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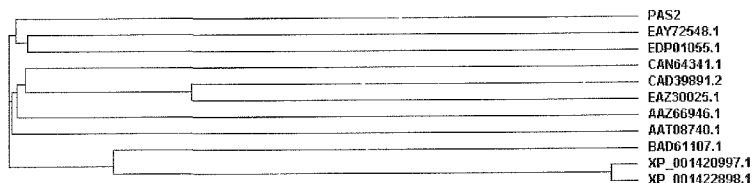


FIGURE I

(57) Abstract: The present invention is relative to a method for the production of Very-Long-Chain Fatty Acids (VLCFA) into a plant cell, comprising culturing a recombinant plant cell in an appropriate medium, wherein said plant cell is transformed with an heterologous gene encoding for an hydroxyacyl-CoA dehydratase. The invention is also relative to a method for producing vegetable oil comprising high levels of VLCFA.

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## RECOMBINANT CELLS AND PLANTS FOR SYNTHESIS OF VERY LONG CHAINS FATTY ACID (VLCFA)

### INTRODUCTION

5 Living organisms synthesize a vast array of different fatty acids which are incorporated into complex lipids. These complex lipids represent both major structural component membranes, and are a major storage product in both plants and animals

Very-long-chain fatty acids (VLCFAs) are components of eukaryotic cells and are composed of 20 or more carbons in length (i.e. >C18). VLCFAs are involved in many  
10 different physiological functions in different organisms. They are abundant constituents of some tissues like the brain (myelin) or plant seed (storage triacylglycerols, TAGs). VLCFAs are components of the lipid barrier of the skin and the plant cuticular waxes. The long acyl chain of certain VLCFAs is necessary for the high membrane curvature, found for instance in the nuclear pore. VLCFAs are also involved in the secretory pathway for  
15 protein trafficking and for the synthesis of GPI lipid anchor. Finally, VLCFAs are components of sphingolipids that are both membrane constituents and signalling molecules.

Very long chain fatty acids are synthesized in the epidermal cells where they are either directly incorporated into waxes, or serve as precursors for other aliphatic  
20 hydrocarbons found in waxes, including alkanes, primary and secondary alcohols, ketones aldehydes and acyl- esters. VLCFAs also accumulate in the seed oil of some plant species, where they are incorporated into triacylglycerols (TAGs), as in the Brassicaceae, or into wax esters, as in jojoba. These seed VLCFAs include the agronomically important erucic acid (C22: 1), used in the production of lubricants, nylon, cosmetics, pharmaceuticals and  
25 plasticizers.

In yeast and mammals, VLCFA synthesis is catalyzed in the Endoplasmic Reticulum by a membrane-bound multi-enzyme protein complex referred as the elongase. The elongase complex catalyzes the cyclic addition of a C2-moiety obtained from malonyl-Coenzyme A to an acyl-CoA. VLCFAs (C20, C22, C24 or higher) are produced from  
30 shorter fatty acids (usually C16 or C18) made by the cytosolic Fatty Acid Synthase complex (FAS). The two-carbon addition during the elongation cycle requires four independent but sequential enzymatic steps.

The first step involves the condensation of the malonyl-CoA with an acyl<sub>n</sub>-CoA precursor resulting in 3-ketoacyl-CoA intermediate that is reduced to form a 3-hydroxy-acyl-CoA. The third enzymatic step is the dehydration of the 3-hydroxy-acyl-CoA to an enoyl-CoA that is finally reduced to yield an acyl<sub>n+2</sub>-CoA. The component members of the  
5 elongase were recently fully described in yeast.

In plants, there is a large family of 3-ketoacyl-CoA synthases (KCS) condensing enzymes exemplified by the Arabidopsis gene Fatty Acid Elongase 1 (FAEI), required in seeds for the synthesis of the C20+ fatty acids such as erucic acid. The Arabidopsis genome encodes 21 FAE-like KCSs and although these enzymes are structurally unrelated  
10 to the ELO class of condensing enzymes, it has been demonstrated that several Arabidopsis FAE-KCSs can rescue the otherwise lethal yeast *elo2A/elo3A* double mutant. Below is presented a list of genes from *Arabidopsis thaliana*, encoding for enzymes belonging to the elongase complex:

	Gene names	Names found in papers	Accession	References (see below)
15	KCCSSII	KCCSSII	AtIgOl 120	1, 2, 20
	KCS2		Atlg04220	16, 21
	KCS3		Atlg07720	1
	KCS4		At IgI 9440	1
	KCS5	CER60	Atlg25450	2, 4, 21
20	KCCSS66	CEERR66,CCUUTT11	Atlg68530	2, 4, 8, 11, 15, 21
	KCS7		Atlg71 160	1
	KCS8		At2gl5090	
	KCS9		At2g 16280	16
	KCS10	FDH	At2g26250	2, 3, 13, 17, 19, 21, 22
25	KCCSSIII		At2g26640	1
	KCS 12		At2g28630	
	KCS 13	HIC	At2g46720	<b>2, 7</b>
	KCS 14		At3gl0280	
	KCS 15		At3g52160	
30	KCCSS 166		At4g34250	1
	KCS 17		At4g34510	1, 12, 21
	KCS 18	KCS2	At4g34520	
	KCS 19	FAEI	At5g04530	1, 16

KCS20		At5g43760	16, 21
KCS21		At5g49070	
KCR		Atlg67730	23
ECR	CERIO	At3g55360	24, 25

5

References :

1, Blacklock and Jaworski (2006); 2, Costaglioli et al. (2005); 3, Efremova et al. (2004); 4, Fiebig et al. (2000); 5, Ghanevati and Jaworski (2001); 6, Ghanevati and Jaworski (2002); 7, Gray et al. (2000); 8, Hooker et al. (2002); 9, James and Dooner (1990); 10, James et al. (1995); 11, Kunst et al. (2000); 12, Kunst and Clemens (2001); 13, Lolle et al. (1997); 14, Millar and Kunst (1997); 15, Millar et al. (1999); 16, Paul et al. (2006); 17, Pruitt et al. (2000); 18, Rossak et al. (2001); 19, Stasolla et al. (2003); 20, Todd et al. (1999); 21, Trenkamp et al. (2004); 22, Yephremov et al. (1999); 23, Beaudoin et al. (2002); 24, Gable et al. (2004); 25, Zheng et al. (2005).

15

**PRIOR ART**

The goal of the present invention is to increase the production of VLCFA into plants. Although the lipid and fatty acid content of seed oil can be modified by the traditional methods of plant breeding, the advent of recombinant DNA technology has allowed for easier manipulation of the oil content of a plant.

20

In order to increase or alter the levels of compounds such as seed oils in plants, nucleic acid sequences and proteins regulating lipid and fatty acid metabolism must be identified.

In yeast, identification of the dehydratase of the elongase complex remained elusive until the recent identification of *PHS1* as encoding this activity. The *phs1* mutant was also characterized as a cell cycle mutant defective in G2/M phase. The biochemical function of Phs1p as an hydroxyacyl-CoA dehydratase was provided by *in vitro* activity of recombinant protein and reconstitution of the elongase complex in proteoliposomes (Denic & Weissman, 2007). However, effects of the surexpression of this gene *in vivo* are unknown.

30

The role of the Arabidopsis *PASTICCINO2* (*PAS2*) gene in regulation of the cellular cycle has been known for a while. Mutations in *PAS2* gene lead to strong developmental defects mainly associated with ectopic cell division (Bellec et al., 2002;

Faure et al., 1998; Haberer et al., 2002). This gene shares a significant similarity with the yeast dehydratase *PHSI* (Bellec et al., 2002). Responses to hormones like auxin and cytokinins that are essential for cell cycle progression and cell differentiation were also altered in *pas2* mutant (Harrar et al., 2003). Finally, PAS2 was demonstrated to be able to  
5 interact with phosphorylated Cyclin dependent kinase and subsequently to prevent its dephosphorylation by CDC25-like phosphatase(s), preventing premature entry in mitosis (Da Costa et al., 2006).

## DESCRIPTION OF THE INVENTION

10 The recent advances in plant molecular biology have made possible genetic engineering of most crop species. The technology has been applied to improving biosynthesis of VLCFAs in plant cells.

Here, inventors showed for the first time that a recombinant plant cell expressing an heterologous gene encoding for an hydroxyacyl-CoA dehydratase is useful for the  
15 production of VLCFA.

In particular, inventors showed that *PAS2* gene from Arabidopsis is associated with lipid biosynthesis and homeostasis. Indeed, PAS2 was found to be associated with ER and to physically interact with the reductase CERIO, which was consistent with a role of dehydratase in the Arabidopsis microsomal elongase complex. An overexpression of the  
20 *PAS2* gene leads to an increased production of VLCFA in recombinant plant cells.

In the present application, a new method for the production of VLCFA into a plant cell is provided, comprising culturing a recombinant plant cell in an appropriate medium, wherein said plant cell comprises an heterologous gene encoding for an hydroxyacyl-CoA dehydratase, such as *PAS2* from *Arabidopsis thaliana*.  
25

## DETAILED DESCRIPTION OF THE INVENTION

The invention is related to a method for the production of VLCFA into a plant cell, comprising culturing a recombinant plant cell in an appropriate medium, wherein said plant cell is transformed with an heterologous gene encoding for an hydroxyacyl coA  
30 dehydratase.

As used herein, the following terms may be used for interpretation of the claims and specification.

According to the invention, the term "VLCFA" refers to very long chain fatty acids, that are composed of 20 or more carbons in length (i.e. >C18).

The term "plant cell" designates an isolated cell obtained from a plant by classical methods known by the man skilled in the art, such as a cell from any organ of a plant (seeds, leaves, roots, flowers) or cells that form *in vitro* grown plant cell cultures.

The term "recombinant plant cell" designates a cell having been transformed with exogenous DNA, and having integrated this DNA.

The term "transformation" refers to the introduction of new genes or extra copies of existing genes into a plant cell. The acquired genes may be incorporated into chromosomal DNA or introduced as extra-chromosomal elements. As an example, for plant cells, a method for transferring DNA into a host organism is inoculation or infiltration of plant cells (from *in vitro* culture), of explants (like hypocotyls, roots) or of organs (like leaves or flowers) with *Agrobacterium tumefaciens* or *Agrobacterium rizhogenes*. Another method is the direct introduction of DNA (like electroporation or PEG mediated transfection) into plant protoplasts.

The term "culturing" includes maintaining and/or growing a living plant cell such that it can perform its intended function, i.e the production of fatty acids. A plant cell may be cultured in liquid media, in solid media, semi-solid media or in soil.

An "appropriate medium" designates a medium (e.g., a sterile, liquid media) comprising nutrients essential or beneficial to the maintenance and/or growth of the cell such as carbon sources or carbon substrate, for example carbohydrate, hydrocarbons, oils, fats, fatty acids, organic acids, and alcohol's; nitrogen sources, for example, peptone, yeast extracts, meat extracts, malt extracts, urea, ammonium sulfate, ammonium chloride, ammonium nitrate and ammonium phosphate; phosphorus sources, for example, monopotassium phosphate or dipotassium phosphate; trace elements (e.g., metal salts), for example magnesium salts, cobalt salts and/or manganese salts; as well as growth factors such as amino acids, vitamins, growth promoters, and the like.

The terms "encoding" or "coding" refer to the process by which a polynucleotide, through the mechanisms of transcription and translation, produces an amino-acid sequence. This process is allowed by the genetic code, which is the relation between the sequence of bases in DNA and the sequence of amino-acids in proteins. One major feature of the genetic code is to be degenerate, meaning that one amino-acid can be coded by more than one triplet of bases (one "codon"). The direct consequence is that the same amino-acid

sequence can be encoded by different polynucleotides. It is well known from the man skilled in the art that the use of codons can vary according to the organisms. Among the codons coding for the same amino-acid, some can be used preferentially by a given microorganism. It can thus be of interest to design a polynucleotide adapted to the codon usage of a particular microorganism in order to optimize the expression of the corresponding protein in this organism.

The terms "enzyme activity" and "enzymatic activity" are used interchangeably and refer to the ability of an enzyme to catalyse a specific chemical reaction.

The term "hydroxyacyl-CoA dehydratase" refers to a polypeptide responsible for an enzyme activity that catalyzes the "third step" of the VLCFA elongation, i.e. the dehydration of a 3-hydroxy-acyl-CoA to an enoyl-CoA. Such an enzyme activity of 3-hydroxy acyl-CoA dehydration was described in plants in (Lessire et al, 1999). Methods to measure this enzyme activity were provided in the same reference and in the recent work of (Kihara et al., 2008).

Inventors showed that this step of dehydration is a limiting step in the full processus of elongation. Therefore, increasing the amount or activity of this specific enzyme, among the four enzymes involved in the VLC fatty acids elongation, lead to a dramatic increase of production of VLCFA.

In a particular embodiment of the invention, the heterologous gene is a gene sharing homology with the PAS2 gene from Arabidopsis, or a gene encoding for a protein sharing homology with the protein PAS2, such can be determined by the man skilled in the art.

A protein sharing homology with the protein PAS2 may be obtained from plants or may be a variant or a functional fragment of a natural protein originated from plants.

The term "variant or functional fragment of a natural protein" means that the amino-acid sequence of the polypeptide may not be strictly limited to the sequence observed in nature, but may contain additional amino-acids. The term "a fragment" means that the sequence of the polypeptide may include less amino-acid than the original sequence but still enough amino-acids to confer hydroxyacyl CoA dehydratase activity. It is well known in the art that a polypeptide can be modified by substitution, insertion, deletion and/or addition of one or more amino-acids while retaining its enzymatic activity. For example, substitution of one amino-acid at a given position by a chemically equivalent amino-acid that does not affect the functional properties of a protein are common. For the

purpose of the present invention, substitutions are defined as exchanges within one of the following groups :

- Small aliphatic, non-polar or slightly polar residues : Ala, Ser, Thr, Pro, Gly
- Polar, negatively charged residues and their amides : Asp, Asn, Glu, Gln
- 5    ▪ Polar, positively charged residues : His, Arg, Lys
- Large aliphatic, non-polar residues : Met, Leu, Ile, Val, Cys
- Large aromatic residues : Phe, Tyr, Trp.

Thus, changes that result in the substitution of one negatively charged residue for another (such as glutamic acid for aspartic acid) or one positively charged residue for another (such as lysine for arginine) can be expected to produce a functionally equivalent product.

The positions where the amino-acids are modified and the number of amino-acids subject to modification in the amino-acid sequence are not particularly limited. The man skilled in the art is able to recognize the modifications that can be introduced without affecting the activity of the protein. For example, modifications in the N- or C-terminal portion of a protein may be expected not to alter the activity of a protein under certain circumstances.

The term "variant" refers to polypeptides submitted to modifications such as defined above while still retaining the original enzymatic activity.

According to the invention, the polypeptide having an hydroacyl-CoA dehydratase enzymatic activity may comprise a sequence having at least 30 % of homology with the sequence of PAS2, preferentially at least 50% of homology, and more preferentially at least 70% of homology.

Methods for the determination of the percentage of homology between two protein sequences are known from the man skilled in the art. For example, it can be made after alignment of the sequences by using the software CLUSTALW available on the website <http://www.cbi.ac.uk/clustalw/> with the default parameters indicated on the website. From the alignment, calculation of the percentage of identity can be made easily by recording the number of identical residues at the same position compared to the total number of residues. Alternatively, automatic calculation can be made by using for example the BLAST programs available on the website <http://www.ncbi.nlm.nih.gov/BLAST/> with the default parameters indicated on the website.

Preferred genes encoding proteins according to the invention are selected among genes presented in figure 1, i.e. genes from *Vitis vinifera* (encoding CAN64341.1 hypothetical protein), *Oryza sativa* (CAD39891.2, EAY72548.1 hypothetical protein



OsI\_000395, EAZ30025.1 hypothetical protein OsJ\_013508 and BAD61 107.1 tyrosine phosphatase-like), *Brassica rapa* (AAZ66946.1), *Hyacinthus orientalis* (AAT08740.1 protein tyrosine phosphatase), *Ostreococcus lucimarinus* (XP\_001420997.1 predicted protein and XP\_001422898.1 predicted protein), *Chlamydomonas reinhardtii* (EDPO 1055.1 predicted protein), and also from *Brassica napus*, *Raphanus sativus*,  
5 *Brassica oleracea*.

In a specific embodiment of the invention, the heterologous gene is the gene PAS2 from *Arabidopsis thaliana*, registered in UniGene databank under number NP\_196610.2, also known as F12B17.170; F12B17\_170; PASTICCINO 2; PEP; and PEPINO.

10 In another specific embodiment of the invention, the heterologous gene is the *PHSI* gene from *Saccharomyces cerevisiae*, registered in gene databanks under number NP\_012438.1

In another embodiment of the invention, the heterologous gene is from the same species than the species of the host plant cell.

15 In a preferred embodiment of the invention, the heterologous gene is under the control of a promoter allowing the expression of said gene in the host plant cell.

Preferentially, said promoter is a seed-specific promoter. This term "seed-specific promoter" means that a gene expressed under the control of the promoter is predominantly expressed in plant seeds with no substantial expression, typically less than 5% of the overall expression level, in other plant tissues.  
20

Seed-specific plant promoters are known to those of ordinary skill in the art and are identified and characterized using seed-specific mRNA libraries and expression profiling techniques. Seed-specific promoters include the napin- gene promoter from rapeseed, the USP-promoter from *Vicia faba*, the oleosin-promoter from *Arabidopsis*, the phaseolin-promoter from *Phaseolus vulgaris*, the Bce4-promoter from *Brassica* or the legumin B4  
25 promoter as well as promoters conferring seed specific expression in monocot plants like maize, barley, wheat, rye, rice etc.

In a specific embodiment of the invention, the promoter is the promoter of the gene Napin from *Arabidopsis* (Accession number: At4g27150); for reference see (Guerche et al., 1990).  
30

In another embodiment of the invention, the promoter used in the invention is an inducible promoter. Chemically inducible promoters are especially suitable if gene expression is desired in a time specific manner. Examples for such promoters are a

salicylic acid inducible promoter, a tetracycline inducible promoter and an ethanol inducible promoter. Promoters responding to biotic or abiotic stress conditions are also suitable promoters such as the pathogen inducible PRP1-gene promoter, the heat inducible hsp80-promoter from tomato, cold inducible alpha-amylase promoter from potato or the wound- inducible pinll-promoter.

In a specific embodiment of the invention, the promoter may be chosen in a way to obtain gene expression in a time specific manner; for example, the man skilled in the art might chose between the following list of Arabidopsis promoters:

Gene name	Accession	Expression pattern	references
ABB	At3g24650	early	1
At2S3	At4g27160	late	2
FUSCA3	At3g26790	late	3
OLEOSIN 1	At4g25140	late	4, 5
OLEOSIN 2	At5g40420	late	4, 5
OLEOSIN 3	At5g50770	late	4, 5
OLEOSIN 4	At3g27660	late	4, 5

References :

1, Despres (2001); 2, Guerche (1990); 3, Luerssen (1998); 4, Plant, (1994); 5, Siloto (2006)

All these promoters could be used for expressing a gene encoding for an hydroxyacyl-CoA dehydratase in the seed.

In a specific embodiment of the invention, at least one another gene involved in the VLCFA biosynthesis is introduced into the plant cell. In particular, this gene encodes for another or several enzyme(s) belonging to the elongase complex.

Preferentially, said at least one gene is encoding for an enzyme selected from the following list: a fatty acid elongase, a reductase and combinations thereof.

In a particular way to realize the invention, the recombinant plant cell is a cell from the seed.

The invention is also related to a method for the production of VLCFA into plants, comprising culturing a plant comprising at least one cell transformed with an heterologous gene encoding for an hydroxyacyl-CoA dehydratase.

In a specific embodiment of the invention, the totality of the cells from the plant  
5 was transformed with the heterologous gene, and the plant is said "transformed plant" or "transgenic plant".

The term « production of VLCFA » designates the fact that the plant biosynthesizes a detectable amount of VLCFA. Quantities that might be obtained are shown in the examples, in particular in Figures 2 and 3, wherein VLCFA productions were analysed and  
10 compared from seeds from different genotypes.

The transformed plant may be chosen among *Arabidopsis thaliana*, *Brassica napus*, *Brassica juncea*, *Helianthus annuus*, and all other plants that may be determined as useful by the man skilled in the art. In particular, the invention could be applied to other plants including rapeseed, canola, linseed, soybean, sunflower, maize, oat, rye, barley, wheat,  
15 rice, pepper, tagetes, cotton, oil palm, coconut palm, flax, castor, and peanut.

Preferentially, the method according to the invention further comprises a step of extraction of the VLCFA from the cell plant or from the plant. Technics for extraction of fatty acids from plants are well known by the man skilled in the art, and comprise in particular gas chromatography; see for reference Baud et al. (2002).

20 This invention is also related to a method for producing vegetable oil, comprising the following steps :

- Culturing a plant comprising at least one cell transformed with an heterologous gene encoding for an hydroxyacyl-CoA dehydratase such as defined previously, and
- 25 - extracting the oil from the plant.

Said vegetable oil is advantageously enriched in VLCFA.

Finally, the invention is also related to a method for identifying plants having a high potential of VLCFA biosynthesis, wherein said plants are selected on their level of  
30 expression or level of activity of hydroxyacyl-CoA dehydratase.

## DRAWINGS

**Figure 1.** Phylogenetic analysis of *PAS2* homologs in plants. *PAS2* protein homologs were identified by BLASTp from plant protein database (BCBI) and the resulting sequences were aligned using CLUSTALw. Graphical representation of sequence identities  
5 is presented as phylogenetic rooted tree.

**Figure 2.** Acyl-CoA dehydratase is an essential and limiting activity.

(A) Total fatty acid levels in the roots of *pas2-1* and *PHSl* expressing plants compared to wild type.

(B) Seed dry weight of *pas2-1* and *PHSl* expressing plants compared to wild type.

10 (C) Total fatty acid levels in *pas2-1* and *PHSl* expressing plants compared to wild type.

Dry weight and fatty acid values are the average of three samples  $\pm$  sd.

**Figure 3.** VLCFA production in plant seeds transformed with *PAS2*, *PHSl*, CoIO or deleted of the *PAS2* gene. VLCFA levels are presented as values of each class (assessed by the length of the acyl chain) relative to the total amount of VLCFA (expressed in % of  
15 absolute values in nmol/mg fresh weight).

## EXAMPLES

**Example 1 : Arabidopsis cells expressing an heterologous gene *PHSl* from yeast produce higher levels of VLCFA**

20 The orthologous yeast *PHSl* gene was introduced into Arabidopsis plant cell to monitor the effect of increasing dehydratase activity on VLCFA levels and on plant development. *PHSl* was cloned under the control of the ubiquitous 35S promoter. Several independent lines expressing *PHSl* showed clear growth retardation associated with abnormal leaf development :

- 25
- Leaves from transgenic lines were smaller and more crinkled than that of control plants.
  - They also showed altered shapes with pronounced serration and often an asymmetric development of the leaf blade leading to a sickled shape.
  - Epidermal cells from *PHSl* expressing transgenic leaves were characterized by a  
30 large heterogeneity in cell sizes and shapes.
  - the surface of *PHSl*-expressing leaf epidermal cells was decorated with wax crystals suggesting an increase in cuticular waxes in contrast to wild type (Fig. 5C).

- Flower development was also modified by *PHSl* expression with for instance misshapen and unfused carpels.
- Detailed analysis of cell surface of unfused carpel showed high accumulation of cuticular waxes.

5

Extraction and analyses of fatty acid methyl esters by gas chromatography were performed as described previously in (Baud et al., 2002) and modified according to (Li et al, 2006).

For lipid extraction, 20 seeds were ground in a glass reaction tube in 250 µl of chloroform/methanol/acetic acid/water (10:10:1:1, v/v/v/v) and incubated at -20 C overnight. Then, 92 µl of chloroform/methanol/water (5:5:1, v/v/v) and 125 µl of Hajra solution (2 M KCl and 0.2 M H<sub>3</sub>PO<sub>4</sub>) were added. After shaking and centrifugation the lower phase, which contains lipids, was transferred to a new glass tube and stored at -20 C. For total fatty acid quantity and composition analyses by gas chromatography of the corresponding fatty acyl methyl esters, extracted lipids were incubated in 1 ml of methanol/sulphuric acid (100:2.5, v/v) at 80 °C for 30 min after addition of 17:0 fatty acid as an internal standard. Fatty acyl methyl esters were then extracted into 450 µl of hexane following the addition of 1.5 ml of water. After vigorous shaking and centrifugation, 1 µl of the upper organic phase was analysed by gas chromatography. Fatty acid methyl esters were separated by GC on a 15-m x 0.53-mm Carbowax column (Alltech, France) and quantified using a flame ionisation detector. The gas chromatograph was programmed for an initial temperature of 160 C for 1 min followed by a 40 C/min ramp to 190 C and a secondary ramp of 4 C/min to 230 C; this final temperature was maintained for 2 min.

Analysis of fatty acid content of roots of young seedlings showed that ectopic expression of *PHSl* modified VLCFA content. Indeed, the *35S:PHSl* seedlings showed significant changes in the relative distribution of VLCFAs with higher levels of 22:0 compared to wild type (Figure 2A). Since VLCFAs are also normally found in mature seeds, we investigated the effect of *PHSl* expression on seed size and total fatty acid levels. Expression of *PHSl* led to slightly larger seeds while *pas2* mutant showed smaller seeds compared to wild type (Figure 2B). Similarly to that observed with seedlings, *PHSl* expressing seeds showed an increase in VLCFAs mostly 22:1 (Figure 2C).

In roots, fatty acid analysis showed a similar effect of *Phsl* in roots compared to seeds. *Phsl* expressing plants do not show any increase of c20 fatty acids (levels are

actually decreased by 20%). The levels of longer fatty acids like 22 :0 and 24 :0 were increased respectively by 54 and 44%.

In conclusion, VLCFA dehydratase, is not only an essential enzyme for plant growth and development but it is also a limiting step for VLCFA synthesis since an  
5 increased dehydratase expression resulted in enhanced levels of VLCFAs in both vegetative and seed tissues.

**Example 2.** Ectopic *PHSI* and *PAS2* expression in mature seeds lead to an increase in VLCFAs mostly C22:0 and C22:1.

10 For seed fatty acid analysis, 20 mature seeds were ground in clean glass tube with 1 ml of methanol/toluene/H<sub>2</sub>SO<sub>4</sub> (1:0,3 v:v plus 0,25 % H<sub>2</sub>SO<sub>4</sub> v/v). Then, samples are incubated at 80 °C checking after 1 or 2 minutes for any leak. After 90 minutes, tubes are removed from heat, and fatty acyl methyl esters were then extracted into 450 µl of hexane following the addition of 1.5 ml of water. After vigorous shaking and centrifugation, 1 µl of the  
15 upper organic phase was analysed by gas chromatography. Fatty acid methyl esters were separated by GC. To estimate the total fatty acids, 10 µg of C17:0 per mL of sulfuric methanol toluene were added.

The VLCFAs seed composition were analyzed for two independent lines expressing either *PHSI* (lines 3.3 and 3.16) or *PAS2* (lines 1 and 2) under the control of the 35S promoter  
20 and compared with wild type (accession Columbia-0, CoIO). Plant cells "*PAS2*" designates mutant cells whose *PAS2* gene is deleted.

Results are presented in figure 3.

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## CLAIMS

1. Method for the production of VLCFA into a plant cell, comprising culturing a recombinant plant cell in an appropriate medium, wherein said plant cell is transformed with an heterologous gene encoding for an hydroxyacyl-CoA dehydratase.  
5
2. A method according to claim 1, wherein said heterologous gene is selected among genes from *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Vitis vinifera*, *Oryza sativa*, *Brassica rapa*, *Hyacinthus orientalis*, *Ostreococcus lucimarinus*,  
10 *Chlamydomonas reinhardtii*, *Brassica napus*, *Raphanus sativus*, and *Brassica oleracea*.
3. A method according to claim 1, wherein the heterologous gene is the gene PAS2 from *Arabidopsis thaliana*.
4. A method according to claim 1, wherein the heterologous gene is the PHS1 gene  
15 from *Saccharomyces cerevisiae*.
5. A method according to anyone of claims 1 to 4, wherein the heterologous gene is under the control of a promoter.
6. A method according to claim 5, wherein the heterologous gene is under the control of a seed-specific promoter.
- 20 7. A method according to claims 5 or 6, wherein the heterologous gene is under the control of an inductible promoter.
8. A method according to anyone of claims 1 to 7, wherein at least one other gene involved in the VLCFA biosynthesis is introduced into the cell.
9. A method according to claim 8, wherein said at least one gene is encoding for an  
25 enzyme selected from the group consisting in : a fatty acid elongase, a reductase, and combinations thereof.
10. A method according to anyone of claim 1 to 9, wherein the plant cell is a seed cell.
11. Method for the production of VLCFA into plants, comprising culturing a plant comprising at least one cell transformed with an heterologous gene encoding for an  
30 hydroxyacyl-CoA dehydratase.

12. Method according to claim 11, wherein the plant is chosen among *Arabidopsis thaliana*, *Brassica napus*, *Brassica juncea* and *Helianthus annuus*.
13. Method according to anyone of claims 1 to 12, comprising a step of extraction of the VLCFA from the plant cell or from the plant.
- 5 14. A method for producing vegetable oil, comprising :
  - culturing a plant comprising at least one cell transformed with an heterologous gene encoding for an hydroxyacyl-CoA dehydratase as disclosed in one of claims 1 to 12, and
  - extracting the oil from the transformed plant.
- 10 15. A method for identifying plants having a high potential of VLCFA biosynthesis, wherein plants are selected on their level of expression or level of activity of hydroxyacyl-CoA dehydratase.

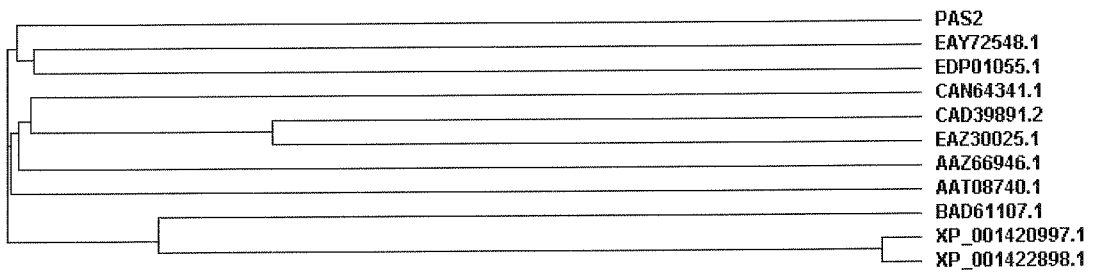


FIGURE 1

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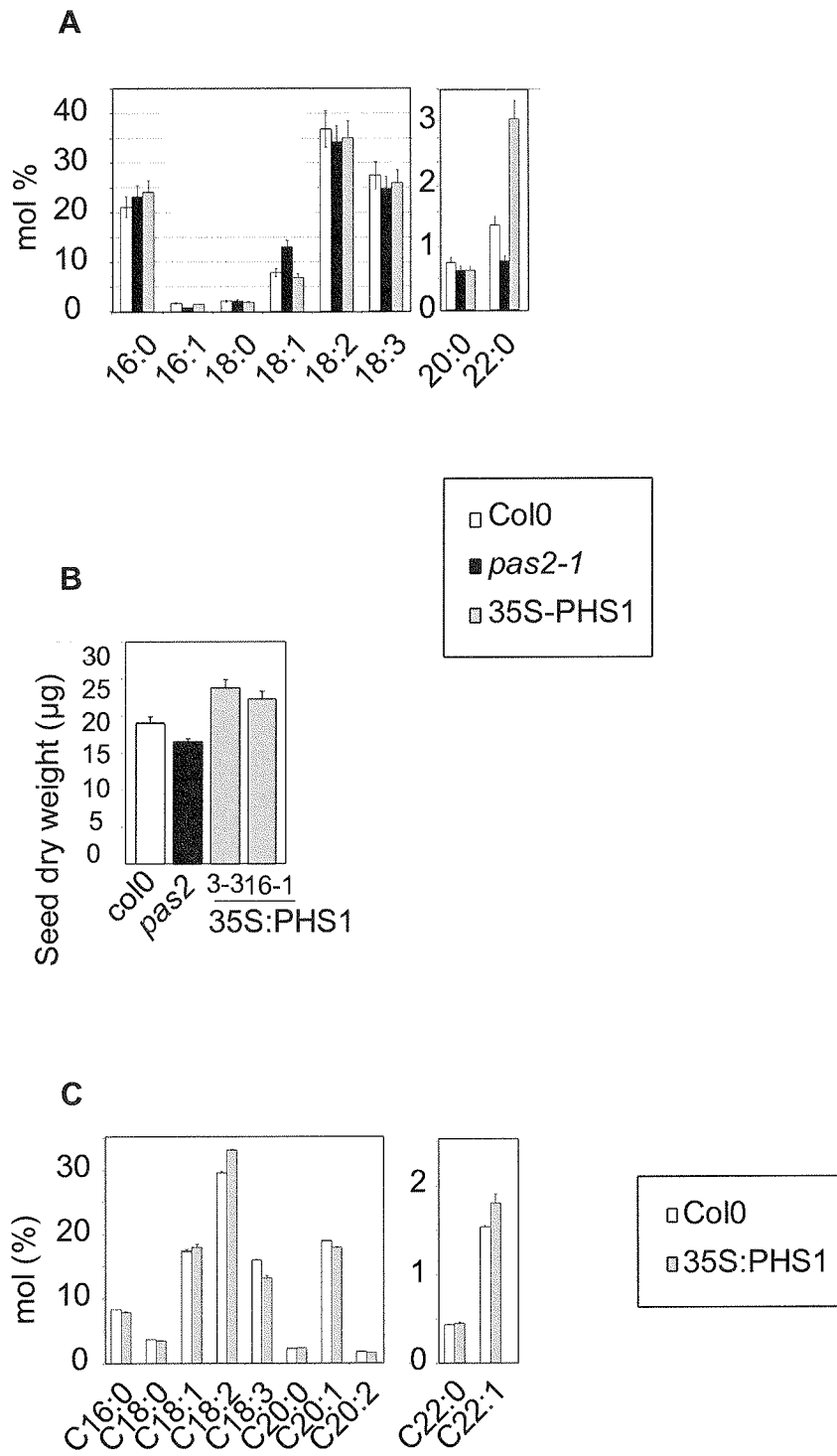
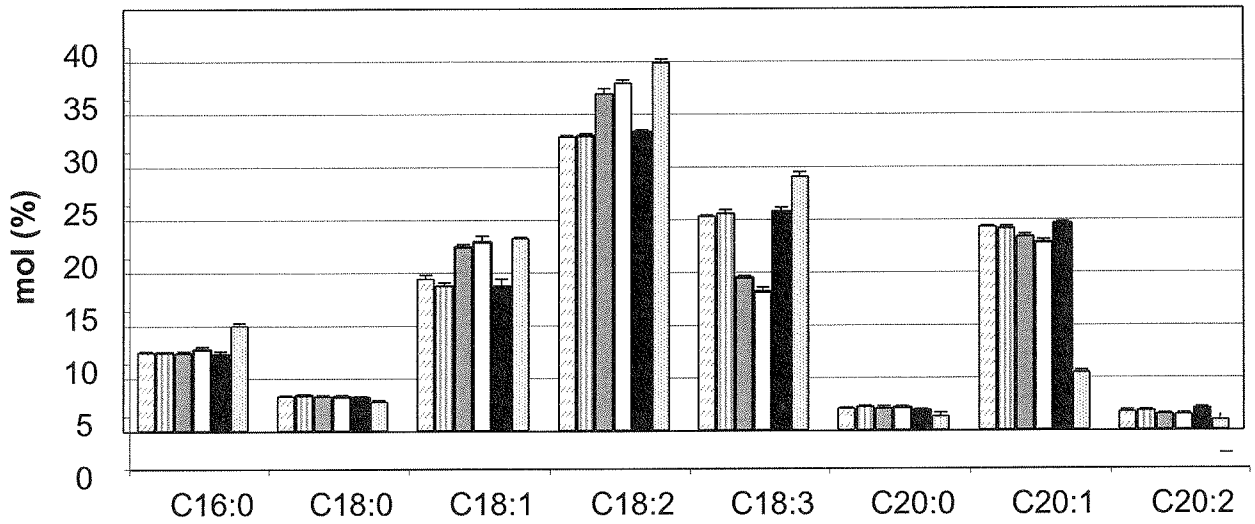
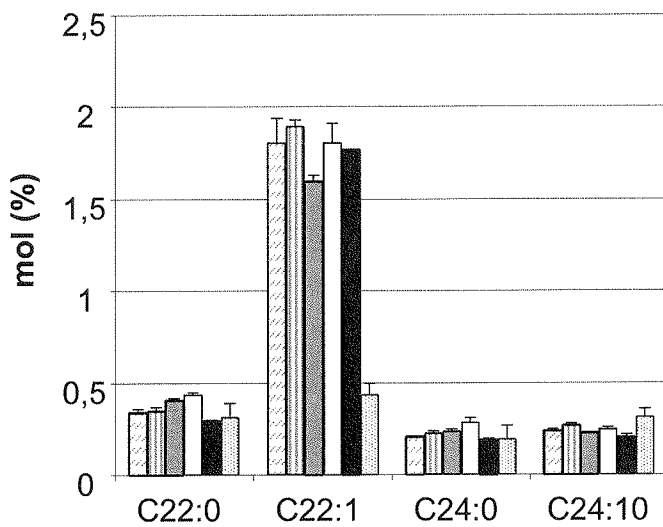
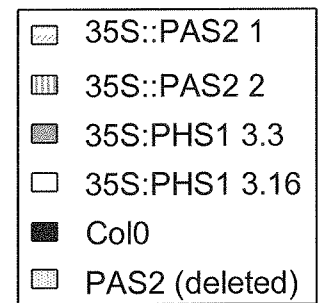


FIGURE 2

3/3



Fatty acids



Fatty acids

FIGURE 3

**INTERNATIONAL SEARCH REPORT**

International application No  
**PCT/EP2009/057964**

**A. CLASSIFICATION OF SUBJECT MATTER**  
INV. C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
**C12N**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and where practical search terms used)  
**EPO-Internal , WPI Data, PAO, BIOSIS, FSTA**

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	WO 2005/052162 A (NAT RES COUNCIL [CA]; MIETKIEWSKA ELZBIETA [CA]; TAYLOR DAVID C [CA];) 9 June 2005 (2005-06-09) claims 1-23	1-5
Y	----- DENIC VLADIMIR ET AL: "A molecular caliper mechanism for determining very long-chain fatty acid length" CELL, vol. 130, no. 4, August 2007 (2007-08), pages 663-677, XP002506523 ISSN: 0092-8674 page 674, right-hand column, paragraph 2 page 664, right-hand column ----- -/--	1-15

Further documents are listed in the continuation of Box C       See patent family annex

Special categories of cited documents

'A' document defining the general state of the art which is not considered to be of particular relevance	'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
'E' earlier document but published on or after the international filing date	'X <sup>1</sup> ' document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	'Y <sup>1</sup> ' document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents such combination being obvious to a person skilled in the art
'O' document referring to an oral disclosure, use, exhibition or other means	'&' document member of the same patent family
'P' document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search <b>4 August 2009</b>	Date of mailing of the international search report <b>14/08/2009</b>
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Name and mailing address of the ISA/ European Patent Office P R 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040 Fax (+31-70) 340-3016	Authorized officer  <b>Marchesini, Patrizia</b>
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## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/057964

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	<p>BELLECC YANNICK ET AL: "Pasticcino2 is a protein tyrosine phosphatase-like Involved in cell proliferation and differentiation in Arabidopsis."            PLANT JOURNAL,            vol. 32, no. 5, December 2002 (2002-12),            pages 713-722, XP002506524            ISSN: 0960-7412            page 718, right-hand column, paragraph 2 -            page 719, right-hand column, paragraph 2            -----</p>	1-15
Y	<p>JOUBES JEROME ET AL: "The VLCFA elongase gene family in Arabidopsis thaliana: phylogenetic analysis, 3D modelling and expression profiling"            PLANT MOLECULAR BIOLOGY,            vol. 67, no. 5, May 2008 (2008-05), pages            547-566, XP002506525            ISSN: 0167-4412            paragraph [INTRODUCTION]            page 564, right-hand column, line 20 -            left-hand column, last paragraph            -----</p>	1-15
Y	<p>PUYAUBERT JULIETTE ET AL: "Acyl-CoA elongase, a key enzyme in the development of high-erucic acid rapeseed?"            EUROPEAN JOURNAL OF LIPID SCIENCE AND TECHNOLOGY,            vol. 107, no. 4, April 2005 (2005-04),            pages 263-267, XP002506526            ISSN: 1438-7697            page 266, left-hand column, lines 19-25            -----</p>	1-15
L	<p>BACH LIN ET AL: "The very-long-chain hydroxy fatty acyl-CoA dehydratase PASTICCINO2 is essential and limiting for plant development"            PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA,            vol. 105, no. 38,            September 2008 (2008-09), pages            14727-14731, XP002506527            ISSN: 0027-8424            the whole document            -----</p>	1-15

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2009/057964

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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