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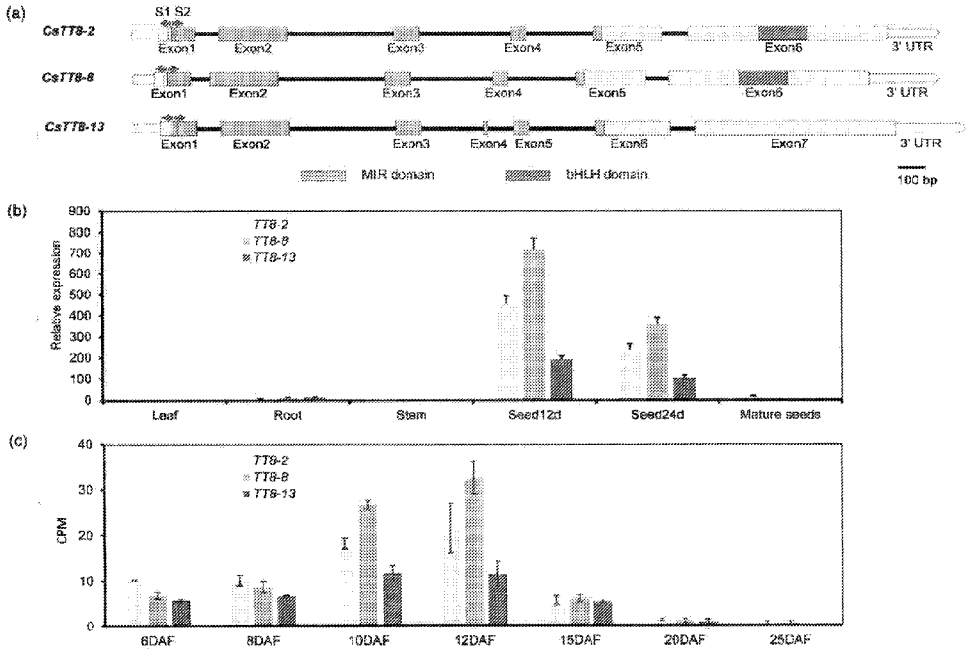
(54) Title: YELLOW-SEED CAMELINA SATIVA WITH ENHANCED OIL ACCUMULATION BY DISRUPTION OF TRANSPARENT TESTA 8

FIG. 1



(57) Abstract: The invention provides Brassicaceae family plants, seeds and germplasm altered in all *Transparent Testa 8* (*TT8*) transcription factor encoding genes to provide a yellow seed variety having increased seed oil content, particularly Camelina variety, particularly *Camelina sativa*. Methods for altering *Transparent Testa 8* (*TT8*) transcription factor encoding genes and generating seeds, plants and germplasm lacking; active *TT8* transcription factor, including nsing CRISPR/Cas9 technology to disrupt all copies of the TT8 genes, are provided. Plants inactivated for *TT8* and seeds, germplasm, and oil bodies derived, therefrom are provided. Three Camelina sativa *TT8* genes were identified and independent *CsTT8* lines containing framesbift edits are provided. Disruption of *TT8* resulted in seed coat color change to yellow, reflecting reduced flavonoid accumulation up to 44% and loss of a well- organized seed coat mucilage layer. Metabolic remodeling with increases in total fatty acid accumulation up to 38.0% of seed weight is provided, and TAG yield is

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[Continued on next page]

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increased by more than 21 %, without significant changes in starch or protein levels.

YELLOW-SEED *Camelina sativa* WITH ENHANCED OIL ACCUMULATION BY DISRUPTION OF *Transparent Testa 8*

GOVERNMENT SUPPORT

[0001] This invention was made with Government support under contract number DE-SC0012704, awarded by the U.S. Department of Energy. The Government has certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] The present application claims priority from U.S. provisional application Serial No. 63/575,496 filed April 5, 2024, the disclosure of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0003] A Sequence Listing conforming to the rules of WIPO Standard ST.26 is hereby incorporated by reference. The Sequence Listing has been filed as an electronic document via PatentCenter encoded as XML in UTF-8 text. The electronic document, created on April 4, 2025, is entitled "369-321_ST26.xml", and is 121,705 bytes in size.

FIELD OF THE INVENTION

[0004] The present invention relates generally to *Brassicaceae* family plants, seeds and germplasm altered in one or all *Transparent Testa 8* (*TT8* hereafter) transcription factor encoding genes resulting in increased seed oil content and to provide a yellow seed variety. Methods for altering *TT8* transcription factor encoding genes and generating seeds, plants and germplasm lacking active TT8 transcription factor are provided. The invention also relates to plants, particularly genetically edited or genetically engineered plants, inactivated for TT8, as well as seeds, germplasm, and oil bodies derived from the plants. A *Brassicaceae* family *Camelina* variety, particularly *Camelina sativa*, having a seed with increased oil content and yellow phenotype is provided.

BACKGROUND OF THE INVENTION

[0001] Liquid biofuels offer considerable promise, however, the reality of utilizing biological material is tempered by competing uses and the quantities available. Oilseed crops are a diverse group of crop species which are utilized for the production of oil that can be used for diverse industries including food, feed, consumer products, and biofuels. Plant oils (triacylglycerol or TAG) are increasingly preferred as sustainable feedstocks for biofuel production because they have a high energy density and they are compatible with the current energy infrastructure (Wang et al., 2022). Oil seed crops

include soybeans, rapeseed, canola, pennycress, sunflowers and sesame. Rapeseed, canola and pennycress are members of the *Brassicaceae* family.

[0002] *Camelina*, a hexaploid member of the *Brassicaceae* family, is an emerging oilseed crop being developed to meet the increasing demand for plant oils as feedstocks for biofuels, feed, and food. *Camelina sativa (Cs)*, an allohexaploid oil crop in the *Brassicaceae* family, has garnered attention for its relatively high oil yield, short generation time, stress resistance, and low resource requirements (Yuan and Li, 2020). Increasing seed oil content is one of the primary targets for improving *camelina* productivity (Marisol Berti, 2016).

[0003] In *Brassicaceae*, dark brown seed coats result from the accumulation of an oxidized flavonoid known as proanthocyanidin within the endothelium layer of the inner integument of the seed coat (Lepiniec et al. 2006). Flavonoid accumulation has been extensively studied in the model plant *Arabidopsis*, revealing two distinct groups of activities. The first group consists of early biosynthetic genes (EBGs), including chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase, and flavanone-3'-hydroxylase. The second group comprises late biosynthetic genes (LBGs), such as dihydroflavonol reductase (DFR), leucocyanidin dioxygenase, and anthocyanidin reductase (ANR or BAN) (Hartmann et al., 2020). In addition to these enzymes, various regulatory proteins play pivotal roles in flavanol cell biology, including TT1, TT2, TT8, TT16, TTG1, TTG2, TT12, TT19, and AHA10, which are likely involved in the compartmentation of flavonoids (Lepiniec et al., 2006). The regulation of gene expression, particularly that of LBGs such as DFR and BAN, is tightly coordinated by a ternary complex of TT2 (R2R3-MYB), TT8 (basic helix–loop–helix, bHLH), and the WD40 regulatory protein encoded by TTG1, referred to as the MYB-bHLH-WD40 (MBW) complex which ultimately regulates the biosynthesis of proanthocyanidins (Baudry et al., 2004; Hichri et al., 2011). The MBW complex activates proanthocyanidin biosynthesis in developing seeds, influencing seed color (Hartmann et al., 2020).

[0004] In other *Brassicas*, high oil content can be associated with a yellow seed phenotype. *Camelina* with yellow seeds are not known. That natural yellow seed variants have not been identified in camelina may be due to the low probability of null mutants arising in all three copies of genes controlling seed color simultaneously.

[0005] The *TT8* gene, known as *Transparent Testa 8*, is a transcriptional repressor that plays a pivotal role in metabolism, specifically in the regulation of proanthocyanin biosynthesis (Baudry et al., 2004). Mutations or variations in *TT8* led to alterations in anthocyanin production, affecting the coloration of plant tissues (Chen et al., 2014; Padmaja et al., 2014). Moreover, TT8 (bHLH42) plays negative roles in regulating seed fatty acid biosynthesis by repressing LEC1, LEC2, and FUS3 (Chen et al., 2014). Knocking out *TT8* not only changes seed coat color but also increases oil content in *Arabidopsis* (Chen et al., 2014) and *Brassica napus* (Li et al., 2023; Zhai et al., 2020).

[0006] Genome editing, particularly by CRISPR/Cas9 technology, has become a useful method for understanding and manipulating gene function (Doudna and Charpentier, 2014). This technology has been

successfully applied to camelina, where knocking out the Fatty Acid Desaturase 2 (*FAD2*) genes increased oleic acid content while reducing the levels of long-chain polyunsaturated fatty acids (Han et al., 2022; Jiang et al., 2017; Lee et al., 2021; Morineau et al., 2017). Deactivating Fatty Acid Elongase1 (*FAE1*) resulted in reduced production of very long-chain fatty acids and increased levels of oleic acid or α-linolenic acid (Ozseyhan et al., 2018). Editing seed storage protein CRUCIFERIN C (CsCRUC) did not affect the total seed protein content but altered the abundance of cruciferin isoforms and other seed storage proteins (Lyzenga et al., 2019). The multiplex editing of genes in camelina enabled the creation of a stable early-flowering trait (Bellec et al., 2022).

[0007] Yellow-seed is a highly desirable trait in *Brassica* oilseed crops (Marles and Gruber, 2004; Meng et al., 1998; Rahman and McVetty, 2011; Tang et al., 1997) because it is often associated with higher oil content compared to dark-seed varieties. In addition, yellow seeds typically have lower pigment content and reduced hull mass. Because the oil-rich interior is a higher proportion of the overall seed mass, oil extraction from yellow-seed varieties is more cost-effective. The reduced pigment also improves oil quality (Meng et al., 1998). *Camelina* has no naturally occurring yellow-seed variants and it is unknown whether their oil contents could be altered or increased by manipulation of genes or altering production of proteins, such as by direct disruption of one or more regulatory proteins in flavanol cell biology. There is a need in the art for *Brassica* oilseed crops, including *Camelina* with higher oil content.

[0008] The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

[0009] Flowering plants efficiently store energy in their seeds through the accumulation of oil, namely triacylglycerol (TAG hereafter), and store it in discreet oil bodies by embedding a phospholipid protein monolayer around the oil body. These seed crops have been used in a variety of agricultural applications as feed and more recently also as a feedstock source for biofuels. On a per weight basis, lipids have approximately double the energy content of either proteins or carbohydrates and substantial focus has been placed on raising the oil content of various plant species. Unfortunately, plant seeds represent a very small percentage of total plant biomass and with the demand for improved agricultural productivity and alternative energies, current oil production from a number of devoted seed crops is insufficient. It is a general object of the invention to provide methods for increasing the level of oil production in plant tissues/organs and/or methods for increasing the production of oil from plants.

[00010] The present invention relates generally to increasing the oil content in a plant seed, the method comprising abolishing the expression or activity of TT8 in said plant. The present invention relates to increasing seed yield and increasing the oil content in a plant seed, the method comprising abolishing the expression or activity of TT8 in said plant. In some embodiments, plants of the *Brassicacea* family are altered at one or more TT8 genes to provide yellow seeds with increased oil

content. In particular embodiments, plants of the *Brassicacea* family are altered at all TT8 genes to provide yellow seeds with increased oil content. In embodiments, seed yield is increased. In an embodiment, all TT8 encoding genes are disrupted. In one embodiment, all TT8 encoding genes are disrupted by gene editing. In an embodiment, gene editing utilizes a CRISPR/Cas system.

[00011] In an embodiment of the invention, fatty acid accumulation and TAG yield are increased and flavonoid accumulation is reduced. In an embodiment, TAG yield is greater than 20%. In an embodiment, TAG yield is about 20% to 40% greater than in wild type or parental plants or species.

[00012] In an embodiment, the seeds are yellow in color and the seeds of wild type or parental plants are not yellow.

[00013] In an embodiment, there are no significant changes in starch or protein levels in the seeds compared to wild type or parental plants. In an embodiment, the seed yield is increased. In an embodiment, the seed germination rate is increased.

[00014] In another embodiment, FAD2 is increased and wherein FAD3 expression is decreased. In one embodiment, lipid types are altered, particularly wherein there is an accumulation or increase of oleic acid (18:2) and a decrease in linolenic acid (18:3). In a further embodiment, oleic acid (18:1) is increased.

[00015] In embodiments of the invention, the plant is a *Brassicacea* family plant and is of the genus selected from *Camelina*, *Thlapsi* (pennycress), *Capsella*, *Cardamine* (bittercress), *Eutrema* and *Arabis*. In some embodiments, the plant is *Camelina* genus and is *Camelina sativa L. (Cs)*.

[00016] In embodiments, the present invention relates to the use of gene editing or gene mutation technology, to disrupt one or more or all genome copies of the *Transparent Testa 8* gene to create a seed phenotype associated with increased seed oil yield in a plant that is a member of the *Brassicacea* family. In embodiments, the present invention relates to the use of CRISPR technology, in an embodiment CRISPR/Cas9 technology, to disrupt one or more or all all genome copies of the *Transparent Testa 8* gene to create a yellow seed phenotype associated with increased seed oil yield in a plant that is a member of the *Brassicacea* family. In embodiments, the plant is a *Brassicacea* family plant and is of the genus selected from *Camelina*, *Thlapsi* (pennycress), *Capsella*, *Cardamine* (bittercress), *Eutrema* and *Arabis*. In an embodiment, the plant is *Camelina* genus and is *Camelina sativa L. (Cs)*. In an embodiment, the plant is *Brassica* genus and is *Brassica napus* or *Brassica carinata*.

[00017] The present invention provides methods relating to the use of CRISPR/Cas9 technology to disrupt one or more *Transparent Testa 8* (*TT8*) transcription factor genes (*CsTT8-2*, *CsTT8-8* and *CsTT8-13*) in *Camelina sativa L.* (*Cs*) to enhance total fatty acid accumulation in the seeds. In an embodiment, the present invention provides methods relating to the use of CRISPR/Cas9 technology to disrupt all *Transparent Testa 8* (*TT8*) transcription factor genes (*CsTT8-2*, *CsTT8-8* and *CsTT8-13*) in *Camelina sativa L.* (*Cs*) to enhance total fatty acid accumulation in the seeds.

[00018] The present discovery relates to a yellow seed, high-oil phenotype Suneson variety of *Camelina sativa* germplasm that can be used directly for oil production. It can also be used as a variety into which breeders can introduce certain other traits. Such traits might include but are not limited to stress resistance, desiccation or heat resistance, fatty acid modification, other yield enhancements, and disease or insect resistance. Alternatively, the TT8 mutated *Camelina sativa* line(s) can be used to breed other commercial *Camelina* varieties to obtain new TT8 deficient commercial camelina varieties.

[00019] The present invention provides a method directed to the use of CRISPR/Cas9 technology to disrupt one or more *TT8* transcription factor genes in a *Brassicacea* family plant to enhance total fatty acid accumulation in the seeds. The present invention provides a method directed to the use of CRISPR/Cas9 technology to disrupt one or more *TT8* transcription factor genes in a *Brassicacea* family plant of the genus selected from *Camelina*, *Thlapsi* (pennycress), *Brassica* (canola, rapeseed), *Capsella, Cardamine* (bittercress), *Eutrema* and *Arabis* to enhance total fatty acid accumulation in the seeds. The present invention provides a method directed to the use of CRISPR/Cas9 technology to disrupt one or more *TT8* transcription factor genes in a *Brassicacea* family plant of the genus selected from *Camelina*, *Thlapsi* (pennycress), *Capsella, Cardamine* (bittercress), *Eutrema* and *Arabis* to enhance total fatty acid accumulation in the seeds. The present invention provides a method directed to the use of CRISPR/Cas9 technology to disrupt one or more *TT8* transcription factor encoding genes in *Camelina sativa L.* (*Cs*) to enhance total fatty acid accumulation in the seeds. The present invention provides a method directed to the use of CRISPR/Cas9 technology to disrupt one or all of the *TT8* transcription factor encoding genes in *Camelina sativa L. (Cs)* (denoted herein as *CsTT8-2, CsTT8-8* and *CsTT8-13*) to enhance total fatty acid accumulation in the seeds. Nucleotide primers have been designed against each of the three *TT8* genes and used CRISPR cas9 to introduce mutations at certain target sites. Plants with these mutations were taken to homogeneity for all three genes and the oil yield and other parameters were tested.

[00020] In other aspects of the invention, the TT8 genes can be targeted for disruption by any of various methods or approaches known in the art to generate null mutants, premature termination, inactive TT8 proteins. In embodiments, one or more or all TT8 genes are targeted in coding or non-coding region(s) of the gene(s). In one embodiment, targeting in the non-coding region can result in failure to transcribe or translate any TT8. In some embodiments, the coding region is target. In an embodiment, the first exon is targeted for disruption or null mutation or premature termination. In an embodiment, each and all of the TT8 encoding genes are targeted, particularly wherein there are at least two distinct TT8 genes. In one embodiment three TT8 genes are targeted. In an embodiment, three TT8 genes are identified and targeted in the *Camelina* genus, in one such embodiment the particular TT8 encoding genes (*CsTT8-2, CsTT8-8* and *CsTT8-13*) in *Camelina sativa*. Different nucleotide sequences can be targeted within the *TT8* target genes. Disruption, selective deletion, or mutation, such as mutation to generate premature termination and/or inactive TT8 polypeptide, can be achieved by any of various alternative methods known in the art. Disruption by CRISPR technology is one such method. Disruption or mutation

by gene knockout approaches is contemplated. Disruption or mutation by knock in approaches or introduction of heterogenous sequence to generate an altered protein that is inactive is contemplated. In one embodiment, these can be achieved via homologous recombination facilitated via overlapping sequence in the TT8 gene sequences. In other embodiments, untargeted mutagenesis such as ethyl methanesulfonate, or radiation to introduce mutations can be utilized, with visual selection of yellow seed phenotype seeds in the progeny

[00021] In some embodiments, the altered TT8 genes may be expressed in other plants or species. In one embodiment, they are expressed in *N. Benthamiana*.

[00022] The present discovery involves the identification of three *CsTT8* isoforms in *C. sativa* and use of CRISPR/Cas9 technology to create null *TT8* mutants. In the *CsTT8* mutants, flavonoid accumulation is reduced by 44%. Carbon allocation was redirected toward enhanced synthesis of fatty acids which accumulated to as high as 38% of dry weight (DW) without changes in starch and protein contents. Disrupting all three *TT8* alleles created yellow seed camelina with increased TAG yield of more than 21%.

[00023] In embodiments, the invention provides a genetically edited plant, or a seed or other germplasm derived therefrom, having increased seed yield and increased seed oil content and oil accumulation, wherein the expression or activity of TT8 is disrupted or abolished.

[00024] In some embodiments, the plant is a *Brassicacea* family plant and is of the genus selected from *Camelina*, *Thlapsi* (pennycress), *Brassica* (canola, rapeseed), *Capsella, Cardamine* (bittercress), *Eutrema* and *Arabis*. In an embodiment, the plant is *Camelina* genus and is *Camelina sativa L. (Cs)*. In some embodiments, the plant is a *Brassicacea* family plant and is of the genus selected from *Camelina*, *Thlapsi* (pennycress), *Capsella, Cardamine* (bittercress), *Eutrema* and *Arabis*. In an embodiment, the plant is *Camelina* genus and is *Camelina sativa L. (Cs)*. In an embodiment, the plant is *Brassica* genus and is *Brassica napus* or *Brassica carinata*.

[00025] In particular embodiments, all TT8 encoding genes are disrupted. In an embodiment, all TT8 encoding genes are disrupted by gene editing. In one embodiment, gene editing utilizes a CRISPR/Cas system.

[00026] In another embodiment, fatty acid accumulation and TAG yield are increased and flavonoid accumulation is reduced. In some embodiments, the triacylglycerol yield increase is at least 20%. In some embodiments, the triacylglycerol yield increase is greater than 20%. In some embodiments, the triacylglycerol yield increase is about 20%-40%.

[00027] In particular embodiments, the seeds are yellow in color and the seeds of wild type or parental plants are not yellow.

[00028] In some embodiments, there are no significant changes in starch or protein levels in the seeds compared to wild type or parental plants.

[00029] In embodiments of the invention the plant is a *Camelina* species. In an embodiment, the plant is *Camelina sativa*. In embodiments of the invention, the *Transparent Testa 8* transcription factor encoding genes are *Camelina sativa* isoforms *CsTT8-2, CsTT8-8* and *CsTT8-13*.

[00030] In an aspect, the invention provides a seed having increased seed oil content and oil accumulation, wherein the expression or activity of TT8 is disrupted or abolished. In an aspect, the seed is a *Camelina* seed. In one aspect, the seed is a yellow *Camelina* seed. In an aspect, the seed is a *Camelina* sativa seed.

[00031] In another embodiment, the invention provides a method of modifying a plant or a seed or other germplasm derived therefrom to increase seed yield, seed oil content and oil accumulation, comprising abolishing the expression or activity of TT8 by disrupting all *TT8* transcription factor encoding genes in said plant. In an embodiment of the method, fatty acid accumulation and TAG yield are increased and flavonoid accumulation is reduced.

[00032] In an embodiment, all TT8 encoding genes are disrupted by gene editing and wherein gene editing utilizes a CRISPR/Cas system.

[00033] In another embodiment, the plant is a member of the *Brassicacea* family and is not an *Arabidopsis* genus plant or a *Brassica* genus plant. In an embodiment, the plant is a *Brassicacea* family plant and is of the genus selected from *Camelina*, *Thlapsi* (pennycress), *Capsella*, *Cardamine* (bittercress), *Eutrema* and *Arabis*. In an aspect, the plant is not an *Arabidopsis* genus plant. In an aspect, the plant is not a *Brassica* genus plant. In an aspect, the plant is selected from the genus. In an aspect, the plant is a *Camelina* genus plant. In one aspect, the plant is *Camelina sativa L. (Cs)*.

[00034] In some aspects of the invention, higher *FAD2* but decreased *FAD3* expression is provided. In an embodiment, higher *FAD2* but decreased *FAD3* expression is provided favoring the accumulation of 18:2 at the expense of 18:3.

[00035] In an embodiment of the invention, mucilage accumulation in the outermost layer of the seed coat is minimized or reduced, leading to a noticeable reduction in seed coat thickness. Thus in an embodiment, the seeds have reduced seed coat thickness. In an embodiment, the seeds demonstrated increased or improved germination, such as germination rate per seed is increased.

[00036] In embodiments of the methods of the invention, all TT8 encoding genes are disrupted by gene editing, wherein gene editing utilizes a CRISPR/Cas system, and the TT8 encoding genes are homologs of *Camelina sativa* isoforms CsTT8-2, CsTT8-8 and CsTT8-13.

[00037] In one embodiment, the homologs of *Camelina sativa* isoforms CsTT8-2, CsTT8-8 and CsTT8-13 have at least 80% amino acid sequence identity to SEQ ID NO:25, 26 and/or 27.

[00038] The invention provides a method of modifying a plant or a seed or other germplasm derived therefrom to increase seed yield, seed oil content and oil accumulation, comprising abolishing the expression or activity of TT8 by disrupting all *TT8* transcription factor encoding genes in said plant.

[00039] In one embodiment, fatty acid accumulation and triacylglyceride (TAG) yield are increased and flavonoid accumulation is reduced.

[00040] The invention provides a method for producing a genetically edited plant or part thereof that comprises a genetically edited plant cell, said genetically edited plant cell comprising

a) disrupting one or more *TT8* transcription factor encoding genes, and

b) enhancing oil accumulation in the plant's seeds.

[00041] The invention provides a method for producing a genetically edited plant or part thereof that comprises a genetically edited plant cell, said genetically edited plant cell comprising

a) disrupting *TT8* transcription factor encoding genes, and

b) enhancing oil accumulation in the plant's seeds.

[00042] The invention provides a method for increasing seed yield and increasing the oil content in a plant seed, the method comprising abolishing the expression or activity of one or more TT8 gene in said plant. The invention provides a method for increasing seed yield and increasing the oil content in a plant seed, the method comprising abolishing the expression or activity of all TT8 genes in said plant. The invention provides a method for increasing seed yield and increasing the oil content in a plant seed, the method comprising abolishing the expression or activity of TT8 in said plant.

[00043] In an aspect, the plant is a plant which natively does not produce yellow seeds. In an aspect, the plant is an oilseed crop plant. In an aspect, the plant is a member of the *Brassicacea* family. In an aspect, the plant is not an *Arabidopsis* genus plant. In an aspect, the plant is not a *Brassica* genus plant. In an aspect, the plant is selected from the genus *Camelina*, *Thlapsi* (pennycress), *Brassica* (canola, rapeseed), *Capsella*, *Cardamine* (bittercress), *Eutrema* and *Arabis*. In an embodiment, the plant is *Brassica* genus and is *Brassica napus* or *Brassica carinata*. In an aspect, the plant is a *Camelina* genus plant.

[00044] In some aspects, abolishing the expression or activity of TT8 is achieved by disruption, inactivation or mutation of one or more copies of a *TT8* gene in a plant, whereby the expression or activity of TT8 protein produced in the plant is significantly reduced. In one such aspect, the expression or activity of at least one TT8 encoding gene is abolished by disruption, inactivation or mutation of said at least one copy of a *TT8* gene in a plant. In some aspects, abolishing the expression or activity of TT8 is achieved by disruption, inactivation or mutation of all copies of *TT8* gene in a plant, whereby no active TT8 protein is produced in the plant.

[00045] In another embodiment, the invention provides a method for increasing seed yield and increasing the oil content of seeds by disruption, inactivation or mutation of all copies of *TT8* gene in a plant, whereby no active TT8 protein is produced in the plant. In an embodiment, the invention provides a method for increasing seed yield, increasing the oil content of seeds, increasing fatty acid accumulation and TAG yield, reducing flavonoid accumulation, by disruption, inactivation or mutation of all copies of *TT8* gene in a plant. In one aspect of the method, there are no significant changes in starch or protein

levels compared to wild type or parental plants. In one embodiment the seeds are yellow in color. In one embodiment, the wild type or parental plant does not have yellow seeds.

[00046] Isolated nucleic acid encoding the disrupted or altered TT8 polypeptide are provided. A recombinant vector comprising the nucleic acid is provided in an aspect. In one such aspect, a host cell comprising the vector is provided, including wherein the host cell is recombinantly engineered or genetically modified to overproduce or heterologously produce the disrupted or altered TT8 polypeptide. Host plants comprising the vector are also provided in an aspect.

[00047] The plants, seed or other germplasm, or host cell of the invention may be further engineered to express a TAG synthesizing enzyme. In some embodiments, these are further engineered to express one or more of diacylglycerol acyltransferase (DGAT), wrinkled1 (WRI1) or medium chain thioesterase (MCT or T). The host cell may be a plant cell.

[00048] In a further embodiment, the plant, seed or other germplasm thereof, is additionally genetically modified to express a TAG synthesizing enzyme. In a further embodiment the TAG synthesizing enzyme is expressed in the same tissue as the disrupted or mutated TT8.

[00049] In a further embodiment, the plant, seed or other germplasm thereof, is genetically modified to comprise a nucleic acid sequence encoding a TAG synthesizing enzyme. In a further embodiment the plant comprises an expression construct including a nucleic acid sequence encoding a TAG synthesizing enzyme. In another aspect the plant may be genetically modified to express or overexpress DGAT2 and/or WRI1.

[00050] In one embodiment, the plant, seed or other germplasm thereof, accumulates more total lipid in its non-photosynthetic tissues/organs than does a control plant. In a further embodiment the plant accumulates at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 100%, more preferably 150%, more preferably 200%, more preferably 250%, more preferably 300%, more preferably 350%, more preferably 400%, more preferably 450%, more preferably 500%, more total lipid in its non-photosynthetic tissues/organs than does a control plant. In one embodiment the plant produces total lipid in its non-photosynthetic tissues/organs in the range 100% to 900%, more preferably 200% to 800%, more preferably 300% to 700%, more preferably 400% to 600%, more than a control plant.

[00051] Suitable control plants include non-transformed, non-genetically modified, or wild-type versions of plant of the same variety and/or species as the transformed or genetically modified plant used in the method of the invention. Suitable control plants also include plants of the same variety and or species as the transformed plant that are transformed with a control construct. Suitable control plants also include plants that have not been disrupted in any one or more TT8 encoding genes.

[00052] Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0005] FIG. 1 depicts identification of three TT8 genes in Camelina. a) Two CsTT8 isoforms comprise six exons (box) separated by five introns (represented by the solid line) and one contains 7 exons with six introns. The N-terminal MYB interaction region (MIR) domain and the bHLH domain in the C terminal region are colored blue and orange, respectively. The vertical dashed lines in the gene model indicate the CRISPR target sites, and the arrows indicate the sgRNA direction. The target sequences are shown with the PAM in red. (b) Expression pattern of three isoforms of CsTT8 in camelina. Relative gene expression of CsTT8 in leaf, root, stem, seeds of 12 days after flowering (12d), 24 days after flowering (24d) and mature seeds of Suneson were determined by qRT-PCR; values are the means ± SE of three biological replicates. (c) Expression of three CsTT8 homologous genes in camelina during seed development with the number of days after flowering (DAF) indicated. Data (normalized counts per million) are derived from RNAseq experiments.

[0006] FIG. 2 shows editing of the *Cs*TT8 changed seed coat color to light yellow. (a) Sequences at the sgRNA target sites of null *Cs*TT8 homozygous mutants. The S1 target sequence is ACAGAGAAATCTGCCGGAGC (SEQ ID NO:1). The S2 target sequence is TCAAGGGCTGCTTAAGGAAG (SEQ ID NO:2). Lines 26-6, 26-7, and 27-9 contained homozygous changes in all three isoforms. The protospacer adjacent motif (PAM) is highlighted in blue, and target site S1 and S2 are labelled. Red letters refer to insertions. The wild type region native sequence at and around the target site (SEQ ID NO:3) is first depicted. The CsTT8-2 locus sequence in lines 26-6, 26-7 and 27-9 (SEQ ID NOs:4-6), the CsTT8-8 locus sequence in lines 26-6, 26-7 and 27-9 (SEQ ID NOs:7-9), and the CsTT8-8 locus sequence in lines 26-6, 26-7 and 27-9 (SEQ ID NOs:10-12) are each then shown. (b) Seed coat color of WT Suneson and three null TT8 modification lines, 26–6, 26–7 and 27–9 respectively.

[0007] FIG. 3 shows the effect of editing TT8 on flavonoid content in *camelina* seeds. (a) Transverse section of camelina seeds. In all *TT8* modification lines containing homozygous disruptions of all three *TT8* genes, seed color changed to a light-yellow hue. Arrowheads indicate the endothelial cells of seed coat. (b) Flavonoid content in camelina seeds is depicted. Total flavonoid was extracted from ground seeds and its content was measured and displayed as mg of rutin equivalents per g FW seeds. The values represent the mean ± standard deviation of three biological replicates. **Student t-test $P < 0.01$. The flavonoid content decreased markedly in all three *CsTT8* mutant lines at 35.5% in line 26-6, 43.9% in line 26-7, and 44.1% in line 27-9, respectively.

[0008] FIG. 4 shows the effect of editing *CsTT8* on seed coat in *camelina* seeds. (a) Differential Interference Contrast (DIC) imaging of camelina seeds. Under a confocal microscope, DIC imaging of wild type seeds showed a well-organized outermost layer of mucilage. In contrast, in all three *CsTT8* mutant lines, the mucilage layer was either partially reduced or completely absent. (b) The seed coat of camelina. Camelina seeds were fixed and sectioned with a cryo microtome, and then stained with

toluidine blue O (TBO) and observed under a dissection microscope. Images represent the dissection of 10–12 seeds from wild type and lines 26–6, 26–7 and 27–9. The wild type seeds consistently exhibited a clearly stained mucilage layer that was notably absent in the seeds of the *TT8*-modified lines. (c) Mucilage content in camelina seeds. Mucilage was extracted from seeds was quantified as a weight percent of dry seed weight. The values represent the mean ± standard deviation of four biological replicates. **Indicates a significant difference from wild type by student t-test $P < 0.01$.

[0009] FIG. 5 shows editing of *TT8* changed fatty acid accumulation and increased oil content in *camelina* seeds. Seed total fatty acid (TFA) and TAG content was quantified by GC of fatty acid methyl esters. TFA (a) and TAG contents (b) in camelina seeds are presented as a proportion of dry seed weight. (c) Fatty acid composition in total fatty acid is expressed as a weight percentage of the total FA. Seed total fatty acid (TFA) contents increased in all three edited lines. In WT *camelina* controls, the TFA content was 32.4% of dry weight (DW). The *CsTT8* edited lines had higher *FAD2* but decreased *FAD3* expression favoring the accumulation of 18:2 at the expense of 18:3. Values represent means (±) standard deviation ($n = 3$). *Student t-test, $P < 0.05$ for differences between wild type and edited lines.

[0010] FIG. 6 shows the effect of targeted TT8 modification on the expression level of genes involved in fatty acid (FA) biosynthesis. Transcript levels of transcription factors (a), fatty acid synthesis (b) and fatty acid desaturation (c) genes were analysed by RT-qPCR in seeds, $n = 3$ biological replicates, and error bars represent SD. The relative expression levels are reported relative to the expression of the Actin transcript. RT-qPCR analysis shows that editing of *CsTT8* led to substantial increases in the expression levels of these genes in *Camelina* seeds. In (c) it is shown that the *FAD2* transcript was elevated while the *FAD3* transcript displayed a small decrease in the *CsTT8* edited lines.

[0011] FIG. 7 depicts [^{14}C]Acetate incorporation assay in developing seeds. [14C]Acetate incorporation into total lipids showed ACCase activity in 11–13-DAF developing seeds of WT, TT8 editing lines. Stars indicate significant differences (*, $P < 0.05$, **,$p < 0.01$) as determined by Student's t test. Values are presented as means ± SD of four biological replicates.

[0012] FIG. 8 provides phylogenetic tree of the *TT8* homologs from camelina and other plants. Phylogenetic analysis shows that all three *CsTT8* copies cluster in a clade with *AtTT8*. Protein sequences are from GenBank with the following accession numbers: AtTT8 (Q9FT81) in *Arabidopsis thaliana*; BnA09.TT8 (MN399821) and BnC09.TT8b (MN399822) in *Brassica napus*; BoTT8a (ADV03944), BoTT8b (ADP76654) in *Brassica oleracea*; BjuA.TT8 (AIN41653.1) in *Brassica juncea*; RsTT8 (ASF79354.1) from *Raphanus sativus*; BrTT8 (XP_009113574) in *Brassica rapa*; LjTT8 (AB490778) in *Lotus japonicus*; RsTT8 (KY651179) in *Raphanus sativus*; LtTT8 (ARK19321.1) in *Lotus tenuis*; LcTT8 (KY196477) in *Lotus corniculatus*; AcbHLH42 (QAT77714) in *Actinidia chinensis*; MtTT8 (KM892777) in *Medicago truncatula*.

[0013] FIG. 9A, 9B and 9C depicts that editing of TT8 in camelina changed protein sequence but did not edit the predicted off target sites. Fig. 9A: Protein sequence changes are shown within the first 66

amino acid of tt8 mutants. The * represents stop codon, - represents deletion. For Cs TT8-2, the first 66 amino acids of the wild type (WT) (SEQ ID NO:13), 26-6 (SEQ ID NO:14), 26-7 (SEQ ID NO:15) and 27-9 (SEQ ID NO:16) mutants are shown. For Cs TT8-8, the first 66 amino acids of the wild type (WT) (SEQ ID NO:17), 26-6 (SEQ ID NO:18), 26-7 (SEQ ID NO:19) and 27-9 (SEQ ID NO:20) mutants are shown. For Cs TT8-13, the first 66 amino acids of the wild type (WT) (SEQ ID NO:21), 26-6 (SEQ ID NO:22), 26-7 (SEQ ID NO:23) and 27-9 (SEQ ID NO:24) mutants are shown. Fig. 9B provides predicted off-target sites. Fig. 9C provides sequencing of predicted off-target sites CCTCCTTCAGCCGCCCTTGA (SEQ ID NO:81) and ATACAGAACTCTGCCGGAGCAGG (SEQ ID NO: 82). No changes were observed in the predicted off-target sites. No editing was seen in the predicted off target sites in the *CsTT8* mutagenesis lines.

[0014] FIG. 10 shows seed coat color changes in some of the T1 transgenic lines. The seeds of T_1 lines 13, 22, 26, and 27 appeared yellow, whereas seeds of other lines (lines 21, 24, 25, 30, 31, 32 and 33) remained brown as was the wild type (WT).

[0015] FIG. 11 shows seed germination and plant growth in the mutated *CsTT8* lines. FIG. 12 shows FIGs. (a) shows seed germination on wet filter paper after 20 hours. Consistent results were observed when 10-month-old seeds were germinated on wet filter paper at similar rates to that of WT. (b) No growth differences were observed in the mutated CsTT8 lines. Overall, there were no discernible alterations in growth or development observed in camelina plants in which all three *CsTT8* genes had been disrupted but were free of the Cas9 and DsRed marker transgenes.

[0016] FIG. 12 shows editing of *TT8* in *camelina* changes the fatty acid (FA) composition in TAG. Fatty acid composition in TAG was expressed as a weight percentage of the total FA. Values represent means (±) standard deviation (n = 3). *Student t-test, $P < 0.05$.

[0017] FIG. 13 shows the effect of editing *TT8* on protein and starch content in *camelina* seeds. Protein content (a) and starch content (b) were expressed as a weight percentage of the total seed weight. Values represent means (±) standard deviation (n = 3). (c) Hundred seed weight. Values represent means (±) standard deviation (n = 4). The protein content in these lines remained largely unaffected. While there was some variation in starch content in the mutant lines, the means were not significantly different from those of WT.

[0018] FIG. 14 depicts the effect of editing *TT8* on expression of GL2 and TTG2.

DETAILED DESCRIPTION

[00053] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art.

[00054] Therefore, if appearing herein, the following terms shall have the definitions set out below.

[00055] "Wild-type" and "normal" are interchangeably used when in reference to any molecule or its level (e.g., amino acid sequence, and nucleic acid sequence, etc.) and/or phenomenon or its level (e.g., expression of a gene, transcription of a DNA sequence, translation of an mRNA molecule to an amino acid sequence) and/or phenotype or its level (e.g., seed yield per plant, amount of total seed fatty acid per seed, amount of a target fatty acid per seed, seed yield per plant, seed germination rate, proportion of a target fatty acid relative to total seed fatty acid per seed, amount of total seed fatty acid per plant, establishment of roots, establishment of plant aerial parts) to mean that the molecule or its level and/or phenomenon or its level and/or phenotype or its level is the same as found in nature without alteration by the hand of man (such as by chemical and/or molecular biological techniques, etc.).

[00056] "Expression" refers to the transcription and stable accumulation of sense or anti-sense RNA derived from a nucleic acid. "Expression" may also refer to translation of mRNA into a polypeptide or protein. As used herein, the term "antisense RNA" refers to an RNA transcript that is complementary to all or a part of a mRNA that is normally produced in a cell. The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-translated sequence, introns, or the coding sequence. As used herein, the term "RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complimentary copy of the DNA sequence, it is referred to as the primary transcript or it may be an RNA sequence derived from post-transcriptional processing of the primary transcript and is referred to as the mature RNA.

[00057] "Transgenic" refers to a cell whose genome has been manipulated by any molecular biological technique, including, for example, the introduction of a transgene, homologous recombination, knocking in of a gene, knockout of a gene, and/or CRISPR gene editing.

[00058] The term "transgene" refers to any nucleic acid sequence that is introduced into the cell by experimental manipulations.

[00059] "Genetically edited" refers to a cell whose genome has been altered or manipulated by any molecular biological technique, including, for example, homologous recombination, knockout of a gene, and/or CRISPR gene editing. In a particular aspect, genetically edited does not include the introduction of a transgene or genetic sequence which is not present in the native or wild type.

[00060] The terms "increase," "elevate," "raise," and grammatical equivalents (including "higher," "greater," *etc.*) when in reference to the level of any molecule (*e.g.*, amino acid sequence, and nucleic acid sequence, etc.) and/or phenomenon (*e.g.*, level of expression of a gene, level of transcription of a DNA sequence, level of translation of an mRNA molecule to an amino acid sequence) and/or phenotype (*e.g.*, seed yield per plant, amount of total seed fatty acid per seed, amount of a target fatty acid per seed, seed yield per plant, seed germination rate, proportion of a target fatty acid relative to total seed fatty acid per seed, amount of total seed fatty acid per plant, establishment of roots, establishment of plant aerial

parts) in a first composition (e.g., first plant cell) relative to a second composition (e.g., second plant cell), mean that the quantity of molecule and/or phenomenon and/or phenotype in the first composition is higher than in the second composition by any amount that is statistically significant using any art-accepted statistical method of analysis. This includes, without limitation, a quantity of molecule and/or phenomenon and/or phenotype in the first composition that is at least 10% greater than, at least 15% greater than, at least 20% greater than, at least 25% greater than, at least 30% greater than, at least 35% greater than, at least 40% greater than, at least 45% greater than, at least 50% greater than, at least 55% greater than, at least 60% greater than, at least 65% greater than, at least 70% greater than, at least 75% greater than, at least 80% greater than, at least 85% greater than, at least 90% greater than, and/or at least 95% greater than the quantity of the same molecule and/or phenomenon and/or phenotype in the second composition.

[00061] The terms "alter" and "modify" when in reference to the level of any molecule (*e.g.*, amino acid sequence, and nucleic acid sequence, etc.) and/or phenomenon (*e.g.*, level of expression of a gene, level of transcription of a DNA sequence, level of translation of an mRNA molecule to an amino acid sequence) and/or phenotype (*e.g.*, seed yield per plant, amount of total seed fatty acid per seed, amount of a target fatty acid per seed, seed yield per plant, seed germination rate, proportion of a target fatty acid relative to total seed fatty acid per seed, amount of total seed fatty acid per plant, establishment of roots, establishment of plant aerial parts) in a first composition (e.g., first plant cell) relative to a second composition (e.g., second plant cell), mean that the quantity of molecule and/or phenomenon and/or phenotype in the first composition refer to an increase and/or decrease in the level of molecule and/or phenomenon and/or phenotype.

[00062] The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. Abbreviations for amino acid residues are in keeping with standard and art-recognized polypeptide nomenclature.

[00063] It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues.

[00064] A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g.,

restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

[00065] An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

[00066] A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[00067] Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

[00068] A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence.

[00069] An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

[00070] A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

[00071] The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

[00072] The term "probe" refers to a short polynucleotide that is used to detect a polynucleotide sequence that is complementary to the probe, in a hybridization-based assay. The probe may consist of a "fragment" of a polynucleotide as defined herein.

[00073] The term "primer" as used herein refers to a short polynucleotide, usually having a free 3'OH group, that is hybridized to a template and used for priming polymerization of a polynucleotide complementary to the target. A "primer" may be an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

[00074] The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

[00075] A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

[00076] Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. It

should be appreciated that also within the scope of the present invention are DNA sequences encoding the same amino acid sequence as provided in a SEQ ID NO: herein, but which are degenerate to SEQ ID NO:. By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid. Codons that can be used interchangeably to code for each specific amino acid are well known and standard for one skilled in the art.

[00077] Mutations can be made in sequences as provided herein such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping).

[00078] Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, preferably at least 85%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions. In a particular aspect, two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, preferably at least 85%, and most preferably at least about 90 or 95%) are identical.

[00079] A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

[00080] A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

[00081] The term "polynucleotide(s)," as used herein, means a single or double-stranded deoxyribonucleotide or ribonucleotide polymer of any length but preferably at least 15 nucleotides, and include as non-limiting examples, coding and non-coding sequences of a gene, sense and antisense sequences complements, exons, introns, genomic DNA, cDNA, pre-mRNA, mRNA, rRNA, siRNA, miRNA, tRNA, ribozymes, recombinant polypeptides, isolated and purified naturally occurring DNA or RNA sequences, synthetic RNA and DNA sequences, nucleic acid probes, primers and fragments.

[00082] A "fragment" of a polynucleotide sequence provided herein is a subsequence of contiguous nucleotides that is capable of specific hybridization to a target of interest, e.g., a sequence that is at least 15 nucleotides in length. The fragments of the invention comprise 15 nucleotides, preferably at least 20 nucleotides, more preferably at least 25 nucleotides, more preferably at least 30 nucleotides, more preferably at least 35 nucleotides, more preferably at least 40 nucleotides, more preferably at least 45 nucleotides, more preferably at least 50 nucleotides, more preferably at least 60 nucleotides, more preferably at least 70 nucleotides, more preferably at least 80 nucleotides, more preferably at least 90 nucleotides, more preferably at least 100 nucleotides, more preferably at least 150 nucleotides, more preferably at least 200 nucleotides, more preferably at least 250 nucleotides, more preferably at least 300 nucleotides, more preferably at least 350 nucleotides, more preferably at least 400 nucleotides, more preferably at least 450 nucleotides and most preferably at least 500 nucleotides of contiguous nucleotides of a polynucleotide disclosed. A fragment of a polynucleotide sequence can be used in antisense, RNA interference (RNAi), gene silencing, triple helix or ribozyme technology, or as a primer, a probe, included in a microarray, or used in polynucleotide-based selection methods of the invention.

[00083] The term "polypeptide", as used herein, encompasses amino acid chains of any length but preferably at least 5 amino acids, including full-length proteins, in which amino acid residues are linked by covalent peptide bonds. Polypeptides of the present invention, or used in the methods of the invention, may be purified natural products, or may be produced partially or wholly using recombinant or synthetic techniques. The term may refer to a polypeptide, an aggregate of a polypeptide such as a dimer or other multimer, a fusion polypeptide, a polypeptide fragment, a polypeptide variant, or derivative thereof.

[00084] A "fragment" of a polypeptide is a subsequence of the polypeptide that performs a function that is required for the biological activity and/or provides three dimensional structure of the polypeptide. The term may refer to a polypeptide, an aggregate of a polypeptide such as a dimer or other multimer, a fusion polypeptide, a polypeptide fragment, a polypeptide variant, or derivative thereof capable of performing the above enzymatic activity.

[00085] The term "isolated" as applied to the polynucleotide or polypeptide sequences disclosed herein is used to refer to sequences that are removed from their natural cellular environment. An isolated molecule may be obtained by any method or combination of methods including biochemical, recombinant, and synthetic techniques.

[00086] The term "recombinant" refers to a polynucleotide sequence that is removed from sequences that surround it in its natural context and/or is recombined with sequences that are not present in its natural context.

[00087] A "recombinant" polypeptide sequence is produced by translation from a "recombinant" polynucleotide sequence.

[00088] The term "derived from" with respect to polynucleotides or polypeptides of the invention being derived from a particular genera or species, means that the polynucleotide or polypeptide has the same sequence as a polynucleotide or polypeptide found naturally in that genera or species. The polynucleotide or polypeptide, derived from a particular genera or species, may therefore be produced synthetically or recombinantly.

[00089] As used herein, the term "variant" refers to polynucleotide or polypeptide sequences different from the specifically identified sequences, wherein one or more nucleotides or amino acid residues is deleted, substituted, or added. Variants may be naturally occurring allelic variants, or non-naturally occurring variants. Variants may be from the same or from other species and may encompass homologues, paralogues and orthologues. In certain embodiments, variants of the inventive polypeptides and polypeptides possess biological activities that are the same or similar to those of the inventive polypeptides or polypeptides. The term "variant" with reference to polypeptides and polypeptides encompasses all forms of polypeptides and polypeptides as defined herein.

[00090] Polynucleotide or polypeptide sequence identity can be determined in the following manner. The subject polynucleotide sequence is compared to a candidate polynucleotide sequence using BLASTN (from the BLAST suite of programs, version 2.2.5 [Nov. 2002]) in bl2seq (Tatiana A. Tatusova, Thomas L. Madden (1999), "Blast 2 sequences--a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250), which is publicly available from NCBI (ftp://ftp.ncbi.nih.gov/blast/).

[00091] Polynucleotide or polypeptide variants of the present invention also encompass those which exhibit a similarity to one or more of the specifically identified sequences that is likely to preserve the functional equivalence of those sequences and which could not reasonably be expected to have occurred by random chance. Such sequence similarity with respect to polypeptides may be determined using the publicly available bl2seq program from the BLAST suite of programs (version 2.2.5 [Nov. 2002]) from NCBI (ftp://ftp.ncbi.nih.gov/blast/).

[00092] The term "variant" with reference to polypeptides encompasses recombinantly and synthetically produced polypeptides. Variant polypeptide sequences preferably exhibit at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 89%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more

preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, and most preferably at least 99% identity to a sequences of the present invention. Identity is found over a comparison window of at least 20 amino acid positions, preferably at least 50 amino acid positions, more preferably at least 100 amino acid positions, and most preferably over the entire length of a polypeptide of the invention.

[00093] Polypeptide variants of the present invention, or used in the methods of the invention, also encompass those which exhibit a similarity to one or more of the specifically identified sequences that is likely to preserve the functional equivalence of those sequences and which could not reasonably be expected to have occurred by random chance. Such sequence similarity with respect to polypeptides may be determined using the publicly available bl2seq program from the BLAST suite of programs (version 2.2.5 [Nov. 2002]) from NCBI (ftp://ftp.ncbi.nih.gov/blast/).

[00094] Conservative substitutions of one or several amino acids of a described polypeptide sequence without significantly altering its biological activity are also included in the invention. A skilled artisan will be aware of methods for making phenotypically silent amino acid substitutions.

[00095] The term "genetic construct" refers to a polynucleotide molecule, usually double-stranded DNA, which may have inserted into it another polynucleotide molecule (the insert polynucleotide molecule) such as, but not limited to, a cDNA molecule. A genetic construct may contain the necessary elements that permit transcribing the insert polynucleotide molecule, and, optionally, translating the transcript into a polypeptide. The insert polynucleotide molecule may be derived from the host cell, or may be derived from a different cell or organism and/or may be a recombinant polynucleotide. Once inside the host cell the genetic construct may become integrated in the host chromosomal DNA. The genetic construct may be linked to a vector.

[00096] The term "vector" refers to a polynucleotide molecule, usually double stranded DNA, which is used to transport the genetic construct into a host cell. The vector may be capable of replication in at least one additional host system, such as E. coli.

[00097] The term "expression construct" refers to a genetic construct that includes the necessary elements that permit transcribing the insert polynucleotide molecule, and, optionally, translating the transcript into a polypeptide. An expression construct typically comprises in a 5' to 3' direction: a) a promoter functional in the host cell into which the construct will be transformed, b) the polynucleotide to be expressed, and c) a terminator functional in the host cell into which the construct will be transformed.

[00098] The term "coding region" or "open reading frame" (ORF) refers to the sense strand of a genomic DNA sequence or a cDNA sequence that is capable of producing a transcription product and/or a polypeptide under the control of appropriate regulatory sequences. The coding sequence may, in some cases, identified by the presence of a 5' translation start codon and a 3' translation stop codon. When

inserted into a genetic construct, a "coding sequence" is capable of being expressed when it is operably linked to promoter and terminator sequences.

[00099] "Operably-linked" means that the sequenced to be expressed is placed under the control of regulatory elements that include promoters, tissue-specific regulatory elements, temporal regulatory elements, enhancers, repressors and terminators.

[000100] As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "µg" mean microgram, "mg" means milligram, "ul" or "µl" mean microliter, "ml" means milliliter, "l" means liter.

[000101] Another feature of this invention is the expression of the DNA sequences disclosed herein. As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host. Such operative linking of a DNA sequence of this invention to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision of an initiation codon, ATG, in the correct reading frame upstream of the DNA sequence.

[000102] A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col El, pCR1, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAS, e.g., the numerous derivatives of phage λ, e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2µ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

[000103] Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, promoters. Promoters suitable for expression in plants are well known and available. A tissue/organ preferred promoter is a promoter that drives expression of an operably linked polynucleotide in a particular tissue/organ at a higher level than in other tissues/organs. A tissue specific promoter is a promoter that drives expression of an operably linked polynucleotide specifically in a particular tissue/organ. Even with tissue/organ specific promoters, there is usually a small amount of expression in at least one other tissue. A tissue specific promoter is by definition also a tissue preferred promoter. Vegetative Tissue Specific Promoters - An example of a vegetative specific promoter is found in U.S. Pat. No. 6,229,067; and 7,629,454; and 7,153,953; and 6,228,643. Pollen Specific Promoters - An example of a pollen specific promoter is found in U.S. Pat. Nos. 7,141,424; and 5,545,546; and 5,412,085; and 5,086,169; and 7,667,097. Seed Specific Promoters - An example of a seed specific promoter is found in U.S. Pat. Nos. 6,342,657; and 7,081,565;

and 7,405,345; and 7,642,346; and 7,371,928. Fruit Specific Promoters - An example of a fruit specific promoter is found in U.S. Pat. Nos. 5,536,653; and 6,127,179; and 5,608,150; and 4,943,674. Non-Photosynthetic Tissue Preferred Promoters - Non-photosynthetic tissue preferred promoters include those preferentially expressed in non-photosynthetic tissues/organs of the plant. Non-photosynthetic tissue preferred promoters may also include light repressed promoters. Light Repressed Promoters - An example of a light repressed promoter is found in U.S. Pat. Nos. 5,639,952 and in 5,656,496. Root Specific Promoters - An example of a root specific promoter is found in U.S. Pat. No. 5,837,848; and US 2004/0067506 and US 2001/0047525. Tuber Specific Promoters - An example of a tuber specific promoter is found in U.S. Pat. No.6,184,443. Bulb Specific Promoters - An example of a bulb specific promoter is found in Smeets et al., (1997) Plant Physiol. 113:765-771. Rhizome Preferred Promoters - An example of a rhizome preferred promoter is found Seong Jang et al., (2006) Plant Physiol. 142:1148-1159. Endosperm Specific Promoters - An example of an endosperm specific promoter is found in U.S. Pat. No. 7,745,697. Photosynthetic Tissue Preferred Promoters - Photosynthetic tissue preferred promoters include those that are preferentially expressed in photosynthetic tissues of the plants. Photosynthetic tissues of the plant include leaves, stems, shoots and above ground parts of the plant. Photosynthetic tissue preferred promoters include light regulated promoters. Light Regulated Promoters - Numerous light regulated promoters are known to those skilled in the art and include for example chlorophyll a/b (Cab) binding protein promoters and Rubisco Small Subunit (SSU) promoters. An example of a light regulated promoter is found in U.S. Pat. No. 5,750,385. Light regulated in this context means light inducible or light induced.

[000104] A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, insect cells, and human cells and plant cells in tissue culture. Host cells may be derived from, for example, bacterial, fungal, yeast, insect, mammalian, algal or plant organisms. Host cells may also be synthetic cells. Preferred host cells are eukaryotic cells. A particularly preferred host cell is a plant cell, particularly a plant cell in a vegetative tissue of a plant.

[000105] A "transgenic plant" refers to a plant which contains new genetic material as a result of genetic manipulation or transformation.The new genetic material may be derived from a plant of the same species as the resulting transgenic plant or from a different species.

[000106] It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector, the host

must be considered because the vector must function in it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, will also be considered.

[000107] In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Suitable unicellular hosts will be selected by consideration of, e.g., their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products.

[000108] Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences of this invention on fermentation or in large scale animal culture.

[000109] The labels most commonly employed for studies with relevance to the present invention are known to one skilled in the art. Examples are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate. The mutant oleosin can also be labeled with a radioactive element or with an enzyme. The radioactive label can be detected by any of the currently available counting procedures. The preferred isotope may be selected from ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re. Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase, ß-glucuronidase, ß-D-glucosidase, ß-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S. Patent Nos. 3,654,090; 3,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods.

[000110] The present invention is directed to mutants of the *Brassicaceae* plant family, particularly oilseed relevant plants, which are altered to provide for yellow seed coat color and increased seed oil content. *Brassicaceae* family plants which are relevant to the invention include *Arabidopsis*, *Brassica napus* (rapeseed or canola) as well as the *Camelina* species, *Capella rubella*, *Eutrema salsugineum*, *Arabis alpha*, *Cardamine amara*, and *Thlapsi arvense* (pennycress). *Camelina* species include *Camelina alyssum*, *Camelina microcarpa*, *Camelina rumelica* and *Camelina sativa*, particularly *Camelina sativa*.

[000111] In an aspect of the invention, each and all of the *TT8* transcription factor encoding genes in a plant are mutated or genetically modified to eliminate production of TT8 protein in the plant, its germplasm, or its seeds. In accordance with the present invention, three *CsTT8* isoforms were identified in *C. sativa* and all of the isoforms were inactivated or mutated to effectively be null mutations/mutants. In a particular aspect, CRISPR/Cas9 technology was utilized to create null mutants.

[000112] Thus, the invention provides a means and approach to generate plants, germplasm or seed of a plant which are yellow in color and increased in oil content. In an aspect, the plant is a *Brassicaceae* family plants. In an aspect, the plant is of the *Camelina* species, *Capella rubella*, *Eutrema salsugineum*, *Arabis alpha*, *Cardamine amara*, and *Thlapsi arvense* (pennycress). *Camelina* species include *Camelina alyssum*, *Camelina microcarpa*, *Camelina rumelica* and *Camelina sativa*, particularly *Camelina sativa*. In an aspect, a plant or crop unsuitable for biofuel production because of its low seed oil content or other seed characteristics is rendered useful and cost effective for oil seed production, such as a biofuel, by manipulation and deletion, disruption, or null mutation of any and all of its *TT8* transcription factor encoding genes.

[000113] The present method relates to the use of a technology to precisely delete nucleotides to disrupt one or more of the *TT8* transcription factor genes (denoted herein *CsTT8-2, CsTT8-8* and *CsTT8-13*) in *Camelina sativa L.* (*Cs*) to enhance total fatty acid accumulation in the seeds. The present method relates to the use of CRISPR/Cas9 technology to disrupt the *TT8* transcription factor genes (*CsTT8-2, CsTT8-8* and *CsTT8-13*) in *Camelina sativa L.* (*Cs*) to enhance total fatty acid accumulation in the seeds. The discovery relates to a high-oil phenotype yellow seed Suneson variety of *Camelina sativa* germplasm that can be used directly for oil production.

[000114] It can also be used as a variety into which breeders can introduce certain other traits. Such traits might include but are not limited to stress resistance, desiccation or heat resistance, fatty acid modification, other yield enhancements, and disease or insect resistance.

[000115] The present disclosure relates to the use of null mutant generating technology or methods, such as CRISPR technology, to disrupt one or more or all copies of *Transparent Testa 8* gene(s) to create a yellow seed phenotype associated with increased seed oil yield in members of the *Brassicacea* family. The present disclosure relates to the use of CRISPR cas9 technology or other null mutant generating technology or methods to disrupt all copies of the *Transparent Testa 8* gene to create a yellow seed phenotype associated with increased seed oil yield in members of the *Brassicacea* family.

[000116] It may be expressed in *N. Benthamiana*. Nucleotide primers have been designed against each of the three *TT8* genes and CRISPR cas9 was used to introduce mutations at certain target sites. Plants with these mutations were taken to homogeneity for all three genes and the oil yield and other parameters were tested.

[000117] In the *CsTT8* mutants, flavonoid accumulation was reduced by 44%. Carbon allocation was redirected toward enhanced synthesis of fatty acids which accumulated to as high as 38% of dry weight (DW) without changes in starch and protein contents. Disrupting all three *TT8* alleles created yellow seed camelina with increased TAG yield of more than 21%.

[000118] Different nucleotide sequences could be targeted within the *TT8* target genes, or the present method could use a form of untargeted mutagenesis such as ethyl methanesulfonate, or radiation to introduce mutations and visual selection of yellow seed phenotype seeds in the progeny of the present experiments.

[000119] In accordance with an aspect of the invention three *TT8* isoforms designated as *CsTT8-2* (*Csa02g028180.1*,), *CsTT8-8* (*Csa08g037600.2*) and *CsTT8-13* (*Csa13g044750.1*) located on chromosomes 2, 8 and 13 were identified and targeted for disruption to generate null mutants thereof. Null mutants are mutants whereby the proteins are not generated in complete or full length form, such as by premature termination, include an internal deletion of amino acids alone or in combination with premature termination, or otherwise are inactive or not produced in any significant or active amount. The amino acid sequences of the three *TT8* proteins in C. sativa are provided below. The region of amino acid sequence and first exon that is targeted for deletion and/or premature termination in an aspect of the invention (corresponding to the first 66 amino acids) are shown in bold.

[000120] Csa 02g028180.1 CsTT8-2 on chromosome 2

MDGSSIIPTEKSAGAEKRELQGLLKEAVQSVEWTYSLFWQFCPQQRVLVWGNGYYNGAIK
TRKTTQPAEVTAEEAALERSQQLRELYQTLLAGESTSEARACTALSPEDLTETEWFYLMCVSFSF
SPPSGIPGKAYARRKHVWLSGANEVDSKSFSRAILAKTVVCIPMLNGVVELGTTKKVKEDVEFV
ELIKSFFIGYSNSIPKPALSEHSTYEVHEEVEEEEEEEVEEEMTMSEEMRLGSPDDDDVSNQNLRS
DLHIESTHTLDTNMDMMNLMEEGGIYSQTVTTLLMSHPTSLLSESVTTSSYVQSSFSTWRVENVR
PSASGVAMDAQTHDLESSYNTKDKSLPREDLNHVVAERRRREKLNEKFLTLRSMVPFLTKMDK
VSILGDTIEYVNHLRKRVHELETTHHEKQHKRTRTSKRKTLEEVEVSIIESDVLLEMRCEYRDGLL
LDILQVLHDLGIETTSVHTAVNDGDFEAEIRAKVRGKKASIAEVKRAIHQVIIHNTNL (SEQ ID
NO:25)

[000121] Csa 08g037600.1 CsTT8-8 on chromosome 8

MDGSINIPTEKSAGADKRELQGLLKEAVQSVEWTYSLFWQFCPQQRVLVWGNGYYNGAIK
TRKTTQPAEVTAEEAALERSQQLRELYETLLAGESTSEARACTALSPEDLTETEWFYLMCVSFSF
SPPSGMPGKAYARRKHVWLSGANEVDSKTFCRAILAKSAKIQTVVCIPMLNGVVELGTTKKVKE
DVEFVELIKSFFIGYSNSIPKPALSEHSTYEVHEEVEEEEEEEEEEEEMTMSGEMRLGSPDDDDV
SNQNLRSDLHIESTHTLDTHMDMMNLMEEGGIYSQTVTTLLMSHPTSLLSDSVSTSSYVQSSFST
WRAENVKDHQRVESQWMLKHMILRVPFLHDNTKDKSLPREDLNHVVAERRRREKLNEKFTTL
RSMVPFMTKMDKVSILGDTIEYVNHLRKRVHELETTHHDKQHKRTRTCKRKTSEEVEVSIIESDV

LLEMRCEYRDGLLLDILQVLHDLGIETTSVHTAVNDGDFEAEIRAKVRGKKASIVEVKRAIHQVII HN (SEQ ID NO:26)

[000122] Csa 13g044750.1 CsTT8-13 on chromosome 13

MDGSSIIPTEKSAGAEKRELQGLLKEAVQSVEWTYSLFWQFCPQQRVLVWGNGYYNGAIK TRKTTQPAEVTAEEATLERSQQLRELYETLLAGESTSEARACTALSPEDLTETEWFYLMCVSFSF SPPSGMPGKAYARRKHVWLSGANEVDSKTFCRAILAKSAKIQTVVCIPMLNGVVELGTTKKVKE DVEFVELVKSFFIGYSNSIPKPALSEHSTYEVHEEEEEEEAEVEEEEEEEEEEEEMTMSEEMRFGSP DDDNVSNQNLRSDLHIESTHTLDTHMDMMNLMEEGGIYSQTVTTLLMSHPTSLLSDSVSTSSYV QSSFSTWRAENVKDHQRVESQWMLKHMILRVPFLHDNTKEKSLPREDLNHVVAERRRREKLNE KFTTLRSMVPFMTKMDKVSILGDTIEYVNHLRKRVHELETTHHDKQHKRTRTCKRKTSEEVEVS IIESDVLLEMRCEYRDGLLLDILQVLHDLGIETSSVHTAVNDGDFEAEIRAKVRGKKASIVEVKRA IHQVIIHN (SEQ ID NO:27)

[000123] The nucleic acid coding sequences of the three *TT8* proteins in C. sativa are provided below. The S1 and S2 regions of nucleic acid sequence targeted in the TT8 editing by CRISPR/Cas constructs herein are shown in bold and underlined, respectively, in each nucleic acid sequence.

[000124] Csa 02g028180.1 CsTT8-2 on chromosome 2

ATGGATGGATCAAGTATTATTCCG**ACAGAGAAATCTGCCGGAGC**TGAGAAAAGGGAGCT**T CAAGGGCTGCTTAAGGAAG**CGGTTCAATCTGTGGAGTGGACTTATAGTCTGTTCTGGCAAT TTTGTCCTCAACAACGGGTATTGGTGTGGGGGAATGGATACTATAACGGTGCAATAAAGACG AGAAAGACAACTCAACCAGCGGAGGTGACGGCAGAAGAGGCTGCACTAGAGAGGAGCCAA CAGCTGAGGGAGCTTTACCAGACGCTTTTAGCGGGAGAGTCAACGTCGGAAGCGAGAGCAT GCACCGCACTGTCGCCGGAGGATTTGACGGAGACTGAATGGTTTTATCTAATGTGTGTCTCT TTCTCTTTCTCTCCTCCTTCAGGGATACCAGGAAAAGCGTATGCGAGGAGGAAGCATGTATG GCTAAGTGGTGCAAATGAGGTTGACAGTAAAAGTTTTTCTAGGGCTATTCTCGCTAAGACAG TGGTTTGCATTCCCATGCTTAATGGTGTTGTGGAACTTGGCACAACGAAGAAGGTAAAAGAA GATGTAGAGTTTGTTGAGCTAATAAAGAGTTTCTTCATTGGCTACTCTAATTCGATCCCAAAG CCTGCTCTTTCTGAACACTCCACCTACGAAGTGCATGAAGAAGTTGAAGAAGAAGAAGAAG AAGAAGTAGAAGAAGAGATGACAATGTCAGAGGAGATGAGGCTTGGCTCTCCTGATGATGA CGACGTCTCCAATCAAAATCTACGCTCTGATCTTCATATAGAATCAACCCACACGTTAGATA CAAACATGGACATGATGAACTTAATGGAGGAAGGTGGAATTTACTCTCAGACAGTAACAAC ACTTCTCATGTCGCATCCCACGAGTCTTCTTTCAGAATCAGTTACCACATCTTCTTACGTCCA ATCATCTTTTTCCACGTGGAGGGTTGAGAATGTCAGACCATCAGCGAGTGGAGTCGCAATGG ATGCTCAAACACATGATCTTGAGAGTTCCTACAACACTAAAGATAAGAGTCTACCGCGGGA AGACCTGAACCACGTAGTGGCTGAGCGACGAAGGAGAGAGAAGTTGAACGAGAAATTCCTA ACGTTGAGATCAATGGTTCCATTTTTGACAAAGATGGATAAAGTATCTATCCTTGGAGACAC CATCGAGTACGTAAATCATCTTCGAAAGAGGGTCCATGAGCTTGAGACTACTCATCATGAGA

AACAACATAAGCGGACACGTACTAGTAAGAGAAAAACATTGGAGGAGGTGGAGGTTTCCAT
CATAGAGAGTGATGTTTTGTTAGAGATGAGATGTGAGTACCGAGATGGTTTGTTGCTTGACA
TTCTTCAGGTTCTTCATGACCTTGGTATAGAGACTACTTCGGTTCATACCGCGGTGAACGACG
GTGATTTCGAGGCGGAGATAAGGGCAAAAGTAAGAGGGAAGAAAGCAAGCATTGCTGAGG
TCAAAAGAGCCATCCACCAAGTCATAATACATAATACTAATCTATAG (SEQ ID NO:28)

[000125] Csa 08g037600.1 CsTT8-8 on chromosome 8

ATGGATGGATCAATTAATATTCCG**ACAGAGAAATCTGCCGGAGC**TGATAAAAGGGAGCT**T**
CAAGGGCTGCTTAAGGAAGCGGTTCAATCTGTGGAGTGGACTTATAGTCTGTTCTGGCAAT
TTTGTCCGCAACAACGGGTATTGGTGTGGGGGAATGGATACTATAACGGTGCAATAAAGAC
GAGAAAGACAACTCAACCAGCGGAGGTGACGGCAGAAGAGGCTGCACTAGAGAGGAGCCA
ACAGCTGAGGGAGCTTTACGAGACGCTTTTAGCGGGAGAGTCAACATCGGAAGCGAGAGCA
TGCACCGCATTATCGCCAGAGGATTTGACGGAGACTGAATGGTTTTATCTAATGTGTGTCTCT
TTCTCTTTCTCTCCTCCTTCAGGGATGCCAGGAAAAGCGTATGCGAGGAGGAAGCATGTATG
GCTAAGTGGTGCAAATGAGGTTGACAGTAAAACTTTTTGTAGGGCTATTCTCGCTAAGAGTG
CCAAAATTCAGACAGTGGTTTGTATTCCCATGCTTAATGGTGTTGTGGAACTTGGCACAACG
AAGAAGGTAAAAGAAGATGTAGAGTTTGTTGAGCTAATAAAGAGTTTCTTCATTGGCTACTC
TAATTCGATCCCAAAGCCTGCTCTTTCTGAACACTCCACCTACGAAGTGCATGAAGAAGTTG
AAGAAGAAGAAGAGGAAGAAGAAGAAGAAGAAGAAGAGATGACAATGTCAGGGGAGATG
AGGCTTGGCTCTCCTGATGATGACGACGTCTCCAATCAAAATCTACGCTCTGATCTTCATATA
GAATCAACCCACACGTTAGATACACACATGGACATGATGAACTTAATGGAGGAAGGTGGAA
TTTACTCTCAGACAGTAACAACACTTCTCATGTCGCATCCCACGAGTCTTCTTTCAGATTCAG
TTTCCACATCTTCTTACGTCCAATCATCTTTTTCCACGTGGAGGGCTGAGAATGTCAAAGACC
ATCAGCGAGTGGAGTCGCAATGGATGCTCAAACACATGATCTTGAGAGTTCCTTTCCTCCAT
GACAACACTAAAGATAAGAGTCTTCCTCGGGAAGACCTGAACCACGTAGTGGCAGAGCGAC
GAAGGAGAGAGAAGTTGAACGAGAAATTTACAACGTTGAGATCAATGGTTCCATTTATGAC
CAAGATGGATAAAGTATCAATCCTTGGAGACACTATCGAGTACGTAAATCATCTTAGAAAG
AGAGTACACGAGCTTGAGACTACTCATCACGATAAACAACATAAGCGCACACGTACTTGTA
AGAGAAAAACATCGGAGGAGGTGGAGGTTTCCATCATAGAGAGCGACGTTTTGTTAGAGAT
GAGATGTGAGTACCGAGATGGTTTGTTGCTTGACATTCTTCAGGTTCTTCATGATCTTGGTAT
AGAGACTACCTCGGTTCATACCGCGGTGAACGACGGTGATTTCGAGGCGGAGATAAGGGCA
AAAGTAAGAGGGAAGAAAGCAAGCATCGTTGAGGTCAAAAGAGCCATCCACCAAGTCATTA
TACATAATTAA (SEQ ID NO:29)

[000126] Csa 13g044750.1 CsTT8-13 on chromosome 13

ATGGATGGATCAAGTATTATTCCG**ACAGAGAAATCTGCCGGAGC**TGAGAAAAGGGAGCT**T**
CAAGGGCTGCTTAAGGAAGCGGTTCAATCTGTGGAGTGGACTTATAGTCTGTTCTGGCAAT
TTTGTCCTCAACAACGGGTATTGGTGTGGGGGAATGGATACTATAACGGTGCAATAAAGACG

AGAAAGACAACTCAACCAGCGGAGGTGACGGCAGAAGAGGCTACACTAGAGAGGAGCCAA
CAGCTGAGGGAGCTTTACGAGACGCTTTTAGCGGGAGAGTCAACGTCGGAAGCGAGAGCAT
GCACCGCATTGTCGCCGGAGGATTTGACGGAGACTGAATGGTTTTATCTAATGTGTGTCTCTT
TCTCTTTCTCTCCTCCTTCAGGGATGCCAGGAAAAGCGTATGCGAGGAGGAAGCATGTATGG
CTAAGTGGTGCAAATGAGGTTGACAGTAAAACTTTTTGTAGGGCTATTCTCGCTAAGAGTGC
CAAAATTCAGACAGTGGTTTGTATTCCCATGCTTAATGGTGTTGTGGAACTTGGCACAACGA
AGAAGGTAAAAGAAGACGTAGAGTTTGTTGAGCTAGTAAAGAGTTTCTTCATTGGCTACTCT
AATTCGATCCCAAAGCCTGCTCTTTCTGAACACTCCACCTACGAAGTGCATGAAGAAGAAGA
AGAAGAAGAAGCAGAAGTAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAGATGA
CAATGTCAGAGGAGATGAGGTTTGGCTCTCCTGATGATGACAACGTCTCCAATCAAAATCTA
CGCTCTGATCTTCATATAGAATCAACCCACACGTTAGATACACACATGGACATGATGAACTT
AATGGAGGAAGGTGGAATTTACTCTCAGACAGTAACAACACTTCTCATGTCGCATCCCACGA
GTCTTCTTTCAGATTCAGTTTCCACATCTTCTTACGTCCAATCATCTTTTTCCACGTGGAGGGC
TGAGAATGTCAAAGACCATCAGCGAGTGGAGTCGCAATGGATGCTCAAACACATGATCTTG
AGAGTTCCTTTCCTCCATGACAACACTAAAGAAAAGAGTCTTCCTCGAGAAGACCTGAACCA
CGTAGTGGCAGAGCGACGAAGGAGAGAGAAGTTGAACGAGAAATTCACAACGTTGAGATC
AATGGTTCCATTTATGACCAAGATGGATAAAGTATCAATCCTTGGAGACACTATCGAGTACG
TAAATCATCTTAGAAAGAGAGTACACGAGCTTGAGACTACTCATCACGATAAACAACATAA
GCGGACACGTACTTGTAAGAGAAAAACATCGGAGGAGGTGGAGGTTTCCATCATAGAGAGC
GATGTTTTGTTAGAGATGAGATGTGAGTACCGAGATGGTTTGTTGCTTGACATTCTTCAGGTT
CTTCATGATCTTGGTATAGAGACTAGCTCGGTTCATACCGCCGTGAACGACGGTGATTTCGA
GGCGGAGATAAGGGCAAAAGTAAGAGGGAAGAAAGCAAGCATCGTTGAGGTCAAAAGAGC
CATCCACCAAGTCATTATACATAATTAA (SEQ ID NO:30)

[000127] As described herein, mutation of TT8 genes in corresponding *Brassicacea* family plants is contemplated to provide other species with increased oil content in their seeds, and also yellow colored seeds. This renders these plant species more suitable for seed oil production and applications to biofuels. Examples of other *Brassicaceae* family plant sequences, particularly homologous TT8 sequences, for alteration and null mutation can be identified by one skilled in the art. Such sequences can be identified including in public sequence databases. For example, a BLAST search of the NCBI sequence protein database with the any of the three TT8 genes of C. sativa identifies homologous gene sequences in other plants, including in other *Brassicacea* family plants.

[000128] Various related or distinct plant species TT8 proteins are therefore known and available. Exemplary sequences from other plants can readily be identified and compared or aligned with the one or more *C. sativa* TT8 sequences hereof so as to provide comparable corresponding amino acids to generate further or alternative TT8 mutants in accordance with the invention.

[000129] In an aspect, TT8 sequences in other *Brassicacea* family members are identified as candidates for mutation of their TT8 sequence(s) to increase oil content in their seeds. TT8 sequences identified in other plants by databasing searching based on the *C. sativa* TT8-2, TT8-8 and TT8-13 amino acid sequences include sequences in *Brassicacea* family members of several plant species. These various TT8 relative sequences can be particularly ordered and characterized by virtue of their percent amino acid sequence identity, the percentage of total amino acids in the full length protein which are identical in comparison to any or each or all of *C. sativa* TT8-2, TT8-8 and/or TT8-13 sequence. In a particular aspect, TT8 sequences having at least 75% identity, at least 78% identity, at least 80% identity, at least 85% identity to *C. sativa* TT8-2, TT8-8 and/or TT8-13 sequence are selected as candidates. In a particular aspect, TT8 sequences having at least 80% identity to *C. sativa* TT8-2, TT8-8 and/or TT8-13 sequence are selected as candidates.

[000130] Among the TT8 sequences in other *Brassicacea* family members in particular are: Pennycress plant *Thlapsi arvense* (CAH2070747.1); *Capsella rubella* (XP_023636554.1, XP_023636553.1, EOA21963.1); *Cardamine amara subsp. amara* (KAL1219929.1, KAL1196018.1, KAL1219930.1, KAL1196019.1); *Eutrema salsugineum* (XP_024009789.1, ESQ38593.1); and *Arabis alpina* (KFK32044.1).

[000131] In addition, various species of the *Arabidopsis* genus, which is a member of the *Brassicacea* family, are identified as candidates, including the scientific study model plant organism *Arabidopsis thaliana*. TT8 sequences in other Arabidopsis genus family members include those of the species *Arabidopsis lyrate subsp.lyrata* (XP002872462.1), *Arabidopsis arenosa* (CAE6129862.1), *Arabidopsis suecica* (KAG7557235.1, KAG7557236.1) and *Arabidopsis helleri* (CAL9232203.1).

[000132] In addition, various species of the *Brassica* genus, which is a member of the *Brassicacea* family, are identified as candidates, including the rapeseed plant *Brassica napus*. TT8 sequences in other Arabidopsis genus family members include those of the species *Brassica carinata* (KAG2330249.1), *Brassica rapa* (RID45596.1, XP_009113574.1) and *Brassica oleracea var. botrytis* (ADP76654.1), *Brassica oleracea var. italica* (QGW62411.1) and *Brassica oleracea var. oleracea* (XP_013609428.1).

[000133] In addition, phylogenetic analysis can be utilized and provides related *TT8* homologs in other plants. For example, Figure 8 provides TT8 target and related sequences n various plants, particularly AtTT8 (Q9FT81) in *Arabidopsis thaliana*; BnA09.TT8 (MN399821) and BnC09.TT8b (MN399822) in *Brassica napus*; BoTT8a (ADV03944), BoTT8b (ADP76654) in *Brassica oleracea*; BjuA.TT8 (AIN41653.1) in *Brassica juncea*; RsTT8 (ASF79354.1) from *Raphanus sativus*; BrTT8 (XP_009113574) in *Brassica rapa*; LjTT8 (AB490778) in *Lotus japonicus*; RsTT8 (KY651179) in *Raphanus sativus*; LtTT8 (ARK19321.1) in *Lotus tenuis*; LcTT8 (KY196477) in *Lotus corniculatus*; AcbHLH42 (QAT77714) in *Actinidia chinensis*; MtTT8 (KM892777) in *Medicago truncatula*.

[000134] Alternative plant TT8 sequences may further be known or identified by those in the art. US 20090133155A1, for example, describes TT8 sequences in white clover (*Trifolium Repens*), and further references medic (*Medicago*), ryegrass (*Lolium*) and fescus (*Festuca*) species. The sequences and polypeptides are described for introduction to or expression of the wild type polypeptide in plants for modification of flavonoid biosynthesis.

[000135] Corresponding N-terminal region sequences which correlate with the exon 1 sequences in the C. sativa TT8 genes can readily be identified. One skilled in the art can similarly target these sequences for null mutation or deletion or premature termination/truncation using approaches and methods known in the art. In an aspect, alternative plant species TT8 sequences are targeted for null mutation or deletion or premature termination/truncation via CRISPR mediated disruption.

[000136] Thus, alternative CRISPR constructs can be designed, either as alternatives targeting the *C. sativa* TT8 sequences provided and disclosed herein, or as alternatives targeting TT8 sequences in other plants, particularly in *Brassicaceae* family plant sequences. In an aspect, alternative CRISPR constructs are designed targeting TT8 sequences in other plants, particularly in *Brassicaceae* family plant sequences, particular in plants which do not have yellow seeds, including wherein no or few yellow seed varieties are known or available. For example, Pennycress *Thlapsi arvense* and *Cardamine amara subsp. amara* have brown to black or brown seeds, respectively. The plant *Capsella rubella* has orange seeds.

[000137] In an aspect of the invention, disrupted or mutated TT8 polypeptide(s) are provided or constructed wherein one or more amino acid substitution and/or one or more amino acid deletion are introduced such that the normal or wild type TT8 is not expressed or produced, particularly wherein any such disrupted or mutated TT8 polypeptide(s) are inactive.

[000138] Disrupted or mutated TT8 polypeptide(s) are provided having one or more amino acids deleted and/or which are prematurely terminated. In one such aspect, one or more amino acids are deleted in the first exon of TT8 and/or the encoded TT8 polypeptide is prematurely terminated, particularly in the first exon.

[000139] Nucleic acids or polynucleotides encoding the disrupted or mutated TT8 polypeptide(s) are also provided. In another aspect, the invention provides a construct containing a polynucleotide that encodes a disrupted or mutated TT8 polypeptide(s) as provided herein. In a further aspect the invention provides an expression construct comprising a polynucleotide of the invention. In one embodiment the polynucleotide in the construct is operably linked to a promoter sequence. In one embodiment the promoter sequence is capable of driving expression of the polynucleotide in a vegetative tissue of a plant. In another embodiment the promoter sequence is capable of driving expression of the polynucleotide in a seed of a plant. In a further embodiment the promoter sequence is capable of driving expression of the polynucleotide in the pollen of a plant. In a further embodiment the promoter sequence is capable of driving expression of the polynucleotide in a bacterial cell or yeast cell.

[000140] In another embodiment the invention provides a host cell comprising a construct of the invention. In an embodiment the invention provides a host cell genetically modified to comprise a polynucleotide of the invention. In a further embodiment the invention provides a host cell genetically modified to express a polynucleotide of the invention.

[000141] In a further embodiment the host cell is also genetically modified to express a TAG synthesizing enzyme. In one such aspect, the host cell is genetically modified to comprise a nucleic acid sequence encoding a TAG synthesizing enzyme. In another aspect the host cell comprises an expression construct including a nucleic acid sequence encoding a TAG synthesizing enzyme.

[000142] In a further embodiment the nucleic acid is operably linked to a promoter sequence. The promoter sequence may capable of driving expression of the nucleic acid sequence in a vegetative tissue of a plant. In one aspect the promoter sequence is capable of driving expression of the nucleic acid sequence in a seed of a plant or in the pollen of a plant. The promoter sequence may be capable of driving expression of the polynucleotide in a bacterial cell or in a yeast cell.

[000143] The host cell may be any suitable type of cell, including a prokaryotic cell or a eukaryotic cell. In one embodiment the host cell is selected from a bacterial cell, a yeast cell, a fungal cell, an insect cell, algal cell, and a plant cell. In a particular embodiment the host cell is a plant cell. The invention further provides a plant comprising a plant cell of the invention. In one aspect the invention provides a plant comprising a construct of the invention. In an aspect the invention provides a plant genetically modified to comprise or to express a polynucleotide of the invention.

[000144] In a further aspect, the plant is also genetically modified to express a TAG synthesizing enzyme. In a further embodiment the TAG synthesizing enzyme is expressed in the same tissue as the modified oleosin.

In a further embodiment the plant is genetically modified to comprise a nucleic acid sequence encoding a TAG synthesizing enzyme. In a further embodiment the plant comprises an expression construct including a nucleic acid sequence encoding a TAG synthesizing enzyme. In another aspect the plant may be genetically modified to express or overexpress DGAT2 and/or WRI1.

[000145] The nucleic acid or polynucleotide of the invention may be operably linked to a promoter sequence. In an aspect, the promoter is suitable and applicable for expression in plants. In an aspect, the promoter is a constitutive promoter. In an aspect, the promoter is an inducible promoter. In an aspect, the promoter is a plant specific promoter, or a promoter directing expression in leaves, tissues or seeds of a plant. In an aspect, the promoter sequence is capable of driving expression of the nucleic acid sequence in a vegetative tissue of a plant. In one embodiment the promoter sequence is capable of driving expression of the nucleic acid sequence in a seed of a plant. In one embodiment the promoter sequence is capable of driving expression of the nucleic acid sequence in the pollen of a plant. In aspects, the

promoter may be the constitutive promoter 35S or may be a seed promoter, particularly a strong seed promoter such as the promoter for the gene phaseolin.

[000146] The promoters suitable for use in the constructs of this invention are functional in a cell, tissue or organ of a monocot or dicot plant and include cell-, tissue- and organ-specific promoters, cell cycle specific promoters, temporal promoters, inducible promoters, constitutive promoters that are active in most plant tissues, and recombinant promoters. Choice of promoter will depend upon the temporal and spatial expression of the cloned polynucleotide, so desired. The promoters may be those normally associated with a transgene of interest, or promoters which are derived from genes of other plants, viruses, and plant pathogenic bacteria and fungi. Those skilled in the art will, without undue experimentation, be able to select promoters that are suitable for use in modifying and modulating plant traits using genetic constructs comprising the polynucleotide sequences of the invention. Examples of constitutive plant promoters include the CaMV 35S promoter, the nopaline synthase promoter and the octopine synthase promoter, and the Ubi 1 promoter from maize. Plant promoters which are active in specific tissues, respond to internal developmental signals or external abiotic or biotic stresses are described in the scientific literature. Exemplary promoters are described, e.g., in WO 02/00894, which is herein incorporated by reference.

[000147] The relative terms, such as increased and reduced as used herein with respect to plants, are relative to a control plant. Suitable control plants include non-transformed or wild-type versions of plant of the same variety and/or species as the transformed plant used in the method of the invention. Suitable control plants also include plants of the same variety and/or species as the transformed plant that are transformed with a control construct. Suitable control constructs include empty vector constructs, known to those skilled in the art. Suitable control plants also include plants that have not been transformed with a polynucleotide encoding a modified oleosin including at least one artificially introduced cysteine. Suitable control plants also include plants that do not express a modified oleosin including at least one artificially introduced cysteine.

[000148] The term "total lipid" as used herein includes fats, oils, waxes, sterols, glycerol lipids, monoglycerides, diglycerides, phospholipids, monogalactolipids, digalactolipids, phosphatidylcholines, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, sulfoguinovosyldiacylglycerol, and triglycerides.

[000149] The term "oil" as used herein preferably refers to triacylglycerol (TAG).

[000150] The term "biomass" refers to the size and/or mass and/or number of vegetative organs of the plant at a particular age or developmental stage. Thus a plant with increased biomass has increased size and/or mass and/or number of vegetative organs than a suitable control plant of the same age or at an equivalent developmental stage. Increased biomass may also involve an increase in rate of growth and/or rate of formation of vegetative organs during some or all periods of the life cycle of a plant relative to a

suitable control. Thus increased biomass may result in an advance in the time taken for such a plant to reach a certain developmental stage.

[000151] The terms "seed yield", "fruit yield" and "organ yield" refer to the size and/or mass and/or number of seed, fruit or organs produced by a plant. Thus a plant with increased seed, fruit or organ yield has increased size and/or mass and/or number of seeds, fruit or organs respectively, relative to a control plant at the same age or an equivalent developmental stage.

[000152] It may be beneficial, when producing a transgenic plant from a particular species, to transform such a plant with a sequence or sequences derived from that species. The benefit may be to alleviate public concerns regarding cross-species transformation in generating transgenic organisms. Additionally when down-regulation of a gene is the desired result, it may be necessary to utilize a sequence identical (or at least highly similar) to that in the plant, for which reduced expression is desired. For these reasons among others, it is desirable to be able to identify and isolate orthologues of a particular gene in several different plant species.

[000153] The invention further provides plant cells which comprise a genetic construct of the invention, and plant cells modified to alter expression of a polynucleotide or polypeptide of the invention, or used in the methods of the invention. Plants comprising such cells also form an aspect of the invention.

[000154] Methods for transforming plant cells, plants and portions thereof with polypeptides are described in Draper et al., 1988, Plant Genetic Transformation and Gene Expression. A Laboratory Manual. Blackwell Sci. Pub. Oxford, p. 365; Potrykus and Spangenburg, 1995, Gene Transfer to Plants. Springer-Verlag, Berlin.; and Gelvin et al., 1993, Plant Molecular Biol. Manual. Kluwer Acad. Pub. Dordrecht. A review of transgenic plants, including transformation techniques, is provided in Galun and Breiman, 1997, Transgenic Plants. Imperial College Press, London.

[000155] A number of plant transformation strategies are available (e.g. Birch, 1997, Ann Rev Plant Phys Plant Mol Biol, 48, 297, Hellens R P, et al (2000) Plant Mol Biol 42: 819-32, Hellens R et al Plant Meth 1: 13). For example, strategies may be designed to increase expression of a polynucleotide/polypeptide in a plant cell, organ and/or at a particular developmental stage where/when it is normally expressed or to ectopically express a polynucleotide/polypeptide in a cell, tissue, organ and/or at a particular developmental stage which/when it is not normally expressed. The expressed polynucleotide/polypeptide may be derived from the plant species to be transformed or may be derived from a different plant species. Transformation strategies may be designed to reduce expression of a polynucleotide/polypeptide in a plant cell, tissue, organ or at a particular developmental stage which/when it is normally expressed. Such strategies are known as gene silencing strategies.

[000156] Exemplary terminators that are commonly used in plant transformation genetic construct include, e.g., the cauliflower mosaic virus (CaMV) 35S terminator, the Agrobacterium tumefaciens

nopaline synthase or octopine synthase terminators, the Zea mays zein gene terminator, the Oryza sativa ADP-glucose pyrophosphorylase terminator and the Solanum tuberosum PI-II terminator.

[000157] Selectable markers commonly used in plant transformation include the neomycin phophotransferase II gene (NPT II) which confers kanamycin resistance, the aadA gene, which confers spectinomycin and streptomycin resistance, the phosphinothricin acetyl transferase (bar gene) for Ignite (AgrEvo) and Basta (Hoechst) resistance, and the hygromycin phosphotransferase gene (hpt) for hygromycin resistance.

[000158] Use of genetic constructs comprising reporter genes (coding sequences which express an activity that is foreign to the host, usually an enzymatic activity and/or a visible signal (e.g., luciferase, GUS, GFP) which may be used for promoter expression analysis in plants and plant tissues are also contemplated. The reporter gene literature is reviewed in Herrera-Estrella et al., 1993, Nature 303, 209, and Schrott, 1995, In: Gene Transfer to Plants (Potrykus, T., Spangenberg. Eds) Springer Verlag. Berline, pp. 325-336.

[000159] The following are representative publications disclosing genetic transformation protocols that can be used to genetically transform the following plant species: Rice (Alam et al., 1999, Plant Cell Rep. 18, 572); apple (Yao et al., 1995, Plant Cell Reports 14, 407-412); maize (U.S. Pat. Nos. 5,177,010 and 5,981,840); wheat (Ortiz et al., 1996, Plant Cell Rep. 15, 1996, 877); tomato (U.S. Pat. No. 5,159,135); potato (Kumar et al., 1996 Plant J. 9, :821); cassaya (Li et al., 1996 Nat. Biotechnology 14, 736); lettuce (Michelmore et al., 1987, Plant Cell Rep. 6, 439); tobacco (Horsch et al., 1985, Science 227, 1229); cotton (U.S. Pat. Nos. 5,846,797 and 5,004,863); grasses (U.S. Pat. Nos. 5,187,073 and 6,020,539); peppermint (Niu et al., 1998, Plant Cell Rep. 17, 165); citrus plants (Pena et al., 1995, Plant Sci. 104, 183); caraway (Krens et al., 1997, Plant Cell Rep, 17, 39); banana (U.S. Pat. No. 5,792,935); soybean (U.S. Pat. Nos. 5,416,011; 5,569,834; 5,824,877; 5,563,04455 and 5,968,830); pineapple (U.S. Pat. No. 5,952,543); poplar (U.S. Pat. No. 4,795,855); monocots in general (U.S. Pat. Nos. 5,591,616 and 6,037,522); brassica (U.S. Pat. Nos. 5,188,958; 5,463,174 and 5,750,871); cereals (U.S. Pat. No. 6,074,877); pear (Matsuda et al., 2005, Plant Cell Rep. 24(1):45-51); Prunus (Ramesh et al., 2006 Plant Cell Rep. 25(8):821-8; Song and Sink 2005 Plant Cell Rep. 2006; 25(2):117-23; Gonzalez Padilla et al., 2003 Plant Cell Rep. 22(1):38-45); strawberry (Oosumi et al., 2006 Planta. 223(6):1219-30; Folta et al., 2006 Planta April 14; PMID: 16614818), rose (Li et al., 2003), Rubus (Graham et al., 1995 Methods Mol. Biol. 1995; 44:129-33), tomato (Dan et al., 2006, Plant Cell Reports V25:432-441), apple (Yao et al., 1995, Plant Cell Rep. 14, 407-412), Canola (Brassica napus L.). (Cardoza and Stewart, 2006 Methods Mol. Biol. 343:257-66), safflower (Orlikowska et al, 1995, Plant Cell Tissue and Organ Culture 40:85-91), ryegrass (Altpeter et al, 2004 Developments in Plant Breeding 11(7):255-250), rice (Christou et al, 1991 Nature Biotech. 9:957-962), maize (Wang et al 2009 In: Handbook of Maize pp. 609-639) and Actinidia eriantha (Wang et al., 2006, Plant Cell Rep. 25, 5: 425-31). Transformation of other species is

also contemplated by the invention. Suitable methods and protocols are available in the scientific literature.

[000160] The term "plant" is intended to include a whole plant, any part of a plant, a seed, a fruit, propagules and progeny of a plant.

[000161] The term "propagule" means any part of a plant that may be used in reproduction or propagation, either sexual or asexual, including seeds and cuttings.

[000162] The plants of the invention may be grown and either selfed or crossed with a different plant strain and the resulting hybrids, with the desired phenotypic characteristics, may be identified. Two or more generations may be grown to ensure that the subject phenotypic characteristics are stably maintained and inherited. Plants resulting from such standard breeding approaches also form an aspect of the present invention.

[000163] The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1

[000164] Camelina (*Camelina sativa* L.), a hexaploid member of the Brassicaceae family, is an emerging oilseed crop being developed to meet the increasing demand for plant oils as biofuel feedstocks. In other Brassicas, high oil content can be associated with a yellow seed phenotype, which is unknown for camelina. We sought to create yellow seed camelina using CRISPR/Cas9 technology to disrupt its *Transparent Testa 8* (TT8) genes and to evaluate the resulting seed phenotype.

[000165] We identified three TT8 genes, one in each of the three camelina subgenomes, and obtained independent CsTT8 lines containing frameshift edits. Disruption of TT8 caused seed coat colour to change from brown to yellow reflecting their reduced flavonoid accumulation of up to 44%, and the loss of a well-organized seed coat mucilage layer. Transcriptomic analysis of CsTT8-edited seeds revealed significantly increased expression of the lipid-related transcription factors LEC1, LEC2, FUS3, and WRI1 and their downstream fatty acid synthesis-related targets. These changes caused metabolic remodelling with increased fatty acid synthesis rates and corresponding increases in total fatty acid (TFA) accumulation from 32.4% to as high as 38.0% of seed weight, and TAG yield by more than 21% without significant changes in starch or protein levels compared to parental line. These data highlight the effectiveness of CRISPR in creating novel enhanced-oil germplasm in camelina. The resulting lines have applicability to future net-zero carbon energy

production or to be combined with other traits to produce desired lipid-derived bioproducts at high yields.

[000166] RESULTS

[000167] Identification of TT8 homologs in Camelina sativa

[000168] Using the sequence of *AtTT8* to BLAST against the camelina genome of *C. sativa* (Kagale, et al., 2014), we identified three *TT8* isoforms designated as *CsTT8-2* (*Csa02g028180.1,*), *CsTT8-8* (*Csa08g037600.2*) and *CsTT8-13* (*Csa13g044750.1*) located on chromosomes 2, 8 and 13, respectively. *CsTT8-2* and *CsTT8-8* each contain six exons, while *CsTT8-13* has an extra exon FIG. 1(a). Like *AtTT8*, the predicted amino acid sequences of all three *CsTT8*s contain a series of conserved domains: the N - terminal MYB interaction region (MIR), the bHLH domain in the C - terminal region (FIG. 1(a)). The *CsTT8-2* protein shares 92.46% and 91.87% identity to *CsTT8-8* and *CsTT8-13*, respectively. *CsTT8-8* and *CsTT8-13* protein are 97.84% identical at the amino acid level. The high identity shared by all three *CsTT8* genes suggests that they encode transcription factors with the same or similar functions. Phylogenetic analysis shows that all three *CsTT8* copies cluster in a clade with *AtTT8* (FIG. 8). In addition, assessment of gene synteny relationships with the GenomicusPlantv49.01 online resource (doi.org/10.1093/nar/gkx1003) showed that *CsTT8-2*, *CsTT8-8* and *CsTT8-13* are syntenic orthologs to *AtTT8*. Taken together this suggests similar, or equivalent functions of these three *TT8* genes in camelina.

[000169] Expression analysis of the CsTT8 genes.

[000170] The tissue specific expression patterns of *CsTT8* isoforms were investigated in camelina variety Suneson using quantitative real - time PCR (qRT - PCR) (FIG. 1(b)). Various amounts of transcript were detected for *CsTT8-2, CsTT8-8* and *CsTT8-13* in leaf, root, stem and seeds of 12, days afer flowering (DAF), 24 DAF and mature seeds. All three *TT8* genes exhibit high expression levels in developing seeds with the highest levels observed at 12 days DAF. Their transcription in roots is much lower than in seeds, but is higher than that in leaves and stems, where it is barely detectable (FIG. 1(b)). These results suggest that *CsTT8*s plays a role in seed development. Overall, *CsTT8-8* had a significantly higher expression level (by 1.5 to 3 fold) than *CsTT8-2 and CsTT8-13* during seed development (FIG. 1(b)). Further analysis of *CsTT8* genes in developing seeds by RNAseq revealed strong expression for all three *TT8* genes (FIG. 1(c)). High expression levels were detected at early stages that peaked at 10-12 DAF, and subsequently decreased from 14-22 DAF. Again, *CsTT8-8* demonstrated the highest expression at the peak stages, followed by *CsTT8-2*, while *CsTT8-13* exhibited the lowest expression level (FIG. 1(c)). These results are consistent with those obtained from RT-qPCR, although the 24 DAF seeds showed higher expression by RT-PCR, possibly due to different growth conditions.

[000171] Creation of CRISPR/Cas9 - targeted mutations in CsTT8s

[000172] To generate Cas9 - induced knockout mutations in all three *CsTT8* genes, two sets of sgRNAs (S1 and S2) were designed using the CRISPR - P algorithm (Lei et al., 2014). Both sets are in the first exon of *TT8*, with S1 close to the start codon, and S2 targeting the MIR domain FIG. 1(a). To facilitate the screening of transgenic lines and achieve transgene-free *CsTT8* mutagenized camelina lines, a *DsRed* expression cassette was inserted into the pHEE401E vector (Wang et al., 2015; Xie et al., 2015). We obtained and planted 33 transgenic seeds with DsRed fluorescence, of which 14 lines were genotyped by DNA sequencing. In the first generation (T_1) (Table 1), lines 13, 22, 26 and 27 showed DNA sequence changes in all three *CsTT8* isoforms, and lines 13 and 22 showed one homozygous target site changed for only a single isoform. Lines 24, 25, 31, 32, and 33 showed mutations in at least one target site in one or two of the three isoforms. But lines 23, 28, 29, 30 and 34 remained unedited in all three isoforms. We then planted dark and red seeds from lines 13, 22, 26, and 27 and genotyped the progeny (T_2) plants (Table 1). Lines 26-6, 26-7, and 27-9 contained homozygous changes in all three isoforms (FIG. 2(a)). Mutations in all three genes resulted in frameshifts that truncate TT8 within the first 65 amino acid resulting in loss of function except for tt8-2 gene in line 26–7, in which deletion of 48 bp of DNA resulted in deletion of 16 amino acid (Figure 9(a)) resulting in loss of function. Moreover, no changes were observed in the predicted off-target sites (Figure 9(b) and (c)). Thus, we obtained three homozygous lines containing edits in all three isoforms of CsTT8 for subsequent studies. Lines 22-1, 22-2, 27-8, 27-10, and 27-11 had at least one homozygous mutation.

Table 1. Genotyping of CRISPR-CsTT8 transgenic T1 and T2 plants

Plant ID	Generation	Genotype at targets of *CsTT8-2*		Genotype at targets of *CsTT8-8*		Genotype at targets of *CsTT8-13*		Seed color
		S1	S2	S1	S2	S1	S2	
13	T1	homo (-3bp)	homo (-4bp)	hetero	hetero	hetero	hetero	yellow
22	T1	hetero	hetero	wt	homo (-4bp)	hetero	hetero	yellow
22-1	T2	homo (-17bp)	homo (-18bp)	wt	homo (-4bp)	hetero	hetero	
22-2	T2	homo (-17bp)	homo (-18bp)	wt	homo	hetero	hetero	
22-3	T2	hetero	hetero	wt	homo (-4bp)	homo (-15bp)	homo (-18bp)	
24	T1	wt	wt	wt	hetero	wt	hetero	brown
25	T1	wt	hetero	wt	wt	hetero	hetero	brown
26	T1	hetero	hetero	hetero	hetero	hetero	hetero	yellow
26-4	T2	hetero	hetero	homo (-17bp)	homo (-18bp)	hetero	hetero	
26-5	T2	hetero	hetero	hetero	hetero	hetero	hetero	
26-6	T2	homo (+1bp)	homo (-4bp)	wt	homo (-4bp)		h(-49bp)	
26-7	T2		h(-48bp)	wt	homo (-4bp)	wt	homo (-1bp)	
27	T1	wt	hetero	hetero	hetero	hetero	hetero	yellow
27-10	T2	wt	hetero	wt	homo (-5bp)	wt	homo (-4bp)	
27-11	T2	wt	homo (+1bp)	hetero	hetero	hetero	hetero	

27-8	T2	wt	hetero	hetero	hetero	homo (-1bp)	homo (-5bp)	
27-9	T2	wt	homo (+1bp)	wt	homo (-5bp)	wt	homo (-5bp)	
31	T1	hetero	hetero	wt	wt	wt	wt	brown
32	T1	hetero	hetero	wt	wt	wt	wt	tan
33	T1	wt	hetero	hetero	hetero	hetero	wt	brown

[000173] The sequences of the qPCR primers and Golden gate clone used in these studies are provided in Table 2 below.

Primers name	Primer sequence
FUS3-F	TCTACGACACGACGGCTTTG (SEQ ID NO:31)
FUS3-R	ATTCTCAACGGAGCCCAAAC (SEQ ID NO:32)
LEC1-F	GTAAAAGCTTACCGGCGCAC (SEQ ID NO:33)
LEC1-R	CCAAGCTTGCTCATAGCCCA (SEQ ID NO:34)
LEC2-F	CCGGGAAGACAAAATGGAGGA (SEQ ID NO:35)
LEC2-R	AGGGTTGGGATAGTGATCGT (SEQ ID NO:36)
FabG-F	GCAATCACAATGGGACGAAGT (SEQ ID NO:37)
FabG-R	GCAGCGTAGTTTGCTTGACC (SEQ ID NO:38)
FabI-F	TGGAGACATCGAGGAAAGGC (SEQ ID NO:39)
FabI-R	TTCCTCCACCATACCCAGGA (SEQ ID NO:40)
FAD2-F	ATCCTGCGTTGCCTCACTAC (SEQ ID NO:41)
FAD2-R	CCACATGTGTGTCCGTGATG (SEQ ID NO:42)
FAD3-F	TTGTGATGTGGTTGGACGCT (SEQ ID NO:43)
FAD3-R	ACGTGAGTTCCAATGTCGTGA (SEQ ID NO:44)
BCCP1-F	AAGAATGGCGTCTTCGTCGT (SEQ ID NO:45)
BCCP1-R	ACAGGGTAGCTACTGCGACT (SEQ ID NO:46)
BCCP2-F	CCCTCCGGTGTATCTCAGAC (SEQ ID NO:47)
BCCP2-R	CACAGTCGAGCTGCTTCAGT (SEQ ID NO:48)
BC-F	GTGCTCTCGGTGCTACATGC (SEQ ID NO:49)
BC-R	AGTACGACTGGTTGCTGGGA (SEQ ID NO:50)
α-CT-F	AAGCAGAGATGGCTGGTGTG (SEQ ID NO:51)
α-CT-R	AGGTTTTCGGATGGGGCAAA (SEQ ID NO:52)
KASI-F	AGAAGGGTCACAGGAGGATT (SEQ ID NO:53)
KASI-R	GCAGCAGCGTAAAAGCAGTAG (SEQ ID NO:54)
KASII-F	TTGGCTCAGCAATGGGAGG (SEQ ID NO:55)
KASII-R	AGCATAGCAGAACCCATGTT (SEQ ID NO:56)
KASIII-F	ACCATAGCCACATCCGGTTT (SEQ ID NO:57)
KASIII-R	CCAAGTTTCCAGTGTTCCTCG (SEQ ID NO:58)
WRI1-F	GGAGGCTCGGATTGGAAGAG (SEQ ID NO:59)
WRI1-R	TGGTTAGCTTGGCTCACAGG (SEQ ID NO:60)
BADC1-F	TCCCTCAGTTGGCGAGTTTC (SEQ ID NO:61)
BADC1-R	CTTCCCCAGCTACATCCGAC (SEQ ID NO:62)
BADC2-F	AGCCACTTCCTCGTCATTGG (SEQ ID NO:63)
BADC2-R	TGGCCACCGAGTTGTTCAAT (SEQ ID NO:64)

BADC3-F	TCGGTGGCCAATTCCCAATA (SEQ ID NO:65)
BADC3-R	TCCCAGGGAAAGAGGGAAGG (SEQ ID NO:66)
CsTT8-2-F	GATCTTGAGAGTTCCTACAACACTAAAG (SEQ ID NO:67)
CsTT8-2-R	AGTAGTCTCAAGCTCATGGACCCTC (SEQ ID NO:68)
CsTT8-8-F	AGGGCTATTCTCGCTAAGACAGTGGTT (SEQ ID NO:69)
CsTT8-8-R	TTCTTCTTCCTCTTCTTCTTCTTCAACTTC (SEQ ID NO:70)
CsTT8-13-F	TTCTCGCTAAGAGTGCCAAAATTCAGAC (SEQ ID NO:71)
CsTT8-13-R	TTCTTCTTCTTCTTCTTCTACTTCTGC (SEQ ID NO:72)
CsTT8CrS1F2	TAGAGTCGAAGTAGTGATTG GCTCCGGCAGATTTCTCTGT GTTTTAGAGCTAGAAATAGC (SEQ ID NO:73)
CsTT8CrS2R2	GCTATTTCTAGCTCTAAAAC CTTCCTTAAGCAGCCCTTGA TGCACCAGCCGGGAATCGAA (SEQ ID NO:74)
CsTT8-F	GGAATGGATACTATAACGGTGCAAT (SEQ ID NO:75)
CsTT8-2R	AAAGTAGATAGATCAGCGTTAGTGTA (SEQ ID NO:76)
CsTT8-8R	ATAGTTATATACGCTATTTTTACATTTTTT (SEQ ID NO:77)
CsTT8-13R	TAGGCTATTTTTACTTTTTTTATATATATG (SEQ ID NO:78)

[000174] Editing CsTT8 produced yellow seed coat

[000175] We hoped that successful mutagenesis of the *CsTT8* by CRISPR could result in lighter color in camelina seeds, as disruption of *AtTT8* in *Arabidopsis* resulted in reduced seed pigmentation (Nesi et al., 2000). The seeds of T_1 lines 13, 22, 26, and 27 appeared yellow, whereas seeds of other lines remained brown (FIG. 10). In lines 21, 30, 31, 32, and 34, seed coat color appeared light brown or tan. A reddish-brown color was observed in the seeds of lines 24 and 33. The seed coat color changed from brown to pale yellow in the T2 lines 26-7, 26-8, and 27-9 (FIG. 2(b)), correlated with their homozygous CRISPR-induced *CsTT8* mutations (FIG. 2(a)). Interestingly, no discernible alterations in growth or development were observed in these camelina plants, in which all three *CsTT8* genes had been disrupted but were free of the Cas9 and DsRed marker transgenes (FIG. 11).

[000176] Effect of CsTT8 mutations on flavonoid content in camelina seeds

[000177] Seeds were sectioned and examined under a dissection microscope. A brown coloration was evident in the wild type (WT) camelina seed coat endothelium layer. However, in all three *TT8* modification lines containing homozygous disruptions of all three *TT8* genes, this color changed to a light-yellow hue, as illustrated in FIG. 3(a). In *Brassicaceae* plants, the dark seed coat is attributed to the accumulation of an oxidized form of a flavonoid known as proanthocyanidin within the endothelium layer of the inner integument of the seed coat (Lepiniec et al., 2006). To investigate the relationship between *CsTT8* and the production of flavonoids in camelina seeds, their flavonoid contents were extracted and quantified. The flavonoid content decreased markedly in all three *CsTT8* mutant lines albeit with minor variation at 35.5% in line 26-6, 43.9% in line 26-7, and 44.1% in line 27-9, respectively FIG. 4(b). These

findings are consistent with the key role of *CsTT8* in the synthesis or regulation of the flavonoids responsible for brown pigmentation in the seed coats of Brassicaceae plants.

[000178] Editing TT8 in camelina changed the seed coat structure

[000179] Under the confocal microscope, Differential Interference Contrast (DIC) imaging of wild type seeds showed a well-organized outermost layer of mucilage. In contrast, in all three *CsTT8* mutant lines, the mucilage layer was either partially reduced or completely absent as shown in FIG. 4(a). To gain further insights into changes in the mucilage layer, seed cross-sections were stained with toluidine blue O, a basic thiazine metachromatic dye (Sridharan and Shankar, 2012) that stains polysaccharides purple and nucleic acid blue. The wild type seeds consistently exhibited a clearly stained mucilage layer that was notably absent in the seeds of the *TT8*-modified lines, as exemplified in FIG. 4(b). Loss of this layer in the seeds of *TT8* mutagenized lines is a visible indicator of the disruption of *TT8* genes on seed coat morphology. We extracted and quantified their mucilage contents. The mucilage content decreased from 2.1% to as low as 0.4%, i.e., an approximately 80% decrease (Figure 4c).

[000180] While changes in the levels of mucilage has been reported to influence seed germination (Arsovski et al., 2010), the *CsTT8* edited lines exhibited robust germination on soil. Consistent results were observed when 10-month-old seeds were germinated on wet filter paper at a similar rate to that of WT (FIG. 12(a). We also observed longer radicles after 20 hrs of imbibition in the *TT8* edited lines compared to those in the WT, suggesting accelerated germination.

[000181] Editing TT8 in camelina increased seed oil content

[000182] To investigate the potential impact of editing the *CsTT8* genes on other seed metabolites, we performed a comprehensive analysis focusing first on TFA and TAG content. As shown in FIG. 5, seed TFA contents increased in all three edited lines. In WT camelina controls, the TFA content was 32.4% of dry weight (DW). However, disruption of *CsTT8* resulted in large increases in TFA content to 37.5%, 38.0%, and 36.1% in the three distinct *CsTT8* mutant lines, respectively. The TAG content also reached levels as high as 34.6% from 28.5% in WT. This concurrent rise of both TFA and TAG in gene edited seeds demonstrates a substantial influence of *CsTT8* on seed lipid metabolism.

[000183] Fatty acid composition was next analyzed by GC-MS and a distinct shift in the fatty acid profile was observed. Notably, the editing of *CsTT8* led to a significant increase in 18:1 and 18:2 fatty acids, along with a corresponding decrease in 18:3 fatty acids (FIG. 12).

[000184] To provide a more comprehensive analysis, we analyzed the protein and starch contents of the *TT8* mutant lines. As shown in FIG. 13(a), the protein content in these lines remained largely unaffected. While there was some variation in starch content in the mutant lines, the means were not significantly different from those of WT (FIG. 13(b)) The lack of significant changes to protein and starch contents underscores the specificity of *TT8* with respect to lipid metabolism. The hundred-seed weight of 26–6 exhibited no significant change, whereas 26–7 and 27–9 showed a slight decrease (FIG. 13(c)).

[000185] Editing CsTT8 changed the expression levels of genes involved in FA biosynthesis in camelina seeds

[000186] Previous studies suggested that TT8 can negatively regulate seed FA biosynthesis by binding to the promoters of the *LEC1*, *LEC2* and *FUS3* transcription factors (Chen et al., 2014). RT-qPCR analysis showed that editing of *CsTT8* led to substantial increases in the expression levels of these genes in camelina seeds (FIG. 6(a). Expression of the transcription factor *WRI1* and several key genes associated with fatty acid synthesis, including α *carboxyltransferase (CT)*, *biotin carboxyl carrier protein (BCCP)1*, *BCCP2*, *3-KETOACYL ACP SYNTHASE (KAS)I*, *KASII*, and *KASIII* displayed an increasing trend in their expression levels whereas KASIII showed a significant 2.6-fold increase in abundance (FIG. 6(b)). This implies that disruption of *TT8* genes has a positive regulatory effect on these downstream targets that contribute to increased fatty acid production within the seeds.

[000187] The disruption of *TT8* also resulted in subtle changes in the expression of fatty acid desaturase genes. Specifically, the *FAD2* transcript was elevated while the *FAD3* transcript displayed a small decrease in the *CsTT8* edited lines (FIG. 6(c). These changes may have contributed to alterations in the fatty acid composition.

[000188] Editing CsTT8 increased FA biosynthesis rate in camelina seeds

[000189] To further elaborate the effect of CsTT8 on seed oil accumulation, developing seeds at 13-15 DAF were collected and their fatty acid synthesis rates were determined by measuring the rate of [^{14}C]acetate incorporation into FAs. As shown in FIG. 7, compared to WT, all three *CsTT8* triple mutants showed a significant increase in its fatty acid synthesis rate relative to the parental line consistent with the observed changes in FAS-related transcripts.

[000190] DISCUSSION

[000191] CRISPR/Cas9-targeted mutations in CsTT8 created yellow-seed camelina

[000192] Within *Brassica* oil crops, yellow seeds are commonly associated with elevated oil content and reduced pigmentation and hull content (Xie et al., 2020; Zhai et al., 2020). In this study, CRISPR camelina lines were successfully created with mutated *CsTT8* genes that resulted in yellow seeds. Consistent with earlier reports for both *Arabidopsis* and *Brassica napus*, the *camelina* mutants exhibited enhanced fatty acid synthesis, leading to a significant 21.4% increase in seed TAG. Furthermore, a close examination of the seeds confirmed a reduction in flavonoid deposition in the seed coat of *CsTT8* mutants, as shown in FIG. 4(a).

[000193] To streamline the selection of transgenics and desired genotypes and enable the removal of CRISPR/Cas from lines with desired edits, a DsRed marker was introduced into the CRISPR/Cas vector. T_1 transgenic seeds exhibiting DsRed fluorescence were planted for genotyping, and yellow seeds edited for CsTT8 genes but lacking the CRISPR/Cas9 were identified by visual screening. These targeted mutations were stably inherited in subsequent generations, resulting in the establishment of a pool of

homozygous mutants harboring loss-of-function alleles of the target genes for subsequent phenotyping (see FIG. 2(a) and Table 1). The yellow seed phenotype initially appeared in four distinct T_1 transgenic plants, demonstrating that CRISP-Cas9 editing is very efficient in the hexaploid *camelina* genome, as previously reported (Ozseyhan et al., 2018).

[000194] *Camelina sativa* is a hexaploid with its three sub-genomes arising from two separate polyploidization events (Kagale et al., 2014). Three *CsTT8* homeologs were identified in *camelina*, with *CsTT8-8* and *CsTT8-13* exhibiting a closer relationship to each other than to *CsTT8-2* (FIG. 9.) Notably, all three isoforms are preferentially expressed during seed development, with *CsTT8-8* demonstrating a higher expression level compared to the other two isoforms (FIGs. 1(b) and 1(c)). This observation is consistent with a general increase of gene expression in the Cs-G3 subgenome compared to the other two sub-genomes that was attributed to the two-stage polyploidization pathway (Kagale et al., 2014). Our results suggest that all three *CsTT8* genes play overlapping roles in controlling seed coat color because mutagenesis of one or two isoforms did not result in a change of seed coat color from brown to yellow. Indeed, the seed color change was only observed when all three *CsTT8* genes were mutated (Table 1, FIGs 2(b) and 10). The low likelihood of loss of function mutations arising simultaneously in all three *CsTT8* genes provides a likely explanation as to why no naturally occurring yellow seeds in *Camelina sativa* populations have been identified to date.

[000195] CsTT8 mutation accelerated fatty acid synthesis by upregulating the expression of genes involved in FA biosynthesis

[000196] Disruption of *TT8* in camelina increased gene expression especially in key lipogenic transcription factor genes, including *LEC1*, *LEC2*, and *FUS3*. This result is consistent with previous reports in *Arabidopsis* and *B. napus* (Chen et al., 2014). We also observed an upregulated expression of *WRI*, the master transcriptional activator of fatty acid synthesis, and correspondingly increased expression levels of its targets, *αCT*, *BCCP1*, *BCCP2*, *KASI*, *KASII*, and *KASIII* (Kuczynski et al., 2022). The increased expression levels of factors in fatty acid biosynthesis is consistent with the enhanced fatty acid production observed in the *TT8*-edited *camelina* lines.

[000197] Interestingly, disruption of *TT8* also had an impact on the expression of genes encoding fatty acid desaturases. Specifically, the *CsTT8* edited lines had higher *FAD2* but decreased *FAD3* expression favoring the accumulation of 18:2 at the expense of 18:3 (FIG. 5(c).) This differs from *TT8* modification in *B. napus* and *Arabidopsis*. *FAD2* was significantly upregulated in the *Bntt8* mutant, which led to increases in 16:0, 18:2, and 18:3, accompanied by decreases in 18:0 and 18:1 (Zhai et al., 2020). In contrast, the *TT8* mutation in *Arabidopsis* resulted in substantial increases in the expression levels of both *FAD2* and *FAD3*, and the *TT8* mutant showed elevated levels of 18:1 and reduced 16:0, 18:2, and 18:3 (Chen et al., 2014). Hence, *CsTT8* appears to exhibit a distinct function compared to its orthologues in *Arabidopsis* and *Brassica napus*. These findings illustrate the complex regulatory networks involved in seed fatty acid biosynthesis, with *TT8* emerging as a player that influences both the content and

composition of fatty acids in oilseeds, thus providing the potential tool for enhancing oil production and tailoring its composition in camelina.

[000198] CsTT8s are involved in mucilage formation

[000199] Mucilage is a gelatinous substance composed of pectins, cellulose, hemicellulose and proteins that can be found in the seeds of many plants, including Arabidopsis (Haughn and Western, 2012). It is produced by specialized epidermal cells in the seed coat and serves various functions in seed dispersal and provides a protective barrier for the seed (Arsovski et al., 2010). Inactivating all three *CsTT8* genes did not visibly change the internal structure of mucilage secretion cells (MSC), which retained their central volcano-shaped cellulosic structures called the columella, but it effectively minimized mucilage accumulation in the outermost layer of the seed coat, leading to a noticeable reduction in seed coat thickness (FIG. 5.) Thus, it can be concluded that *CsTT8*, a *bHLH* transcription factor, is a positive regulator of seed coat mucilage synthesis. In contrast to camelina, in Arabidopsis the mucilage biosynthetic pathway seems to be redundantly controlled by multiple *bHLH* transcription factors. In *Arabidopsis*, mutants of the *TT8* locus alone do not display any defect in columellae development and mucilage production (Nesi et al., 2000; Zhang et al., 2003), reflecting a possible functional redundancy between *TT8* and ENHANCER OF GLABRA3 (EGL3) because *egl3 TT8* double mutants have collapsed columellae with no releasable mucilage (Zhang et al., 2003).

[000200] The reduction in mucilage biosynthesis in our *CsTT8* mutants might free up carbon resources for FA and TAG synthesis, and therefore could explain the increased oil contents (FIG. 6) based on carbon re-allocation. The relationship between mucilage and oil content in seeds is complex and varies among plant species. For example, in flax, seed coat mucilage was positively correlated with seed oil content (Miart et al., 2021). The transcriptional regulation associated with seed oil and fatty acid metabolism appears to occur in the seed coat during the mid-stage of seed development. (Arsovski et al., 2010). However, an inverse relationship between mucilage and oil content has been reported for other cases including legumes (Tookey and Jones, 1965), such as *Medicago orbicularis* (Tonnet and Snudden, 1974), and *M. truncatula* (Song et al., 2017). These differences have been attributed to resource allocation in seeds of different species based on their reproductive and ecological strategies (Song et al., 2017), that involve various transcription factors. For example, GL2 promotes the formation of non-glandular trichomes and regulates the production of mucilage in seed coat cells. It is also an inhibitor of oil content, impacting the balance between oil and mucilage production (Cheng et al., 2021). AtMIF1 functions as a positive regulator, enhancing oil content by attenuating GL2 inhibition. Its interaction with MYB domain protein 5 (MYB5) disrupts normal transcriptional activation of the MBW complex. Consequently, the expression of GL2, a target of MBW, is reduced. Seeds of the AtMIF1-overexpressing plants no longer secrete mucilage normally, but the oil content is significantly enhanced (Cheng et al., 2021). The MBW complex is implicated in seed coat mucilage and the biosynthesis of flavonoids in the endothelium (Zumajo-Cardona et al., 2023). Downstream targets of TTG1/MYB/bHLH complexes include other

transcription factors such as the Glabra2 (GL2) and Transparent Testa Glabra2 (TTG2) (Gonzalez et al., 2009; Johnson et al., 2002; Morohashi et al., 2007; Rerie et al., 1994). Both GL2 and TTG2 play positive roles in seed coat mucilage production. *CsTT8* editing may disrupt the formation of the *camelina* MBW complex leading to decreased transcription of GL2 and TTG2 (FIG. 14) and consequently resulting in a reduction of mucilage. Studies also suggest that the *TT8* genes within this complex play a role in overcoming postzygotic hybridization barriers, specifically the triploid block (Baudry et al., 2006; Nesi et al., 2000).

[000201] TT8 mediates pleiotropic effects, but most significantly, editing of *CsTT8* in *camelina* results in a 44% decrease in flavonoid production. This reflects its ancestral role in the biosynthesis of flavonoids (Zumajo-Cardona et al., 2023) involving in the final stages of this pathway that regulate the synthesis of anthocyanins and proanthocyanins (Baudry et al., 2006; Nesi et al., 2000). While mucilage has the potential to influence seed germination (Arsovski et al., 2010), it is perhaps surprising, that the *CsTT8* edited lines exhibited robust germination on soil. The 10-month-old seeds of the *CsTT8*-edited lines also displayed a germination rate comparable to that of the WT, as depicted in FIG. 11(b). The absence of mucilage in these edited lines could have facilitated more rapid water absorption by the seeds, thereby accelerating germination. Consistent with this is our finding that the edited seeds can more rapidly absorb water, thereby accelerating the germination process, resulting in longer radicles in the edited lines compared to those in the WT.

[000202] In summary, disruption of *CsTT8* via gene editing resulted in yellow seeds associated with reduced flavonoid accumulation and mucilage formation. Significantly, it caused substantial reprogramming of seed metabolism that led to increased TFA and TAG contents, along with changes in fatty acid composition. Our results demonstrate the potential of creating new germplasm in camelina by manipulating *TT8* to enhance lipid biosynthesis. Understanding the regulation of lipid metabolism by *TT8* and other lipogenic factors may provide additional gene targets that can be manipulated to increase oil yields. The use of materials described herein with increased FA and TAG content, and others derived from them, have the potential to increase the yield of feedstocks for biofuels and bioproducts that can contribute towards a net-zero carbon bioeconomy.

[000203] This highlights the effectiveness of site-directed mutagenesis and gene alteration in creating enhanced-oil germplasm in *camelina*. The resulting lines can contribute to future net-zero carbon energy production or be combined with other traits to produce desired lipid-derived bioproducts at high yields.

[000204] MATERIALS AND METHODS

[000205] **Plant Material**

[000206] *Camelina sativa* Suneson are grown in walk-in growth chambers at 22°C with 16-h photoperiod and photon flux density of 70 μmol/m^2/s. Flowers on the primary inflorescence were marked

at anthesis, and the seeds at 12 days after flowering (DAF), 24 DAF and mature seeds were collected for RT-qPCR and acetate incorporation experiment.

[000207] Construction of the CRISPR/Cas9 vector and camelina transformation

[000208] The DsRed expression cassette was amplified from phas-DsRed with its BsaI site removed by site-directed mutagenesis. The PCR fragments and *Eco*R1 linearized pHEE401E (Wang et al., 2015; Xie et al., 2015) were used to assemble pHEE401E-DRM by Gibson assembly. Two sgRNA sites from CsTT8 coding region were selected to create pHEE401E-DRM-CsTT8 (Wang et al., 2015; Xie et al., 2015). All primers used are listed in Table 2.

[000209] The resulting constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into *camelina* via vacuum infiltration (Lu and Kang, 2008). Transgenic *camelina* seeds were screened for DsRed fluorescence as previously described (Pidkowich et al., 2007) and planted.

[000210] Sanger sequencing analysis of target sites

[000211] Genomic DNA of *C. sativa* was extracted from young leaves with the extraction buffer (200 mM Tris-HCL, 0.5% SDS, and 25 mM EDTA, pH 8.0) and precipitated with two volumes of 100% ethanol. PCR was used to amplify the genomic regions encompassing the specific targets of the three *TT8* genes and the PCR products were sequenced. Primers used for vector construction and *TT8* gene amplification are listed in Table 2.

[000212] Microscopic Examination of Seed Coat

[000213] Seeds of the WT and three *TT8* mutant lines were fixed in a FAA buffer (4% formalin, 5% glacial acetic acid, 50% ethanol, 41% water, V/V) within a vacuum for a duration of 25 minutes, as per our prior methodology (Cao et al., 2023). After fixation, those seeds were embedded in Embedding Molds filled with OCT compound (Tissue-Tek) and carefully submerged in a beaker containing 2-Methylbutane chilled by liquid nitrogen. Following this, transverse sections of the seeds with 80 µm thickness, were prepared using freezing-microtome (Leica CM 1950; Leica Biosystems Nussloch GmbH Inc., Heidelberger, Germany) at a temperature of -30 °C. To ascertain the seed coat thickness, Differential Interference Contrast (DIC) imaging was conducted using a Leica TCS SP5 laser scanning confocal microscope. For a more comprehensive understanding of the seed coat composition, images of seed sections were captured without staining or with 0.02% (w/v) Toluidine Blue O (TBO) staining, employing a Leica M125 light microscope.

[000214] Mucilage extraction and quantification

[000215] Mucilage was extracted from the seeds following Zhao et al.(2017). Briefly, 1 mL of water was added to 10 mg of seeds and incubated for 5 min with shaking at 100 rpm. The supernatants were then transferred to a new tube. Subsequently, the seeds were treated with ultrasonication for 20 s after adding 1 mL of water, and the resulting supernatants were combined with those from the previous step.

The seeds were washed with 1 mL of water, and the supernatants were pooled. Finally, the pooled supernatants were subjected to freeze-drying and the dried mucilage samples were weighed.

[000216] Seed germination experiment

[000217] *Camelina* seeds were arranged on filter paper saturated with 4 ml of distilled water (ddH_2O) within a sterile petri dish. Subsequently, the petri dish was placed in a dark environment and maintained at room temperature. After a period of 20 hours, the progression of seed germination was captured through photography. To assess seed germination in soil, it was moistened and firmly packed into pots. Subsequently, seeds were placed on the soil surface and the pots were transferred to an enclosed environment with 25% humidity. Seed germination was recorded at 3 days.

[000218] RNA extraction and qRT-PCR

[000219] RNAs from leaves, roots, stems and seeds of development stages of camelina plants were extracted according to Schultz *et al.* (Schultz, 1994). RNA quality and concentration was examined by gel electrophoresis and nanodrop spectroscopy. cDNAs were prepared using SuperScript IV VILO Master Mix (with ezDNase exzyme; Invitrogen). RT-qPCR was carried out on the CFX96 Real-time PCR Detection System (Bio-Rad) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Gene-specific primers used in the analysis are listed in Table 2. CsActin was used as the internal control (Yu et al., 2019). Statistical analysis of RT-qPCR data was carried out with REST2009 (Pfaffl et al., 2002).

[000220] Fatty acid analyses

[000221] Fatty acid (FA) analyses were carried out as described (Broadwater et al., 2002). Lipids were extracted in methanol/chloroform (2:1) from seeds and heptadecanoic acid (17:0) was added as an internal standard. Total seed lipids were converted into fatty acid methyl esters (FAMEs) in 3M BCL_3 at 90°C for 1 hr and extracted with hexane. Lipid profiles and acyl group identification were analyzed on a Hewlett Packard 6890 gas chromatograph equipped with a 5973 mass selective detector and an Agilent J&W DB 23 capillary column as previously reported (Yu et al., 2018). The FA percentage values were presented as a mean of at least three biological replicates.

[000222] [^{14}C]Acetate Incorporation Assay

[000223] Developing seeds at 11-13 DAF were collected for [^{14}C]Acetate Incorporation Assay following our former protocol (Yu et al., 2021). Approximately 30 mg fresh developing seeds were labeled by incubating in 0.2 mCi of [^{14}C]acetate for 15 min. Cells were subsequently washed and total lipids were extracted and suspended in Ultima Gold liquid scintillation cocktail (PerkinElmer) for incorporated radioactivity measurement with a scintillation counter (Packard BioScience).

[000224] Accession numbers

[000225] CsTT8-2 (Csa02g028180.1,), CsTT8-8 (Csa08g037600.2) and CsTT8-13 (Csa13g044750.1)

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[000284] This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrate and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

[000285] Various references are cited throughout this Specification, each of which is incorporated herein by reference in its entirety.

WHAT IS CLAIMED IS:

1. A genetically edited plant, or a seed or other germplasm derived therefrom, having increased seed yield and increased seed oil content and oil accumulation, wherein the expression or activity of *Transparent Testa 8* (TT8) is disrupted or abolished.

2. The plant, seed or other germplasm of claim 1, wherein the plant is a *Brassicacea* family plant.

3. The plant, seed or other germplasm of claim 1, wherein the plant is a *Brassicacea* family plant of the genus selected from *Camelina*, *Thlapsi* (pennycress), *Brassica*, *Capsella*, *Cardamine* (bittercress), *Eutrema* and *Arabis*.

4. The plant, seed or other germplasm of claim 3, wherein the plant is *Camelina* genus and is *Camelina sativa L. (Cs)*.

5. The plant, seed or other germplasm of any of claims 1-4, wherein one or more TT8 encoding genes are disrupted.

6. The plant, seed or other germplasm of any of claims 1-4, wherein all TT8 encoding genes are disrupted.

7. The plant, seed or other germplasm of any of claims 1-6, wherein TT8 encoding genes are disrupted by gene editing.

8. The plant, seed or other germplasm of claim 7, wherein gene editing utilizes a CRISPR/Cas system.

9. The plant, seed or other germplasm of any of claims 1-8, wherein fatty acid accumulation and triacylglycerol (TAG) yield are increased and flavonoid accumulation is reduced.

10. The plant, seed or other germplasm of claim 9, wherein triacylglycerol yield is greater than 20%.

11. The plant, seed or other germplasm of any of claims 1-9, wherein FAD2 expression is increased and wherein FAD3 expression is decreased.

12. The plant, seed or other germplasm of any of claims 1-11, wherein lipid types are altered, particularly wherein there is an accumulation or increase of oleic acid (18:1) and linoleic acid (18:2) and a decrease in linolenic acid (18:3).

13. The plant, seed or other germplasm of claim 12, wherein oleic acid (18:1) is increased.

14. The plant, seed or other germplasm of any of claims 1-13, wherein the seeds are yellow in color and the seeds of wild type or parental plants are not yellow.

15. The plant, seed or other germplasm of any of claims 1-14, wherein there are no significant changes in starch or protein levels in the seeds compared to wild type or parental plants.

16. The plant, seed or other germplasm of any of claims 1-15, seed yield is increased.

17. The plant, seed or other germplasm of any of claims 1-16, wherein the *Transparent Testa 8* transcription factor gene is one or more or all *Camelina sativa* isoforms denoted *CsTT8-2, CsTT8-8* and *CsTT8-13.*

18. A seed of any of claims 1-17, which is a yellow *Camelina* seed.

19. The seed of claim 18, which is a *Camelina sativa* seed.

20. A method of modifying a plant or a seed or other germplasm derived therefrom to increase seed yield, seed oil content and oil accumulation, comprising abolishing the expression or activity of TT8 by disrupting one or more *TT8* transcription factor encoding genes in said plant.

21. The method of claim 20, wherein fatty acid accumulation and triacylglycerol (TAG) yield are increased and flavonoid accumulation is reduced.

22. The method of claim 20 or 21, wherein all TT8 encoding genes are disrupted by gene editing and wherein gene editing utilizes a CRISPR/Cas system.

23. The method of any of claims 20-22, wherein the plant is a member of the *Brassicacea* family and is not an *Arabidopsis* genus plant

24. The method of any of claims 20-22, wherein the plant is a *Brassicacea* family plant and is of the genus selected from *Camelina*, *Thlapsi* (pennycress), *Brassica*, *Capsella*, *Cardamine* (bittercress), *Eutrema* and *Arabis*.

25. The method of claim 23 or 24, wherein the plant is a *Camelina* genus plant.

26. The method of claim 25, wherein the plant is *Camelina sativa L. (Cs)*.

27. The method of any of claims 20-26, wherein all TT8 encoding genes are disrupted by gene editing and wherein gene editing utilizes a CRISPR/Cas system and the TT8 encoding genes are homologs of *Camelina sativa* isoforms CsTT8-2, CsTT8-8 and CsTT8-13.

28. The method of claim 27, wherein the homologs of *Camelina sativa* isoforms CsTT8-2, CsTT8-8 and CsTT8-13 have at least 80% amino acid sequence identity to SEQ ID NO:25, 26 and/or 27.

FIG. 1

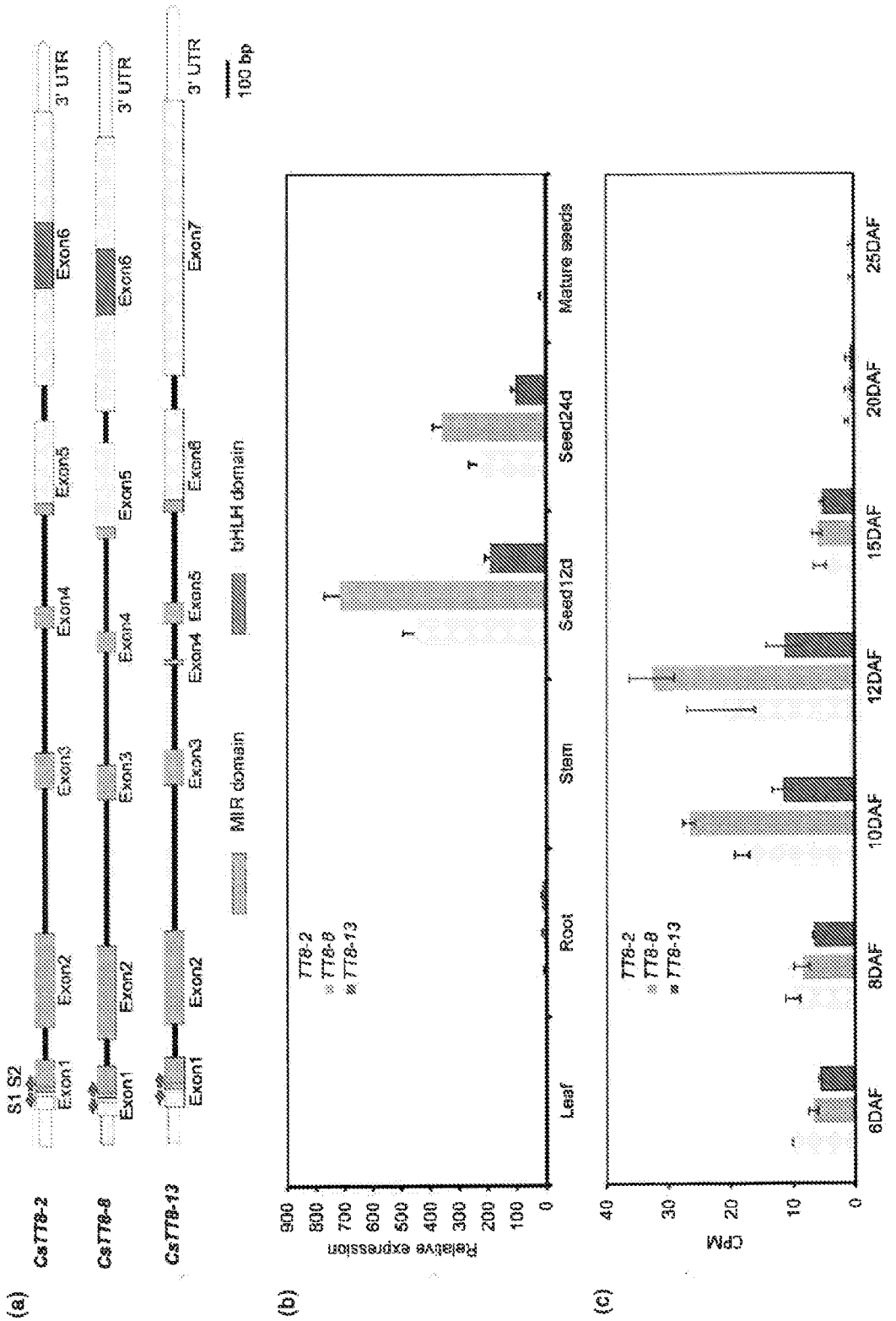

(a)
CsTT8-2
CsTT8-8
CsTT8-13
S1 S2
Exon1
Exon2
Exon3
Exon4
Exon5
Exon6
Exon7
Exon8
3' UTR
MIR domain
bHLH domain
100 bp
(b)
Relative expression
TT8-2
TT8-8
TT8-13
Leaf
Root
Stem
Seed12d
Seed24d
Mature seeds
(c)
CPM
6DAF
8DAF
10DAF
12DAF
15DAF
20DAF
25DAF

FIG. 2

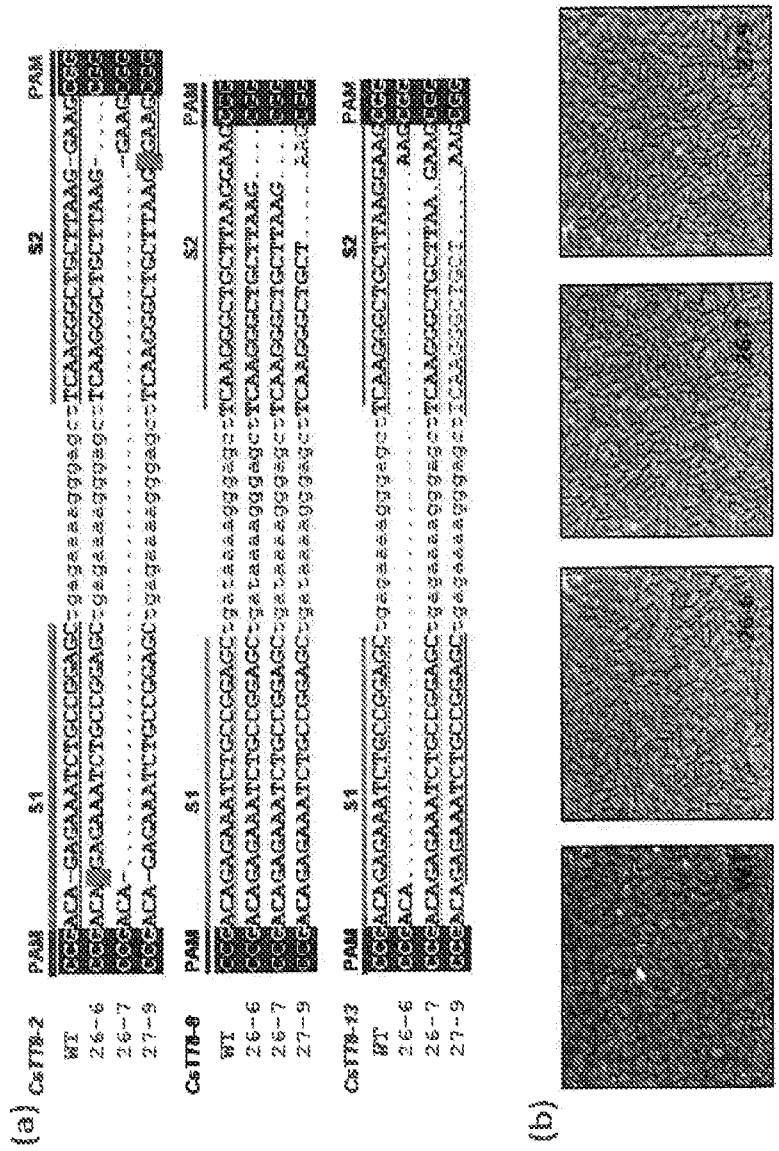

(a)
CsTT8-2
PAM
S1
S2
PAM
WT
26-6
26-7
27-9
CsTT8-8
PAM
S1
S2
PAM
WT
26-6
26-7
27-9
CsTT8-13
PAM
S1
S2
PAM
WT
26-6
26-7
27-9
(b)
WT
26-6
26-7
27-9

FIG. 3

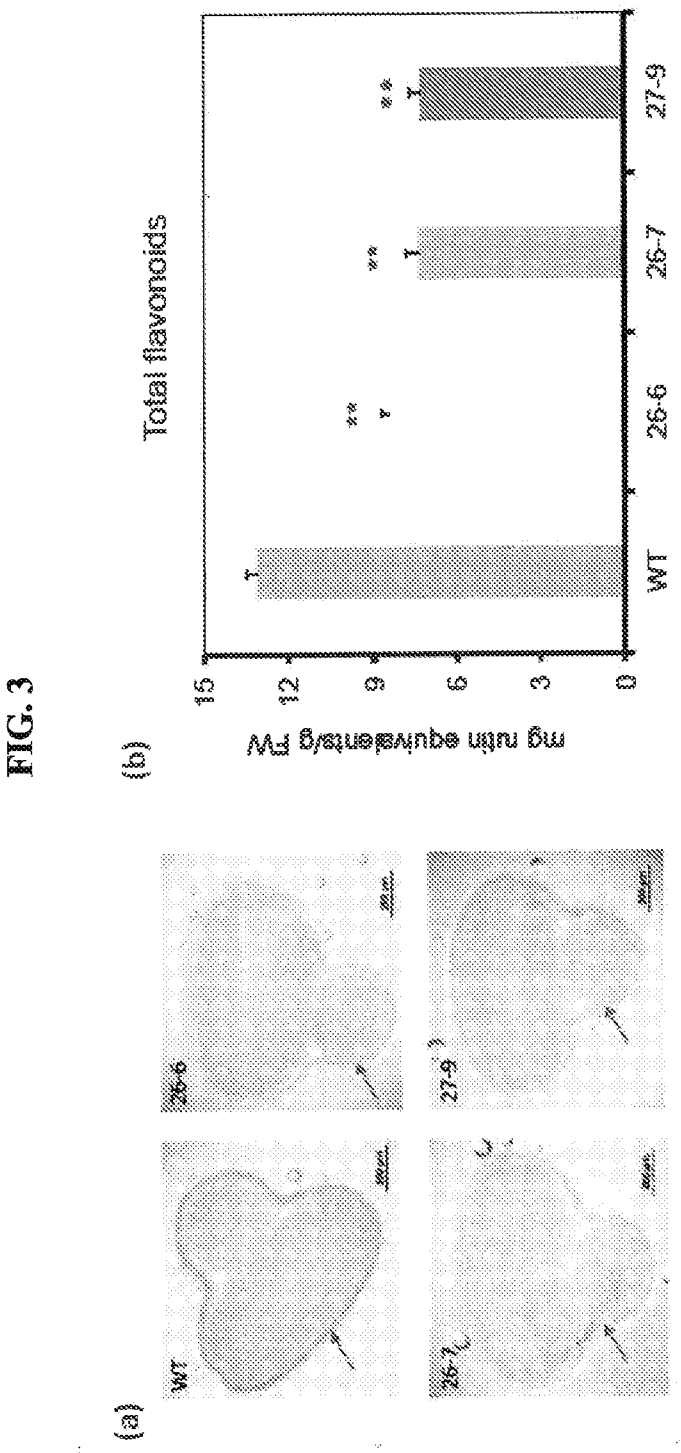

(a)
WT
26-6
26-7
27-9
(b)
Total flavonoids
mg rutin equivalents/g FW
0
3
6
9
12
15
WT
26-6
26-7
27-9
a
b
b
b
**
**
**

FIG. 4

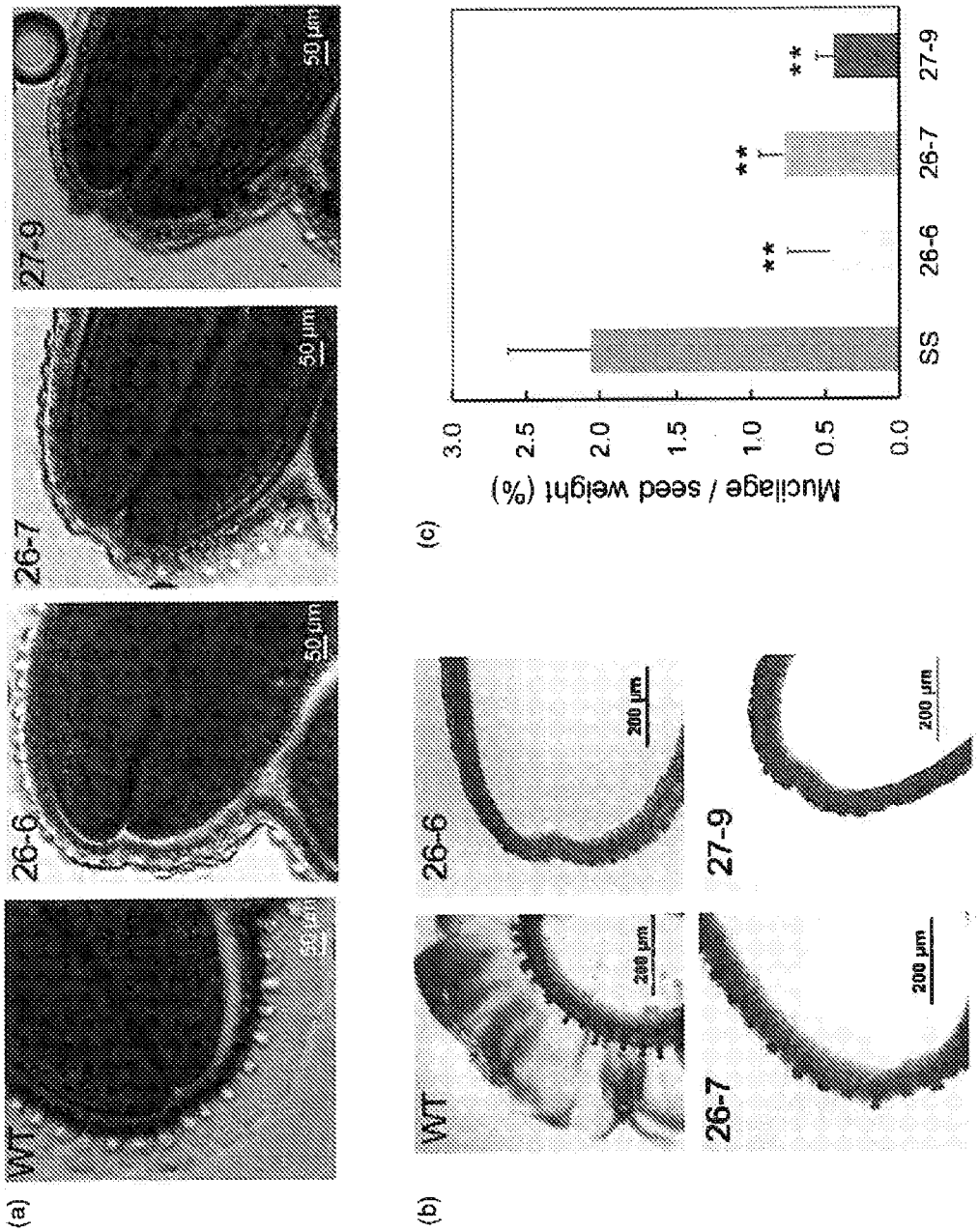

(a)
WT
26-6
26-7
27-9
50 μm
(b)
WT
26-6
26-7
27-9
200 μm
(c)
Mucilage / seed weight (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
SS
26-6
26-7
27-9
**

FIG. 5

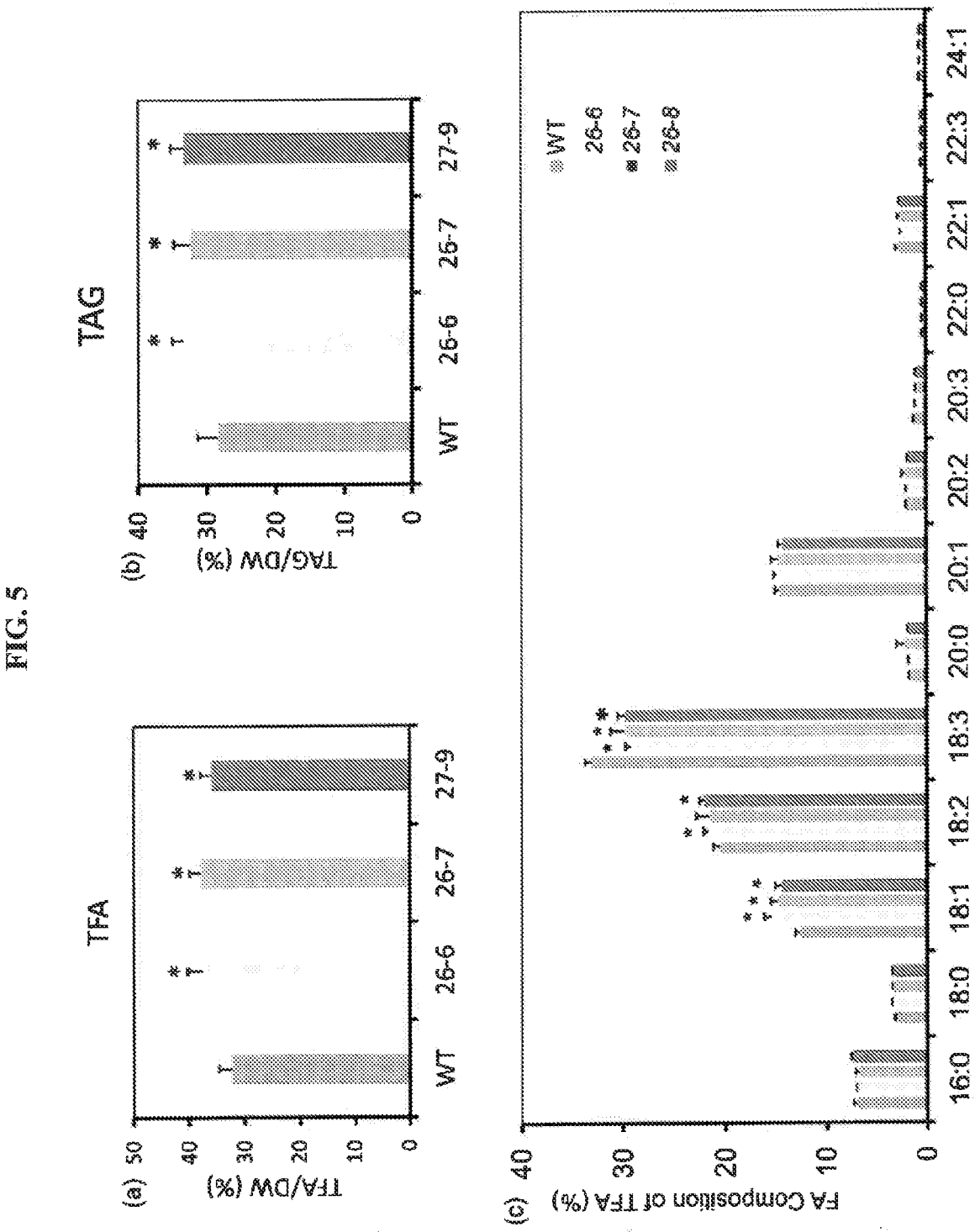

TFA
(a)
TFA/DW (%)
WT
26-6
26-7
27-9
TAG
(b)
TAG/DW (%)
(c)
FA Composition of TFA (%)
WT
26-6
26-7
26-8
16:0
18:0
18:1
18:2
18:3
20:0
20:1
20:2
20:3
22:0
22:1
22:3
24:1

FIG. 6

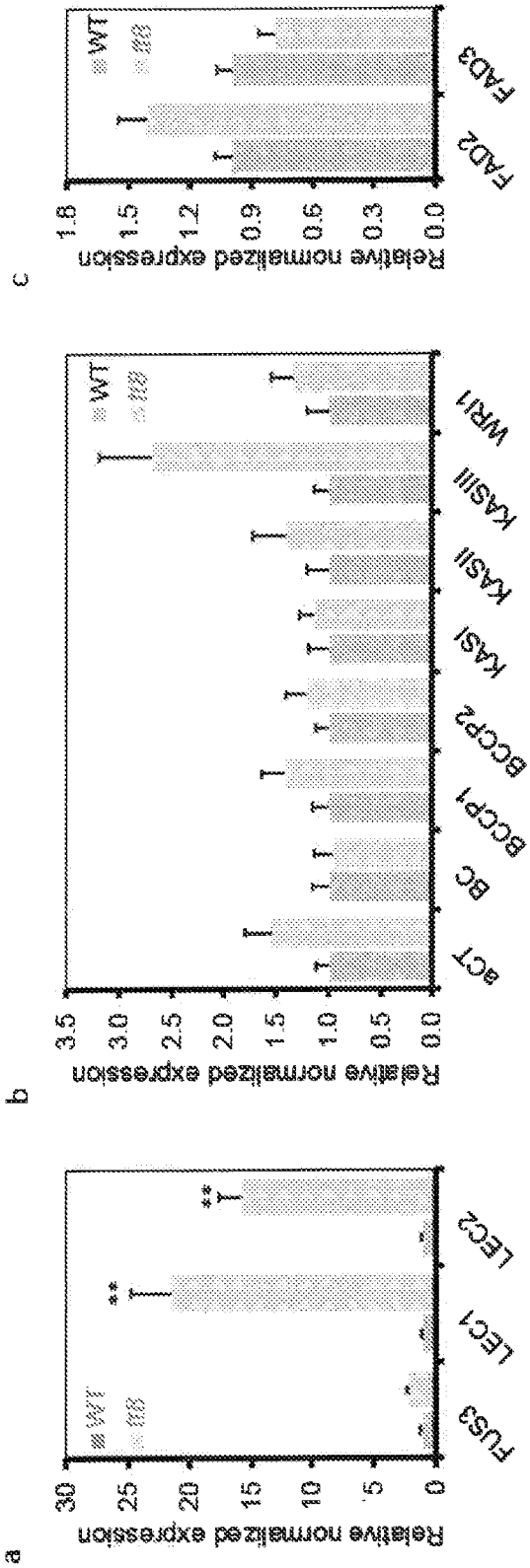

a
Relative normalized expression
WT
fab
FUS3
LEC1
LEC2
b
Relative normalized expression
WT
fab
aCT
BC
BCCP1
BCCP2
KASI
KASII
KASIII
WRI1
c
Relative normalized expression
WT
fab
FAD2
FAD3

FIG. 7

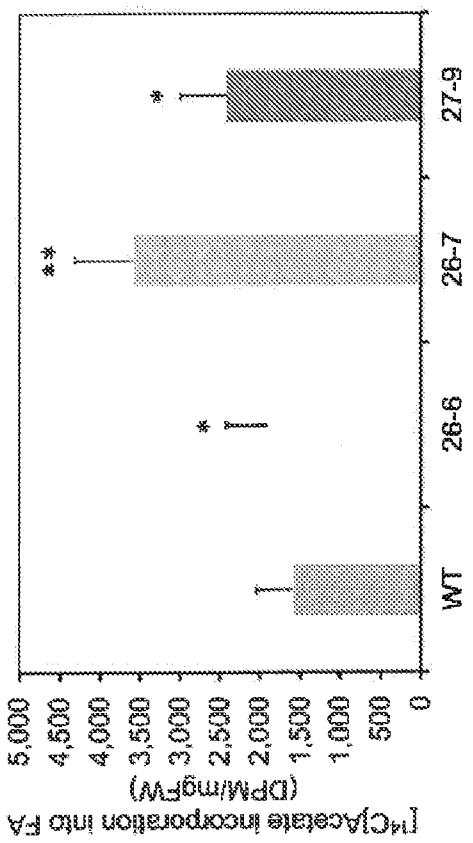

[14C]Acetate incorporation into FA
(DPM/mgFW)
5,000
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
WT
26-6
26-7
27-9
*
**
*

FIG. 8

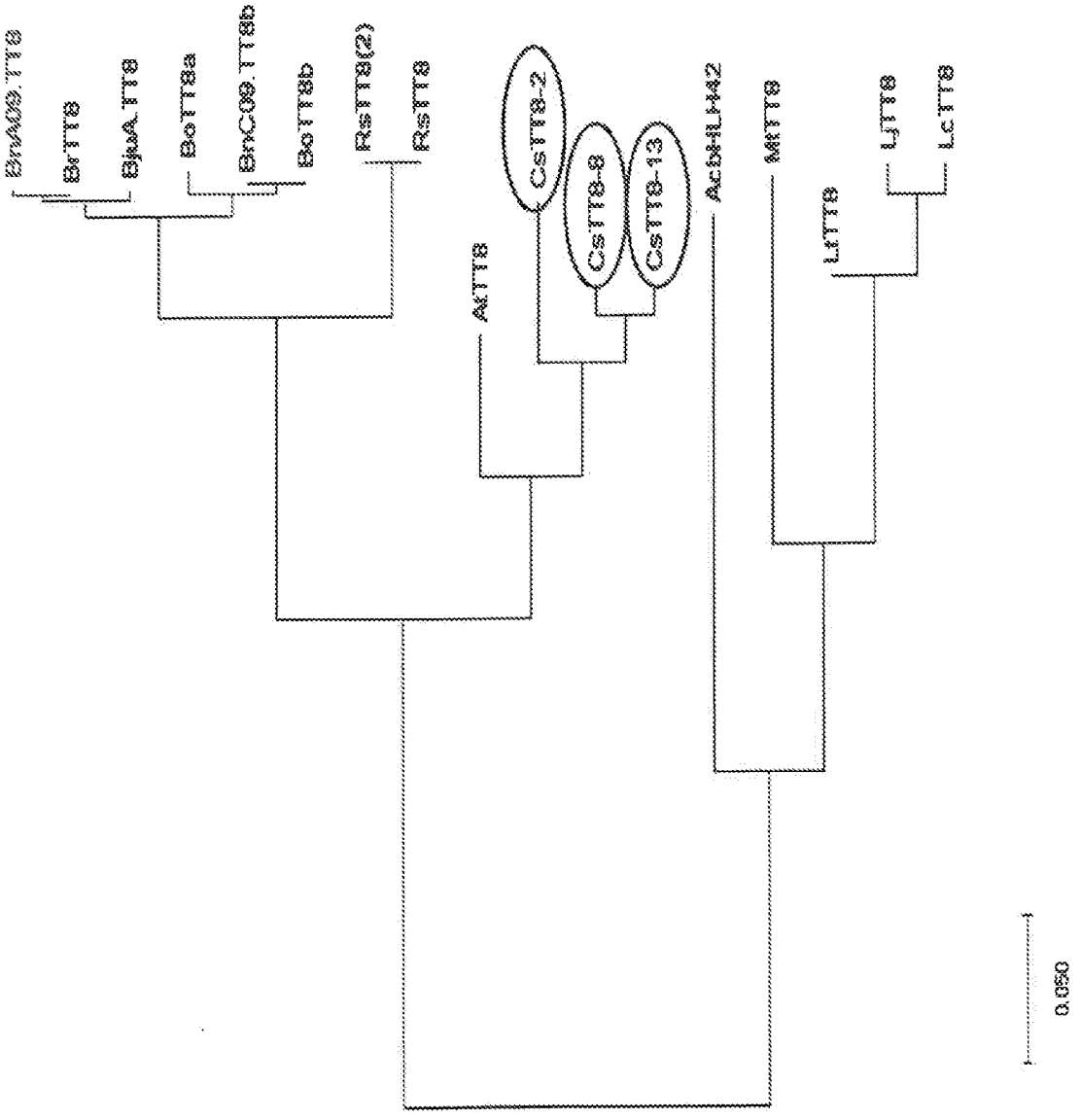

BnA09.TT8
BrTT8
BjuA.TT8
BoTT8a
BnC09.TT8b
BoTT8b
RsTT8(2)
RsTT8
AtTT8
CsTT8-2
CsTT8-8
CsTT8-13
AcbHLH42
MtTT8
LjTT8
LyTT8
LcTT8
0.050

FIG. 9-A

```
CsTTG-2
  WT     MDGSSIIPTEKSAGAEKRELQGLLKEAVQSVEWTYSLFWQFCPQQRVLVWGNGYYNGAIKTRKTTQ   66
  26-6   MDGSSIIPTREICRS*-------------------------------------------------   15
  26-7   MDGSSIIPT----------------EAVQSVEWTYSLFWQFCPQQRVLVWGNGYYNGAIKTRKTTQ   49
  27-9   MDGSSIIPTEKSAGAEKRELQGLLKEGGSICGVDL*-------------------------------   35

CsTTG-8
  WT     MDGSINIPTEKSAGADKRELQGLLKEAVQSVEWTYSLFWQFCPQQRVLVWGNGYYNGAIKTRKTTQ   66
  26-6   MDGSINIPTEKSAGADKRELQGLLKRFN---------LWSGLIVCSGNFVRNNGYWCGGMDTITVQ*   57
  26-7   MDGSINIPTEKSAGADKRELQGLLKRFN---------LWSGLIVCSGNFVRNNGYWCGGMDTITVQ*   57
  27-9   MDGSINIPTEKSAGADKRELQGLLSGSICGVDL*-----------------------------------   33

CsTTG-13
  WT     MDGSSIIPTEKSAGAEKRELQGLLKEAVQSVEWTYSLFWQFCPQQRVLVWGNGYYNGAIKTRKTTQ   66
  26-6   MDGSSIIP----------------TKRFN--------LWSGLIVCSGNFVLNNGYWCGGMDTITVQ*   42
  26-7   MDGSSIIPTEKSAGAEKRELQGLLKKRFN--------LWSGLIVCSGNFVLNNGYWCGGMDTITVQ*   58
  27-9   MDGSSIIPTEKSAGAEKRELQGLLSGSICGVDL*-----------------------------------   33
```

FIG. 9-B

Bulge Type	Target	Chromosome	Position	Direction	Mismatches	Bulge Size
X	crRNA: ACAGAGAAATCTGCCGGAGCNGG DNA: ATACAGAAATCTGCCGGAGCAGG	Chr6	2884836	+	3	0

FIG. 9-C

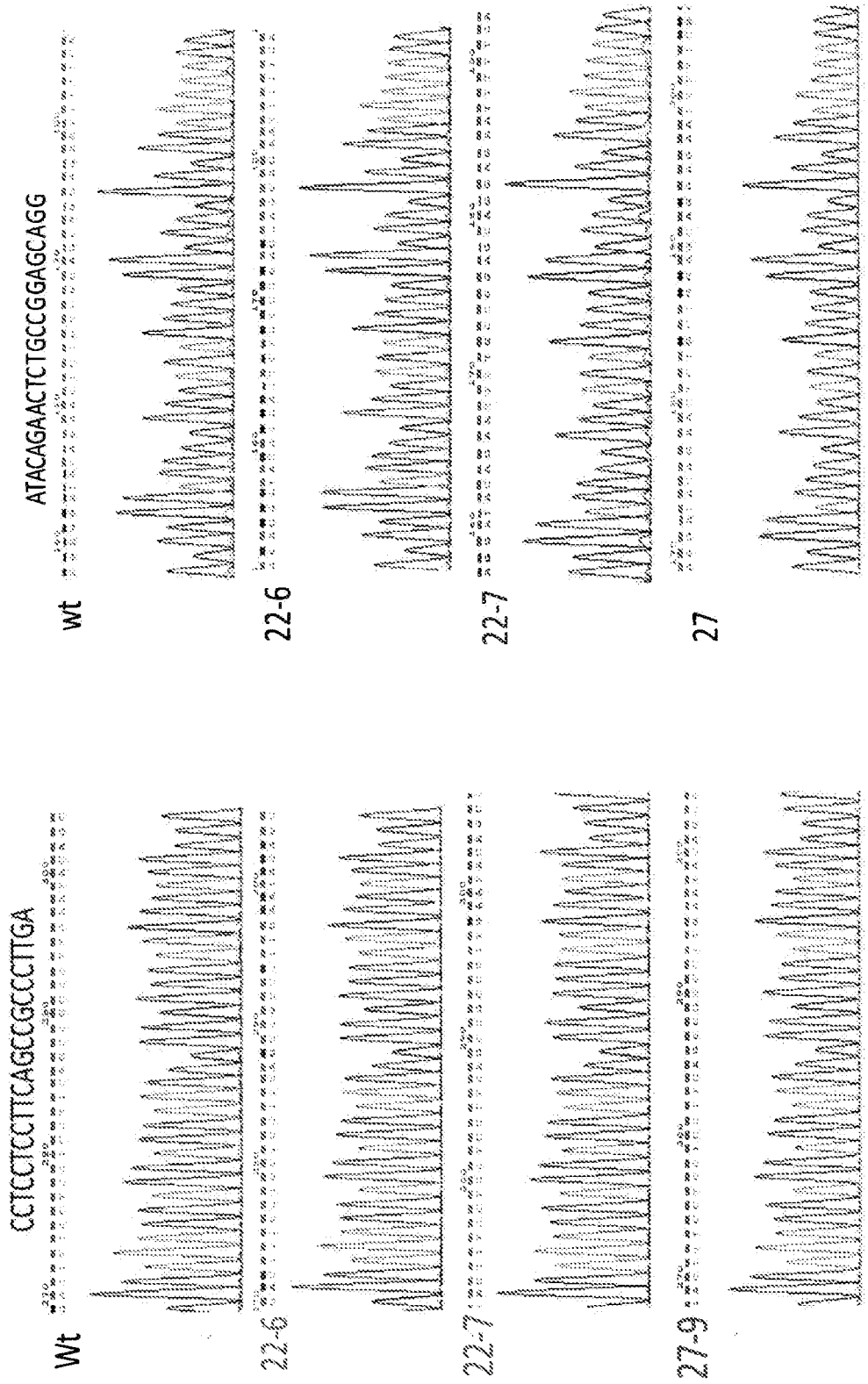

ATACAGAACTCTGCCGGAGCAGG
wt
22-6
22-7
27
CCTCCTCCTTCAGCCGCCCTTGA
Wt
22-6
22-7
27-9

FIG. 10

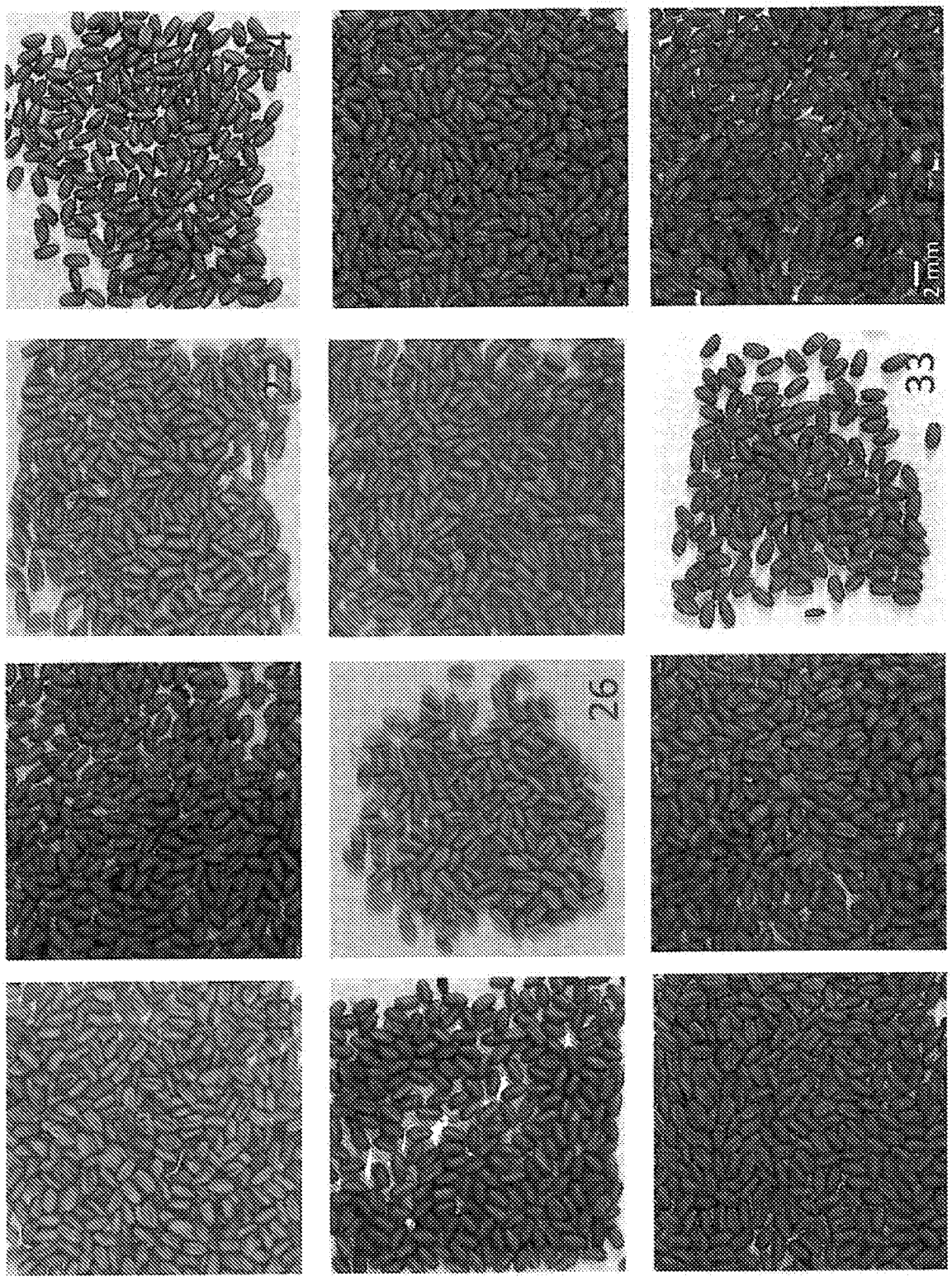


FIG. 11

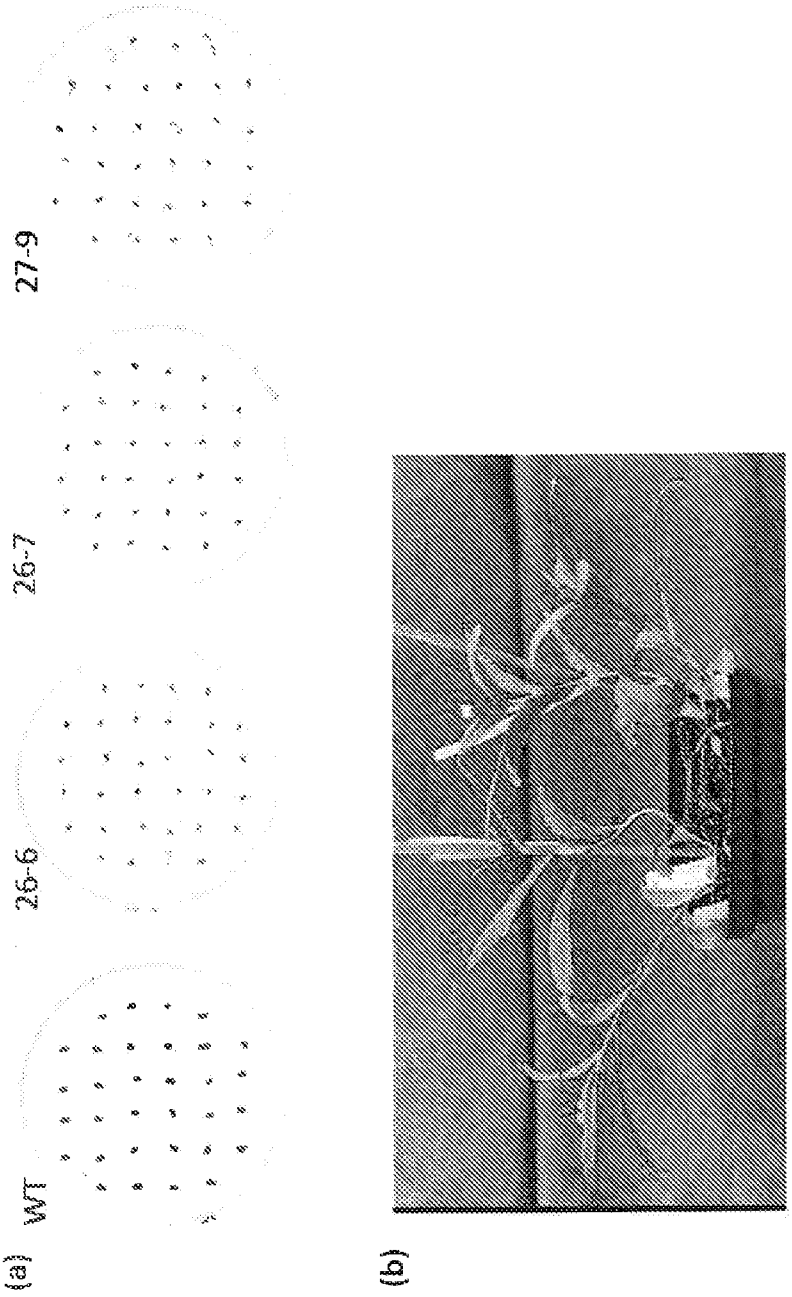


FIG. 12

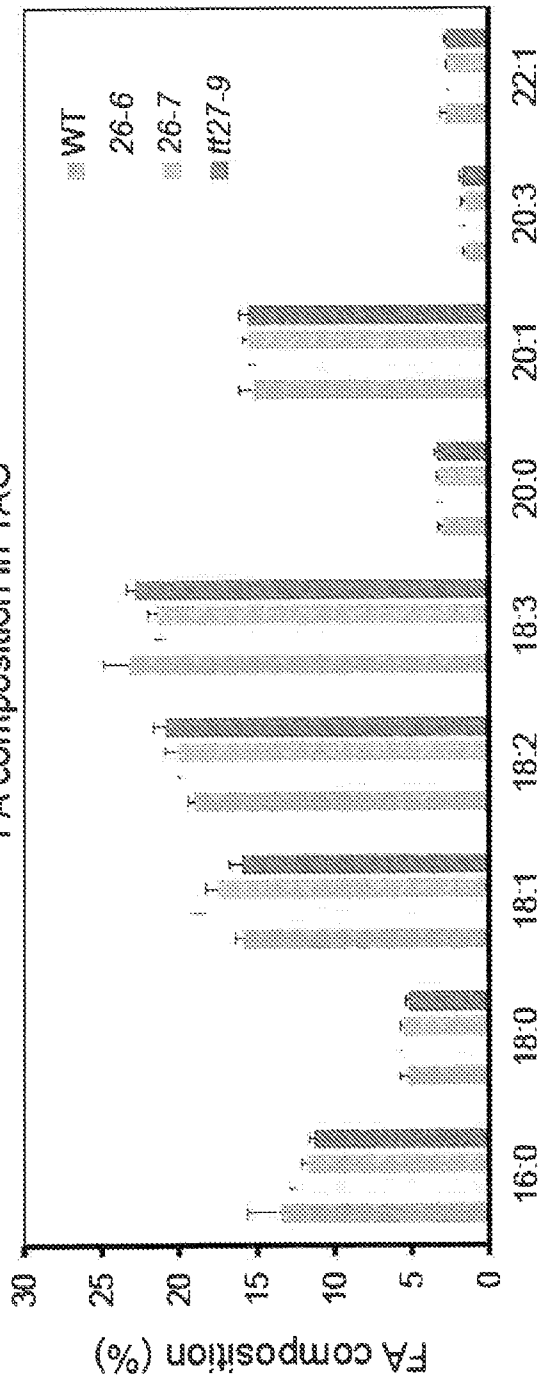

FA composition in TAG
WT
26-6
26-7
tt27-9
FA composition (%)
30
25
20
15
10
5
0
16:0
18:0
18:1
18:2
18:3
20:0
20:1
20:3
22:1

FIG. 13

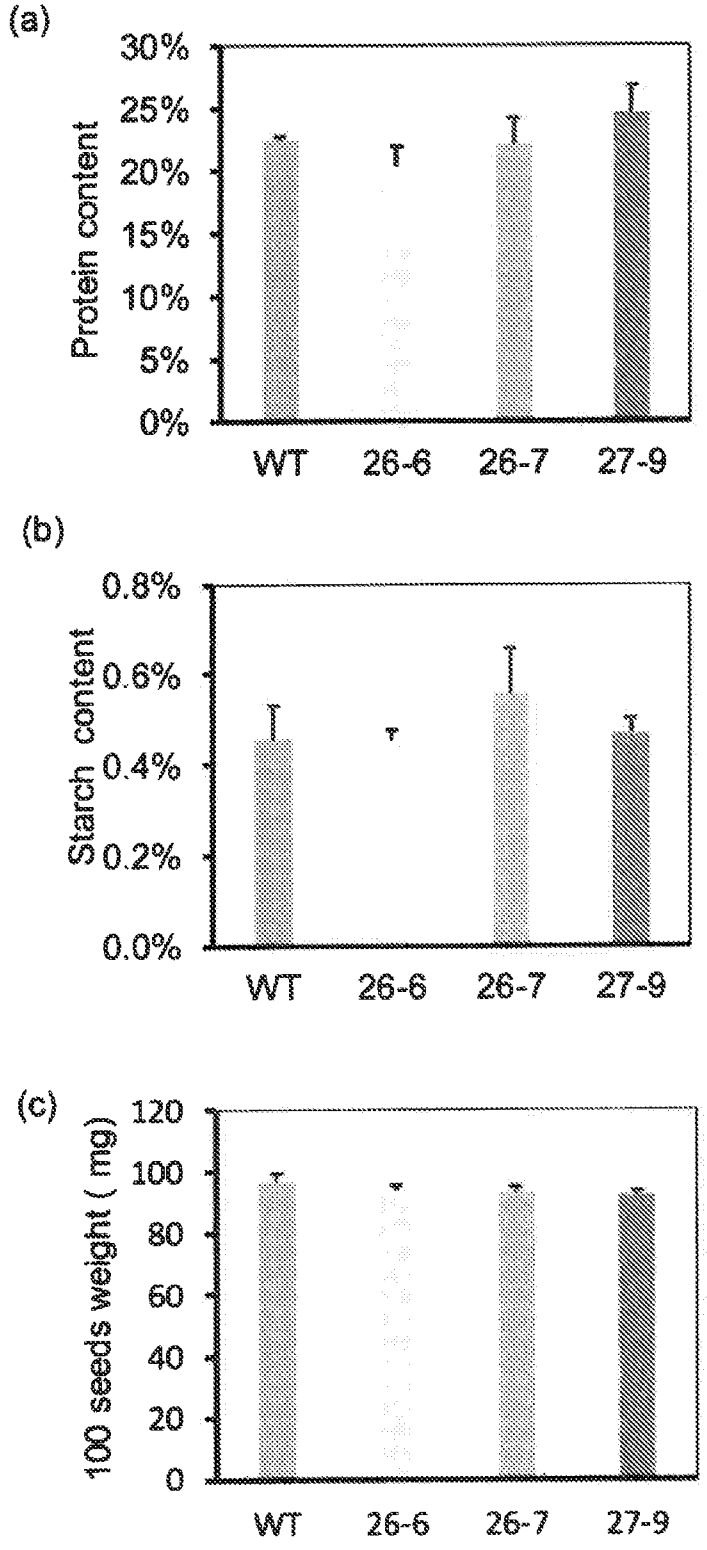
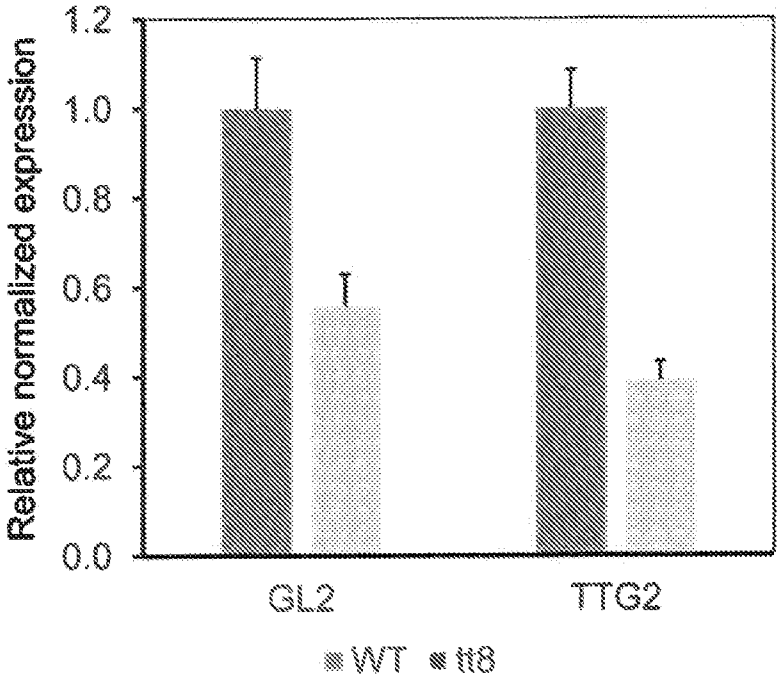

(a)
Protein content
30%
25%
20%
15%
10%
5%
0%
WT
26-6
26-7
27-9
(b)
Starch content
0.8%
0.6%
0.4%
0.2%
0.0%
WT
26-6
26-7
27-9
(c)
100 seeds weight (mg)
120
100
80
60
40
20
0
WT
26-6
26-7
27-9

FIG. 14


Relative normalized expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GL2
TTG2
WT
tt8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2025/023245

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C12N 15/84*** (2025.01); ***A01H 5/00*** (2025.01); ***A01H 6/20*** (2025.01); ***C12N 15/10*** (2025.01); *C12P 19/34* (2025.01)

CPC: ***C12N 15/8247; A01H 5/00; A01H 6/20; C12N 15/8213; C12N 15/102;*** *C12N 2310/20; Y02A 40/146; C12P 19/34*

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)

See Search History Document

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

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	ZHAI et al. "Targeted mutagenesis of BnTT8 homologs controls yellow seed coat development for effective oil production in Brassica napus L." Plant Biotechnology Journal. 2020. Vol. 18, Pgs. 1153-1168	
X	entire document	1-3, 5, 6, 20-22
Y	entire document	4
	XIE et al. "Targeted Knockout of BnTT2 Homologues for Yellow-Seeded Brassica napus with reduced flavonoids and Improved Fatty Acid Composition" J. Agric. Food Chem. 12 May 2020. Vol. 68, Pgs. 5676-5690	
Y	entire document	4
	CAI et al. "Creating yellow seed Camelina sativa with enhanced oil accumulation by CRISPR-mediated disruption of Transparent Testa 8" Plant Biotechnology Journal. 10 June 2024. Vol. 22, Pgs. 2773-2784	
P, X	entire document	1-6, 20-22

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"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
12 May 2025 (12.05.2025)	**27 May 2025 (27.05.2025)**

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2025/023245

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.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2025/023245

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.: **7-19, 23-28**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).