

FUNCTIONAL ANALYSIS OF *JATROPHA CURCAS* GENES

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present invention claims priority to U.S. provisional patent application Serial No. 61/143,484 filed on 9 January 2009, incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] The present invention relates to the field of functional analysis of *Jatropha* genes on a genomic scale. More specifically, the present invention relates to a method for high-throughput functional analysis of *Jatropha curcas* genes on a genomic scale using virus-induced gene silencing.

[0003] The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the Bibliography.

[0004] The world is facing dwindling supply of fossil fuel and worsening Green House Effect. There is an urgent demand to increase production and consumption of renewable energy. Biofuels have been recognized as a national priority for many countries in their search for alternative sources to meet their energy security needs and at the same time help reduce CO₂ emissions that cause the Green House Effect. The demand for biofuel has put increasing pressure on food production. For example, to satisfy the biofuel need for Germany in 2017 as mandated by the German government the entire farm land of this country would have to be used for growing bioenergy crops with no land left for food production. To ease this competition for land and to satisfy our need for renewable fuels, there is a strong need to utilize marginal land for bio-energy production.

[0005] *Jatropha curcas* is a small woody plant belonging to the *Euphorbiaceae* family. Several unique characters of *Jatropha curcas* make it an ideal plant for biodiesel production. These include its rapid growth, easy propagation, low cost of seeds, high oil content, short gestation period, wide adaptability, drought tolerance and the ability to thrive on degraded soils. Moreover, its plant size renders convenient collection of seed (Jones, 1991; Sujatha et al., 2008).

[0006] However, *Jatropha* suffers from several shortcomings that may limit its wide adoption. The productivity of the plant is constrained by the unfavourable male to female flower ratio and its oil content has not been optimized by breeding. This plant is also sensitive to biotic stresses such as viral (Narayanna et al., 2007), fungal and bacterium pathogens and abiotic stresses, especially cold and drought (<http://www.jatropha.org>). The

presence of several toxic components (e.g. the protein toxin, curcin, and the cancer-causing agent phorbol esters) in seeds and leaves of the plant possess health hazards for farmers and bioprocess workers in the *Jatropha* industry.

[0007] An important strategy to improve agronomic and quality traits of *Jatropha curcas* is by genetic modification. Transgenic *Jatropha* plants can be generated expressing homologous or heterologous gene sequences. In many instances, over-expression or silencing by RNAi of one or more homologous genes of defined function is desired. Gene sequences of *Jatropha* can be obtained from cDNA and genomic libraries and functions of genes can be tentatively assigned by sequence homology with other plant genes of known function. However, such assignment at best is only provisional and tentative. Quite often functional assignment is confounded by the presence of a gene family whose members are highly related in sequences. Gene function can be more accurately determined by over-expression or RNAi-mediated silencing of candidate genes in transgenic plants; however, such procedure is laborious and time consuming and not suitable for high throughput analysis on a genomic scale.

[0008] Thus, it is desired to develop a method for the high-throughput functional analysis of *Jatropha curcas* genes on a genomic scale.

SUMMARY OF THE INVENTION

[0009] The present invention relates to the field of functional analysis of *Jatropha* genes on a genomic scale. More specifically, the present invention relates to a method for high-throughput functional analysis of *Jatropha curcas* genes on a genomic scale using virus-induced gene silencing.

[0010] The present invention relates to the use of virus-induced gene silencing (VIGS) to evaluate gene function in *Jatropha curcas* reliably and rapidly, and in a high-throughput manner. In one aspect, the present invention provides an efficient and reproducible system and procedure for VIGS in *J. curcas*. In one embodiment, the present invention provides for the co-silencing of DCL4 to enhance the VIGS efficiency. In another embodiment, the present invention provides for the further re-synthesis of the whole tobacco rattle virus (TRV) viral genomes. In an additional embodiment, the present invention demonstrates that these vectors have similar efficiency as the original vectors. In a further embodiment, the present invention provides for the use of TRV VIGS system for cloning and functional identifying several important fatty acid biogenesis pathway genes. These important genes can be used to improve

fuel properties of biodiesel such as cold-temperature flow characteristics, oxidative stability and NOx emissions.

[0011] Thus in a first aspect, the present invention provides a method of virus-induced gene silencing (VIGS) in *Jatropha*. In accordance with the present invention, the method comprises:

[0012] (a) inserting a nucleic acid comprising a sequence of a first desired gene to be silenced into a vector comprising a tobacco rattle virus (TRV) RNA2 sequence to produce a modified TRV RNA2 vector;

[0013] (b) preparing a mixed culture of *Agrobacterium* comprising *Agrobacterium* containing a vector comprising a TRV RNA1 sequence and *Agrobacterium* containing the modified TRV RNA2 vector;

[0014] (c) introducing the mixed culture of *Agrobacterium* into plant tissue of *Jatropha* by vacuum infiltration to produce infected plants; and

[0015] (d) growing the infected plants for a sufficient time to induce gene silencing of the desired gene.

[0016] In one embodiment, the vector comprising TRV RNA2 and the vector comprising TRV RNA1 are synthetic plant vectors. In another embodiment, the sequence of the first desired gene is the sequence of a sense strand of the gene. In an additional embodiment, the sequence of the first desired gene is the sequence of an antisense strand of the gene. In a further embodiment, the nucleic acid further comprises a sequence of a second desired gene to be silenced. In one embodiment, the second desired gene is virus resistance gene. In another embodiment, the virus resistance gene is selected from the group consisting of *RNase III Dicer-like 4 (DCL4)* gene, *RNase III Dicer-like 2 (DCL2)* gene, *RNase III Dicer-like 3 (DCL3)* gene, *ARGONAUTE1 (AGO1)*, *ARGONAUTE71 (AGO7)*, *RNA-dependent RNA polymerase 1 (RDR1)*, *RNA-dependent RNA polymerase 6 (RDR6)*, *Suppressor of gene silencing 1 (SGS1)*, *Suppressor of gene silencing 3 (SGS3)*, and *Silencing defective 3 (SDE3)*. In a further embodiment, the nucleic acid comprises sequences of more than two desired genes to be silenced.

[0017] In some embodiments, the desired gene is a candidate gene in fatty acid biosynthesis, such as a *β -ketoacyl-acyl carrier protein synthase II (KASII)* gene, an *acyl-acyl carrier protein thioesterase B (FATB)* gene, a *stearoyl-acyl carrier protein desaturase (SAD)* gene, and a *fatty acid desaturase (FAD)* gene. In other embodiments, the desired gene is a candidate transcription factor gene. In one embodiment, the desired gene is a candidate gene in small RNA (smRNA) biosynthesis. In another embodiment, the desired gene is a candidate gene in biosynthesis of toxic agents.

[0018] Thus in a first aspect, the present invention provides a method of analyzing gene function in *Jatropha*. In accordance with the present invention, the method comprises:

[0019] (a) inserting a nucleic acid comprising a sequence of a candidate gene to be silenced into a vector comprising a tobacco rattle virus (TRV) RNA2 sequence to produce a modified TRV RNA2 vector;

[0020] (b) preparing a mixed culture of *Agrobacterium* comprising *Agrobacterium* containing a vector comprising a TRV RNA1 sequence and *Agrobacterium* containing the modified TRV RNA2 vector;

[0021] (c) introducing the mixed culture of *Agrobacterium* into plant tissue of *Jatropha* by vacuum infiltration to produce infected plants;

[0022] (d) growing the infected plants for a sufficient time to induce gene silencing of the candidate gene; and

[0023] analyzing the phenotypic effect of the silenced candidate gene on the infected plant.

[0024] In one embodiment, the vector comprising TRV RNA2 and the vector comprising TRV RNA1 are synthetic plant vectors. In another embodiment, the sequence of the first desired gene is the sequence of a sense strand of the gene. In an additional embodiment, the sequence of the first desired gene is the sequence of an antisense strand of the gene. In a further embodiment, the nucleic acid further comprises a sequence of a second desired gene to be silenced. In one embodiment, the second desired gene is virus resistance gene. In another embodiment, the virus resistance gene is selected from the group consisting of *RNase III Dicer-like 4 (DCL4)* gene, *RNase III Dicer-like 2 (DCL2)* gene, *RNase III Dicer-like 3 (DCL3)* gene, *ARGONAUTE1 (AGO1)*, *ARGONAUTE71 (AGO7)*, *RNA-dependent RNA polymerase 1 (RDR1)*, *RNA-dependent RNA polymerase 6 (RDR6)*, *Suppressor of gene silencing 1 (SGS1)*, *Suppressor of gene silencing 3 (SGS3)*, and *Silencing defective 3 (SDE3)*. In a further embodiment, the nucleic acid comprises sequences of more than two desired genes to be silenced.

[0025] In some embodiments, the desired gene is a candidate gene in fatty acid biosynthesis, such as a *β -ketoacyl-acyl carrier protein synthase II (KASII)* gene, an *acyl-acyl carrier protein thioesterase B (FATB)* gene, a *stearoyl-acyl carrier protein desaturase (SAD)* gene, and a *fatty acid desaturase (FAD)* gene. In other embodiments, the desired gene is a candidate transcription factor gene. In one embodiment, the desired gene is a candidate gene in smRNA biosynthesis. In another embodiment, the desired gene is a candidate gene in biosynthesis of toxic agents.

BRIEF DESCRIPTION OF THE FIGURES

[0026] Figure 1 illustrates a method for *J. curcas* virus-induced gene silencing (VIGS) in accordance with the present invention.

[0027] Figures 2A-2E show the VIGS effect on *CH42* gene and the insert length effect of the *CH42* gene. Cultures of *Agrobacterium tumefaciens* strains carrying pTRV1 or pTRV2 (Vector in Fig. 2A, TRV2-CH42-440 in Fig. 2B and TRV2-CH42-680 in Fig. 2C) were mixed in 1:1 ratio. Mixed culture was injected into *Jatropha* plants at 2-3 leaf stage plants using a 1 ml needle-less syringe. The CH42 enzyme is responsible for adding Mg into the porphyrin ring during chlorophyll biosynthesis. Silencing of the CH42 gene blocked chlorophyll synthesis in newly emerging leaves which lost their green color but appeared yellow owing to the presence of carotenoids. Note that the inoculated leaves still remained green because of the stability of pre-formed chlorophyll. TRV2-CH42S and TRV2-CH42L contained inserts of CH42 gene fragment of 440bp and 689bp, respectively. Fig. 2D: The silencing signal can travel downwards from the inoculated leaf (indicated by a black arrow) to produce gene silencing effects in a newly emerged lateral leaf (indicated by a white arrow) Fig. 2E: RT-PCR analysis to determine *J. curcas* CH42 RNA levels in the upper leaves of longer CH42 insertion TRV-CH42L infected (lane 1), short CH42 insertion TRV-CH42S infected (lane 2-3) and vector control infected (lane CK) infected *J. curcas* plants. The first strand cDNA was generated from total RNA isolated from silenced and non-silenced plants using oligo (dT) primer and reverse transcriptase. The first strand cDNA was used in a PCR reaction using gene specific primers. Ethidium bromide-stained agarose gels showing RT-PCR products after 30 cycles. PCR products of the *rbcL* gene transcript were used as loading controls.

[0028] Figures 3A and 3B show silencing efficiency is similar with the synthetic TRV vector compared with the original TRV vectors. Fig. 3A: CH42 silencing using original TRV vector. Fig. 3B: CH42 silencing using the synthetic TRV vector.

[0029] Figures 4A-4G show silencing of the *J. curcas* *PDS* gene. Infection of *J. curcas* plants with recombinant TRV alone (Fig. 4A) or TRV carrying a *J. curcas* *PDS* gene fragment (Figs. 4B, 4C, 4D). TRV2-PDS-300 and TRV2-PDS-700 refer to insert size of 300bp and 700bp of the *PDS* gene in the TRV2 vector. Infection with TRV-PDS-300 (Fig. 4C) and TRV-PDS-700 (Figs. 4B, 4D) silenced endogenous *PDS* gene in *J. curcas* plants and inhibited carotenoid biosynthesis resulting in various photo-bleaching phenotypes. Using the time-consuming agro-injection method, only a small region (leave vein) showed photo-bleaching phenotypes. The vacuum-infiltration method described here considerably increased the

silencing efficiency (compare Fig. 4B and Fig. 4D). In addition, the treatment time is reduced to 1/5 that used for agro-injection. Fig. 4E: Quantitative PT-PCR analysis to show the relative *PDS* RNA level of silenced *J. curcas* leaves in Fig. 4C and Fig. 4D. Average values from three independent repeats are shown along with error bars. ** indicates lower *PDS* RNA levels of plants silenced with TRV-PDS-600 compared to that silenced with TRV-PDS-300. Vacuum infiltration helps better silencing efficiency in CH42 gene silencing (compare agro-injection data in Fig. 4F with vacuum infiltration data in Fig. 4G).

[0030] Figures 5A-5E show co-silencing of two genes in one vector. Silencing of the meristemic-specific gene *PCNA* alone (Fig. 5A) and co-silencing of *CH42* and *PCNA* (Figs. 5B and 5C: adaxial side of leaf; and Fig. 5D: abaxial side of leaf). *J. curcas* plants were infected with recombinant TRV-PCNA or TRV-PCNA co-CH42 by vacuum infiltration. TRV-PCNA co-CH42 carried an insert containing both the *J. curcas PCNA* and *CH42* gene fragments. Silencing by vacuum infiltration showed a better silencing effect than that of syringe-based agro-injection. Fig. 5E: Quantitative RT-PCR analysis to show the relative *PCNA* and *CH42* RNA levels of silenced *J. curcas* leaves. Average values of three independent experiments are shown along with error bars.

[0031] Figures 6A-6C show co-silencing of *DCL4* and *CH42* to enhance VIGS efficiency. Silencing of the *CH42* gene alone (Fig. 6A) and co-silencing of *DCL4* and *CH42* (Fig. 6B). Fig. 6C: Quantitative RT-PCR analysis to show the relative *DCL4* and *CH42* RNA levels of silenced *J. curcas* leaves. Average values of 3 independent experiments are shown along with error bars. ** indicates a higher silencing efficiency in leaves co-silenced for both the *CH42* and *DCL4* genes as compared to leaves silenced for the *CH42* gene alone ($p < 0.01$).

[0032] Figures 7A-7C show silencing of the *Jatropha KASII* gene encoding β -keto acyl-acyl carrier protein synthase II by TRV VIGS.

[0033] Figure 7A: Phenotypes of silenced *J. curcas* plants infected with different TRV constructs 18 days post-TRV infiltration. Panel (a): *J. curcas* plant treated with empty TRV vector. Panel (b): *J. curcas* plants infected with TRV:*KASII* with mild yellowish phenotypes. Panels (c) and (d): *J. curcas* plants infected with TRV:*KASII* with severe phenotypes. Treated plants showed downwards curling of newly emerged leaves, brownish leaves blade and reduced shoot extension. Panels (e) and (f): Antagonist effects of the *J. curcas FATB1* gene and the *KASII* gene. The *FATB1* gene encodes acyl-ACP thioesterase. We used the vector, TRV:*KASII* Co-*FATB1*, to co-silence *Jatropha KASII* as well as *FATB1*. Leaves of infected plants displayed

yellowish phenotype along leaf veins very similar to the mild phenotypes found in TRV:*KASII* only infected leaves. Scale bars = 10 mm.

[0034] Figure 7B: Quantitative RT-PCR analysis of *KASII* and *FATB* gene expression levels in silenced *J. curcas* leaves. Panel (a): Vector control. Panel (b): plant silenced in *KASII* expression. Panel (c): plant silenced in *KASII* expression with severe phenotype in Fig. 2A. Panel (d) same as Panel (b), except injected plants showed severe phenotype. Panels (e) and (f): leaves from plants co-silenced in *KASII* and *FATB* gene expression. *KASII* and *FATB* transcript levels were quantified by amplification using gene specific primers. No significant difference was found in *FATB* gene expression level between vector control plant and *KASII*-silenced plant.

[0035] Figure 7C: Gas chromatograms of fatty acid methyl esters from silenced *J. curcas* leaves: vector, vector control; *KASII*-mild, leaves from plants silenced in *KASII* expression displaying mild phenotype. These plants showed mild yellowish phenotype and modest increase in 16:0 accumulation (*KASII* mild); *KASII*-severe, plants silenced in *KASII* expression but with severe phenotype. Note the significant increase in 16:0 accumulation (*KASII* severe); *KASII* CO *FATB*, plants co-silenced in *KASII* and *FATB* gene expression. Methyl esters are labeled: 16:0 (peak 1), 16:1³ (peak 2), *cis*-16:1⁹ (peak 3), 16:3 (peak 4), 18:0 (peak 5), *cis*-18:1 Δ^9 (peak 6), 18:1 Δ^{11} (peak 7), *cis*-18:2^{9,12} (peak 8), and *cis*-18:3^{9,12,15} (peak 9).

[0036] Figures 8A-8C show silencing of expression of several desaturase genes by TRV VIGS system in *J. curcas*.

[0037] Figure 8A: Phenotypes of *J. curcas* plants with silenced expression of individual members of the stearoyl-ACP desaturase (SAD) gene family. Plant infiltrated with TRV constructs were analyzed 18 days post-infiltration. Panel (a): Empty vector-treated *J. curcas* plant. Panels (b)-(d): *J. curcas* plants infected with TRV:*SAD1* showed severe phenotypes at the early stage (12 dpi) with downward curled, narrow leaf blades and brownish leaf vein shown in (panel (b)); and later stages (18 dpi) in (panels (c) and (d)). Panel (e): TRV:*SAD2* treatment did not produced any apparent phenotype. Panels (f) and (g): TRV:*SAD3* caused much weaker phenotype than that of plants infected with TRV:*SAD1*. Scale bar = 10 mm.

[0038] Figure 8B: Real-time RT-PCR quantification of *SAD* gene expression levels infected *J. curcas* plants. Relative *SAD1* gene transcript level in TRV: *SAD1* early stage samples (*SAD1-E*).

[0039] Figure 8C: Gas chromatograms of fatty acid methyl esters from leaf samples of *Jatropha* plants with silenced expression of three *SAD* gene family members and three oleate

desaturase genes: Vector, empty vector control ; *SAD1-E*, samples from plants at early stage of TRV:*SAD1* infection. Leaf phenotypes of plants are shown in Fig. 3A; *SAD1-L*, samples from plants at late stage of TRV:*SAD1* infection; *FAD2-1*, samples from plants treated with TRV-*FAD2-1*; *FAD2-2*, samples from plants treated with TRV-*FAD2-2*; *FAD6*, samples from plants treated with TRV-*FAD6*. Note that *FAD6* is located in the ER. Methyl esters are labeled: 16:0 (peak 1), 16:1³ (peak 2), *cis*-16:1⁹ (peak 3), 16:3 (peak 4), 18:0 (peak 5), *cis*-18:1 Δ ⁹ (peak 6), *cis*-18:2^{9, 12} (peak 7), and *cis*-18:3^{9, 12, 15} (peak 8). Unknown peak is noted as peak 9.

[0040] Figures 9A-9I show *J. curcas* plants with silenced expression of key genes regulating plant development.

[0041] Figure 9A: Phenotypes of *J. curcas* plants infected with TRV VIGS constructs at 27 dpi except i and j were taken at 42 dpi. Panels (a) – (d): *J. curcas* plants infected with TRV:*AS1*. (panel (a)) adaxial side and (panel (b)) abaxial side of downward curled leaves from infected plants. Panel (c): Adaxial ectopic leaf blade along the leaf vein on leaf of treated *J. curcas* plants. Panel (d): High magnification view of the adaxial ectopic leaf blade and bubble structure in leaves of treated *J. curcas* plants. Panels (e) – (j): Leaves of *J. curcas* plant infected with TRV:*AGO1*. Panel (e) adaxial side and panel (f) abaxial side of upward curled leaves of treated *J. curcas* plants. Panel (g): High magnification view of the abaxial ectopic leaf blade along the leaf vein of leaves of treated *J. curcas* plants. Panel (h): Typical serrate leaf phenotype of plants silenced in *AGO1* expression. Panels (i) and (j): Abnormal leaf shape in plants with silenced *AGO1* expression. (k) adaxial (m) abaxial vector control leaves. Scale bar = 10 mm.

[0042] Figure 9B: Scanning electron micrograph of leaves with silenced *AS1* expression. Note the adaxial ectopic leaf blade. PB: primary blade; EB: ectopic blade; ebad: ectopic blade adaxial; ebab: ectopic blade abaxial.

[0043] Figure 9C: Transverse sections of ectopic blade showing differentiation of palisade tissue (ectopic blade palisade, ebp) and lateral vein (ectopic blade vein, ebv).

[0044] Figure 9D: Scanning electron micrograph of leaves with silenced *AGO1* expression. Note the abaxial ectopic leaf blade along the primary veins (V).

[0045] Figure 9E: Transverse sections of leaf with silenced *AGO1* expression. Note that the ectopic abaxial blade is contiguous with the lower epidermis (E) and the spongy parenchyma (sp).

[0046] Figure 9F: Transverse sections of adaxial bubbles of leaf with silenced *AGO1* expression. Note the mosaic composition of vascular-like structure (xylem, x; phloem, p) and two layers palisade parenchyma (pp).

- [0047] Figure 9G: Transverse sections of *J. curcas* leaves infected with empty vector control.
- [0048] Figure 9H: Real time RT-PCR analysis of expression levels of *ASI* and class I *KNOX* gene (*KNAT1-like*) in *J. curcas* leaves with silenced *ASI* expression.
- [0049] Figure 9I: Real time RT-PCR analysis of expression levels of *AGO1* and *iPHAVOLUTA* (*PHAV*) in *J. curcas* leaves with silenced *AGO1* expression. Scale bar = 50 μ m (C, E, F, G), 200 μ m in B and D.
- [0050] Figure 10 shows silencing of the gene encoding curcin toxin protein in *J. curcas*. Western blot analysis of leaves of *J. curcas* with silenced curcin expression. Top panel: the curcin proteins were detected with anti-curcin antibody. Bottom panel: Coomassie Bright Blue staining of the RUBL (the large subunit of RUBISCO) which serves as a loading control. Lane1: empty vector-infected plant leaves, lanes 2-4, TRV:*curcin* infected *J. curcas* leaves.
- [0051] Figures 11A-11E show some putative host plants (Dinesh Kumar et al. (2007) for plants shown in Figs. 11A, 11C, 11D and 11E) that have been treated with sTRV:JcCH42. Fig. 11A: *Arachis hypogaea*; Fig 11B: *Phaseolus aureus*; Fig. 11C: *Glycine max*; Fig. 11D: *Phaseolus vulgaris*; Fig. 11E: *Ricinus communis*. Scale bar: 10mm.
- [0052] Figure 12 shows TRV viral RNA analysis (RNA1 and RNA2) of some putative host plants (Dinesh Kumar et al. (2007) for plants for which data shown in lanes 1-5, 11, 12 and 14-17) that have been treated with sTRV:JcCH42 or mock control. Lanes 1-5: *Arachis hypogaea* (lanes 1-3, treated with sTRV:JcCH42, lanes 4 and 5 from mock control); lanes 6-10: *Phaseolus aureus* (lanes 6-8, treated with sTRV:JcCH42, lanes 9 and 10 from mock control); lanes 11-12 and 14-19: *Ricinus communis* (lanes 11-12 and 14-17, treated with sTRV:JcCH42, lanes 18 and 19 from mock control), lane 13 is 1kb DNA ladder marker. "*" indicate proposed PCR amplified bands.

DETAILED DESCRIPTION OF THE INVENTION

[0053] The present invention relates to the field of functional analysis of *Jatropha* genes on a genomic scale. More specifically, the present invention relates to a method for high-throughput functional analysis of *Jatropha curcas* genes on a genomic scale using virus-induced gene silencing.

[0054] Virus-induced gene silencing (VIGS) (Ruiz et al., 1998; Burch-Smith et al., 2004) system offers the possibility to determine the biological function of gene products without the need to genetically transform the plant. Both RNA and DNA viruses induce RNA silencing resulting in the production of virus-related siRNAs (Baulcombe, 2004). Recombinant viruses

can be constructed carrying an inserted partial sequence of a candidate plant gene. Such recombinant viruses can move systemically in whole plants producing siRNA which can mediate degradation of the endogenous candidate gene transcripts (Brigneti et al., 2004; Burch-Smith et al., 2004) resulting in silencing of the candidate gene expression in inoculated plants.

[0055] VIGS approach offers several opportunities:

[0056] (1) an efficient reverse genetics tool to gene/gene family knock-down;

[0057] (2) a rapid and high-throughout (Liu et al., 2002b) -- whole genome ORF knock-out in less than one month;

[0058] (3) transient, reversible and so called "inducible" knock-out phenotype (Liu et al., 2002a); and

[0059] (4) different organs suitable for silencing, offer a chance to knock-out genes in the roots (Valentine et al., 2004; Kaloshian, 2007), the flowers (Liu et al., 2004; Chen et al., 2005), the leaves (Liu et al., 2002a; Liu et al., 2002b; Burch-Smith et al., 2006), or the fruit (Fu et al., 2005), by different infected methods.

[0060] The TRV VIGS system has been successfully applied in some plants such as *Arabidopsis* (Burch-Smith et al., 2006), *Capsicum annuum* (Chung et al., 2004), *Lycopersicon esculentum* (Liu et al., 2002; Dinesh Kumar et al., 2007), *Petunia hybrida* (Chen et al., 2005), *Nicotiana benthamiana* (Liu et al., 2002), *Solanum tuberosum* (Brigneti et al., 2004). Most of these plants have been experimentally proven to be susceptible hosts of some strain of TRV (Plant Virus Online, <http://colon.backslash.backslash.image.dot.fs.dot.uidaho.dot.edu.backslash.vide.backslash.descr808.dot.htm>). More importantly, this TRV VIGS system cannot reasonably be expected to inevitably work in all plants. For example, Dinesh Kumar et al. (2007) contains a list of plants for which it is stated that the TRV VIGS system may work. However, this TRV VIGS system cannot work in *Arachis hypogaea* and *Glycine max*, because they are not hosts to TRV despite their inclusion in the list in Dinesh Kumar et al. (2007). In fact, as demonstrated herein, the TRV VIGS system does not work in all plants listed as being susceptible to Tobacco Rattle Virus (TRV) in Dinesh Kumar et al. (2007) or in the online virus databases. Furthermore, systemic infection is required for the TRV VIGS system to be useful for functional gene analysis. Some plants may be susceptible to TRV locally but not systemically, and thus the TRV VIGS system will not work in those plants. Prior to the present invention, *Jatropha* was not known to be host to TRV or known to be susceptible in any degree to TRV either locally or systemically. The finding that *Jatropha* is susceptible to TRV and that the TRV VIGS system can be used in *Jatropha* was discovered after screening many viral vectors and thus was

unexpected. The unexpected nature of the present invention is further evidenced by the inability of the TRV VIGS system to work in all plants which are susceptible to TRV or are listed as host plants for TRV.

[0061] In one aspect, the present invention provides an efficient and reproducible system and procedure for VIGS in *J. curcas*. In one embodiment, the present invention provides for the co-silencing of DCL4 to enhance the VIGS efficiency. In another embodiment, the present invention provides for the further re-synthesis of the whole TRV viral genomes. In an additional embodiment, the present invention demonstrates that these vectors have similar efficiency as the original vectors. In a further embodiment, the present invention provides for the use of TRV VIGS system for cloning and functionally identifying several important fatty acid biogenesis pathway genes. These important genes can be used to improve fuel properties of biodiesel such as cold-temperature flow characteristics, oxidative stability and NOx emissions.

[0062] Thus in a first aspect, the present invention provides a method of virus-induced gene silencing (VIGS) in *Jatropha*. In accordance with the present invention, the method comprises:

[0063] (a) inserting a nucleic acid comprising a sequence of a first desired gene to be silenced into a vector comprising a tobacco rattle virus (TRV) RNA2 sequence to produce a modified TRV RNA2 vector;

[0064] (b) preparing a mixed culture of *Agrobacterium* comprising *Agrobacterium* containing a vector comprising a TRV RNA1 sequence and *Agrobacterium* containing the modified TRV RNA2 vector;

[0065] (c) introducing the mixed culture of *Agrobacterium* into plant tissue of *Jatropha* by vacuum infiltration to produce infected plants; and

[0066] (d) growing the infected plants for a sufficient time to induce gene silencing of the desired gene.

[0067] In one embodiment, the vector comprising TRV RNA2 and the vector comprising TRV RNA1 are synthetic plant vectors. The results and the phenotypic data shown herein indicate that the synthetic STRV-VIGS systems can be used as effectively as TRV-VIGS systems to induce silencing of desirable endogenous *J. curcas* genes. In another embodiment, the sequence of the first desired gene is the sequence of a sense strand of the gene. In an additional embodiment, the sequence of the first desired gene is the sequence of an antisense strand of the gene. As shown herein, the VIGS system of the present invention can be used to rapidly characterize individual members of a multiple gene family which may share overlapping functions. In a further embodiment, the nucleic acid further comprises a sequence of a second

desired gene to be silenced. In one embodiment, the second desired gene is virus resistance gene. In another embodiment, the virus resistance gene is selected from the group consisting of *RNase III Dicer-like 4 (DCL4)* gene, *RNase III Dicer-like 2 (DCL2)* gene, *RNase III Dicer-like 3 (DCL3)* gene, *ARGONAUTE1 (AGO1)*, *ARGONAUTE71 (AGO7)*, *RNA-dependent RNA polymerase 1 (RDR1)*, *RNA-dependent RNA polymerase 6 (RDR6)*, *Suppressor of gene silencing 1 (SGS1)*, *Suppressor of gene silencing 3 (SGS3)*, and *Silencing defective 3 (SDE3)*. The phenotypic and molecular analyses shown herein demonstrate that co-silencing virus resistance genes can confer a higher VIGS efficiency in treated *J. curcas* plants. Furthermore, co-silencing of the viral-resistant system also can be used to increase the efficiency of other VIGS systems in other plant species. In a further embodiment, the nucleic acid comprises sequences of more than two desired genes to be silenced. The data shown herein indicate that VIGS system of the present invention can be used to knock down the expression of two or more genes thus allowing the deciphering of gene function in the event of functional redundancy.

[0068] In some embodiments, the desired gene is a candidate gene in fatty acid biosynthesis, such as a *β -ketoacyl-acyl carrier protein synthase II (KASII)* gene, an *acyl-acyl carrier protein thioesterase B (FATB)* gene, a *stearoyl-acyl carrier protein desaturase (SAD)* gene, and a *fatty acid desaturase (FAD)* gene. In other embodiments, the desired gene is a candidate transcription factor gene. In one embodiment, the desired gene is a candidate gene in smRNA biosynthesis. In another embodiment, the desired gene is a candidate gene in biosynthesis of toxic agents. The results shown herein demonstrate that the VIGS system of the present invention can efficiently suppress targeted host genes and can be used as a rapid means to assay the role of candidate genes in fatty acid biosynthesis or in the biosynthesis of toxic genes, as well as to study the role of regulatory genes, such a transcription factor genes or the role of genes involved in the small RNA biogenesis pathways. As shown herein, seed suppression of *SAD1* gene can be used to produce *Jatropha* oil containing a high stearic acid content. As further shown herein, seed suppression of *FAD2-1* gene and *FAD-2* gene can be used to produce *Jatropha* oil containing higher oleic acid content with lower 16-carbon fatty acid content.

[0069] The results and the phenotypes data shown herein indicate that the VIGS system can be successfully used to induce silencing of other desirable endogenous *J. curcas* genes. We have generated expressed sequence tags (EST) for several different *Jatropha* tissues such as seeds and flowers. These expressed sequence tags (ESTs) provide a wealth of information for functional genomics study of *Jatropha*. Therefore, the VIGS assay described herein offers a means to test the function of *Jatropha* gene sequences in a homologous system. Using a

normalized cDNA library it is possible to conduct large scale screens of gene function with the VIGS system of the present invention.

[0070] In a second aspect, the present invention provides a method of analyzing gene function in *Jatropha*. In accordance with the present invention, the method comprises:

[0071] (a) inserting a nucleic acid comprising a sequence of a candidate gene to be silenced into a vector comprising a tobacco rattle virus (TRV) RNA2 sequence to produce a modified TRV RNA2 vector;

[0072] (b) preparing a mixed culture of *Agrobacterium* comprising *Agrobacterium* containing a vector comprising a TRV RNA1 sequence and *Agrobacterium* containing the modified TRV RNA2 vector;

[0073] (c) introducing the mixed culture of *Agrobacterium* into plant tissue of *Jatropha* by vacuum infiltration to produce infected plants;

[0074] (d) growing the infected plants for a sufficient time to induce gene silencing of the candidate gene; and

[0075] analyzing the phenotypic effect of the silenced candidate gene on the infected plant.

[0076] In one embodiment, the vector comprising TRV RNA2 and the vector comprising TRV RNA1 are synthetic plant vectors. In another embodiment, the sequence of the first desired gene is the sequence of a sense strand of the gene. In an additional embodiment, the sequence of the first desired gene is the sequence of an antisense strand of the gene. In a further embodiment, the nucleic acid further comprises a sequence of a second desired gene to be silenced. In one embodiment, the second desired gene is virus resistance gene. In another embodiment, the virus resistance gene is selected from the group consisting of *RNase III Dicer-like 4 (DCL4)* gene, *RNase III Dicer-like 2 (DCL2)* gene, *RNase III Dicer-like 3 (DCL3)* gene, *ARGONAUTE1 (AGO1)*, *ARGONAUTE71 (AGO7)*, *RNA-dependent RNA polymerase 1 (RDR1)*, *RNA-dependent RNA polymerase 6 (RDR6)*, *Suppressor of gene silencing 1 (SGS1)*, *Suppressor of gene silencing 3 (SGS3)*, and *Silencing defective 3 (SDE3)*. In a further embodiment, the nucleic acid comprises sequences of more than two desired genes to be silenced.

[0077] In some embodiments, the desired gene is a candidate gene in fatty acid biosynthesis, such as a *β -ketoacyl-acyl carrier protein synthase II (KASII)* gene, an *acyl-acyl carrier protein thioesterase B (FATB)* gene, a *stearoyl-acyl carrier protein desaturase (SAD)* gene, and a *fatty acid desaturase (FAD)* gene. In other embodiments, the desired gene is a candidate transcription factor gene. In one embodiment, the desired gene is a candidate gene in smRNA

biosynthesis. In another embodiment, the desired gene is a candidate gene in biosynthesis of toxic agents.

[0078] Once the function of a *Jatropha* gene or *Jatropha* genes has been characterized, transgenic plants can be prepared using conventional techniques to alter expression patterns of the gene or genes.

[0079] The DNA that is inserted (the DNA of interest) into plants of the genera *Jatropha* is not critical to the transformation process. Generally the DNA that is introduced into a plant is part of a construct. The DNA may be a gene of interest, e.g., a coding sequence for a protein, or it may be a sequence that is capable of regulating expression of a gene, such as an antisense sequence, a sense suppression sequence or a micro-RNA (miRNA) sequence. The construct typically includes regulatory regions operatively linked to the 5' side of the DNA of interest and/or to the 3' side of the DNA of interest. 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. 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 and 2006/0248616. The expression cassette may additionally contain selectable marker genes. See, U.S. Patent No. 7,205,453 and U.S. Patent Application Publication Nos. 2006/0218670 and 2006/0248616.

[0080] Generally, the expression cassette will 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, WO 02/36782, U.S. Patent No. 7,205,453 and U.S. Patent Application Publication Nos. 2006/0248616 and 2007/0143880, and those references cited therein. This list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used.

[0081] 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. Patent No. 6,072,050); the core CaMV^{35S} promoter (Odell et al., 1985); rice actin (McElroy et al., 1990); ubiquitin (Christensen and Quail, 1989 and Christensen et al., 1992); pEMU (Last et al., 1991); MAS (Velten et al., 1984); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, those disclosed in U.S. Patent 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.

[0082] 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. Patent 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.

[0083] Where appropriate, the DNA of interest may be optimized for increased expression in the transformed plant. That is, the coding sequences can be synthesized using plant-preferred codons for improved expression. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, 5,436,391, and 7,205,453 and U.S. Patent Application Publication Nos. 2006/0218670 and 2006/0248616.

[0084] 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

[0085] The present invention is described by reference to the following Examples, which is offered by way of illustration and is not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Experimental Procedures

[0086] *J. curcas* seedlings: *J. curcas* seeds purchased from India were germinated in a greenhouse. *J. curcas* seedlings after 10-14 days post germination were used for VIGS assays. At this stage, seedlings have 2-3 true leaves.

[0087] *Original TRV vectors*: pTRV1 (GenBank No. AF406990) and pTRV2 (GenBank No. AF406991) were kindly given by Dr. Dinesh Kumar (Yale University).

[0088] *Synthetic TRV RNA1 expression vector*: Synthetic TRV1 vector full length (7756bp) sequence including: SphI site, T-DNA right border sequence (152bp), the duplicated cauliflower mosaic virus (CaMV) 35S enhancer region (752bp) (Shi et al., 1997) the TRV Ppk20 strain RNA1 (6791bp), Subterranean Clover Mottle Virus satellite RNA ribozyme sequence (46bp) and SmaI site sequence. This full length sequence was divided into two parts by an endogenous SalI site. The two parts were separately synthesized and cloned into pGH vector to give two vectors pGH-YeJ-V1-1 and pGH-YeJ-V1-2. The synthetic TRV RNA1 fragments, V1-1, released from pGH-YeJ-V1-1 by treatment with SphI and SalI enzymes, and V1-2, released from pGH-YeJ-V1-2 by treatment with SalI and SmaI enzymes, were linked with the pBIN121 vector treated with SphI and EcoRI enzymes. The new synthetic TRV RNA1 vector was named psTRV1001. The sequence of the synthetic psTRV1001 is set forth in SEQ ID NO:1. The synthetic TRV RNA1 sequence is the same as the published TRV RNA1 sequence.

[0089] *Synthetic TRV RNA2 expression vector*: Synthetic TRV2 vector full length (2915bp) sequence including: HindIII site, the duplicated cauliflower mosaic virus (CaMV) 35S enhancer region (752bp) (Shi et al., 1997) the TRV strain ppk20 RNA2 5'-sequence (1639bp), multiple cloning site (61bp), the TRV strain ppk20 RNA2 3'-sequence (396bp), HpaI site. The full length sequence was synthesized and cloned into pGH vector give pGH-YeJ-V2. The synthetic TRV RNA2 fragment V2 was linked into the pCAMBIA0390 by HindIII and HpaI sites. The new synthetic TRV RNA2 vector was named psTRV2001. The sequence of the synthetic sTRV2 is set forth in SEQ ID NO:2. The synthetic TRV RNA2 sequence is the same as the published TRV RNA2 sequence.

[0090] *Gene cloning and VIGS vector cloning*: Most of the gene sequences were obtained from the GenBank and a sequence data base of cDNA library prepared from *J. curcas* seeds. *CH42*, *PDS*, *PCNA*, *ASI* and *DCL4* genes were cloned by PCR using designed primers of conserved regions shared by homologous gene sequences of other *Euphorbiaceae* plants such as castor bean and cassava. For single gene VIGS, all candidate genes were amplified by PCR

from cDNA products of *J. curcas* seeds, roots, flower and leaves samples, and cloned into the original TRV vectors pTRV2 or the XbaI and BamHI sites of the synthetic vector psTRV2001. For co-silencing vectors, cDNA fragment of the second gene was inserted into BamHI and KpnI sites of the vector. The primers used in cloning the genes are set forth in Table 1, which also includes reference to the sequence of the cloned gene.

TABLE 1
Gene Primers and Gene Sequences

Gene	Primers	SEQ ID NO:	Cloned Gene
KASII	5' cctgtgtggatccagtttcaagg	3	GenBank: DQ987700 829 bp
	3' ttatctagactgcatgccacaaaacctccc	4	
PCNA	5' tctagaccatggattccagccacgttgc	5	T-easy vector, then XbaI and BamHI SEQ ID NO:7 614 bp
	3' ggatccctcagcaatctgtactcaacc	6	
PDS- shorter	5' cccggggcmatgtcaaaggcdknaakt	8	T-easy vector 302 bp
	3' ggatcctcaaaccatatmtgvacmttwat	9	
PDS-longer	5' aattctctagaggccagagaaagtaagttgcaattgg	10	SEQ ID NO:12 786 bp
	3' tgaattggatcctctgacagcagatatttcacaggaaa	11	
CH42- shorter	5' tctagagaagarggrgagctccggccccagc	13	T-easy vector 446 bp
	3' ggatccaatttctcaatgacaaagcaaac	14	
CH42- longer	5' aatccatctagaagagaggcaatttctattccaccggcagc	15	SEQ ID NO:17 696 bp
	3' aatatagatctattcattctctcaatttagtac	16	
Curcin	5' gcgtctagaggaaagatgaggatattacagg	18	GenBank AY069946 836 bp
	3' taaagatctaagagaagcatttggc	19	
FAD2-1	5' aactttctagaccaccattcacacttggtcag	20	GenBank DQ157776 862 bp
	3' ttattggatcccatgagtgtctgtaattgtatg	21	
FAD2-2	5' aactttctagacgactcactataggcgca	22	SEQ ID NO:24 711 bp
	3' ttattggatcccaagtgttagagttccat	23	
FAD6	5' aactttctagaaccattctcagacagtgc	25	GenBank EU106889 835 bp
	3' ttattggatccagggtagtcacaatgaac	26	
FATB	5' tggatccacagtaggctata	27	GenBank EU106891 796 bp
	3' aattggatccagccaatgtacttcacattg	28	
SAD1	5' atctctagatgaatgggctgag	29	GenBank DQ08449 862 bp
	3' tgtttagatctacaaacgtaatcctgagc	30	
SAD2	5' aatatctagacgccacctctattgaagc	31	SEQ ID NO:33 821 bp
	3' atatggatcctactccaagccgctgagc	32	
SAD3	5' tataatctagaccacagcctgagtctgaa	34	SEQ ID NO:36 847 bp
	3' tgattggatccgatgtgtcttcttgactc	35	
AGO1	5' ttatttctagacttgacaaatgtgtcat	37	SEQ ID NO:39 854 bp
	3' tagaaggatccaggaagtatatattccac	38	
AS1	5' acatatctagattgaggagagatgctgaagcaaa	40	SEQ ID NO:42 629 bp
	3' tggatggatccgcttcagcatctctctcaa	41	
DCL4	5' aataacictagacaggtatatatactgatcaaccatt	43	T-easy vector cloning SEQ ID NO:45 678 bp
	3' ttattggatccatacctgatagcagcctctcc	44	
DCL4 co- silencing	5' aataaggtaccatacctgatagcagcctcc	46	
	3' gtagtgagtgttctaatactcgagaataa	47	

[0091] *Agrobacterium* infiltration: pTRV1, pTRV2, synthetic psTRV vectors and its derivatives were introduced into *Agrobacterium* strain AGL1 by electroporation. A 3 ml culture was grown for 24 hr at 28° C in 50 mg/L kanamycin and 25 mg/L rifampicin. On the following day, the culture was inoculated into LB medium containing 50 mg/L kanamycin, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 20 µM acetosyringone and grown overnight in a 28° C shaker. *Agrobacterial* cells were collected by centrifugation and resuspended in MMA solution (10 mM MES, 10 mM MgCl₂, 200 µM acetosyringone) to a final OD₆₀₀ of 1.5. The agrobacterial suspension was left at room temperature for 3-4hr without shaking. Before infiltration, *Agrobacterium* cultures containing the pTRV1/psTRV1 or pTRV2/psTRV2 vectors were mixed in a 1:1 ratio. *Jatropha* plants were infiltrated with cultures either by syringe infiltration or by vacuum infiltration. For syringe infiltration, agrobacterial-inocula were delivered into the underside of two or three youngest fully-expanded leaf using a 1 ml needleless syringe. For vacuum infiltration, whole plants were submerged into agrobacterial-inocula and subjected to 80-90 kPa vacuum for 5 min, and then quickly releasing the vacuum, letting the inoculum rapidly enter plant tissues. After infiltration, excess agrobacterial cell suspension was used to drench the root system of infiltrated plants. Infiltrated plants were grown in a growth chamber at 25° C with 16 hr light/8 hr dark photoperiod cycle. The same method was also used in experiments testing VIGS in putative host plants.

[0092] *Gas chromatography*: 100-200 mg fresh *Jatropha* leaf samples were homogenized in liquid nitrogen, and 1 ml of 2:1 chloroform:methanol (v/v) was added to samples. Tripentadecanoin (50 µg; SIGMA) was added to the mixture as an internal standard. Plant tissue was further broken with MIXER MILL MM300 (Retsch, Germany) then frozen in liquid nitrogen again. After thawing, the suspension was centrifuged at 12,000 g for 10 min. The pellet was discarded and 0.3 ml of 1% KCl (w/w) was added to the supernatant and mixed. After centrifugation, the bottom phase was transferred into a new tube and the content concentrated in Concentrator 5301 (Eppendorf, Germany). The lipid extract was re-extracted three times by 1 ml of hexane each and centrifuged to discard the pellet. Lipids were transmethylated to fatty acid methyl esters by incubation with an equal volume 3N methanolic-HCL (SIGMA) plus 400 µL 2,2-dimethoxypropane (2,2-DMP, SIGMA) and kept at 70° C in a water bath for 10 min. The samples were cooled at 4° C for 10 min, and 1 ml water and 1 ml hexane were added and the content mixed vigorously. The upper ester layer was collected and concentrated or analysis by GC using Agilent 6890 (Palo Alto, CA, USA) employing two 7 m × 0.53-mm Supelcowax columns (SPB50, Supelco, Bellefonte, PA, USA) and using helium

as the carrier gas. The GC analytical method was 140° C for 50 sec, 30° C min⁻¹ ramp to 240° C, and the final temperature was maintained for 50 sec for a total run time of 32 min.

[0093] *Scanning Electron Microscope (SEM)*: SEM (JSM-6360LV, JEOL, USA) was used to observe fresh *J. curcas* leaf samples. Fresh leaves were detached and fixed with tape inside the sample chamber, following freezing in liquid N₂.

[0094] *Light microscopy*: Leaf discs were excised from silenced *J. curcas* leaves and fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Leaf discs were rinsed three times in 0.1 M phosphate buffer for 15 min each, and were then post-fixed in 1% (w/v) aqueous OsO₄ for 1 hr. Tissues were dehydrated in an ethanol series and embedded in Spurr's resin. Semi-thin sections 500 nm thick were stained in 0.1% toluidine blue and photographed with a Zeiss Axiophot microscope (Carl Zeiss, Germany).

[0095] *RNA extraction and analysis*: 100 mg leaf tissue was ground in liquid N₂ and extracted with plant RNA purification reagent (Invitrogen) or Trizol (Invitrogen). RNA concentration was measured by Nanodrop (Thermo, USA). DNase treatment and reverse transcription (RT) reaction were performed as described (Qu et al., 2007). For TRV RNA1 analysis, first strand cDNA synthesis reaction was performed with TRV1-P3 primer (5'-TTAAGACGAGTTTTTCTTATTAGACGCTCTCT-3'; SEQ ID NO:82), followed by full-length movement protein gene (MP, length in 756nt) PCR amplification with TRV1-P5 (5'-ATGGAAGACAAGTCATTGGTCACCTTGAAGAA-3'; SEQ ID NO:83) and TRV1-P3 primers. For TRV RNA2 analysis, first strand cDNA synthesis reaction was performed with TRV2-P3 primer (5'-GATCAATCAAGATCAGTCGAGAATG-3'; SEQ ID: NO:84), followed by further PCR amplification with TRV2-P5 (5'-GATGGACATTGTTACTCAAGGAAGC-3'; SEQ ID NO:85) and TRV2-P3 primers. Theoretical PCR amplified TRV RNA2 band should be 939 bps (including the inserted partial *JcCH42* gene). Real-time PCR was performed with Power SYBR® Green PCR Master (Applied Biosystems, USA) and run in ABI7900HT. All samples were run in triplicates and data was analyzed with RQ manager at a pre-set Ct value (Applied Biosystems, USA). The *J. curcas* rbcL mRNA served as an internal control. The real-time PCR primer sequences are set forth in Table 2.

TABLE 2

Real-Time PCR Primers

Primer	Sequence (5'-3')	SEQ ID NO:
CH42-F	CTGTGCAGATAGATCATG	48
CH42-R	ATGACAGTAGCAACATCC	49

FAD6-F	TGGTGCATCATACGGCTC	50
FAD6-R	ATGTGAACATTGATATCATG	51
AGO1-F	GGCAAGGAGCTTGACCTGC	52
AGO1-R	TCAGCGCTACATTGGCTAG	53
AS1-F	AGAGCTGAAGAGGACGCC	54
AS1-R	CAGGCTTGAGGTAGTTCTTC	55
DCL4-F	GCCAGTCAAGTCAGATGC	56
DCL4-R	CCACACTGATGCCAGCC	57
FATB-F	TGTGGATGAGGATGGCAG	58
FATB-R	CCAGCCAAATGTACTTCAC	59
SAD1-F	CAGCTGTTGCACAGCGGC	60
SAD1-R	GGAGGTAGCCGACAAACG	61
SAD2-F	GTGGACTGGGCCTGGACT	62
SAD2-R	TCTGATCCTGGGTCCATG	63
SAD3-F	TTCGTTCCAGGAGAGAGC	64
SAD3-R	TCGACCTCAAATAACTTCTC	65
KNOT1-F	GGTTTGGACCAGAAACAAATAAAT	66
KNOT1-R	CACCCATGTAGTGACCATCC	67
PHV-F	GGGTGGTGGTTCAATCATT	68
PHV-R	TCTCCGCTAGTCTCTTGTC	69
KASII-F	CCTTGTGTGGATCCAGTTTTCAAGG	70
KASII-R	GCTTCTCCAGAAAGAGCGAGCCGA	71
PCNA-R	CAACAGTAGACAAGCCTGAAGAGG	72
PCNA-F	GCAATCTTGTACTIONCAACCACAACAG	73
PDS-R	CTCCTGAAGAAATCGGTTCAAGT	74
PDS-F	TGACCGTTCAAGAAATGGATGAG	75
RUBL-R	CTTCTCCAGCAACGGGCTC	76
RUBL-F	GGAGTTCCGCCTGAGGAAG	77
FAD2-1-R	GGTTGAGGAAGGAGGTGGAAG	78
FAD2-1-F	CCACCATTACACTTGGTCAG	79
FAD2-2-F	AGCAATCAAGCCTATATTGGGC	80
FAD2-2-R	CCAGAGAACTCCTCGGTTGG	81

[0096] *Antibodies and protein gel blot analysis:* Curcin protein antibody was prepared by Dr. Yin zhongcao's lab. Total plant proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. ECL peroxidase conjugated donkey anti-rabbit immunoglobulin G was used as a secondary antibody. Immunoreactive bands were visualized using ECL Western blotting Detection Reagents (GE healthcare). Coomassie blue-stained rbcL band was used as a loading control.

EXAMPLE 2

Development of a VIGS System in *J. curcas* Using CH42 as a Marker Gene

[0097] This example describes the construction of a tobacco rattle virus (TRV) based vector and its use for gene silencing in *J. curcas*. Virus induce gene silencing (VIGS) is initiated when a recombinant virus carrying a sequence from a host gene infects the plant. The endogenous

gene transcripts with sequence homology to the insert in the VIGS vector are degraded by a post-transcriptional gene silencing mechanism (PTGS) (Baulcombe, 2004).

[0098] Initially, TRV vectors kindly provided by Dr. Dinesh Kumar (Yale University) were used in this study. TRV is a bipartite positive sense RNA virus. TRV RNA1 encodes 134 kDa and 194 kDa replicase proteins from the genomic RNA, a 29-kDa movement protein and 16-kDa cysteine-rich protein from subgenomic RNAs. TRV RNA2 encodes the coat protein from the genomic RNA and two non-structural proteins from the subgenomic RNAs. TRV RNA1 can replicate and move systemically without RNA2. In the TRV RNA2 cDNA construct, the non-structural genes were replaced with a multiple cloning site (MCS) useful for cloning the target gene sequences for VIGS (MacFarlane et al., 1999).

[0099] We assessed the gene silencing efficiency of the TRV-VIGS clones to suppress *CH42* gene expression in *J. curcas*. The enzyme encoded by the *CH42* gene is responsible for adding magnesium into the porphyrin ring during chlorophyll biosynthesis. When this gene is silenced chlorophyll synthesis is blocked and consequently leaves lose their green color but appear yellow instead owing to the presence of carotenoids.

[0100] To amplify the *CH42* homolog from *J. curcas*, PCR primers were designed targeting the conserved sequence motifs of *CH42* from different species of the *Euphorbiaceae* family. First, we obtained a 440-bp *CH42* cDNA of *J. curcas* by PCR, and the identity of this clone was verified by sequencing. This *CH42* fragment was inserted into the TRV2 MCS site to give pTRV2-*CH42S*. We used this short *CH42* sequence as a seed to find longer EST sequences of different *Euphorbiaceae* family *CH42* genes in GenBank by BLASTN. We then further designed PCR primers to clone a 689-bp fragment of the *J. curcas* *CH42* cDNA to give pTRV2-*CH42L*. The sequence of *CH42L* was also verified by sequencing.

[0101] Cultures of *Agrobacterium* carrying pTRV1 was mixed with cultures of *Agrobacterium* carrying either pTRV2-*CH42S* or pTRV2-*CH42L*. The mixed culture was injected onto *J. curcas* plants with 2-3 true leaves (for details see Example 1). Three to four days post injection, upper leaves of the treated plants were examined for silencing effects. We observed that the gene silencing effect persisted indefinitely in 3-5 leaves younger than the injected leaves (Figs. 2B and 2C). TRV-*CH42L* which contained a *CH42* insertion, induced a better silencing effect than the shorter one, TRV-*CH42S* (compare Fig. 2B with Fig. 2C). Moreover, the *CH42L* suppression effect was visible uniformly throughout the entire leaf (Figs. 2B and 2C). Uniform silencing of target gene is helpful for high-throughout study and rapid analysis using VIGS since it allows easy sampling and collection of reproducible data. We

performed RT-PCR, using total RNA extracted from plants treated with different TRV vectors to confirm the VIGS of the *CH42* gene at the molecular and the results are shown in Fig. 2E. *J. curcas* *CH42* RNA accumulation in the upper leaves of TRV-*CH42L* infected plant (lane 3) was much lower than that of plants infected with the empty TRV vector (lane 1) and lower than that of plants infected with TRV-*CH42-S* (lane 2) and of plants infected with the non-related vector control TRV-*JcKASII* (lane 4).

[0102] We further synthesized full-length TRV1 and TRV2 cDNA sequences and cloned into plant expression vectors to give psTRV1001 and psTRV2001. The *CH42L* gene fragment was inserted into psTRV2001 to generate psTRV2002. Mixture cultures of *Agrobacteria* cells containing pTRV1 and pTRV2-*CH42L* or psTRV1001 and psTRV2002 was agro-injected onto *J. curcas* plants with 2-3 true leaf. Four to 5 days post agro-injection, we observed similar silencing effects on plant leaves which turned yellowish (compare Fig. 3A with Fig. 3B).

[0103] These results and the phenotypic data indicated that the synthetic sTRV-VIGS systems could be used to induce silencing of desirable endogenous *J. curcas* genes. The data of the *CH42* gene demonstrates that the same silencing efficiency is achieved with the synthetic vectors as with the original vectors.

EXAMPLE 3

Optimization of the VIGS System by Vacuum Infiltration

[0104] After the *CH42* gene, we next chose to silence another marker gene *phytoene desaturase* (*PDS*) which encodes a key enzyme involved in carotenoid biosynthesis. Silencing of the *PDS* gene would inhibit carotenoid biosynthesis leading to chlorophyll photooxidation and destruction at high light intensity and resulting in photo-bleached leaves.

[0105] To amplify the *PDS* homolog from *J. curcas*, PCR primers were designed to target conserved sequences of *PDS* from different species of the *Euphorbiaceae* family. First, we obtained a 302-bp *PDS* cDNA of *J. curcas* by PCR and inserted this fragment into pTRV2 to give pTRV2-PDSS. This short *PDS* sequence was used as a seed to find longer EST sequences of different *Euphorbiaceae* family *PDS* genes in GenBank by BLASTN. We further designed PCR primers to clone a 786-bp fragment of the *J. curcas* *PDS* cDNA to generate pTRV2-PDSL.

[0106] A mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2-PDSS or pTRV2-PDSL was infiltrated onto *J. curcas* plants with 2-3 true leaves (for details see Example 1). Ten days post agro-injection very weak silencing effect was observed in veins of the third and fourth leaf of plants treated with either TRV-PDSS (data not shown) or TRV-PDSL (Fig.

3B). For VIGS in *Jatropha*, we found that the agro-injection method is not only time-consuming but also does not work very well and reproducible for some genes such as *PDS*. Therefore, we explored the use of vacuum infiltration to increase the VIGS efficiency and reproducibility. Using the same *Agrobacterium* culture mix, the silencing efficiency was much improved with the application of vacuum using a pump (compare Fig. 4B with Fig. 4D). Moreover, the treatment time could be reduced to 1/5 that of agro-injection. The effect of *PDS* insert size on the silencing efficiency was also found (compare Figs. 4C and 4D). We also performed real-time PCR analysis to determine the relative *PDS* mRNA levels in silenced *J. curcas* leaves shown in Figs. 4C and 4D. The *PDS* mRNA level was reduced by more than 95% for TRV-*PDSS* and 98% for *PDSL* compared to the vector control (Fig. 4E). In these experiments, the *rbcL* mRNA was used as a loading control. Statistically lower *PDS* mRNA level indicates that a longer in TRV-*PDSL* rendered a more efficacious silencing effect which was visible uniformly throughout the entire leaf.

[0107] We found the vacuum infiltration also worked better than the injection method for other genes, such as the *CH42* gene (compare agro-injection data in Fig. 4F with vacuum infiltration data in Fig. 4G). Taken together, the vacuum infiltration method is more superior to the agro-injection method as it saves time as well as gives a better silencing effect. These results and the phenotypic data provide further evidence that the TRV-VIGS system could be successfully used to induce efficient silencing of desired endogenous *J. curcas* genes.

EXAMPLE 4

Co-silencing Multiple Genes in One Vector

[0108] Higher plants often contain complex and big gene family to render them more resistant to mutation or to provide gene functional specialization, e.g. use under biotic or abiotic stress. Because of functional redundancy it is quite common to find no obvious phenotypes when a single gene of a multigene family is silenced. Therefore, silencing of multiple genes could provide a valuable approach to studying functions that depend on the independent expression of two or more genes. We assessed the gene silencing efficiency of the TRV-VIGS by co-silencing the expression of the *CH42* gene and the *PCNA* gene. The latter encodes the proliferating cell nuclear antigen (PCNA) in meristems of *J. curcas*.

[0109] In Examples 2 and 3, we have proven the silencing of the *CH42* gene by TRV-*CH42S* and TRV-*CH42L*. To determine whether the TRV vector can be used to silence a gene expressed in meristems, we targeted the *PCNA* gene. PCNA is a highly conserved processivity

factor for DNA polymerase δ , which is required for DNA replication and repair, and is highly expressed in dividing cells such as shoot apical meristems (Kelman, 1997).

[0110] We amplified the *PCNA* homolog from *J. curcas*. PCR primers were designed to target the conserved 614-bp cDNA of *PCNA* from different species of *Euphorbiaceae* family. This partial *PCNA* cDNA was cloned into pTRV2 to give pTRV2-*PCNA* and it was also inserted into pTRV2-*CH42* vector to give pTRV2-*CH42 co-PCNA*.

[0111] A mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2-*PCNA* or pTRV2-*CH42 co-PCNA* was vacuum-infiltrated into *J. curcas* plants with 2-3 true leaves. Eight days post-infiltration severe symptoms appeared, and at 18 dpi, shoot extension ceased in all tested plants and leaves curled downward typical of leaf phenotype with silenced *PCNA* expression. At 21-28 dpi, the curled leaves became detached from the tree trunk and the main shoot never resumed normal growth for at least one month. TRV *CH42-co-PCNA* infected plants displayed co-silencing phenotypes with both yellow leaves and stunted shoots (Figs. 5B, 5C, 5D), suggesting that the two endogenous genes, *CH42* and *PCNA*, were both silenced. These data demonstrate that the TRV VIGS system can be used to knock down the expression of two or more genes thus allowing the deciphering of gene function in the event of functional redundancy.

EXAMPLE 5

Co-Silencing of the Host Viral Resistance System Increases the Silencing Efficiency

[0112] RNA silencing is one of the natural plant defense mechanisms against virus infection. We hypothesized that the co-silencing of the host viral resistance system using TRV-VIGS should result in more efficient VIGS.

[0113] A current model for antiviral silencing in higher plants, using *Arabidopsis thaliana* as an example, suggests that double-stranded (ds) RNA replication intermediates of viral genomic RNAs or highly structured regions within single-stranded viral RNAs are first cleaved by RNase III-type Dicer-like 4 (DCL4) or alternatively by DCL2 to produce 21- or 22-nucleotide (nt) small interfering RNAs (siRNAs) (Baulcombe, 2004). This model implies that *DCL4* is an important *Arabidopsis* viral resistance gene (Deleris et al., 2006). We tested the ability of the TRV-VIGS system to silence *DCL4* and also explored the possibility of increasing silencing efficiency of a candidate gene by co-silencing of *DCL4*. We used the marker gene *CH42* to examine this possibility.

[0114] First, we have shown that infection with TRV-*CH42L* alone can result in knockdown of *CH42* gene expression. Next, we investigated whether the silencing efficiency can be increased by co-silencing of *Jatropha DCL4*. To amplify the *DCL4* homolog from *J. curcas*, PCR primers were designed to target conserved sequence motifs of *DCL4* from different species of the *Euphorbiaceae* family. Using PCR, applicants cloned an anti-sense 678-bp fragment of the *J. curcas DCL4* cDNA and inserted this fragment into pTRV2-*CH42L* to give pTRV2-*CH42L co-DCL4*. A mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2-*CH42L co-DCL4* was vacuum-infiltrated onto *J. curcas* plants and phenotypes were observed at 18 dpi. Results showed that co-silencing of *DCL4* and *CH42* gave a better silencing effect compared silencing of *CH42* gene alone (compare Fig. 6B with Fig. 6A).

[0115] We also used real-time PCR to analyze the relative *DCL4* and *CH42* RNA levels of silenced *J. curcas* leaves. *CH42* mRNA levels were lower in the co-silenced plants compared to plants silenced only in the expression of *CH42* alone ($p < 0.01$) (Fig. 6C).

[0116] Taken together, the phenotypic and molecular analyses demonstrate that co-silencing *DCL4* can confer a higher VIGS efficiency in treated *J. curcas* plants. It is obvious that this strategy can also be extended to co-silencing of other host genes involved in viral resistance. Furthermore, co-silencing of the viral-resistant system also can be used to increase the efficiency of other VIGS systems in other plant species.

[0117] These results and the phenotypes data indicate that the TRV-VIGS system could be successfully used to induce silencing of other desirable endogenous *J. curcas* genes. We have generated expressed sequence tags (EST) for several different *Jatropha* tissues such as seeds and flowers. These expressed sequence tags (ESTs) provide a wealth of information for functional genomics study of *Jatropha*. Therefore, the VIGS assay described here will offer a means to test the function of *Jatropha* gene sequences in a homologous system. Using a normalized cDNA library it will be possible to conduct large scale screens of gene function with the TRV-based VIGS system.

EXAMPLE 6

Knock-Down of *KASII* mRNA Levels Using TRV VIGS System

[0118] Triacylglycerols produced by plants are one of the most energy-rich and abundant forms of reduced carbon available from nature. Given their chemical similarities, plant oils represent a logical substitute for conventional diesel, a non-renewable energy source. The

functional properties of both animal fats and vegetable oils are largely determined by the properties of the fatty acids that are esterified to triacylglycerol.

[0119] We tested the ability of the TRV-VIGS system for high-throughout functional analysis of genes involved in fatty acid biogenesis. *Jatropha* seed oil contains predominantly unsaturated 18-carbon (18C) fatty acids (more than 80%) (Jones et al., 1991), whereas palm oil contain a higher proportion (about 50%) of 16-carbon (16C) saturated fatty acids. We hypothesized that the modulation of *KASII* level using TRV-VIGS should result in the conversion of C18 to C16 fatty acids in *J. curcas* leaves. In the model plant *Arabidopsis*, the *KASII* gene encoding β -ketoacyl-acyl carrier protein synthase II is responsible for the elongation of 16:0-ACP to 18:0-ACP (Pidkowich et al., 2007). First, we investigated whether TRV infection of *J. curcas* alone has an effect on its leaf fatty acid composition. A mixture of *Agrobacterium* containing pTRV1 and pTRV2 was infiltrated into *J. curcas* plants (Fig. 7A, panel (a)). Eighteen days post infiltration, the upper leaves of these plants showed no obvious phenotypes and no change in fatty acid profile was observed when compared with mock-treated *J. curcas* (Fig. 7C for vector and not show the mock-inoculation data). These results, together with the above-described phenotypic data, confirm that TRV infection alone has no effect on *Jatropha* leaf fatty acid profile and therefore the TRV-VIGS system can be used to study genes involved in fatty acid biogenesis.

[0120] To silence *KASII* function using TRV-VIGS, we used PCR to clone an antisense 829-bp fragment of the *J. curcas* *KASII* cDNA corresponding to nt 240-1063 (GenBank No. DQ987700). This fragment was inserted into pTRV2 to give pTRV2-*KASII*. A mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2-*KASII* was infiltrated into *J. curcas* plants (Fig. 7A, panels (b), (c), (d)). Eighteen days post-infiltration, the upper leaves showed two kinds of phenotypes: some leaves showed mild yellowish phenotypes (Fig. 7A, panel (b)) whilst other newly emerged leaves curled downward with brownish leaf blade, and shoot extension ceased (Fig. 7A, panels (c) and (d)). We performed real-time quantitative RT-PCR, using total RNA extracted from *J. curcas* plants infected with TRV-*KASII* or TRV alone. In TRV-*KASII* infected plants showing severe phenotypes, the *KASII* mRNA was reduced by more than 76% compared to the TRV infected control (Fig. 7B) when *rbcL* mRNA was used to normalize loading of the samples. These results showed that the TRV-based VIGS system can efficiently silence targeted host genes and can be used as a rapid means to assay the role of candidate genes in fatty acid biogenesis pathway.

[0121] We used gas chromatograms to examine fatty acid methyl esters from control and silenced *J. curcas* leaves. Lipids were extracted with 2:1 chloroform: methanol and transmethylated to fatty acid methyl esters by incubation with equal volume of 3N methanolic-HCL (SIGMA). An Agilent 6890 GC machine was used to analysis fatty acid methyl esters profile. As expected, higher 16C and lower 18C were found in *KASII* VIGS plants compared to control plants (Fig. 7C and Table 3). The 18C to 16C conversion was more obvious in samples with severe phenotypes and the degree of conversion correlated with phenotypic severity. The amount of 16C:0 (palm oil-like fatty acid) can reach as high as 41% in leaf samples with severe phenotype. This level of C16:0 is nearly 50% level of that in palm oil. Not only the fatty acid chain length, but also the unsaturated/saturated fatty acid ratio was highly dependent on the *KASII* expression levels (Table 3). On the other hand, the vaccenic acid (18:1 *trans*-11) level increased when *KASII* expression was down-regulated. Mammals convert vaccenic acid it into rumenic acid, a conjugated linoleic acid, which shows anticarcinogenic properties (Banni et al., 2001). Our VIGS results in *Jatropha* show very similar fatty acid profile modification resulting by stable *KASII* RNAi silencing method in model plant *Arabidopsis* (Pidkowich et al., 2007), that indicates we functionally identified *KASII* gene in this biodiesel plants.

TABLE 3

Fatty Acid Composition of Vector CK,
KASII Mild, *KASII* Severe and *KASII co-FATB J. curcas* (mol%)

	16:0	16:1 ³	16:1 ⁹	18:0	18:1 ⁹	18:1 ¹¹	18:2	18:3	16:18	S/US
CK	16.5±0.1	2.2±0.4	2.0±0.1	3.9±0.1	2.1±0.1	0.0±0.0	15.1.1.1±	51.1±1.2	0.39	0.25
<i>KASII</i> Mild	26.6±0.1	2.3±0.1	3.6±0.1	4.6±0.3	1.7±0.1	2.7±0.1	12.9±1.2	40.8±1.1	0.60	0.44
<i>KASII</i> Severe	41.2±0.3	0.0±0.0	3.9±0.2	6.1±0.0	0.0±0.0	4.8±1.3	12.1±0.7	24.0±0.1	1.12	0.90
<i>KASII co-FATB</i>	26.5±10.	3.0±0.2	3.8±0.2	4.6±0.1	0.4±0.3	3.7±0.1	9.9±1.2	43.5±1.2	0.61	0.44

Notes: Means and standard of error of three independent samples are presented. 16:18 means the ratio of 16-carbon fatty acid to 18-carbon fatty acid. S/US is the saturated/unsaturated fatty acid ratio.

[0122] We have demonstrated the co-silencing of 2 genes in *J. curcas* with the TRV VIGS system. We next examined whether the TRV VIGS system can be used to silence multiple genes in one vector. We hypothesized that the modulation of *KASII* and other enzyme levels using the TRV VIGS system should result in a fatty acid composition different from that obtained with silencing *KASII* alone. In *Arabidopsis*, *KASII* elongates 16:0-ACP to 18:0-ACP in the plastid,

where it competes with three other enzymes at the first major branch point in fatty acid biosynthesis. Acyl-ACP thioesterase B (FATB), one of three enzymes, remove the acyl-ACP from 16:0-ACP and has been shown to be important for saturated fatty acid biogenesis such as 16:0 and 18:0 (Jones et al., 1995). Preventing the release of saturated fatty acids from ACP by down-regulating *FATB* lowered saturated fatty acid levels, which can improve biodiesel characteristics with regard to cold-temperature flow properties and NO emissions (Hawkins and Kridl, 1998). Using PCR we cloned a sense 796-bp fragment of the *J. curcas* *FATB* cDNA corresponding to nt 174-976 (GenBank No. EU106891) and inserted this fragment into pTRV2-*KASII* to give pTRV2-*KASII* co-*FATB*. A mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2-*KASII* co-*FATB* was infiltrated onto *J. curcas* plants. Eighteen days post-infiltration, the upper leaves showed a weaker phenotype (only mild yellowish color) than that of leaves of plants treated with TRV-*KASII*. We performed real-time quantitative RT-PCR, using total RNA extracted from *J. curcas* plants infected with TRV-*KASII* co-*FATB* to confirm down-regulation of both the *KASII* and *FATB* genes in the same leaf sample. In TRV-*KASII* co-*FATB* infected plants, the mRNA were reduced by more than 81% for *KASII* and 84% for *FATB* compared to the TRV infected control (Fig. 7B) when *rbcL* mRNA was used to normalize RNA loading. We further used gas chromatography to examine fatty acid methyl esters from the co-silenced *J. curcas* leaves. The 18C to 16C conversion seen in knock-down of the *KASII* gene alone was dampened upon co-silencing of the *FATB* gene. As we expected according to the *FATB* T-DNA insertion mutant work in model plant *Arabidopsis* (Bonaventure et al., 2003), co-silencing *FATB* with *KASII* gene leads to less 18:0 and 16:0 level compared with the *KASII* only silencing plants. Not only the 18C fatty acid ratio, but also the unsaturated fatty acid ratio was much higher in *FATB* and *KASII* co-silencing than the single *KASII* silencing plants. Therefore, co-silencing *FATB* gene can reverse the *KASII* single gene silencing effect on fatty acid metabolism, which further indicates that manipulation the balance of these two genes expression level is one potential method to get ideal bio-diesel fatty acid composition in *Jatropha* industry.

[0123] These results also show that the TRV based VIGS system can efficiently suppress targeted host genes and can be used as a rapid means to assay the role of candidate genes in fatty acid biosynthesis. Therefore, we set out to silence putative *J. curcas* desaturase genes *SAD1*, *SAD2*, *SAD3*, *FAD2-1*, *FAD2-2* and *FAD6* homologs in *J. curcas* to investigate their role in fatty acid biosynthesis.

EXAMPLE 7

Rapid Functional Analysis of Several Stearoyl-ACP
Desaturase Genes by the TRV VIGS system in *J. curcas*

[0124] One method of altering the unsaturated fatty acid content of oilseeds is by manipulating the expression of desaturase genes of these plants. Manipulating the expression of desaturase genes in transgenic crops such as soybean, maize and canola (oilseed rape) has led to oils with altered functionality and nutrition.

[0125] Stearoyl-acyl carrier protein (Stearoyl-ACP) desaturase (SAD) catalyzes the first desaturation step in fatty acid biosynthesis, converting stearoyl-ACP to oleoyl-ACP. Therefore, SAD plays important roles in determining the chain length of fatty acids and the ratio of saturated to unsaturated fatty acids (Shanklin and Somerville, 1991). It has been shown that stearic acid content of oil in seed can be increased by downregulation of *SAD* expression level (Knutzon et al., 1992). Furthermore, the stearyl desaturase-catalyzed enzymatic step leads to the synthesis of jasmonic acid (JA), a plant hormone important for abiotic and biotic stresses in plants (Kachroo et al., 2003). At the same time, SAD is also encoded by a gene family, and seven SAD genes are present in the *Arabidopsis* genome. We have isolated three *J. curcas* SAD genes by sequencing a *Jatropha* seed cDNA library. The functions of these 3 different SAD genes were investigated using the TRV-VIGS system.

[0126] Using PCR, we cloned a sense 796-bp fragment of the *J. curcas* *SAD1* cDNA (GenBank No. DQ084491) to form pTRV2-SAD1. We also cloned a sense 868-bp and 847-bp fragments of the *J. curcas* *SAD2* and *SAD3* cDNA (SEQ ID NO:33 and SEQ ID NO:36, respectively). These cDNA fragments were inserted into pTRV2 to give pTRV2-SAD2 and pTRV2-SAD3. Mixtures of *Agrobacterium* cultures containing pTRV1 and pTRV2-SAD1, pTRV2-SAD2 or pTRV2-SAD3 were infiltrated onto different *J. curcas* plants. Four dpi after infections, newly emerged leaves showed yellowish vein phenotype (data not shown). At 10 dpi the vein phenotype was more obvious in TRV-SAD1 plants which displayed brownish veins and severe narrowing of leaf blades (Fig. 8A, panel (b)). Leaves and stems withering can be found in most of the TRV-SAD1 plants at 18 dpi (Fig. 8A, panels (c) and (d)). No phenotypes were seen in TRV-SAD2 plants (Fig. 8A, panel (e)). Much weaker phenotypes, such as partial leaf vein yellowing and leaf curling were found in TRV-SAD3 plant at 18 dpi (Fig. 8A, panels (f) and (g)). To confirm the down-regulation of the appropriate *SAD* gene, we performed real-time quantitative RT-PCR using total RNA extracted from infected *J. curcas* plants. Compared to the

TRV-infected control plants, the *SAD1* mRNA was reduced by 91% in TRV-*SAD1* plants, the *SAD3* mRNA was reduced by 75% in TRV-*SAD3* plants (FIG. 8B).

[0127] We further used gas chromatography to examine fatty acid methyl esters from leaves of silenced *J. curcas* plants. At 10 dpi plants silenced in *SAD1* expression (10 dpi in Fig. 8A, panel (b)), contained 10.8% stearic acid which is about 2.8 fold higher than that of vector control plants. On the other hand, these *SAD1*-silenced plants displayed little changes in their C16 fatty acid profile (Fig. 8C and Table 4). Meanwhile, all 18C unsaturated fatty acids were down-regulated. A higher saturated to unsaturated fatty acid ratio also indicated higher levels of saturated fatty acids in *SAD1*-silenced leaves (Table 4). At a later stage (*SAD1L*), dramatic changes were found in the levels of 16C fatty acid as well as 18C fatty acids (Table 4). These data provide the first genetic evidence that not only 18:0-ACP but also 16:0-ACP can serve as substrates of the *SAD* genes. In fact, the *SAD1* gene should be named as Δ^9 -ACP desaturase gene. For plants silenced in *SAD3* expression changes in GC profile are consistent with the much weaker phenotypes. Leaves from these plants contained only 4.5% stearic acid, which was only 0.6% higher than that in the vector control plants.

TABLE 4
Fatty Acid Composition of Several
Desaturase Gene VIGS *J. curcas* Leaves (mol%)

	16:0	16:1 ³	16:1 ⁹	18:0	18:1	18:2	18:3	16:18	S/US
CK	16.5±0.1	2.2±0.4	2.0±0.1	3.9±0.1	2.1±0.1	15.1±1.1	51.1±1.2	0.39	0.25
<i>SAD1-E</i>	17.9±0.5	1.6±0.1	1.0±0.0	10.8±0.6	0.8±0.4	14.7±1.4	45.0±2.5	0.37	0.42
<i>SAD1-L</i>	23.8±1.1	0.0±0.0	0.0±0.0	14.6±1.4	0.0±0.0	10.3±1.8	28.2±2.5	0.67	0.62
<i>SAD3</i>	16.5±0.0	2.2±0.1	1.7±0.5	4.5±0.1	1.7±0.0	16.9±0.8	51.6±0.1	0.32	0.27
<i>FAD2-1</i>	13.6±0.6	2.4±0.3	1.7±0.0	3.9±0.5	17.8±2.1	5.4±2.0	49.0±1.8	0.30	0.21
<i>FAD2-2</i>	15.7±0.6	2.6±0.3	1.6±0.1	3.7±0.4	1.2±0.6	15.8±1.3	50.9±1.0	0.43	0.23
<i>FAD6</i>	15.4±1.5	2.1±0.3	1.5±0.3	3.4±0.1	5.0±0.2	17.8±1.9	45.5±2.3	0.29	0.23

Notes: Means and standard error of three independent samples are presented. 16:18 means the ration of 16-carbon fatty acid to 18-carbon fatty acid. S/US is the saturated/unsaturated fatty acid ratio. The samples of *SAD1-E* are the leaves of *SAD1* VIGS *J. curcas* at 12 dpi.

[0128] These biochemical results along with the above-described phenotypic data indicate that TRV VIGS could be used to rapidly characterize individual members of a multiple gene family, which may share overlapping functions. Also seed-specific suppression of the *SAD1*

(Δ^9 -ACP desaturase) gene can be used to produce *Jatropha* oil containing high-stearic acid content.

EXAMPLE 8

Manipulation of *FAD2-1* and *FAD6* Expression Level Can Be Used to Produce High Oleic Acid Content in *Jatropha* Plants

[0129] Oleic acid (18:1) content of plant oils is a quantitative trait with importance for human and animal nutrition and also for use of plant oil as a source of biodiesel (Durrett et al., 2008). Oils with higher oleic acid content have more oxidative stability, healthier and present beneficial cooking properties. Several QTLs for seed oil content of many plant species such as *Arabidopsis*, canola, peanut and maize, mapped to *fad2* genes (Patel et al., 2004; Belo et al., 2008). *FAD2* encodes an endoplasmic reticulum-localized Δ^{12} desaturase required for converting the monounsaturated oleic acid (18:1) to the polyunsaturated linoleic acid (18:2), itself the precursor to linolenic acid (18:3; Okuley et al., 1994). On the other hand, the *fatty acid desaturase-6* gene (*FAD6*) encodes a delta-12 desaturase that functions in plastids. By sequencing the *Jatropha* cDNA library we found at least 2 *FAD2* genes (*FAD2-1* and *FAD2-2*) in the *J. curcas* genome. These two *FAD2* genes share high nucleotide and amino acid sequence similarity. We used the TRV-VIGS system to analyze the function of these two genes in fatty acid biosynthesis.

[0130] Using PCR, we cloned a sense 862-bp and 711-bp fragments of the *J. curcas FAD2-1* and *FAD2-2* cDNA, respectively (JcFAD2-1, GenBank No. DQ157776). These fragments were inserted into pTRV2 to give pTRV2-*FAD2-1* and pTRV2-*FAD2-2*. We also used PCR to clone a sense 835-bp fragment of the *J. curcas FAD6* cDNA (GenBank No. EU106889), and this fragment was inserted into pTRV2 to give pTRV2-*FAD6*. Mixtures of *Agrobacterium* cultures containing pTRV1 and pTRV2-*FAD2-1*, pTRV2-*FAD2-2* or pTRV2-*FAD6* were separately infiltrated into different *J. curcas* plants. No obvious morphological phenotype was seen in plants infected with these recombinant viruses for more than one month (data not shown). We also used real-time PCR to examine mRNA levels in silenced *J. curcas* plants. The mRNA level was reduced by 83% for *FAD2-1* in TRV-*FAD2-1* plants and 82% for *FAD2-2* in TRV-*FAD2-2* (Fig. 8B), whereas the *FAD6* mRNA level in TRV-*FAD6* plant was only 8% that of vector control plants. Although there were no obvious morphological phenotypes for plants silenced in one of these 3 Δ^{12} desaturase genes, dramatic changes were found in the GC profile of *FAD2-1* silenced plants (Fig. 8C and Table 4). In *FAD2-1* VIGS plants, the linoleic acid (18:2) was

down-regulated to 5.4% from 15.4% found in vector control plants. By contrast the oleic acid content was up-regulated to 17.8% from 2.1%. In *FAD2-2* VIGS plants, the oleic acid was even lower than that found in vector control plants. Data obtained by the applicants also showed that the *FAD6* gene has minor contributions to oleic acid accumulation in *J. curcas*. In the *FAD6*-silenced plants, the oleic acid content was up-regulated to 5.0% from 2.1%, whereas the linoleic acid (18:2) content was up-regulated slightly. Most interestingly, both the ratio of 16C to 18C and the saturated to unsaturated fatty acids were down-regulated, which means that knock-down of *FAD2-1* and *FAD6* genes can result in the production of longer fatty acids and unsaturated fatty acids.

[0131] These results provide further evidence that TRV VIGS could be used to rapidly screen for function of members of a multiple gene family, which may share similar functions. Also seed-specific suppression of *FAD2-1* and *FAD6* (Δ^{12} -ACP desaturase) gene expression can be used to produce plant oil with higher oleic oil and lower 16C in seed of *J. curcas*.

EXAMPLE 9

Using VIGS to Analyze Function of Transcription Factor Genes in *J. curcas*

[0132] Transcription factors (TFs)-mediated regulation of mRNA production is a major mode of regulation for plants mounting responses to developmental signals and environmental cues, and transcriptional regulation has been widely studied in model plants, such as *Arabidopsis* and rice. We tested the utility of the TRV-VIGS system for high-throughput analysis TF gene functions.

[0133] The *ASYMMETRIC LEAVES 1 (AS1)* gene in *Arabidopsis* and its ortholog the *ROUGH SHEATH2 (RS2)* gene in maize (*Zea mays*) and *PHAN* in *Antirrhinum* seem to play an evolutionarily conserved role in shoot apical meristem, leaf and fruit development (Sun et al., 2002; Alonso-Cantabrana et al., 2007). Both AS1 and its orthologs belong to the R2R3 MYB type of TF proteins. AS1 functions as a transcriptional repressor by binding to the promoter of class I *KNOTTED1*-like homeobox (*KNOX*) genes. This factor promotes stem cell function through the regulation of phytohormone activities (Alonso-Cantabrana et al., 2007). AS1 operates as a negative regulator of inducible resistance against these pathogens by selectively binding to the promoters of genes controlled by the immune activator, jasmonic acid (JA), damping the defense response. By contrast, AS1 is a positive regulator of salicylic acid (SA)-independent extracellular defenses against bacterial pathogens (Nurmburg et al., 2007).

[0134] To amplify the *ASI* homolog from *J. curcas*, PCR primers were designed to target conserved sequence motifs of *ASI* from different species of the *Euphorbiaceae* family. Using PCR we cloned a sense 630-bp fragment of the *J. curcas ASI* cDNA which was inserted in to pTRV2 to give pTRV2-*ASI*. A mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2-*ASI* vector was infiltrated into different *J. curcas* plants. After 27 dpi, obvious phenotypes can be seen in newly emerged leaves. Severe downward curling was the most obvious phenotype both from the adaxial side (Fig. 9A, panel (a)) as well as from the abaxial side (Fig. 9A, panel (b)) of leaves. Leaves silenced in *ASI*-expression had normal adaxial/abaxial polarity but displayed a specific disruption in the adaxial domain, leading to the formation of ectopic leaf blades on the lateral flanks of vein (Fig. 9A, panels (a), (b), (c), (d)). Ectopic leaf blades (EB) emerged from the primary blade (PB) and showed a fixed polarity with the adaxial surface facing away from the rib (Fig. 9A, panels (c), (d)).

[0135] We further investigated the morphological changes in detail by microscopy. Using scanning electron microscopy (SEM), we found ectopic adaxial leaf blade structures developed directly from the primary leaf blade (Fig. 9B). Moreover, the adaxial surface was facing away from the rib and the abaxial surface with a large number of stomata was covered by the ectopic leaf blades, just like a withering flower. Transverse sections through ectopic blades confirmed that these are differentiated leaf blades, with a recognizable palisade layer in the upper mesophyll, polarized vascular tissue, and a spongy mesophyll. Quantitative RT-PCR data confirmed that the *ASI* mRNA level was reduced by 69% (Fig. 9H).

[0136] We also checked the expression level of a putative *J. curcas KNAT1-like* gene in *ASI*-silenced leaves. Quantitative RT PCR data showed that *J. curcas KNAT1-like* gene expression was 3.28 fold that of the vector control plants. This result provides evidence that the *KNAT1-like* gene is negatively regulated by *ASI* (Fig. 9H). In *Arabidopsis*, ectopic expression of *KNAT1* leads to production of ectopic adaxial leaf blade (Sun et al., 2002; Theodoris et al., 2003).

[0137] These molecular data and the adaxial leaf blade phenotype provide further evidence that TRV VIGS could be used in *J. curcas* to rapidly screen for function of TF genes. Such genes may be important for plant fruit development and oil body formation in developing seed. More importantly, recent evidence shows that common networks regulate leaf and fruit patterning in *Arabidopsis* (Nurmeberg et al., 2007). Thus, one can use the leaf as a model system for rapid assessment of TF gene functions and to make use of such information to further to make use of these genes to modify or enhance the quality and quantity of *J. curcas* oil. In

certain embodiments, the invention also provides plant products obtained from transgenic plants of the invention. The term "plant product" is intended to include anything that may be obtained from a particular plant, including, for example, fruits, seeds, pollen, ovules, plant embryos, oils, juices, waxes, proteins, lipids, fatty acids, vitamins, plant tissues in whole or in part, (e.g. roots, leaves, stems, flowers, bark), cells, cell suspensions, tubers and stolons.

EXAMPLE 10

Functional Analysis of Small RNA Pathway Genes in *J. curcas* by VIGS

[0138] Small RNAs (smRNAs) regulate processes as diverse as plant resistance to viruses, and plant development and differentiation. We tested the ability of the TRV-VIGS system for high-throughput analysis of functions of genes involved in smRNA biogenesis pathways.

[0139] All RNA-silencing pathways require the genesis of 18- to 26-nt smRNAs from the cleavage of double-stranded RNA (dsRNA) or highly structured regions within single-stranded viral RNAs. MicroRNA is one important kind of smRNAs. Bound to ARGONAUTE1 (AGO1) protein, miRNAs guide RNA-induced silencing complexes (RISCs) to cleave mRNAs with partial or complete sequence complementarity. Accordingly, *Arabidopsis* AGO1 binds miRNAs and displays slicer activity toward miRNA targets, and strong *ago1* loss-of-function mutants overaccumulate miRNA target transcripts (Baulcombe, 2004; Baumberger and Baulcombe, 2005). AGO1 has also proven to bind viral-derived siRNA and *ago1* mutant show hypersusceptibility to virus (Beclin et al., 2002; Morel et al., 2002). By sequencing the cDNA library, we found four cDNA sequences encoding putative AGO1 in *J. curcas*.

[0140] Using PCR we cloned a sense 855-bp fragment of the *J. curcas* AGO1 cDNA which was inserted into pTRV2 to give pTRV2-AGO1. A mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2-AGO1 infiltrated into different *J. curcas* plants. After 27 dpi, diverse and varied phenotypes can be seen in new emerged leaves. Severe upward curling was the most obvious phenotype both from the adaxial side (Fig. 9A, panel (e)) and the abaxial side (Fig. 9A, panels (f), (g)) of leaves. AGO1-silenced leaves showed a specific disruption in the abaxial domain, leading to the formation of abaxial ectopic leaf blades (Fig. 9A, panels (f), (g)). Typical serrate phenotypes, including deep serration some even into the bottom of the leaf blade, were found in the AGO1-silenced plants (Fig. 9A, panels (h), (i), (j)).

[0141] We further observed the morphological changes in detail by microscopy. Using SEM, ectopic abaxial leaf blades structure emerged along with the leaf vein and faced toward the vein

(Fig. 9D). Transverse sections through ectopic blades confirmed that these were differentiated leaf blades, with a recognizable palisade layer in the upper mesophyll and a spongy mesophyll.

[0142] There were some bubbles in the adaxial domain of *AGO1*-silenced leaves (Fig. 9A, panel (e)). Transverse sections through these bubbles confirmed that one ectopic leaf vein-leaf blade mixed structure with polarized vascular tissue and parenchyma, a two-layer blade palisade in the upper mesophyll and a spongy mesophyll (Fig. 9F).

[0143] Real time RT-PCR data confirmed that *AGO1* mRNA levels were reduced by 69% (Fig. 9I). We also checked the expression level of one putative *J. curcas* miR155/156 regulated gene *PHAVOLUTA* (*PHAV*) in *AGO1*-silenced leaves. Real time RT-PCR data shows that *J. curcas* *PHAV* gene expression was 2.82 fold that of the vector control plants. This result shows that *PHAV* gene expression is negatively correlated with *AGO1* mRNA levels (FIG. 9H). In Arabidopsis, ectopic *PHAV* expression leads to the formation of ectopic abaxial leaf blade (Kidner and Martienssen, 2004).

[0144] These molecular data and the adaxial leaf blade phenotype provide further evidence that TRV VIGS could be used in *J. curcas* to rapidly screen for function of genes involved in smRNA biogenesis pathway.

EXAMPLE 11

Using VIGS to Functionally Analyze Genes Involved in Toxin Biosynthesis in *J. curcas*

[0145] Curcin is a toxic protein specifically expressed in *Jatropha* seeds. It is one of the several toxic components which prevent *Jatropha* seeds and protein meal to be used as animal feed (Thomas et al., 2008). We tested the ability of the TRV-VIGS system to knock-out or knock-down *curcin* gene expression.

[0146] Using PCR, we cloned an anti-sense 836-bp fragment of the *J. curcas* *curcin* cDNA (*curcin* GenBank No. AY069946). This fragment was inserted into pTRV2 to give pTRV2-*curcin*. A mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2-*curcin* was infiltrated into *J. curcas* plants. After 18 dpi, no obvious phenotype was found in *curcin*-silenced leaves. Silenced leaves were collected and curcin antibody was used to detect the curcin protein. Western blot analysis showed that silenced leaves contained only 2-3% of curcin found in leaves of vector control plant. Coomassie Bright Blue staining of the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase indicates comparable loading of the samples.

[0147] These data show the TRV VIGS system can be used for analysis of gene function in biogenesis pathways of toxic agents in *Jatropha*.

EXAMPLE 12

Analysis of TRV VIGS System in Other Plants

[0148] According to the description from the International Committee on Taxonomy of Viruses (<http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/007201001.htm>; hereinafter “ICTV”), TRV has a fairly wide host range. In addition, Dinesh Kumar et al. (2007) contains an extensive list of susceptible hosts. In order to determine whether the TRV VIGS system can be used for plants within the susceptibility lists, we tested the synthetic TRV VIGS (sTRV VIGS) system of the present invention in other putative hosts of TRV (ICTV; Dinesh Kumar et al. (2007)). We noted that different kinds of beans were independently listed as susceptible hosts in Dinesh Kumar et al. (2007) instead of a family or genus. Therefore, we chose some plants from different genera, but all in single *Faboideae* subfamily in the *Fabaceae* family within the *Fabales* order: common bean (*Phaseolus vulgaris*), peanut (*Arachis hypogaea*) and soybean (*Glycine max*). In addition, we also chose castor bean (*Ricinus communis*) which is from the same family (*Euphorbiaceae*) but a different subfamily with *Jatropha*. We found that the sTRV VIGS system does work in castor bean, although less potently than in *Jatropha*. After 18 dpi, CH42 silencing effect can be found in both inoculated leaf and systemic leaf (Fig. 11E). Recombinant TRV RNA1 and RNA2 can also be detected in almost all tested castor bean plants leaves (Fig. 12, lanes 11, 12, 14-17). A few necrotic spots (indicated by dark arrow in Fig. 11D) were seen in the inoculated leaf of the treated common bean. However, no necrotic spots were seen in systemic leaf of the treated common bean (indicated by white arrow in Fig. 11D). This fewer necrotic spot phenotype is also correlated with very low viral RNA1 and RNA2 accumulation in the inoculated leaves of the common bean (Fig. 12, lane 20). It appears that the virus replication was heavily limited by host plant cell death and thus can not move systemically. Both the phenotypic data and molecular data in common bean suggest that this host is a local necrotic host of the TRV strain. ICTV also describes common bean as a “necrotic local lesions; no systemic infection”. Systemic leaves are always required for functional gene analysis in VIGS. Common bean, as a local necrotic host, therefore is not suitable as a host of the TRV VIGS system.

[0149] We also chose another bean species from the same *Phaseolus* genus with common bean, namely mung bean (*Phaseolus aureus*) (http://en.wikipedia.org/wiki/Mung_bean), to demonstrate that there is no

reasonable expectation that the host range could be extended from one listed-plant species to the whole genus of plants or even to the whole family of plants. No local necrotic lesion and no viral RNA were found in mung bean plants (Figure 12, lanes 6-8). This finding demonstrates that mung bean is not a host for TRV, although it is a near relative of common bean which is a local host of TRV.

[0150] We did not observe any silencing effect in other two listed host plants, peanut (*Arachis hypogaea*) and soybean (*Glycine max*), after treating with sTRV2:JcCH42 (Fig. 11A and Fig. 11C). No viral RNA can be detected in peanut leaf (Fig. 12, lanes 1-3).

[0151] The data obtained from this example highly suggests that the TRV VIGS system can work in some plants in the TRV susceptible lists (ICTV; Dinesh Kumar et al. (2007)), but cannot work in all of the listed plants. The data obtained in this example also shows the unpredictability of which plants may be hosts for TRV and for which plants the TRV VIGS system can be used for functional gene analysis. For example, the data shows the unpredictability even between plants having a near evolutionary relationship, such as common bean and mung bean which are both in the same genus. The data shows that mung bean is not a host for TRV, but that common bean is a local necrotic host. Even though common bean is a local necrotic host, it is not suitable for functional gene analysis by the TRV VIGS system. Thus, the present example demonstrates the inherent unpredictability of plant-viral interactions, for example the unpredictability as to the susceptibility of any given plant species to TRV and to the ability of the TRV VIGS system to be useful for functional gene analysis in any given plant species.

[0152] 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. For example, if the range 10-15 is disclosed, then 11, 12, 13, and 14 are also disclosed. 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.

[0153] It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. 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.

BIBLIOGRAPHY

- [0154] Alonso-Cantabrana, H. et al. (2007). Common regulatory networks in leaf and fruit patterning revealed by mutations in the Arabidopsis ASYMMETRIC LEAVES1 gene. *Development* **134**:2663-2671.
- [0155] Angell, S.M. and Baulcombe, D.C. (1999). Technical advance: potato virus X amplicon-mediated silencing of nuclear genes. *Plant J* **20**:357-362.
- [0156] Banni, S. et al. (2001). Vaccenic acid feeding increases tissue levels of conjugated linoleic acid and suppresses development of premalignant lesions in rat mammary gland. *Nutr Cancer* **41**:91-97.
- [0157] Baulcombe, D. (2004). RNA silencing in plants. *Nature* **431**:356-363.
- [0158] Baumberger, N. and Baulcombe, D.C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc Natl Acad Sci USA* **102**:11928-11933.
- [0159] Beclin, C. et al. (2002). A branched pathway for transgene-induced RNA silencing in plants. *Curr Biol* **12**:684-688.
- [0160] Belo, A. et al. (2008). Whole genome scan detects an allelic variant of fad2 associated with increased oleic acid levels in maize. *Mol Genet Genomics* **279**:1-10.
- [0161] Bonaventure, G. et al. (2003). Disruption of the FATB gene in Arabidopsis demonstrates an essential role of saturated fatty acids in plant growth. *Plant Cell* **15**:1020-1033.
- [0162] Brigneti, G. et al. (2004). Virus-induced gene silencing in Solanum species. *Plant J* **39**:264-272.

- [0163] Burch-Smith, T.M. et al. (2004). Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant J* **39**:734-746.
- [0164] Burch-Smith, T.M. (2006). Efficient virus-induced gene silencing in Arabidopsis. *Plant Physiol* **142**:21-27.
- [0165] Chen, J.C. et al. (2005). Silencing a prohibitin alters plant development and senescence. *Plant J* **44**:16-24.
- [0166] Chung, E. et al. (2004). A method of high frequency virus-induced gene silencing in chili pepper (*Capsicum annuum* L. cv. Bukang). *Mol Cells* **17**:377-380.
- [0167] Deleris, A. et al. (2006) Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* **313**:68-71.
- [0168] Dinesh Kumar et al. (2007). Tobacco rattle virus vectors and related compositions and methods. U. S. No. Patent 7,229,829 B2.
- [0169] Durrett, T.P. et al. (2008). Plant triacylglycerols as feedstocks for the production of biofuels. *Plant J* **54**:593-607.
- [0170] Fu, D.Q. et al. (2005). Virus-induced gene silencing in tomato fruit. *Plant J* **43**:299-308.
- [0171] Hawkins, D.J. and Kridl, J.C. (1998). Characterization of acyl-ACP thioesterases of mangosteen (*Garcinia mangostana*) seed and high levels of stearate production in transgenic canola. *Plant J* **13**:743-752.
- [0172] Jones, A. et al. (1995). Palmitoyl-acyl carrier protein (ACP) thioesterase and the evolutionary origin of plant acyl-ACP thioesterases. *Plant Cell* **7**:359-371.
- [0173] Jones, N.M.J. (1991). *Jatropha curcas* - a multipurpose species for problematic sites. *Land Resources Series* **1**:1-12.
- [0174] Kaloshian, I. (2007). Virus-induced gene silencing in plant roots. *Methods Mol Biol* **354**:173-181.
- [0175] Kelman, Z. (1997). PCNA: structure, functions and interactions. *Oncogene* **14**:629-640.
- [0176] Kidner, C.A. and Martienssen, R.A. (2004). Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. *Nature* **428**:81-84.
- [0177] Knutzon, D.S. et al. (1992). Modification of Brassica seed oil by antisense expression of a stearyl-acyl carrier protein desaturase gene. *Proc Natl Acad Sci USA* **89**:2624-2628.
- [0178] Liu, Y. et al. (2002a). Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant J* **30**:415-429.
- [0179] Liu, Y. et al. (2002b). Virus-induced gene silencing in tomato. *Plant J* **31**:777-786.
- [0180] Liu, Y. et al. (2004). Virus induced gene silencing of a DEFICIENS ortholog in *Nicotiana benthamiana*. *Plant Mol Biol* **54**:701-711.
- [0181] MacFarlane, S.A. et al. (1999). Similarities in the genome organization of tobacco rattle virus and pea early-browning virus isolates that are transmitted by the same vector nematode. *J Gen Virol* **80** (Pt 1):273-276.
- [0182] Morel, J.B. et al. (2002). Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* **14**:629-639.

- [0183] Narayana, D.S.A et al. (2007). Distinct Begomoviruses Closely Related to Cassava Mosaic Viruses cause Indian *Jatropha* Mosaic Disease. *Int'l J Virol* **3**:1-11.
- [0184] Nurmberg, P.L. et al. (2007). The developmental selector AS1 is an evolutionarily conserved regulator of the plant immune response. *Proc Natl Acad Sci USA* **104**:18795-18800.
- [0185] Okuley, J. et al. (1994). Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *Plant Cell* **6**:147-158.
- [0186] Patel, M. et al. (2004). High-oleate peanut mutants result from a MITE insertion into the FAD2 gene. *Theor Appl Genet* **108**:1492-1502.
- [0187] Pidkowich, M.S. et al. (2007). Modulating seed beta-ketoacyl-acyl carrier protein synthase II level converts the composition of a temperate seed oil to that of a palm-like tropical oil. *Proc Natl Acad Sci USA* **104**:4742-4747.
- [0188] Qu, J. et al. (2007). Artificial microRNA-mediated virus resistance in plants. *J Virol* **81**:6690-6699.
- [0189] Ruiz, M.T. et al. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant Cell* **10**:937-946.
- [0190] Shanklin, J. and Somerville, C. (1991). Stearoyl-acyl-carrier-protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. *Proc Natl Acad Sci USA* **88**:2510-2514.
- [0191] Shi, B.J. et al. (1997). Plasmid vector for cloning infectious cDNAs from plant RNA viruses: high infectivity of cDNA clones of tomato aspermy cucumovirus. *J Gen Virol* **78** (Pt 5):1181-1185.
- [0192] Sujatha, M. et al. (2008). Role of biotechnological interventions in the improvement of castor (*Ricinus communis* L.) and *Jatropha curcas* L. *Biotechnol Adv* **26**:424-435.
- [0193] Sun, Y. et al. (2002). ASYMMETRIC LEAVES1, an Arabidopsis gene that is involved in the control of cell differentiation in leaves. *Planta* **214**:694-702.
- [0194] Theodoris, G. et al. (2003). Conservation and molecular dissection of ROUGH SHEATH2 and ASYMMETRIC LEAVES1 function in leaf development. *Proc Natl Acad Sci USA* **100**:6837-6842.
- [0195] Thomas, R. et al. (2008). Therapeutic biology of *Jatropha curcas*: a mini review. *Curr Pharm Biotechnol* **9**:315-324.
- [0196] Valentine, T. et al. (2004). Efficient virus-induced gene silencing in roots using a modified tobacco rattle virus vector. *Plant Physiol* **136**:3999-4009.

WHAT IS CLAIMED IS:

1. A method of virus-induced gene silencing (VIGS) in *Jatropha* comprising:
 - (a) inserting a nucleic acid comprising a sequence of a first desired gene to be silenced into a vector comprising a tobacco rattle virus (TRV) RNA2 sequence to produce a modified TRV RNA2 vector;
 - (b) preparing a mixed culture of *Agrobacterium* comprising *Agrobacterium* containing a vector comprising a TRV RNA1 sequence and *Agrobacterium* containing the modified TRV RNA2 vector;
 - (c) introducing the mixed culture of *Agrobacterium* into plant tissue of *Jatropha* by vacuum infiltration to produce infected plants; and
 - (d) growing the infected plants for a sufficient time to induce gene silencing of the desired gene.
2. A method of analyzing gene function in *Jatropha* comprising
 - (a) inserting a nucleic acid comprising a sequence of a first candidate gene the function of which is to be analyzed into a vector comprising a tobacco rattle virus (TRV) RNA2 sequence to produce a modified TRV RNA2 vector;
 - (b) preparing a mixed culture of *Agrobacterium* comprising *Agrobacterium* containing a vector comprising a TRV RNA1 sequence and *Agrobacterium* containing the modified TRV RNA2 vector;
 - (c) introducing the mixed culture of *Agrobacterium* into plant tissue of *Jatropha* by vacuum infiltration to produce infected plants;
 - (d) growing the infected plants for a sufficient time to induce gene silencing of the candidate gene; and.
 - (e) analyzing the phenotypic effect of the silenced candidate gene on the infected plant.
3. The method of claim 1 or 2, wherein the vector comprising TRV RNA2 and the vector comprising TRV RNA1 are synthetic plant vectors.
4. The method of any one of claims 1 to 3, wherein the sequence of the first desired gene is the sequence of a sense strand of the gene.

5. The method of any one of claims 1 to 3, wherein the sequence of the first desired gene is the sequence of an antisense strand of the gene.
6. The method of any one of claims 1 to 5, wherein the nucleic acid further comprises a sequence of a second desired gene to be silenced.
7. The method of claim 6, wherein the second desired gene is virus resistance gene.
8. The method of claim 7, wherein the virus resistance gene is selected from the group consisting of *RNase III Dicer-like 4 (DCL4)* gene, *RNase III Dicer-like 2 (DCL2)* gene, *RNase III Dicer-like 3 (DCL3)* gene, *ARGONAUTE1 (AGO1)*, *ARGONAUTE71 (AGO7)*, *RNA-dependent RNA polymerase 1 (RDR1)*, *RNA-dependent RNA polymerase 6 (RDR6)*, *Suppressor of gene silencing 1 (SGS1)*, *Suppressor of gene silencing 3 (SGS3)*, and *Silencing defective 3 (SDE3)*.
9. The method of any one of claims 1 to 3, wherein the nucleic acid comprises sequences of multiple desired genes to be silenced.
10. The method of any one of claims 1 to 3, wherein the desired gene is a candidate gene in fatty acid biosynthesis.
11. The method of claim 10, wherein the desired gene is selected from the group consisting of a *β -ketoacyl-acyl carrier protein synthase II (KASII)* gene, an *acyl-acyl carrier protein thioesterase B (FATB)* gene, a *stearoyl-acyl carrier protein desaturase (SAD)* gene, and a *fatty acid desaturase (FAD)* gene.
12. The method of any one of claims 1 to 3, wherein the desired gene is a candidate transcription factor gene.
13. The method of any one of claims 1 to 3, wherein the desired gene is a candidate gene in smRNA biosynthesis.

14. The method of any one of claims 1 to 3, wherein the desired gene is a candidate gene in biosynthesis of toxic agents.