has been confirmed. There is public concern about the presence of selectable markers in the transgenic crops and these worries have especially been raised in respect of antibiotic selectable markers. Marker-free selection would therefore ease people's concern related to these antibiotic selectable markers and also herbicide resistance markers and their potential to be spread to other species like bacteria or weeds. However, EFSA (European Safety Authority) recommends that resistance genes towards antibiotics that are not in clinical use should be allowed in commercial GM plants (EFSA document ENV/04/27). Therefore EC recommendations for the use of selectable markers might change in the future.

The ability of plant biotechnologists to modify more complex metabolic pathways is accompanied by the transfer of multiple traits and genes to the plant. This calls for an increasing number of different selectable markers in order to distinguish the different traits from each other.

The above issues can be solved by using a marker-free selection strategy. Several alternatives exist such as the Cre/Lox system (Corneille *et al*., 2001), using a transiently cointegrated selection gene (Klaus *et al*., 2004) or combining an inducible site-specific recombinase for the precise elimination of undesired, introduced DNA sequences with a bifunctional selectable marker gene used for the initial positive selection of transgenic tissue and subsequent negative selection for fully marker-free plants (Schaart *et al*., 2004).

4.2.2 Risk mitigation of GM

To minimise risks in using a genetic engineering strategy for the production of wax esters, this report suggests certain steps.

To reduce the risks of mixing an industrial feedstock into the food chain, establishing this production in a non-food crop is regarded as the preferred strategy. It is not expected that *Crambe* can cross easily with other related species. Inter-specific crosses between the most closely related species *C. hispanica* and *C. abyssinica* gave nearly sterile offspring, associated with imbalances in chromosome number (Wang and Peng, 1998). Outcrossing of a GM *Crambe* with wild *Crambe* species and/or other *crucifereae* species is therefore unlikely. However, when growing the GM *Crambe* it is recommended to use the same cultivation practices as when growing GM rapeseed, e.g. non-GM barrier crop, minimum distance to nearby non-GM *Crambe* field and prevention of volunteer GM-*Crambe* by tillage and/or herbicide treatment the following season should be utilised to help ensure identity preservation of the crop.

Considering the types of wax esters and fatty acids that will be produced in the GM *Crambe*, i.e. novel branched fatty acids and hydroxyl fatty acids in waxes, it should be noted that the same or similar compound already exists in nature. For example hydroxy fatty acids are produced in seed oils of castor bean and *Lesquerella sp*. (van de Loo *et al*., 1995; Broun, *et al*., 1998) while wax esters containing branched fatty acids are found in the fluids that certain birds use to impregnate their feathers (Kolattukudy *et al*., 1985).

4.3 Economic case study

4.3.1 Specific marketing information on wax esters

Size of market

Presently the amount of wax esters such as the jojoba type, that is used worldwide is very small due to high price and low production (see below). However, if the synthetic ester segment of the lubricant market is considered, then the market is quite substantial where polyalfaolefins is around 0.5 million tonnes per year and organic esters 0.05 million tonnes per year.

Specific, extant end uses for wax esters are mainly low-volume, high cost specialty markets where plant waxes capture a significant portion. The cosmetic industry is the largest user of wax esters today. Wax ester provides excellent properties to applications such as cosmetic formulations for dry and sensitive skin and in lipsticks. Jojoba is a main source for wax esters in this industry, utilising around 2,000 tonnes per year (total production estimated to 3,500 tonnes per year). The other major industry using jojoba oil is the pharmaceutical sector.

Due to the high price of jojoba oil (5000 €/t), lubricant applications provide a market for only around 100 tonnes of jojoba oil annually. These are special applications such as high pressure, high temperature lubricants for use in gearboxes, differentials, crankcase lubricant; cutting oils, etc.

4.3.2 Production cost of a GM wax esters feed stocks

The cost of production of GM *Crambe* wax ester oils has been calculated and is compared with alternative methods of providing wax esters. The assumptions that have been made are:

- Petroleum oil is the dominating raw material for lubricants presently. Cost and availability suggest a need to develop alternatives.

- Vegetable oil has very good lubrication properties but needs improvement to be considered for bulk market lubricants.
- Wax esters is a target oil that in itself can function as an excellent lubricant for many applications but also function as an additive in vegetable-base oils and improve their stability.
- The current price and availability of wax esters make them unsuitable for widespread use for the lubricant market.
- To make wax ester available in the volume required and at an acceptable price this report has considered production of this target oil in the crop *Crambe*.
- The production of plant-derived wax esters in Europe will necessitate the use of a genetically modified crop.
- As stated above the importance of using a non-food oil crop for the feedstock production disqualifies e.g. rapeseed, even though it is a higher yielding crop compared to *Crambe*.
- The calculations take no account of either the cost of developing a GM variety or the regulatory cost associated with approval of the GM variety. Neither does the calculation include a set-aside payment (subsidy) for *Crambe*.

The production costs calculated for *Crambe* crude oil are based on 2005 German prices and the real output of a 1996 through 1999 test study (Troegel, 2000) of non-GM *Crambe* grown in different parts of Germany. It assumes that the yield of oil for a GM *Crambe* will be the same as for the non-GM *Crambe*. The case includes three different harvest scenarios and the use of two differently sized production facilities i.e. oilseed crushing plants, one small with a conversion capacity of 30,000 t/year and one larger at 100,000 t/year. The production facilities are assumed to be situated in the centre of the *Crambe* cultivation area. Depreciation time has been set to 20 years and the interest rates are assumed to be 6%[1].

Being a crucifer, *Crambe* is grown in a four-year crop rotation scheme. For an arable area of 1,000 ha this means that a maximum of 250 ha will be available each

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[1] Today's call money rate in Germany (Oct 2006)

year for *Crambe* cultivation. Assuming that only 50% of this possible maximum land area is used for *Crambe* and the remaining for growing other crops, then 125 ha would be utilised, equivalent to 12.5% of the total available arable land.

Three different yield scenarios have been chosen based on what has been reported from previous *Crambe* field trials (Lagetti *et al*., 1995; Fontana *et al.*, 1998; Troegel, 2000; Fila *et al*., 2002) and the costs of the necessary inputs to grow *Crambe* are shown in Table 5. Farmers' earnings have been set to an opportunity level i.e. to make *Crambe* cultivation attractive as an alternative investment in comparison to other crops.

Table 5 Input costs for different cultivation scenarios and the assumed output.

Input		**Scenario**			
		best case	**normal case**	**worst case**	**rapeseed**
Output					
Yield	[t/ha]	4.0	2.4	1.2	4.0
Farmers' earnings (opportunity)	[€/ha]	350.0	350.0	350.0	350
Variable cost					
Seed cost	[€/kg]	60.5	60.5	60.5	
Var. machine	[€/ha]	74.0	90.0	100.0	
Fertiliser N	[€/ha]	67.5	67.5	67.5	
Plant protection	[€/ha]	38.0	65.0	75.0	
Drying (standard)	[€/t]	14.0	14.0	14.0	
Land (standard)	[€/ha]	50.0	50.0	150.0[a]	

[a] opportunity cost

The radius of the cultivated area depends on the assumed yields and the size of the crushing facilities fed with the *Crambe* seed. This case study assumes that the seeds are dried but not dehulled before storage. As can be seen in Table 6 the transportation and storage costs are comparably low and therefore a dehulling of the seeds will not have a significant effect on the total cost. In fact, the option analysed to crush the seeds with the hull and afterwards burn the meal and hull, will produce electricity and/or heat that can be used in the production process. In addition to reducing the energy cost it also helps to avoid fossil CO_2 emissions generated by the production process. Extraction of the *Crambe* oil uses the hexane solvent process that currently is a more efficient option to e.g. cold-press extraction that leaves as much as up to 10% of the original oil in the meal. Assumed costs for extraction are also shown in Table 6.

Table 6 Transport, storage and extraction costs [€/t] for *Crambe* in a big and small production facility.

	Big facility (100,000 t/y)			Small facility (30,000 t/y)		
	best case	normal case	worst case	best case	normal case	worst case
Radius (km)	25	33	46	14	18	25
Transportation cost	4.4	5.6	8.0	2.4	3.1	4.4
Storage cost	5.4	5.4	5.4	5.4	5.4	5.4
Extraction costs	43.8	43.8	43.8	65.7	65.7	65.7

The results of the analysis are shown in Table 7.

Table 7 Production costs [€/t] for *Crambe* oil in two different sized factories. Cost comparison is presented on using the meal and hull in energy production or not.

	Without using the meal and hull			Burning the meal and hull		
	best case	normal case	worst case	best case	normal case	worst case
Big factory	683	1,062	2,267	504	954	2,213
Small factory	742	1,119	2,321	562	1,011	2,267

The best case scenario shows the influence of a higher yield and therefore a lower total production cost per tonne of oil. Worst case assumes a lower yield and therefore higher total production cost per tonne of oil. Under the worst case scenario the oil production cost, ranging from 2,213 – 2,321 €/t, compares favourably with current import prices in "free Europe"[2] of about 5,000 €/t for jojoba oil.

An alternative way to produce wax esters is to use an enzymatic or chemical catalytic process (Nieschlag *et al*., 1977; Steinke *et al*., 2001; Hills, 2003). This requires long chain fatty alcohols that can be transesterified with long chain fatty acids to produce a mixture of C40-42 jojoba-type wax esters. The minimum input cost for the raw materials needed in this process is 1,800 €/t (current market price for alcohols and fatty acids). From this it can be concluded that the production costs for this alternative have to be lower than 521 €/t (worst case scenario in Table 7, 2,321 minus 1,800 €/t) in order to compete with the worst case wax ester production in a genetically modified *Crambe*. In fact, the assessment shows that in order to compete with *Crambe*-produced wax esters, the alternative process has to produce wax esters at a price equal or lower than 1,119 €/t (Table 7, normal case). Taken that only the raw materials for the alternative process adds to 1,800 €/t, it can be concluded that wax ester production for bulk consumption by an enzymatically or chemical catalyst process, is not an economically viable process to invest in.

Under the above presented assumptions and current costs it can be concluded that production of plant-derived wax ester oils in a genetically modified *Crambe* has a considerable economic potential compared to both wax esters from jojoba and/or an enzymatic or chemical process. These wax esters also have the potential to compete on a price basis with not only synthetic-base oils but also mineral-base oils.

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[2] Free Europe is defined as free on board Singapore + transportation costs by ship

5 GENE DISCOVERY FOR WAX PRODUCTION

Waxes fulfil very important properties for plants when being part of the cuticle. The plant cuticle reduces non-stomata transpiration, protects from ultraviolet radiation and has an important role in defence against bacterial and fungal pathogens (Kunst and Samuels, 2003 review). Waxes found in the cuticle of plants are mainly composed of alkenes, fatty alcohols, secondary alcohols, ketones and fatty acids. They consist of carbon chains predominately of a length between C20 and C35 and are derived from very long chain fatty acids (VLCFAs). VLCFAs are produced by elongating fatty acids such as C16 and C18 through the action of fatty acid elongase (FAE).

Waxes are also found as oxygen esters between primary alcohols and fatty acids. Wax esters fill important functions such as energy storage and buoyant density regulation among others. A well known example of the later is the accumulation of liquid wax esters and triacylglycerols (66 and 30 % w/w) in sperm whales (*Physeter macrocephalus*) as spermaceti oil (Metz *et al*., 2000). Up until 1971 when the hunting for sperm whale was banned, the sulphurised form[3] of spermaceti oil was used as an excellent additive in many lubricant applications (Nieschlag *et al*., 1977).

Wax esters are also present at rather high levels in deep-sea fish such as orange roughy (*Hoplostethus atlanticus*), black oreo (*Allocyttus sp.*) and smooth oreo (*Pseudocyttus maculatur*). The catching of these fish species from sea depths of up to 1,200 m is done on a commercialised scale in New Zealand. Unlike inshore/mid-water fish that have no wax esters, 95% of the lipid fraction is comprised of wax esters in the deep-sea fish species (Buisson and Hannan, 1998).

Some plants accumulate liquid wax esters in the seed and the most well known example is jojoba (*Simmondsia chinensis* L. Schneider). Jojoba is a slow-growing native plant from the American Southwest that tolerates high temperature and

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[3] Polysulphates are produced by reacting the olefin with sulfur or sulfur monochloride.

drought (Ash *et al.*, 2005). Unlike most seed-bearing plants, the oil accumulating in the jojoba seed (50% of dry weight of seed) contains almost all wax esters (97%) and no triacylglycerols. The main wax esters in jojoba oil are C38-C44, which is more similar to the range of wax esters in deep sea fishes C34-C42 (orange roughy) in contrast to the C28-C36 wax esters in the sperm whale (Buisson and Hannan, 1998).

Wax esters are found in insects as surface waxes. For example the red harvester ant (*Pogonomyrmex barbatus*) has a range of wax esters from C19 to C31 that consist of both odd- and even-chain-length alcohols, some of which are branched. Fatty acids range from C8 to C18 and the alcohols from C8 to C17 (Nelson *et al*., 2001). The honey bee (*Apis mellifera*) produces cuticular wax that consists of more than 55% hydrocarbons and about 23% wax esters. The wax esters range between C36 – C50 with predominately fatty acids of C16 and C18 and primary alcohols of C20 to C32 (Blomqvist *et al*., 1980). This wax is produced by epidermal cells closely associated with the cuticle. Honey bees also produce a wax that is used to make the honeycomb. This is produced by wax glands on the ventral abdominal side of the bee, and unlike the cuticle wax it contains only 15% hydrocarbons and 35% wax esters. The wax esters have an average length of 40 and contain methyl groups as branching side chains (Basson and Reynhardt, 1988).

Bacteria accumulate high levels of wax esters as a common storage lipid in intracellular inclusion bodies that can occupy a significant part of the cell volume (Ishige *et al*., 2003). Especially bacteria of the genus *Acinetobacter* store wax esters up to 14% of the cell dry weight as C30 to C36 mono esters composed of saturated and monounsaturated fatty acid and fatty alcohol moieties (Kalscheuer *et al*., 2006).

Wax esters constitute around 30% of the sebum and meibum that is produced in the sebaceous and meibomian gland and are secreted onto the surface of the skin and eye of some mammals (Downing, 1974; Nikkari, 1974). Sebum has important functions in lubricating the skin and contributes to the development of a water barrier (Nicolaides, 1981; McCulley, 2002) while meibum prevents the evaporation and spreading of the tear film in the eye (Driver, 1996).

In some birds, specialised tissues like the uropygial glands in mallard ducks (*Anus platyrhynchos*) produce a liquid that is high in wax esters (Kolattukudy *et al*., 1985). Interestingly these wax esters consist of short chain fatty acids (predominately C6) that are highly branched with 1 or 2 methyl groups. The alcohol portion of the wax ester is C16 to C19 with more than 50% of C18 as the dominating chain length. A small portion of mono-, di- and tri-methylated branched fatty alcohols are also present.

5.1 Brief description of three target waxes

This report address three different types of wax esters as potential target oils produced in the oil crop *Crambe* using genetic engineering. They are similar to wax esters that are naturally present in different organisms where they fill important biological functions. It is assumed that due to the structural differences the suggested wax ester types will have different kinds of material characteristics and properties. They should therefore have significant potential for use in various lubrication applications either as base oils or as additives.

5.1.1 Long chain wax esters

A jojoba-type of wax ester – C_{40-42}.

The potential world production of jojoba oil (2002) is around 3,500 metric tons per year. The oil commands a high price mainly because of the slow production and high demand. It has been estimated that the demand for jojoba oil currently is 64,000 and 200,000 metric tons/year. The cost is one main reason why this outstanding lubrication oil is restricted in use to limited application areas such as cosmetics, specialty lubricants, and other high value products. However, if the cost of producing these types of waxes could be substantially reduced while at the same time increasing the volume of production, these types of wax esters could immediately be considered for much larger marketing applications such as automotive, transmission and hydraulic fluids.

Addition of jojoba oil (15-20%) to soybean oil has shown to effectively improve oxidation stability of the plant oil (Torres *et al*., 2006). However, due to a high price on wax esters from jojoba the final price on the improved soy oil will not compete with petroleum-based oils and would have difficulty to compete with synthetic oils as well.

Hence, an alternative production of wax esters in a high-yielding oil crop constitutes an attractive approach to provide the market with outstanding lubrication oil at an affordable price level.

Target genes to modify and redirect the flow of carbon chain in the genetically modified *Crambe* to produce novel wax esters include:

For a jojoba type of wax ester

- Fatty acid reductase (FAR) with specificity towards C22:1. *Crambe* already has high levels of up to 60% of the long chain erucic fatty acid (22:1). Consequently this fatty acid is a good substrate for the alcohol-producing FAR enzyme.
- Acyl-CoA:fatty alcohol acyltransferase (WS) with specificity towards 22:1 alcohol and C18 or C20 fatty acyl-CoA substrates. The 22:1 alcohol is used by WS for production of wax esters with a C18 or a C20 fatty acid. The desired wax product would have a structure of C_{40-44} consisting of a long chain fatty alcohol C_{20-22} esterified to a long chain fatty acid C_{18-22} and contain not more than 1 double bond per carbon chain.

5.1.2 Branched chain wax esters

Branched wax esters, $C_{22 - 30}$

The target molecule consists of a long chain fatty alcohol C_{18} with none or one methyl branch and a branched, medium chain fatty acid C_{8-12} with not more than one double bond. This mimics the wax esters that birds use to impregnate their feathers with and which confers high water repellent properties to the plumage.

The presence of a branched chain fatty acid as well as a double bond on the hydrocarbon chain disrupts the lipid packing of the wax and broadens the liquid-solid phase transition by reducing the melting temperature. In this respect it is much more effective if this irregularity along the chain is positioned internally rather than terminally (Gibbs and Pomonis, 1995). Double bonds are even more superior in lowering the melting point of hydrocarbons but with the drawback of adding oxidative instability to the oil. It is this effect of lowering the melting temperature of the fatty acids and thereby the pour point of base oils without adding oxidative instability that makes branched chain fatty acids very interesting in formulating lubricants.

Target genes to modify and redirect the flow of carbon chain length in the genetically modified *Crambe* producing:

Wax ester with branched medium-chained fatty acids

- Fatty acid reductase (FAR) with specificity towards C18
- Acyl-CoA:fatty alchol acyltransferase (WS) with specificity to methyl branched medium chained fatty acids
- Thioesterase with specificity towards either of C8, C10 or C12
- Methyltransferase for introducing a methyl branch in the fatty acid chain

Short chained and branched wax ester

- Fatty acid reductase (FAR) with specificity towards C18
- Acyl-CoA:fatty alchol acyltransferase (WS) with specificity to branched short chained fatty acids
- Aminotransferase and a branched-chain oxoacid dehydrogenase complex for producing C4 and C5 branched fatty acids from leucine or valine

5.1.3 Unusual fatty acid chain wax esters

Hydroxy-type of wax esters $C_{38\text{-}40}$-OH

The target molecular structure consists of a long chain fatty alcohol $C_{20\text{-}22}$ and a hydroxy fatty acid C_{18}-OH (ricinoleic fatty acid).

Hydroxy fatty acids are presently used in lubrication formulas as additives in order to improve low temperature, improve viscosity index and oxidative stability properties in way very similar to branched fatty acids. Bio-based hydroxy fatty acids are synthetically produced by introducing hydroxy groups in the hydrocarbon chains of common plant oils by a chemical catalyst. Another natural source of hydroxy fatty acids is the seed oil from castor bean, which contains up to 90% ricinoleic acid (C18:1OH). Cultivation of castor bean is cumbersome with laborious production methods and has a high toxicity of the seed due to the presence of ricin (Tyson *et al.*, 2004) and allergenic proteins. To transgenically produce hydroxy fatty acids as wax esters in a high-yielding agricultural crop therefore presents a highly interesting alternative.

The hydroxy wax esters can be used as a value-added wax ester base oil or the hydroxy fatty acids can be isolated from the wax ester and used as an additive in lubricant formulations. For example, treating hydroxy fatty acids with alkali and high temperature will cleave the fatty acid to capryl alcohols and sebacic acid. Sebacic acid, a 10-carbon dicarboxylic acid, are used in jet lubricants and in air-cooled combustion motors (Ogunniyi, 2005).

Target genes to modify and redirect the flow of carbon chain in the genetically modified *Crambe* producing:

Wax esters containing hydroxy groups

- Fatty acid reductase (FAR) with specificity towards C22
- Acyl-CoA:fatty alcohol acyltransferase (WS) with specificity to hydroxy fatty acids
- Δ12-desaturase-like hydroxygenase

5.2 Genes/enzymes for production of waxes

Lardizabal and co-workers (Lardizabal *et al*., 2000) have shown that insertion of two genes from jojoba and one from *Lunaria annua* in *Arabidopsis* resulted in transgenic plants producing seed oil with up to 70% of wax esters instead of the common triacylglycerols. The two genes from jojoba were fatty acyl-CoA reductase (FAR) converting fatty acids to fatty alcohols, and fatty acyl-CoA:fatty alcohol acyltransferase (wax synthase, WS) which catalyses the esterification of the fatty alcohols with fatty acids from acyl-CoA. Since both the jojoba FAR and WS enzymes have preference for carbon chain lengths of 20 or longer, a FAE (fatty acid elongase) gene from *L. annua* was also transferred to *Arabidopsis,* which normally has only 15% of fatty acids longer than 18 carbons in their seeds. However, an alternative approach that would bypass the need to use an elongase gene is to use plants that naturally contain high amounts of erucic acid (C22:1) such as *Crambe* (*Crambe abyssinica*). *Crambe* has 60% of erucic acid in its seed oil and thus, has an ideal fatty acid composition for production of jojoba type wax esters.

5.2.1 Wax synthases

Genes coding for wax esters synthesising enzymes (WS) have been cloned and characterised from jojoba, bacteria and mammals (Lardizabal *et al.*, 2000; Kalscheuer and Steinbüchel, 2003; Cheng and Russell, 2004a). Jojoba WS shows significant activity to both saturated and unsaturated acyl-CoAs ranging from 14 to 24 carbons with C20:1 as the preferred substrate and the highest activity with C18:1 and 18:2 alcohols (Lardizabal *et al.*, 2000). In *Arabidopsis* there are 12 sequences that have considerable sequence similarity to the jojoba WS protein (Kunst and Samuels, 2003) and therefore represent potential candidates for additional WS activity.

The WS/DGAT in *Acinetobacter calcoaceticus* was identified as a bifunctinal enzyme that catalyzed the acyl-CoA dependent acylation of DAG and fatty alcohols to produce TAG and wax esters, respectively. It has no sequence homology to other known acyltransferases (Wälterman *et al.*, 2006) and represents a new class of acyl-CoA-dependent acyltransferase (Stöveken *et al.*, 2005). Even though the enzyme accepts alcohols of a broad range from C2 to C30, its highest activity is with chain lengths from C14 to C18 and 18:1 and it has a preference towards C14 and C16 acyl-CoAs. Nearly a hundred WS/DGAT homologues from more than 20 different microorganisms have so far been identified in the genome databases (Wältermann *et al.*, 2006) as well as 10 putative protein sequences in the *Arabidopsis* genome with some similarities to the WS/DGAT (Kalscheuer and Steinbüchel, 2003).

In mammals, WS enzymes have been identified and characterised from mice and humans and shown to belong to the acyltransferase family with highest sequence identity towards DGAT2 (46.5%), MGAT2 (40.5%) and MGAT1 (38.7%) proteins (Cheng and Russell, 2004). These enzymes efficiently acylate alcohols shorter than C20 with a preference towards C18:1 and C18:2. Further, they have higher activity with shorter acyl-CoA chains such as C16:0, C16:1, C18:1, C14 and C12.

5.2.2 Fatty alcohol reductases

The production of primary alcohols from very long chained acyl-CoA esters likely proceeds through a two-step reaction. Reduction of the acyl-CoA ester to an aldehyde is carried out by an NADH-dependent acyl-CoA reductase activity and the further reduction of the aldehyde to an alcohol is executed by an NADPH-dependent fatty aldehyde reductase activity. At least in plants this whole process is carried out by a single fatty acyl-CoA reductase (FAR) and with no aldehyde intermediate released (Kunst and Samuels, 2003). In contrast, microorganisms seem to carry out the alcohol production with two separate enzymes. In *Acinetobacter baylyi* the acyl-CoA reductase (Acr1) produces an aldehyde that is reduced to the corresponding fatty alcohol by an NADPH-dependent fatty aldehyde reductase (Reiser and Somerville, 1997; Ishige *et al*., 2002). However, a gene encoding an aldehyde reductase has so far not been identified (Wältermann *et al.*, 2006).

Genes with homology to the jojoba alcohol-generating fatty acyl-CoA reductase (FAR) are common in the plant kingdom and are present in species such like corn (*Zea maize*), rice (*Oryza sativa*), cotton (*Gossypium hirsutum*), rapeseed (*Brassica napus*) and *Arabidopsis*. Only the *Arabidopsis* genome has been entirely sequenced and notably, there are 8 sequences with homology to jojoba FAR. It has been shown that the jojoba FAR has a preference for long-chain fatty acyl-CoA substrates (C20, C22:1 and C24:1). Preliminary results suggest that the substrate specificity among the 8 *Arabidopsis* FAR homologs varies from short-chain to long-chain fatty acyl-CoA substrates (unpublished results, Peter Olson, SLU).

Homologues to the jojoba FAR are also found in *Caenorhabditis elegans*, *Drosophila melanogaster* and in mammals (Metz *et al.*, 2000; Cheng and Russell, 2004b). Recently two fatty alcohol-producing acyl-CoA reductases were identified in mice and humans with specificity toward long chain acyl-CoAs (C16:0 – C18:1).

5.2.3 Fatty acid elongases

In plants, *de novo* fatty acid synthesis (FAS) in the chloroplast produces C16:0, C18:0 and C18:1 fatty acids that are exported to the cytoplasm and converted into Acyl-CoA. The acyl-CoAs (mainly C18:1) are further elongated with malonyl-CoA by the membrane-bound fatty acid elongase β ketoacyl-CoA synthetase (FAE KCS) to produce very long-chained fatty acids (VLCFA) (Ohlrogge and Browse, 1995; Millar and Kunst, 1997).

The condensing enzymes control not only the amount of VLCFA produced but also the chain lengths of the VLCFA produced (Millar and Kunst, 1997). When jojoba KCS and the *Arabidopsis* FAE1 were expressed in *Arabidopsis*, the KCS produced more of the longer chain fatty acids (62.7% C20, 27.6% C22 and 9.6% C24 of total VLCFA) (Lassner *et al*., 1996) than what FAE1 produced (approximately 81.6% of C20, 17.7% of C22 and very low level C24 of total VLCFA).

KCS enzymes with unusual fatty acid specificity have been cloned, such as the KCS from *Limnathes alba*. This enzyme use 16:0 as the substrate for the elongation of the fatty acid. The Δ5-desaturase from the same species use 20:0 as the substrate for the Δ5 desaturation. Co-expression of both the *Limnathes* KCS and the Δ5-desaturase in soybean resulted in an accumulation of up to 12% Δ5-monounsaturated C20 and C22 in soybean (Cahoon *et al*., 2000).

5.2.4 Enzymes for production of unusual oxidised fatty acids

Although a few oil qualities for industrial use, such as e.g. wax esters and medium chain fatty acid, have already been produced in relatively high amounts in transgenic plants (Voelker *et al*., 1996; Lardizabal *et al*., 2000;), this is not the case for most of the potentially valuable oils with unusual fatty acid structures. Thus in spite of the many genes encoding enzymes synthesising a variety of these fatty acids have been cloned. As a matter of fact, identifying genes encoding enzymes catalysing the synthesis of unusual fatty acids is today, in most cases, not a significant scientific challenge. However, transgenic plants expressing these genes have disappointingly produced only a few percentage of the desired fatty acid in the oil whereas the original host plants of the genes have up to 95% (for excellent reviews see Jaworski and Cahoon, 2003; Drexler *et al*., 2003). There are numerous bottlenecks in the production of these unusual fatty acids in transgenic plants that need to be removed. Therefore, a substantial amount of research will be needed in the following areas.

- In plant oil biosynthesis, as well as for the very last step of assembling oil, the TAG biosynthesis, there is not a thorough understanding of the various enzymes involved.
- For many other steps e.g. handling the phosphatidylcholine (PC), the acyl-CoA synthetases and movement of acyl chains, a good understanding of the relative contributions of these alternative steps is lacking.
- Furthermore, it is not known how the unusual fatty acids move from the membrane lipid PC, where they are made and how they are inserted into the TAG. A greater understanding of the area of transcription factors and transcriptional regulation are also of utmost importance in order to increase oil yield.

5.2.4.1 Hydroxylase

A gene, coding for a Δ12-hydroxylase, was identified and characterised from castor bean (*Ricinus communis*) (van de Loo *et al.*, 1995). The enzyme uses oleate (18:1) as substrate and produce ricinoleic acid with a hydroxy group at position 12 on the carbon chain. When the castor bean hydroxylase was expressed in rapeseed as well as in *Arabidopsis,* four different hydroxy group-containing fatty acids were produced. Besides ricinoleic acid (18:1-OH), also densipolic acid (18:2-OH), lesquerolic acid (20:1-OH) and auricolic acid (20:2-OH) were identified (Broun and Somerville, 1997; Smith *et al.*, 2003). While castor bean accumulates up to 90% ricinoleic acid (18:1-OH) in the seed oil, species of the genus *Lesquerella* accumulate up to 60% lesquerolic acid (20:1-OH). A gene for a bifunctional oleate

12-hydroxylase:-desaturase was isolated and characterised from *L. fendleri*. Unlike the hydroxylase from castor bean, the *L. fendleri* gene has both hydroxylase and desaturase activity (Broun *et al*., 1998). Recently two divergent Δ12 oleic acid-desaturases were characterised from *Dimorphotheca sinuate. Dimorphotheca* species accumulates up to 60% of the seed oil of dimorphecolic acid (9-OH-18:2 $\Delta^{10trans,\ 12trans}$) that is an unusual fatty acid containing one hydroxy and two conjugated bonds (Cahoon and Kinney, 2004).

5.2.4.2 Other enzymes that produce unusual fatty acids with functional groups

In addition to the hydroxylase mentioned above there are epoxygenase (epoxy group), acetylenase (acetylenic bond) and conjugase enzymes (conjugated double bonds) that produce industrially important fatty acids. These enzymes share similarity with FAD2 and are therefore named divergent FAD2 or Δ12-desaturase-like enzymes. Other examples of enzymes producing unusual fatty acids which have been characterised is a P450-type epoxygenase from the spurge plant (*Euphorbia lagasque*) that produced up to 13% vernolic acid in transgenic plants (Cahoon *et al*., 2002). Recently production of cyclopropane fatty acids has been shown in transgenic plants using a gene from *Sterculia foetida* (Bao *et al*., 2002).

Many attempts have been made to express the genes for divergent FAD2 enzymes in transgenic plants, but with poor results when compared to fatty acid levels in the native plants. This area is excellently covered in several recent reviews in which also the bottlenecks behind the low production of unusual fatty acids in transgenic plants are addressed and discussed (Thelen and Ohlrogge, 2002; Drexler *et al*., 2003; Dyer and Mullen, 2005; Cahoon and Kinney, 2005)

5.2.4.3 Branched fatty acid

Branched fatty acids of various kinds can be found in many animals and bacteria and in some plants. Usually they exist as saturated *iso* (*i*)- and *anteiso* (*ai*)-methyl branched and saturated mid-chain methyl-branched fatty acids. The branch can consist of a methyl group attached to a carbon chain of 10 to more than 30 carbons, usually 14 to 18 carbons, but also ethyl and propyl branches are found. The *i-* and *ai* compounds are synthesised via the conventional mechanisms for the synthesis of saturated fatty acids, from 2-methylbutanyl-CoA or 2-metylpropanyl-CoA instead of acetyl-CoA. This results in a carbon chain with the branch at the methyl end. Saturated mid-chain methyl-branched fatty acid are synthesised from oleic fatty acid while attached to a phospholipid. The methyl donors here as well as for the *i-* and *ai* compound are various amino acids (Van der Hoeven and Steffens, 2000).

An enzyme that can introduce methyl groups on the fatty acid chain is referred to as a methyltransferase. A recent example of a successful cloning and characterisation of a methyltransferase is the work by Bao and co-workers (2002).

Glandular trichomes produce exudates that are rich in short-chained branched fatty acids C4 and C5 acids, and branched and straight medium chain C10, C11, and C12 acids (Walters and Steffens, 1990). These fatty acids and the short-chained branched fatty acids (*ai*6:0 and *ai*7:0) found in e.g. petunia (*Petunia hybrida*) and species in the genus *Nicotiana* are derived from branched chained amino acid metabolism. Leucine, valine, isoleucine and threonine are converted into short fatty acids such as 2-methylpropionic and 3-methylbutyric acid that can be elongated further (Van der Hoeven and Steffens, 2000).

An attempt to develop a bio-based production of branched fatty acids by the engineering of oilseeds was the REFLAX project funded by the European Commission (REFLAX, 2006). The general conclusion of the project was that it seems feasible to promote synthesis of branched chain fatty acids in plants. However, to achieve high levels of these compounds more research is needed to target the bottlenecks still present. (Detailed results from this project are confidential until the end of 2006.)

Another example of the interest in branched chain fatty acids is an ongoing ARS project focused on developing bio-based products from animal fats and vegetable oils (Foglia *et al*., 2006). One of their objectives is to develop methods to produce branched fatty acids synthetically from bio-based fats and oils.

5.2.5 Enzymes for production of medium chain fatty acids

5.2.5.1 Thioesterases

Plants like the California bay (*Umbellularia californi*a) and species in the genus *Cuphea*, accumulate high levels of medium-chain fatty acids in their seed oils (C8-C12). This is due to the activity of an acyl-acyl carrier protein (ACP) thioesterase (TE) with specificity to medium chain fatty acids (Dehesh, 2001). A C12:0-ACP TE was cloned from the California bay plant and when expressed in canola this resulted in an accumulation of up to 45% lauric fatty acid and constituted the first genetically engineered medium chain triacylglycerol-producing oil crop (Voelker *et al*., 1996). Transgenic rapeseed plants in which C12-ACP TE was combined with a medium-chain-specific LPAAT from coconut (Knutzon *et al*. 1999), a C8/C10-ACP TE from *Cuphea hookeriana* was expressed alone (Dehesh *et al*., 1996) or together with a *C. hookeriana* 3-keto-acyl-ACP synthase, a medium-chain-specific condensing enzyme (Dehesh et al., 1998) was analysed for their oil composition. This showed that the transgenic rapeseed accumulated up to 7% C8:0, 29% C10:0 and 63% C12:0 medium-chain fatty acid. The results also showed that the transgenic seeds could exclude and/or remove the medium-chain fatty acids from their membrane and that this mechanism(s) was more effective with the shorter-chain fatty acids (Wiberg *et al.*, 2000). This is different from transgenic plants expressing Δ12-desaturase like enzymes like hydroxylase, acetylenase and epoxygenase. These transgenics accumulate high levels of the unusual fatty acids in the membrane lipids (Thomæus *et al*., 2001).

5.3 Strategy for increased percentage of wax esters in seeds

Waxes are synthesised from a long-chain fatty acid like erucic acid (C22:1) and a fatty alcohol. A high concentration of C22:1 in the oil may increase the amount of wax produced in transgenic *Crambe*. Selection of elevated erucic acid levels by breeding seems to be possible by selecting for a low content of the saturated fatty acids. Alternatively, knocking out the fatty acid desaturase (fad2) gene by either molecular mutation breeding or gene silencing may raise the level of C18:1 and C22:1 and reduce the level of unwanted polyunsaturated fatty acids in the *Crambe* oil.

Several enzymes are known that are involved in the specific synthesis of the triacylglycerols by adding fatty acids to the glycerol backbone. Molecular mutation breeding or gene silencing can be employed to silence these enzymes.

5.3.1 Carbon flux into wax/TAG pathways

Crambe oil contains about 14% of polyunsaturated fatty acids and these fatty acids are undesired in the wax esters since they make the wax esters more vulnerable to oxidation (Bondioli *et al*; 1998). A reduction in the polyunsaturated fatty acids can be achieved by abolishing the Δ12-desaturase activity in the *Crambe* seeds with the same RNAi technique as the DGAT genes below using the *Crambe* Δ12-desaturase. This technique has been used to drastically reduce linoleic and linolenic acids in *Arabidopsis* seeds (Stoutjesdijk *et al*., 2002).

5.3.2 Downregulation of TAG pathways

Lardizabal and co-workers (Lardizabal *et al*., 2000) reported that about 70% of the molecules in the oil of wax-producing transgenics were wax esters with the remaining being triacylglycerols. The value of the oil would increase if the proportion of triacylglycerols in the oil could be lowered as much as possible. It should be noted that wax ester-accumulating jojoba plant contains only wax esters and no triacylglycerols.

It is believed that most triacylglycerols in seeds are synthesised from diacylglycerols by two different DGAT (acyl-CoA:diacylglycerol acyltransferase) enzymes DGAT1 and DGAT2 (Hobbs *et al*., 1999: Cases, *et al*., 2001). Eliminating these enzyme activities in *Crambe* seeds in combination with the introduction of FAR and WS enzymes will likely shift the percentage of wax in the oil to near 100%. Constructs that contain inverted repeat of these genes (Smith *et al*., 2000; Wesley *et al*., 2001) can be used for knocking out the enzyme activities.

5.3.3 Mobilisation of wax esters during germination

Although no reports about germination of transgenic seeds accumulating wax esters has been published, problems in mobilising the energy stored in the wax esters for promoting seedling growth can be anticipated. ESTs from germinating jojoba seeds have revealed a number of candidate genes, such as wax ester lipases and fatty alcohol oxidases that might alleviate such germination problems (unpublished results). If severe germination problems are encountered in the wax-producing *Crambe* seeds, biochemical studies have to be performed to identify the bottlenecks and relevant jojoba gene(s) has to be transferred.

6 EPOBIO RECOMMENDATIONS

The finite supply and increasing cost of mineral oil present considerable risks to a society dependent on fossil reserves for energy and products. Plant oils offer a sustainable alternative to mineral oil. Plant oils have similar structures and properties to mineral oils and can be used in many of the applications currently dependent on mineral oils.

Lubricants represent a major global market for petroleum-based products with an annual demand of 35 billion metric tonnes. The use of plant-based lubricants offers significant opportunities in terms of environmental and economic benefits as well as addressing the supply chain issues of mineral oil. To date, these alternative lubricants have had a limited impact in the current market.

The major bottleneck preventing uptake has been cost. Economically, plant-based alternatives to mineral oil-based lubricants cannot compete unless oil prices are in excess of $60/barrel. Since current prices are at this level, there is a strong probability that an economic case can be increasingly made. This necessitates research and development to fully optimise the qualities of plant oils for the many different applications in the lubrication market met by mineral oil.

In terms of plant oils, wax esters have recognised qualities and functionalities as lubricants. Wax esters can be used in the high volume/low price base oils and in the high price/low volume additive segment of the markets. Typically, wax esters can be produced through enzymatic processes from many different plant oils, but this is too costly for the bulk lubricant market. One plant species, jojoba, produces wax esters, but the production is insufficient to meet global demand.

These issues are strong indicators that alternative sources of wax esters should be investigated. In principle, the cultivation of jojoba could be substantially increased, but the species is unsuitable as a mainstream agricultural crop in Europe. Therefore, there is an immediate need to establish a new cost-effective supply of plant-derived wax esters. This will necessitate the use of genetic modification (GM). For risk mitigation, the agricultural crop used for GM production of wax esters should not be used for food purposes, nor be able to outcross to indigenous species.

Nearly any new commercial use of plant biotechnology is bound to be dependent on access to pre-existing IPR. This is true for both enabling technologies like transformation methods, vectors, promoters, etc., as well as for more specific gene traits. It is therefore recommended that an investigation of the patent landscape for the production of the different wax qualities in *Crambe* should be undertaken and ways to avoid major IP blocks should be identified in order to facilitate commercialisation of the developed products.

The EPOBIO study has revealed the potential utility of *Crambe* (*Crambe abyssinica*) as an oil crop for the agricultural production of wax esters in Europe. This crop and jojoba are also useful targets for collaborative investigations with developing countries. For cultivation in the US, additional oil crops such as soybean can be considered for GM modification.

6.1 Research and development needs

Targets for delivery of specific wax esters from *Crambe*

1. Three classes of wax esters have been identified on the basis of their potential utility in lubricant formulations: long-chain (jojoba type), medium/branched-chain and hydroxy-containing wax esters. A gene discovery programme is required to identify the relevant enzymes for production of these wax ester classes in high yield.

2. A transformation system for *Crambe* must be optimised for routine use.

3. Potential agronomic impacts of increasing wax ester levels in *Crambe* seeds, such as the possibility of reduced germination efficiency, need to be investigated and reconciled for widespread cultivation of the transgenic crop.

4. The processing of *Crambe* seed with high levels of wax esters needs to be optimised and integrated into a biorefining process that offers new opportunities for increasing the industrial value of the non-food/non-feed by-products.

Targets for alternative sources of wax esters

1. In collaboration with developing countries, there is scope for improvement of current jojoba varieties in terms of increasing yield.

2. In collaboration with the US, information gained from the *Crambe* programme will be useful in GM programmes with alternative oil crops such as soybean.

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