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(71) Applicants: **BAYER CROPSCIENCE NV** [BE/BE]; J.E. Mommaertslaan 14, BE-1831 Diegem (BE). **WASHINGTON STATE UNIVERSITY** [US/US]; Lighty 280, PO Box 641060, Pullman, Washington 99164-1060 (US).

(72) Inventors: **DENOLF, Peter**; Schonenberg 6, BE-9620 Velzeke (BE). **ENGELEN, Steven**; Bastelare 62, BE-9080 Lochristi (BE). **BROWSE, John**; 41 McKenzie Road, Palouse, Washington 99161 (US). **BAI, Shuangyi**; 1075 SW Latour Peak, Pullman, Washington 99163 (US).

(74) Agents: **FIELDS, Whitney, C.** et al.; Whitham, Curtis & Cook, PC, 11491 Sunset Hills Road, Suite 340, Reston, VA 20190 (US).

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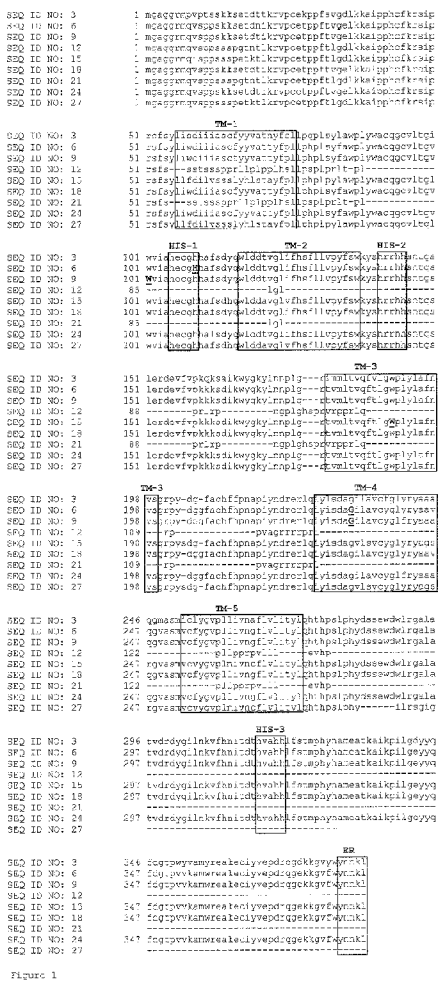

Figure 1

(57) Abstract: The present invention relates to *Brassica* plants comprising mutant *FAD2* genes, *FAD2* nucleic acid sequences and proteins, as well as methods for generating and identifying said plants and alleles, which can be used to plants with increased levels of C18:1 in the seed oil.

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BRASSICA PLANTS WITH MODIFIED SEED OIL COMPOSITION

FIELD OF THE INVENTION

The invention relates to the field of agronomy. Methods and means are provided to modulate fatty acid composition in *Brassica* seeds, such as to increase levels of unsaturated fatty acids in *Brassica* seeds by modification of *FAD2* genes in various manners, including provision of partial knock-out *FAD2* alleles.

BACKGROUND OF THE INVENTION

Many plant species store triacylglycerols (TAGs) in their seeds as a carbon reserve. These TAGs are the major source of energy and carbon material that supports seedling development during the early stages of plant life. Vegetable oils from soybean (*Glycine max*), Brassica (*Brassica napus* or *B. rapa*), sunflower (*Helianthus annuus*) and many other oilseed crops are also an important source of oil for the human diet or industrial applications including, but not limited to biofuels, biolubricants, nylon precursors, and detergent feedstocks. The degree and/or amount of polyunsaturated fatty acids of vegetable oils are characteristic and determinative properties with respect to oil uses in food or non-food industries. More specifically, the characteristic properties and utilities of vegetable oils are largely determined by their fatty acyl compositions in TAG.

Major vegetable oils are comprised primarily of palmitic (16:0), stearic (18:0), oleic (18:1*cis* Δ^{9}), linoleic (18:2*cis* $\Delta^{9, 12}$), and α -linolenic (18:3*cis* $\Delta^{9, 12, 15}$ or C18:3) acids. Palmitic and stearic acids are, respectively, 16 and 18 carbon-long, saturated fatty acids. Oleic, linoleic, and linolenic acids are 18-carbon-long, unsaturated fatty acids containing one, two, and three double bonds, respectively. Oleic acid is referred to as a mono-unsaturated fatty acid, while linoleic and linolenic acids are referred to as poly-unsaturated fatty acids. Modifications of the fatty acid compositions have been sought after for at least a century in order to provide optimal oil products for

human nutrition and chemical (*e.g.*, oleochemical) uses (Gunstone, 1998, Prog Lipid Res 37:277; Broun et al., 1999, Annu Rev Nutr 19:107; Jaworski et al, 2003, Curr Opin Plant Biol 6:178). In particular, the polyunsaturated fatty acids (18:2 and 18:3) have received considerable attention because they are major factors that affect nutritional value and oil stability. However, while these two fatty acids provide essential nutrients for humans and animals, they increase oil instability because they comprise multiple double bonds that may be easily oxidized during processing and storage.

The desaturation of 18:1 into 18:2 is a critical step for synthesizing polyunsaturated fatty acids. During storage lipid biosynthesis, this reaction is known to be catalyzed by the fatty acid desaturase, FAD2, a membrane-bound enzyme located on the endoplasmic reticulum (ER) (Browse and Somerville, 1991, Annu Rev Plant Physiol Plant Mol Biol 42:467), which has delta-12 fatty acid desaturase activity. The FAD2 substrate 18:1 must be esterified on the *sn*-2 position of phosphatidylcholine (PC) (Miquel and Browse, 1992, J Biol Chem 267:1502; Okuley et al., 1994, Plant Cell 6:147), which is the major membrane phospholipid of plant cells. Not surprisingly, therefore, down-regulation of *FAD2* (and *FAD3*) genes has become a preferred strategy for avoiding the need to hydrogenate vegetable oils and the concomitant production of undesirable *trans* fatty acids. For example, soybean has both seed-specific and constitutive *FAD2* desaturases, so that gene silencing of the seed-specific isoform has allowed the production of high-oleate cultivars (>88% 18:1 in the oil) in which membrane unsaturation and plant performance are largely unaffected.

There are several reports on silencing of FAD2 genes in order to increase the levels of oleic acid. Stoutjesdijk et al., 2000 (Biotech Soc Trans 28:938) discloses *B. napus* plants carrying a Δ12-desaturase (FAD2) co-suppression contstruct having oleic acid levels of up to 89%. Chen et al., 2006 (J Plant Physiol Mol Biol 32: 665) report seed-specific FAD2 gene silencing in Brassica napus, which results in oleic acid content in transgenic plant seeds of 83.9%. They further report that the transgenic plants with high oleic acid grow normally and without disadvantageous agronomic traits. Peng et al., 2010, Plant Cell Rep 29:317 disclose *Brassica napus* plants in which FAD2 and the fatty acid elongase 1 (FAE1) genes are simultaneously silenced, reaching oleic acid levels of up to

85%. WO1994/011516 report gene silencing of FAD2 genes in *Brassica napus* resulting in levels of oleic acid of up to 85%. WO2013/112523.

There are also several mutant *Brassica* plants described with increased levels of oleic acid: WO97/21340 and WO98/56239 disclose *Brassica* lines with increased levels of oleic acid, comprising amino acid substitutions in the FAD2 proteins; WO2006/079567 describes a high oleic *Brassica napus* line comprising a nucleotide deletion in a FAD2 gene, leading to a premature translation stop, whereas WO2013/049356 also describes a high oleic *Brassica napus* line comprising a premature translation stopcodon in the FAD2 gene leading to a truncated protein; WO2007138444, WO2007/099459, WO2007/107590 and WO2008/084107 describe several mutations in FAD2 genes in *Brassica* lines with high levels of oleic acid.
Wells et al., 2014 (Mol Breeding 33: 349) and WO2012/117256 describe oilseed rape cultivars with a lower than usual polyunsaturated fatty acids content, which has non-functional alleles at three of the four orthologous *FAD2* loci. Further mutations in the remaining functional FAD2 copy, leading to amino acid substitutions or premature stopcodons, result in a polyunsaturated fatty acids content of about 6%, and an oleic acid content of about 84%.

Significantly, however, canola and other oilseed plants have only constitutive *FAD2* enzymes. Therefore, in canola and other such constitutive *FAD2* crops, silencing or down-regulation of *FAD2* not only alters the fatty acid composition of the storage triacylglycerol (TAG) in seeds, but also of the cellular membranes. For example, the defective *FAD2* in the *Arabidopsis* mutant *fad2* alters fatty acid compositions of seeds as well as vegetable tissues, and severely compromises plant growth (Browse and Somerville, *supra*). *FAD2* mutations and silencing that produce the highest 18:1 levels in the oil also reduce membrane unsaturation in vegetative and seed tissues, resulting in plants that germinate and grow poorly. As a result, only partial downregulation of FAD2 expression is possible, producing approximately 70-75% 18:1 in the oil of commercial cultivars such as Nexera/Natreon (Dow AgroSciences) and Clear Valley 75 (Cargill).

The object of the current invention is to provide *Brassica* FAD2 alleles for the production of plants with high levels of oleic acids while maintaining normal agronomic development.

SUMMARY OF THE INVENTION

It is a first embodiment of the invention to provide a *Brassica* plant, or a cell, part, seed or progeny thereof, comprising at least one FAD2 gene, wherein at least one allele of said FAD2 gene is a partial knock-out *fad2* allele, said partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, wherein said plant does not contain a FAD2 gene of which the *FAD2* alleles encode a wild-type functional protein. In a further embodiment, said *Brassica* plant, or a cell, part, seed or progeny thereof, comprises at least a second, or a second and a third, or a second, a third and a fourth FAD2 gene, wherein the *fad2* alleles of said FAD2 genes are full knock-out *fad2* alleles, such as a *Brassica* plant, or a cell, part, seed or progeny thereof, wherein the full knock-out *fad2* allele of said second, or second and third, or second and third and fourth *FAD2* gene is a *fad2* allele encoding a protein in which the His at a position corresponding to position 109 of SEQ ID NO: 6 is substituted with another amino acid, or comprising a stop codon mutation at the codon encoding the Trp at a position corresponding to position 101 of SEQ ID NO: 9, or comprising a deletion mutation of the nucleotides corresponding to nucleotides 2036-2042 of SEQ ID NO: 13, or comprising a stop codon mutation in the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15. In yet another embodiment, the partial knock-out *fad2* allele of the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention, is an allele of a FAD2-A1 or a FAD2-C1 gene. In another embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is a *Brassica napus* plant, or a cell, part, seed or progeny thereof, comprising a second, a third and a fourth FAD2 gene, wherein the partial knock-out *fad2* allele is an allele of a FAD2-A1 gene, and wherein the second FAD2 gene is a FAD2-C1 gene, the third FAD2 gene is a FAD2-C2 gene, and the fourth FAD2 gene is a FAD2-A2 gene, or wherein the partial knock-out *fad2* allele is an allele of a FAD2-C1 gene, and wherein the second FAD2 gene is a FAD2-

A1 gene, the third FAD2 gene is a FAD2-C2 gene, and the fourth FAD2 gene is a FAD2-A2 gene. In yet another embodiment, the partial knock-out *fad2* allele in the plants according to the invention encodes a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp.

In a further embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is a *Brassica* plant, or a cell, part, seed or progeny thereof, wherein a fisrt FAD2 gene is a FAD2-C1 gene of which at least one allele is said partial knock-out *fad2* allele which encodes a FAD2 protein of which the Gly at a position corresponding to position 232 of SEQ ID NO: 9 is substituted with Ser or with Asp; said second FAD2 gene is a FAD2-A1 gene of which at least one allele encodes a FAD2 protein of which the His at a position corresponding to position 109 of SEQ ID NO: 6 is substituted with Tyr; and the third FAD2 gene is a FAD2-C2 gene of which at least one allele contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15. In yet another embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is derivable or obtainable from seeds selected from the group consisting of a) seed comprising HIOL101 having been deposited at NCIMB under accession number NCIMB 42376, b) seed comprising HIOL120 having been deposited at NCIMB under accession number NCIMB 42377, and c) seed comprising HIOL109 having been deposited at NCIMB under accession number NCIMB 42375.

In yet another embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is a *Brassica* plant, or a cell, part, seed or progeny thereof, wherein a fisrt FAD2 gene is a FAD2-A1 gene of which at least one allele is said partial knock-out *fad2* allele which encodes a FAD2 protein of which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp; the second FAD2 gene is a FAD2-C1 gene of which at least one allele contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 101 of SEQ ID NO: 9; and the third FAD2 gene is a FAD2-C2 gene of which at least one allele contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15. In yet another embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof

according to the invention is derivable or obtainable from seeds selected from the group consisting of a) seed comprising HIOL116 having been deposited at NCIMB under accession number NCIMB 42373, b) seed comprising HIOL103 having been deposited at NCIMB under accession number NCIMB 42374, and c) seed comprising HIOL109 having been deposited at NCIMB under accession number NCIMB 42375.

In a further aspect, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is homozygous for any one of the knock-out *fad2* alleles, and in yet another aspect, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention has increased levels of C18:1 in the seed oil, such as a level of C18:1 in the seed oil of about 75%, and which maintains normal agronomic development. Also provided is oil from the seeds according to the invention.

Another aspect of the invention provides a method for increasing the levels of C18:1 in seed oil while maintaining normal agronomic development, said method comprising introducing a partial knock-out *fad2* allele of a *FAD2* gene, said partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, and, optionally, introducing one or more full knock-out *fad2* alleles of one or more FAD2 genes, into a *Brassica* plant and selecting a *Brassica* plant comprising said partial knock-out *fad2* allele which does not contain another *FAD2* gene of which the *FAD2* alleles encode a wild-type functional FAD2 protein. In a further embodiment, the *Brassica* plant produced by said method comprises at least a second, or a second and a third, or a second, a third and a fourth FAD2 gene, wherein the *fad2* alleles of said FAD2 genes are full knock-out *fad2* alleles. In another embodiment, the method according to the invention comprises the step of selecting said *Brassica* plant comprising said partial knock-out *fad2* allele which does not contain another *FAD2* gene of which the *FAD2* alleles encode a wild-type functional FAD2 protein by analyzing genomic DNA from said plant for the presence of at least one molecular marker, wherein said at least one molecular marker is linked to said partial knock-out *fad2* allele and, optionally, to one or more full knock-out *fad2* alleles.

In a further embodiment, a method is provided to determine the presence or absence of a knock-out *fad2* allele in a biological sample, comprising providing genomic DNA

from said biological sample, and analyzing said DNA for the presence of at least one molecular marker, wherein the at least one molecular marker is linked to said knock-out *fad2* allele. Yet another embodiment provides a kit for the detection of a knock-out *fad2* allele in *Brassica* DNA samples, wherein said kit comprises one or more PCR primer pairs, which are able to amplify a DNA marker linked to said knock-out *fad2* allele. Another embodiment provides a method for determining the zygosity status of a knock-out *fad2* allele in a plant, or a cell, part, seed or progeny thereof, comprising determining the presence of a knock-out and/or a corresponding wild type *FAD2* specific region in the genomic DNA of said plant, or a cell, part, seed or progeny thereof.

In yet another aspect of the invention, a method is provided for transferring at least one partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, from one plant to another plant comprising the steps of: (a) identifying a first plant comprising at least one partial knock-out *fad2* allele, (b) crossing the first plant with a second plant not comprising the at least one partial knock-out *fad2* allele and collecting F1 hybrid seeds from the cross, (c) optionally, identifying F1 plants comprising the at least one partial knock-out *fad2* allele, (d) backcrossing F1 plants comprising the at least one partial knock-out *fad2* allele with the second plant not comprising the at least one partial knock-out *fad2* allele for at least one generation (x) and collecting BCx seeds from the crosses, and (e) identifying in every generation BCx plants comprising the at least one partial knock-out *fad2* allele by analyzing genomic DNA of said BCx plants for the presence of at least one molecular marker, wherein the at least one molecular marker is linked to said partial knock-out *fad2* allele.

In another embodiment, a method is provided for combining at least one partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, with at least one full knock-out *fad2* allele in a single *Brassica* plant, said method comprising a) generating and/or identifying two or more plants each comprising one or more selected partial and/or full knock-out *fad2* alleles; b) crossing a first plant comprising one or more selected partial and/or full knockout *fad2* alleles

with a second plant comprising one or more other selected partial and/or full knockout *fad2* alleles; c) collecting seeds from the cross, and, optionally, identifying a plant comprising at least one partial knock-out *fad2* allele and at least one full knockout *fad2* allele; and, optionally d) repeat steps b) and c) until a plant comprising at least one partial knock-out *fad2* allele and at least one full knockout *fad2* allele, is obtained, wherein said plant does not contain a *FAD2* gene of which the *FAD2* alleles encode a wild-type functional protein.

In a further aspect of the invention, a partial knock-out *fad2* allele of a FAD2 gene is provided, wherein the partial knock-out *fad2* allele is a mutated version of the native *FAD2* gene, wherein the native *FAD2* gene is selected from the group consisting of: (a) a nucleic acid molecule which comprises at least 90% sequence identity to SEQ ID No. 4 or 7; (b) a nucleic acid molecule comprising a coding sequence which comprises at least 90% sequence identity to SEQ ID No. 5 or 8; and (c) a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID No. 6 or 9, and wherein said partial knock-out *fad2* allele encodes a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, such as a partial knock-out *fad2* allele which encodes a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp.

In a further embodiment, a method is provided for producing oil, comprising harvesting seeds from the plants according to the invention, and extracting the oil from said seeds.

In yet a further embodiment, a method is provided of producing food or feed, such as oil, meal, grain, starch, flour or protein, or an industrial product, such as biofuel, fiber, industrial chemicals, a pharmaceutical or a neutraceutical, comprising obtaining the plant or a part thereof according to the invention, and preparing the food, feed or industrial product from the plant or part thereof.

Another embodiment provides the use of the partial knock-out *fad2* allele according to the invention to increase the level of C18:1 in the seed oil of a *Brassica* plant while maintaining normal agronomic development. Yet another embodiment provides a

method to produce a *Brassica* plant comprising an increased level of C18:1 in the seed oil and which maintains normal agronomic development, said method comprising sowing seeds according to the invention and growing plants from said seeds.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Alignment of the Arabidopsis and Brassica FAD2 protein sequences. Boxes indicate the conserved domains: TM = transmembrane or membrane-associated domain; HIS = Histidine box; ER = ER retrieval motif. Amino acids of which the codons are mutated in the mutant *fad2* alleles of the current invention are indicated bold, underlined capitals.

Figure 2. Relative expression of *Brassica napus* FAD2 genes. Diamonds: BnFAD2-A1; squares: BnFAD2-C1; triangles: BnFAD2-A2; crosses: BnFAD2-C2. 1: root, 2 weeks old plant; 2: Cotyledons, 10 days after sowing (DAS); 3: stem 15 DAS; 4: stem, 33 DAS; 5: young leaf, 33 DAS; 6: apical meristem + smallest leaf, 33 DAS; 7: small flowerbud, 42 DAS; 8: big flower bud, 42 DAS > 5mm; 9: open flower, 52 DAS; 10: pod, 14-20 DAF; 11: pod, 21-25 DAF; 12: seeds, 14-20 DAF; 13: seeds, 21-25 DAF; 14: seeds, 26-30 DAF; 15: seeds, 31-35 DAF; 16: seeds, 42 DAF; 17: seeds, 49 DAF.

GENERAL DEFINITIONS

A "*FAD2* gene" or "*FAD2* allele", as used herein, is a gene or allele comprising a sequence having at least 60% sequence identity to the coding sequence of the *FAD2* gene of *Arabidopsis thaliana*, accession number At3G12120, as depicted in SEQ ID NO: 2, nts 177-1328.

A *FAD2* gene or *FAD2* allele can, but does not need to encode a functional FAD2 protein. Functionality of the FAD2 protein can be tested, for example, by complementation of the *Arabidopsis fad2-1* mutant as described by Okuley et al., 1994, Plant Cell 6: 147, or by expression and activity analysis in yeast as described by

Peyou-Ndi et al., Arch Biochem Biophys 376:399. "*FAD2* genes" or "*FAD2* alleles" encompass, but are not limited to, *BnFAD2-A1, BnFAD2-A2, BnFAD2-C1, BnFAD2-C2, BrFAD1-1, BrFAD2-2, BoFAD2-1* and *BoFAD2-2* genes or alleles.

A "knock-out *fad2* gene" or "knock-out *fad2* allele" as used herein is a *fad2* gene or a *fad2* allele which encodes no functional FAD2 protein, or which encodes a FAD2 protein with reduced activity. Said "knock-out *fad2* gene" can be a full knock-out *fad2* gene, encoding no functional FAD2 protein, or can be a partial knock-out *fad2* gene, encoding a FAD2 protein with reduced activity. Said "knock-out *fad2* gene" or "knock-out *fad2* allele" can be a mutant *fad2* allele or a mutant *fad2* gene, which may encode no functional FAD2 protein, or which may encode a mutant FAD2 protein with reduced activity. The gene or allele may also be referred to as an inactivated gene or allele. A knock-out *fad2* gene or allele can be a wild-type *FAD2* gene, i.e. a wild-type FAD2 gene which encodes no functional FAD2 protein, or which encodes a FAD2 protein with reduced activity, or can be a mutant *fad2* gene or allele.

A "partial knock-out" *fad2* allele, as used herein, refers to a *fad2* allele, which encodes an FAD2 protein having a reduced biological activity, or a significantly reduced biological activity, as compared to the corresponding wild-type functional FAD2 protein. Such a "partial knock-out *fad2* allele" is, for example, a wild-type *fad2* allele, which comprises one or more mutations in its nucleic acid sequence, for example, one or more mis-sense mutations. In particular, such a partial knockout *fad2* allele is a wild-type FAD2 allele, which comprises a mutation that preferably result in the production of a FAD2 protein wherein at least one conserved and/or functional amino acid is substituted with another amino acid, such that the biological activity is significantly reduced but not completely abolished.

A "full knock-out" or "null" *fad2* allele, as used herein, refers to a *fad2* allele, which encodes a FAD2 protein having no biological activity as compared to a functional FAD2 protein (such as, for example, the wild-type BnFAD2-A1 protein of SEQ ID NO: 6, or the wild-type BnFAD2-C1 protein of SEQ ID NO: 9), or no detectable biological activity in a yeast assay as described by Peyou-Ndi et al., Arch Biochem Biophys 376:399, or which encodes no protein at all. Such a "full knock-out *fad2* allele" is, for example, a wild-type *fad2* allele, which comprises one or more

mutations in its nucleic acid sequence, for example, one or more non-sense or mis-sense mutations. In particular, such a full knock-out *fad2* allele is a wild-type *fad2* allele, which comprises a mutation that preferably result in the production of a FAD2 protein lacking at least one functional domain, such as at least one of the three Histidine boxes, or at least one of the five transmembrane or membrane-associated domains (TM domains), or the ER retrieval motif, such that the biological activity of the FAD2 protein is completely abolished, or whereby the mutation(s) preferably result in no production of a FAD2 protein.

A "functional *FAD2* gene" or "functional *FAD2* allele" as used herein is a *FAD2* gene or a *FAD2* allele which encodes a functional FAD2 protein.

A "mutant *fad2* gene" or "mutant *fad2* allele" as used herein refers to any *fad2* gene or *fad2* allele which is not found in plants in the natural population or breeding population, but which is produced by human intervention such as mutagenesis or gene targeting. A mutant *fad2* allele comprises knock-out *fad2* alleles, and functional *FAD2* alleles. A:mutant *fad2* allele can also be referred to as an "induced mutant *fad2* allele".

Functional FAD2 protein is a FAD2 protein which has at least 10%, or at least 15%, or at least 20%, or at least 25%, or at least 30%, or at least 50%, or at least 80% of the activity of the protein encoded by a reference *Brassica napus FAD2-A1* or *FAD2-C1* gene, as tested, for example, in yeast as described by by Peyou-Ndi et al., Arch Biochem Biophys 376:399, wherein the reference *Brassica napus FAD2-A1* and *FAD2-C1* gene encodes the protein with the amino acid sequence as depicted in SEQ ID No. 6 and SEQ ID No. 9, respectively. A functional FAD2 protein may be a FAD2 protein with "full functionality", which can be 100% functionality of the reference *Brassica napus* FAD2-A1 and FAD2-C1 proteins. A functional FAD2 protein may also be a FAD2 protein with reduced functionality. The activity of a functional FAD2 protein should however not be completely abolished. For example, a functional FAD2 protein has detectable FAD2 activity in a yeast assay as described by Peyou-Ndi et al., Arch Biochem Biophys 376:399.

A "wild-type functional protein" or a "wild-type functional FAD2 protein" as used herein is a functional FAD2 protein which has the functionality of the reference wild-type FAD2 protein, such as the wild-type FAD2-A1, FAD2-C1, or FAD2-C2 protein.

"Biological activity" of a FAD2 protein as used herein is delta-12 desaturation of oleic acid (C18:1) to form linoleic acid (C18:2). The biological activity of a FAD2 protein can, for example, be determined in a yeast assay as described by Peyou-Ndi et al., Arch Biochem Biophys 376:399.

A mutant FAD2 protein with reduced functionality is a FAD2 protein encoded by a mutant *fad2* gene which has reduced activity or a reduction in the biological activity, or a significant reduction in the biological activity, as compared to the corresponding wild-type FAD2 protein encoded by the wild-type *FAD2* gene, but not completely abolished. Said activity may be a reduced with at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 93%, or at least 95%, but wherein the activity is not completely abolished. For example, a mutant FAD2 protein with reduced functionality has detectable FAD2 activity in a yeast assay as described by Peyou-Ndi et al., Arch Biochem Biophys 376:399. A significant reduction in the biological activity of the FAD2 protein refers herein to a reduction in the delta-12 fatty acid desaturase activity, such that the levels of C18:1 in a plant are increased as compared to a plant expressing the corresponding wild type FAD2 protein.

The term "nucleic acid sequence" (or nucleic acid molecule) refers to a DNA or RNA molecule in single or double stranded form, particularly a DNA encoding a protein or protein fragment according to the invention. An "endogenous nucleic acid sequence" refers to a nucleic acid sequence within a plant cell, e.g. an endogenous allele of an *FAD2* gene present within the nuclear genome of a *Brassica* cell. An "isolated nucleic acid sequence" is used to refer to a nucleic acid sequence that is no longer in its natural environment, for example *in vitro* or in a recombinant bacterial or plant host cell.

The term "gene" means a DNA sequence comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. into a pre-mRNA, comprising intron

sequences, which is then spliced into a mature mRNA, or directly into a mRNA without intron sequences) in a cell, operably linked to regulatory regions (e.g. a promoter). A gene may thus comprise several operably linked sequences, such as a promoter, a 5' leader sequence comprising e.g. sequences involved in translation initiation, a (protein) coding region (cDNA or genomic DNA) and a 3' non-translated sequence comprising e.g. transcription termination sites. "Endogenous gene" is used to differentiate from a "foreign gene", "transgene" or "chimeric gene", and refers to a gene from a plant of a certain plant genus, species or variety, which has not been introduced into that plant by transformation (i.e. it is not a "transgene"), but which is normally present in plants of that genus, species or variety, or which is introduced in that plant from plants of another plant genus, species or variety, in which it is normally present, by normal breeding techniques or by somatic hybridization, e.g., by protoplast fusion. Similarly, an "endogenous allele" of a gene is not introduced into a plant or plant tissue by plant transformation, but is, for example, generated by plant mutagenesis and/or selection or obtained by screening natural populations of plants.

"Expression of a gene" or "gene expression" refers to the process wherein a DNA region, which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an RNA molecule. The RNA molecule is then processed further (by post-transcriptional processes) within the cell, e.g. by RNA splicing and translation initiation and translation into an amino acid chain (polypeptide), and translation termination by translation stop codons. The term "functionally expressed" is used herein to indicate that a functional protein is produced; the term "not functionally expressed" to indicate that a protein with significantly reduced or no functionality (biological activity) is produced or that no protein is produced (see further below).

The terms "protein" or "polypeptide" are used interchangeably and refer to molecules consisting of a chain of amino acids, without reference to a specific mode of action, size, 3-dimensional structure or origin. A "fragment" or "portion" of a FAD2 protein may thus still be referred to as a "protein". An "isolated protein" is used to refer to a protein that is no longer in its natural environment, for example *in vitro* or in a recombinant bacterial or plant host cell. "Amino acids" are the principal building blocks of proteins and enzymes. They are incorporated into proteins by transfer RNA

according to the genetic code while messenger RNA is being decoded by ribosomes. During and after the final assembly of a protein, the amino acid content dictates the spatial and biochemical properties of the protein or enzyme. The amino acid backbone determines the primary sequence of a protein, but the nature of the side chains determines the protein's properties. "Similar amino acids", as used herein, refers to amino acids that have similar amino acid side chains, i.e. amino acids that have polar, non-polar or practically neutral side chains. "Non-similar amino acids", as used herein, refers to amino acids that have different amino acid side chains, for example an amino acid with a polar side chain is non-similar to an amino acid with a non-polar side chain. Polar side chains usually tend to be present on the surface of a protein where they can interact with the aqueous environment found in cells ("hydrophilic" amino acids). On the other hand, "non-polar" amino acids tend to reside within the center of the protein where they can interact with similar non-polar neighbors ("hydrophobic" amino acids"). Examples of amino acids that have polar side chains are arginine, asparagine, aspartate, cysteine, glutamine, glutamate, histidine, lysine, serine, and threonine (all hydrophilic, except for cysteine which is hydrophobic). Examples of amino acids that have non-polar side chains are alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, and tryptophan (all hydrophobic, except for glycine which is neutral).

As used herein, the term "allele(s)" means any of one or more alternative forms of a gene at a particular locus. In a diploid (or amphidiploid) cell of an organism, alleles of a given gene are located at a specific location or locus (loci plural) on a chromosome. One allele is present on each chromosome of the pair of homologous chromosomes.

As used herein, the term "homologous chromosomes" means chromosomes that contain information for the same biological features and contain the same genes at the same loci but possibly different alleles of those genes. Homologous chromosomes are chromosomes that pair during meiosis. "Non-homologous chromosomes", representing all the biological features of an organism, form a set, and the number of sets in a cell is called ploidy. Diploid organisms contain two sets of non-homologous chromosomes, wherein each homologous chromosome is inherited from a different parent. In amphidiploid species, essentially two sets of diploid genomes exist,

whereby the chromosomes of the two genomes are referred to as "homeologous chromosomes" (and similarly, the loci or genes of the two genomes are referred to as homeologous loci or genes). A diploid, or amphidiploid, plant species may comprise a large number of different alleles at a particular locus.

As used herein, the term "heterozygous" means a genetic condition existing when two different alleles reside at a specific locus, but are positioned individually on corresponding pairs of homologous chromosomes in the cell. Conversely, as used herein, the term "homozygous" means a genetic condition existing when two identical alleles reside at a specific locus, but are positioned individually on corresponding pairs of homologous chromosomes in the cell.

As used herein, the term "locus" (loci plural) means a specific place or places or a site on a chromosome where for example a gene or genetic marker is found. For example, the "FAD2-*A1* locus" and the "FAD2-A2 locus" refers to the position on a chromosome of the A genome where the *FAD2-A1* gene (and two *FAD2-A1* alleles) or the *FAD2-A2* gene (and two *FAD2-A2* alleles) may be found, such as the position on the chromosome of the A genome of the BnFAD2-A1 locus, the BnFAD2-A2 locus, the BrFAD2-1 locus, and the BnFAD2-2 locus, while the"FAD2-*C1* locus" and the "*FAD2-C2* locus" refers to the position on a chromosome of the C genome where the FAD2-*C1* gene (and two FAD2-*C1* alleles) or the BnFAD2-*C2* gene (and two FAD2-*C2* alleles) may be found, such as the position on the chromosome of the C genome of the BnFAD2-C1 locus, the BnFAD2-C2 locus, the BoFAD2-1 locus, and the BoFAD2-2 locus.

"Wild type" (also written "wildtype" or "wild-type"), as used herein, refers to a typical form of a plant or a gene as it most commonly occurs in nature. A "wild type plant" refers to a plant in the natural population or in a breeding population. A "wild type allele" refers to an allele of a gene occurring in wild-type plants.

"Mutant" as used herein refers to a form of a plant or a gene which is different from such plant or gene in the natural population, and which is produced by human intervention, e.g. by mutagenesis, and a "mutant allele" refers to an allele which is not

found in plants in the natural population or breeding population, but which is produced by human intervention such as mutagenesis or gene targeting.

Whenever reference to a "plant" or "plants" according to the invention is made, it is understood that also plant parts (cells, tissues or organs, seed pods, seeds, severed parts such as roots, leaves, flowers, pollen, etc.), progeny of the plants which retain the distinguishing characteristics of the parents (especially the increased levels of C18:1), such as seed obtained by selfing or crossing, e.g. hybrid seed (obtained by crossing two inbred parental lines), hybrid plants and plant parts derived there from are encompassed herein, unless otherwise indicated.

"Creating propagating material", as used herein, relates to any means know in the art to produce further plants, plant parts or seeds and includes inter alia vegetative reproduction methods (e.g. air or ground layering, division, (bud) grafting, micropropagation, stolons or runners, storage organs such as bulbs, corms, tubers and rhizomes, striking or cutting, twin-scaling), sexual reproduction (crossing with another plant) and asexual reproduction (e.g. apomixis, somatic hybridization).

A "molecular assay" (or test) refers herein to an assay that indicates (directly or indirectly) the presence or absence of one or more particular *FAD2* alleles at one or more *FAD2* loci (e.g. at one or both of the FAD2-A1, FAD2-A2, FAD2-C1 or FAD2-C2 loci). In one embodiment it allows one to determine whether a particular (wild type or mutant) FAD2 allele is homozygous or heterozygous at the locus in any individual plant.

"Mutagenesis", as used herein, refers to the process in which plant cells (e.g., a plurality of *Brassica* seeds or other parts, such as pollen, etc.) are subjected to a technique which induces mutations in the DNA of the cells, such as contact with a mutagenic agent, such as a chemical substance (such as ethylmethylsulfonate (EMS), ethylnitrosourea (ENU), etc.) or ionizing radiation (neutrons (such as in fast neutron mutagenesis, etc.), alpha rays, gamma rays (such as that supplied by a Cobalt 60 source), X-rays, UV-radiation, etc.), T-DNA insertion mutagenesis (Azpiroz-Leehan et al. (1997) Trends Genet 13:152-156), transposon mutagenesis (McKenzie et al. (2002) Theor Appl Genet 105:23-33), or tissue culture mutagenesis (induction of

somaclonal variations), or a combination of two or more of these. Thus, the desired mutagenesis of one or more *FAD2* alleles may be accomplished by one of the above methods. While mutations created by irradiation are often large deletions or other gross lesions such as translocations or complex rearrangements, mutations created by chemical mutagens are often more discrete lesions such as point mutations. For example, EMS alkylates guanine bases, which results in base mispairing: an alkylated guanine will pair with a thymine base, resulting primarily in G/C to A/T transitions. Following mutagenesis, *Brassica* plants are regenerated from the treated cells using known techniques. For instance, the resulting *Brassica* seeds may be planted in accordance with conventional growing procedures and following self-pollination seed is formed on the plants. Alternatively, doubled haploid plantlets may be extracted to immediately form homozygous plants, for example as described by Coventry *et al.* (1988, Manual for Microspore Culture Technique for *Brassica napus*. Dep. Crop Sci. Techn. Bull. OAC Publication 0489. Univ. of Guelph, Guelph, Ontario, Canada). Additional seed that is formed as a result of such self-pollination in the present or a subsequent generation may be harvested and screened for the presence of mutant *fad2* alleles. Several techniques are known to screen for specific mutant alleles, e.g., Deleteagene™ (Delete-a-gene; Li *et al.*, 2001, Plant J 27: 235-242) uses polymerase chain reaction (PCR) assays to screen for deletion mutants generated by fast neutron mutagenesis, TILLING (targeted induced local lesions in genomes; McCallum *et al.*, 2000, Nat Biotechnol 18:455-457) identifies EMS-induced point mutations, etc. Additional techniques to screen for the presence of specific mutant *fad2* alleles are described in the Examples below.

The term “gene targeting” refers herein to directed gene modification that uses mechanisms such as homologous recombination, mismatch repair or site-directed mutagenesis. The method can be used to replace, insert and delete endogenous sequences or sequences previously introduced in plant cells. Methods for gene targeting can be found in, for example, WO 2006/105946 or WO2009/002150. Gene targeting can be used to create mutant *fad2* alleles, such as knock-out *fad2* alleles.

As used herein, the term "non-naturally occurring" or “cultivated” when used in reference to a plant, means a plant with a genome that has been modified by man. A transgenic plant, for example, is a non-naturally occurring plant that contains an

exogenous nucleic acid molecule, e.g., a chimeric gene comprising a transcribed region which when transcribed yields a biologically active RNA molecule capable of reducing the expression of an endogenous gene, such as a FAD2 gene, and, therefore, has been genetically modified by man. In addition, a plant that contains a mutation in an endogenous gene, for example, a mutation in an endogenous FAD2 gene, (e.g. in a regulatory element or in the coding sequence) as a result of an exposure to a mutagenic agent is also considered a non-naturally plant, since it has been genetically modified by man. Furthermore, a plant of a particular species, such as *Brassica napus*, that contains a mutation in an endogenous gene, for example, in an endogenous FAD2 gene, that in nature does not occur in that particular plant species, as a result of, for example, directed breeding processes, such as marker-assisted breeding and selection or introgression, with a plant of the same or another species, such as *Brassica juncea* or *rapa*, of that plant is also considered a non-naturally occurring plant. In contrast, a plant containing only spontaneous or naturally occurring mutations, i.e. a plant that has not been genetically modified by man, is not a "non-naturally occurring plant" as defined herein and, therefore, is not encompassed within the invention. One skilled in the art understands that, while a non-naturally occurring plant typically has a nucleotide sequence that is altered as compared to a naturally occurring plant, a non-naturally occurring plant also can be genetically modified by man without altering its nucleotide sequence, for example, by modifying its methylation pattern.

The term "ortholog" of a gene or protein refers herein to the homologous gene or protein found in another species, which has the same function as the gene or protein, but is (usually) diverged in sequence from the time point on when the species harboring the genes diverged (i.e. the genes evolved from a common ancestor by speciation). Orthologs of the *Brassica napus FAD2* genes may thus be identified in other plant species (e.g. *Brassica juncea,* etc.) based on both sequence comparisons (e.g. based on percentages sequence identity over the entire sequence or over specific domains) and/or functional analysis.

A "variety" is used herein in conformity with the UPOV convention and refers to a plant grouping within a single botanical taxon of the lowest known rank, which grouping can be defined by the expression of the characteristics resulting from a given genotype or combination of genotypes, can be distinguished from any other plant

grouping by the expression of at least one of the said characteristics and is considered as a unit with regard to its suitability for being propagated unchanged (stable).

The term "comprising" is to be interpreted as specifying the presence of the stated parts, steps or components, but does not exclude the presence of one or more additional parts, steps or components. A plant comprising a certain trait may thus comprise additional traits.

It is understood that when referring to a word in the singular (e.g. plant or root), the plural is also included herein (e.g. a plurality of plants, a plurality of roots). Thus, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

For the purpose of this invention, the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e., a position in an alignment where a residue is present in one sequence but not in the other, is regarded as a position with non-identical residues. The "optimal alignment" of two sequences is found by aligning the two sequences over the entire length according to the Needleman and Wunsch global alignment algorithm (Needleman and Wunsch, 1970, J Mol Biol 48(3):443-53) in The European Molecular Biology Open Software Suite (EMBOSS, Rice *et al.*, 2000, Trends in Genetics 16(6): 276—277; see e.g. http://www.ebi.ac.uk/emboss/align/index.html) using default settings (gap opening penalty = 10 (for nucleotides) / 10 (for proteins) and gap extension penalty = 0.5 (for nucleotides) / 0.5 (for proteins)). For nucleotides the default scoring matrix used is EDNAFULL and for proteins the default scoring matrix is EBLOSUM62.

"Substantially identical" or "essentially similar", as used herein, refers to sequences, which, when optimally aligned as defined above, share at least a certain minimal percentage of sequence identity (as defined further below).

"Stringent hybridization conditions" can be used to identify nucleotide sequences, which are substantially identical to a given nucleotide sequence. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequences at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically stringent conditions will be chosen in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least 60°C. Lowering the salt concentration and/or increasing the temperature increases stringency. Stringent conditions for RNA-DNA hybridizations (Northern blots using a probe of e.g. 100nt) are for example those which include at least one wash in 0.2X SSC at 63°C for 20min, or equivalent conditions.

"High stringency conditions" can be provided, for example, by hybridization at 65°C in an aqueous solution containing 6x SSC (20x SSC contains 3.0 M NaCl, 0.3 M Na-citrate, pH 7.0), 5x Denhardt's (100X Denhardt's contains 2% Ficoll, 2% Polyvinyl pyrollidone, 2% Bovine Serum Albumin), 0.5% sodium dodecyl sulphate (SDS), and 20 µg/ml denaturated carrier DNA (single-stranded fish sperm DNA, with an average length of 120 - 3000 nucleotides) as non-specific competitor. Following hybridization, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridization temperature in 0.2-0.1× SSC, 0.1% SDS.

"Moderate stringency conditions" refers to conditions equivalent to hybridization in the above described solution but at about 60-62°C. Moderate stringency washing may be done at the hybridization temperature in 1x SSC, 0.1% SDS.

"Low stringency" refers to conditions equivalent to hybridization in the above described solution at about 50-52°C. Low stringency washing may be done at the hybridization temperature in 2x SSC, 0.1% SDS. See also Sambrook *et al.* (1989) and Sambrook and Russell (2001).

DETAILED DESCRIPTION

The current invention is based on the identification of two *FAD2* genes in *Brassica rapa* and in *Brassica oleracea*, and of 4 *FAD2* genes in *Brassica napus*, and of the role of the Brassica *FAD2* gene products in fatty acid desaturation.

It is a first embodiment of the invention to provide a *Brassica* plant, or a cell, part, seed or progeny thereof, comprising at least one FAD2 gene, wherein at least one allele of said FAD2 gene is a partial knock-out *fad2* allele, said partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, wherein said plant does not contain a FAD2 gene of which the *FAD2* alleles encode a wild-type functional protein. In a further embodiment, said *Brassica* plant, or a cell, part, seed or progeny thereof, comprises at least a second, or a second and a third, or a second, a third and a fourth FAD2 gene, wherein the *fad2* alleles of said FAD2 genes are full knock-out *fad2* alleles, such as a *Brassica* plant, or a cell, part, seed or progeny thereof, wherein the full knock-out *fad2* allele of said second, or second and third, or second and third and fourth *FAD2* gene is a *fad2* allele encoding a protein in which the His at a position corresponding to position 109 of SEQ ID NO: 6 is substituted with another amino acid, or comprising a stop codon mutation at the codon encoding the Trp at a position corresponding to position 101 of SEQ ID NO: 9, or comprising a deletion mutation of the nucleotides corresponding to nucleotides 2036-2042 of SEQ ID NO: 13, or comprising a stop codon mutation in the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15. In yet another embodiment, the partial knock-out *fad2* allele of the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention, is an allele of a FAD2-A1 or a FAD2-C1 gene. In another embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is a *Brassica napus* plant, or a cell, part, seed or progeny thereof, comprising a second, a third and a fourth FAD2 gene, wherein the partial knock-out *fad2* allele is an allele of a FAD2-A1 gene, and wherein the second FAD2 gene is a FAD2-C1 gene, the third FAD2 gene is a FAD2-C2 gene, and the fourth FAD2 gene is a FAD2-A2 gene, or wherein the partial knock-out *fad2* allele is an allele of a FAD2-C1 gene, and wherein the second FAD2 gene is a FAD2-A1 gene, the third FAD2 gene is a FAD2-C2 gene, and the fourth FAD2 gene is a FAD2-A2 gene. In yet another embodiment, the partial knock-out *fad2* allele in the plants according to the invention encodes a FAD2 protein in which the Gly at a

position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp.

In a further embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is a *Brassica* plant, or a cell, part, seed or progeny thereof, wherein a fisrt FAD2 gene is a FAD2-C1 gene of which at least one allele is said partial knock-out *fad2* allele which encodes a FAD2 protein of which the Gly at a position corresponding to position 232 of SEQ ID NO: 9 is substituted with Ser or with Asp; said second FAD2 gene is a FAD2-A1 gene of which at least one allele encodes a FAD2 protein of which the His at a position corresponding to position 109 of SEQ ID NO: 6 is substituted with Tyr; and the third FAD2 gene is a FAD2-C2 gene of which at least one allele contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15. In yet another embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is derivable or obtainable from seeds selected from the group consisting of a) seed comprising HIOL101 having been deposited at NCIMB under accession number NCIMB 42376, b) seed comprising HIOL120 having been deposited at NCIMB under accession number NCIMB 42377, and c) seed comprising HIOL109 having been deposited at NCIMB under accession number NCIMB 42375.

In yet another embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is a *Brassica* plant, or a cell, part, seed or progeny thereof, wherein a fisrt FAD2 gene is a FAD2-A1 gene of which at least one allele is said partial knock-out *fad2* allele which encodes a FAD2 protein of which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp; the second FAD2 gene is a FAD2-C1 gene of which at least one allele contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 101 of SEQ ID NO: 9; and the third FAD2 gene is a FAD2-C2 gene of which at least one allele contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15. In yet another embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is derivable or obtainable from seeds selected from the group consisting of a) seed comprising HIOL116 having been deposited at NCIMB under accession number NCIMB 42373, b) seed comprising HIOL103 having been

deposited at NCIMB under accession number NCIMB 42374, and c) seed comprising HIOL109 having been deposited at NCIMB under accession number NCIMB 42375.

In a further aspect, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention is homozygous for any one of the knock-out *fad2* alleles.

A *Brassica* plant, as used herein, can be *Brassica napus* (AACC, 2n=38), *Brassica juncea* (AABB, 2n=36), *Brassica carinata* (BBCC, 2n=34), *Brassica rapa* (syn. *B. campestris*) (AA, 2n=20), *Brassica oleracea* (CC, 2n=18) or *Brassica nigra* (BB, 2n=16). Said *Brassica* plant can be a crop plant species cultivated as a crop.

Nucleic acid sequences according to the invention

Provided are both wild type *FAD2* nucleic acid sequences encoding functional FAD2 proteins and mutant *fad2* nucleic acid sequences (comprising one or more mutations, preferably mutations which result in no or a significantly reduced biological activity of the encoded FAD2 protein or in no FAD2 protein being produced) of *FAD2* genes from *Brassica*, especially from *Brassica napus, Brassica rapa,* and *Brassica oleracea.*

However, isolated *FAD2* and *fad2* nucleic acid sequences (e.g. isolated from the plant by cloning or made synthetically by DNA synthesis), as well as variants thereof and fragments of any of these are also provided herein, as these can be used to determine which sequence is present endogenously in a plant or plant part, whether the sequence encodes a functional, a non-functional or no protein (e.g. by expression in a recombinant host cell as described below) and for selection and transfer of specific alleles from one plant into another, in order to generate a plant having the desired combination of functional and mutant alleles.

Nucleic acid sequences of *FAD2-A1, FAD2-C1, FAD2-A2,* and *FAD2-C2* have been isolated from *Brassica napus,* nucleic acid sequences of *FAD2*-1, and *FAD2*-2 have been isolated from *Brassica oleracea* and from *Brassica rapa* as depicted in the sequence listing. The wild type *FAD2* sequences are depicted, while the mutant *fad2* sequences of these sequences, and of sequences essentially similar to these, are described herein below and in the Examples, with reference to the wild type *FAD2* sequences. The genomic FAD2 protein-encoding DNA from *Brassica napus,*

Brassica oleracea, and Brassica rapa do comprise any introns. The coding sequences or cDNA sequences, of the *Brassica FAD2* genes, not comprising the introns, are also depicted in the sequence listing.

A "*Brassica napus FAD2-A1* gene", "*BnFAD2-A1* gene", *Brassica napus FAD2-A1* allele", "*BnFAD2-A1* allele" or "*FAD2-A1* from *Brassica napus*", or variant nucleic acid sequences thereof as used herein refers to a gene, allele or a sequence having at least 90%, or at least 95%, or at least 98%, or at least 99%, or 100% sequence identity SEQ ID No. 4.

A "*Brassica napus FAD2-C1* gene", "*BnFAD2-C1* gene", *Brassica napus FAD2-C1* allele", "*BnFAD2-C1* allele" or "*FAD2-C1* from *Brassica napus*", or variant nucleic acid sequences thereof as used herein refers to a gene, allele or a sequence having at least 90%, or at least 95%, or at least 98%, or at least 99%, or 100% sequence identity SEQ ID No. 7.

A "*Brassica napus FAD2-A2* gene", "*BnFAD2-A2* gene", *Brassica napus FAD2-A2* allele", "*BnFAD2-A2* allele" or "*FAD2-A2* from *Brassica napus*", or variant nucleic acid sequences thereof as used herein refers to a gene, allele or a sequence having at least 90%, or at least 95%, or at least 98%, or at least 99%, or 100% sequence identity SEQ ID No. 10.

A "*Brassica napus FAD2-C2* gene", "*BnFAD2-C2* gene", *Brassica napus FAD2-C2* allele", "*BnFAD2-C2* allele" or "*FAD2-C2* from *Brassica napus*", or variant nucleic acid sequences thereof as used herein refers to a gene, allele or a sequence having at least 90%, or at least 95%, or at least 98%, or at least 99%, or 100% sequence identity SEQ ID No. 13.

A "*Brassica rapa FAD2-1* gene", "*BrFAD2-1* gene", *Brassica rapa FAD2-1* allele", "*BrFAD2-1* allele" or "*FAD2-1* from *Brassica rapa*", or variant nucleic acid sequences thereof as used herein refers to a gene, allele or a sequence having at least 90%, or at least 95%, or at least 98%, or at least 99%, or 100% sequence identity SEQ ID No. 16.

A "*Brassica rapa FAD2-2* gene", "*BrFAD2-2* gene", *Brassica rapa FAD2-2* allele", "*BrFAD2-2* allele" or "*FAD2-2* from *Brassica rapa*", or variant nucleic acid sequences thereof as used herein refers to a gene, allele or a sequence having at least 90%, or at least 95%, or at least 98%, or at least 99%, or 100% sequence identity SEQ ID No. 19.

A "*Brassica oleracea FAD2-1* gene", "*BoFAD2-1* gene", *Brassica oleracea FAD2-1* allele", "*BoFAD2-1* allele" or "*FAD2-1* from *Brassica oleracea*", or variant nucleic acid sequences thereof as used herein refers to a gene, allele or a sequence having at least 90%, or at least 95%, or at least 98%, or at least 99%, or 100% sequence identity SEQ ID No. 22.

A "*Brassica oleracea FAD2-2* gene", "*BoFAD2-2* gene", *Brassica oleracea FAD2-2* allele", "*BoFAD2-2* allele" or "*FAD2-2* from *Brassica oleracea*", or variant nucleic acid sequences thereof as used herein refers to a gene, allele or a sequence having at least 90%, or at least 95%, or at least 98%, or at least 99%, or 100% sequence identity SEQ ID No. 25.

A "*FAD2-A1* gene" or a "*FAD2-A1* allele" can be a *BnFAD2-A1* gene or allele, or can be a *BrFAD2-1* gene or allele.

A "*FAD2-C1* gene" or a "*FAD2-C1* allele" can be a *BnFAD2-C1* gene or allele, or can be a *BoFAD2-1* gene or allele.

A "*FAD2-A2* gene" or a "*FAD2-A2* allele" can be a *BnFAD2-A2* gene or allele, or can be a *BrFAD2-2* gene or allele.

A "*FAD2-C2* gene" or a "*FAD2-C2* allele" can be a *BnFAD2-C2* gene or allele, or can be a *BoFAD2-2* gene or allele.

Thus the invention provides both nucleic acid sequences encoding wild type, functional FAD2 proteins, including variants and fragments thereof (as defined further below), as well as mutant nucleic acid sequences of any of these, whereby the mutation in the nucleic acid sequence preferably results in one or more amino acids

being inserted, deleted or substituted in comparison to the wild type FAD2 protein. Preferably the mutation(s) in the nucleic acid sequence result in one or more amino acid changes (i.e. in relation to the wild type amino acid sequence one or more amino acids are inserted, deleted and/or substituted) whereby the biological activity of the FAD2 protein is significantly reduced or completely abolished.

Functionality of the FAD2 protein can be tested, for example, by expression and activity analysis in yeast as described by Peyou-Ndi et al., Arch Biochem Biophys 376:399.

Both endogenous and isolated nucleic acid sequences are provided herein. Also provided are fragments of the *FAD2* sequences and *FAD2* variant nucleic acid sequences defined above, for use as primers or probes and as components of kits according to another aspect of the invention (see further below). A "fragment" of a *FAD2* or *fad2* nucleic acid sequence or variant thereof (as defined) may be of various lengths, such as at least 10, 12, 15, 18, 20, 50, 100, 200, 500, 600 contiguous nucleotides of the *FAD2* or *fad2* sequence (or of the variant sequence).

Wild-type nucleic acid sequences encoding wild-type FAD2 proteins

The nucleic acid sequences depicted in the sequence listing encode wild type FAD2 proteins from *Brassica napus,* from *Brassica rapa,* and from *Brassica oleracea.* Thus, these sequences are endogenous to the *Brassica* plants from which they were isolated.

Other *Brassica* crop species, varieties, breeding lines or wild accessions may be screened for other *FAD2* alleles, encoding the same FAD2 proteins or variants thereof. For example, nucleic acid hybridization techniques (e.g. Southern blot analysis, using for example stringent hybridization conditions) or nucleic acid amplification-based techniques such as PCR techniques may be used to identify *FAD2* alleles endogenous to other *Brassica* plants, such as various *Brassica napus*, *Brassica rapa*, or *Brasssica oleracea* varieties, lines or accessions. To screen such plants, plant organs or tissues for the presence of *FAD2* alleles, the *FAD2* nucleic acid sequences provided in the sequence listing, or variants or fragments of any of these, may be used. For example whole sequences or fragments may be used as probes or primers. For example specific or degenerate primers may be used to amplify nucleic acid sequences encoding FAD2 proteins from the genomic DNA of the plant, plant organ or tissue. These *FAD2* nucleic acid sequences may be isolated and sequenced using standard molecular biology techniques. Bioinformatics analysis may then be used to characterize the allele(s), for example in order to determine which *FAD2* allele the sequence corresponds to and which FAD2 protein or protein variant is encoded by the sequence.

In addition, it is understood that *FAD2* nucleic acid sequences and variants thereof (or fragments of any of these) may be identified *in silico*, by screening nucleic acid databases for essentially similar sequences. Likewise, a nucleic acid sequence may be synthesized chemically. Fragments of nucleic acid molecules according to the invention are also provided, which are described further below.

Wild-type *FAD2* nucleic acid sequences may encompass knock-out *FAD2* nucleic acid sequences, such as the full knock-out Bn*FAD2-A2* and *BrFAD2-2* nucleic acid sequence of SEQ ID NO: 10 and 19, respectively, as described herein, which contain a deletion in the coding sequence leading to a truncated protein of 136 amino acids (SEQ ID NO: 12 and 21, respectively) which lack the five TM domains, the three Histidine boxes, and the ER retrieval motif (see Figure 1), and such as the knock-out *BoFAD2-2* nucleic acid sequence of SEQ ID NO: 25 as described herein, which contains a 1 nt deletion deletion at a position corresponding to position 2608 of the BnFAD2-C2 gene (SEQ ID NO: 13) (i.e. the position after position 2726 of the BoFAD2-2 gene, resulting in a frameshift mutation, leading to a truncated protein of 290 amino acids (SEQ ID NO: 27) which lacks third Histidine box and the ER retrieval motif (see Figure 1). Thus, knock-out *fad2* alleles of the FAD2-A2 gene can be wild-type *FAD2* alleles of the FAD2-A2 gene.

Nucleic acid sequences encoding mutant FAD2 proteins

Nucleic acid sequences comprising one or more nucleotide deletions, insertions or substitutions relative to the wild type nucleic acid sequences are another embodiment of the invention, as are fragments of such mutant nucleic acid molecules. Such mutant nucleic acid sequences (referred to as *fad2* sequences) can be generated and/or identified using various known methods, as described further below. Again, such nucleic acid molecules are provided both in endogenous form and in isolated form. In one embodiment, the mutation(s) result in one or more changes (deletions, insertions and/or substitutions) in the amino acid sequence of the encoded FAD2 protein (i.e. it is not a "silent mutation"). In another embodiment, the mutation(s) in the nucleic acid sequence result in a significantly reduced or completely abolished biological activity of the encoded FAD2 protein relative to the wild type protein.

Basically, any mutation in the wild type FAD2 nucleic acid sequences which results in a FAD2 protein comprising at least one amino acid insertion, deletion and/or substitution relative to the wild type FAD2 protein can lead to significantly reduced or no biological activity. It is, however, understood that certain mutations in the FAD2 protein are more likely to result in a complete abolishment of the biological activity of the FAD2 protein, such as mutations whereby significant portions of conserved domains, such as one or more of the Histidine boxes are lacking, or whereby certain

critical amino acid residues within these domains are lacking or substituted, preferably substituted by non-similar or non-conservative amino acids, while other mutations such as for example amino acid substitutions outside of the Histidine boxes or within the TM domain, are more likely to result in a reduction of the biological activity of the FAD2 protein without completely abolishing the biological activity of the encoded FAD2 protein.

The conserved first, second and third Histidine boxes are at a position corresponding to position 105-109, 141-145, and 314-319, respectively, of the Arabidopsis FAD2 protein of SEQ ID NO: 3. The conserved first, second, third, fourth and fifth TM domains are at a position corresponding to position 56-76, 117-137, 179-199, 225-245, and 252-272, respectively, of the Arabidopsis FAD2 protein of SEQ ID NO: 3. The conserved ER retrieval motif is at a position corresponding to position 379-383 of the Arabidopsis FAD2 protein of SEQ ID NO: 3.

Optimal alignment of the *Arabidopsis FAD2* nucleic acids (SEQ ID NO: 1 and 2) and amino acid (SEQ ID NO: 3) sequences with *Brassica FAD2* sequences of the present invention, allows to determine the positions of the corresponding conserved domains and amino acids in these *Brassica* sequences.

Thus, the conserved first Histidine box is at a position corresponding to position 105-109 of SEQ ID NO: 6, 9, 15, 18, 24 and 27; the conserved second Histidine box is at a position corresponding to position 141-145 of SEQ ID NO: 6, 9, 15, 18, 24 and 27; the conserved third Histidine box is at a position corresponding to position 316-320 of SEQ ID NO: 6, 9, 15, 18, and 24; the conserved first TM domain is at a position corresponding to position 56-76 of SEQ ID NO: 6, 9, 15, 18, 24 and 27; the conserved second TM domain is at a position corresponding to position 117-137 of SEQ ID NO: 6, 9, 15, 18, 24 and 27; the conserved third TM domain is at a position corresponding to position 179-199 of SEQ ID NO: 6, 9, 15, 18, 24 and 27; the conserved fourth TM domain is at a position corresponding to position 226-246 of SEQ ID NO: 6, 9, 15, 18, 24 and 27; the conserved fifth TM domain is at a position corresponding to position 253-273 of SEQ ID NO: 6, 9, 15, 18, 24 and 27; and the ER retrieval motif is at a position corresponding to position 380-384 of SEQ ID NO: 6, 9, 15, 18, and 24.

The present invention describes full knockout *fad2* alleles, in particular e.g. the HIOL101 allele, encoding a FAD2 protein in which the conserved Histidine (His, H) at position 5 of the first Histidine box (i.e. at a position corresponding to position 109 of SEQ ID NO: 3) is substituted with a Tyrosine (Tyr, Y); the HIOL103 allele, encoding a FAD2 protein which is truncated after the amino acid at a position corresponding to position 100 of SEQ ID NO: 3, and lacks the three Histidine boxes, the second, the third, the fourth and the fifth TM domain, and the ER retrieval motif; and the HIOL109 allele, encoding a FAD2 protein which is truncated after the amino acid at a position corresponding to position 189 of SEQ ID NO: 3, and lacks the third Histidine box, part of the third, and the complete fourth and fifth TM domain, and the ER retrieval motif. Further full knockout *fad2* alleles are, e.g., the HIOL112 and HIOL 119 alleles, encoding a FAD2 protein in which the conserved Serine (Ser, S) at the 21st (or last) position of the third TM domain (i.e. at a position corresponding to position 199 of SEQ ID NO: 3) is substituted with a Leucine (Leu, L); the HIOL113 allele, encoding a FAD2 protein in which the conserved Glycine (Gly, G) at a position immediately following the third TM domain (i.e. at a position corresponding to position 200 of SEQ ID NO: 3) is substituted with an Arginine (Arg, R), the HIOL117 allele, encoding a FAD2 protein in which the conserved Proline (Pro, P) at a position 17 of the second TM domain (i.e. at a position corresponding to position 133 of SEQ ID NO: 3) is substituted with a Leucine (Leu, L).

Full knockout *fad2* alleles can thus be *fad2* alleles which encode a FAD2 protein of which at least one of the conserved Histidine boxes is completely or partially deleted, such as a *fad2* allele encoding a truncated FAD2 protein of which at least the third Histidine box is deleted. Examples of such a *fad2* allele is a *fad2* allele containing a stop codon mutation upstream of the codon encoding the first amino acid of the third Histidine box (i.e. the amino acid corresponding to amino acid 315 of SEQ ID NO: 3, or to amino acid 316 of SEQ ID NO: 6, 9 or 15), such as a *fad2* allele containing a stop codon mutation upstream of the codon at a position corresponding to position 2992 of SEQ ID NO: 4, or to position 3866 of SEQ ID NO: 7, or to position 2704 of SEQ ID NO: 13. Full knockout *fad2* alleles can also be *fad2* alleles which encode a FAD2 protein in which at least one amino acid in at least one of the conserved Histidine boxes is substituted with another amino acid, such as *fad2* alleles encoding FAD2 proteins of which one or more of the amino acids at a position corresponding to

position 105, 106, 107, 108, 109, 141, 142, 143, 144, 145, 315, 316, 317, 318 or 319 of SEQ ID NO: 3, or corresponding to position 105, 106, 107, 108, 109, 141, 142, 143, 144, 145, 316, 317, 318, 319 or 320 of SEQ ID NO: 6, 9, 15, 18 or 24, is substituted, preferably substituted with a non-conservative amino acid.

Full knockout *fad2* alleles can also be alleles which do not produce FAD2 protein, such as *fad2* alleles with deletions or mutations in the promoter region produce no FAD2 mRNA and no FAD2 protein, or alleles of which (part of) the FAD2 coding sequence is deleted.

Full knockout *fad2* alleles can also be alleles which produce a FAD2 protein in which an amino acid in a TM domain is substituted, such as a FAD2 protein in which the Serine at a position corresponding to position 199 of SEQ ID NO: 3 is substituted, preferably substituted with a non-conservative amino acid, such as a Leucine, or such as a FAD2 protein in which the Proline at a position corresponding to position 133 of SEQ ID NO: 3 is substituted, preferably substituted with a non-conservative amino acid, such as a Leucine. Full knockout *fad2* alleles can also be alleles which produce a FAD2 protein in which the Glycine at a position corresponding to position 133 of SEQ ID NO: 3 is substituted, preferably substituted with a non-conservative amino acid, such as an Arginine.

The present invention also describes partial knockout *fad2* alleles, in particular e.g. the HIOL116 and the HIOL120 alleles, encoding a FAD2 protein in which the Glycine at a position corresponding to position 231 of SEQ ID NO: 3 (i.e. at a position corresponding to position 232 of SEQ ID NO: 6, 9, 15, 18 or 24), positioned in the fourth TM domain, is substituted with a Serine or for an Aspartic Acid.
The knock-out *FAD2* genes may, thus, comprise one or more mutations, such as:
(a) a "missense mutation", which is a change in the nucleic acid sequence that results in the substitution of an amino acid for another amino acid;
(b) a "nonsense mutation" or "STOP codon mutation", which is a change in the nucleic acid sequence that results in the introduction of a premature STOP codon and thus the termination of translation (resulting in a truncated protein); plant genes contain the translation stop codons "TGA" (UGA in RNA), "TAA" (UAA in RNA) and "TAG" (UAG in RNA); thus any nucleotide substitution, insertion, deletion

which results in one of these codons to be in the mature mRNA being translated (in the reading frame) will terminate translation;
(c) an "insertion mutation" of one or more amino acids, due to one or more codons having been added in the coding sequence of the nucleic acid;
(d) a "deletion mutation" of one or more amino acids, due to one or more codons having been deleted in the coding sequence of the nucleic acid;
(e) a "frameshift mutation", resulting in the nucleic acid sequence being translated in a different frame downstream of the mutation. A frameshift mutation can have various causes, such as the insertion, deletion or duplication of one or more nucleotides;
(f) a splice site mutation, resulting in altered splicing, which results in an altered mRNA processing and may, consequently, in an altered encoded protein or the production of no protein.

Thus in one embodiment, nucleic acid sequences comprising one or more of any of the types of mutations described above are provided. In another embodiment, *fad2* sequences comprising one or more stop codon (nonsense) mutations, one or more missense mutations, one or more frameshift mutations, and/or one or more splice site mutations are provided. Any of the above mutant nucleic acid sequences are provided *per se* (in isolated form), as are plants and plant parts comprising such sequences endogenously. In the tables herein below *fad2* alleles are described comprising one or more mutations.

A nonsense mutation in a *FAD2* allele, as used herein, is a mutation in a *FAD2* allele whereby one or more translation stop codons are introduced into the coding DNA and the corresponding mRNA sequence of the corresponding wild type *FAD2* allele. Translation stop codons are TGA (UGA in the mRNA), TAA (UAA) and TAG (UAG). Thus, any mutation (deletion, insertion or substitution) that leads to the generation of an in-frame stop codon in the coding sequence will result in termination of translation and truncation of the amino acid chain. In one embodiment, a knockout *FAD2* allele is provided comprising a nonsense mutation wherein an in-frame stop codon is introduced in the *FAD2* codon sequence by a single nucleotide substitution, such as the mutation of CAG to TAG, TGG to TAG, TGG to TGA, or CAA to TAA. In another embodiment, a knockout *FAD2* allele is provided comprising a nonsense mutation wherein an in-frame stop codon is introduced in the *FAD2* codon sequence

by double nucleotide substitutions, such as the mutation of CAG to TAA, TGG to TAA, or CGG to TAG or TGA. In yet another embodiment, a knockout *FAD2* allele is provided comprising a nonsense mutation wherein an in-frame stop codon is introduced in the *FAD2* codon sequence by triple nucleotide substitutions, such as the mutation of CGG to TAA. The truncated protein lacks the amino acids encoded by the coding DNA downstream of the mutation (i.e. the C-terminal part of the FAD2 protein) and maintains the amino acids encoded by the coding DNA upstream of the mutation (i.e. the N-terminal part of the FAD2 protein).

A range of possible EMS stop codon mutations in the *BnFAD2-A1, BnFAD2-C1, BnFAD2-C2, BoFAD2-1,* and *BrFAD2-* genes are shown in Tables 1a-e, respectively.

Table 1a: possible stop codon mutations in BnFAD2-A1

position relative to genomic sequence (SEQ ID NO: 4)	WT		position relative to protein sequence (SEQ ID NO: 3)	stop codon
	codon	AA		
2068-2070	CAA	Gln	8	TAA
2218-2220	TGG	Trp	58	TAG, TGA, TAA
2302-2304	TGG	Trp	86	TAG, TGA, TAA
2314-2316	TGG	Trp	90	TAG, TGA, TAA
2323-2325	CAG	Gln	93	TAG, TAA
2347-2349	TGG	Trp	101	TAG, TGA, TAA
2392-2394	CAG	Gln	116	TAG, TAA
2395-2397	TGG	Trp	117	TAG, TGA, TAA
2455-2457	TGG	Trp	137	TAG, TGA, TAA
2470-2472	CGA	Arg	142	TGA, TAA
2545-2547	TGG	Trp	167	TAG, TGA, TAA
2599-2601	CAG	Gln	185	TAG, TAA
2614-2616	TGG	Trp	190	TAG, TGA, TAA
2719-2721	CAG	Gln	225	TAG, TAA
2785-2787	CAA	Gln	247	TAA
2866-2868	CAG	Gln	274	TAG, TAA
2908-2910	TGG	Trp	288	TAG, TGA, TAA
2914-2916	TGG	Trp	290	TAG, TGA, TAA

3082-3084	CAG	Gln	346	TAG, TAA
3115-3117	TGG	Trp	357	TAG, TGA, TAA
3157-3159	CAA	Gln	371	TAA
3181-3183	TGG	Trp	379	TAG, TGA, TAA

Table 1b: possible stop codon mutations in BnFAD2-C1

position relative to genomic sequence (SEQ ID NO: 7)	WT		position relative to protein sequence (SEQ ID NO: 9)	stop codon
	codon	AA		
2942-2944	CAA	Gln	8	TAA
3092-3094	TGG	Trp	58	TAG, TGA, TAA
3176-3178	TGG	Trp	86	TAG, TGA, TAA
3188-3190	TGG	Trp	90	TAG, TGA, TAA
3197-3199	CAA	Gln	93	TAA
3221-3223	TGG	Trp	101	TAG, TGA, TAA
3266-3268	CAG	Gln	116	TAG, TAA
3269-3271	TGG	Trp	117	TAG, TGA, TAA
3329-3331	TGG	Trp	137	TAG, TGA, TAA
3344-3346	CGA	Arg	142	TGA, TAA
3419-3421	TGG	Trp	167	TAG, TGA, TAA
3473-3475	CAG	Gln	185	TAG, TAA
3488-3490	TGG	Trp	190	TAG, TGA, TAA
3593-3595	CAG	Gln	225	TAG, TAA
3659-3661	CAG	Gln	247	TAG, TAA
3740-3742	CAG	Gln	274	TAG, TAA
3782-3784	TGG	Trp	288	TAG, TGA, TAA
3788-3790	TGG	Trp	290	TAG, TGA, TAA
3956-3958	CAG	Gln	346	TAG, TAA
3989-3991	TGG	Trp	357	TAG, TGA, TAA
4031-4033	CAA	Gln	371	TAA
4055-4057	TGG	Trp	379	TAG, TGA, TAA

Table 1c: possible stop codon mutations in BnFAD2-C2

position relative to genomic sequence (SEQ ID NO: 13)	WT		position relative to protein sequence (SEQ ID NO: 15)	stop codon
	codon	AA		
1780-1782	CAA	Gln	8	TAA
2014-2016	TGG	Trp	86	TAG, TGA, TAA
2026-2028	TGG	Trp	90	TAG, TGA, TAA
2035-2037	CAA	Gln	93	TAA
2059-2061	TGG	Trp	101	TAG, TGA, TAA
2104-2106	CAG	Gln	116	TAG, TAA
2107-2109	TGG	Trp	117	TAG, TGA, TAA
2167-2169	TGG	Trp	137	TAG, TGA, TAA
2182-2184	CGA	Arg	142	TGA, TAA
2257-2259	TGG	Trp	167	TAG, TGA, TAA
2311-2313	CAG	Gln	185	TAG, TAA
2326-2328	TGG	Trp	190	TAG, TGA, TAA
2431-2433	CAG	Gln	225	TAG, TAA
2497-2499	CGA	Arg	247	TGA, TAA
2578-2580	CAG	Gln	274	TAG, TAA
2620-2622	TGG	Trp	288	TAG, TGA, TAA
2626-2628	TGG	Trp	290	TAG, TGA, TAA
2794-2796	CAG	Gln	346	TAG, TAA
2827-2829	TGG	Trp	357	TAG, TGA, TAA
2869-2871	CAA	Gln	371	TAA
2893-2895	TGG	Trp	379	TAG, TGA, TAA

Table 1d: possible stop codon mutations in BrFAD2-1

position relative to genomic sequence (SEQ ID NO: 16)	WT		position relative to protein sequence (SEQ ID NO: 18)	stop codon
	codon	AA		
2029-2031	CAA	Gln	8	TAA
2179-2181	TGG	Trp	58	TAG, TGA, TAA
2263-2265	TGG	Trp	86	TAG, TGA, TAA

2275-2277	TGG	Trp	90	TAG, TGA, TAA
2284-2286	CAG	Gln	93	TAG, TAA
2308-2310	TGG	Trp	101	TAG, TGA, TAA
2353-2355	CAG	Gln	116	TAG, TAA
2356-2358	TGG	Trp	117	TAG, TGA, TAA
2416-2418	TGG	Trp	137	TAG, TGA, TAA
2431-2433	CGA	Arg	142	TGA, TAA
2506-2508	TGG	Trp	167	TAG, TGA, TAA
2560-2562	CAG	Gln	185	TAG, TAA
2575-2577	TGG	Trp	190	TAG, TGA, TAA
2680-2682	CAG	Gln	225	TAG, TAA
2746-2748	CAA	Gln	247	TAA
2827-2829	CAG	Gln	274	TAG, TAA
2869-2871	TGG	Trp	288	TAG, TGA, TAA
2875-2877	TGG	Trp	290	TAG, TGA, TAA
3043-3045	CAG	Gln	346	TAG, TAA
3076-3078	TGG	Trp	357	TAG, TGA, TAA
3118-3120	CAA	Gln	371	TAA
3142-3144	TGG	Trp	379	TAG, TGA, TAA

Table 1e: possible stop codon mutations in BoFAD2-1

position relative to genomic sequence (SEQ ID NO: 22)	WT		position relative to protein sequence (SEQ ID NO: 24)	stop codon
	codon	AA		
2913-2915	CAA	Gln	8	TAA
3063-3065	TGG	Trp	58	TAG, TGA, TAA
3147-3149	TGG	Trp	86	TAG, TGA, TAA
3159-3161	TGG	Trp	90	TAG, TGA, TAA
3168-3170	CAA	Gln	93	TAA
3192-3194	TGG	Trp	101	TAG, TGA, TAA
3237-3239	CAG	Gln	116	TAG, TAA
3240-3242	TGG	Trp	117	TAG, TGA, TAA
3300-3302	TGG	Trp	137	TAG, TGA, TAA

3315-3317	CGA	Arg	142	TGA, TAA
3390-3392	TGG	Trp	167	TAG, TGA, TAA
3444-3446	CAG	Gln	185	TAG, TAA
3459-3461	TGG	Trp	190	TAG, TGA, TAA
3564-3566	CAG	Gln	225	TAG, TAA
3630-3632	CAG	Gln	247	TAG, TAA
3711-3713	CAG	Gln	274	TAG, TAA
3753-3755	TGG	Trp	288	TAG, TGA, TAA
3759-3761	TGG	Trp	290	TAG, TGA, TAA
3927-3929	CAG	Gln	346	TAG, TAA
3960-3962	TGG	Trp	357	TAG, TGA, TAA
4002-4004	CAA	Gln	371	TAA
4026-4028	TGG	Trp	379	TAG, TGA, TAA

Obviously, mutations are not limited to the ones shown in the above tables and it is understood that analogous STOP mutations may be present in *fad2* alleles other than those depicted in the sequence listing and referred to in the tables above.

Suitable to the invention are full knockout mutant *FAD2* allele comprising a stopcodon mutation of the codon encoding the Tryptophan (Trp, W) at position 101 of the FAD2 protein in SEQ ID NO: 9, or a sequence essentially similar thereto, such as the HIOL103 allele (Table 2b), or comprising a stopcodon mutation of the codon at position 2057-2059 of SEQ ID NO: 10, or a sequence essentially similar thereto, such as the HIOL111 allele (Table 2c), or comprising a stopcodon mutation of the codon encoding the Tryptophan (Trp, W) at position 190 of the FAD2 protein in SEQ ID NO: 15, or a sequence essentially similar thereto, such as the HIOL109 allele (Table 2d).

Not only stopcodon mutations, but also mutations resulting in an amino acid substitution may lead to proteins with reduced functionality or with no detectable activity.

A missense mutation in an *FAD2* allele, as used herein, is any mutation (deletion, insertion or substitution) in a *FAD2* allele whereby one or more codons are changed

into the coding DNA and the corresponding mRNA sequence of the corresponding wild type *FAD2* allele, resulting in the substitution of one or more amino acids in the wild type FAD2 protein for one or more other amino acids in the mutant FAD2 protein. In one embodiment, a partial knockout mutant *FAD2* allele is provided comprising a missense mutation resulting in a substitution of a glycine (Gly) residue at position 232 of the FAD2 protein in SEQ ID NO: 6, or SEQ ID NO: 9, or a sequence essentially similar thereto, in particular by an aspartic acid (Asp, D) or by a serine (Ser, S) residue, such as the HIOL116 allele (Table 2a) and the HIOL120 allele (Table 2b). In another embodiment, a full knockout mutant *FAD2* allele is provided comprising a missense mutation resulting in a substitution of a Histidine (His) residue at position 109 of the FAD2 protein in SEQ ID NO: 6, or a sequence essentially similar thereto, in particular by a tyrosine (Tyr) residue, such as the HIOL101 allele (Table 2a), or comprising a missense mutation resulting in a substitution of a Serine (Ser) residue at position 199 of the FAD2 protein in SEQ ID NO: 6 or in SEQ ID NO: 9, or a sequence essentially similar thereto, in particular by a Leucine (Leu) residue, such as the HIOL112 and HIOL19 alleles (Tables 2a and 2b), comprising a missense mutation resulting in a substitution of a Glycine (Gly) residue at position 200 of the FAD2 protein in SEQ ID NO: 6, or a sequence essentially similar thereto, in particular by a Arginine (Arg) residue, such as the HIOL113 allele (Table 2a), or comprising a missense mutation resulting in a substitution of a Proline (Pro) residue at position 133 of the FAD2 protein in SEQ ID NO: 9, or a sequence essentially similar thereto, in particular by a Leucine (Leu) residue, such as the HIOL117 allele (Table 2b).

In another embodiment, said knock-out *fad2* gene in *Brassica napus* is selected from the group consisting of nucleic acids comprising:

- a C to T mutation at position 2371 of SEQ ID No. 4;
- a C to T mutation at position 2642 of SEQ ID No. 4;
- a G to A mutation at position 2644 of SEQ ID No. 4;
- a G to A mutation at position 2740 of SEQ ID No. 4;
- a G to A mutation at position 3223 of SEQ ID No. 7;
- a C to T mutation at position 3318 of SEQ ID No. 7;
- a C to T mutation at position 3516 of SEQ ID No. 7;
- a G to A mutation at position 3614 of SEQ ID No. 7;
- a G to A mutation at position 2327 of SEQ ID No. 13.

Wild-type and mutant FAD2 nucleic acid sequences from the A-genome as described herein, such as *BnFAD2-A1*, *BnFAD2-A2*, *BrFAD2-1*, and *BrFAD2-2* are also suitable to use in other *Brassica* species comprising an A genome, such as *Brassica juncea.*

Wild-type and mutant FAD2 nucleic acid sequences from the C-genome as described herein, such as *BnFAD2-C1*, *BnFAD2-C2*, *BoFAD2-1*, and *BoFAD2-2* are also suitable to use in other *Brassica* species comprising a C genome, such as *Brassica carinata.*

Amino acid sequences according to the invention

Provided are both wild type FAD2 amino acid sequences and mutant FAD2 amino acid sequences (comprising one or more mutations, preferably mutations which result in a significantly reduced or no biological activity of the FAD2 protein) from *Brassicaceae*, particularly from *Brassica* species, especially from *Brassica napus, Brassica rapa*, and *Brassica oleracea.* In addition, mutagenesis methods can be used to generate mutations in wild type *FAD2* alleles, thereby generating mutant alleles which can encode further mutant FAD2 proteins. In one embodiment the wild type and/or mutant FAD2 amino acid sequences are provided within a *Brassica* plant (i.e. endogenously). However, isolated FAD2 amino acid sequences (e.g. isolated from the plant or made synthetically), as well as variants thereof and fragments of any of these are also provided herein.

Amino acid sequences of FAD2-A1, FAD2-C1, FAD2-A1 and FAD2-C2 proteins from Brassica napus, FAD2-1 and FAD2-2 proteins from *Brassica rapa*, and FAD2-1 and FAD2-2 proteins from *Brassica oleracea*, have been isolated as depicted in the sequence listing. The wild type *FAD2* sequences are depicted, while the mutant *FAD2* sequences of these sequences, and of sequences essentially similar to these, are described herein below, with reference to the wild type *FAD2* sequences.

"*Brassica napus* FAD2-A1 amino acid sequences" or "BnFAD2-A1 amino acid sequences" or variant amino acid sequences thereof according to the invention are amino acid sequences having at least 95%, 98%, 99% or 100% sequence identity with SEQ ID NO: 6. These amino acid sequences may also be referred to as being

"essentially similar" or "essentially identical" to the *FAD2* sequences provided in the sequence listing.

"*Brassica napus* FAD2-C1 amino acid sequences" or "BnFAD2-C1 amino acid sequences" or variant amino acid sequences thereof according to the invention are amino acid sequences having at least 95%, 98%, 99% or 100% sequence identity with SEQ ID NO: 9. These amino acid sequences may also be referred to as being "essentially similar" or "essentially identical" to the *FAD2* sequences provided in the sequence listing.

"*Brassica napus* FAD2-A2 amino acid sequences" or "BnFAD2-A2 amino acid sequences" or variant amino acid sequences thereof according to the invention are amino acid sequences having at least 95%, 98%, 99% or 100% sequence identity with SEQ ID NO: 12. These amino acid sequences may also be referred to as being "essentially similar" or "essentially identical" to the *FAD2* sequences provided in the sequence listing.

"*Brassica napus* FAD2-C2 amino acid sequences" or "BnFAD2-C2 amino acid sequences" or variant amino acid sequences thereof according to the invention are amino acid sequences having at least 95%, 98%, 99% or 100% sequence identity with SEQ ID NO: 15. These amino acid sequences may also be referred to as being "essentially similar" or "essentially identical" to the *FAD2* sequences provided in the sequence listing.

"*Brassica rapa* FAD2-1 amino acid sequences" or "BrFAD2-1 amino acid sequences" or variant amino acid sequences thereof according to the invention are amino acid sequences having at least 95%, 98%, 99% or 100% sequence identity with SEQ ID NO: 18. These amino acid sequences may also be referred to as being "essentially similar" or "essentially identical" to the *FAD2* sequences provided in the sequence listing.

"*Brassica rapa* FAD2-2 amino acid sequences" or "BrFAD2-2 amino acid sequences" or variant amino acid sequences thereof according to the invention are amino acid sequences having at least 95%, 98%, 99% or 100% sequence identity with SEQ ID

NO: 21. These amino acid sequences may also be referred to as being "essentially similar" or "essentially identical" to the *FAD2* sequences provided in the sequence listing.

"*Brassica oleracea* FAD2-1 amino acid sequences" or "BoFAD2-1 amino acid sequences" or variant amino acid sequences thereof according to the invention are amino acid sequences having at least 95%, 98%, 99% or 100% sequence identity with SEQ ID NO: 24. These amino acid sequences may also be referred to as being "essentially similar" or "essentially identical" to the *FAD2* sequences provided in the sequence listing.

"*Brassica oleracea* FAD2-2 amino acid sequences" or "BoFAD2-2 amino acid sequences" or variant amino acid sequences thereof according to the invention are amino acid sequences having at least 95%, 98%, 99% or 100% sequence identity with SEQ ID NO: 27. These amino acid sequences may also be referred to as being "essentially similar" or "essentially identical" to the *FAD2* sequences provided in the sequence listing.

A "*FAD2-A1* protein" can be a *BnFAD2-A1* protein, or can be a *BrFAD2-1* protein.
A "*FAD2-C1* protein" can be a *BnFAD2-C1* protein, or can be a *BoFAD2-1* protein.
A "*FAD2-A2* protein" can be a *BnFAD2-A2* protein, or can be a *BrFAD2-2* protein.
A "*FAD2-C2* protein" can be a *BnFAD2-C2* protein, or can be a *BoFAD2-2* protein.

Thus, the invention provides both amino acid sequences of wild type proteins, including variants and fragments thereof (as defined further below), as well as mutant amino acid sequences of any of these, whereby the mutation in the amino acid sequence preferably results in a significant reduction in or a complete abolishment of the biological activity of the FAD2 protein as compared to the biological activity of the corresponding wild type FAD2 protein.

Both endogenous and isolated amino acid sequences are provided herein. Also provided are fragments of the FAD2 amino acid sequences and FAD2 variant amino acid sequences defined above. A "fragment" of a FAD2 amino acid sequence or variant thereof (as defined) may be of various lengths, such as at least 10, 12, 15, 18,

20, 50, 100, 150, 175, 180 contiguous amino acids of the FAD2 sequence (or of the variant sequence).

Amino acid sequences of wild-type FAD2 proteins

The amino acid sequences depicted in the sequence listing are wild type FAD2 proteins from *Brassica napus*. Thus, these sequences are endogenous to the *Brassica napus* plants from which they were isolated. Other *Brassica*, varieties, breeding lines or wild accessions may be screened for other functional FAD2 proteins with the same amino acid sequences or variants thereof, as described above.

In addition, it is understood that FAD2 amino acid sequences and variants thereof (or fragments of any of these) may be identified *in silico*, by screening amino acid databases for essentially similar sequences. Fragments of amino acid molecules according to the invention are also provided.

Wild-type *FAD2* amino acid sequences may encompass amino acid sequences of non-functional FAD2 proteins, such as Bn*FAD2-A2* and *BrFAD2-2* amino acid sequence of SEQ ID NO: 12 and 21, respectively, as described herein, are truncated proteins of 136 amino acids which lack the five TM domains, the three Histidine boxes, and the ER retrieval motif (see Figure 1), and such as the *BoFAD2-2* amino acid sequence of SEQ ID NO: 27 as described herein, which is a truncated protein of 290 amino acids which lacks third Histidine box and the ER retrieval motif (see Figure 1).

Amino acid sequences of mutant FAD2 proteins

Amino acid sequences comprising one or more amino acid deletions, insertions or substitutions relative to the wild type amino acid sequences are another embodiment of the invention, as are fragments of such mutant amino acid molecules. Such mutant amino acid sequences can be generated and/or identified using various known methods, as described above. Again, such amino acid molecules are provided both in endogenous form and in isolated form.

In one embodiment, the mutation(s) in the amino acid sequence result in a significantly reduced or completely abolished biological activity of the FAD2 protein relative to the wild type protein. As described above, basically, any mutation which

results in a protein comprising at least one amino acid insertion, deletion and/or substitution relative to the wild type protein can lead to significantly reduced or no biological activity.

Thus in one embodiment, mutant FAD2 proteins are provided comprising one or more deletion or insertion mutations, whereby the deletion(s) or insertion(s) result(s) in a mutant protein which has significantly reduced or no activity. Such mutant FAD2 proteins are FAD2 proteins wherein at least 1, at least 2, at least 3, at least 4, at least 5, at least 10, at least 20, at least 30, at least 50, at least 100, at least 150, at least 200, at least 250, or more amino acids are deleted, inserted or substituted as compared to the wild type FAD2 protein, whereby the deletion(s) or insertion(s) result(s) in a mutant protein which has significantly reduced or no activity.

In another embodiment, mutant FAD2 proteins are provided which are truncated whereby the truncation results in a mutant protein that has significantly reduced or no activity. Truncated FAD2 proteins with significantly reduced or no activity may be truncated FAD2 proteins lacking at least the third Histidine box, such as FAD2 proteins truncated at a position corresponding to position 315 of SEQ ID NO: 3, or at a position corresponding to position 316 of SEQ ID NO: 6, 9, 15, 18, or 24, or at any position upstream of a position corresponding to position 315 of SEQ ID NO: 3, or at a position corresponding to position 316 of SEQ ID NO: 6, 9, 15, 18, or 24.

In yet another embodiment, mutant FAD2 proteins are provided comprising one or more substitution mutations, whereby the substitution(s) result(s) in a mutant protein that has significantly reduced or no activity. FAD2 proteins with one or more substitution mutations with significantly reduced or no activity may be FAD2 proteins in which at least one amino acid in at least one of the conserved Histidine boxes is substituted with another amino acid, such as *fad2* alleles encoding FAD2 proteins of which one or more of the amino acids at a position corresponding to position 105, 106, 107, 108, 109, 141, 142, 143, 144, 145, 315, 316, 317, 318 or 319 of SEQ ID NO: 3, or corresponding to position 105, 106, 107, 108, 109, 141, 142, 143, 144, 145, 316, 317, 318, 319 or 320 of SEQ ID NO: 6, 9, 15, 18 or 24, is substituted, preferably substituted with a non-conservative amino acid.

Also suitable are mutant FAD2 proteins in which an amino acid in a TM domain is substituted, such as FAD2 proteins encoded by full knock-out mutant *fad2* alleles in which the Serine at a position corresponding to position 199 of SEQ ID NO: 3 is substituted, preferably substituted with a non-conservative amino acid, such as a Leucine, or such as a FAD2 protein in which the Proline at a position corresponding to position 133 of SEQ ID NO: 3 is substituted, preferably substituted with a non-conservative amino acid, such as a Leucine. Also suitable are FAD2 proteins in which the Glycine at a position corresponding to position 133 of SEQ ID NO: 3 is substituted, preferably substituted with a non-conservative amino acid, such as an Arginine. Also suitable are mutant FAD2 proteins in which an amino acid in a TM domain is substituted, such as FAD2 proteins encoded by partial knock-out mutant *fad2* alleles in which the Glycine at a position corresponding to position 232 of SEQ ID NO: 3 is substituted with another amino acid, such as a Serine (Ser, S), or with a Aspartic acid (Asp, D).

Examples of conservative amino acid substitutions are:

Residue	Conservative Substitutions	Residue	Conservative Substitutions
Ala	Ser	Leu	Ile, Val
Arg	Lys	Lys	Arg, Gln
Asn	Gln, His	Met	Leu, Ile
Asp	Glu	Phe	Met, Leu, Tyr
Gln	Asn	Ser	Thr, Gly
Cys	Ser	Thr	Ser, Val
Glu	Asp	Trp	Tyr
Gly	Pro	Tyr	Trp, Phe
His	Asn, Gln	Val	Ile, Leu
Ile	Leu, Val		

Non-conservative amino acids are thus amino acids other than the conservative amino acids.

In a further embodiment, said mutant FAD2 proteins from *Brassica napus* are selected from the group consisting of proteins comprising:

- a H to Y substitution at position 109 of SEQ ID No. 6;
- a S to L substitution at position 199 of SEQ ID No. 6 or of SEQ ID No. 9;

- a G to R substitution at position 200 of SEQ ID No. 6;
- a G to S substitution at position 232 of SEQ ID No. 6;
- a G to D substitution at position 232 of SEQ ID No. 6 or SEQ ID No. 9;
- SEQ ID No. 9 truncated after the amino acid at position 100;
- a P to L substitution at position 133 of SEQ ID No. 6;
- a G to S substitution at position 232 of SEQ ID No. 6;
- SEQ ID No. 15 truncated after the amino acid at position 189.

In a further embodiment, the *Brassica* plant, or a cell, part, seed or progeny thereof according to the invention has increased levels of C18:1 in the seed oil, such as a level of C18:1 in the seed oil of about 75%, and which maintains normal agronomic development.

In a further embodiment, seeds are provided from the plants according to the invention, i.e. plants comprising a knock-out *FAD2* gene. In yet another embodiment, oil from the seeds of the plants according to the invention is provided.

Another aspect of the invention provides a method for increasing the levels of C18:1 in seed oil while maintaining normal agronomic development, said method comprising introducing a partial knock-out *fad2* allele of a *FAD2* gene, said partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, and, optionally, introducing one or more full knock-out *fad2* alleles of one or more FAD2 genes, into a *Brassica* plant and selecting a *Brassica* plant comprising said partial knock-out *fad2* allele which does not contain another *FAD2* gene of which the *FAD2* alleles encode a wild-type functional FAD2 protein. In a further embodiment, the *Brassica* plant produced by said method comprises at least a second, or a second and a third, or a second, a third and a fourth FAD2 gene, wherein the *fad2* alleles of said FAD2 genes are full knock-out *fad2* alleles. In another embodiment, the method according to the invention comprises the step of selecting said *Brassica* plant comprising said partial knock-out *fad2* allele which does not contain another *FAD2* gene of which the *FAD2* alleles encode a wild-type functional FAD2 protein by analyzing genomic DNA from said plant for the presence of at least one molecular

marker, wherein said at least one molecular marker is linked to said partial knock-out *fad2* allele and, optionally, to one or more full knock-out *fad2* alleles.

"C18:1", also referred to as "oleic acid", "cis-9-octadecenoic", "18:1", "18:1 (n-9)", "9*c*-18:1" or "18:1*cis* Δ^9"as used herein, refers to a monounsaturated omega-9 fatty acid, with the IUPAC name (9*Z*)-Octadec-9-enoic acid.

Increasing the C18:1 levels or increased C18:1 levels in seed oil can be an increase of C18:1 levels with at least 2%, or at least 5%, or at least 8%, or at least 10%, or at least 12%. Said increase is an increase with respect to C18:1 levels as obtained in control plants.

C18:1 levels of about 75% can, for example, be C18:1 levels of between 70 and 80%, or between 71 and 79%, or between 72 and 78%, or between 73 and 77%, or between 74 and 76%, or of 75%.

C18:1 levels in the seed oil can be measured as described herein, such as, for example, using the methods as described in Example 5.

The "control plant" as used herein is generally a plant of the same species which has wild-type levels of FAD2. "Wild-type levels of FAD2" as used herein refers to the typical levels of FAD2 protein in a plant as it most commonly occurs in nature, of which the *FAD2* genes are wild-type *FAD2* genes.

"Maintaining normal agronomic development" as used herein refers to having agronomic parameters which are not significantly different from a control plant. "Maintaining normal agronomic development" can, for example, be having a vigor score which is not significantly different from a control plant. "Maintaining normal agronomic development" can also be having a score for establishment, vigor, flowering start, flowering end, plant height, maturity, or protein content, or any combination thereof, which is not significantly different from a control plant. Agronomic parameters can be determined, for example, as described herein in Example 6.

A method to introduce a knock-out *fad2* allele, such as a partial knock-out *fad2* allele, or a full knock-out *fad2* allele may comprise the steps of treating seeds or plant material with a mutagenic chemical substance or with ionizing radiation; identifying plants with a mutated *fad2* gene, wherein the *FAD2* gene, prior to being mutated, encodes a polypeptide having at least 90% sequence identity to SEQ ID No. 6, to SEQ ID No. 9, to SEQ ID No. 15, SEQ ID NO: 18 or to SEQ ID No. 24; and selecting a plant with an increased level of C18:1 in the seeds compared to a plant in which the *FAD2* gene is not mutated.

Said *FAD2* gene, prior to being mutated, can be, for example, a *FAD2* gene having at least 90% sequence identity, or at least 95% sequence identity, or at least 98% sequence identity or having 100% sequence identity to SEQ ID No. 4, to SEQ ID No. 7, to SEQ ID No. 13, SEQ ID NO: 16 or to SEQ ID No. 22, or can be a *FAD2* gene of which the cDNA has at least 90% sequence identity, or at least 95% sequence identity, or at least 98% sequence identity or has 100% sequence identity to SEQ ID No. 5, to SEQ ID No. 8, to SEQ ID No. 14, SEQ ID NO: 17 or to SEQ ID No. 23.

Introducing said knock-out allele of *FAD2* can also occur through introduction of a knock-out *FAD2* allele from one plant into another, for example by crossing a plant comprising said knock-out *FAD2* allele with a plant not comprising said knock-out *FAD2* allele and identifying progeny plants comprising said knock-out *FAD2* allele, optionally using one or more molecular markers.

Said partial knock-out *fad2* allele can be introduced into a *Brassica* plant which does not contain another *FAD2* gene. Alternatively, said partial knock-out *fad2* allele can be a partial knock-out allele of a first *FAD2* gene, and the *Brassica* plant into which the partial knock-out *fad2* allele of said first *FAD2* gene is introduced may comprise a second, a second and a third, or a second, a third, and a fourth *FAD2* gene. The alleles of said second, third and/or fourth *FAD2* genes of said *Brassica* plant in which said partial knock-out *fad2* allele is introduced may be full knock-out *FAD2* alleles. Alternatively, the alleles of said second, third and/or fourth *FAD2* genes of said *Brassica* plant in which said partial knock-out *fad2* allele is introduced may be *FAD2* alleles encoding a functional protein, and said method comprises introducing said

partial knock-out *fad2* allele of said first *FAD2* gene one, and introducing or more full knock-out *fad2* alleles of said second, third and/or fourth *FAD2* genes.

In a further embodiment, a method is provided to determine the presence or absence of a knock-out *fad2* allele in a biological sample, comprising providing genomic DNA from said biological sample, and analyzing said DNA for the presence of at least one molecular marker, wherein the at least one molecular marker is linked to said knock-out *fad2* allele.

Said genomic DNA can be provided by isolating genomic DNA from said biological sample. Isolating genomic DNA refers to isolating a biological sample comprising genomic DNA from, such as isolating part of a tissue, such as, for example part of a leaf. Isolating genomic DNA from said biological sample can, but does not need to comprise, purification of genomic DNA from said sample.

Yet another embodiment provides a kit for the detection of a knock-out *fad2* allele in *Brassica* DNA samples, wherein said kit comprises one or more PCR primer pairs, which are able to amplify a DNA marker linked to said knock-out *fad2* allele.

Another embodiment provides a method for determining the zygosity status of a knock-out *fad2* allele in a plant, or a cell, part, seed or progeny thereof, comprising determining the presence of a knock-out and/or a corresponding wild type *FAD2* specific region in the genomic DNA of said plant, or a cell, part, seed or progeny thereof.

In yet another aspect of the invention, a method is provided for transferring at least one partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, from one plant to another plant comprising the steps of: (a) identifying a first plant comprising at least one partial knock-out *fad2* allele, (b) crossing the first plant with a second plant not comprising the at least one partial knock-out *fad2* allele and collecting F1 hybrid seeds from the cross, (c) optionally, identifying F1 plants comprising the at least one partial knock-out *fad2* allele, (d) backcrossing F1 plants comprising the at least one partial knock-out *fad2* allele with the second plant not

comprising the at least one partial knock-out *fad2* allele for at least one generation (x) and collecting BCx seeds from the crosses, and (e) identifying in every generation BCx plants comprising the at least one partial knock-out *fad2* allele by analyzing genomic DNA of said BCx plants for the presence of at least one molecular marker, wherein the at least one molecular marker is linked to said partial knock-out *fad2* allele.

A molecular marker which is linked to said knock-out allele of a *FAD2* gene or said mutant *FAD2* allele can comprise on or more primers or probes that specifically detect said knock-out allele of said *FAD2* gene as described herein below.

Methods according to the invention

Methods are provided for generating and selecting seed plants, and cells, parts, seeds and progeny thereof, having increased levels of C18:1 in the seeds, containing at least one partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, and wherein said plant does not contain a FAD2 gene of which the *FAD2* alleles encode a wild-type functional protein. In particular, methods are provided for generating and selecting *Brassica* plants comprising at least one *FAD2* gene, in particular *Brassica napus* plants, and cells, parts, seeds and progeny thereof, containing at least one partial knock-out *fad2* allele at at least one of the FAD2 loci in the genome, for example at at least one of the two different loci of the *Brassica napus* FAD2-A1 and FAD2-C1 gene, and to distinguish between the presence of full knockout *fad2* alleles, partial knockout *fad2* alleles and wild type *FAD2* alleles in a plant or plant part having increased C18:1 levels in the seeds. Thus methods are provided (such as mutagenesis and/or marker assisted selection) for generating and/or identifying partial knockout *fad2* alleles and/or full knockout *fad2* alleles or seed plants or plant parts comprising such *fad2* alleles and for combining a suitable number of partial knockout *fad2* alleles and/or full knockout *fad2* alleles and/or different types of partial knockout *fad2* alleles and/or full knockout *fad2* alleles in a single seed plant to alter the levels of C18:1 in the seeds of the plants while maintaining normal agronomic development.

Mutant *fad2* alleles may be generated (for example induced by mutagenesis) and/or identified using a range of methods, which are conventional in the art, for example using nucleic acid amplification based methods to amplify part or all of the *fad2* genomic or cDNA.

Following mutagenesis, plants are grown from the treated seeds, or regenerated from the treated cells using known techniques. For instance, mutagenized seeds may be planted in accordance with conventional growing procedures and following self-pollination seed is formed on the plants. Alternatively, doubled haploid plantlets may be extracted from treated microspore or pollen cells to immediately form homozygous plants, for example as described by Coventry *et al.* (1988, Manual for Microspore Culture Technique for *Brassica napus*. Dep. Crop Sci. Techn. Bull. OAC Publication 0489. Univ. of Guelph, Guelph, Ontario, Canada). Additional seed which is formed as a result of such self-pollination in the present or a subsequent generation may be harvested and screened for the presence of mutant *FAD2* alleles, using techniques which are conventional in the art, for example nucleic acid amplification based techniques, such as polymerase chain reaction (PCR) based techniques (amplification of the *fad2* alleles) or hybridization based techniques, e.g. Southern blot analysis, BAC library screening, and the like, and/or direct sequencing of *fad2* alleles. To screen for the presence of point mutations (so called Single Nucleotide Polymorphisms or SNPs) in mutant *FAD2* alleles, SNP detection methods conventional in the art can be used, for example oligoligation-based techniques, single base extension-based techniques or techniques based on differences in restriction sites, such as TILLING.

As described above, mutagenization (spontaneous as well as induced) of a specific wild-type *FAD2* allele results in the presence of one or more deleted, inserted, or substituted nucleotides (hereinafter called "mutation region") in the resulting mutant *FAD2* allele. The mutant *FAD2* allele can thus be characterized by the location and the configuration of the one or more deleted, inserted, or substituted nucleotides in the wild type *FAD2* allele. The site in the wild type *FAD2* allele where the one or more nucleotides have been inserted, deleted, or substituted, respectively, is herein also referred to as the "mutation region or sequence". A "5' or 3' flanking region or sequence" as used herein refers to a DNA region or sequence in the mutant (or the

corresponding wild type) *FAD2* allele of at least 20 bp, preferably at least 50 bp, at least 750 bp, at least 1500 bp, and up to 5000 bp of DNA different from the DNA containing the one or more deleted, inserted, or substituted nucleotides, preferably DNA from the mutant (or the corresponding wild type) *FAD2* allele which is located either immediately upstream of and contiguous with (5' flanking region or sequence") or immediately downstream of and contiguous with (3' flanking region or sequence") the mutation region in the mutant *FAD2* allele (or in the corresponding wild type *FAD2* allele). A "joining region" as used herein refers to a DNA region in the mutant (or the corresponding wild type) *FAD2* allele where the mutation region and the 5' or 3' flanking region are linked to each other. A "sequence spanning the joining region between the mutation region and the 5' or 3' flanking region thus comprises a mutation sequence as well as the flanking sequence contiguous therewith.

The tools developed to identify a specific mutant *FAD2* allele or the plant or plant material comprising a specific mutant *FAD2* allele, or products which comprise plant material comprising a specific mutant *FAD2* allele are based on the specific genomic characteristics of the specific mutant *FAD2* allele as compared to the genomic characteristics of the corresponding wild type *FAD2* allele, such as, a specific restriction map of the genomic region comprising the mutation region, molecular markers comprising primers and/or probes as described below, or the sequence of the flanking and/or mutation regions.

Once a specific mutant *FAD2* allele has been sequenced, molecular markers, such as primers and probes can be developed which specifically recognize a sequence within the 5' flanking, 3' flanking and/or mutation regions of the mutant *FAD2* allele in the nucleic acid (DNA or RNA) of a sample by way of a molecular biological technique. For instance an amplification method can be developed to identify the mutant *FAD2* allele in biological samples (such as samples of plants, plant material or products comprising plant material). Such an amplification is based on at least two specific "primers": one recognizing a sequence within the 5' or 3' flanking region of the mutant *FAD2* allele and the other recognizing a sequence within the 3' or 5' flanking region of the mutant *FAD2* allele, respectively; or one recognizing a sequence within the 5' or 3' flanking region of the mutant *FAD2* allele and the other recognizing a sequence within the mutation region of the mutant *FAD2* allele; or one recognizing a

sequence within the 5' or 3' flanking region of the mutant *FAD2* allele and the other recognizing a sequence spanning the joining region between the 3' or 5' flanking region and the mutation region of the specific mutant *FAD2* allele (as described further below), respectively.

The primers preferably have a sequence of between 15 and 35 nucleotides which under optimized amplification conditions "specifically recognize" a sequence within the 5' or 3' flanking region, a sequence within the mutation region, or a sequence spanning the joining region between the 3' or 5' flanking and mutation regions of the specific mutant *FAD2* allele, so that a specific fragment ("mutant *FAD2* specific fragment" or discriminating amplicon) is amplified from a nucleic acid sample comprising the specific mutant *FAD2* allele. This means that only the targeted mutant *FAD2* allele, and no other sequence in the plant genome, is amplified under optimized amplification conditions.

PCR primers suitable for the invention may be the following:

- oligonucleotides ranging in length from 17 nt to about 200 nt, comprising a nucleotide sequence of at least 17 consecutive nucleotides, preferably 20 consecutive nucleotides selected from the 5' or 3' flanking sequence of a specific mutant *FAD2* allele or the complement thereof (i.e., for example, the sequence 5' or 3' flanking the one or more nucleotides deleted, inserted or substituted in the mutant *FAD2* alleles of the invention, such as the sequence 5' or 3' flanking the non-sense, mis-sense, frameshift or splice site mutations described above or the sequence 5' or 3' flanking the STOP codon mutations indicated in the above Tables or the substitution mutations indicated above or the complement thereof) (primers recognizing 5' flanking sequences); or
- oligonucleotides ranging in length from 17 nt to about 200 nt, comprising a nucleotide sequence of at least 17 consecutive nucleotides, preferably 20 nucleotides selected from the sequence of the mutation region of a specific mutant *FAD2* allele or the complement thereof (i.e., for example, the sequence of nucleotides inserted or substituted in the *FAD2* genes of the invention or the complement thereof) (primers recognizing mutation sequences) .

The primers may of course be longer than the mentioned 17 consecutive nucleotides, and may e.g. be 18, 19, 20, 21, 30, 35, 50, 75, 100, 150, 200 nt long or even longer. The primers may entirely consist of nucleotide sequence selected from the mentioned nucleotide sequences of flanking and mutation sequences. However, the nucleotide sequence of the primers at their 5' end (i.e. outside of the 3'-located 17 consecutive nucleotides) is less critical. Thus, the 5' sequence of the primers may consist of a nucleotide sequence selected from the flanking or mutation sequences, as appropriate, but may contain several (e.g. 1, 2, 5, 10) mismatches. The 5' sequence of the primers may even entirely consist of a nucleotide sequence unrelated to the flanking or mutation sequences, such as e.g. a nucleotide sequence representing restriction enzyme recognition sites. Such unrelated sequences or flanking DNA sequences with mismatches should preferably be no longer than 100, more preferably not longer than 50 or even 25 nucleotides.

Moreover, suitable primers may comprise or consist of a nucleotide sequence spanning the joining region between flanking and mutation sequences (i.e., for example, the joining region between a sequence 5' or 3' flanking one or more nucleotides deleted, inserted or substituted in the mutant *FAD2* alleles of the invention and the sequence of the one or more nucleotides inserted or substituted or the sequence 3' or 5', respectively, flanking the one or more nucleotides deleted, such as the joining region between a sequence 5' or 3' flanking non-sense, missense, frameshift or splice site mutations in the *FAD2* genes of the invention described above and the sequence of the non-sense, missense, frameshift or splice site mutations, or the joining region between a sequence 5' or 3' flanking a potential STOP codon mutation as indicated in the above Tables or the substitution mutations indicated above and the sequence of the potential STOP codon mutation or the substitution mutations, respectively), provided the nucleotide sequence is not derived exclusively from either the mutation region or flanking regions.

It will also be immediately clear to the skilled artisan that properly selected PCR primer pairs should also not comprise sequences complementary to each other.

For the purpose of the invention, the "complement of a nucleotide sequence represented in SEQ ID No: X" is the nucleotide sequence which can be derived from

the represented nucleotide sequence by replacing the nucleotides through their complementary nucleotide according to Chargaff's rules (A↔T; G↔C) and reading the sequence in the 5' to 3' direction, i.e. in opposite direction of the represented nucleotide sequence.

Examples of primers suitable to identify specific mutant *FAD2* alleles are described in the Examples.

As used herein, "the nucleotide sequence of SEQ ID No. Z from position X to position Y" indicates the nucleotide sequence including both nucleotide endpoints.

Preferably, the amplified fragment has a length of between 50 and 1000 nucleotides, such as a length between 50 and 500 nucleotides, or a length between 100 and 350 nucleotides. The specific primers may have a sequence which is between 80 and 100% identical to a sequence within the 5' or 3' flanking region, to a sequence within the mutation region, or to a sequence spanning the joining region between the 3' or 5' flanking and mutation regions of the specific mutant *FAD2* allele, provided the mismatches still allow specific identification of the specific mutant *FAD2* allele with these primers under optimized amplification conditions. The range of allowable mismatches however, can easily be determined experimentally and are known to a person skilled in the art.

Detection and/or identification of a "mutant *FAD2* specific fragment" can occur in various ways, e.g., via size estimation after gel or capillary electrophoresis or via fluorescence-based detection methods. The mutant *FAD2* specific fragments may also be directly sequenced. Other sequence specific methods for detection of amplified DNA fragments are also known in the art.

Standard nucleic acid amplification protocols, such as PCR protocols are described in the art, such as in 'PCR Applications Manual" (Roche Molecular Biochemicals, 2nd Edition, 1999) and other references. The optimal conditions for the amplification, including the sequence of the specific primers, is specified in a "PCR identification protocol" for each specific mutant *FAD2* allele. It is however understood that a

number of parameters in the PCR identification protocol may need to be adjusted to specific laboratory conditions, and may be modified slightly to obtain similar results. For instance, use of a different method for preparation of DNA may require adjustment of, for instance, the amount of primers, polymerase, $MgCl_2$ concentration or annealing conditions used. Similarly, the selection of other primers may dictate other optimal conditions for the PCR identification protocol. These adjustments will however be apparent to a person skilled in the art, and are furthermore detailed in current PCR application manuals such as the one cited above.

Alternatively, specific primers can be used to amplify a mutant *FAD2* specific fragment that can be used as a "specific probe" for identifying a specific mutant *FAD2* allele in biological samples. Contacting nucleic acid of a biological sample, with the probe, under conditions that allow hybridization of the probe with its corresponding fragment in the nucleic acid, results in the formation of a nucleic acid/probe hybrid. The formation of this hybrid can be detected (e.g. labeling of the nucleic acid or probe), whereby the formation of this hybrid indicates the presence of the specific mutant *FAD2* allele. Such identification methods based on hybridization with a specific probe (either on a solid phase carrier or in solution) have been described in the art. The specific probe is preferably a sequence that, under optimized conditions, hybridizes specifically to a region within the 5' or 3' flanking region and/or within the mutation region of the specific mutant *FAD2* allele (hereinafter referred to as "mutant *FAD2* specific region"). Preferably, the specific probe comprises a sequence of between 10 and 1000 bp, 50 and 600 bp, between 100 to 500 bp, between 150 to 350bp, which is at least 80%, preferably between 80 and 85%, more preferably between 85 and 90%, especially preferably between 90 and 95%, most preferably between 95% and 100% identical (or complementary) to the nucleotide sequence of a specific region. Preferably, the specific probe will comprise a sequence of about 13 to about 100 contiguous nucleotides identical (or complementary) to a specific region of the specific mutant *FAD2* allele.

Specific probes suitable for the invention may be the following:

- oligonucleotides ranging in length from 13 nt to about 1000 nt, comprising a nucleotide sequence of at least 13 consecutive nucleotides selected from the 5' or 3' flanking sequence of a specific mutant *FAD2* allele or the complement thereof

(i.e., for example, the sequence 5' or 3' flanking the one or more nucleotides deleted, inserted or substituted in the mutant *FAD2* alleles of the invention, such as the sequence 5' or 3' flanking the non-sense, mis-sense, frameshift or splice site mutations described above or the sequence 5' or 3' flanking the potential STOP codon mutations indicated in the above Tables or the substitution mutations indicated above), or a sequence having at least 80% sequence identity therewith (probes recognizing 5' flanking sequences); or

- oligonucleotides ranging in length from 13 nt to about 1000 nt, comprising a nucleotide sequence of at least 13 consecutive nucleotides selected from the mutation sequence of a specific mutant *FAD2* allele or the complement thereof (i.e., for example, the sequence of nucleotides inserted or substituted in the *FAD2* genes of the invention, or the complement thereof), or a sequence having at least 80% sequence identity therewith (probes recognizing mutation sequences).

The probes may entirely consist of nucleotide sequence selected from the mentioned nucleotide sequences of flanking and mutation sequences. However, the nucleotide sequence of the probes at their 5' or 3' ends is less critical. Thus, the 5' or 3' sequences of the probes may consist of a nucleotide sequence selected from the flanking or mutation sequences, as appropriate, but may consist of a nucleotide sequence unrelated to the flanking or mutation sequences. Such unrelated sequences should preferably be no longer than 50, more preferably not longer than 25 or even no longer than 20 or 15 nucleotides.

Moreover, suitable probes may comprise or consist of a nucleotide sequence spanning the joining region between flanking and mutation sequences (i.e., for example, the joining region between a sequence 5' or 3' flanking one or more nucleotides deleted, inserted or substituted in the mutant *FAD2* alleles of the invention and the sequence of the one or more nucleotides inserted or substituted or the sequence 3' or 5', respectively, flanking the one or more nucleotides deleted, such as the joining region between a sequence 5' or 3' flanking non-sense, mis-sense, frameshift or splice site mutations in the *FAD2* genes of the invention described above and the sequence of the non-sense, mis-sense, frameshift or splice site mutations, or the joining region between a sequence 5' or 3' flanking a potential STOP codon mutation as indicated in the above Tables or the substitution mutations indicated above and the sequence of

the potential STOP codon or substitution mutation, respectively), provided the mentioned nucleotide sequence is not derived exclusively from either the mutation region or flanking regions.

Examples of specific probes suitable to identify specific mutant *fad2* alleles are described in the Examples.

Detection and/or identification of a "mutant *FAD2* specific region" hybridizing to a specific probe can occur in various ways, e.g., via size estimation after gel electrophoresis or via fluorescence-based detection methods. Other sequence specific methods for detection of a "mutant *FAD2* specific region" hybridizing to a specific probe are also known in the art.

Alternatively, plants or plant parts comprising one or more mutant *fad2* alleles can be generated and identified using other methods, such as the "Delete-a-gene™" method which uses PCR to screen for deletion mutants generated by fast neutron mutagenesis (reviewed by Li and Zhang, 2002, Funct Integr Genomics 2:254-258), by the TILLING (Targeting Induced Local Lesions IN Genomes) method which identifies EMS-induced point mutations using denaturing high-performance liquid chromatography (DHPLC) to detect base pair changes by heteroduplex analysis (McCallum *et al.*, 2000, Nat Biotech 18:455, and McCallum *et al.* 2000, Plant Physiol. 123, 439-442), etc. As mentioned, TILLING uses high-throughput screening for mutations (e.g. using Cel 1 cleavage of mutant-wildtype DNA heteroduplexes and detection using a sequencing gel system). Thus, the use of TILLING to identify plants or plant parts comprising one or more mutant *fad2* alleles and methods for generating and identifying such plants, plant organs, tissues and seeds is encompassed herein. Thus in one embodiment, the method according to the invention comprises the steps of mutagenizing plant seeds (e.g. EMS mutagenesis), pooling of plant individuals or DNA, PCR amplification of a region of interest, heteroduplex formation and high-throughput detection, identification of the mutant plant, sequencing of the mutant PCR product. It is understood that other mutagenesis and selection methods may equally be used to generate such mutant plants.

Instead of inducing mutations in *FAD2* alleles, natural (spontaneous) mutant alleles may be identified by methods known in the art. For example, ECOTILLING may be used (Henikoff *et al.* 2004, Plant Physiology 135(2):630-6) to screen a plurality of plants or plant parts for the presence of natural mutant *fad2* alleles. As for the mutagenesis techniques above, preferably *Brassica* species are screened which comprise an A and/or a C genome, so that the identified *fad2* allele can subsequently be introduced into other *Brassica* species, such as *Brassica napus*, by crossing (inter- or intraspecific crosses) and selection. In ECOTILLING natural polymorphisms in breeding lines or related species are screened for by the TILLING methodology described above, in which individual or pools of plants are used for PCR amplification of the *fad2* target, heteroduplex formation and high-throughput analysis. This can be followed by selecting individual plants having a required mutation that can be used subsequently in a breeding program to incorporate the desired mutant allele.

The identified mutant alleles can then be sequenced and the sequence can be compared to the wild type allele to identify the mutation(s). Optionally functionality can be tested as indicated above. Using this approach a plurality of mutant *fad2* alleles (and *Brassica* plants comprising one or more of these) can be identified. The desired mutant alleles can then be combined with the desired wild type alleles by crossing and selection methods as described further below. Finally a single plant comprising the desired number of mutant *fad2* and the desired number of wild type *FAD2* alleles is generated.

Oligonucleotides suitable as PCR primers or specific probes for detection of a specific mutant *FAD2* allele can also be used to develop methods to determine the zygosity status of the specific mutant *FAD2* allele.

To determine the zygosity status of a specific mutant *FAD2* allele, a nucleic acid amplification-based assay can be developed to determine the presence of a mutant and/or corresponding wild type *FAD2* specific allele:

To determine the zygosity status of a specific mutant *FAD2* allele, two primers specifically recognizing the wild-type *FAD2* allele can be designed in such a way that

they are directed towards each other and have the mutation region located in between the primers. These primers may be primers specifically recognizing the 5' and 3' flanking sequences, respectively. This set of primers allows simultaneous diagnostic amplification of the mutant, as well as of the corresponding wild type *FAD2* allele.

Alternatively, to determine the zygosity status of a specific mutant *FAD2* allele, two primers specifically recognizing the wild-type *FAD2* allele can be designed in such a way that they are directed towards each other and that one of them specifically recognizes the mutation region. These primers may be primers specifically recognizing the sequence of the 5' or 3' flanking region and the mutation region of the wild type *FAD2* allele, respectively. This set of primers, together with a third primer which specifically recognizes the sequence of the mutation region in the mutant *FAD2* allele, allow simultaneous diagnostic amplification of the mutant *FAD2* gene, as well as of the wild type *FAD2* gene.

Alternatively, to determine the zygosity status of a specific mutant *FAD2* allele, two primers specifically recognizing the wild-type *FAD2* allele can be designed in such a way that they are directed towards each other and that one of them specifically recognizes the joining region between the 5' or 3' flanking region and the mutation region. These primers may be primers specifically recognizing the 5' or 3' flanking sequence and the joining region between the mutation region and the 3' or 5' flanking region of the wild type *FAD2* allele, respectively. This set of primers, together with a third primer which specifically recognizes the joining region between the mutation region and the 3' or 5' flanking region of the mutant *FAD2* allele, respectively, allow simultaneous diagnostic amplification of the mutant *FAD2* gene, as well as of the wild type *FAD2* gene.

Alternatively, the zygosity status of a specific mutant *FAD2* allele can be determined by using alternative primer sets that specifically recognize mutant and wild type *FAD2* alleles.

If the plant is homozygous for the mutant *FAD2* gene or the corresponding wild type *FAD2* gene, the diagnostic amplification assays described above will give rise to a single amplification product typical, preferably typical in length, for either the mutant

or wild type *FAD2* allele. If the plant is heterozygous for the mutant *FAD2* allele, two specific amplification products will appear, reflecting both the amplification of the mutant and the wild type *FAD2* allele.

Identification of the wild type and mutant *FAD2* specific amplification products can occur e.g. by size estimation after gel or capillary electrophoresis (e.g. for mutant *FAD2* alleles comprising a number of inserted or deleted nucleotides which results in a size difference between the fragments amplified from the wild type and the mutant *FAD2* allele, such that said fragments can be visibly separated on a gel); by evaluating the presence or absence of the two different fragments after gel or capillary electrophoresis, whereby the diagnostic amplification of the mutant *FAD2* allele can, optionally, be performed separately from the diagnostic amplification of the wild type *FAD2* allele; by direct sequencing of the amplified fragments; or by fluorescence-based detection methods.

Examples of primers suitable to determine the zygosity of specific mutant *FAD2* alleles are described in the Examples.

Alternatively, to determine the zygosity status of a specific mutant *FAD2* allele, a hybridization-based assay can be developed to determine the presence of a mutant and/or corresponding wild type *FAD2* specific allele:

To determine the zygosity status of a specific mutant *FAD2* allele, two specific probes recognizing the wild-type *FAD2* allele can be designed in such a way that each probe specifically recognizes a sequence within the *FAD2* wild type allele and that the mutation region is located in between the sequences recognized by the probes. These probes may be probes specifically recognizing the 5' and 3' flanking sequences, respectively. The use of one or, preferably, both of these probes allows simultaneous diagnostic hybridization of the mutant, as well as of the corresponding wild type *FAD2* allele.

Alternatively, to determine the zygosity status of a specific mutant *FAD2* allele, two specific probes recognizing the wild-type *FAD2* allele can be designed in such a way that one of them specifically recognizes a sequence within the *FAD2* wild type allele

upstream or downstream of the mutation region, preferably upstream of the mutation region, and that one of them specifically recognizes the mutation region. These probes may be probes specifically recognizing the sequence of the 5' or 3' flanking region, preferably the 5' flanking region, and the mutation region of the wild type *FAD2* allele, respectively. The use of one or, preferably, both of these probes, optionally, together with a third probe which specifically recognizes the sequence of the mutation region in the mutant *FAD2* allele, allow diagnostic hybridization of the mutant and of the wild type *FAD2* gene.

Alternatively, to determine the zygosity status of a specific mutant *FAD2* allele, a specific probe recognizing the wild-type *FAD2* allele can be designed in such a way that the probe specifically recognizes the joining region between the 5' or 3' flanking region, preferably the 5' flanking region, and the mutation region of the wild type *FAD2* allele. This probe, optionally, together with a second probe that specifically recognizes the joining region between the 5' or 3' flanking region, preferably the 5' flanking region, and the mutation region of the mutant *FAD2* allele, allows diagnostic hybridization of the mutant and of the wild type *FAD2* gene.

Alternatively, the zygosity status of a specific mutant *FAD2* allele can be determined by using alternative sets of probes that specifically recognize mutant and wild type *FAD2* alleles.

If the plant is homozygous for the mutant *FAD2* gene or the corresponding wild type *FAD2* gene, the diagnostic hybridization assays described above will give rise to a single specific hybridization product, such as one or more hybridizing DNA (restriction) fragments, typical, preferably typical in length, for either the mutant or wild type *FAD2* allele. If the plant is heterozygous for the mutant *FAD2* allele, two specific hybridization products will appear, reflecting both the hybridization of the mutant and the wild type *FAD2* allele.

Identification of the wild type and mutant *FAD2* specific hybridization products can occur e.g. by size estimation after gel or capillary electrophoresis (e.g. for mutant *FAD2* alleles comprising a number of inserted or deleted nucleotides which results in a size difference between the hybridizing DNA (restriction) fragments from the wild

type and the mutant *FAD2* allele, such that said fragments can be visibly separated on a gel); by evaluating the presence or absence of the two different specific hybridization products after gel or capillary electrophoresis, whereby the diagnostic hybridization of the mutant *FAD2* allele can, optionally, be performed separately from the diagnostic hybridization of the wild type *FAD2* allele; by direct sequencing of the hybridizing DNA (restriction) fragments; or by fluorescence-based detection methods.

Furthermore, detection methods specific for a specific mutant *FAD2* allele that differ from PCR- or hybridization-based amplification methods can also be developed using the specific mutant *FAD2* allele specific sequence information provided herein. Such alternative detection methods include linear signal amplification detection methods based on invasive cleavage of particular nucleic acid structures, also known as Invader™ technology, (as described e.g. in US patent 5,985,557 "Invasive Cleavage of Nucleic Acids", 6,001,567 "Detection of Nucleic Acid sequences by Invader Directed Cleavage, incorporated herein by reference), RT-PCR-based detection methods, such as Taqman, or other detection methods, such as SNPlex. Briefly, in the Invader™ technology, the target mutation sequence may e.g. be hybridized with a labeled first nucleic acid oligonucleotide comprising the nucleotide sequence of the mutation sequence or a sequence spanning the joining region between the 5' flanking region and the mutation region and with a second nucleic acid oligonucleotide comprising the 3' flanking sequence immediately downstream and adjacent to the mutation sequence, wherein the first and second oligonucleotide overlap by at least one nucleotide. The duplex or triplex structure that is produced by this hybridization allows selective probe cleavage with an enzyme (Cleavase®) leaving the target sequence intact. The cleaved labeled probe is subsequently detected, potentially via an intermediate step resulting in further signal amplification.

A "kit", as used herein, refers to a set of reagents for the purpose of performing the method of the invention, more particularly, the identification of a specific mutant *FAD2* allele in biological samples or the determination of the zygosity status of plant material comprising a specific mutant *FAD2* allele. More particularly, a preferred embodiment of the kit of the invention comprises at least two specific primers, as described above, for identification of a specific mutant *FAD2* allele, or at least two or three specific primers for the determination of the zygosity status. Optionally, the kit

can further comprise any other reagent described herein in the PCR identification protocol. Alternatively, according to another embodiment of this invention, the kit can comprise at least one specific probe, which specifically hybridizes with nucleic acid of biological samples to identify the presence of a specific mutant *FAD2* allele therein, as described above, for identification of a specific mutant *FAD2* allele, or at least two or three specific probes for the determination of the zygosity status. Optionally, the kit can further comprise any other reagent (such as but not limited to hybridizing buffer, label) for identification of a specific mutant *FAD2* allele in biological samples, using the specific probe.

The kit of the invention can be used, and its components can be specifically adjusted, for purposes of quality control (e.g., purity of seed lots), detection of the presence or absence of a specific mutant *FAD2* allele in plant material or material comprising or derived from plant material, such as but not limited to food or feed products.

The term "primer" as used herein encompasses any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process, such as PCR. Typically, primers are oligonucleotides from 10 to 30 nucleotides, but longer sequences can be employed. Primers may be provided in double-stranded form, though the single-stranded form is preferred. Probes can be used as primers, but are designed to bind to the target DNA or RNA and need not be used in an amplification process.

The term "recognizing" as used herein when referring to specific primers, refers to the fact that the specific primers specifically hybridize to a nucleic acid sequence in a specific mutant *FAD2* allele under the conditions set forth in the method (such as the conditions of the PCR identification protocol), whereby the specificity is determined by the presence of positive and negative controls.

The term "hybridizing", as used herein when referring to specific probes, refers to the fact that the probe binds to a specific region in the nucleic acid sequence of a specific mutant *FAD2* allele under standard stringency conditions. Standard stringency conditions as used herein refers to the conditions for hybridization described herein or to the conventional hybridizing conditions as described by Sambrook et al., 1989

(Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, NY) which for instance can comprise the following steps: 1) immobilizing plant genomic DNA fragments or BAC library DNA on a filter, 2) prehybridizing the filter for 1 to 2 hours at 65°C in 6 X SSC, 5 X Denhardt's reagent, 0.5% SDS and 20 µg/ml denaturated carrier DNA, 3) adding the hybridization probe which has been labeled, 4) incubating for 16 to 24 hours, 5) washing the filter once for 30 min. at 68°C in 6X SSC, 0.1 %SDS, 6) washing the filter three times (two times for 30 min. in 30ml and once for 10 min in 500ml) at 68°C in 2 X SSC, 0.1 %SDS, and 7) exposing the filter for 4 to 48 hours to X-ray film at -70°C.

As used in herein, a "biological sample" is a sample of a plant, plant material or product comprising plant material. The term "plant" is intended to encompass plant tissues, at any stage of maturity, as well as any cells, tissues, or organs taken from or derived from any such plant, including without limitation, any seeds, leaves, stems, flowers, roots, single cells, gametes, cell cultures, tissue cultures or protoplasts. "Plant material", as used herein refers to material that is obtained or derived from a plant. Products comprising plant material relate to food, feed or other products that are produced using plant material or can be contaminated by plant material. It is understood that, in the context of the present invention, such biological samples are tested for the presence of nucleic acids specific for a specific mutant *FAD2* allele, implying the presence of nucleic acids in the samples. Thus the methods referred to herein for identifying a specific mutant *FAD2* allele in biological samples, relate to the identification in biological samples of nucleic acids that comprise the specific mutant *FAD2* allele.

In another embodiment, a method is provided for combining at least one partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, with at least one full knock-out *fad2* allele in a single *Brassica* plant, said method comprising a) generating and/or identifying two or more plants each comprising one or more selected partial and/or full knock-out *fad2* alleles; b) crossing a first plant comprising one or more selected partial and/or full knockout *fad2* alleles with a second plant comprising one or more other selected partial and/or full knockout *fad2* alleles; c) collecting seeds from the cross, and, optionally, identifying a plant

comprising at least one partial knock-out *fad2* allele and at least one full knockout *fad2* allele; and, optionally d) repeat steps b) and c) until a plant comprising at least one partial knock-out *fad2* allele and at least one full knockout *fad2* allele, is obtained, wherein said plant does not contain a *FAD2* gene of which the *FAD2* alleles encode a wild-type functional protein. Said single *Brassica* plant can be a *Brassica napus* plant comprising four FAD2 genes, wherein two partial knock-out *fad2* alleles of a first FAD2 genes are introduced, and two full knock-out *fad2* alleles each of a second, a third, and a fourth *FAD2* gene are introduced. Said first FAD2 gene can be a FAD2-A1 gene of which at two alleles are said partial knock-out *fad2* alleles which encode a FAD2 protein of which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp; said second FAD2 gene can be a FAD2-C1 gene of which two alleles contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 101 of SEQ ID NO: 9; said third FAD2 gene can be a FAD2-C2 gene of which two alleles contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15, and said fourth FAD2 gene can be a FAD2-A2 gene of which two alleles comprising a deletion mutation of the nucleotides corresponding to nucleotides 2036-2042 of SEQ ID NO: 13. Alternatively, Said first FAD2 gene can be a FAD2-C1 gene of which at two alleles are said partial knock-out *fad2* alleles which encode a FAD2 protein of which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp; said second FAD2 gene can be a FAD2-A1 gene of which two alleles encodes a FAD2 protein of which the His at a position corresponding to position 109 of SEQ ID NO: 6 is substituted with Tyr; said third FAD2 gene can be a FAD2-C2 gene of which two alleles contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15, and said fourth FAD2 gene can be a FAD2-A2 gene of which two alleles comprising a deletion mutation of the nucleotides corresponding to nucleotides 2036-2042 of SEQ ID NO: 13.

Levels of C18:1 in seed of a *Brassica* plant can be increased by generating and/or selecting a *Brassica* plant comprising at least one *FAD2* gene, wherein at least two alleles of the at least one FAD2 gene are partial knockout *fad2* alleles, as described above, and selecting a plant with increased levels of C18:1 in the seeds, while maintaining normal agronomic development.

The *Brassica* plant comprising at least one *FAD2* gene may be a *Brassica napus* plant comprising a *FAD2-A1,* a *FAD2-C1,* a *FAD2-A2,* and a *FAD2-C2* gene, wherein the alleles of one of said *FAD2* genes, such as the FAD2-A1 or the FAD2-C1 genes, are partial knock-out *fad2* alleles, and wherein the alleles of the other three *FAD2* genes are full knock-out *fad2* alleles.

A hybrid *Brassica* crop plant or seed, comprising at least one FAD2 gene, in particular a hybrid *Brassica napus* plant or seed comprising four FAD2 genes having increased levels of C18:1 while maintaining normal agronomic development, can be made by generating and/or identifying a first plant comprising a first partial knockout *fad2* allele in homozygous state and a second plant comprising a second partial knockout *fad2* allele in homozygous state, as described above, crossing the first and the second plant and collecting F1 hybrid seeds from the cross comprising two partial knockout *fad2* alleles.

The first plant may additionally comprise a first full knockout *fad2* allele in homozygous state and the second plant additionally comprises a second full knockout *fad2* allele in homozygous state, as described above, and F1 hybrid seeds comprising two partial knockout *fad2* alleles and two full knockout *fad2* alleles of the four FAD2 genes are collected.

The first and the second partial knockout *fad2* alleles may be the same, such that the F1 hybrid seeds are homozygous for a partial knockout *fad2* allele. The first and the second full knockout *fad2* alleles may also be the same, such that the F1 hybrid seeds are homozygous for a full knockout *fad2* allele.

Full knockout *fad2* alleles and/or partial knockout *fad2* alleles (i.e., *FAD2* alleles the functional expression of which is partially abolished) according to the invention can be combined according to standard breeding techniques.

Partial and/or full knockout *fad2* alleles can, for example, be transferred from one *Brassica* plant to another by a) generating and/or identifying a first plant comprising one or more selected partial and/or full knockout *fad2* alleles, as described above, or

generating the first plant by combining the one or more selected partial and/or full knockout *fad2* alleles in one plant, as described above (wherein the first plant is homozygous or heterozygous for the one or more partial and/or full knockout *fad2* alleles), b) crossing the first plant comprising the one or more partial and/or full knockout *fad2* alleles with a second plant not comprising the one or more partial and/or full knockout *fad2* alleles, collecting F1 seeds from the cross (wherein the seeds are heterozygous for a partial and/or full knockout *fad2* allele if the first plant was homozygous for that partial and/or full knockout *fad2* allele, and wherein half of the seeds are heterozygous and half of the seeds are azygous for, i.e. do not comprise, a partial and/or full knockout *fad2* allele if the first plant was heterozygous for that partial and/or full knockout *fad2* allele), and, optionally, identifying F1 plants comprising one or more selected partial and/or full knockout *fad2* alleles, as described above, c) backcrossing F1 plants comprising one or more selected partial and/or full knockout *fad2* alleles with the second plant not comprising the one or more selected partial and/or full knockout *fad2* alleles for one or more generations (x), collecting BCx seeds from the crosses, and identifying in every generation BCx plants comprising the one or more selected partial and/or full knockout *fad2* alleles, as described above, d) optionally, generating BCx plants which are homozygous for the one or more selected partial and/or full knockout *fad2* alleles by performing one of the following steps:

- extracting doubled haploid plants from treated microspore or pollen cells of BCx plants comprising the one or more desired partial and/or full knockout *fad2* allele(s), as described above,
- selfing the BCx plants comprising the one or more desired partial and/or full knockout *fad2* allele(s) for one or more generations (y), collecting BCx Sy seeds from the selfings, and identifying BCx Sy plants, which are homozygous for the one or more desired partial and/or full knockout *fad2* allele, as described above.

The first and the second *Brassica* plant can be *Brassica napus* plants or plants from another *Brassica* crop species. Alternatively, the first plant can be a *Brassica napus* plant or a plant from another *Brassica* crop species, and the second plant can be a *Brassica napus* breeding line or from a breeding line from another *Brassica* crop species. "Breeding line", as used herein, is a preferably homozygous plant line

distinguishable from other plant lines by a preferred genotype and/or phenotype that is used to produce hybrid offspring.

In a further aspect of the invention, a partial knock-out *fad2* allele of a FAD2 gene is provided, wherein the partial knock-out *fad2* allele is a mutated version of the native *FAD2* gene, wherein the native *FAD2* gene is selected from the group consisting of: (a) a nucleic acid molecule which comprises at least 90% sequence identity to SEQ ID No. 4 or 7; (b) a nucleic acid molecule comprising a coding sequence which comprises at least 90% sequence identity to SEQ ID No. 5 or 8; and (c) a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID No. 6 or 9, and wherein said partial knock-out *fad2* allele encodes a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, such as a partial knock-out *fad2* allele which encodes a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp.

In a further embodiment, a method is provided for producing oil, comprising harvesting seeds from the plants according to the invention, i.e. plants comprising a partial knock-out *FAD2* gene, and extracting the oil from said seeds.

In yet a further embodiment, a method is provided of producing food or feed, such as oil, meal, grain, starch, flour or protein, or an industrial product, such as biofuel, fiber, industrial chemicals, a pharmaceutical or a neutraceutical, comprising obtaining the plant or a part thereof according to the invention, and preparing the food, feed or industrial product from the plant or part thereof.

Another embodiment provides the use of the partial knock-out *fad2* allele according to the invention to increase the level of C18:1 in the seed oil of a *Brassica* plant while maintaining normal agronomic development. Yet another embodiment provides a method to produce a *Brassica* plant comprising an increased level of C18:1 in the seed oil and which maintains normal agronomic development, said method comprising sowing seeds according to the invention and growing plants from said seeds.

Plants according to the invention, such as plants comprising at least one partial knock-out *FAD2* gene can further be used to produce seeds, such as seeds with increased levels of C18:1, or to produce seed oil with increased levels of C18:1.

The plants according to the invention may additionally contain an endogenous or a transgene, which confers herbicide resistance, such as the bar or pat gene, which confer resistance to glufosinate ammonium (Liberty®, Basta® or Ignite®) [EP 0 242 236 and EP 0 242 246 incorporated by reference]; or any modified EPSPS gene, such as the 2mEPSPS gene from maize [EP0 508 909 and EP 0 507 698 incorporated by reference], or glyphosate acetyltransferase, or glyphosate oxidoreductase, which confer resistance to glyphosate (RoundupReady®), or bromoxynitril nitrilase to confer bromoxynitril tolerance, or any modified AHAS gene, which confers tolerance to sulfonylureas, imidazolinones, sulfonylaminocarbonyltriazolinones, triazolopyrimidines or pyrimidyl(oxy/thio)benzoates, such as oilseed rape imidazolinone-tolerant mutants PM1 and PM2, currently marketed as Clearfield® canola. Further, the plants according to the invention may additionally contain an endogenous or a transgene which confers increased oil content or improved oil composition, such as a 12:0 ACP thioesteraseincrease to obtain high laureate, which confers pollination control, such as barnase under control of an anther-specific promoter to obtain male sterility, or barstar under control of an anther-specific promoter to confer restoration of male sterility, or such as the Ogura cytoplasmic male sterility and nuclear restorer of fertility.

The plants and seeds according to the invention may be further treated with a chemical compound, such as a chemical compound selected from the following lists:
Herbicides: Clethodim, Clopyralid, Diclofop, Ethametsulfuron, Fluazifop, Glufosinate, Glyphosate, Metazachlor, Quinmerac, Quizalofop, Tepraloxydim, Trifluralin.
Fungicides / PGRs: Azoxystrobin, N-[9-(dichloromethylene)-1,2,3,4-tetrahydro-1,4-methanonaphthalen-5-yl]-3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide (Benzovindiflupyr, Benzodiflupyr), Bixafen, Boscalid, Carbendazim, Carboxin, Chlormequat-chloride, Coniothryrium minitans, Cyproconazole, Cyprodinil, Difenoconazole, Dimethomorph, Dimoxystrobin, Epoxiconazole, Famoxadone, Fluazinam, Fludioxonil, Fluopicolide, Fluopyram, Fluoxastrobin, Fluquinconazole,

Flusilazole, Fluthianil, Flutriafol, Fluxapyroxad, Iprodione, Isopyrazam, Mefenoxam, Mepiquat-chloride, Metalaxyl, Metconazole, Metominostrobin, Paclobutrazole, Penflufen, Penthiopyrad, Picoxystrobin, Prochloraz, Prothioconazole, Pyraclostrobin, Sedaxane, Tebuconazole, Tetraconazole, Thiophanate-methyl, Thiram, Triadimenol, Trifloxystrobin, Bacillus firmus, Bacillus firmus strain I-1582, Bacillus subtilis, Bacillus subtilis strain GB03, Bacillus subtilis strain QST 713, Bacillus pumulis, Bacillus. pumulis strain GB34.
Insecticides: Acetamiprid, Aldicarb, Azadirachtin, Carbofuran, Chlorantraniliprole (Rynaxypyr), Clothianidin, Cyantraniliprole (Cyazypyr), (beta-)Cyfluthrin, gamma-Cyhalothrin, lambda-Cyhalothrin, Cypermethrin, Deltamethrin, Dimethoate, Dinetofuran, Ethiprole, Flonicamid, Flubendiamide, Fluensulfone, Fluopyram,Flupyradifurone, tau-Fluvalinate, Imicyafos, Imidacloprid, Metaflumizone, Methiocarb, Pymetrozine, Pyrifluquinazon, Spinetoram, Spinosad, Spirotetramate, Sulfoxaflor, Thiacloprid, Thiamethoxam, 1-(3-chloropyridin-2-yl)-N-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-3-{[5-(trifluoromethyl)-2H-tetrazol-2-yl]methyl}-1H-pyrazole-5-carboxamide, 1-(3-chloropyridin-2-yl)-N-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-3-{[5-(trifluoromethyl)-1H-tetrazol-1-yl]methyl}-1H-pyrazole-5-carboxamide, 1-{2-fluoro-4-methyl-5-[(2,2,2-trifluorethyl)sulfinyl]phenyl}-3-(trifluoromethyl)-1H-1,2,4-triazol-5-amine, (1E)-N-[(6-chloropyridin-3-yl)methyl]-N'-cyano-N-(2,2-difluoroethyl)ethanimidamide,
Bacillus firmus, Bacillus firmus strain I-1582, Bacillus subtilis, Bacillus subtilis strain GB03, Bacillus subtilis strain QST 713, Metarhizium anisopliae F52.

In some embodiments, the plant cells of the invention, i.e. a plant cell comprising a knock-out *fad2* gene, as well as plant cells generated according to the methods of the invention, may be non-propagating cells.

The obtained plants according to the invention can be used in a conventional breeding scheme to produce more plants with the same characteristics or to introduce the characteristic according to the invention in other varieties of the same or related plant species, or in hybrid plants. The obtained plants can further be used for creating propagating material. Plants according to the invention can further be used to produce gametes, seeds (including crushed seeds and seed cakes), seed oil, embryos, either zygotic or somatic, progeny, or to produce food or feed, such as oil, meal, grain,

starch, flour or protein, or an industrial product, such as biofuel, fiber, industrial chemicals, a pharmaceutical or a neutraceutical, or to produce hybrids of plants obtained by methods of the invention.

All patents, patent applications, and publications or public disclosures (including publications on internet) referred to or cited herein are incorporated by reference in their entirety.

The sequence listing contained in the file named „BCS15-2005-WO1_ST25.txt", which is 126 kilobytes (size as measured in Microsoft Windows®), contains 57 sequences SEQ ID NO: 1 through SEQ ID NO: 57 is filed herewith by electronic submission and is incorporated by reference herein.

In the description and examples, reference is made to the following sequences:

SEQUENCES

SEQ ID No. 1: Genomic DNA sequence of *FAD2* from *Arabidopsis thaliana.*
SEQ ID No. 2: cDNA sequence of *FAD2* from *Arabidopsis thaliana.*
SEQ ID No. 3: protein sequence of FAD2 from *Arabidopsis thaliana.*
SEQ ID No. 4: Genomic DNA sequence of *FAD2-A1* from *Brassica napus.*
SEQ ID No. 5: cDNA sequence of *FAD2-A1* from *Brassica napus.*
SEQ ID No. 6: protein sequence of FAD2-A1 from *Brassica napus.*
SEQ ID No. 7: Genomic DNA sequence of *FAD2-C1* from *Brassica napus.*
SEQ ID No. 8: cDNA sequence of *FAD2-C1* from *Brassica napus.*
SEQ ID No. 9: protein sequence of FAD2-C1 from *Brassica napus.*
SEQ ID No. 10: Genomic DNA sequence *of FAD2-A2* from *Brassica napus.*
SEQ ID No. 11: cDNA sequence of *FAD2-A2* from *Brassica napus.*
SEQ ID No. 12: protein sequence of FAD2-A2 from *Brassica napus.*
SEQ ID No. 13: Genomic DNA sequence of *FAD2-C2* from *Brassica napus.*
SEQ ID No. 14: cDNA sequence of *FAD2-C2* from *Brassica napus.*
SEQ ID No. 15: protein sequence of FAD2-C2 from *Brassica napus.*
SEQ ID No. 16: Genomic DNA sequence of *FAD2-1* from *Brassica rapa.*
SEQ ID No. 17: cDNA sequence of *FAD2-1* from *Brassica rapa.*
SEQ ID No. 18: protein sequence of FAD2-1 from *Brassica rapa.*
SEQ ID No. 19: Genomic DNA sequence of *FAD2-2* from *Brassica rapa.*
SEQ ID No. 20: cDNA sequence of *FAD2-2* from *Brassica rapa.*
SEQ ID No. 21: protein sequence of FAD2-2 from *Brassica rapa.*
SEQ ID No. 22: Genomic DNA sequence of *FAD2-1* from *Brassica oleracea.*
SEQ ID No. 23: cDNA sequence of *FAD2-1* from *Brassica oleracea.*
SEQ ID No. 24: protein sequence of FAD2-1 from *Brassica oleracea.*
SEQ ID No. 25: Genomic DNA sequence of *FAD2-2* from *Brassica oleracea.*
SEQ ID No. 26: cDNA sequence of *FAD2-2* from *Brassica oleracea.*
SEQ ID No. 27: protein sequence of FAD2-2 from *Brassica oleracea.*
SEQ ID No. 28: FAM primer HIOL101.
SEQ ID No. 29: VIC primer HIOL101.
SEQ ID No. 30: Reverse primer HIOL101.

SEQ ID No. 31: FAM primer HIOL112.
SEQ ID No. 32: VIC primer HIOL112.
SEQ ID No. 33: Reverse primer HIOL112.
SEQ ID No. 34: FAM primer HIOL113.
SEQ ID No. 35: VIC primer HIOL113.
SEQ ID No. 36: Reverse primer HIOL113.
SEQ ID No. 37: FAM primer HIOL114.
SEQ ID No. 38: VIC primer HIOL114.
SEQ ID No. 39: Reverse primer HIOL114.
SEQ ID No. 40: FAM primer HIOL116.
SEQ ID No. 41: VIC primer HIOL116.
SEQ ID No. 42: Reverse primer HIOL116.
SEQ ID No. 43: FAM primer HIOL103.
SEQ ID No. 44: VIC primer HIOL103.
SEQ ID No. 45: Reverse primer HIOL103.
SEQ ID No. 46: FAM primer HIOL117.
SEQ ID No. 47: VIC primer HIOL117.
SEQ ID No. 48: Reverse primer HIOL117.
SEQ ID No. 49: FAM primer HIOL119.
SEQ ID No. 50: VIC primer HIOL119.
SEQ ID No. 51: Reverse primer HIOL119.
SEQ ID No. 52: FAM primer HIOL120.
SEQ ID No. 53: VIC primer HIOL120.
SEQ ID No. 54: Reverse primer HIOL120.
SEQ ID No. 55: FAM primer HIOL109.
SEQ ID No. 56: VIC primer HIOL109.
SEQ ID No. 57: Reverse primer HIOL109.

EXAMPLES

Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor

Laboratory Press, NY, in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA and in Volumes I and II of Brown (1998) Molecular Biology LabFax, Second Edition, Academic Press (UK). Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) PCR - Basics: From Background to Bench, First Edition, Springer Verlag, Germany. Standard procedures for AFLP analysis are described in Vos et al. (1995, NAR 23:4407-4414) and in published EP patent application EP 534858.

Example 1 – Isolation of the DNA sequences of Brassica *FAD2* genes

A TBLASTN homology search using the *A. thaliana FAD2* gene sequence (At3G12120) was used as the query in a BLAST homology search of in-house databases of *Brassica rapa* sequences and of *Brassica oleracea* sequences. The BLAST analyses resulted in the identification of 2 *FAD2* gene homologs for *B. rapa* (*BrFAD2-1* (SEQ ID No. 16), *BrFAD2-2* (SEQ ID No. 19), and 2 *FAD2* gene homologs for *B. oleracea (BoFAD2-1* (SEQ ID No. 22), *BoFAD2-2* (SEQ ID No. 25). cDNAs corresponding to these sequences were predicted using FgeneSH software, and are depicted in SEQ ID No. 17, SEQ ID No. 20, SEQ ID No. 23, and SEQ ID No. 26, respectively. A BLAST homology search of an in-house database containing *Brassica napus* mRNA sequences using the *B. rapa BrFAD2* gene sequences resulted in the identification of the cDNA sequences of *B. napus BnFAD2-A1* (SEQ ID No. 5), and *BnFAD2-A2* (SEQ ID No. 11). Based on gene structure predictions using the Fgenesh software or mRNA derived sequencing read abundance the corresponding coding sequences were identified. Similarly, a BLAST homology search of the in-house database containing *Brassica napus* mRNA sequences using the *B. oleracea BoFAD2* gene sequences as a query resulted in the identification of the cDNA sequences of *BnFAD2-C1* (SEQ ID No. 8), and *BnFAD2-C2* (SEQ ID No. 14). The corresponding coding sequences were obtained following the above-mentioned gene structure prediction methods.

In order to retrieve the *B. napus FAD2* gene sequences a BAC library was screened. Following standard GS-FLX sequencing of the positive library clones and *de novo* contig assembly using the 454 assembly software Newbler the gene sequences for *BnFAD2-A1* (SEQ ID No. 4), *BnFAD2-A2* (SEQ ID No. 10), *BnFAD2-C1* (SEQ ID No. 7) and *BnFAD2-C2* (SEQ ID No. 13) were identified.

The *B. napus BnFAD2-A1, BnFAD2-C1*, and *BnFAD2-C2* genes, *B. rapa BrFAD2-1* gene, and the *B. oleracea BoFAD2-1* gene, encode FAD2 proteins of 384 amino acids (SEQ ID NOs: 6, 9, 15, 18, and 24, respectively).

The *B. oleracea* BoFAD2-2 gene contains a 1 nt deletion at a position corresponding to position 2608 of the BnFAD2-C2 gene (SEQ ID NO: 13) (i.e. the position after position 2726 of the BoFAD2-2 gene (SEQ ID NO: 25), resulting in a frameshift mutation, leading to a truncated protein of 290 amino acids (SEQ ID NO: 27).

The *B. napus BnFAD2-A2* and *B. rapa BrFAD2-2* genes contain missing nucleotides at a position corresponding to positions 2036-2042 of BnFAD2-C2 gene (SEQ ID NO: 13), leading to a frameshift and premature stopcodon in the coding sequence, resulting in truncated proteins of 136 amino acids (SEQ ID NO: 12 (BnFAD2-A2 protein and SEQ ID NO: 21 (BrFAD2-2 protein). The *BnFAD2-A2* and *BrFAD2-2* genes therefore represent nonfunctional pseudo-genes.

Alignment of the proteins encoded by the *Brassica FAD2* genes as described herein are shown in Figure 1.

Example 2 – Expression analysis of Brassica napus *FAD2* genes

The relative gene expression levels of *Brassica napus FAD2* genes were determined through analysis of Illumina mRNAseq derived transcriptome databases obtained for multiple tissues and developmental stages. Gene expression levels were calculated taking into account a normalization step for the sequencing depth per database (target reads per million reads in the database) and for the target gene length (reads per kilobase per million reads in the database) [RPKM; Mortazavi A, Williams BA,

McCue K, Schaeffer L, Wold B: Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature Methods (2008), 5(7):621-628].

The result of the expression analysis is shown in Figure 2. From this figure it can be seen that *BnFAD2-A1* and *BnFAD2-C1* have the highest levels of expression, and that the expression is highest in seeds.

Example 3 – Generation and isolation of mutant *Brassica napus fad2* alleles

Mutations in the *FAD2* genes from *Brassica napus* identified in Example 1 were generated and identified as follows:

- 30,000 seeds from an elite spring oilseed rape breeding line (M0 seeds) were preimbibed for two hours on wet filter paper in deionized or distilled water. Half of the seeds were exposed to 0.8% EMS and half to 1% EMS (Sigma: M0880) and incubated for 4 hours.
- The mutagenized seeds (M1 seeds) were rinsed 3 times and dried in a fume hood overnight. 30,000 M1 plants were grown in soil and selfed to generate M2 seeds. M2 seeds were harvested for each individual M1 plant.
- Two times 4800 M2 plants, derived from different M1 plants, were grown and DNA samples were prepared from leaf samples of each individual M2 plant according to the CTAB method (Doyle and Doyle, 1987, Phytochemistry Bulletin 19:11-15).
- The DNA samples were screened for the presence of point mutations in the *FAD2* genes causing the introduction of STOP codons in the protein-encoding regions of the *FAD2* genes, or amino acid substitutions, by direct sequencing by standard sequencing techniques and analyzing the sequences for the presence of the point mutations using the NovoSNP software.
- The following mutant *fad2* alleles were thus identified:

Table 2a: mutations in *Bn FAD2-A1*

Plant name	Nt pos Genomic SEQ ID 4	Nt pos cDNA SEQ ID 5	AA pos. SEQ ID 6	WT → mut codon	WT → mut AA
HIOL101*	2371	615	109	CAC→TAC	H→Y

HIOL112	2642	886	199	TCG→TTG	S→L
HIOL113	2644	888	200	GGG→AGG	G→R
HIOL114	2651	895	202	CCT→CTT	P→L
HIOL116*	2740	984	232	GGC→AGC	G→S

Table 2b: mutations in *BnFAD2-C1*

Plant name	Nt pos Genomic SEQ ID 7	Nt pos cDNA SEQ ID 8	AA pos. SEQ ID 9	WT → mut codon	WT → mut AA
HIOL103*	3223	620	101	TGG→TGA	W→STOP
HIOL117	3318	715	133	CCT→CTT	P→L
HIOL119	3516	913	199	TCG→TTG	S→L
HIOL120*	3614	1011	232	GGC→AGC	G→S

Table 2c: mutations in *BnFAD2-C2*

Plant name	Nt pos Genomic SEQ ID 13	Nt pos cDNA SEQ ID 14	AA pos. SEQ ID 15	WT → mut codon	WT → mut AA
HIOL109*	2327	721	190	TGG→TAG	W→STOP

Footnotes*: Seeds comprising a mutant *BnFAD1-A1* allele comprising the HIOL101 mutation, a mutant *BnFAD1-A1* allele comprising the HIOL116 mutation, a mutant *BnFAD1-C1* allele comprising the HIOL103 mutation, a mutant *BnFAD1-C1* allele comprising the HIOL120 mutation, or a mutant *BnFAD1-C2* allele comprising the HIOL109 mutation have been deposited at the NCIMB (NCIMB Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen AB21 9YA, Scotland, UK) on 26 February 2015, under accession number NCIMB 42376, NCIMB 42373, NCIMB 42374, NCIMB 42377, and NCIMB 42375, respectively.

Example 4 – Activity of FAD2 mutants in a yeast activity assay

A FAD2 cDNA was created containing a mutation leading to a substitution of the Glycine (Gly, G) at a position corresponding to position 232 of the *Brassica* FAD2 protein of SEQ ID NO: 9 for an Aspartic Acid (Asp, D) (G232D mutation). The cDNA, and a cDNA encoding the wild-type FAD2 gene, was cloned into a vector for expression in yeast, the sequence was confirmed by sequencing, and the vectors

containing the FAD2 coding sequences, as well as an empty vector, were transformed in yeast. For desaturation analysis of each FAD2 protein, at least three different yeast single colonies were grown in culture at 20°C or at 30°C, the C18:1 and C18:2 levels were determined essentially as described by Peyou-Ndi et al., Arch Biochem Biophys 376:399, and the percentage of C18:1 conversion was determined. As can be seen from Table 3, no C18:1 conversion was observed for the empty vector; the wild-type FAD2 protein had a C18:1 conversion of almost 77%, whereas the C18:1 conversion of the G232D mutant was significantly reduced to 7% at 30°C and to 41.3% at 20°C, but not completely abolished. The G232D thus has a C18:1 conversion level of 9% (30°C) or 54% (20°C) of the wild-type FAD2 protein.

Table 3. C18:1 conversion of wild-type and mutant FAD2 protein.

Sample	% of 18:1 Conversion	
	48h growth at 30°C	48h growth at 20°C
Empty vector	0	0
G232D	7.0±1.4	41.3±5.4
FAD2 WT	76.8±0.9	76.9±1.5

Example 5 – Oil composition in seeds from *Brassica napus* comprising *BnFAD2-A1, BnFAD2-C1* and *BnFAD2-C2* knock-out alleles grown in the greenhouse

Brassica plants comprising mutant *BnFAD2-A1. BnFAD2-C1* and BnFAD2-*C2* alleles were crossed. Following selfing seeds from plants homozygous for *FAD2-A1, FAD2-C1* or *FAD2-C2* mutations and combinations thereof, or wild type segregants (i.e. not comprising any mutant FAD2 allele that would impact the normal function of a FAD2 protein) were obtained based on molecular marker based selection of plants (see below).

The fatty acid composition of the seed oil of the above *Brassica* plants grown in the greenhouse was determined by extracting the fatty acyls from the seeds and analyzing

their relative levels in the seed oil by capillary gas-liquid chromatography as described in WO09/007091.

Fatty acid composition was determined from plants grown in the greenhouse in seeds of the *Brassica* lines with mutant *BnFAD2-A1*, *BnFAD2-C1* or *BnFAD2-C2* alleles, and combinations thereof, and in wild-type segregants. Wild type check refers to a reference *B. napus* genotype that was not subject to EMS treatment.

Table 4. C18:1, C18:2 and C18:3 levels (% of oil weight in seed) in seeds of *Brassica napus* plants with different combinations of mutations in the *BnFAD2-A1*, *BnFAD2-C1*, and *BnFAD2-C2* genes, grown in the greenhouse. A1, C1, C2 refers to presence of mutant alleles; -- refers to presence of wild-type alleles. An asterisk indicates that there is no significant difference between the mutant and the corresponding wild type segregant.

GENOTYPES	C18:1	C18:2	C18:3
	%	%	%
HIOL112/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	87.14	1.25	2.32
HIOL112/HIOL103/HIOL109 (A1A1/C1C1/--)	85.47	1.76	3.5
HIOL112/HIOL103/HIOL109 (A1A1/--/C2C2)	73.97	8.51	7.85
HIOL112/HIOL103/HIOL109 (--/C1C1/C2C2)	71.17	11.25	8.04
HIOL112/HIOL103/HIOL109 (A1A1/--/--)	72.58	10.14	8.15
HIOL112/HIOL103/HIOL109 (--/C1C1/--)	69	12.68	8.45
HIOL112/HIOL103/HIOL109 (--/--/C2C2)	63.29	18.38	9.06*
HIOL112/HIOL103/HIOL109 (--/--/--)	61.83	18.85	9.16
HIOL113/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	86.46	1.31	2.7
HIOL113/HIOL103/HIOL109 (A1A1/C1C1/--)	85.58	1.7	3.71
HIOL113/HIOL103/HIOL109 (A1A1/--/C2C2)	74.96	7.9	8.12
HIOL113/HIOL103/HIOL109 (--/C1C1/C2C2)	70.88	10.75	8.9
HIOL113/HIOL103/HIOL109 (A1A1/--/--)	73.12	9.13	8.57
HIOL113/HIOL103/HIOL109 (--/C1C1/--)	70.33	12.01	8.51
HIOL113/HIOL103/HIOL109 (--/--/C2C2)	63.39	17.22	10.03
HIOL113/HIOL103/HIOL109 (--/--/--)	62.38	18.47	9.71
HIOL114/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	70.58	11.48	8.98
HIOL114/HIOL103/HIOL109 (A1A1/C1C1/--)	68.79	12.46	9.63
HIOL114/HIOL103/HIOL109 (A1A1/--/C2C2)	62.44	18.31	10.05*
HIOL114/HIOL103/HIOL109 (--/C1C1/C2C2)	70.37	11.73	8.91
HIOL114/HIOL103/HIOL109 (A1A1/--/--)	61.03	19.33	10.65
HIOL114/HIOL103/HIOL109 (--/C1C1/--)	68.02	14.24	8.77
HIOL114/HIOL103/HIOL109 (--/--/C2C2)	61.3	19.25	10.33*
HIOL114/HIOL103/HIOL109 (--/--/--)	60.44	19.79	10.25
HIOL116/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	77.22	6.58	7.3
HIOL116/HIOL103/HIOL109 (A1A1/C1C1/--)	75.42	7.83	7.82
HIOL116/HIOL103/HIOL109 (A1A1/--/C2C2)	74.32	8.45	8.32
HIOL116/HIOL103/HIOL109 (--/C1C1/C2C2)	70.84	11.3	8.55
HIOL116/HIOL103/HIOL109 (A1A1/--/--)	66.77	14.74	9.25
HIOL116/HIOL103/HIOL109 (--/C1C1/--)	70.16	12.03	8.6
HIOL116/HIOL103/HIOL109 (--/--/C2C2)	69.15	12.55	9.02
HIOL116/HIOL103/HIOL109 (--/--/--)	68.19	13.56	8.97
wild-type check	63.83	17.36	9.46

Table 4 shows that the levels of C18:1 are significantly increased in mutant lines as compared to wild-type segregants. When the HIOL114 mutation (Pro to Leu substitution at position 202 of the protein) is present in the BnFAD2-A1 gene, the effect on the level of C18:1 is weakest; the HIOL116 mutation (Gly to Ser at position 232) has an intermediate effect on C18:1, and the HIOL112 mutation (Ser to Leu at position 199) and HIOL113 mutation (Gly to Arg at position 200) have the strongest

effect on C18:1 levels. This indicates that the HIOL116 mutation (Gly to Ser at position 232) results in a partial reduction in activity of the encoded FAD2 protein. Table 4 also shows that the levels of C18:2 and, for most combinations, the levels of C18:3, are significantly reduced in mutant lines as compared to wild type segregants.

Example 6 – Oil composition in seeds from *Brassica napus* comprising *BnFAD2-A1, BnFAD2-C1,* and *BnFAD2-C2* knock-out alleles grown in the field

Fatty acid composition and plant performance parameters were determined from plants grown in the field as described above in seeds of the *Brassica* lines with mutant *BnFAD2-A1, BnFAD2-C1* or *BnFAD2-C2* alleles, and combinations thereof, and in wild type segregants not comprising any of the mutant BnFAD2 alleles. The mutant genotypes were tested at 3 different geographic locations.

Fatty acid composition in the seed oil was determined as described above. The following plant performance parameters were determined: Establishment (EST1) at the 2-3 leaf stage on a scale 1-9, wherein 1 = very thin, 5 = average, 9 = very thick; Vigor (VIG1) at the 5-6 leaf stage on a scale 1-9, wherein 1 = poor, 5 = average, 9 = vigorous; Flowering – Start (DTF): the stage (in days after seeding) at which 10% is in flower; Flowering – End (EOF): the stage (in days after seeding) at which 10% remains in flower; Plant Height (HICM) at the stage of flowering end in cm; Lodging Resistance at Maturity (LOM) at the maturity stage on a scale 1-9 wherein 1 = 0 degrees (flat), 5 = 45 degrees, 9 = 90 degrees (upright); Maturity (MAT) on a scale 1-9 wherein 1 = late, 5 = average, 9 = early; Glucosinolate content (GLUN) in the seed in µmoles/gram seeds; Oil content (OILN) in the seed in % of whole seed; protein content (PRON) in the seed in % of whole seed. Seed quality parameters were obtained through GC analysis. For the statistical analysis an ANOVA test was run. Contrasts between the mutant lines versus the corresponding null-segregants were subject to significance testing. Wild type check refers to a reference *B. napus* genotype that was not subject to EMS treatment.

Tables 5a and b show the plant performance parameters of the plants with the different combinations of the BnFAD2 mutant alleles, and Tables 6a and b show the

fatty acid composition in the seed oil of plants with the different combinations of the BnFAD2 mutant alleles.

Table 5a. Agronomic performance of FAD2 mutants. A1, C1, C2 refers to presence of mutant alleles; -- refers to presence of wild-type alleles. *: mutant significantly different from wild-type segregant; ** wild-type segregant significantly different from wild-type check.

GENOTYPES	EST1 (1-9)	VIG1 (1-9)	DTF days	EOF days	HICM cm	MAT (1-9)	LOM (1-9)	OILN %	PRON %	GLUN µmol/g
HIOL101/HIOL117/HIOL109 (A1A1/C1C1/C2C2)	3.8*	2.8*	48*	71.1*	97.8*	3*	4.2	34.5*	35*	11.8
HIOL101/HIOL117/HIOL109 (A1A1/C1C1/--)	4.3*	3.5*	47.3*	70.5*	97.1*	3*	3.7*	35*	34.5*	11.6
HIOL101/HIOL117/HIOL109 (A1A1/--/C2C2)	5.1	6.8	46.6	67.5	119.4	4.8	5	41.7	28.8	9.4
HIOL101/HIOL117/HIOL109 (--/C1C1/C2C2)	4.8	6.3	46.7	68.1	120.1	4.4	5.2	41.1	29.2	10.5
HIOL101/HIOL117/HIOL109 (--/--/--)	5	6.3**	46.3	67.7	115.3	4.4**	4.9	40.8**	29	10.3
HIOL101/HIOL119/HIOL109 (A1A1/C1C1/C2C2)	4.1*	3.2*	47.4*	70.5*	94.3*	3*	4.3	34.7*	34.6*	14.2*
HIOL101/HIOL119/HIOL109 (A1A1/C1C1/--)	4.2*	3.2*	47.8*	70.6*	97.5*	3*	4.3	35.7*	34*	12.2
HIOL101/HIOL119/HIOL109 (A1A1/--/C2C2)	5	6.6	46.6	67.5	120.5	5.2	5.4	40.8	29	10.8
HIOL101/HIOL119/HIOL109 (--/C1C1/C2C2)	4.8	6.6	46.8	68	123.4	5.1	5.4	40.9	29	10.8
HIOL101/HIOL119/HIOL109 (--/--/--)	4.9	6.5**	46.7**	67.6	120	5.1	5.2	40.5**	29.1	11.1
HIOL101/HIOL120/HIOL109 (A1A1/C1C1/C2C2)	5	6.7	46.4	67	117.8	5.2	4.9	42.2*	28.1	10
HIOL101/HIOL120/HIOL109 (A1A1/C1C1/--)	4.9	6.4	46.3	67	112.4	5.2	5	41.3	28.5	10.4
HIOL101/HIOL120/HIOL109 (A1A1/--/C2C2)	5.2	6.6	46.4	67.6	117.3	5.3	5	41.7	28.2	10.8
HIOL101/HIOL120/HIOL109 (--/C1C1/C2C2)	4.9	6.6	46.7	67.6	120	5	4.8	41.9	28.4	11.5
HIOL101/HIOL120/HIOL109 (--/--/--)	5	6.8**	46.4	67.6	119.8	4.9	4.6	40.6**	28.4	10.7
wild-type check	5	7.4	45.9	66.5	120.1	5.3	5.3	42.3	27.6	10.2

Table 5b. Agronomic performance of FAD2 mutants. A1, C1, C2 refers to presence of mutant alleles; -- refers to presence of wild-type alleles. *: mutant significantly different from wild-type segregant; ** wild-type segregant significantly different from wild-type check.

GENOTYPES	EST1 (1-9)	VIG1 (1-9)	DTF days	EOF days	HICM cm	MAT (1-9)	LOM (1-9)	OILN %	PRON %	GLUN µmol/g
HIOL112/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	3.9*	2.2*	47.9*	71.8*	93.3*	3*	3.5*	38.2*	33.8*	13.9*
HIOL112/HIOL103/HIOL109 (A1A1/C1C1/--)	4.1*	2.8*	47.7*	71.8*	98.1*	3.1*	3.8*	36.8*	34.4*	12.6
HIOL112/HIOL103/HIOL109 (A1A1/--/C2C2)	5	6.6	46.7	68.3	120	4.9	4.8	42	29.3	7.6*
HIOL112/HIOL103/HIOL109 (--/C1C1/C2C2)	4.8	6.5	46.8	68.1	124	5.1	5	42.7	28.6	10.6
HIOL112/HIOL103/HIOL109 (--/--/--)	4.9	6.8**	46.6**	67.9	122.4	4.8	5.1	41.6	29.2	10.4
HIOL113/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	4.1*	2.3*	47.8*	71.7*	90.4*	3.1*	3.7*	38.1*	32.9*	15.1*
HIOL113/HIOL103/HIOL109 (A1A1/C1C1/--)	4.3*	2.8*	47.5*	70.6*	96.8*	3.4*	3.9*	38.6*	32.3*	14.1*
HIOL113/HIOL103/HIOL109 (A1A1/--/C2C2)	4.8	5.7*	46.6	67.8	121.3	5.3	4.7	42.3	28.4	10.3
HIOL113/HIOL103/HIOL109 (--/C1C1/C2C2)	5	6.1	46.5	67.7	122.3	5.3	4.8	42.1	28.7	11.8
HIOL113/HIOL103/HIOL109 (--/--/--)	5.1	6.3**	46.6**	67.8	117.8	5	4.9	42.5	27.8	9.9
HIOL116/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	5	6.7	46.9	67.9	122.8	5.4	5*	41	29.2	10.8
HIOL116/HIOL103/HIOL109 (A1A1/C1C1/--)	4.8*	6.5	46.5*	67.8	116.9	5.8	5.7	40.6	29.5	11.2
HIOL116/HIOL103/HIOL109 (A1A1/--/C2C2)	4.9	6.5	47.4	68.7	123	5.1	5.3	39.9	30.3	11.5
HIOL116/HIOL103/HIOL109 (--/C1C1/C2C2)	4.8*	6.4	46.8	68.2	120.6	5.3	5.4	39.6	30.3	12.3
HIOL116/HIOL103/HIOL109 (--/--/--)	5.3	6.7**	47.2**	68.3	118.6	5.5	5.8	40.1**	29.8	11
wild-type check	5.2	7.3	45.9	67.4	123.8	5.3	5.3	42.2	28.1	9.4

Table 6a. C18:1, C18:2, C18:3 levels and total levels of saturated fatty acids (SATS) of FAD2 mutants. A1, C1, C2 refers to presence of mutant alleles; -- refers to presence of wild-type alleles. *: mutant significantly different from wild-type segregant; ** wild-type segregant significantly different from wild-type check.

GENOTYPES	C18:1	C18:2	C18:3	SATS
	%	%	%	%
HIOL101/HIOL117/HIOL109 (A1A1/C1C1/C2C2)	81.05*	4.58*	4.68*	6.54*
HIOL101/HIOL117/HIOL109 (A1A1/C1C1/--)	80.76*	4.65*	5.06*	6.7*
HIOL101/HIOL117/HIOL109 (A1A1/--/C2C2)	74.13*	9.6*	7.15*	6.56*
HIOL101/HIOL117/HIOL109 (--/C1C1/C2C2)	71.24*	12.08*	7.52*	6.71*
HIOL101/HIOL117/HIOL109 (--/--/--)	62.99**	19**	8.69	7.01
HIOL101/HIOL119/HIOL109 (A1A1/C1C1/C2C2)	80.96*	4.59*	4.63*	6.8*
HIOL101/HIOL119/HIOL109 (A1A1/C1C1/--)	81.76*	3.71*	4.76*	6.89*
HIOL101/HIOL119/HIOL109 (A1A1/--/C2C2)	73.22*	9.88*	7.52*	6.87*
HIOL101/HIOL119/HIOL109 (--/C1C1/C2C2)	70.13*	12.76*	7.58*	7.02*
HIOL101/HIOL119/HIOL109 (--/--/--)	61.7	20.38	8.35**	7.18**
HIOL101/HIOL120/HIOL109 (A1A1/C1C1/C2C2)	74.61*	9.16*	7.75*	6.34*
HIOL101/HIOL120/HIOL109 (A1A1/C1C1/--)	72.71*	10.32*	8.09*	6.58*
HIOL101/HIOL120/HIOL109 (A1A1/--/C2C2)	73.36*	9.89*	7.73*	6.61*
HIOL101/HIOL120/HIOL109 (--/C1C1/C2C2)	64.11*	18.71*	8.43*	6.63*
HIOL101/HIOL120/HIOL109 (--/--/--)	60.54**	21.46**	8.87	6.93
wild type check	61.88	20.19	8.67	6.94

Table 6b. C18:1, C18:2, C18:3 levels and total levels of saturated fatty acids (SATS) of FAD2 mutants. A1, C1, C2 refers to presence of mutant alleles; -- refers to presence of wild-type alleles. *: mutant significantly different from wild-type segregant; ** wild-type segregant significantly different from wild-type check.

GENOTYPES	C18:1	C18:2	C18:3	SATS
	%	%	%	%
HIOL112/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	83.01*	3.56*	3.92*	6.48*
HIOL112/HIOL103/HIOL109 (A1A1/C1C1/--)	82.49*	3.75*	4.13*	6.76*
HIOL112/HIOL103/HIOL109 (A1A1/--/C2C2)	72.38*	10.7*	7.21*	7.21*
HIOL112/HIOL103/HIOL109 (--/C1C1/C2C2)	69.2*	13.89*	7.31*	7.14*
HIOL112/HIOL103/HIOL109 (--/--/--)	60.82	21.31**	8.07**	7.44**
HIOL113/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	81.12*	4.62*	4.59*	6.64*
HIOL113/HIOL103/HIOL109 (A1A1/C1C1/--)	81.65*	3.72*	4.97*	6.51*
HIOL113/HIOL103/HIOL109 (A1A1/--/C2C2)	72.74*	10.49*	7.64*	6.53*
HIOL113/HIOL103/HIOL109 (--/C1C1/C2C2)	69.21*	13.33*	7.97*	6.84*
HIOL113/HIOL103/HIOL109 (--/--/--)	60.96	20.74	8.81	7.03
HIOL116/HIOL103/HIOL109 (A1A1/C1C1/C2C2)	75.99*	7.92*	7.01*	6.5*
HIOL116/HIOL103/HIOL109 (A1A1/C1C1/--)	73.58*	9.68*	7.57*	6.6*
HIOL116/HIOL103/HIOL109 (A1A1/--/C2C2)	72.86*	10.18*	7.78*	6.53*
HIOL116/HIOL103/HIOL109 (--/C1C1/C2C2)	68.22*	14.26*	8.13	6.84
HIOL116/HIOL103/HIOL109 (--/--/--)	65.73**	16.6**	8.41	6.78
wild-type check	61.67	20.38	8.71	6.89

From Tables 5 and 6, it can be seen that ultra high oleic acid levels (of at least 80%) can be achieved for the A1/C1 combinations HIOL101/HIOL117, HIOL101/HIOL119, HIOL112/HIOL103, and HIOL113/HIOL103. There is no added value of the C2 mutant HIOL109 on top of these combinations at such high values of oleic acid. These results indicate that the H109Y (HIOL101), the P133L (HIOL117), S199L (HIOL119 and HIOL112), W101STOP (HIOL103) and G200R (HIOL113) are mutations that (almost) completely abolish the FAD2 function.

The ultra high oleic acid profile is however linked with a severe reduction in biomass (represented by VIG1 ratings; see Table 5).

The mutant combinations HIOL116/HIOL103/HIOL109, and HIOL101/HIOL120/HIOL109 display a high oleic acid profile of 75% without an agronomic penalty. However, for the HIOL116/HIOL103/HIOL109 combinations, it can also be seen that the wild type segregant population is already characterized by a higher oleic acid content, indicating another factor in these mutants which may contribute to the high levels of oleic acid. As HIOL103 (W101STOP) and HIOL109 (W190STOP) encode truncated proteins representing full knock-out FAD2 alleles (see also above) and HIOL101 (H109Y) also represents a full knock-out FAD2 allele (see above), these results indicate that the G232S mutation (HIOL116 and HIOL120) results in a partial or intermediate level of reduction of the FAD2 protein activity.

In summary, these results show that combination of a mutant FAD2 allele encoding a protein with an amino acid substitution at position G232, more particularly the G232S mutation on the BnFAD2-A1 or the BnFAD2-C1 gene, with a full knock-out FAD2 allele on the other FAD2 genes (on the BnFAD2-C1 or BnFAD2-A1 allele, and on the BnFAD2-C2 allele), results in plants with high levels of oleic acid without agronomic penalty. More specifically, combination of the G232S mutation with full knock-out mutations on the remaining FAD2 alleles can lead to levels of oleic acid of 75% without agronomic penalty.

Example 7 – Detection and/or transfer of mutant *FAD2* alleles into (elite) *Brassica* lines

The mutant *FAD2* genes are transferred into (elite) *Brassica* breeding lines by the following method: A plant containing a mutant *FAD2* gene (donor plant), is crossed with an (elite) *Brassica* line (elite parent / recurrent parent) or variety lacking the mutant *FAD2* gene. The following introgression scheme is used (the mutant *FAD2* allele is abbreviated to *fad2* while the wild type is depicted as *FAD2*):

BC1 cross: *fad2 / fad2* (donor plant) X *FAD2 / FAD2* (elite parent)

F1 plant: *FAD2 / fad2*

BC2 cross: *FAD2 / fad2* X *FAD2 / FAD2* (recurrent parent)

BC2 plants: 50% *FAD2 / fad2* and 50% *FAD2 / FAD2*

The 50% *FAD2 / fad2* are selected using molecular markers (e.g. AFLP, PCR, Invader™, TaqMan®, KASP assay, and the like; see also below) for the mutant *FAD2* allele (*fad2*).

BC3 cross: *FAD2 / fad2* (BC1 plant) X *FAD2 / FAD2* (recurrent parent)

BC3 plants: 50% *FAD2 / fad2* and 50% *FAD2 / FAD2*

The 50% *FAD2 / fad2* are selected using molecular markers for the mutant *FAD2* allele (*fad2*).

Backcrossing is repeated until BC4 to BC7.

BC4-7 plants: 50% *FAD2 / fad2* and 50% *FAD2 / FAD2*

The 50% *FAD2 / fad2* are selected using molecular markers for the mutant *FAD2* allele (*fad2*). To reduce the number of backcrossings (e.g. until BC4 instead of BC7), molecular markers can be used specific for the genetic background of the elite parent.

BC4-7 S1 cross: *FAD2 / fad2* X *FAD2 / fad2*

BC4-7 S1 plants: 25% *FAD2* / FAD2 and 50% *FAD2 / fad2* and 25% *fad2 / fad2*

Plants containing *fad2* are selected using molecular markers for the mutant *FAD2* allele (*fad2*). Individual BC4-7 S1 or BC4-7 S2 plants that are homozygous for the mutant *FAD2* allele (*fad2 / fad2*) are selected using molecular markers for the mutant and the wild-type *FAD2* alleles. These plants are then used for seed production.

To select for plants comprising a point mutation in a *FAD2* allele, direct sequencing by standard sequencing techniques known in the art can be used.

Alternatively, Invader™ technology (Third Wave Agbio) can be used to discriminate plants comprising a specific point mutation in an *FAD2* allele from plants not comprising that specific point mutation. Discriminating Invader™ probes are thus developed to detect the presence or absence and the zygosity status of mutant alleles identified in Example 3, based on the single nucleotide difference between the mutant and wildtype allele. Briefly, probes specific for the mutant or corresponding wild-type target *FAD2* gene and "invading" probes which can be used in combination with them are developed. Generally, each probe set consists of one probe specific for the mutant or the wild type target gene of which the first nucleotide after the "5' flap" sequence matches with the nucleotide difference (the so-called "primary probe") and one probe specific for the nucleotides upstream of the nucleotide difference (the so-called "invader® oligo"). The last nucleotide of the latter primer may match with the nucleotide difference in the mutant, but other nucleotides may be used as well for this last nucleotide as long as the primary probe and the invader® oligo are still able to form a single base overlap when hybridized to the target DNA to generate the specific invasive structure recognized by the Cleavase® enzymes (Third Wave Agbio). The Invader™ assay procedure and interpretation of the data are performed as prescribed by the manufacturer (Third Wave Agbio). Briefly, 5' "flap" nucleotide sequences (flap1 for the mutant allele and flap2 for the wild-type allele) are cleaved from the primary probes in the primary phase of the Invader™ assay and are complementary to sequences in FRET™ cassette 1 and 2, respectively, and not complementary to the target mutant or wild type sequences. If the primary probes are cleaved in the primary phase and the flap1-probe and/or flap2-probe hybridise to FRET™ cassette 1 and 2, respectively, in the secondary phase, a signal is generated indicative of the presence in the sample of the mutant or corresponding wild-type target *FAD2* gene, respectively.

Alternatively, KASP assays (KBioscience) can be used to discriminate plants comprising a specific point mutation in an *FAD2* allele from plants not comprising that specific point mutation. Discriminating primers were developed to detect the presence or absence and the zygosity status of mutant alleles identified in Example 3.
Briefly, forward primers specific for the mutant or corresponding wild-type target *FAD2* gene and a reverse primer that can be used in combination with them were developed.

The nucleotide at the 3' end of the forward primers corresponds to the nucleotide which differs between the mutant and the corresponding wild-type allele. The primers can be used in combination with fluorescent dyes, such as FAM and VIC according to the protocol as described by the manufacturer (KBioscience).

Primers to detect the presence or absence and the zygosity status of the mutant *FAD2* alleles are shown in Table 7.

Table 7: Forward (Fw) and reverse (Rv) primers to detect mutant *FAD2* alleles and the corresponding wild-type alleles. FAM probe: wild-type allele, VIC probe: mutant allele

Name		Primer	SEQ ID
HIOL101	Fw FAM	GAAGGTGACCAAGTTCATGCTGTAGTCGCTGAA GGCGTGGTG	28
	Fw VIC	GAAGGTCGGAGTCAACGGATTGTAGTCGCTGAA GGCGTGGTA	29
	Rv	TCTCTACTGGGCCTGCCAG	30
HIOL112	Fw FAM	GAAGGTGACCAAGTTCATGCTCCGTCGTAAGGT CTCCCCG	31
	Fw VIC	GAAGGTCGGAGTCAACGGATTGCCGTCGTAAGG TCTCCCCA	32
	Rv	CAGTTCACTCTCGGCTGGCCT	33
HIOL113	Fw FAM	GAAGGTGACCAAGTTCATGCTGTACTTAGCCTTC AACGTCTCGG	34
	Fw VIC	GAAGGTCGGAGTCAACGGATTGTACTTAGCCTT CAACGTCTCGA	35
	Rv	AAGCCGCCGTCGTAAGGTCTC	36
HIOL114	Fw FAM	GAAGGTGACCAAGTTCATGCTGCGAAGCCGCCG TCGTAAG	37
	Fw VIC	GAAGGTCGGAGTCAACGGATTAAGCGAAGCCGC CGTCGTAAA	38
	Rv	TTAGCCTTCAACGTCTCGGGG	39
HIOL116	Fw FAM	GAAGGTGACCAAGTTCATGCTCCAGATATACATC TCCGACGCTG	40
	Fw VIC	GAAGGTCGGAGTCAACGGATTCCAGATATACAT CTCCGACGCTA	41

	Rv	CGAGGCAACTCCTTGGAC	42
HIOL103	Fw FAM	GAAGGTGACCAAGTTCATGCTCGCACTCGTGGG CTATGACC	43
	Fw VIC	GAAGGTCGGAGTCAACGGATTCCGCACTCGTGG GCTATGACT	44
	Rv	TCTACTGGGCCTGCCAAGGG	45
HIOL117	Fw FAM	GAAGGTGACCAAGTTCATGCTGACTGTACTTCCA GGAGAAGTAAG	46
	Fw VIC	GAAGGTCGGAGTCAACGGATTATGACTGTACTT CCAGGAGAAGTAAA	47
	Rv	GGCTTGACGACACCGTCGGT	48
HIOL119	Fw FAM	GAAGGTGACCAAGTTCATGCTGTTGTACTTAGCC TTCAACGTCTC	49
	Fw VIC	GAAGGTCGGAGTCAACGGATTCGTTGTACTTAG CCTTCAACGTCTT	50
	Rv	AAACCATTGACAATCAGAAGCGGG	51
HIOL120	Fw FAM	GAAGGTGACCAAGTTCATGCTCCAGATATACATC TCCGACGCTG	52
	Fw VIC	GAAGGTCGGAGTCAACGGATTCCAGATATACAT CTCCGACGCTA	53
	Rv	TGCGCGGCGGCGTAACGGA	54
HIOL109	Fw FAM	GAAGGTGACCAAGTTCATGCTGTCCAGTTCACG CTCGGCTG	55
	Fw VIC	GAAGGTCGGAGTCAACGGATTCGTCCAGTTCAC GCTCGGCTA	56
	Rv	CCGTCGCTGTAAGGTCTTCCA	57

CLAIMS

1. A *Brassica* plant, or a cell, part, seed or progeny thereof, comprising at least one FAD2 gene, wherein at least one allele of said FAD2 gene is a partial knock-out *fad2* allele, said partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid,
wherein said plant does not contain a *FAD2* gene of which the *FAD2* alleles encode a wild type functional protein.
2. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to claim 1, comprising at least a second, or a second and a third, or a second, a third and a fourth FAD2 gene, wherein the *fad2* alleles of said FAD2 genes are full knock-out *fad2* alleles.
3. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to claim 2, wherein the full knock-out *fad2* allele of said second, or second and third, or second and third and fourth FAD2 gene is selected from the group consisting of:
    a) a *fad2* allele encoding a protein in which the His at a position corresponding to position 109 of SEQ ID NO: 6 is substituted with another amino acid,
    b) a *fad2* allele comprising a stop codon mutation in the codon encoding the Trp at a position corresponding to position 101 of SEQ ID NO: 9,
    c) a *fad2* allele comprising a deletion mutation of the nucleotides corresponding to nucleotides 2036-2042 of SEQ ID NO: 13, and
    d) a *fad2* allele comprising a stop codon mutation in the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15.
4. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to any one of the preceding claims, wherein the partial knock-out *fad2* allele is an allele of a FAD2-A1 or a FAD2-C1 gene.
5. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to claim 4, which is a *Brassica napus* plant, or a cell, part, seed or progeny thereof, and which comprises a second, a third and a fourth FAD2 gene, wherein

a) the partial knock-out *fad2* allele is an allele of a FAD2-A1 gene, and wherein the second FAD2 gene is a FAD2-C1 gene, the third FAD2 gene is a FAD2-C2 gene, and the fourth FAD2 gene is a FAD2-A2 gene, or

b) the partial knock-out *fad2* allele is an allele of a FAD2-C1 gene, and wherein the second FAD2 gene is a FAD2-A1 gene, the third FAD2 gene is a FAD2-C2 gene, and the fourth FAD2 gene is a FAD2-A2 gene.

6. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to any one of the preceding claims, wherein said partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp.

7. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to claim 5 or 6, which is a *Brassica napus* plant, or a cell, part, seed or progeny thereof, wherein

a) a first FAD2 gene is a FAD2-C1 gene of which at least one allele is said partial knock-out *fad2* allele which encodes a FAD2 protein of which the Gly at a position corresponding to position 232 of SEQ ID NO: 9 is substituted with Ser or with Asp;

b) said second FAD2 gene is a FAD2-A1 gene of which at least one allele encodes a FAD2 protein of which the His at a position corresponding to position 109 of SEQ ID NO: 6 is substituted with Tyr; and

c) said third FAD2 gene is a FAD2-C2 gene of which at least one allele contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15.

8. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to claim 7, which is derivable or obtainable from seeds selected from the group consisting of:

a) seed comprising HIOL101 having been deposited at NCIMB under accession number NCIMB 42376,

b) seed comprising HIOL120 having been deposited at NCIMB under accession number NCIMB 42377, and

c) seed comprising HIOL109 having been deposited at NCIMB under accession number NCIMB 42375.

9. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to claim 5 or 6, wherein

a) a first FAD2 gene is a FAD2-A1 gene of which at least one allele is said partial knock-out *fad2* allele which encodes a FAD2 protein of which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp;

b) said second FAD2 gene is a FAD2-C1 gene of which at least one allele contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 101 of SEQ ID NO: 9; and

c) said third FAD2 gene is a FAD2-C2 gene of which at least one allele contains a stop codon mutation at the codon encoding the Trp at a position corresponding to position 190 of SEQ ID NO: 15.

10. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to claim 9, which is derivable or obtainable from seeds selected from the group consisting of:

a) seed comprising HIOL116 having been deposited at NCIMB under accession number NCIMB 42373,

b) seed comprising HIOL103 having been deposited at NCIMB under accession number NCIMB 42374, and

c) seed comprising HIOL109 having been deposited at NCIMB under accession number NCIMB 42375.

11. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to any one of claims 1-10 which is homozygous for any one of the knock-out *fad2* alleles.

12. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to any one of claims 1-11, which has increased levels of C18:1 in the seed oil and which maintains normal agronomic development.

13. The *Brassica* plant, or a cell, part, seed or progeny thereof, according to claim 12, which has a level of C18:1 in the seed oil of about 75%.

14. Oil from the seeds according to any one of claims 1-13.

15. A method for increasing the levels of C18:1 in seed oil while maintaining normal agronomic development, said method comprising introducing a partial knock-out *fad2* allele of a *FAD2* gene, said partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, and, optionally, introducing one or more full knock-out *fad2* alleles of one or more FAD2 genes into a *Brassica* plant, and selecting a *Brassica* plant comprising said partial knock-out *fad2* allele which does

not contain another *FAD2* gene of which the *FAD2* alleles encode a wild-type functional FAD2 protein.

16. The method according to claim 15, wherein the *Brassica* plant produced by said method comprises at least a second, or a second and a third, or a second, a third and a fourth FAD2 gene, wherein the *fad2* alleles of said FAD2 genes are full knock-out *fad2* alleles.

17. The method according to claim 15 or 16, wherein said *Brassica* plant comprising said partial knock-out *fad2* allele which does not contain another *FAD2* gene of which the *FAD2* alleles encode a wild-type functional FAD2 protein is selected by analyzing genomic DNA from said plant for the presence of at least one molecular marker, wherein said at least one molecular marker is linked to said partial knock-out *fad2* allele and, optionally, to one or more full knock-out *fad2* alleles.

18. A method to determine the presence or absence of a knock-out *fad2* allele in a biological sample, comprising providing genomic DNA from said biological sample, and analyzing said DNA for the presence of at least one molecular marker, wherein the at least one molecular marker is linked to said knock-out *fad2* allele.

19. A kit for the detection of a knock-out *fad2* allele in *Brassica* DNA samples, wherein said kit comprises one or more PCR primer pairs, which are able to amplify a DNA marker linked to said knock-out *fad2* allele.

20. A method for determining the zygosity status of a knock-out *fad2* allele in a plant, or a cell, part, seed or progeny thereof, comprising determining the presence of a knock-out and/or a corresponding wild type *FAD2* specific region in the genomic DNA of said plant, or a cell, part, seed or progeny thereof.

21. A method for transferring at least one partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, from one plant to another plant comprising the steps of:

(a) identifying a first plant comprising at least one partial knock-out *fad2* allele,

(b) crossing the first plant with a second plant not comprising the at least one partial knock-out *fad2* allele and collecting F1 hybrid seeds from the cross,

(c) optionally, identifying F1 plants comprising the at least one partial knock-out *fad2* allele,

(d) backcrossing F1 plants comprising the at least one partial knock-out *fad2* allele with the second plant not comprising the at least one partial knock-out *fad2* allele for at least one generation (x) and collecting BCx seeds from the crosses, and

(e) identifying in every generation BCx plants comprising the at least one partial knock-out *fad2* allele by analyzing genomic DNA of said BCx plants for the presence of at least one molecular marker, wherein the at least one molecular marker is linked to said partial knock-out *fad2* allele.

22. A method for combining at least one partial knock-out *fad2* allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid, with at least one full knock-out *fad2* allele in a single *Brassica* plant, said method comprising

a) generating and/or identifying two or more plants each comprising one or more selected partial and/or full knock-out *fad2* alleles;

b) crossing a first plant comprising one or more selected partial and/or full knockout *fad2* alleles with a second plant comprising one or more other selected partial and/or full knockout *fad2* alleles;

c) collecting seeds from the cross, and, optionally, identifying a plant comprising at least one partial knock-out *fad2* allele and at least one full knockout *fad2* allele; and, optionally

d) repeat steps b) and c) until a plant comprising at least one partial knock-out *fad2* allele and at least one full knockout *fad2* allele, is obtained, wherein said plant does not contain a *FAD2* gene of which the *FAD2* alleles encode a wild-type functional protein.

23. A partial knock-out *fad2* allele of a *FAD2* gene, wherein the partial knock-out *fad2* allele is a mutated version of the native *FAD2* gene, wherein the native *FAD2* gene is selected from the group consisting of:

(a) a nucleic acid molecule which comprises at least 90% sequence identity to SEQ ID No. 4 or 7;

(b) a nucleic acid molecule comprising a coding sequence which comprises at least 90% sequence identity to SEQ ID No. 5 or 8; and

(c) a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID No. 6 or 9, and

wherein said partial knock-out *fad2* allele encodes a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid.

24. The partial knock-out *fad2* allele of claim 23, which encodes a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with Ser or with Asp.

25. Method for producing oil, comprising harvesting seeds from the plants according to any one of claims 1-13 and extracting the oil from said seeds.

26. A method of producing food, feed, or an industrial product comprising

(a) obtaining the plant or a part thereof, of any one of claims 1-13; and

(b) preparing the food, feed or industrial product from the plant or part thereof

27. The method of claim 26 wherein

(a) the food or feed is oil, meal, grain, starch, flour or protein; or

(b) the industrial product is biofuel, fiber, industrial chemicals, a pharmaceutical or a nutraceutical.

28. Use of the partial knock-out *fad2* allele of claim 23 or 24 to increase the level of C18:1 in the seed oil of a *Brassica* plant while maintaining normal agronomic development.

29. A method to produce a *Brassica* plant comprising an increased level of C18:1 in the seed oil and which maintains normal agronomic development, said method comprising sowing seeds according to any one of claims 1-13 and growing plants from said seeds.

```
SEQ ID NO: 3          1 mgaggrmpvptsskksetdttkrvpcekppfsvgdlkkaipphcfkrsip
SEQ ID NO: 6          1 mgaggrmqvsppskksetdnikrvpcetppftvgelkkaipphcfkrsip
SEQ ID NO: 9          1 mgaggrmqvsppskksetdtikrvpcetppftvgelkkaipphcfkrsip
SEQ ID NO: 12         1 mgaggrmqvsppssspgtntlkrvpcetppftlgdlkkaipphcfkrsip
SEQ ID NO: 15         1 mgaggrmqisppssspetktlkrvpcetppftlgdlkkaipphcfkrsip
SEQ ID NO: 18         1 mgaggrmqvsppskksetdtikrvpcetppftvgelkkaipphcfkrsip
SEQ ID NO: 21         1 mgaggrmqvsppssspgtntlkrvpcetppftlgdlkkaipphcfkrsip
SEQ ID NO: 24         1 mgaggrmqvsppskksetdtikrvpcetppftvgelkkaipphcfkrsip
SEQ ID NO: 27         1 mgaggrmqvsppssspetktlkrvpcetppftlgdlkkaipphcfkrsip

                                TM-1
SEQ ID NO: 3         51 rsfsylisdiiiascfyyvatnyfsllpqplsylawplywacqqgcvltgi
SEQ ID NO: 6         51 rsfsyliwdiiiascfyyvattyfpllphplsyfawplywacqqgcvltgv
SEQ ID NO: 9         51 rsfsyliwdiiiascfyyvattyfpllphplsyfawplywacqqgcvltgv
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SEQ ID NO: 15        51 rsfsyllfdilvssslyhlstayfpllphplpylawplywacqqgcvltgl
SEQ ID NO: 18        51 rsfsyliwdiiiascfyyvattyfpllphplsyfawplywacqqgcvltgv
SEQ ID NO: 21        51 rsfs--sstssspprllplpplhsllpsplprlt-pl------------
SEQ ID NO: 24        51 rsfsyliwdiiiascfyyvattyfpllphplsyfawplywacqqgcvltgv
SEQ ID NO: 27        51 rsfsyllfdilvssslyhlstayfpllphplpylawplywacqqgcvltgl

                          HIS-1                TM-2                HIS-2
SEQ ID NO: 3        101 wviahecghhafsdyqwlddtvglifhsfllvpyfswkyshrrhhsntgs
SEQ ID NO: 6        101 wviahecgHhafsdyqwlddtvglifhsfllvpyfswkyshrrhhsntgs
SEQ ID NO: 9        101 Wviahecghhafsdyqwlddtvglifhsfllvpyfswkyshrrhhsntgs
SEQ ID NO: 12        85 ----------------lgl-----------------------------
SEQ ID NO: 15       101 wviahecghhafsdhqwlddavglvfhsfllvpyfswkyshrrhhsntgs
SEQ ID NO: 18       101 wviahecghhafsdyqwlddtvglifhsfllvpyfswkyshrrhhsntgs
SEQ ID NO: 21        85 ----------------lgl-----------------------------
SEQ ID NO: 24       101 wviahecghhafsdyqwlddtvglifhsfllvpyfswkyshrrhhsntgs
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                                                        TM-3
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SEQ ID NO: 15       151 lerdevfvpkkksdikwygkylnnplg---rtvmltvqftlgWplylafn
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SEQ ID NO: 21        88 --------prlrp---------ngplghsprvrpprlq------------
SEQ ID NO: 24       151 lerdevfvpkkksdikwygkylnnplg---rtvmltvqftlgwplylafn
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                    TM-3                                TM-4
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SEQ ID NO: 12       109 ---rp--------------pvagrrrrprl-------------------
SEQ ID NO: 15       198 vsgrpysdg-fachfhpnapiyndrerlqiyisdagvlsvcyglyryags
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SEQ ID NO: 21       109 ---rp--------------pvagrrrrprl-------------------
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```

Figure 1

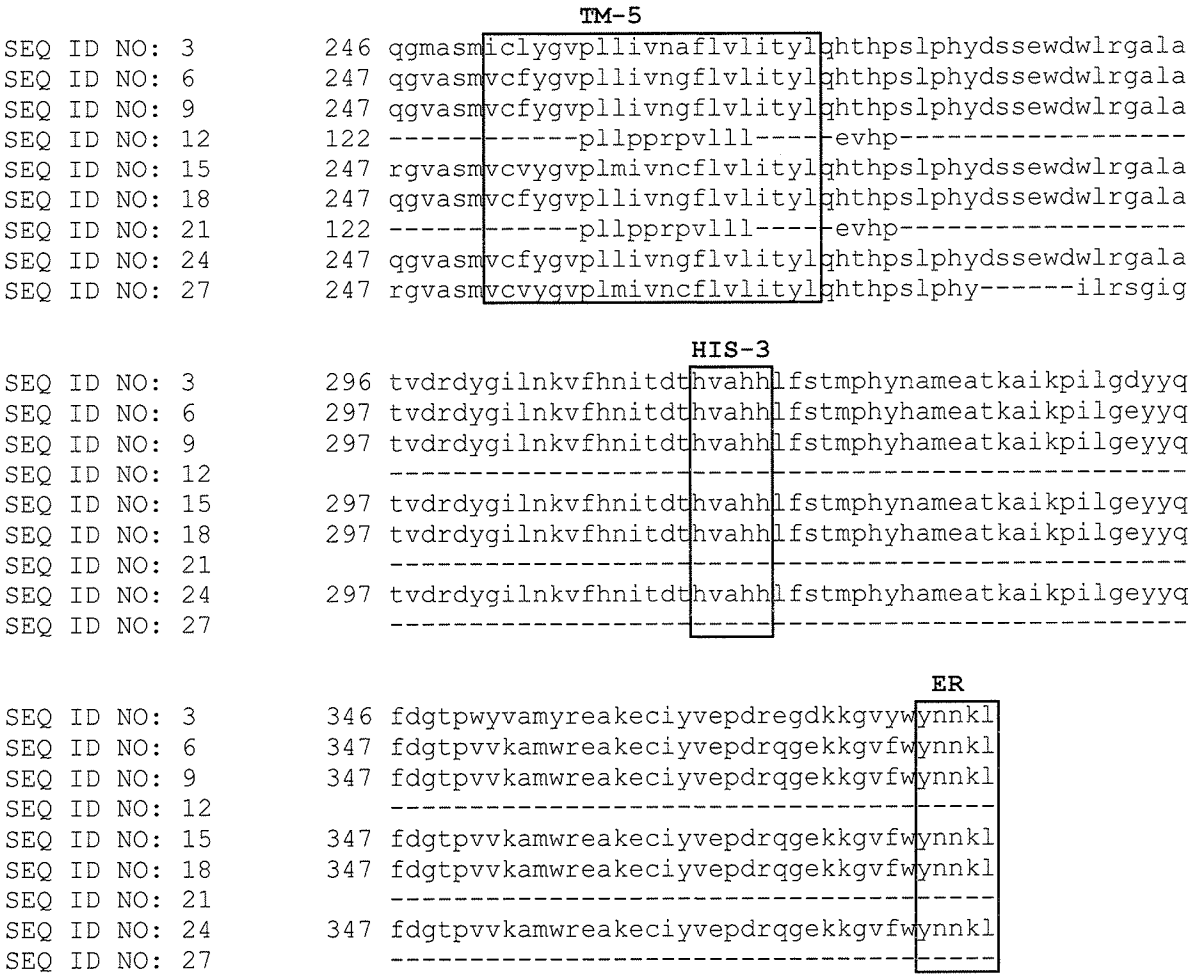

Figure 1, continued

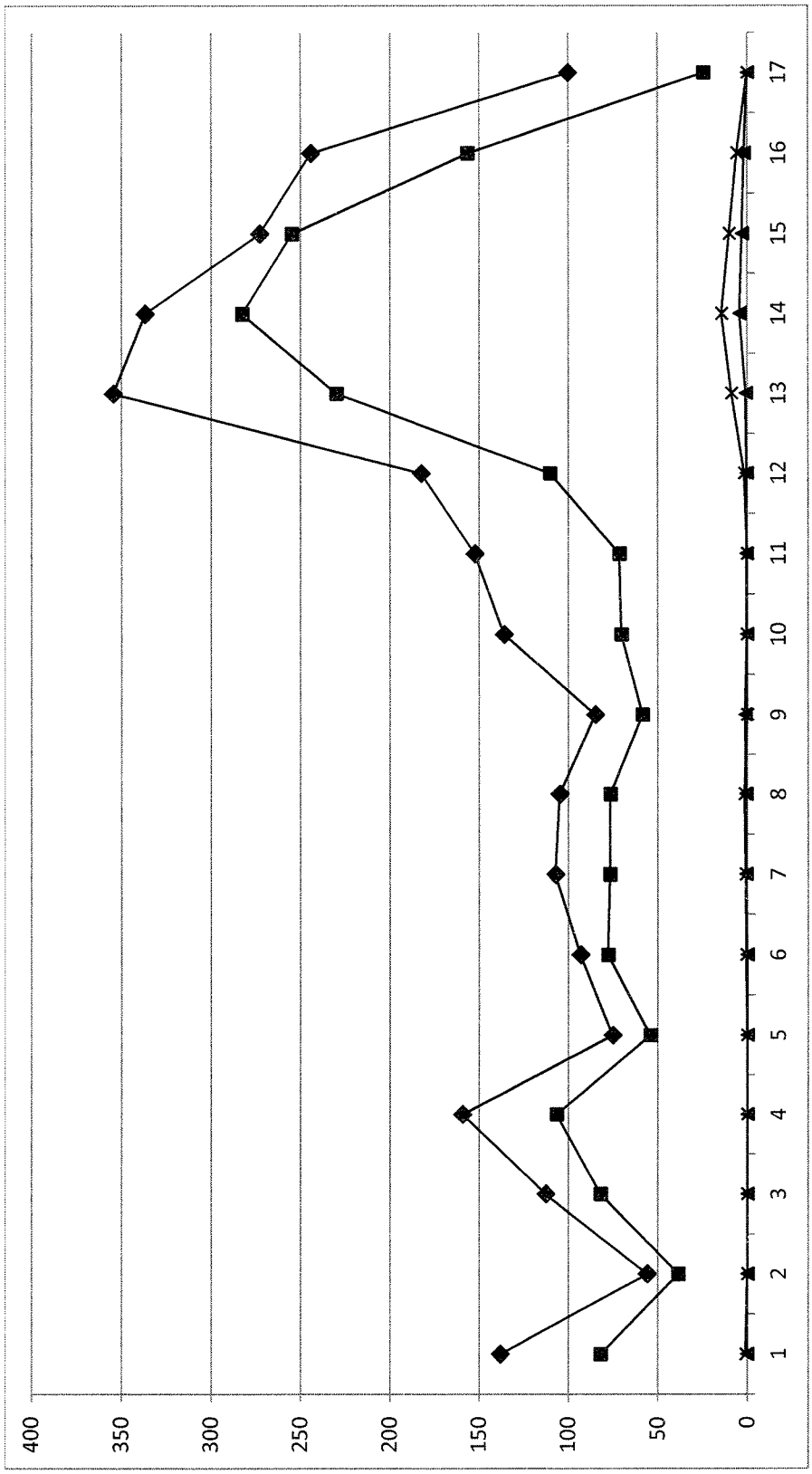

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/29828

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/29828

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-14, 25-27, 29
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: claims 1-5, 15-17, 21-24, 28, directed to a Brassica plan comprising at least one partial knock-out fad2 allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid (claims 1-5); a method comprising introducing into a plant said partial knock-out fad2 allele encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO:6 is substituted with another amino acid (claims 15-17, 21, 22); a nucleic acid comrpising a knock-out fad2 allele of a FAD2 gene encoding a FAD2 protein in which the Gly at a position corresponding to position 232 of SEQ ID NO: 6 is substituted with another amino acid (claims 23 ,24, 28).

********** See Supplemental Sheet to continue **********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5, 15-17, 21-24, 28

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/29828

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01H 5/00, A01H 1/00, A23K 1/00 (2016.01)
CPC - A01H 1/04, C11C 1/002, A01H 5/10
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)
IPC(8) - A01H 5/00, A01H 1/00, A23K 1/00 (2016.01)
CPC - A01H 1/04, C11C 1/002, A01H 5/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 800/306, 426/615, 800/264

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); PatBase, Google/Scholar: partial loss-of-function mutation, rapeseed, Brassica napus, rape, oilseed rape, rapa, rappi, FAD2, oleate desaturase, B. napus delta-12 fatty acid desaturase 2 D form, BnFAD2-AI, BnFAD2-CI, defective FAD2 genes deactivation, partial loss of function, knockout, knockdown, high oleic acid.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 7,109,392 B1 (Broglie, et al.) 19 September 2006 (19.09.2006) SEQ ID NO 2, 99.5% to SEQ ID NO:6	1-5, 15-17, 21-24, 28
A	WO 2014/039872 A1 (Dow Agrosciences LLC) 13 March 2014 (13.03.2014) SEQ ID NO 1, nucleotides 44349-40633, 94.2% identity to SEQ ID NO: 4; SEQ ID NO 4, nucleotides 7153-8953, 83.2% identity to SEQ ID NO:8	23, 24, 28
A	US 2002/0092038 A1 (Kodali, et al.) 11 July 2002 (11.07.2002) para [0018], SEQ ID NO:13, SEQ ID NO:14, 100% identity to SEQ ID NO:9	23, 24, 28

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"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
"X" 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
"Y" 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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 August 2016 (24.08.2016)	15 SEP 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/29828

In Continuation of Box III. Observations where unity of invention is lacking:

Group II: claims 18-20, directed to a method comprising determining the presence of a knock-out fad2 allele in a plant (claims 18, 20); a kit for the detection of a knock-out fad2 allele in Brassica DNA samples, wherein said kit comprises one or more PCR primer pairs, which are able to amplify a DNA marker linked to said knock-out fad2 allele (claim 19).

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features
The inventions of Group II do not include the shared or common technical feature of a FAD2 protein from Brassica napa having the Gly at a position corresponding to position 232 of SEQ ID NO: 6 substituted with another amino acid, as required by Group I.

The inventions of Group I do not include the shared or common technical feature of a method comprising analyzing genomic DNA for the presence of a molecular marker linked to a knock-out fad2 allele, or a kit for the detection of a knock-out fad2 allele in Brassica DNA samples, as required by Group II.

Common Technical Features
The inventions of Groups I a II share the technical feature of a Brassica plant comprising a knock-out fad2 allele. Some inventions of Group I and some inventions of Group II share the technical feature of a method comprising selecting a Brassica plant comprising said partial knock-outfad2 allele which does not contain another FAD2 gene of which the FAD2 alleles encode a wild-type functional FAD2 protein. However, these shared technical features do not represent a contribution over prior art as being anticipated by US 2014/0090100 A1 to Dow Agrosciences LLC (hereinafter "Dow").

Dow discloses a Brassica plant comprising a knock-out fad2 allele (para [0159], "Once genes are obtained/identified, mutant FAD genes are transferred into B. juncea plants..."; para [0160], "Down regulation of FAD2B and FAD3B enzymes is accomplished by deletion, insertion mutagenesis in the coding regions or regulatory domains within the native sequences").

Dow also discloses a method comprising selecting a Brassica plant comprising said partial knock-out fad2 allele (para [0152]-[0155], "A) Molecular Markers... Three different methods are used for the determination of B genome in the self-pollinated and DH progeny from B. napus and B. juncea interspecific crosses exhibiting desired seed oil profile... B) Fluorescence In Situ Hybridization... [0154] ... (FISH) technique is used to determine the presence and enrichment of B genome in the progeny. The progeny plants are used both for marker analysis... C) ... [0155] Genome In Situ Hybridization (GISH) technique is used where chromosomes of the candidate progeny plants can be probed with total nuclear BB genome DNA using total B. juncea DNA as the competing unlabeled probe. A strong hybridization to BB genome chromosomes in B. juncea indicates the presence of BB genome in the progeny"). As said technical features were known in the art at the time of the invention, this cannot be considered special technical feature that would otherwise unify the groups.

Groups I and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.