

A METHOD TO ENHANCE PLANT VIRUS RESISTANCE

[0001] SEQUENCE SUBMISSION

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577233PCTSequenceListing.txt, was created on 3 January 2014 and is 55 kb in size. The information in the electronic format of the Sequence Listing is part of the present application and is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0003] The present invention relates to the field of modifying *Jatropha* disease resistance. More specifically, the present invention relates to a method for enhancing *Jatropha curcas* virus resistance by transgenic expression of a hairpin construct, which comprises sequences homologous to two or more key geminivirus DNA-A genes encoding replication associated protein (*Rep*; *AC1*), transcriptional activator protein (*TrAP*; *AC2*), replication enhancer protein (*REn*; *AC3*), gene silencing suppressor (*AC4*) and coat protein (*CP*, *AVI*). The plants having virus resistance can be used for *Jatropha* breeding.

[0004] 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.

[0005] Due to the rapid depletion of world crude oil reserves, increasing prices and global warming concerns, the demand for biofuels have soared up greatly, along with government subsidies and mandates for sustainable energy. The increasing demand for biofuels, however, is an additional pressure on food production by increasing the competition between biofuel crops and food crops for arable land.

[0006] *Jatropha curcas*, a small woody plant belonging to the *Euphorbiaceae* family, is a non-food crop mainly grown in the tropical and subtropical regions. This plant possesses several properties rendering it suitable for biodiesel production; such as its rapid growth, ease of propagation, short gestation period, low seed cost, high oil content, wide adaptability, drought tolerance and the ability to thrive on degraded soils. The latter characteristics are important, as they would allow the use of marginal or non-arable wasteland for the development of large-scale *J. curcas* plantations.

[0007] However, the productivity of *Jatropha* in the field is limited by the occurrence of *Jatropha curcas* mosaic disease (JcMD) (Raj et al., 2008; Aswatha Narayana et al., 2007; Gao et al., 2010). The disease incidence is particularly significant in the Indian subcontinent, about 25%

in Northern India (Raj et al., 2008) and up to 47% in Southern India (Aswatha Narayana et al., 2007).

[0008] We reported the first full-length genome sequence of one geminivirus, a strain of India Mosaic virus- ICMV-Dha, as the causative pathogen of JcMD found in Southern India (Gao et al., 2010). Following our report, 3 other related geminiviruses were isolated from *Jatropha* plants in African and Asia (Shehi et al., 2012; Ramkat et al., 2011; Kashima et al., 2013). Geminiviruses, which are single-stranded DNA viruses infecting a range of economically important crop species (e.g., cassava, maize, cotton and tomato) in tropical and subtropical regions, have become a major threat to world agriculture in the past decade (Brown et al., 2012). Based on genome organization, insect vector and host range, the family *Geminiviridae* can be classified into four genera: *Begomovirus*, *Mastrevirus*, *Curtovirus* and *Topocuvirus*. So far, all the 5 *Jatropha* viral pathogens belong to one genera: *Begomovirus*. These viruses each contain two genomic components termed DNA A and DNA B (~ 2.7 - 3.0 kb) and they are exclusively transmitted via the whitefly, *Bemisia tabaci*. The virus DNA-A positive strand encodes the coat protein (CP/AV1) involved in the encapsidation of viral DNA, virus movement and viral transmission by whiteflies (*Bemisia tabaci*). Among other encoded proteins, the replication associated - protein (Rep/AC1) is absolutely required for the replication of both genomic components. The transcriptional activator protein (TrAP/AC2) is needed for transcriptional activation of viral gene transcription and plant host gene expression. The replication enhancer protein (REN; AC3) greatly enhances viral DNA accumulation by interacting with Rep. Another viral protein AC4 acts as a gene silencing suppressor to compromise the host defense system. All these five genes are essential for the virus life cycle and pathogenesis (Brown et al., 2012).

[0009] Because of the capacity of geminiviruses to evolve rapidly by mutation, recombination and pseudorecombination, the development of plants with durable virus resistance is still a major challenge. One strategy involves genetic crossing of resistant and susceptible *Jatropha* germplasms. This strategy has the advantage that segregation patterns can be clearly observed between resistant and susceptible lines (Ahuja et al., 2007). However, germplasm-mediated resistance via cross breeding is time-consuming and requires a large number of progeny plants (i.e. large scale field tests) to ascertain segregation patterns in future generations (Ahuja et al., 2007). Therefore, transgenic technology has been considered as a method of choice for improving *Jatropha* traits. Recently, we have established a transformation platform which facilitates transfer of foreign genes into *Jatropha* genome and we used this method to produce virus-resistant transgenic *Jatropha* (Qu et al., 2012).

[0010] A major strategy to produce transgenic plants with virus resistance is based on the concept of pathogen-derived resistance (PDR) in which the transgene is derived from viral sequences. The mechanism of PDR includes protein-mediated resistance and RNA interference (RNAi). Both mechanisms have been shown to confer geminivirus resistance in transgenic tomato, bean, and cassava etc (Vanderschuren et al., 2009; Bonfim et al., 2007; Abhary et al., 2006; Zhang and Gruissem, 2003; Vanderschuren et al., 2007b). Here, we report the production of several JcMD-resistant *Jatropha* lines by expressing a hairpin dsRNA that targets 5 key geminivirus DNA-A genes. Some of the transgenic lines displayed broad resistance to other geminiviruses, with 94% nucleotide identity to the transgene sequences.

[0011] It is desired to modify disease resistance in *Jatropha* in order to improve the efficiency and use of *Jatropha* for producing biofuels.

SUMMARY OF THE INVENTION

[0012] The present invention relates to the field of modifying *Jatropha* disease resistance. More specifically, the present invention relates to a method for enhancing *Jatropha curcas* virus resistance by transgenic expression of a hairpin construct, which comprises sequences homologous to two or more key geminivirus DNA-A genes encoding replication initiator protein (*Rep*; *AC1*), transcriptional activator protein (*TrAP*; *AC2*), replication enhancer protein (*REN*; *AC3*), gene silencing suppressor (*AC4*) and coat protein (*CP*, *AV1*). The plants having virus resistance can be used for *Jatropha* breeding.

[0013] The present invention provides methods and compositions for obtaining *Jatropha* plants resistant to geminiviruses. In some embodiments, the geminiviruses are members of the geminivirus genera *Begomovirus*. In other embodiments, the *Begomovirus* causes *Jatropha* mosaic disease, such as *J. curcas* mosaic disease. In certain embodiments, the resistance provided to the plant virus species is provided by expression of a nucleic acid construct that produces dsRNA. In some embodiments the resistance provided to the plant virus species is provided by expression of a dsRNA fusion construct. In some embodiments of the invention, the dsRNA interferes with expression of one or more viral genes described herein. In one embodiment RNA-mediated gene suppression can be conferred by the expression of an inverted-repeat transgene cassette that generates a population of small interfering RNAs (siRNAs) derived from the dsRNA region of a transgene transcript.

[0014] Thus in one aspect, the present invention provides a nucleic acid construct which comprises one or more nucleic acid fragments. In one embodiment, the nucleic acid construct

comprises one nucleic acid fragment. In another embodiment, the nucleic acid construct comprises two nucleic acid fragments. In a further embodiment, the nucleic acid construct comprises three nucleic acid fragments. In some embodiments, two or more nucleic acid fragments are linked together in the nucleic acid construct. In other embodiments, each nucleic acid fragment comprises sequences homologous to a geminivirus DNA-A region (target regions) encoding one, two or more genes (target genes) for silencing the target genes. In some embodiments, the one, two or more key geminivirus DNA-A genes encode the replication initiator protein (*Rep*; *AC1*), the transcriptional activator protein (*TrAP*; *AC2*), the replication enhancer protein (*REN*; *AC3*), the gene silencing suppressor (*AC4*) and the coat protein (*CP*; *AV1*).

[0015] In one embodiment, a first nucleic acid fragment comprises sequences homologous to genes encoding the AV1 protein and the AC5 protein (uncharacterized protein; Raghaven et al., 2004). In an additional embodiment, a second nucleic acid fragment comprises sequences homologous to genes encoding the AC2 protein and the AC3 protein. In a further embodiment, a third nucleic acid fragment comprises sequences homologous to genes encoding the AC1 protein and the AC4 protein. In another embodiment, a fourth nucleic acid fragment comprises two or more of these fragments linked together.

[0016] In some embodiments, nucleic acids of the present invention include (a) a nucleic acid fragment that is substantially homologous to nucleotides 1 to 514 of a gene encoding the AV1 protein and that is substantially homologous to nucleotides 3 to 483 of a gene encoding the AC5 protein; (b) a nucleic acid fragment that is substantially homologous to nucleotides 4 to 405 of a gene encoding AC2 protein and that is substantially homologous to nucleotides 1 to 376 of a gene encoding AC3 protein; and (c) a nucleic acid fragment that is substantially homologous to nucleotides 324 to 932 of a gene encoding AC1 protein and that is substantially homologous to nucleotides 167 to 300 of a gene encoding AC4 protein. In some embodiments, nucleic acids of the present invention include (d) a nucleic acid fragment that is substantially homologous to nucleotides 227 to 476 of a gene encoding the AV1 protein and that is substantially homologous to nucleotides 41 to 290 of a gene encoding the AC5 protein; (e) a nucleic acid fragment that is substantially homologous to nucleotides 156 to 405 of a gene encoding AC2 protein and that is substantially homologous to nucleotides 8 to 257 of a gene encoding AC3 protein; and (f) a nucleic acid fragment that is substantially homologous to nucleotides 324 to 932 of a gene encoding AC1 protein and that is substantially homologous to nucleotides 167 to 300 of a gene encoding AC4 protein. In other embodiments, a linked nucleic acid fragment comprises two or

more of nucleic acid fragments (d), (e) and (f) linked together, preferably all three fragments linked together. In some embodiments, the first nucleic acid fragment comprises 563 bp, the second nucleic acid fragment comprises 521 bp and the third nucleic acid fragment comprises 609 bp. In other embodiments, the first nucleic acid fragment comprises 250 bp, the second nucleic acid fragment comprises 250 bp and the third nucleic acid fragment comprises 609 bp. In some embodiments, the linked nucleic acid fragment comprises 1109 bp comprising 250 bp of the first nucleic acid fragment, 250 bp of the second nucleic acid fragment and 609 bp of the third nucleic acid fragment.

[0017] In some embodiments, the one or more nucleic acid fragments are present in the nucleic acid construct such that a hairpin construct is produced upon expression of the one or more nucleic acid fragments. In one embodiment, the nucleic acid construct comprises one or more nucleic acid fragments in a sense orientation with respect to the promoter, a spacer sequence that can form a loop and the one or more nucleic acid fragments in an antisense orientation with respect to the promoter. In another embodiment, the nucleic acid construct comprises one or more nucleic acid fragments in an antisense orientation with respect to the promoter, a spacer sequence that can form a loop and the one or more nucleic acid fragments in a sense orientation with respect to the promoter. In an additional embodiment, the nucleic acid construct comprises one or more nucleic acid fragments in a sense orientation with respect to the promoter, an intron and the one or more nucleic acid fragments in an antisense orientation with respect to the promoter. In a further embodiment, the nucleic acid construct comprises one or more nucleic acid fragments in an antisense orientation with respect to the promoter, an intron and the one or more nucleic acid fragments in a sense orientation with respect to the promoter. In some embodiments, the intron is removed from the nucleic acid construct during transcription to produce a loop-less hairpin (Smith et al., 2000).

[0018] In one embodiment, a hairpin construct, which is sometimes referred to herein as hp101, is produced that contains the first nucleic acid fragment. In another embodiment, a hairpin construct, which is sometimes referred to herein as hp102, is produced that contains the second nucleic acid fragment. In a further embodiment, a hairpin construct, which is sometimes referred to herein as hp103, is produced that contains the third nucleic acid fragment. In an additional embodiment, a hairpin construct, which is sometimes referred to herein as hp300, is produced that contains the first, second and third nucleic acid fragments linked together. In some embodiments, the sizes of hp101, hp102 and hp103 are 1297 bp, 1213 bp and 1389 bp, respectively. In other embodiments, the size of hp300 is 2389 bp.

[0019] In some embodiments, the nucleic acid construct further comprises a plant operable promoter operably linked to the one or more nucleic acid fragments. In one embodiment, a plant operable promoter is linked to each nucleic acid fragment. In another embodiment, a plant operable promoter is linked to two or more nucleic acid fragments that are linked together. In some embodiments, the plant operable promoter is heterologous to the one or more nucleic acid fragments. In some embodiments, the nucleic acid construct further comprises a plant operable terminator. In one embodiment, a plant operable terminator is linked to each nucleic acid fragment. In another embodiment, a plant operable terminator is linked to two or more nucleic acid fragments that are linked together. In some embodiments, the plant operable terminator is heterologous to the one or more nucleic acid fragments.

[0020] In other embodiments, the nucleic acid construct further comprises a selectable marker. In some embodiments, the selectable marker is part of a recombination marker free system. In one embodiment, the recombination marker free system is a Cre-lox recombination marker free system. In some embodiments, the recombination marker free system is positioned between the plant operable promoter and the one or more nucleic acid fragments.

[0021] In a second aspect, the present invention provides a transgenic plant comprising one or more nucleic acid constructs described herein in which the transgenic plant is resistant to geminiviruses described herein. In one embodiment, the transgenic plant is a *Jatropha* plant. In a further embodiment, the transgenic plant is a *Jatropha curcas* plant. In some embodiments, the present invention provides any generation of *Jatropha* plant resistant to the geminiviruses that infect *Jatropha* plants, including *Jatropha curcas* plants, including those described herein. In other embodiments, the present invention provides transgenic *Jatropha* seed of any resistant generation of the *Jatropha* plants. In some embodiments, the present invention provides a method to prepare geminivirus resistant transgenic *Jatropha* plants and seeds described herein.

[0022] In a third aspect, the present invention provides a method for conferring resistance in a *Jatropha* plant to geminiviruses described herein, the method comprising expressing in the plant at least one nucleic acid construct that expresses dsRNA targeting geminivirus genes described herein. In some embodiments, one, two or three nucleic acid constructs that express dsRNA targeting the described geminivirus genes are used to confer resistance. In certain embodiments, the resistance comprises resistance against a *Begomovirus*.

BRIEF DESCRIPTION OF THE FIGURES

[0023] FIGS. 1A-1E show schematic diagrams of transformation vectors and experimental procedures. FIG. 1A: The selection of three fragments targeting different viral genome region. Each fragment targets two or three genes. Fragment 1 targets AV1 and AC5. Fragment 2 targets AC2 and AC3. Fragment 3 targets AC1 and AC4. FIG. 1B: A single target hairpin RNAi structure (hp101, fragment 1; hp102, fragment 2; hp103, fragment 3) to target one of three chosen region on the virus. FIG. 1C: Three fragments were ligated and constructed into a hairpin RNAi structure (hp300). FIG. 1D: Schematic diagram of marker-free vector based hairpin RNAi structure. P1 to P4 denote primers used for PCR analysis to detect recombination event or hygromycin antibiotic resistant selection marker free event shown in Fig. 3. FIG. 1E: A flowchart of experimental procedure for screening virus resistant event either by *Agrobacterium*-mediated infection method or transmission vector-whitefly inoculation method.

[0024] FIGS. 2A-2C show virus resistant lines generated by single target construct. FIG. 2A: Wild type (WT) infected plants. FIG. 2B: Virus resistant lines (mixed primary transformed T0 *J. curcas* plants with expression of one of hairpin structure: hp101, hp102 or hp103). FIG. 2C: Heritable virus resistance in T1 transgenic *J. curcas* plants expressing hairpin structure hp103. Bar: 10 cm.

[0025] FIGS. 3A and 3B show PCR analysis of genomic DNA prepared from wild type (WT) and transgenic plants. Upper bar; the expected PCR product using P1 and P2 primers indicates the occurrence of marker-excision-event. CK- means WT control, H₂O control means no plant genomic DNA was added. Lower bar; the PCR product using P3 and P4 primers indicate the presence of hygromycin resistant gene. FIG. 3A: WT and Lines 1-54. FIG. 3B: WT and resistant lines after first virus challenge. Notes: All of the lines area chimeric, since the hygromycin gene product is also positive. The PCR product (P1-P2) of lines 13, 16, 19, 25, 31, 38, 50 and 52 were gel purified and cloned into a T-vector. The sequencing results confirmed that the positive bands are real marker-free products. The transgenic plants with positive P1-P2 product were used for the next virus challenge.

[0026] FIG. 4 shows quantitative PCR analysis of virus titers in transgenic plants and WT plants after vacuum infiltration of IcmV-Dha infection clones (first virus challenge). Uninfected plants were used as control and the average control value was set as 1. U, uninfected WT plants; I, infected WT plants. The virus titer correlated well with the virus symptoms.

[0027] FIGS. 5A-5C show virus symptoms after the first challenge. FIG. 5A: WT and susceptible lines with virus symptoms. FIG. 5B: Putative resistant transgenic lines. FIG. 5C:

Comparison of susceptible and resistant lines. Left, infected WT plants with virus symptoms; middle and right, two T0 transgenic plants from same transgenic event #82 with virus resistance. Bar: 10 cm.

[0028] FIGS. 6A and 6B show Southern blot analysis to determine the transgene copy number. FIG. 6A: Schematic diagram of marker-free vector based hairpin RNAi structure. FIG. 6B: Southern blot using a probe carrying the *CaMV 35S* double enhancers. Transgene or WT control plant DNA was digested with *EcoR* V. The Southern blot using probe prepared from 35S promoter detects the copy number of transgene irrespective of the marker-free status.

[0029] FIGS. 7A-7C show virus symptoms after the second challenge by a natural emerged geminivirus infection in the field. FIG. 7A: A control WT (CK) plant with virus symptoms. Bar: 1 cm. FIG. 7B: Resistant transgenic lines (#82) with similar whitefly density. Bar: 1 cm. FIG. 7C: Comparison of CK and resistant (R) lines. Bar: 10 cm. Note: the pathogen was isolated and identified as a new geminivirus which shared 94% nucleotide similarity with the infection clone used for the first virus challenge. Each plant and a similar whitefly density.

[0030] FIG. 8 shows quantitative PCR analysis of virus titers in control WT and transgenic plants in the field test after a naturally emerged virus challenge. Un-infected (U1-U3) and infected (I1-I9) plants were used as controls and the average control value of uninfected WT plants was set as 1.

[0031] FIGS. 9A-9D show heritable virus resistance trait in T1 transgenic plants. FIG. 9A: Virus symptoms in WT control plants and progeny plants of transgenic line #82-1 after vacuum infiltration of ICMV infection clones. Bar: 1 cm. FIG. 9B: Quantitative PCR analysis of virus titers after virus challenge. Un-infected (U1-U4) plants were used as control and the average control value was set as 1. FIG. 9C: PCR analysis of genomic DNA prepared from WT and transgenic plants. Upper panel: PCR product using P1 and P2 primers. Lower panel: PCR product using P3-1 and P4-1 primers. FIG. 9D: Southern blot analysis of transgenic T1 progeny plants derived from selfing of T0 #81. Note that #82-3 was a null-segregant. M, DNA size markers in bp.

[0032] FIG. 10 shows a phylogenetic relationship of viral isolates recovered from infected *Jatropha* plants. Four *Jatropha*-isolates formed one clade in a diagram showing the phylogenetic relationship among various gemini viruses. Transgenic lines generated from this report are supposed to be resistant to geminiviruses from this clade. ACMV, *African cassava mosaic virus*; EACMCV, *East African cassava mosaic Cameroon virus*; EACMV, *East African cassava mosaic virus*; SACMV, *South African cassava mosaic virus*; ICMV, *Indian cassava mosaic*

virus; SLCMV, *Sri Lankan cassava mosaic virus*; GPMLCuV, *Gossypium punctatum mild leaf curl virus*. The database accession number of each sequence is given.

DETAILED DESCRIPTION

[0033] The present invention relates to the field of modifying *Jatropha* disease resistance. More specifically, the present invention relates to a method for enhancing *Jatropha curcas* virus resistance by transgenic expression of a hairpin construct, which comprises sequences homologous to two or more key geminivirus DNA-A genes encoding replication associated protein (*Rep*; *AC1*), transcriptional activator protein (*TrAP*; *AC2*), replication enhancer protein (*REn*; *AC3*), gene silencing suppressor (*AC4*) and coat protein (*CP*, *AVI*). The plants having virus resistance can be used for *Jatropha* breeding.

[0034] In accordance with the present invention, several JcMD resistant *Jatropha* lines based on an elite germplasm with high yield have been developed. The strategy involved the expression of a construct, which comprises of either single target or combined targets of 5 key geminivirus DNA-A genes encoding replication associated protein (*Rep*; *AC1*), transcriptional activator protein (*TrAP*; *AC2*), replication enhancer protein (*REn*; *AC3*), gene silencing suppressor (*AC4*) and coat protein (*CP*). The data presented herein supports a broad viral resistance for some genetically modified lines, as shown by testing these lines to natural infection by another geminivirus, which shows 94% nucleotide identities to the transgene sequence.

[0035] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention belongs.

[0036] The term "about" or "approximately" means within a statistically meaningful range of a value. Such a range can be within an order of magnitude, preferably within 50%, more preferably within 20%, more preferably still within 10%, and even more preferably within 5% of a given value or range. The allowable variation encompassed by the term "about" or "approximately" depends on the particular system under study, and can be readily appreciated by one of ordinary skill in the art.

[0037] A "dsRNA" or "RNAi molecule," as used herein in the context of RNAi, refers to a compound, which is capable of down-regulating or reducing the expression of a gene or the activity of the product of such gene to an extent sufficient to achieve a desired biological or physiological effect. A dsRNA may be a hairpin construct that comprises a loop or spacer

sequence which joins the two strands of the double stranded portion of the hairpin construct. The loop or spacer may be derived from cleavage of an intron during transcription.

[0038] The term "down regulated," as it refers to genes inhibited by the subject RNAi method, refers to a diminishment in the level of expression of a gene(s) in the presence of one or more RNAi construct(s) when compared to the level in the absence of such RNAi construct(s). The term "down regulated" is used herein to indicate that the target gene expression is lowered by 1-100%. For example, the expression may be reduced by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%. To produce a geminivirus resistant transgenic plant of the present invention, the target gene expression is typically lowered by at least 90%.

[0039] The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of an RNA results from transcription of a polynucleotide. Similarly as will be clear from the context, expression of a protein results from transcription and translation of a polynucleotide.

[0040] As used herein, "gene" refers to a nucleic acid sequence that encompasses a 5' promoter region associated with the expression of the gene product, any intron and exon regions and 3' or 5' untranslated regions associated with the expression of the gene product.

[0041] The term "gene silencing" refers to the suppression of gene expression, e.g., transgene, heterologous gene and/or endogenous gene expression. Gene silencing may be mediated through processes that affect transcription and/or through processes that affect post-transcriptional mechanisms. Gene silencing may be allele-specific wherein specific silencing of one allele of a gene occurs.

[0042] The term "heterologous" or "exogenous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous or exogenous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

[0043] As used herein, the term “nucleic acid fragment” refers to a polynucleotide that comprises a portion of a larger polynucleotide. Typically, a nucleic acid fragment comprises about 100 to about 1000 nucleotides.

[0044] “Operable linkage” or “operably linked” as used herein is understood as meaning, for example, the sequential arrangement of a promoter and the nucleic acid to be expressed and, if appropriate, further regulatory elements such as, for example, a terminator, in such a way that each of the regulatory elements can fulfill its function in the recombinant expression of the nucleic acid to make dsRNA. This does not necessarily require direct linkage in the chemical sense. Genetic control sequences such as, for example, enhancer sequences, can also exert their function on the target sequence from positions which are somewhat distant, or indeed from other DNA molecules (cis or trans localization). Preferred arrangements are those in which the nucleic acid sequence to be expressed recombinantly is positioned downstream of the sequence which acts as promoter, so that the two sequences are covalently bonded with one another.

[0045] As used herein, “phenotype” refers to the detectable characteristics of a cell or organism, which characteristics are the manifestation of gene expression.

[0046] The terms “polynucleotide,” “nucleic acid” and “nucleic acid molecule” are used interchangeably herein to refer to a polymer of nucleotides which may be a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, including deoxyribonucleic acid, ribonucleic acid, and derivatives thereof. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role. Unless otherwise indicated, nucleic acids or polynucleotide are written left to right in 5' to 3' orientation. Nucleotides are referred to by their commonly accepted single-letter codes. Numeric ranges are inclusive of the numbers defining the range. The “nucleic acid” may also optionally contain non-naturally occurring or altered nucleotide bases that permit correct read through by a polymerase and do not reduce expression of the nucleic acid.

[0047] The terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Amino acids may be referred to by their commonly known three-letter or one-letter symbols. Amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range.

[0048] As used herein, the term "substantially homologous" or "substantial homology," with reference to a nucleic acid sequence, includes a nucleotide sequence that hybridizes under stringent conditions to a referenced SEQ ID NO., or a portion or complement thereof, are those that allow an antiparallel alignment to take place between the two sequences, and the two sequences are then able, under stringent conditions, to form hydrogen bonds with corresponding bases on the opposite strand to form a duplex molecule that is sufficiently stable under conditions of appropriate stringency, including high stringency, to be detectable using methods well known in the art. Substantially homologous sequences may have from about 70% to about 80% sequence identity, or more preferably from about 80% to about 85% sequence identity, or most preferable from about 90% to about 95% sequence identity, to about 99% sequence identity, to the referent nucleotide sequences as set forth the sequence listing, or the complements thereof.

[0049] As used herein, the term "sequence identity," "sequence similarity" or "homology" is used to describe sequence relationships between two or more nucleotide sequences. The percentage of "sequence identity" between two sequences is determined by comparing two optimally aligned sequences over a comparison window such as the full length of a referenced SEQ ID NO., wherein the portion of the sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. A sequence that is identical at every position in comparison to a reference sequence is said to be identical to the reference sequence and vice-versa. A first nucleotide sequence when observed in the 5' to 3' direction is said to be a "complement" of, or complementary to, a second or reference nucleotide sequence observed in the 3' to 5' direction if the first nucleotide sequence exhibits complete complementarity with the second or reference sequence. As used herein, nucleic acid sequence molecules are said to exhibit "complete complementarity" when every nucleotide of one of the sequences read 5' to 3' is complementary to every nucleotide of the other sequence when read 3' to 5'. A nucleotide sequence that is complementary to a reference nucleotide sequence will exhibit a sequence identical to the reverse complement sequence of the reference nucleotide

sequence. These terms and descriptions are well defined in the art and are easily understood by those of ordinary skill in the art.

[0050] As used herein, a "comparison window" or "window of comparison" refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150, in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Those skilled in the art should refer to the detailed methods used for sequence alignment, such as in the Wisconsin Genetics Software Package Release 7.0 (Genetics Computer Group, 575 Science Drive Madison, Wis., USA).

[0051] As used herein, a "target gene" refers to a gene whose expression is to be down regulated or silenced. "A target region" refers to a genomic region that includes two or more target genes.

[0052] The present invention provides methods and compositions for obtaining *Jatropha* plants resistant to geminiviruses. In some embodiments, the geminiviruses are members of the geminivirus genera *Begomovirus*. In other embodiments, the *Begomovirus* causes *Jatropha* mosaic disease, such as *J. curcas* mosaic disease. In certain embodiments, the resistance provided to the plant virus species is provided by expression of a nucleic acid construct that produces dsRNA. In some embodiments the resistance provided to the plant virus species is provided by expression of a dsRNA fusion construct. In some embodiments of the invention, the dsRNA interferes with expression of one or more viral genes described herein. In one embodiment RNA-mediated gene suppression can be conferred by the expression of an inverted-repeat transgene cassette that generates a population of small interfering RNAs (siRNAs) derived from the dsRNA region of a transgene transcript.

[0053] Thus in one aspect, the present invention provides a nucleic acid construct which comprises one or more nucleic acid fragments. Each nucleic acid fragment is capable of being transcribed in a cell of a *Jatropha* plant. In one embodiment, the nucleic acid construct comprises one nucleic acid fragment. In another embodiment, the nucleic acid construct comprises two nucleic acid fragments. In a further embodiment, the nucleic acid construct comprises three nucleic acid fragments. In some embodiments, two or more nucleic acid fragments are linked together in the nucleic acid construct. In some embodiments, each nucleic acid fragment comprises sequences homologous to a geminivirus DNA-A region (target regions)

encoding two or more genes (target genes) for silencing the target genes. In some embodiments, the two or more key geminivirus DNA-A genes encode the replication initiator protein (*Rep*; *AC1*), the transcriptional activator protein (*TrAP*; *AC2*), the replication enhancer protein (*REn*; *AC3*), the gene silencing suppressor (*AC4*) and the coat protein (*CP*, *AV1*).

[0054] In one embodiment, a first nucleic acid fragment comprises sequences homologous to genes encoding the AV1 protein and the AC5 protein (uncharacterized protein; Raghaven et al., 2004). In an additional embodiment, a second nucleic acid fragment comprises sequences homologous to genes encoding the AC2 protein and the AC3 protein. In a further embodiment, a third nucleic acid fragment comprises sequences homologous to genes encoding the AC1 protein and the AC4 protein. In another embodiment, a fourth nucleic acid fragment comprises two or more of these fragments linked together.

[0055] In some embodiments, nucleic acids of the present invention include (a) a nucleic acid fragment that is substantially homologous to nucleotides 1 to 514 of a gene encoding the AV1 protein and that is substantially homologous to nucleotides 3 to 483 of a gene encoding the AC5 protein; (b) a nucleic acid fragment that is substantially homologous to nucleotides 4 to 405 of a gene encoding AC2 protein and that is substantially homologous to nucleotides 1 to 376 of a gene encoding AC3 protein; and (c) a nucleic acid fragment that is substantially homologous to nucleotides 324 to 932 of a gene encoding AC1 protein and that is substantially homologous to nucleotides 167 to 300 of a gene encoding AC4 protein. In some embodiments, nucleic acids of the present invention include (d) a nucleic acid fragment that is substantially homologous to nucleotides 227 to 476 of a gene encoding the AV1 protein and that is substantially homologous to nucleotides 41 to 290 of a gene encoding the AC5 protein; (e) a nucleic acid fragment that is substantially homologous to nucleotides 156 to 405 of a gene encoding AC2 protein and that is substantially homologous to nucleotides 8 to 257 of a gene encoding AC3 protein; and (f) a nucleic acid fragment that is substantially homologous to nucleotides 324 to 932 of a gene encoding AC1 protein and that is substantially homologous to nucleotides 167 to 300 of a gene encoding AC4 protein. In other embodiments, a linked nucleic acid fragment comprises two or more of nucleic acid fragments (d), (e) and (f) linked together, preferably all three fragments linked together. In some embodiments, the first nucleic acid fragment comprises 563 bp, the second nucleic acid fragment comprises 521 bp and the third nucleic acid fragment comprises 609 bp. In some embodiments, the first nucleic acid fragment comprises 563 bp, the second nucleic acid fragment comprises 521 bp and the third nucleic acid fragment comprises 609 bp. In other embodiments, the first nucleic acid fragment comprises 250 bp, the second nucleic acid

fragment comprises 250 bp and the third nucleic acid fragment comprises 609 bp. In some embodiments, the linked nucleic acid fragment comprises 1109 bp comprising 250 bp of the first nucleic acid fragment, 250 bp of the second nucleic acid fragment and 609 bp of the third nucleic acid fragment.

[0056] In some embodiments, the one or more nucleic acid fragments are present in the nucleic acid construct such that a hairpin construct is produced upon expression of the one or more nucleic acid fragments. In one embodiment, the nucleic acid construct comprises one or more nucleic acid fragments in a sense orientation with respect to the promoter, a spacer sequence that can form a loop and the one or more nucleic acid fragments in an antisense orientation with respect to the promoter. In another embodiment, the nucleic acid construct comprises one or more nucleic acid fragments in an antisense orientation with respect to the promoter, a spacer sequence that can form a loop and the one or more nucleic acid fragments in a sense orientation with respect to the promoter. The spacer sequence may comprise, for example, a sequence of nucleotides of at least about 10-100 nucleotides in length, or alternatively at least about 100-200 nucleotides in length, at least 200-400 about nucleotides in length, or at least about 400-500 nucleotides in length. In an additional embodiment, the nucleic acid construct comprises one or more nucleic acid fragments in a sense orientation with respect to the promoter, an intron and the one or more nucleic acid fragments in an antisense orientation with respect to the promoter. In a further embodiment, the nucleic acid construct comprises one or more nucleic acid fragments in an antisense orientation with respect to the promoter, an intron and the one or more nucleic acid fragments in a sense orientation with respect to the promoter. In some embodiments, the intron is removed from the nucleic acid construct during transcription to produce a loop-less hairpin dsRNA (Smith et al., 2000).

[0057] In one embodiment, a hairpin construct, which is sometimes referred to herein as hp101, is produced that contains the first nucleic acid fragment. In another embodiment, a hairpin construct, which is sometimes referred to herein as hp102, is produced that contains the second nucleic acid fragment. In a further embodiment, a hairpin construct, which is sometimes referred to herein as hp103, is produced that contains the third nucleic acid fragment. In an additional embodiment, a hairpin construct, which is sometimes referred to herein as hp300, is produced that contains the first, second and third nucleic acid fragments linked together. In some embodiments, the sizes of hp101, hp102 and hp103 are 1297 bp, 1213 bp and 1389 bp, respectively. In other embodiments, the size of hp300 is 2389 bp. In further embodiments, the

nucleotide sequences of hp101, hp102, hp103 and hp300 are set forth in SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52 and SEQ ID NO:53, respectively.

[0058] RNAi molecules, particularly dsRNA molecules described herein, can be prepared by the skilled artisan using techniques well known in the art, including techniques for the selection and testing of RNAi molecules that are useful for down regulating geminivirus genes described herein. See, for example, Wesley et al. (2001), Kalantidis et al. (2002), Mysara et al. (2011), Yan et al. (2012) and Qu et al. (2012). It has typically been found that dsRNA of 200-700 bp are particularly suited for inducing RNAi in plants. It has also been found that hairpin RNAs containing an intron, for example, a construct comprising an RNA encoding sequence in a sense direction operably linked to an intron operably linked to an RNA encoding sequence in an antisense direction or *vice versa* which is capable of forming an intron-hairpin RNA (ihpRNA), is suitable for inducing RNAi in plants. See, for example, Wang et al. (2000), Fuentes et al. (2006), Bonfim et al. (2007) Vanderschuren et al. (2007a, 2007b), Zrachya et al. (2007). The ability of ihpRNA or other dsRNA to down regulate geminivirus genes is shown herein. For example, a nucleic acid construct can be prepared that includes a nucleic that is transcribed into an RNA that can anneal to itself, e.g., a double stranded RNA having a stem-loop structure.

[0059] The size of the nucleic acid fragment is selected so that it targets two or more genes of geminiviruses described herein and is capable of forming dsRNA that is cleaved to form small interfering (si) RNAs. In some embodiments, the dsRNA is a hairpin having a loop. In other embodiments, the dsRNA is a loop-less hairpin. In one embodiment, the length of the double stranded portion of the dsRNA comprises about 100 bp to about 1200 bp. In another embodiment, the length of the double stranded portion of the dsRNA comprises about 150 bp to about 1200 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 200 bp to about 1200 bp. In an additional embodiment, the length of the double stranded portion of the dsRNA comprises about 250 bp to about 1200 bp. In another embodiment, the length of the double stranded portion of the dsRNA comprises about 300 bp to about 1200 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 350 bp to about 1200 bp. In an additional embodiment, the length of the double stranded portion of the dsRNA comprises about 400 bp to about 1200 bp. In another embodiment, the length of the double stranded portion of the dsRNA comprises about 450 bp to about 1200 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 500 bp to about 1200 bp. In an additional embodiment, the length of the double stranded portion of the dsRNA comprises about 600 bp to about 1200 bp. In another

embodiment, the length of the double stranded portion of the dsRNA comprises about 650 bp to about 1200 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 700 bp to about 1200 bp. In an additional embodiment, the length of the double stranded portion of the dsRNA comprises about 750 bp to about 1200 bp. In another embodiment, the length of the double stranded portion of the dsRNA comprises about 800 bp to about 1200 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 850 bp to about 1200 bp. In an additional embodiment, the length of the double stranded portion of the dsRNA comprises about 900 bp to about 1200 bp. In another embodiment, the length of the double stranded portion of the dsRNA comprises about 950 bp to about 1200 bp. In an additional embodiment, the length of the double stranded portion of the dsRNA comprises about 1000 bp to about 1200 bp. In another embodiment, the length of the double stranded portion of the dsRNA comprises about 1050 bp to about 1200 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 1100 bp to about 1200 bp. In another embodiment, the length of the double stranded portion of the dsRNA comprises about 1150 bp to about 1200 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 200 bp to about 300 bp. In an additional embodiment, the length of the double stranded portion of the dsRNA comprises about 500 bp to about 550 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 550 bp to about 600 bp. In another embodiment, the length of the double stranded portion of the dsRNA comprises about 600 bp to about 650 bp. In one embodiment, the length of the double stranded portion of the dsRNA comprises about 250 bp. In another embodiment, the length of the double stranded portion of the dsRNA comprises about 521 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 563 bp. In a further embodiment, the length of the double stranded portion of the dsRNA comprises about 609 bp. In another embodiment, the length of the double stranded portion of the dsRNA is 1109 bp.

[0060] In one embodiment, a nucleic acid fragment comprises sequences homologous to genes encoding the AV1 protein and the AC5 protein. In other embodiments, this nucleic acid fragment comprises the nucleotide sequence set forth in SEQ ID NO:1. In an additional embodiment, a nucleic acid fragment comprises sequences homologous to genes encoding the AC2 protein and the AC3 protein. In some embodiments, this nucleic acid fragment comprises the nucleotide sequence set forth in SEQ ID NO:2. In a further embodiment, a nucleic acid fragment comprises sequences homologous to genes encoding the AC1 protein and the AC4

protein. In some embodiments, this nucleic acid fragment comprises the nucleotide sequence set forth in SEQ ID NO:3. In some embodiments, the nucleic acid comprises the fragments linked together in which the fragments comprise (a) sequences homologous to genes encoding the AV1 protein and the AC5 protein, (b) sequences homologous to genes encoding the AC2 protein and the AC3 protein and (c) sequences homologous to genes encoding the AC1 protein and the AC4 protein. In one embodiment, the linked fragments comprise the nucleotide sequence set forth in SEQ ID NO:4. In one embodiment, one strand of the dsRNA region should be at least 90% identical, preferably at least 95% identical, and more preferably at least 98% identical or 99% identical or 100% identical to the corresponding region of the geminivirus genes described herein. It is appreciated that the longer the double stranded region, the lower the degree of identity may be, provided that the siRNA that is produced by processing of the dsRNA is able to silence the target gene(s) and provide disease resistance to the *Jatropha* plant or cell.

[0061] Although specific nucleic constructs and nucleic acid fragments for use in the present invention are shown in the Examples, it is understood that other constructs and fragments can be prepared using the techniques described herein. Such other constructs include those with variations of the target region. In one embodiment, a variation of the target region is one in which the target region is shifted 5' of a disclosed or designed target region of a gene described herein. In another embodiment, a variation of the target region is one in which the target region is shifted 3' of a disclosed or designed target region of a gene described herein. In an additional embodiment, the variation of a target region can be sequence variations for more effective down regulation of genes described herein or homologs thereof.

[0062] In designing additional constructs, the following considerations are utilized for selecting and preparing the constructs. First, the target region should be an essential sequence for the virus' life cycle, such as replication and envelopment. Second, the virus genomes of different strains are aligned and conserved regions are chosen to make RNAi constructs. Third, it is more efficient if the dsRNA targets more than one gene. In the context of the present application, the nucleotide sequence for the DNA-A of ICMV-Dha is set forth in GenBank Accession No. GQ924760, which also identifies regions of the sequence encoding AV1, AC1, AC2, AC3 and AC4. The nucleotide sequence for the DNA-A of ICMV-SG is set forth in GenBank Accession No. JX518289, which also identifies regions of the sequence encoding AV1, AC1, AC2, AC3 and AC4. The nucleotide sequences for these two virus strains are set forth in SEQ ID NO:36 and SEQ ID NO:37, respectively. The nucleotide sequences encoding AV1, AC1, AC2, AC3, AC4 and AC5 from ICMV-Dha are set forth in SEQ ID NO:38, SEQ ID

NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46 and SEQ ID NO:48, respectively. The corresponding amino acid sequences for AV1, AC1, AC2, AC3, AC4 and AC5 from ICMV-Dha are set forth in SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47 and SEQ ID NO:49, respectively.

[0063] In some embodiments, nucleic acid fragments of the present invention include those which hybridize under stringent conditions to the sequences provided as SEQ ID NOs:1-4. As used herein, stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDodSO₄ at 50° C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C.; or (3) employ 50% formamide, 5xSSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5xDenhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS and 10% dextran sulfate at 42° C. in 0.2xSSC and 0.1% SDS. For stringency conditions, see also U.S. Patent Nos. 8,455,716 and 8,536,403.

[0064] Upon expression of the nucleic acid construct to RNA and contact with a plant virus achieves suppression of target viral genes or viral replication or symptomatology (i.e. expression of symptoms) as described herein. Methods to express a gene suppression molecule in plants are known (e.g., U.S. Patent Application Publication No. 2006/0200878; U.S. Patent Application Publication No. 2006/0174380; U.S. Patent Application Publication No. 2008/0066206; Niu et al., 2006), and may be used to express a nucleotide sequence of the present invention.

[0065] In some embodiments, the nucleic acid construct further comprises a plant operable promoter operably linked to the one or more nucleic acid fragments. In one embodiment, a plant operable promoter is linked to each nucleic acid fragment. In another embodiment, a plant operable promoter is linked to two or more nucleic acid fragments that are linked together. In one embodiment, a plant operable promoter is linked to three nucleic acid fragments that are linked together. In some embodiments, the plant operable promoter is heterologous to the one or more nucleic acid fragments.

[0066] 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; U.S. Patent No. 5,850,019); rice actin (McElroy et al., 1990); ubiquitin (Christensen and Quail, 1989; Christensen et al., 1992); pEMU (Last et al., 1991); MAS (Velten et al., 1984); ALS promoter (U.S. 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; 5,608,142 and 8,455,716. In some embodiments, the promoter is a duplicated CaMV 35S promoter.

[0067] Other promoters include inducible promoters, particularly from a pathogen-inducible promoter. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. Other promoters include those that are expressed locally at or near the site of pathogen infection. In further embodiments, the promoter may be a wound-inducible promoter. In other embodiments, chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. The promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. In addition, tissue-preferred promoters can be utilized to target enhanced expression of a polynucleotide of interest within a particular plant tissue. Each of these promoters are described in U.S. Pat. Nos. 6,506,962, 6,575,814, 6,972,349 and 7,301,069 and in U.S. Patent Application Publication Nos. 2007/0061917 and 2007/0143880.

[0068] In some embodiments, the nucleic acid construct further comprises a plant operable terminator. In one embodiment, a plant operable terminator is linked to each nucleic acid fragment. In another embodiment, a plant operable terminator is linked to two or more nucleic acid fragments that are linked together. In some embodiments, the plant operable terminator is heterologous to the one or more nucleic acid fragments.

[0069] In some embodiments, plant operable terminators include those from the nopaline synthase gene of *A. tumefaciens* (NOS), octopine synthase gene of *A. tumefaciens* (OCS), the terminator for the T7 transcript from the octopine synthase gene of *A. tumefaciens*, and the pea RUBISCO synthase E9 gene (E9 3') 3' non-translated transcription termination and polyadenylation sequence. These and other terminator and 3' end regulatory sequences are well known in the art. See, e.g., U.S. Patent Nos. 8,344,209, 8,373,022 and 8,569,583.

[0070] A nucleic acid construct that comprises a plant operable promoter or a plant operable promoter and a plant operable terminator may also be referred to herein as an expression

cassette. The expression cassette may include other transcriptional regulatory regions as are well known in the art.

[0071] In other embodiments, the nucleic acid construct or expression cassette further comprises a selectable marker. Selectable marker genes are utilized for the selection of transformed cells or tissues. Usually, the plant selectable marker gene will encode antibiotic resistance, with suitable genes including at least one set of genes coding for resistance to the antibiotic spectinomycin, the streptomycin phosphotransferase (spt) gene coding for streptomycin resistance, the neomycin phosphotransferase (nptII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (hpt or aphiv) gene encoding resistance to hygromycin, acetolactate synthase (als) genes. Alternatively, the plant selectable marker gene will encode herbicide resistance such as resistance to the sulfonylurea-type herbicides, glufosinate, glyphosate, ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D), including genes coding for resistance to herbicides which act to inhibit the action of glutamine synthase such as phosphinothricin or basta (e.g., the bar gene). See generally, International Publication No. WO 02/36782, U.S. Patent No. 7,205,453 and U.S. Patent Application Publication Nos. 2006/0218670, 2006/0248616, 2007/0143880 and 2009/0100536, and the references cited therein. See also, Jefferson et al. (1991); De Wet et al. (1987); Goff et al. (1990); Kain et al. (1995) and Chiu et al. (1996). This list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used. The selectable marker gene is also under control of a promoter operable in the plant species to be transformed. Such promoters include those described in International Publication No. WO 2008/094127 and the references cited therein.

[0072] In some embodiments, the selectable marker is part of a recombination marker free system. In one embodiment, the recombination marker free system is a Cre-lox recombination marker free system, such as described by Zuo et al. (2001). Such a system is useful for producing selection marker free transgenic plants, including transgenic *Jatropha* plants. In some embodiments, the recombination marker free system is positioned between the plant operable promoter and the one or more nucleic acid fragments. In this embodiment, the removal of the marker gene by the recombination event places the plant operable promoter in operable linkage with the one or more nucleic acid fragments as described herein.

[0073] In preparing the nucleic acid construct or an expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be

employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g. transitions and transversions may be involved.

[0074] Nucleic acids of the present invention may also be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences, by methods known in the art. Thus, all or a portion of the nucleic acids of the present invention may be synthesized using codons preferred by a selected host. Species-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a particular host species. Other modifications of the nucleotide sequences may result in mutants having slightly altered activity.

[0075] In a second aspect, the present invention provides a transgenic plant comprising one or more nucleic acid constructs described herein in which the transgenic plant is resistant to geminiviruses described herein. In one embodiment, the transgenic plant is a *Jatropha* plant. In a further embodiment, the transgenic plant is a *Jatropha curcas* plant. In some embodiments, the present invention provides any generation of *Jatropha* plant resistant to the geminiviruses that infect *Jatropha* plants, including *Jatropha curcas* plants, including those described herein. In other embodiments, the present invention provides transgenic *Jatropha* seed of any resistant generation of the *Jatropha* plants. In some embodiments, the present invention provides a method to prepare geminivirus resistant transgenic *Jatropha* plants and seeds described herein.

[0076] A nucleic acid construct or an expression vector of the present invention may be introduced into a plant cell using conventional transformation procedures. The term "plant cell" is intended to encompass any cell derived from a plant including undifferentiated tissues such as callus and suspension cultures, as well as plant seeds, pollen or plant embryos. Plant tissues suitable for transformation include leaf tissues, root tissues, meristems, protoplasts, hypocotyls, cotyledons, scutellum, shoot apex, root, immature embryo, pollen, and anther. "Transformation" means the directed modification of the genome of a cell by the external application of recombinant DNA from another cell of different genotype, leading to its uptake and integration into the subject cell's genome. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained.

[0077] DNA or nucleic acid constructs described herein can be used to transform any *Jatropha* plant. In one embodiment, the plant is a *Jatropha curcas* plant. The constructs may be introduced into the genome of the desired plant host by a variety of conventional techniques.

Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. Transformation protocols may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation, as is well known to the skilled artisan. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment. Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. Thus, any method, which provides for effective transformation/transfection may be employed. See, for example, U.S. Patent Nos. 7,241,937, 7,273,966 and 7,291,765 and U.S. Patent Application Publication Nos. 2007/0231905 and 2008/0010704 and references cited therein. See also, International Publication Nos. WO 2005/103271 and WO 2008/094127 and references cited therein. Techniques which have been used to transform *Jatropha* include *Agrobacterium*-mediated transformation. See, for example, U.S. Patent Application Publication Nos. 2011/0247099, 2012/0073018, 2012/0246759 and 2012/0272403.

[0078] Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype, e.g., a transgenic plant. A "transgenic plant" is a plant into which foreign DNA has been introduced. A "transgenic plant" encompasses all descendants, hybrids, and crosses thereof, whether reproduced sexually or asexually, and which continue to harbor the foreign DNA. Regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. See for example, International Publication No. WO 2008/094127 and references cited therein. See also, U.S. Patent Application Publication Nos. 2010/0304488, 2011/0117652, 2011/0247099, 2012/0073018, 2012/0246759 and 2012/0272403.

[0079] The foregoing methods for transformation are typically used for producing a transgenic variety in which the nucleic acid construct or expression cassette is stably incorporated. The resistance trait from the resistant transgenic plants produce in accordance with the present invention can be transferred to other plants by sexual crossing. In one

embodiment, the resistant transgenic variety could be crossed with another (non-transformed or transformed) variety in order to produce a new resistant transgenic variety. Alternatively, the resistance trait could be moved into another line using traditional backcrossing techniques that are well known in the plant breeding arts. For example, a backcrossing approach could be used to move the resistance trait from a public, non-elite variety into an elite variety, or from a variety containing a foreign gene in its genome into a variety or varieties which do not contain that gene. As used herein, "crossing" can refer to a simple X by Y cross, or the process of backcrossing, depending on the context. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

[0080] The resistant transgenic plants produced in accordance with the present invention can be cultivated in accordance with conventional procedures. Transgenic seeds can, of course, be recovered from the transgenic plants. These seeds can then be planted in the soil and cultivated using conventional procedures to produce resistant transgenic plants.

[0081] In a third aspect, the present invention provides a method for conferring resistance in a *Jatropha* plant to geminiviruses described herein, the method comprising expressing in the plant at least one nucleic acid construct that expresses dsRNA targeting geminivirus genes described herein. In some embodiments, one, two or three nucleic acid constructs that express dsRNA targeting the described geminiviruses genes are used to confer resistance. In certain embodiments, the resistance comprises resistance against a *Begomovirus*.

[0082] As shown herein, several JcMD resistant *Jatropha* lines based on an elite germplasm with high yield have been developed. The strategy involved the expression of a construct, which comprises of either single target or combined targets of 5 key geminivirus DNA-A genes encoding replication initiator protein (*Rep*; *AC1*), transcriptional activator protein (*TrAP*; *AC2*), replication enhancer protein (*REN*; *AC3*), gene silencing suppressor (*AC4*) and coat protein (CP). The data presented herein supports a broad viral resistance for some genetically modified lines, as shown by testing these lines to natural infection by another geminivirus, which shows 94% nucleotide identities to the transgene sequence.

[0083] 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); Green and Sambrook, 2012, *Molecular Cloning*, 4th 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; Rio et al., 2011, *RNA: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); 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

[0084] The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Materials and Methods

[0085] *Plasmid construction:* To generate a single target region hairpin structure (hp101, hp102 and hp103) shown in Fig. 1A and 1B, the following primers were used to generate hairpin structure with the pSKint vector as described previously (Guo et al., 2003):

[0086] 101 antisense: CCTAAGGATCCGCATGTTCTTCACAGTAGCTG (SEQ ID NO:5) and TTAGGGAATTCGGTTACGATTTAATCAGGGAT (SEQ ID NO:6);

[0087] 101 sense: GGACCAAGCTTGGTTACGATTTAATCAGGG (SEQ ID NO:7) and TTACCTCTCGAGGCATGTTCTTCACAGTAGCTG (SEQ ID NO:8);

[0088] 102 antisense: GGTAAGGATCCCAACCTTCATCTCCCTCACAG (SEQ ID NO:9) and TTCAGGAATTCAGTCTCAACACTCTCAGTCC (SEQ ID NO:10);

[0089] 102 sense: TATCCAAGCTTACTGCTCAACACTCTCAGTCC (SEQ ID NO:11) and CCGGACTCGAGCAACCTTCATCTCCCTCACAG (SEQ ID NO:12);

[0090] 103 antisense: AAGTAGGATCCAGCTGGAATTGGGCCCTGGATT (SEQ ID NO:13) and TGGACGAATTCCGATACCTTGAATGGGGAAC (SEQ ID NO:14);

[0091] 103 sense: AGTCCAAGCTTCGATACCTTGAATGGGGAAC (SEQ ID NO:15) and GGAATCTCGAGTAGCTGGAATTGGGCCCTGG (SEQ ID NO:16).

[0092] These hairpin structures were further subcloned into modified pCAMBIA1300 vector. These hairpin structures were driven by *CaMV 35S* promoter and followed by *NOS* terminator.

[0093] To generate a three target region hairpin structure (hp300) shown in Fig. 1C, the ligated fragment (Fragments 1, 2 and 3) was generated by two rounds of overlap extension PCR using the following primers:

[0094] F1-5Xho: AGCGCTCGAGCGTTTGAATCTAGACACGATGTGCTC (SEQ ID NO:17);

[0095] F1-3: TGCAGTGATGAGTTCCCCTGTGCGTGAACATGTTAAACACCTCACCG AAATCCT (SEQ ID NO:18);

[0096] F2-5: GATTCGGTGAGGTGTTTAACATGTTACGCACAGGGGAACATCATCA CTGCAGC (SEQ ID NO:19);

[0097] F2-3: GGAATGTTCCCCATTCCAAGGTATCGAATACCCTCAAGAAACGCCAG GTCTGAG (SEQ ID NO:20);

[0098] F3-5: CAGACCTGGCGTTTCTTGAGGGTATTCGATACCTTGAATGGGGAAC ATTCCAG (SEQ ID NO:21); and

[0099] F3-3Hind: AGCGCAAGCTTTAGCTGGAATTGGGCCCTGGATTGCAGA (SEQ ID NO:22).

[0100] The PCR fragment was inserted in the sense orientation into the *XhoI/HindIII* sites of the pSKint vector as described previously [15] to generate pSK-int-sense ICMV. Another fragment, amplified with forward primer AGCGCGAATTCTAGCTGGAATTGGGCCCTGGA TTGCAGA (SEQ ID NO:23) and reverse primer AGCGCACTAGTCGTTTGAATCTAGACA CGATGTGCTCCA (SEQ ID NO:24), was subsequently placed in the antisense orientation into the *EcoRI/SpeI* sites of pSK-int-sense ICMV to form pSK-int-ICMV RNAi. To generate marker free vector with a strong constitutive promoter, we replaced the G10-90 promoter in pX7-GFP with CaMV 35S promoter harbouring double enhancer. The resulting vector was named pX9-GFP. Finally, the entire RNAi cassette comprising the sense and antisense fragments interspersed by the *Arabidopsis* actin II intron was excised from pSK-int using the flanking *XhoI/SpeI* sites and inserted into the *XhoI/SpeI* site of pX9-GFP vector, yielding the construct pX9-hpICMV RNAi (hp300).

[0101] *Explant material for transformation:* Seeds were obtained from *Jatropha curcas* (Jc-MD) elite plants which were pre-selected by Drs. Yan Hong and Chengxin Yi (Yi et al., 2010). Seeds were germinated on ½ Murashige and Skoog salt medium. Cotyledons were harvested from 5-7 day-old seedlings, cut into small pieces (5 X 5 mm) and used as explants.

[0102] *PCR analysis of genomic DNA:* Genomic DNAs were prepared with DNeasy plant mini kits (Qiagen) according to the manufacturer's instructions. Approximately 50 ng of genomic DNA were used for PCR. The reactions were subjected to 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min for 40 cycles. Primers P1 (5'-ATCTCCACTGACGTAAGGGATGAC-3'; SEQ ID NO:25) and P2 (5'-GTTTAAGATCTACTTACGTAATCAAGC-3'; SEQ ID NO:26) were used to check the occurrence of marker excision events. DNA fragments containing the hygromycin resistance gene were amplified by either P3-1 (5'-GAGGGCGAAGAATCTCGTG CTTTC-3'; SEQ ID NO:27) and P4-1 (5'-TACTTCTACACAGCCATCGGTCCA-3'; SEQ ID NO:28) or P3-2 (5'-GAAGAATCTCGTGCTTTCAG-3'; SEQ ID NO:29) and P4-2 (5'-CAA CCAAGCTCTGATAGAGT-3'; SEQ ID NO:30). The product amplified by primers P3-1 and P4-1 was 885 bp whereas the product amplified by primers P3-2 and P4-2 was 745 bp.

[0103] *Virus challenge assay:* Infectious clones of ICMV-Dha tandem repeat DNA-A and DNA-B had been constructed earlier (Gao et al., 2010). Virus challenge assay was performed by *Agrobacterium*-mediated vacuum-infiltration as reported earlier (Ye et al., 2009). For hp101, hp102 and hp103, single vacuum-infiltration was used for the virus challenge assay. For hp300

transgenic plants and WT control plants, two vacuum-infiltrations were performed with a 10 days interval between the vacuum-infiltrations. For the second natural virus challenge in the field, random placed transgenic *J. curcas* plants expressing hp300 and WT control were infected by viruliferous B-type whitefly (*Bemisia tabaci*).

[0104] *Real time PCR to detect viral titers in challenged plants:* Total genomic DNA was isolated from the leaves of glasshouse-grown transgenic or control plants using the DNeasy plant mini kit (Qiagen). The concentration of genomic DNA for each sample was measured and equal amounts of genomic DNA were used for the analysis. Real-time PCR was performed with Power SYBR® Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) and run in ABI7900HT. Forward primer CTGCACAATGTGGGACCCTTTG (SEQ ID NO:31) and reverse primer CTTGCCCTGATGACAGAGATC (SEQ ID NO:32) were used for the amplification of viral DNA A. All samples were run in triplicate and the data was analyzed with RQ manager at a pre-set threshold cycle value (Applied Biosystems). The *Jatropha rbc* transcript served as an internal control using forward primer GGAGTTCCGCCTGAGGAAG (SEQ ID NO:33) and reverse primer CTTCTCCAGCAACGGGCTC (SEQ ID NO:34). As described by Prisco et al. (2011), the relative quantification of virus titers was determined based on the value of the cycle threshold (Ct), using the comparative C_T method and the formula $2^{-\Delta\Delta C_t}$.

[0105] *Southern blot:* Genomic DNA was digested with restriction enzymes and separated on 0.8% agarose gels. The gels were processed and transferred to a nylon Hybond-N⁺ membrane (GE Biosciences, Buckinghamshire, UK) following standard procedures [30]. Membranes were hybridized with a *CaMV* 2 X 35S promoter probe (SEQ ID NO:35) which included the double enhancers with 3' ends at -76 (A) of 35S promoter. The probes were DIG-dUTP-labeled by PCR using a PCR DIG probe synthesis kit (Roche) according to the manufacturer's instructions and signals were detected by autoradiography.

EXAMPLE 2

hpRNA Construct

[0106] Hairpin dsRNA-mediated RNAi was used to silence the viral coding genes. A multi-target region approach was used to produce stable and durable virus resistance. The causal pathogen for the JcMD that happened in Southern India has been identified as a strain of India Cassava Mosaic virus, ICMV-Dha (Gao et al., 2010). The sequence of this ICMV-Dha strain was chosen to target virus resistance. Three hairpin structures each with single target region

were made (FIG. 1B, hp101, hp102 and hp103). As shown in FIG. 1A, fragment 1 is 653 bp and simultaneously targets genes encoding the coat protein (CP, AV1) and the AC5. Fragment 2 is 521 bp and simultaneously targets genes encoding the transcriptional activator protein (TrAP, AC2) and the replication enhancer protein (Ren, AC3). Fragment 3 is 609 bp and simultaneously targets genes encoding the replication-associated protein (Rep, AC1) and the gene silencing suppressor (AC4). The fragment size of hp101, hp102 and hp103 is 563 bp, 521 bp and 609 bp respectively. Partial fragment of hp101 (250) bp, partial fragment of hp102 (250 bp) and full 609 bp of hp103 were linked together directly without any linking sequence. Each fragment was generated into an intron-spliced hpRNA structure.

[0107] A second strategy is to target three regions in the ICMV-Dha strain DNA. Three gene fragments were ligated to generate the sense and antisense arms in the hairpin dsRNA. As shown in FIG. 1A, fragment 1 is 250 bp and simultaneously targets genes encoding the coat protein (CP, AV1) and the AC5. Fragment 2 is also 250 bp and simultaneously targets genes encoding the transcriptional activator protein (TrAP, AC2) and the replication enhancer protein (Ren, AC3). Fragment 3 is 609 bp and simultaneously targets genes encoding the replication-associated protein (Rep, AC1) and the gene silencing suppressor (AC4). The ligated fragment (Fragment 1, 2 and 3) was then generated into an intron-spliced hpRNA structure (Figure 1C). The siRNA pool, produced from the hpRNA, was intended to silence five key viral genes encoding Rep, TrAP, Ren, CP and AC4.

[0108] This hpRNA fragment was inserted into a chemical inducible marker-free vector which has been shown to work in *Jatropha* (Qu et al., 2012). The original promoter was then replaced with 35S CaMV promoter and named it pX9-hpIcMV RNAi. Upon induction, Cre-lox mediated recombination excises the DNA fragment containing the hygromycin gene. As this is the only recombination event that happens, it results in the hpRNA structure being situated immediately downstream of the 35S CaMV promoter. The PCR product using primers 1 and 2 should be ~6 kb before induction and ~1.2 kb after induction. However, the ~6 kb product could not be amplified using the PCR program. The absence of PCR products using primers 3 and 4 indicated whether the antibiotic hygromycin phosphotransferase (HPT) gene has been removed (FIG. 1D).

Example 3

Plant Transformation

[0109] The expression vector of hp101, hp102, hp103 or hp300 were transformed into *Agrobacterium*. Cotyledons were used as explants for transformation [9]. More than 100 T0 transgenic *J. curcas* plants for each hairpin expression transformation vector were generated. A total of 133 T0 transgenic *J. curcas* plants for hp300 were generated.

Example 4

Single Target Region hpRNA Confers Virus Resistance

[0110] Vacuum infiltration with *Agrobacterium*-mediated virus infection method was used to inoculate virus into the transgenic plants. After single virus infection, 50 out of 300 transgenic single target region transgenic *J. curcas* expressing hp101, hp102 or hp103 were found to be free of virus symptoms (FIGS. 2A and 2B). T1 seeds from these putative virus resistance plants were collected and further germinated to produce T1 transgenic plants. The T1 plants were further virus-challenged and found to be virus resistant (FIG. 2C).

Example 5

Virus Resistance *J. curcas* by Expression of Three Target Region hpRNA

[0111] As shown in FIG. 1E, the genomic DNAs was first extracted and PCR analysis was done using two pairs of primers (P1-P2 and P3-P4). Fifty-three out of the 133 plants obtained from the hp300 transformation showed a 1.2 kb product using primers P1 and P2, indicating the occurrence of marker-free event and successful marker excision. However, the presence of PCR products of P3 and P4 corresponding to the HPT gene suggests that all of the plants are chimeric marker free. FIG. 3A shows the results of lines #1 to #54. The lanes between 1 and 11 and between lanes 34 and 35 are molecular markers. The PCR product using P1-P2 primers from lines 13, 16, 19, 25, 31, 38, 50 and 52 was gel purified and cloned into a T-vector. The PCR products were sequenced and the results confirmed that the positive bands are marker-free products. The transgenic plants with positive P1-P2 PCR product were used for virus challenge (Fig. 3B). To inoculate the virus into the plants, vacuum infiltration with *Agrobacterium*-mediated virus infection was used.

[0112] Two and half months after the virus challenge, the inoculated WT and susceptible transgenic lines showed very obvious curling and mosaic phenotype on systemic leaves. Genomic DNA was extracted and quantitative PCR was performed to analyze the virus titers in

transgenic and WT plants. Un-infected WT plants were used as controls and the average control value was set as 1. There was no difference in virus titers between the resistant transgenic lines and the un-infected control lines. However, we detected very high virus titers in susceptible transgenic lines which were similar to those found in infected WT plants (FIG. 4). The virus titers correlate very well with the virus symptoms. Several months later, the susceptible transgenic plants became obviously stunted with malformations and plant size reduction, whereas the resistant transgenic plants grew normally (FIGS. 5A-5C). PCR analysis was performed on the resistant lines to confirm the occurrence of a marker excision event (FIG. 3B). Next, Southern blot analysis was performed to test the copy number of transgenes in resistant lines (FIGS. 6A and 6B). As there are two *EcoR* V sites around the two loxP sites, the genomic DNAs were digested with *EcoR* V. Southern blots using probe prepared from the 35S promoter detects the copy number of transgene irrespective of the marker status. Figure 5B shows that resistant lines #7, #20, #25, #50, #82, #113, #131 and #133 contained a single copy of transgene. Along with quantitative PCR analysis of viral titers and symptomatic observations, we considered these transgenic lines to be the best positive resistant lines.

EXAMPLE 6

Second Round of Virus Challenge

[0113] We grew resistant transgenic *Jatropha* plants in pots outside of a greenhouse of our institute in Singapore, together with some uninfected WT plants. Unexpectedly, there was an outbreak of whitefly and whitefly-transmitted viral disease around our institute. All of the WT (100%) plants were infected by this virus (Fig 7A). However, the resistant transgenic lines showed substantial virus resistance with no virus disease symptoms although they experienced similar whitefly density (Fig. 7B). We collected virus-infected leaf samples and cloned the virus genome. Our sequencing results showed the new virus recovered in Singapore (named ICMV-SG, accession no.: JX518289) has 94% nt homology with the ICMV-Dha (unpublished data). We also performed real-time PCR to analyze the virus titers. Several transgenic lines which were resistant in the first round of virus challenge with ICMV-Dha, succumbed to this new ICMV-SG, but they accumulated lower virus titers compared to the susceptible control plants. Nevertheless, 8 transgenic lines remained resistant to this new virus (Fig. 8). This natural outbreak of virus was an excellent field test for the virus resistant transgenic *Jatropha* plants.

EXAMPLE 7

Heritable Virus Resistance in T1 Plants.

[0114] Three T1 seeds derived from line #82 (which was from the hp300 transformation event) were germinated together with WT control. *Jatropha* plants with 3-4 true leaves were inoculated with ICMV-Dha. Two months after virus challenge, WT plants and transgenic line #82-3 showed very obvious virus disease symptom whereas the transgenic plants #82-1 and #82-2 grew normal as un-infected control plants (Fig. 9A). Real-time PCR analysis showed the absence of detectable virus in transgenic plants #82-1 and #82-2 but high virus titers were obtained in #82-3 and WT plants (Fig. 9B). To analyze the unexpected virus resistant phenotype, we performed PCR analysis and Southern blot using genomic DNAs isolated from leaf samples taken before the virus challenge. We found no band amplified from both P1-P2 and P3-P4, indicating line #82-3 was likely a null segregant (Fig. 9C). Southern blot analysis showed lines #82-1 and #82-2 had the same bands as T0 transgenic plants but no signal was seen for #82-3, confirming the latter is a null segregant (Fig. 9D). Overall, our results show that transgenic plants based on the multi-target dsRNA approach can confer durable heritable resistance.

EXAMPLE 8

Broad Range Resistance to Multiple ICMV Strains Isolating from *Jatropha curcas*.

[0115] The fact that the transgenic plants are resistant to two ICMV strains with 94% sequence identity is evidence that the resistant transgenic plants would also be resistant to multiple ICMV strains which are located within a similar evolution clade (FIG. 10). Multiple sequence alignment of full-length DNA-A was carried out using the ClustalV program with default parameters. Phylogenetic trees were constructed from multiple alignments using the neighbor-joining method in MEGA4 program, and a bootstrap analysis with 1000 replicates was performed. Only values above 70 were reported on the trees shown in FIG. 10. The phylogenetic tree of DNA-A showed the sequence relationship between ICMV-SG and those of other cassava related mosaic geminiviruses. ACMV, *African cassava mosaic virus*; EACMCV, *East African cassava mosaic Cameroon virus*; EACMV, *East African cassava mosaic virus*; SACMV, *South African cassava mosaic virus*; ICMV, *Indian cassava mosaic virus*; SLCMV, *Sri Lankan cassava mosaic virus*; GPMLCuV, *Gossypium punctatum mild leaf curl virus*. The database accession number of each sequence is given.

[0116] 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. The term “about” is used to indicate that a value includes the standard deviation of error for the device or method being used to determine the value. 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.

[0117] 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

- [0118] Abhary, M.K. et al. (2006). Post-transcriptional gene silencing in controlling viruses of the Tomato yellow leaf curl virus complex. *Archives of Virology* **151**:2349-2363.
- [0119] Ahuja, S.L. et al. (2007). Genetics of resistance to cotton leaf curl disease in *Gossypium hirsutum* L. under field conditions. *The Journal of Heredity* **98**:79-83.
- [0120] Aswatha Narayana, D.S. et al. (2007). Distinct begomoviruses closely related to Cassava Mosaic Viruses cause Indian Jatropha Mosaic Disease. *Int J Virol* **3**:1-11.

- [0121] Bonfim, K. et al. (2007). RNAi-mediated resistance to Bean golden mosaic virus in genetically engineered common bean (*Phaseolus vulgaris*). *Mol Plant Microbe Interact* **20**:717-726.
- [0122] Brown, J. et al. (2012). Geminiviridae. In *Virus Taxonomy - Ninth Report of the International Committee on Taxonomy of Viruses* (King, A., Adams, M., Carstens, E. and Lefkowitz, E. eds). London, Waltham, San Diego: Associated Press, Elsevier Inc., pp. 351-373.
- [0123] Chiu, W. et al. (1996). Engineered GFP as a vital reporter in plants. *Current Biology* **6**:325-330.
- [0124] Christensen, A.H. and Quail, P.H. (1989). Sequence analysis and transcriptional regulation by heat shock of polyubiquitin transcripts from maize. *Plant Mol Biol* **12**:619-632.
- [0125] Christensen, A.H. et al. (1992). Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol Biol* **18**:675-689.
- [0126] De Wet, J.R. et al. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol* **7**:725-737.
- [0127] Fuentes, A. et al. (2006). Intron-hairpin RNA derived from replication associated protein C1 gene confers immunity to tomato yellow leaf curl virus infection in transgenic tomato plants. *Transgenic Res* **15**:291-304.
- [0128] Gao, S. et al. (2010). A new strain of *Indian cassava mosaic virus* causes a mosaic disease in the biodiesel crop *Jatropha curcas*. *Archives of Virology* **155**:607-612.
- [0129] Guo, H.S. et al. (2003). A chemical-regulated inducible RNAi system in plants. *Plant J* **34**:383-392.
- [0130] Goff, S.A. et al. (1990). Transactivation of anthocyanin biosynthetic genes following transfer of B regulatory genes into maize tissues. *EMBO J* **9**:2517-2522.
- [0131] Jefferson, R.A. et al. (1991). *Plant Molecular Biology Manual*, ed. Gelvin et al., Kluwer Academic Publishers, pp.1-33.
- [0132] Kain, S.R. et al. (1995). Green fluorescent protein as a reporter of gene expression and protein localization. *Biotechniques* **19**:650-655.
- [0133] Kalantidis, et al. (2002). The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. *Mol Plant Microbe Interact* **15**:826-833.
- [0134] Kashina, B.D. et al. (2013). Molecular identification of a new begomovirus associated with mosaic disease of *Jatropha curcas* L. in Nigeria. *Archives of Virology* **158**:511-514.
- [0135] Last, D. I. et al. (1991). pEmu: an improved promoter for gene expression in cereal cells. *Theor Appl Genet* **81**:581-588.
- [0136] McElroy, D. et al. (1990). Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* **2**:163-171.
- [0137] Mysara, M. et al. (2011). MysiRNA-designer: a workflow for efficient siRNA design. *PLOS one* **6**(10):e25642.
- [0138] Niu, Q.W. et al. (2006). Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nature Biotechnology* **24**:1420-1428.

- [0139] Odell, J. T. et al. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* **313**:810-812.
- [0140] Prisco, G.D. et al. (2011). Dynamics of persistent and acute deformed wing virus infections in honey bees, *Apis mellifera*. *Viruses* **3**:2425-2441.
- [0141] Qu, J. et al. (2012). Development of marker-free transgenic *Jatropha* plants with increased levels of seed oleic acid. *Biotechnol Biofuels* **5**:10. doi: 10.1186/1754-6834-5-10.
- [0142] Raghaven, V. et al. (2004). The DNA-A Component of a Plant Geminivirus (Indian Mung Bean Yellow Mosaic Virus) Replicates in Budding Yeast Cells. *J Virol* **78**:2405-2413.
- [0143] Raj, S.K. et al. (2008). First molecular identification of a begomovirus in India that is closely related to Cassava mosaic virus and causes mosaic and stunting of *Jatropha curcas* L. *Australasian Plant Disease Notes* **3**:69-72.
- [0144] Ramkat, R.C. et al. (2011). Biotechnological approaches to determine the impact of viruses in the energy crop plant *Jatropha curcas*. *Virology Journal* **8**:386.
- [0145] Shehi, S. et al. (2012). Biological Characterization and Complete Genome Sequence of a Possible Strain of Indian cassava mosaic virus from *Jatropha curcas* in India. *Journal of Phytopathology* **160**:547-553.
- [0146] Smith, N.A. et al. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature* **407**:319-320.
- [0147] Vanderschuren, H. et al. (2007a). Transgenic cassava resistance to African cassava mosaic virus is enhanced by viral DNA-A bidirectional promoter-derived siRNAs. *Plant Mol Biol* **64**:549-557.
- [0148] Vanderschuren, H. et al. (2007b). Engineering resistance to geminiviruses--review and perspectives. *Plant Biotechnology Journal* **5**:207-220.
- [0149] Vanderschuren, H. et al. (2009). Dose-dependent RNAi-mediated geminivirus resistance in the tropical root crop cassava. *Plant Molecular Biology* **70**:265-272.
- [0150] Velten, J. et al. (1984). Isolation of a dual plant promoter fragment from the Ti plasmid of *Agrobacterium tumefaciens*. *EMBO J* **3**:2723-2730.
- [0151] Wang, M.B. et al. (2000). A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. *Mol Plant Pathol* **1**:347-356.
- [0152] Wesley, S.V. et al. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* **27**:581-590.
- [0153] Yan, P. et al. (2012). High-throughput construction of intron-containing hairpin RNA vectors for RNAi in plants. *PLOS one* **7**(5):e38186.
- [0154] Ye, J. et al. (2009). Rapid analysis of *Jatropha curcas* gene functions by virus-induced gene silencing. *Plant Biotechnology Journal* **7**:964-976.
- [0155] Yi, C. et al. (2010). Does epigenetic polymorphism contribute to phenotypic variances in *Jatropha curcas* L.? *BMC Plant Biol* **2010**, 10.
- [0156] Zhang, P. and Gruissem, W. (2003). Efficient replication of cloned African cassava mosaic virus in cassava leaf disks. *Virus Research* **92**:47-54.

[0157] Zrachya, A. et al. (2007). Production of siRNA targeted against TYLCV coat protein transcripts leads to silencing of its expression and resistance to the virus. *Transgenic Res* **16**:385-398.

[0158] Zuo, J. et al. (2001). Chemical-regulated, site-specific DNA excision in transgenic plants. *Nat Biotechnol* **19**:157-161.

WHAT IS CLAIMED IS:

1. A nucleic acid construct comprising a heterologous plant operable promoter operatively linked to a nucleic acid fragment selected from the group consisting of:

- (a) a nucleic acid fragment comprising two of nucleic acid fragments (i), (ii) and (iii) linked together and

- (b) a nucleic acid fragment comprising nucleic acid fragments (i), (ii) and (iii) linked together, wherein nucleic acid fragment (i) is:

- (A) substantially homologous to nucleotides 1 to 514 of a gene encoding the AV1 protein having the amino acid sequence set forth in SEQ ID NO:39 and that is substantially homologous to nucleotides 3 to 483 of a gene encoding the AC5 protein having the amino acid sequence set forth in SEQ ID NO:49; or

- (B) substantially homologous to nucleotides 227 to 476 of a gene encoding the AV1 protein having the amino acid sequence set forth in SEQ ID NO:39 and that is substantially homologous to nucleotides 41 to 290 of a gene encoding the AC5 protein having the amino acid sequence set forth in SEQ ID NO:49;

- nucleic acid fragment (ii) is:

- (C) substantially homologous to nucleotides 4 to 405 of a gene encoding the AC2 protein having the amino acid sequence set forth in SEQ ID NO:43 and that is substantially homologous to nucleotides 1 to 376 of a gene encoding the AC3 protein having the amino acid sequence set forth in SEQ ID NO:45; or

- (D) substantially homologous to nucleotides 156 to 405 of a gene encoding the AC2 protein having the amino acid sequence set forth in SEQ ID NO:43 and that is substantially homologous to nucleotides 8 to 257 of a gene encoding the AC3 protein having the amino acid sequence set forth in SEQ ID NO:45; and

- nucleic acid fragment (iii) is:

- (E) substantially homologous to nucleotides 324 to 932 of a gene encoding the AC1 protein having the amino acid sequence set forth in SEQ ID NO:41 and that is substantially homologous to nucleotides 167 to 300 of a gene

encoding the AC4 protein having the amino acid sequence set forth in SEQ ID NO:47;

and wherein substantially homologous is at least 94% sequence identity.

2. The nucleic acid construct of claim 1, which further comprises the nucleic acid fragment (a) or (b) operatively linked to a heterologous plant operable terminator.
3. The nucleic acid construct of claim 1, wherein the nucleic acid fragment (a) or (b) is operatively linked in a sense orientation to the promoter and is further linked to the same nucleic acid fragment (a) or (b) in an antisense orientation.
4. The nucleic acid construct of claim 1, wherein the nucleic acid fragment (a) or (b) is operatively linked in an antisense orientation to the promoter and is further linked to the same nucleic acid fragment (a) or (b) in a sense orientation.
5. The nucleic acid construct of claim 3 or 4, wherein the nucleic acid fragment (a) or (b) in the sense orientation is linked by a spacer nucleotide sequence to the same nucleic acid fragment (a) or (b) in the antisense orientation.
6. The nucleic acid construct of claim 3 or 4, wherein the nucleic acid fragment (a) or (b) in the sense orientation is linked by an intron to the nucleic acid fragment (a) or (b) in the antisense orientation.
7. The nucleic acid construct of claim 6, wherein the intron is removed during transcription of the nucleic acid construct.
8. A vector comprising the nucleic acid construct of any one of claims 1 to 7.
9. A transgenic plant cell comprising the nucleic acid construct of any one of claims 1 to 7, preferably a transgenic *Jatropha* plant cell, more preferably a transgenic *Jatropha curcas* plant cell.

10. A transgenic plant seed comprising the nucleic acid construct of any one of claims 1 to 7, preferably a transgenic *Jatropha* plant seed, more preferably a transgenic *Jatropha curcas* plant seed.
11. A transgenic plant comprising the nucleic acid construct of any one of claims 1 to 7, preferably a transgenic *Jatropha* plant, more preferably a transgenic *Jatropha curcas* plant.
12. A transgenic *Jatropha* plant having resistance to a geminivirus, preferably a geminivirus causing mosaic disease, wherein the resistance is achieved by introducing into a cell of the plant the nucleic acid construct of any one of claims 3 to 7 that when expressed produces two or more dsRNA molecules that suppress or down regulate four or more genes of the geminivirus.
13. The transgenic plant of claim 12, wherein each of the dsRNA molecules is an ihpRNA.
14. The transgenic plant of claim 12 or 13, wherein the geminivirus causes *Jatropha curcas* mosaic disease and the transgenic plant is a transgenic *Jatropha curcas* plant.
15. The transgenic plant of claim 14, wherein the four or more genes of the geminivirus that causes *Jatropha curcas* mosaic disease are selected from the group consisting a gene encoding the AV1 protein, a gene encoding the AC5 protein, a gene encoding the AC2 protein, a gene encoding the AC3 protein, a gene encoding the AC1 protein and a gene encoding the AC4 protein.
16. A method of preparing a transgenic *Jatropha* plant having resistance to a geminivirus, preferably a geminivirus causing mosaic disease, wherein expression of four or more genes of the geminivirus is down regulated in the transgenic plant, the method comprises stably incorporating the nucleic acid construct of any one of claims 3 to 7 in the genome of a plant, wherein the nucleic acid construct encodes two or more dsRNA molecules that when expressed in the transgenic plant down regulates or suppresses expression of four or more genes of the geminivirus.

17. The method of claim 16, wherein each of the dsRNA molecules is an ihpRNA.
18. The method of claim 16 or 17, wherein the geminivirus causes *Jatropha curcas* mosaic disease and the transgenic plant is a transgenic *Jatropha curcas* plant.
19. The method of claim 18, wherein the four or more genes of the geminivirus that causes *Jatropha curcas* mosaic disease are selected from the group consisting a gene encoding the AV1 protein, a gene encoding the AC5 protein, a gene encoding the AC2 protein, a gene encoding the AC3 protein, a gene encoding the AC1 protein and a gene encoding the AC4 protein.
20. A method of conferring resistance to geminivirus, preferably a geminivirus causing mosaic disease, in a *Jatropha* plant, the method comprising expressing the nucleic acid construct of any one of claims 3 to 7 in the plant, wherein the nucleic acid construct encodes two or more dsRNA molecules that when expressed in the transgenic plant down regulates or suppresses expression of four or more genes of the geminivirus.
21. The method of claim 20, wherein each of the dsRNA molecules is an ihpRNA.
22. The method of claim 20 or 21, wherein the geminivirus causes *Jatropha curcas* mosaic disease and the transgenic plant is a transgenic *Jatropha curcas* plant.
23. The method of claim 22, wherein the four or more genes of the geminivirus that causes *Jatropha curcas* mosaic disease are selected from the group consisting a gene encoding the AV1 protein, a gene encoding the AC5 protein, a gene encoding the AC2 protein, a gene encoding the AC3 protein, a gene encoding the AC1 protein and a gene encoding the AC4 protein.