

GENE SILENCING OF SUGAR-DEPENDENT 1 IN JATROPHA CURCAS

CROSS-REFERNCE TO RELATED APPLICATION

[0001] The present application is related to and claims priority to U.S. Provisional Patent Application Serial No. 61/773,386, filed 6 March 2013. This application is incorporated herein by reference.

SEQUENCE SUBMISSION

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577229PCTSequencListing.txt, was created on 18 February 2014 and is 43 kb in size. The information in the electronic format of the Sequence Listing is part of the present application and is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0003] The present invention relates to the field of plant molecular biology and gene silencing. More particularly, the present invention relates to gene silencing of *Sugar-dependent 1 (JcSDP1)* in *Jatropha curcas*. *JcSDP1* encodes a patatin-domain triacylglycerol lipase. Silencing of *JcSDP1* enhances seed oil accumulation in *J. curcas*.

[0004] The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended bibliography.

[0005] The diminishing stock of fossil fuel has catalyzed a soaring demand for renewable energy sources world-wide. To meet this demand active research has been initiated relating to solar, wind, tidal and geothermal power generations during the last several decades. Likewise, there is also an increasing focus on biofuels, which are energy sources derived from renewable biomass. There are two main types of biofuels: bioethanol and biodiesel, which are generally used as gasoline and diesel additives, respectively. Bioethanol is mainly produced by fermentation using sugar or starch derived from crops such as sugar cane and corn. Biodiesel, on the other hand, is obtained by trans-esterification of plant oils and animal fats. In 2010, the global biofuel production reached 105 billion liters and provided 2.7% of the world's energy needs for transportation. It has been forecast that biofuel may account for more than a quarter of the world's demand for transportation fuels by 2050 (1).

[0006] Biodiesel is generally produced from oil seed crops such as rape in temperate countries and oil palm in the tropics. In the past several years, a small tree called *Jatropha curcus* grown in the tropical and subtropical regions has emerged as an attractive candidate crop for biodiesel production. *Jatropha* has several interesting attributes making it suitable for consideration as a biodiesel plant. *Jatropha* seeds contain up to 40% oil consisting of approximately 75% unsaturated fatty acids (2, 3), with a high level (around 47%) of linoleic acid (C18:2) (4). In addition to having a high oil content and favorable oil composition for biodiesel, *Jatropha* plants have a short gestation period and adapt well to a wide range of agro-climatic conditions (5, 6). Moreover, its ability to grow on marginal land reduces the possibility that *Jatropha* may compete with food crops for arable land.

Because of *Jatropha* has just been recently been domesticated much work remains to be done to improve its agronomic traits either by traditional breeding or by gene technology.

[0007] Given the commercial interest in *Jatropha* seed oil, it is not surprising that the immediate focus is on seed oil content and quality. With respect to the latter trait, Qu et al. (7) have recently reported that gene silencing of *JcFAD2* can greatly enhance the proportion of oleic acids in seeds of transgenic *Jatropha*. Here, we addressed the issue of increasing levels of oil accumulation in *Jatropha* seeds by genetic modification.

[0008] Plant oil in seeds is stored as triacylglycerol (TAG) consisting of three fatty acid chains (usually C16 or C18) covalently linked to glycerol. Depending on the plant source, TAGs may contain fatty acids of different chain lengths and degree of saturation and the fatty acids may be decorated with diverse modifications. Plant TAGs are generally stored in small organelles called oil bodies which are assembled in developing seeds, flower petals, pollen grains and fruits of a huge number of plant species (8, 9). During seed germination TAGs are hydrolyzed into fatty acids and glycerol and this reaction is catalyzed by TAG lipases, which are widely distributed in plants but also found in animals and microorganisms (10). Among the known lipases, the unorthodox patatin-like TAG lipases (PTLs) are oil body-associated enzymes that play a major role in the initiation of TAG degradation in yeast, mammals and insects (11, 12). During seed germination, TAG lipases initiate TAG degradation into glycerol and free fatty acids and the released fatty acids are consumed through the beta-oxidation pathway which releases energy for early seedling growth (13, 14).

[0009] Recently, Eastmond (15, 16) has shown that the *sugar-dependent 1* (*SDP1*) of *Arabidopsis* encodes a patatin-like acyl hydrolase domain. The encoded protein, *SDP1*, is specifically responsible for the first step of TAG degradation during *Arabidopsis* seed germination. This enzyme is also able to associate with oil body surface as well as the other

reported PTLs. A T-DNA insertion *SDP1* mutant allele, *sdp1-5*, displayed growth retardation on a sugar-deficient medium due to the deficiency of glycerol and free fatty acids, which are products of TAG degradation (15, 16). The study of Eastmond (15, 16) showed an accumulation of clustered oil bodies in seedling cotyledons and a higher TAG levels in *sdp1-5* than WT (Col-0).

[0010] It is desired to increase seed oil content in *Jatropha* which would be an important agronomic trait for this biodiesel crop.

SUMMARY OF THE INVENTION

[0011] The present invention relates to the field of plant molecular biology and gene silencing. More particularly, the present invention relates to gene silencing of *Sugar-dependent 1* (*JcSDP1*) in *Jatropha curcas*. *JcSDP1* encodes a patatin-domain triacylglycerol lipase. Silencing of *JcSDP1* enhances seed oil accumulation in *J. curcas*.

[0012] Thus, in one aspect, the present invention provides for In a first aspect, the present invention provides an isolated nucleic acid encoding a *JcSDP1* protein comprising the amino acid sequence set forth in SEQ ID NO:2. In one embodiment, the nucleic acid comprises the nucleotide sequence set forth in SEQ ID NO:1. In another embodiment, the nucleic acid comprises the nucleotide sequence set forth as nucleotides 874-3450 of SEQ ID NO:1. In an additional embodiment, the nucleic acid comprises the nucleotide sequence set forth as nucleotides 874-3447 of SEQ ID NO:1. In a further embodiment, the nucleic acid further comprises a plant operable promoter operably linked to a nucleic acid comprising the coding sequence, or a nucleic acid comprising the 5' UTR (or portion thereof) and the coding sequence, or a nucleic acid comprising the coding sequence and the 3' UTR (or portion thereof) or a nucleic acid comprising the 5' UTR (or portion thereof), coding sequence and the 3' UTR (or portion thereof). In one embodiment, the promoter is a seed specific promoter. In another embodiment, the seed specific promoter is a *Jatropha* seed specific promoter. In a further embodiment, the *Jatropha* seed specific promoter is the *JcSDP1* promoter. In one embodiment, the *JcSDP1* promoter comprises nucleotides 1-722 of SEQ ID NO:1. In another embodiment, functional fragments of the *JcSDP1* promoter can be used.

[0013] In a second aspect, the present invention provides a construct or vector comprising an isolated nucleic acid as described herein. In one embodiment, the construct or vector is an expression construct or vector. In another embodiment, the construct or vector further comprises a selectable marker. In a further embodiment, the construct or vector comprises a Cre-lox recombination marker free system.

[0014] In a third aspect, the present invention provides a transgenic plant comprising a nucleic acid or vector described herein. In one embodiment, the transgenic plant is a *Jatropha* plant.

[0015] In a fourth aspect, the present invention provides for the down regulation of a *Jatropha SDP1* gene. In one embodiment, the down regulation of a *Jatropha SDP1* gene involves using RNA interference (RNAi), including double stranded RNA, siRNA, microRNA, and hairpin RNA. In another embodiment, the down regulation of a *Jatropha SDP1* gene involves using viral induced gene silencing (VIGS). In one embodiment, a nucleic acid is provided which down regulates the *Jatropha SDP1* gene. In another embodiment, a nucleic acid is provided which down regulates the *Jatropha curcas SDP1* gene. In some embodiments, the nucleic acid comprises nucleotides 3161 to 3533 of SEQ ID NO:1. In another embodiment, a suitable nucleic acid for use in down regulating the *Jatropha SDP1* gene is selected using well known RNAi considerations and algorithms. In some embodiments, the nucleic acid further comprises a plant operable promoter operably linked to the nucleic acid. In one embodiment, the promoter is a seed specific promoter. In another embodiment, the seed specific promoter is a *Jatropha* seed promoter. In a further embodiment, the *Jatropha* seed specific promoter is the *JcSDP1* promoter. In one embodiment, the *JcSDP1* promoter comprises nucleotides 1-722 of SEQ ID NO:1. In another embodiment, functional fragments of the *JcSDP1* promoter can be used. In an additional embodiment, the promoter comprises the *JcSDP1* promoter and the 3' UTR (or portion thereof). In one embodiment, a nucleic acid suitable for down regulating *Jatropha SDP1* gene is set forth in SEQ ID NO:3. According to this aspect, the present invention also provides a vector comprising an isolated nucleic acid as described herein. In one embodiment, the vector is an expression vector. In another embodiment, the vector further comprises a selectable marker. In a further embodiment, the vector comprises a Cre-lox recombination marker free system. According to this aspect, the present invention further provides a transgenic or infected plant or plant cell comprising a nucleic acid or vector described herein. In one embodiment, the transgenic or infected plant or plant cell is a *Jatropha* plant or plant cell. In some embodiments, the down regulation of a *Jatropha SDP1* gene provides enhanced seed oil accumulation in a transgenic or infected *Jatropha* plant, such as *Jatropha curcas*.

[0016] In a fifth aspect, the present invention provides methods of increasing seed oil accumulation in *Jatropha*, such as *Jatropha curcas*. In one embodiment, a method involves modulating the level of activity of a *Sugar-dependent 1 (SDP1)* gene, such as *JcSDP1* gene, which encodes a patatin-domain triacylglycerol lipase in the host *Jatropha* cell or *Jatropha* plant.

The level of activity of the lipase can be reduced by reducing expression of the *SDP1* gene. In one embodiment, the modulation of the level of activity of the lipase is a reduction in the activity of the lipase. The level of activity of the lipase can be reduced by using RNAi techniques described herein in which the lipase is the target for the RNAi. Alternatively, the level of activity of the lipase can be reduced using VIGS techniques as described herein in which at least a partial fragment of the target gene is used. In one embodiment, *Jatropha* RNAi plants accumulate about 13% to about 30% higher seed oil than control plants.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figures 1A-1G show *sdp1-5* mutant accumulates a higher TAG levels than WT (Col-0) in mature seeds. Fig. 1A: Scanning electron microscopy (SEM) showing the seed surface structure of WT (Col-0) and *sdp1-5*. Fig. 1B: Comparative dry seed weight of WT (Col-0) and *sdp1-5*. Fig. 1C: Total amount of fatty acids per seed of WT (Col-0) and *sdp1-5*. Fig. 1D: Relative fatty acids of dried seeds of WT (Col-0) and *sdp1-5*. Fig. 1E: Fatty acid profiles of WT (Col-0) and *sdp1-5* seeds. Fig. 1F: Seed eicosenoic acid (20:1) content. Fig. 1G: TLC separation of neutral lipid fractions from WT (Col-0) and three lines of *sdp1-5* null mutant. 300 μ g of neutral lipids were fractionated by TLC on silica gel plates. TAG, Triacylglycerol (Triolein, Sigma T7140); FFA, Free Fatty Acid (Oleic acid, Sigma 75090), Mix, mix TAG and FFA, SE, sterol ester; FFA, free fatty acid; DAG, diacylglycerol. H) Profiling of relative amounts of FFA and TAG by GC/MS. The absolute amount was calculated using the C15:0 as an internal control by comparing their peak areas. * $P < 0.05$ or ** $P < 0.01$ versus WT (Col-0). Each experiment was performed with 100 seeds per line with 5 biological replicates. Error bar shows SD, standard deviation (n=5). DW, Dry weight

[0018] Figures 2A-2C show *sdp1-5* mutant seeds produce more oil bodies. Fig. 2A: TEM analysis of oil body distribution in WT (Col-0) and *sdp1-5* mature seeds. The scale bar is 5 μ m. PSV; protein storage vacuole, CW; cell wall, OB; oil body. Fig. 2B: Numbers of oil bodies per cell in mature seeds of WT (Col-0) and *sdp1-5*. A cell has an average of 196 μ m² cross-sectional area (Mean value with SD; n=5). Fig. 2C: Expression levels of *AtOLE1* (At4g25140), *AtOLE2* (At5g40420), and *AtOLE3* (At5g51210) in early developing stages of seeds (3 to 5 DAP) from WT (Col-0) and *sdp1-5*. *Actin1* (At2g37620) was used as an internal control. * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$ versus WT (Col-0). There were 5 biological replicates. SD, standard deviation (n=5). DAP: Day after pollination.

[0019] Figures 3A and 3B show cloning of *JcSDP1* gene from *Jatropha* plants and complementation of the *Arabidopsis sdp1-5* mutant. Fig. 3A: Deduced amino acid sequence of

JcSDP1 (SEQ ID NO:2) and alignment to the AtSDP1 amino acid sequence (SEQ ID NO:4). Bold line; patatin domain, star (*****); site-1 protease (S1P) target sequence, RXXL (SEQ ID NO:5), GXGXXG (SEQ ID NO:6); oxyanion hole motif, GX SXG (SEQ ID NO:7); lipase consensus motif with catalytic serine. Fig. 3B: Complementation of *sdp1-5* with *JcSDP1*. WT (Col-0), *sdp1-5*, and CaMV35S:*JcSDP1/sdp1-5* transgenic plants were grown on MS medium with or without sucrose (1%).

[0020] Figures 4A-4E show *JcSDP1* promoter and its expression analysis in *ProJcSDP1:GUS* transgenic plants. Fig. 4A: Composition of putative *cis*-elements in *JcSDP1* promoter. Fig. 4B: Transient *ProJcSDP1:GUS* expression in *Jatropha* fruit and leaf. Fig. 4C: Heterologous expressions of *ProJcSDP1:GUS* in transgenic *Arabidopsis* plants. a) 14 day-old seedling, b-c) inflorescence, d-e) rosette leaf, f) siliques, g) seeds at different developing stages, h) young seeds (3 to 4 DAP, globular-stage embryo), i) mid-stage seeds (9 to 10 DAP, mature green embryo). DAP: Days after pollination. Fig. 4D: Sugar-dependent expression of *ProJcSDP1:GUS* transgenic plants (14 day-old seedling). 1% or 3% fructose, glucose, and sucrose were used as a sugar source and mannitol was used as a control for osmotic stress. Fig. 4E: *JcSDP1* gene expression profiles using quantitative real-time PCR at different seed developing stages (S1; 1WAF, S2; 2 to 3WAF, S3; 4 to 6WAF, and S4; 7 to 8WAF, WAF: weeks after fertilization *JcTubulin* expression levels were used as an internal control. Mean values are given with SD (n=3).

[0021] Figures 5A-5E show construction of *ProJcSDP1:JcSDP1-RNAi* transgenic *Jatropha* plants and analysis of seed oils. Fig. 5A: Structure of the inducible *ProJcSDP:SDP1-RNAi* marker-free construction. Fig. 5B: *ProJcSDP:SDP1-RNAi* marker-free transgenic plants; a-c) T1 generation of dried seeds. d) Overall phenotype of T1 transgenic plants generated from T1 embryos. e) Size comparison of the 5th leaf between control (CK) plants and *JcSDP1-RNAi* transgenic line # 158. Fig. 5C: Total fatty acid analysis in mature endosperm of T1 seed. Fig. 5D: Fatty acid profile in *JcSDP1-RNAi* transgenic lines. Fig. 5E: *JcSDP1* transcript levels in transgenic lines. *JcTubulin* expression levels were used as an internal control. Numbers refer to transgenic line numbers. Asterisks indicated statistically significant difference compared with the control, **P*<0.05, ***P*<0.01, or ****P*<0.001 versus CK, in different biological replicates. CK, 35S:GFP control.

[0022] Figures 6A-6C show schematic diagram of *SDP1* gene structure and insertion position of T-DNA in the *sdp1-5* allele. Fig. 6A: The T-DNA is inserted in the first exon of *SDP1* resulting in a null mutation. Fig. 6B: Isolation of the homozygous *sdp1-5* mutant by RT-PCR. Fig. 6C: Expression levels of *AtSDP1* in early developing seeds (3 to 5 DAP). *Actin1*

(At2g37620) expression levels were used as an internal control. * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$ versus Col-0 for 5 biological replicates. SD, standard deviation ($n=5$). DAP: Days after pollination.

[0023] Figure 7 shows expression profiles of fatty acid synthesis-related genes in early developing seeds of WT (Col-0) and *sdp1-5*. The cDNA library was synthesized from total mRNA derived from seeds (3 to 5 DAP). DAP: Days after pollination. Mean values are given with SD ($n=3$). ACP1(acyl carrier protein; At3g05020), FAD2(oleate desaturase; At3g12120), FAD3(linoleate desaturase; At2g29980), FAE1(fatty acid elongase; At4g34520), Cy-PK(cytosol pyruvate kinase; At5g52920), Ch-PK(chloroplast pyruvate kinase; At3g22960), KASI(ketoacyl-ACP Synthase I; At5g46290), KASIII(ketoacyl-ACP synthase III; At1g62640), BCCP2(biotin carboxyl carrier protein; At5g15530), and SUS2(sucrose synthase 2; At5g49190).

[0024] Figures 8A and 8B shows analysis of lipid composition of mature dried endosperm of *JcSDP1-RNAi* by TLC and GC/MS. Fig. 8A: Total lipids were extracted from mature endosperm of CK and *JcSDP1-RNAi* transgenic line #158 and 300 μ g of total neutral lipids were separated by TLC on silica gel plate and stained with Iodine (I_2). SE, sterol ester; TAG, triacylglycerol; FFA, free fatty acid; DAG, diacylglycerol. Fig. 8B: Profiling of relative amounts of FFA and TAG by GC/MS. The absolute amount was calculated using the C15:0 as an internal control by comparing their peak areas.

[0025] Figure 9 shows a schematic diagram of a *JcSDP1-RNAi* sequence in accordance with one embodiment of the invention. A sequence for this construction is shown in SEQ ID NO:3

[0026] Figures 10A and 10B show Southern blot analysis of T0 and T1 transgenic plants expressing *JcSDP1-RNAi*. Fig. 10A: Total genomic DNA were digested with *XhoI* restriction enzyme and hybridized with *HPT* probe. Fig. 10B: Total lipid content (% w/w) in individual transgenic line carrying the *JcSDP1-RNAi* construct.

DETAILED DESCRIPTION OF THE INVENTION

[0027] The present invention relates to the field of plant molecular biology and gene silencing. More particularly, the present invention relates to gene silencing of *Sugar-dependent 1* (*JcSDP1*) in *Jatropha curcas*. *JcSDP1* encodes a patatin-domain triacylglycerol lipase. Silencing of *JcSDP1* enhances seed oil accumulation in *J. curcas*.

[0028] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention belongs.

[0029] As used herein, “allele” refers to any of one or more alternative forms of a gene locus, all of which alleles relate to a trait or characteristic. In a diploid cell or organism, the two alleles of a given gene occupy corresponding loci on a pair of homologous chromosomes.

[0030] As used herein, “gene” refers to a nucleic acid sequence that encompasses a 5' promoter region associated with the expression of the gene product, any intron and exon regions and 3' or 5' untranslated regions associated with the expression of the gene product.

[0031] As used herein, “genotype” refers to the genetic constitution of a cell or organism.

[0032] As used herein, “phenotype” refers to the detectable characteristics of a cell or organism, which characteristics are the manifestation of gene expression.

[0033] The terms “polynucleotide,” “nucleic acid” and “nucleic acid molecule” are used interchangeably herein to refer to a polymer of nucleotides which may be a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, including deoxyribonucleic acid, ribonucleic acid, and derivatives thereof. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role. Unless otherwise indicated, nucleic acids or polynucleotide are written left to right in 5' to 3' orientation. Nucleotides are referred to by their commonly accepted single-letter codes. Numeric ranges are inclusive of the numbers defining the range.

[0034] The terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Amino acids may be referred to by their commonly known three-letter or one-letter symbols. Amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range.

[0035] Thus, in one aspect, the present invention provides for In a first aspect, the present invention provides an isolated nucleic acid encoding a *JcSDP1* protein comprising the amino acid sequence set forth in SEQ ID NO:2. In one embodiment, the nucleic acid comprises the nucleotide sequence set forth in SEQ ID NO:1. In another embodiment, the nucleic acid comprises the nucleotide sequence set forth as nucleotides 874-3450 of SEQ ID NO:1. In an additional embodiment, the nucleic acid comprises the nucleotide sequence set forth as nucleotides 874-3447 of SEQ ID NO:1. In a further embodiment, the nucleic acid further comprises a plant operable promoter operably linked to a nucleic acid comprising the coding sequence, or a nucleic acid comprising the 5' UTR (or portion thereof) and the coding sequence, or a nucleic acid comprising the coding sequence and the 3' UTR (or portion thereof) or a

nucleic acid comprising the 5' UTR (or portion thereof), coding sequence and the 3' UTR (or portion thereof). In another embodiment, the seed specific promoter is a *Jatropha* seed specific promoter. In a further embodiment, the *Jatropha* seed specific promoter is the *JcSDPI* promoter. In one embodiment, the *JcSDPI* promoter comprises nucleotides 1-722 of SEQ ID NO:1. In another embodiment, functional fragments of the *JcSDPI* promoter can be used. Useful functional fragments of the *JcSDPI* promoter can be identified by the skilled artisan using conventional techniques well known in the art including, but not limited to promoter analysis software, promoter cis-element software and the like.

[0036] In a second aspect, the present invention provides a construct or vector comprising an isolated nucleic acid as described herein. In one embodiment, the construct or vector is an expression construct or vector. In another embodiment, the construct or vector further comprises a selectable marker. In a further embodiment, the construct or vector comprises a Cre-lox recombination marker free system.

[0037] In a third aspect, the present invention provides a transgenic plant comprising a nucleic acid or vector described herein. In one embodiment, the transgenic plant is a *Jatropha* plant.

[0038] In a fourth aspect, the present invention provides for the down regulation of a *Jatropha SDPI* gene. In one embodiment, the down regulation of a *Jatropha SDPI* gene involves using RNA interference (RNAi), including double stranded RNA, siRNA, microRNA, and hairpin RNA. In another embodiment, the down regulation of a *Jatropha SDPI* gene involves using viral induced gene silencing (VIGS). In one embodiment, a nucleic acid is provided which down regulates the *Jatropha SDPI* gene. In another embodiment, a nucleic acid is provided which down regulates the *Jatropha curcas SDPI* gene. In some embodiments, the nucleic acid comprises nucleotides 3161 to 3533 of SEQ ID NO:1. In another embodiment, a suitable nucleic acid for use in down regulating the *Jatropha SDPI* gene is selected using well known RNAi considerations and algorithms. In some embodiments, the nucleic acid further comprises a plant operable promoter operably linked to the nucleic acid. In one embodiment, the promoter is a seed specific promoter. In another embodiment, the seed specific promoter is a *Jatropha* seed promoter. In a further embodiment, the *Jatropha* seed specific promoter is the *JcSDPI* promoter. In one embodiment, the *JcSDPI* promoter comprises nucleotides 1-722 of SEQ ID NO:1. In another embodiment, functional fragments of the *JcSDPI* promoter can be used. In an additional embodiment, the promoter comprises the *JcSDPI* promoter and the 3' UTR (or portion thereof). In one embodiment, a nucleic acid suitable for down regulating *Jatropha SDPI* gene is set forth in SEQ ID NO:3. According to this aspect, the present

invention also provides a vector comprising an isolated nucleic acid as described herein. In one embodiment, the vector is an expression vector. In another embodiment, the vector further comprises a selectable marker. In a further embodiment, the vector comprises a Cre-lox recombination marker free system. According to this aspect, the present invention further provides a transgenic or infected plant or plant cell comprising a nucleic acid or vector described herein. In one embodiment, the transgenic or infected plant or plant cell is a *Jatropha* plant or plant cell. In some embodiments, the down regulation of a *Jatropha SDPI* gene provides enhanced seed oil accumulation in a transgenic or infected *Jatropha* plant, such as *Jatropha curcas*.

[0039] According to this aspect, the nucleic acid is selected to inhibit expression of the native gene or to silence the native gene within a plant's tissues to achieve a desired phenotype. In one embodiment, expression of the native gene is inhibited. Such inhibition might be accomplished, for example, with transformation of a plant cell to comprise a promoter linked to an antisense nucleotide sequence, hairpin, RNA interfering molecule, double stranded RNA, microRNA or other nucleic acid molecule, such that tissue-preferred expression of the molecule interferes with translation of the mRNA of the native DNA sequence or otherwise inhibits expression of the native DNA sequence in plant cells. For further description of RNAi techniques or microRNA techniques, see, e.g., U.S. Patent Nos. 5,034,323; 6,326,527; 6,452,067; 6,573,099; 6,753,139; and 6,777,588. See also International Publication Nos. WO 97/01952, WO 98/36083, WO 98/53083, WO 99/32619 and WO 01/75164; and U.S. Patent Application Publication Nos. 2003/0175965, 2003/0175783, 2003/0180945, 2004/0214330, 2005/0244858, 2005/0277610, 2006/0130176, 2007/0265220, 2008/0313773, 2009/0094711, 2009/0215860, 2009/0308041, 2010/0058498 and 2011/0091975. RNAi molecules or microRNA molecules can be prepared by the skilled artisan using techniques well known in the art, including techniques for the selection and testing of RNAi molecules and microRNA molecules that are useful for down regulating a *Jatropha SDPI* gene. In another embodiment, the native gene may be silenced by using VIGS. Such silencing may be accomplished by infecting a *Jatropha* plant with a VIGS system that contains at least a partial fragment of a candidate gene to be silenced. For further description of VIGS systems, see International Publication Nos. WO 2010/080071 and WO 2010/144058.

[0040] The construct typically includes regulatory regions operatively linked to the 5' side of the nucleic acid described herein (such as a nucleic acid encoding a *Jatropha SDPI* protein or a nucleic acid encoding an RNAi molecule to down regulate a *Jatropha SSDPI* gene) and/or to the 3' side of the nucleic acid. A cassette containing all of these elements is also referred to herein

as an expression cassette. The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the polynucleotide encoding a signal anchor may be native/analogous to the host cell or to each other. The promoters and tissue-specific promoters, such as seed promoters and especially *Jatropha* seed promoters, are particularly useful for preparing constructs for the transformation of *Jatropha*. Alternatively, the regulatory regions and/or the polynucleotide encoding a signal anchor may be heterologous to the host cell or to each other. See, U.S. Patent No. 7,205,453 and U.S. Patent Application Publication Nos. 2006/0218670, 2006/0248616 and 20090100536, and the references cited therein. The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include those described in International Publication No. WO 2008/094127 and the references cited therein.

[0041] A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. That is, the nucleic acids can be combined with constitutive, tissue-preferred, or other promoters for expression in the host cell of interest. Such constitutive promoters include, for example, the core promoter of the Rsyn7 (WO 99/48338 and U.S. Pat. No. 6,072,050); the core CaMV 35S promoter (Odell et al., 1985); rice actin (McElroy et al., 1990); ubiquitin (Christensen and Quail, 1989; Christensen et al., 1992); pEMU (Last et al., 1991); MAS (Velten et al., 1984); ALS promoter (U.S. Pat. No. 5,659,026), and the like. Other constitutive promoters include, for example, those disclosed in U.S. Pat. Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

[0042] Other promoters include inducible promoters, particularly from a pathogen-inducible promoter. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. Other promoters include those that are expressed locally at or near the site of pathogen infection. In further embodiments, the promoter may be a wound-inducible promoter. In other embodiments, chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. The promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. In addition, tissue-preferred promoters can be utilized to target enhanced expression of a polynucleotide of interest within a particular plant tissue. Each of these promoters are described in U.S. Pat. Nos. 6,506,962, 6,575,814, 6,972,349 and 7,301,069 and in

U.S. Patent Application Publication Nos. 2007/0061917 and 2007/0143880. Any other recourse seed specific promoter can be used to, for example, *Jatropha* oleosin promoter (Popluechai et al., 2011), 2S storage protein promoter, the *Jatropha SDP1* promoter described herein, and the like.

[0043] Generally, the expression cassette may additionally comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Usually, the plant selectable marker gene will encode antibiotic resistance, with suitable genes including at least one set of genes coding for resistance to the antibiotic spectinomycin, the streptomycin phosphotransferase (spt) gene coding for streptomycin resistance, the neomycin phosphotransferase (nptII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (hpt or aphiv) gene encoding resistance to hygromycin, acetolactate synthase (als) genes. Alternatively, the plant selectable marker gene will encode herbicide resistance such as resistance to the sulfonylurea-type herbicides, glufosinate, glyphosate, ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D), including genes coding for resistance to herbicides which act to inhibit the action of glutamine synthase such as phosphinothricin or basta (e.g., the bar gene). See generally, International Publication No. WO 02/36782, U.S. Patent No. 7,205,453 and U.S. Patent Application Publication Nos. 2006/0218670, 2006/0248616, 2007/0143880 and 2009/0100536, and the references cited therein. See also, Jefferson et al. (1991); De Wet et al. (1987); Goff et al. (1990); Kain et al. (1995) and Chiu et al. (1996). This list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used. The selectable marker gene is also under control of a promoter operable in the plant species to be transformed. Such promoters include those described in International Publication No. WO 2008/094127 and the references cited therein.

[0044] Alternatively, the expression cassette may additionally comprise a Cre-lox recombination marker free system, such as described by Zuo et al. (2001). Such a system is useful for producing selection marker free transgenic *Jatropha* plants.

[0045] In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g. transitions and transversions may be involved.

[0046] Once a nucleic acid has been cloned into an expression vector, it may be introduced into a plant cell using conventional transformation procedures. The term "plant cell" is intended to encompass any cell derived from a plant including undifferentiated tissues such as callus and suspension cultures, as well as plant seeds, pollen or plant embryos. Plant tissues suitable for transformation include leaf tissues, root tissues, meristems, protoplasts, hypocotyls, cotyledons, scutellum, shoot apex, root, immature embryo, pollen, and anther. "Transformation" means the directed modification of the genome of a cell by the external application of recombinant DNA from another cell of different genotype, leading to its uptake and integration into the subject cell's genome. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained.

[0047] DNA constructs containing the promoters of the present invention can be used to transform any plant and particularly cotton plants. The constructs may be introduced into the genome of the desired plant host by a variety of conventional techniques. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. Transformation protocols may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation, as is well known to the skilled artisan. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment. Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. Thus, any method, which provides for effective transformation/transfection may be employed. See, for example, U.S. Patent Nos. 7,241,937, 7,273,966 and 7,291,765 and U.S. Patent Application Publication Nos. 2007/0231905 and 2008/0010704 and references cited therein. See also, International Publication Nos. WO 2005/103271 and WO 2008/094127 and references cited therein. Techniques which have been used to transform oil palm include biolistic-mediated transformation and *Agrobacterium*-mediated transformation. See, for example, Masli et al. (2009); Omidvar et al. (2008); Parveez et al. (2008); Abdullah et al. (2005); Parveez et al. (2000); Chowdhury, et al. (1997); U.S. Patent Application Publication No. 2009/0038032; and International Publication No. WO 2010/071608.

[0048] Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype, e.g., a transgenic plant. A "transgenic plant" is a plant into which foreign DNA has been introduced. A "transgenic plant" encompasses all descendants, hybrids, and crosses thereof, whether reproduced sexually or asexually, and which continue to harbor the foreign DNA. Regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. See for example, International Publication Nos. WO 2008/094127 and WO 2010/071608 and references cited therein.

[0049] The foregoing methods for transformation are typically used for producing a transgenic variety in which the expression cassette is stably incorporated. After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. In one embodiment, the transgenic variety could then be crossed, with another (non-transformed or transformed) variety, in order to produce a new transgenic variety. Alternatively, a genetic trait which has been engineered into a particular *Jatropha* line using the foregoing transformation techniques could be moved into another line using traditional backcrossing techniques that are well known in the plant breeding arts. For example, a backcrossing approach could be used to move an engineered trait from a public, non-elite variety into an elite variety, or from a variety containing a foreign gene in its genome into a variety or varieties which do not contain that gene. As used herein, "crossing" can refer to a simple X by Y cross, or the process of backcrossing, depending on the context. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

[0050] Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedures. Transgenic seeds can, of course, be recovered from the transgenic plants. These seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants. The cultivated transgenic plants will express the DNA of interest in a tissue-preferred or tissue-specific manner as described herein.

[0051] In a fifth aspect, the present invention provides methods of increasing seed oil accumulation in *Jatropha*, such as *Jatropha curcas*. In one embodiment, a method involves modulating the level of activity of a *Sugar-dependent 1 (SDP1)* gene, such as *JcSDP1* gene, which encodes a patatin-domain triacylglycerol lipase in the host *Jatropha* cell or *Jatropha* plant. The level of activity of the lipase can be reduced by reducing expression of the *SDP1* gene. In

one embodiment, the modulation of the level of activity of the lipase is a reduction in the activity of the lipase. The level of activity of the lipase can be reduced by using RNAi techniques described herein in which the lipase is the target for the RNAi. Alternatively, the level of activity of the lipase can be reduced using VIGS techniques as described herein in which at least a partial fragment of the target gene is used. . In one embodiment, *Jatropha* RNAi plants accumulate about 13% to about 30% higher seed oil than control plants.

[0052] The present invention shows that *SDPI*-deficiency enhances seed oil accumulation in the model plant *Arabidopsis*. Based on this result, RNAi technology and the native *JcSDPI* promoter was used to generate transgenic *Jatropha* plants with reduced endogenous *SDPI* expression. The present invention shows that *SDPI*-deficient transgenic *Jatropha* plants, such as those produce by RNAi technology, accumulate about 13% to about 30% higher seed oil than control plants. Free fatty acid content in seed was reduced from about 27% in WT to about 8.5% in *SDPI*-deficient transgenic *Jatropha* plants, such as those produce by RNAi technology. This transgenic technology leads to an enhanced agronomic trait for this biodiesel crop.

[0053] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992), *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Russell, 1984, *Molecular biology of plants: a laboratory course manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu *et al.* eds.), *Immunochemical Methods In Cell And*

Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Fire et al., *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge University Press, Cambridge, 2005; Schepers, *RNA Interference in Practice*, Wiley-VCH, 2005; Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology*, DNA Press, 2003; Gott, *RNA Interference, Editing, and Modification: Methods and Protocols (Methods in Molecular Biology)*, Human Press, Totowa, NJ, 2004; Sohail, *Gene Silencing by RNA Interference: Technology and Application*, CRC, 2004.

EXAMPLES

[0054] The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized. The following abbreviations are used: DAP: Days After Pollination; FAME: Fatty Acid Methyl Ester; FFA: Free Fatty Acid; GC: Gas Chromatography; GC/MS: Gas Chromatography/Mass Spectrometry; GUS: beta-glucuronidase; PTLs: Patatin-like TAG Lipases; qRT-PCR: Quantitative real time PCR; RNAi: RNA interference; SEM: Scanning Electron Microscopy; SDP1: Sugar-dependent1; TAGs: Triacylglycerols; TEM: Transmission Electron Microscopy; TLC: Thin Layer Chromatography; WAF: Weeks After Fertilization.

EXAMPLE 1

Materials and Methods

[0055] *Plant materials and growth condition:* Arabidopsis Col-0 (*Arabidopsis thaliana* ecotype Columbia-0) was grown on soil with sand. Homozygous *sdp1-5* mutant (*Salk_076697*, Arabidopsis Biological Resource Center, Ohio State University, Columbus, USA) seeds were stratified at 4° C for 3 days on soil and cultured in a growth chamber under 16hr-light/ 8hr-dark cycles, 23° C $\pm$ 3° C and white light (100-150 μ Em⁻²s⁻¹ photosynthetically active radiation). Rosette leaves were harvested from 3 or 4 week-old plants and other organs such as inflorescence stems, cauline leaves, flowers, siliques, and developing seeds were harvested from 6 week-old plants. Seeds from siliques of 3 to 5 DAP (DAP: day after pollination) were used as the early stage of seed maturation in Arabidopsis. Leaves and developing fruits (S1; 1WAF, S2; 2 to 3WAF, S3; 4 to 6WAF, and S4; 7 to 8WAF, WAF: weeks after fertilization) were collected from *Jatropha* plants grown in a greenhouse.

[0056] *RNA isolation and quantitative real-time PCR:* Total mRNA was isolated from plant samples using TRIzol reagent (Invitrogen, St. Louis, MO, USA) according to the manufacturer's instructions. cDNA was synthesized with 1 μ g total mRNA using MMLV Superscript II (Promega, Madison, WI, USA) after DNase I treatment (Roche Applied Science, Mannheim, Germany). Quantitative real-time PCR assay was performed with an ABI 7900 sequence Detection System (Applied-Biosystems, Foster City, CA, USA). Power SYBR Green PCR Master Mix (Applied-Biosystems) reagent protocol was used but reduced the volume to 10 μ L per reaction. As controls, the species-specific *tubulin* and *actin* primer sets for *Jatropha curcas* and *Arabidopsis thaliana*, respectively, were used. Fold change values of the target gene transcripts were subsequently normalized by dividing the Δ Ct values with the Δ Ct values of each control gene transcript. All real time PCR experiments were performed in triplicates using different biological samples.

[0057] *Isolation of JcSDP1 full length cDNA and its promoter:* A partial *SDP1* sequence in our *Jatropha curcas* EST database was found. Using this partial sequence, 5' or 3' cDNA RACE primers were designed and RACE experiments were performed using BD SMART™ RACE cDNA Amplification Kit (BD Bioscience Clontech, Franklin Lakes, NJ, USA). One μ g total RNA derived from developing seeds (S2 to S3) was used for the generation of 5' or 3' RACE pools. The 5' and 3' cDNA ends were obtained by touchdown PCR with advantage 2 PCR Enzyme System (BD Bioscience Clontech) following the manufacturer's instructions. To specifically amplify the products, the primary RACE products were diluted and mixed with a nested gene specific primer (GSP2) and the nested universal primer mixture (AP2). The PCR products were gel purified, cloned into pDrive Cloning Vector (Qiagen, Düsseldorf, Germany) to obtain full-length *JcSDP1* cDNA (2577-bp). The Universal GenomeWalker kit (BD Bioscience Clontech) was used to isolate the promoter fragment of the *JcSDP1* gene. Genomic DNA was restricted by *Dra*I, *Eco*RV, *Pvu*II, *Ssp*I, and *Stu*I endonuclease and five libraries of adaptor-ligated genomic fragments were constructed. These genomic DNA libraries were used as templates for the PCR reactions for promoter isolation. For each round of genome walking, the primary PCR products were amplified by a gene-specific primer (GSP1) and the outer adaptor primer (AP1). To the second PCR reaction mix, the primary products were diluted and used as templates with a nested gene specific primer (GSP2) and the nested adaptor primer (AP2). The secondary PCR products were then analyzed on agarose gels and the relevant DNA fragment purified with QIAEXII Gel Extraction Kit (Qiagen), cloned into pDrive Cloning Vector (Qiagen) and sequenced. Potential *cis*-elements in the promoter region were analyzed using computational analytical methods available on two public web-sites, PlantCare

(<http://bioinformatics.psb.ugent.be/webtools/plantcare>) and PLACE
(<http://www.dna.affrc.go.jp>).

[0058] *Construction of Pro JcSDP1:GUS fusion gene and promoter analysis:* pKGWFS7 destination vector including *GFP* or *GUS* reporter gene was used for promoter analysis. The 5'-flanking region of *JcSDP1* was amplified using two specific primers, JcSDP1-PF1T and JcSDP1-PR1. This promoter was 873-bp in length, including 149-bp of 5' untranslated region (UTR). The amplified PCR product was inserted into TOPO donor vector using the pENTR™/D TOPO Cloning Kit (Invitrogen), and then inserted into the destination vector, pKGWFS7, using the Gateway LR Clonase™ II enzyme mix (Invitrogen). For plant transformation, the constructs were introduced into *Agrobacterium tumefaciens* strain AGL1 using electroporation. Constructs were transformed into WT (Col-0) or *sdp1-5* mutant (*Salk_076697*) background through the floral dip method described by Clough and Bent (38).

[0059] For transient assay of *JcSDP1* promoter expression, 10 µg of *ProJcSDP1:GUS* in pKGWFS7 plasmid DNA was coated with 1 µm diameter gold particles (2.5 mg gold particles, 200 µL of 2.5 M CaCl₂ and 100 µL of 0.1 M Spermidine). After incubating on ice for 30 min, the pellet of DNA/gold particle was washed twice with 70% ethanol and re-suspended in 100% ethanol. *Jatropha* fruits and leaves were centered in a petri-dish containing MS agar medium (39). Fruits and leaves were bombarded at 1,350 psi with a biolistic helium gun device (Bio-rad PDS-1000/He, USA). After incubation for 2 days at 25° C the bombarded tissues were analyzed by histochemical assays as described by Jafferson et al. (40). The tissues were incubated in GUS staining buffer [0.1 M Sodium phosphate (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-D-glucuronide (Sigma, St Louis, MO, USA), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM Na₂EDTA, and 0.1% Triton X-100] for 20 hrs at 37° C. Stained tissues were rinsed with 70% to 80% ethanol until pigments such as chlorophylls had been cleared. Selected organs of *ProJcSDP1:GUS* transgenic *Arabidopsis* lines were analyzed for GUS expression. To investigate the sugar-responsive expression of *JcSDP1* promoter, we used 14 days-old seedlings of T2 lines and incubated for 24 hrs in an MS media with 1% or 3% of a sugar source: sucrose, fructose, and glucose. Mannitol was used as a control for osmotic stress.

[0060] *Complementation of the JcSDP1/sdp1-5 mutation in Arabidopsis:* Full-length *JcSDP1* cDNA was cloned into a vector harboring a tandem CaMV35S promoter and a Nos polyA addition sequence from pCAMBIA 1300 and transformed into *Arabidopsis sdp1-5* mutant (*Salk_076697*). T1 transformants were selected on 40 µg/mL hygromycin and T2 seedlings were assayed for sugar responses on MS agar plates without sucrose.

[0061] *Construction of inducible marker-free ProJcSDP1:JcSDP1-RNAi vector and transformation to Jatropha plants:* The pCCreloxP (pCCLB3) inducible marker free vector system was used as described by Qiu et al. (41). The T-DNA of pCCreloxP vector harbors a *loxP* fragment that consists of CRE-int, HPT and XVE genes (Figure 5A). To silence JcSDP1 expression using RNAi a 373-bp fragment was selected that includes 290-bp 3' coding region of *JcSDP1* cDNA with a stop codon and 83-bp of the 3' UTR region. The 373-bp *JcSDP1* fragment was amplified with two primer sets, JcSDP1-RNAiF-*XhoI* and JcSDP1-RNAiR2-*HindIII* or JcSDP1-RNAiF-*BamHI* and JcSDP1-RNAiR2-*PstI*, used for the sense orientation or antisense orientation, respectively, and the fragments were then cloned into pBluescript SK intron vector (pBS-Ski) which has an intron (156-bp) to generate p*JcSDP1-RNAi* as previously described (42). *ProJcSDP1* was amplified with two primers, JcSDP1-PF1-*ApaI* and JcSDP1-PR1-*XhoI* and then restricted with *ApaI/XhoI*. To insert the *ProJcSDP1* (*JcSDP1* promoter) fragment into the p*JcSDP1-RNAi* construct, we used *ApaI* and *XhoI* restriction enzyme sites in the p*JcSDP1-RNAi*. A 226-bp fragment containing the CaMV35S polyA addition sequence (T35S) was amplified with T35S-F-*XbaI* and T35S-R-*Pml-SacII* primers. The amplified T35S fragment was cleaved with *XbaI/SacII* restriction enzymes and then cloned into *ProJcSDP1-RNAi*. Finally, the *ProJcSDP1:JcSDP1-RNAi:T35S* fragment from the *ProJcSDP1-RNAi* construct was inserted into the pCCreloxP marker free vector at the *ApaI* and *PmlI* sites. All constructs were introduced into *Agrobacterium tumefaciens* strain AGL1 using electroporation and then transformed into *Jatropha* using cotyledon explants (43).

[0062] *Fatty acid analysis in Arabidopsis and Jatropha:* We used gas chromatography (GC) to analyze fatty acid profiles of the *sdp1-5* mutant. (44). 100 dried seeds of each line were weighed and samples were transmethylated at 85°C for 2 hrs in a reaction buffer (1 mL of 3 M HCl-methanol (Sigma), 25 µL of BHT solution (Sigma), and 300 µL of toluene (Sigma)). 50 µg of pentadecanoic acid (C15:0, Sigma) was added to each sample as an internal standard. After being cooled down to room temperature, 1.5 mL of 0.9% NaCl (w/v) was added to the mix and the fatty acid methyl esters (FAMES) were extracted two times with 1 mL of hexane. Extracts were evaporated under nitrogen and then dissolved in 100 µL of the hexane. The final extracts were analyzed with GC using a flame ionization detector (FID) on Agilent 6890 (Agilent, Santa Clara, CA, USA) employing helium as the carrier gas. Total fatty acids were estimated by comparing the total FAME peak area (pA*sec) to that of the C15:0.

[0063] To analyze fatty acid content in *Jatropha* transgenic lines, dried endosperm of seed was used as described previously (7). *Jatropha* samples were transmethylated at 70° C for 20 min in a reaction buffer (1 mL of 3 N methanolic-HCl, 400 µL of 2, 2, dimethoxypropane

(Sigma) and 50 µg of pentadecanoic acid (C15:0)). After being cooled down to room temperature, the fatty acid methyl esters (FAMES) were washed with 1 mL of water and 1 mL hexane twice. The extracts were evaporated under nitrogen and then dissolved in 500 µL of hexane. The final samples were analyzed by GCMS-QP2010 (Shimadzu Corporation, Japan). Standard deviation was calculated based on several different plants.

[0064] *Analysis of Lipids by TLC and Quantification by GC/MS:* Seed lipids were extracted with hexane three times (7, 44). After determination of total lipid amount, 300 µg of neutral lipid were fractionated by TLC (Thin Layer Chromatography) on silica gel plates in a running solvent mixture, hexane: ethyl acetic acid: acetic acid (90:10:1 by vol). Triolein (Sigma T7140) and Oleic acid (Sigma 75090) were used as a standard of TAG and of FFA, respectively. TLC plates were exposed to iodine (I₂) vapor for visualization. The separated neutral lipid species including TAG and FFA were recovered from the plates using hexane and quantified by GC/MS after conversion to their corresponding methyl esters by methanolic-HCl method according to Li et al. (44). The absolute amount was calculated against the C15:0 internal standard by comparing their relative peak areas.

[0065] *TEM and SEM analysis:* TEM was performed with mature dried seeds of WT (Col-0) and *sdp1-5* mutant. Seeds were embedded in resin and sectioned by an ultramicrotome (Leica Ultracut UCT; Leica, Germany) set at 70-nm thickness. Sectioned samples were placed onto 300 mesh cooper grids. Sections were observed and pictures were taken with transmission electron microscope (JEM-1230; JEOL, Japan) at 120kV. For SEM analysis dried seeds were mounted directly and observed using a JSM-6360LV (JEOL, Japan) with an acceleration voltage of 20 kV.

[0066] *Seed weight and size measurement:* Mature seeds were harvested from WT (Col-0) and *sdp1-5* mutant grown under the same conditions. Sample size of 100 seeds per WT (Col-0) or *sdp1-5* mutant was used to obtain an average seed weight with at least five times biological replications. Average values are given along with SD (n=5). The Leica DM5000B microscope (Germany) and ImageJ analysis software were used to measure seed sizes. Average values are given along with SD (n=10).

[0067] *Primers:* Sequence information on primers used in the experiments is set forth in Table 1 The SEQ ID NO: is set forth in parenthesis.

TABLE 1
Primer Sequences

Promoter analysis	(SEQ ID NO:)
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	JcSDP1-PF1T	CACCTacatccagtagattgcacgtcacacacta (8)
	JcSDP1-PR1	ctctaaaagtctcgtgttttgatgattgct (9)
Gene construction		
	JcSDP1-CF-Sal	tggctcgacatggatataagtaatgaggccaatgt (10)
	JcSDP1-CR-Nhe	attgctagcaccatccacagaactttgatcctgtc (11)
	JcSDP1-RNAiF-Xho	atactcgagtcataatgggattgtgttgaaacgtcgt (12)
	JcSDP1-RNAiR2-H3	tcgaagcttgctagtggcacttgatattaaggtgat (13)
	JcSDP1-RNAiF-Bm	ataggatcctccataatgggattgtgttgaaacgtcgt (14)
	JcSDP1-RNAiR2-Pst	tcttctgcagctagtggcacttgatattaaggt (15)
	JcSDP1-PF1-ApaI	tatggccctacatccagtagattgcacgtcacaca (16)
	JcSDP1-PR1-Xho	atgctcgagctctaaaagtctcgtgttttgatgatt (17)
	T35S-F-Xba	acttctagacggccatgctagagtccgcaa (18)
	T35S-R-Pml-SacII	ttaccgcggcacgtgaggtcactggatttt (19)
MF genotyping		
	HygF	aaaaagcctgaactcacgcgacgtct (20)
	HygR	tacttctacacagccatcggtcca (21)
	MF-P1	ctgaattgtcgaggtcgaagatc (22)
	JcSDP1-R16	agcagccaaatgggtctgtcc (23)
	T35S-R	cttctcattatcggtgtgaacat (24)
qRT-PCR		
At2g37620	AtActin F	ctctgtctggattggagggtc (25)
	AtActin R	gcttgagaaatggtcggaaa (26)
At4g25140	AtOleo1-F	tgccgctataaccgtttctcttgga (27)
	AtOleo1-R	atgttccccaccagtatgttgctgtcc (28)
At5g40420	AtOleo2-F	atgcggttggctacgcaggacaa (29)
	AtOleo2-R	tcatgcagccgtcgtctccct (30)
At5g51210	AtOleo3-F	tggaatagccgccattaccgcctt (31)
	AtOleo3-R	agaaacttgttggtgttgactccaatg (32)
At5g04040	AtSDP1-QF	atgccatcgattgggaacca (33)
	AtSDP1-QR	caccggttctgttaaaccggaat (34)
	JcTubulin F	gaggctggatctggcaaacacgtt (35)
	JcTubulin R	tgtgtaatgacctctagcaaaatta (36)
	JcSDP1-QF	cataatgggattgtgttgaaacgtcgt (37)
	JcSDP1-QR	gtcatcatcgatccccagcata (38)
Fatty acid related genes in Arabidopsis		
At3g12120	AtFAD2-F	cctcagcctctcttacttggtt (39)
	AtFAD2-R	ccaggagaagtaaggacgag (40)
At2g29980	AtFAD3-F	ctacttgttggtccataatgttcgtca (41)
	AtFAD3-R	cgtagataactccattccttgcct (42)

At4g34520	AtFAE1-F	gtcttaaccaactttttcaacctctgtt (43)
	AtFAE1-R	ccaaaccgaaaacagtgaagcaaagagt (44)
At3g05020	AtACP1-F	aatctatccttcaacctccgccgt (45)
	AtACP1-R	agagaatctgctccaaggtcagct (46)
At5g15530	AtBCCP2-F	ggatctcctttccctccgatgtt (47)
	AtBCCP2-R	ataaattcagagagctcggcgggt (48)
At3g22960	AtCh-PK-F	tggcatcactggctcctccgatgt (49)
	AtCh-PK-R	tcataaccttggatttcagtaacgaga (50)
At5g52920	AtCy-PK-F	agtcactatcgtccttccggcacia (51)
	AtCy-PK-R	cgtctgtacgattgctatttctct (52)
At5g49190	AtSUS2-F	tcaagtcagttcactgcagatcta (53)
	AtSUS2-R	caactcggtaaagaccaggcatagtga (54)
At5g46290	AtKAS1-F	tcgcaaacacacatcacacac (55)
	AtKAS1-R	gtgattgacgattgatggaag (56)
At1g62640	AtKASIII-F	tggtcttctgcttctgcc (57)
	AtKASIII-R	ccgcttctcactgcctcat (58)

[0068] *Protein and Carbohydrate analysis:* Protein content in *Jatropha* transgenic lines was analyzed according to Focks and Benning (45) with 50 mg of dried endosperm. Protein amounts were measured by the Lowry DC protein assay (Bio-Rad) using gamma-globulin as a standard. To analyze carbohydrate content, 50 mg of dried endosperm were homogenized in 200 μ L of assay buffer and centrifuged at full speed. The extracted supernatant was used for carbohydrate quantification using Total Carbohydrate Assay Kit (Sigma). D-glucose was used as a standard for calibration.

[0069] *gDNA isolation and Southern blot:* Total genomic DNA was isolated from leaves from transgenic or control (CK; 35S:*GFP*) plants grown in a greenhouse. We used CTAB (cetyltrimethylammonium bromide, Sigma) method (46). Genomic DNA was digested with restriction enzymes and separated on 0.8% agarose gels. The gels were processed and blotted onto Hybond-N⁺ membranes (Roche, Germany) following standard procedures (47). Probes were prepared with PCR DIG Labelling Mix using specific primer sets for Hygromycin phosphotransferase (*HPT*) gene and *JcSDP1* gene. Hybridization was performed using the PCR DIG detection kit following the supplier's instructions (Roche, Germany).

EXAMPLE 2

sdp1-5 Accumulates TAG in Mature Dried Seeds

[0070] We obtained from the ABRC stock center an Arabidopsis mutant (*Salk_076697*) with T-DNA insertion in the *SDP1* (At5g04040) locus and this mutant was designated *sdp1-5* [15]. Using RT-PCR as a screen we obtained 3 homozygous lines (#24, 30 and 33) of the *sdp1-5* null allele and one line (#33) was chosen for further experiments (Figure S1B). *SDP1* transcript levels in *sdp1-5* (#33) were 20-fold lower than in WT (Col-0) during early stages of seed development (3 to 5 DAP, DAP: Day after pollination) (Figure 6C).

[0071] We first examined the effect of *SDP1* deficiency on seed development, total fatty acid content and fatty acid profile. Scanning Electron Microscopy (SEM) analysis showed that *sdp1-5* seeds were slightly larger than those of WT (Col-0) in length and width (Figure 1A; Table 2). In addition, *sdp1-5* seeds displayed an increase in dry weight of around 11.5% as compared to WT. To investigate the role of *SDP1* in seed oil accumulation, total oil content and fatty acid composition in dried seeds were compared between WT (Col-0) and the *sdp1-5* mutant. Figure 1 B and C show that the average dry seed weight of WT (Col-0) was about 19 µg containing approximately 5.54 µg of total fatty acids. The seed lipid content obtained by us is very similar to those reported by others, which is around 30-35% of dry seed weight. On the other hand, *sdp1-5* seeds had an average dry weight of around 22 µg per seed containing 7.17 µg of total fatty acids. Therefore, *sdp1-5* seeds have about 10% higher levels of total fatty acids compared to WT (Col-0) seeds (Figure 1D). In addition, there was a clear increase in the relative proportion of unsaturated fatty acids, such as linolenic acid (C18:3) and eicosenoic acid (C20:1) in *sdp1-5* seeds (Figures 1E and 1F). To characterize TAG accumulation in mature dried seeds, we analyzed total neutral lipid from WT (Col-0) and 3 homozygous lines of the *sdp1-5* null allele by thin layer chromatography on silica gel plates. Figure 1G shows reduced levels of free fatty acids (FFA) in *sdp1-5* compared to WT (Col-0). Triolein and oleic acid were used as standards of TAG and FFA, respectively. To obtain quantitative data, we analyzed the samples using GC/MS and pentadecanoic acid (C15:0) was used as an internal control for quantitation. The *sdp1-5* mutant had about 4.25% FFA and 95.75% TAG compared to 13.35% FFA and 86.65% TAG in WT (Col-0) (Figure 1H).

TABLE 2
Seed Weight and Size of WT (Col-0) and *sdp1-5*

Name	Weight of seed (µg/seed) ^a		Length of seed (µm/seed) ^b		Width of seed (µm/seed) ^b	
Col-0	19.00	±1.10	481.12	±3.36	276.70	±10.58
<i>sdp1-5</i>	21.00	±0.57 ** ^c	522.86	±7.56***	294.29	±15.12 *

^aSeed weight determination using 100 mature seeds. Average values are given along with SD (n=5). ^bThe length and width of seed were measured using mature dried seeds. Average values are given along with SD (n=10). ^c**P*<0.05, ***P*<0.01 or ****P*<0.001 versus WT (Col-0) seed.

[0072] To examine the molecular basis of these changes in lipid content and fatty acid profile, we analyzed transcript levels of key genes involved in fatty acid biosynthesis. We found *FAD3* was up-regulated in *sdp1-5* (Figure 7). Previously, Puttick et al. (17) reported that in *Arabidopsis* over-expression of *FAD3* leads to an accumulation of high levels of linolenic acid and that is inversely correlated with linoleic acid (18:2) levels. We also observed an inverse correlation between linolenic acid and linoleic acids levels in *sdp1-5* seeds. In addition, we found an increased accumulation of eicosenoic acid, a very-long-chain fatty acid that has been used as a metabolic marker for storage TAGs formation in *Arabidopsis* seeds (18). We found *sdp1-5* seeds accumulated 1.3 µg of eicosenoic acid per seed which was 40% higher than that of WT (Col-0) seeds. The increased eicosenoic acid level in *sdp1-5* seeds was likely due to the up-regulated *Fatty Acids Elongase 1 (FAE1)* (Figure 7). Our results suggest that *SDP1*-deficiency is closely correlated with seed size augmentation, the relative proportion of unsaturated fatty acids and the accumulation of TAGs in mature seeds.

EXAMPLE 3

sdp1-5 Mutant Has Increased Numbers of Oil Bodies in Dried Seeds

[0073] Plant storage lipids, predominantly triacylglycerols (TAGs), are sequestered by monolayer phospholipids with embedded small proteins, such as oleosin, to form oil bodies (19). Previous work has highlighted the importance of oleosins for oil body structure and TAG accumulation in mature seeds. We used Transmission Electron Microscopy (TEM) to analyze the formation of oil bodies in *sdp1-5* seeds. Mutant *sdp1-5* seeds contained increased number of oil bodies but they were relatively smaller in size compared to those in WT (Col-0) seeds

(Figure 2A). The cross-sectional area of one mature seed cell was about $196 \mu\text{m}^2$. Within this area, there were around 175 oil bodies in WT (Col-0) but about 216 oil bodies in *sdp1-5*, representing an increase of approximately 23% (Figure 2B). Because oleosins are important proteins for seed oil body formation we investigated expression levels of 3 different oleosin genes in *sdp1-5* by qRT-PCR. Figure 2C shows transcript levels of *AtOLE1* (At4g25140) and *AtOLE2* (At5g40420) were two fold higher in *sdp1-5* compared to WT (Col-0). These results suggest the increased number of oil bodies in *sdp1-5* is possibly due to the enhanced expression of oleosins.

EXAMPLE 4

JcSDP1 an Ortholog of *AtSDP1* Rescues the *SDP1*-Deficiency in Arabidopsis

[0074] To investigate the impact of *SDP1* deficiency in an oil seed crop, we isolated full-length *JcSDP1* cDNA from *Jatropha* seed RNA samples using 5' and 3' cRACE technology. *JcSDP1* encodes a protein of 858 amino acids with a molecular mass of approximately 96 kDa. A BLAST search revealed that *JcSDP1* has high sequence homology (76%) to the Arabidopsis *SDP1* (At5g04040). *JcSDP1* has at least three predicted trans-membrane domains and four Site-1 protease (S1P) target sequences at the N terminus (RXXL; SEQ ID NO:5). Moreover, *JcSDP1* has a conserved patatin domain with lipase activity sequences such as the oxyanion hole motif (GXGXXG; SEQ ID NO:6) and a lipase consensus motif with a catalytic serine (GX SXG; SEQ ID NO:7) (Figure 3A). The high similarity of sequences and domains between *JcSDP1* and *AtSDP1* implied a possible similar function of *JcSDP1* in TAGs metabolism. To investigate the function of *JcSDP1* in TAG degradation during early stages of seed germination, we transformed *JcSDP1* under the control of a CaMV35S promoter into the Arabidopsis *sdp1-5* mutant. The Arabidopsis *sdp1-5* showed retarded growth on sugar depleted MS medium and but the retarded growth can be rescued by the supplementation of 1% sucrose (15). Seven day-old seedlings of *sdp1-5* plants expressing the heterologous *JcSDP1* displayed normal growth on MS medium without sucrose whereas *sdp1-5* mutant displayed retarded growth (Figure 3B). This result indicates that *JcSDP1* is able to rescue the retarded growth phenotype of *sdp1-5* providing evidence *JcSDP1* is an ortholog of *AtSDP1*.

EXAMPLE 5

Characterization of *JcSDP1* Promoter

[0075] To specifically silence *JcSDP1* gene expression in *Jatropha*, we isolated a native *JcSDP1* promoter fragment from *Jatropha* genomic DNAs using a GenomeWalker kit

(Clontech). We cloned an approximately 0.7 kb fragment of *JcSDP1* proximal to the 5'-UTR, which contained several putative *cis*-elements for gene expression and regulation. This region of the *JcSDP1* promoter contains a TATA box and a CAAT box, located at -13 to -16 and -66 to -69, respectively. The promoter region also contains two putative sugar-responsive elements found in the alpha amylase gene, TATCCA and TAACAAA (20,21), located at -154 to -159 and -139 to -145, respectively. In addition the promoter fragment includes four E-box motifs, CANNTG (22, 23), which are likely involved in seed specific expression (Figure 4A).

[0076] To see if the *JcSDP1* promoter would show seed specific expressions, we generated a *ProJcSDP1:GUS* expression clone using the pKGWFS7 gateway vector system. The expression patterns of *JcSDP1* promoter were examined in homologous and heterologous systems. To transiently analyze *JcSDP1* expression in a homologous system, we introduced the *ProJcSDP1:GUS* fusion gene into developing fruits and leaf of *Jatropha*. We found the *GUS* gene was transiently expressed in developing *Jatropha* fruits, especially the endosperm part of the seed whereas leaf tissues did not show any *GUS* expression (Figure 4B). This result suggests that the *JcSDP1* promoter has seed-specific expression in *Jatropha* plant. We also analyzed *JcSDP1*-GUS expression in developing seeds of transgenic *Arabidopsis* plants expressing *ProJcSDP1:GUS* transgene. Figure 4C shows that *GUS* expression was highly elevated at 3-4 days (globular embryos) after pollination (DAP) and also at 9-10 DAP (mature green embryos) (Figure 4C-h and i) following the developmental stages described by Le et al. (24). These expression patterns were consistent with the expression pattern of *JcSDP1* which was highly activated at stage S2 (3 WAF) (Figure 4E). These results imply that the *JcSDP1* promoter is controlled in a seed-specific and development dependent manner.

[0077] Using a *cis*-element prediction program we found that the *JcSDP1* promoter carries two sugar-responsive alpha amylase elements, such as TATCCA and TAACAAA (20, 21). To investigate sugar responses of *JcSDP1*, we used 14 day-old transgenic *Arabidopsis* seedlings harboring the *ProJcSDP1:GUS* transgene. We examined the effects on *GUS* expression of sucrose, glucose, or fructose (1% or 3% concentration) supplementation in MS(-) media. As a control, mannose was used as a source of osmotic stress. Figure 4D shows that the *JcSDP1* promoter showed sugar-dependent expression, and the expression was especially responsive to sucrose and fructose.

EXAMPLE 6

JcSDP1-RNAi Transgenic *Jatropha* Plants
Accumulate Increased Storage Lipids in Their Endosperm

[0078] We were interested to see if the reduction of *SDP1* expression in *Jatropha* plants would also lead to an increased seed oil accumulation. To this end, we generated *JcSDP1-RNAi* transgenic *Jatropha* plants. To specifically control lipid accumulation in the mature seeds without ectopic gene silencing, we placed the *JcSDP1-RNAi* transgene under the control of a native *JcSDP1* promoter (Figure 9; SEQ ID NO:3) which is specifically expressed in *Jatropha* seeds. Through a two step selection process using hygromycin and β -estradiol, marker free or non-marker free transgenic plants were screened. These plants were further confirmed by genotyping with specific primer sets for *HygF* and *HygR* for the hygromycin gene and *P1* and *R16* or *P1* and *T35S-R* for the marker free transgene. Based on the genotyping results, we obtained chimeric (heterozygote) marker-free plants in which the antibiotic selection marker was partially removed by homologous recombination. The transgenic lines displayed normal growth patterns and growth rates, leaf number and leaf size (Figure 5B, panels d and e). In contrast to the enlarged seed size found in *Arabidopsis sdp1-5*, T1 mature seeds from *JcSDP1-RNAi* plants were normal in size (Figure 5B, panels a, b, and c). To investigate oil content in T1 mature seeds, we carefully separated endosperms from embryos. Whereas the endosperms were used for further molecular and biochemical analysis, the embryos were regenerated to maintain the transgenic lines. After transgenic plants were fully grown and mature, we harvested mature seeds from four individual transgenic plants for further studies. Compared to control (CK) plants (35S:GFP lines), all tested transgenic lines accumulated increased oil content per dry seed weight in their endosperm (Figure 5C). Endosperm of the best transgenic line (#158) accumulated raw oil content to about 54% of the dry seed weight; this represented a 30% increase of total oil in transgenic plants compared to 35S:GFP control (CK) plants. We analyzed *JcSDP1-RNAi* transgenic line #158 to investigate possible changes in protein and carbohydrate content along with the increased in lipid accumulation in endosperm. The protein content per endosperm of *JcSDP1-RNAi* decreased about 7% compared to the CK (35S:GFP) plant as shown in Table 3. However, no difference in the carbohydrate content was detected between the two.

TABLE 3

Protein and Carbohydrate Contents in Endosperm of
Control (CK; 35S:*GFP*) and *JcSDPI-RNAi* Transgenic Plants

Genotype	Total Lipid Content (%, w/w)	Protein Content (%, w/w)	Carbohydrate Content (%, w/w)
CK (35S: <i>GFP</i>)	41.41 ± 2.19	14.93 ± 0.16	13.89 ± 0.12
<i>JcSDPI-RNAi</i> #158	54.17 ± 2.13	13.99 ± 0.19	13.92 ± 0.13

Values are the mean ± SE of measurements on endosperms from individual seed (n=4) of *JcSDPI-RNAi* #158 T1 transgenic and control (CK; 35S:*GFP*) plants grown in a greenhouse.

[0079] To characterize TAG accumulation in mature endosperm, we analyzed total neutral lipid from CK and 3 individual lines of #158 transgenic plants by thin layer chromatography on silica gel plates. Figure S3A shows reduced levels of free fatty acids (FFA) in the #158 transgenic line compared to CK. In addition, the #158 transgenic plants had about 8.49% FFA and 91.50% TAG compared to 26.83% FFA and 73.17% TAG in CK plants (Figure 8B). We verified the number of T-DNA insertions in the best transgenic lines by Southern blot analysis (See Additional file 4). Among the T1 progeny plants of the parental line #158, we recovered a line (#158-8) that carried a single T-DNA insert and another line (#158-11) with multiple inserts. Regardless of the copy number of insertion, both #158-8 and #158-11 showed a very similar level of total seed lipid accumulation (Figures 10A and 10B).

[0080] To determine possible alterations in fatty acid profiles of *JcSDPI-RNAi* transgenic lines, we analyzed extracts by gas chromatography-mass spectrometry (GCMS-QP2010). Figure 5D shows that *JcSDPI-RNAi* transgenic plants displayed a very similar fatty acid profile to that of CK plants. In contrast to the changes of fatty acids in the Arabidopsis mutant *sdpl-5*, we were unable to find any significant accumulation of C20:1 (less than 0.5% of total TAGs) in the transgenic *Jatropha* lines. The observed differences between the effects of *SDPI* knock-down in Arabidopsis and *Jatropha* might be caused by physiological differences in oil accumulation. Indeed, reductions of *JcSDPI* transcript levels were observed in *JcSDPI-RNAi* transgenic lines (Figure 5E). Moreover, the increased oil accumulation in seeds was inversely correlated to the residual *JcSDPI* transcript levels as assayed by quantitative real-time PCR (qRT-PCR). Taken together, we conclude that that suppression of *JcSDPI* expression using *RNAi* technology can lead to enhanced oil accumulation in mature dried *Jatropha* seeds.

EXAMPLE 7

SDP1-Deficiency Enhances Total Seed Oil Accumulation in Arabidopsis and Jatropha

[0081] The breakdown of TAGs into fatty acids is initiated by lipases many of which have been identified in eukaryotes including plants, yeast and animals (11, 12, 25). One of the characterized lipases is encoded by the Arabidopsis *SUGAR DEPENDENT1* (*SDP1*). This lipase contains a patatin-like domain and is tightly associated with the oil body (13, 26, 27). Three *SDP1* homologous genes have been identified in Arabidopsis and all 3 play important roles in storage oil mobilization (13, 14, 28). Besides its role in seed germination *SDP1* is also active during the seed desiccation stage to hydrolyze TAGs (15).

[0082] Seed development in Arabidopsis consists of two major phases: early embryogenesis which is completed within 6 days after pollination (DAP) and seed maturation phase. The latter process can be further defined into three sub stages; early-maturation, mid-maturation and late-maturation. In the early-maturation stage embryos accumulate increasing amount of starch and begin to synthesize storage oils and proteins (7-10 DAP). During the mid-maturation stage (11-16 DAP) the amount of starch is dramatically reduced and this is inversely related to the accumulation of oils and proteins. Finally, in the late-maturation stage which is also known as desiccation stage, embryos gradually become metabolically quiescent with the exception that they synthesize sugars such as sucrose and trehalose to maintain integrity during the desiccation processes (29, 30). Chia et al. (14) reported that TAGs in oil bodies are important carbon sources for the proceeding of desiccation in late-maturation stage. During the desiccation process, around 10-14% of TAGs are consumed as carbon sources. Many enzymes, such as malate synthase (MS), isocitrate lyase (ICL), 3-ketoacyl CoA thiolase (KAT), hydroxyacyl CoA dehydrogenase (HD), enoyl hydratase (EH), and phosphoenolpyruvate carboxykinase (PEPCK), are involved to metabolize TAG (11, 12, 25). The Arabidopsis *sdp1/sdp1L* double knockout mutant display a similar phenotype as the glyoxylate cycle mutant *icl* or *icl2*, which lacks isocitrate lyase. These mutants are unable to synthesize carbohydrates from fatty acids (28, 31). In the *sdp1-5*, TAG degradation is almost completely blocked because *SDP1* is the first enzyme for TAG metabolism in the desiccation process of the late-maturation stage (15, 28).

[0083] Based on these studies, we hypothesized that *SDP1*-deficiency might block TAG metabolism during the desiccation process thereby reducing the loss of TAG in the late-maturation stage. To test this hypothesis we isolated the Jatropha *SDP1* homolog and showed that it is functional equivalent to the Arabidopsis *SDP1* by genetic complementation of the Arabidopsis *sdp1-5* mutant (Figure 3B). Moreover, we showed *SDP1*-deficiency generated by RNAi technology produced a notable increase of seed oil accumulation in transgenic Jatropha

plants. Our results suggest that the technology can be applied to enhance seed oil accumulation in other oil seed crops as well. It is possible that seeds of *SDP1*-deficient transgenic plants may be partially blocked in the desiccation process which may negatively impact their long-term shelf life. Moreover, the inhibition of TAG degradation may retard seedling growth and reduce seedling vigor. However, in the case of *Jatropha*, these issues can be mitigated by the industrial scale production of clonal transgenic plants by tissue culture. In addition, the issue of reduced seedling vigor can be obviated by germinating seeds in a sucrose-supplemented medium and selling germinated seedlings.

[0084] In this work, we have used the seed-specific promoter of *JcSDP1* for regulated expression of the *JcSDP1*-related RNAi construct. The *JcSDP1* promoter was chosen because we showed its activity peaks in the early stage of developing seed and gradually returns to basal levels in the late stage (Figure 4E). Moreover, the *JcSDP1* promoter is responsive to sugars, especially sucrose and fructose (Figure 5). Based on these findings, we used the *JcSDP1* promoter to express *JcSDP1*-related RNAi sequence so as to establish a feed-back inhibition system. For instance, if the gene silencing is not strong enough to knock down *JcSDP1* expression, the residual *JcSDP1* would degrade TAGs into free fatty acids which can be converted into sucrose, trehalose, and proteins. Once sucrose is elevated to a certain critical level in the endosperm, it can activate the *JcSDP1* promoter which then in turn enhances the suppression of *JcSDP1* gene expression. The gene silencing strategy we showed here has several advantages over the use of a CaMV35S promoter: 1) using the cognate promoter of a target gene can offer more specific regulation of the RNAi-dependent gene silencing against the target gene itself; 2) the end product of the target gene can be used for its own feed-back suppression; and 3) reduced ectopic expression of transgene.

EXAMPLE 8

SDP1-Deficient Transgenic *Jatropha* Plants Are Advantageous for the Process of Biodiesel Production

[0085] Biodiesel is commonly produced from crude oil by alkaline or acid-treatment processes which are known as trans-esterification (32, 33). Owing to the short reaction time and reduced energy consumption, the alkaline-treatment process is the preferred method for trans-esterification. For the alkaline-treatment process, crude oil should contain a very low levels of free fatty acids (FFAs) and moisture because a high level of FFA and water can transform trans-esterification into saponification leading to easy depletion of catalysts (34, 35). For crude oil containing a high amount of FFAs and water acid treatment is more suitable; however, the

reaction time of acid treatment is very long and there is a greater requirement of alcohol. This dilemma has prompted studies to improve trans-esterification processes by chemical methods (36, 37). The deficiency of seed-specific *SDPI* in *Jatropha* plant provides a solution to the dilemma. FFA levels of mature seeds are mainly determined by *SDPI* during the desiccation step at the late-maturation stage (15, 28). Here, we showed that in mature dry seeds *JcSDPI-RNAi* transgenic lines contain higher TAG levels and lower FFA levels compared to control *Jatropha* plants (Figure 1C-1D and Figure 5C). Therefore, crude oils derived from *SDPI*-deficient transgenic *Jatropha* plants are a better substrate compared to WT crude oil for alkaline trans-esterification in biodiesel production.

[0086] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0087] Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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WHAT IS CLAIMED IS:

1. A method of increasing seed oil accumulation in *Jatropha* comprising reducing the activity of a patatin-domain triacylglycerol lipase encoded by a *sugar-dependent-1* (*SDP1*) gene in a *Jatropha* plant to cause an increased seed oil accumulation, wherein the reduction in activity is achieved by an RNAi molecule comprising the nucleotide sequence set forth in SEQ ID NO: 3, and wherein the reduction in *SDP1* expression levels is at least 20-fold compared to wild type.
2. The method of claim 1, wherein the lipase has the amino acid sequence set forth in SEQ ID NO: 2.
3. The method of claim 2, wherein the lipase is encoded by a nucleic acid having a nucleotide sequence set forth in SEQ ID NO: 1, or nucleotides 874-3450 of SEQ ID NO: 1, or nucleotides 874-3447 of SEQ ID NO: 1.
4. The method of any one of claims 1-3, wherein the activity of the lipase is reduced in seeds of the *Jatropha* plant.
5. The method of claim 4, wherein the reduction in activity in *Jatropha* seeds is accomplished using a *Jatropha* seed specific promoter.
6. The method of claim 5, wherein the *Jatropha* seed specific promoter is a *Jatropha curcas* *SDP1* promoter.
7. The method of claim 6, wherein the *Jatropha curcas* *SDP1* promoter has the nucleotide sequence set forth in nucleotides 1-722 of SEQ ID NO: 1 or promoter functional fragment thereof.
8. The method of any one of claims 1-7, wherein the seed oil accumulation is increased by about 13% to about 30%.

9. An isolated nucleic acid consisting of nucleotides 3161-3533 of SEQ ID NO: 1, wherein the nucleic acid, when used in RNAi reduces expression of the *sugar-dependent 1 (SDP1)* gene.
10. The isolated nucleic acid of claim 9, wherein the nucleic acid consists of nucleotides 3161-3528 of SEQ ID NO: 1.
11. The isolated nucleic acid of claim 9 or 10 which further comprises a plant operable promoter operably linked to the nucleic acid.
12. The isolated nucleic acid of claim 11, wherein the promoter is a seed specific promoter.
13. The isolated nucleic acid of claim 12, wherein the seed specific promoter is a *Jatropha curcas SDP1* promoter.
14. The isolated nucleic acid of claim 13, wherein the *Jatropha curcas SDP1* promoter comprises nucleotides 1-722 of SEQ ID NO: 1.
15. An isolated nucleic acid consisting of the nucleotide sequence set forth in SEQ NO: 3 wherein the nucleic acid used in RNAi reduces expression of the *sugar-dependent 1 (SDP1)* gene.
16. A nucleic acid construct or a vector comprising the isolated nucleic acid of any one of claims 9-15.
17. A transgenic plant cell or transgenic plant having the isolated nucleic acid of any one of claims 9-15 stably integrated in its genome.
18. A method of making a transgenic plant cell comprising introducing the isolated nucleic acid of any one of claims 9-15 into a plant cell and selecting a transgenic plant cell in which the isolated nucleic acid is stably integrated in its genome.
19. A method of making a transgenic plant comprising introducing the isolated nucleic acid of any one of claims 9-15 into a plant cell, selecting a transgenic plant cell in which the

isolated nucleic acid is stably integrated in its genome and regenerating a transgenic plant from the selected transgenic plant cell.

20. An isolated nucleic acid comprising a heterologous plant operable promoter operably linked to a nucleic acid encoding a sugar-dependent 1 protein consisting of the amino acid sequence set forth in SEQ ID NO: 2.
21. The isolated nucleic acid of claim 20 wherein the nucleic acid encoding the sugar-dependent 1 protein consists of the nucleotide sequence set forth in SEQ ID NO: 1, or nucleotides 874-3450 of SEQ ID NO: 1 or nucleotides 874-3447 of SEQ ID NO: 1.
22. The isolated nucleic acid of claim 20 or 21, wherein the promoter is a heterologous seed-specific promoter.