

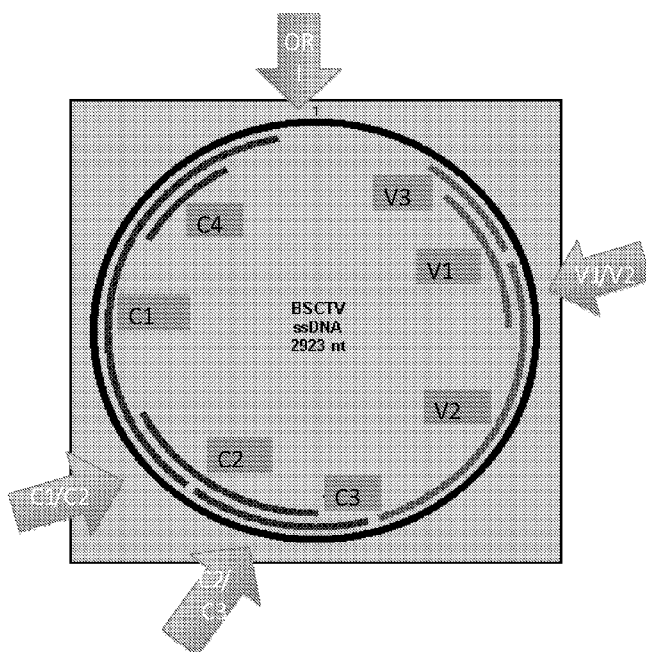


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[Continued on next page]

(54) **Title:** VACCINE FOR CONTROL OF BEET CURLY TOP VIRUS INFECTION OF PLANTS

FIG. 1



(57) **Abstract:** The disclosure provides plant vaccine compositions and methods for inducing immunity to Beet Curly Top Virus (BCTV) and related curtoviruses in a susceptible plant.



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VACCINE FOR CONTROL OF BEET CURLY TOP VIRUS INFECTION OF PLANTS

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application USSN 61/707,868, filed September 28, 2012, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The invention relates to virus-induced gene silencing in plants.

BACKGROUND OF THE INVENTION

[0003] Curly top disease, is caused by Beet curly top virus (BCTV) and related viruses in the genus Curtovirus, family Geminiviridae. BCTV severely impacts vegetable, dry bean, and sugar beet production throughout large portions of the western United States from e.g., California and Oregon to Texas, Oklahoma, and Nebraska. Transmitted by the beet leafhopper, *Circulifer tenellus*, the virus infects a broad host range from many plant families causing disease in over 300 species in 44 plant families.

[0004] The virus is typically restricted to broad-leafed plants, as no monocotyledonous plants have been identified as hosts for this virus. The most commonly infected hosts include sugar beets (for which the disease was first named), tomatoes, peppers, beans, potatoes, spinach, cucurbits, cabbage, alfalfa, and many ornamentals. The virus also survives in many weeds, such as Russian thistle (tumbleweed) and mustard.

[0005] The wide host range of curtoviruses, the abundance of the beet leafhopper, and increasing acreage of uncultivated rangeland where weeds (e.g., tumble weed) are allowed to grow unchecked are making curly top management increasingly difficult.

[0006] For over a century management of curly top disease in the western United States has focused on the large-scale application of insecticides to beet leafhopper overwintering grounds, as well as insecticide application to crops in efforts to reduce populations of the leafhopper vector. Unfortunately, since leafhopper transmission of

curtoviruses requires only a brief feeding interval to introduce the virus into a healthy plant, treating *e.g.*, sugarbeet plantings with insecticides does not effectively block virus all virus transmission. Furthermore, insecticide application has potential for unintended negative biological and environmental consequences.

[0007] Resistance to curly top has been identified in a select number of crops, but unfortunately, none of these eliminate the ability of the virus to infect, and some resistant crops exhibit non-desirable traits that are difficult to separate through plant breeding.

[0008] Therefore, what is needed in the art are effective means to control beet curly top virus. Fortunately, as will be clear from the following disclosure, the present invention provides for these and other needs.

SUMMARY OF THE INVENTION

[0009] In an exemplary aspect, the disclosure provides a plant vaccine composition for inducing immunity to Beet Curly Top Virus (BCTV) and related curtovirus species in a susceptible plant, the plant vaccine comprising: an expression vector comprising an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of nucleic acid sequences having at least about 90% sequence identity to SEQ ID NO: 10, at least about 90% sequence identity SEQ ID NO:14 and at least about sequence identity 90% SEQ ID NO:17, operably linked to a promoter sequence. In one exemplary embodiment, the expression vector comprises an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of nucleic acid sequences having at least about 95% sequence identity to SEQ ID NO:10, at least about 95% sequence identity to SEQ ID NO:14 and at least about 95% sequence identity to SEQ ID NO:17, operably linked to a promoter sequence. In another exemplary embodiment, the expression vector comprises an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of nucleic acid sequences having at least about 99% sequence identity to SEQ ID NO:10, at least about 99% sequence identity to SEQ ID NO:14 and at least about 99% sequence identity to SEQ ID NO:17, operably linked to a promoter sequence. In another exemplary embodiment, the expression vector comprises an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of SEQ ID NO:10, SEQ ID NO:14 and SEQ ID NO:17, operably linked to a promoter sequence. In another exemplary embodiment, the expression vector comprises an isolated BCTV vaccine nucleic acid sequence that

forms a hairpin structure, wherein the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure consists essentially of smaller BCTV vaccine nucleic acid sequences that are arranged sequentially to form the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure, wherein the smaller BCTV vaccine nucleic acid sequences are arranged so that a first sequence having at least about 99% sequence identity to SEQ ID NO:10 is placed next to a second sequence consisting of an intron, and the intron is placed next to a third sequence having at least about 99% sequence identity to SEQ ID NO:11, wherein the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure is operably linked to a promoter sequence. In another exemplary embodiment, the vaccine induces immunity to Beet Curly Top Viruses that are members selected from the group consisting of beet severe curly top virus (BSCTV) and beet mild curly top virus (BMCTV). In another exemplary embodiment, the susceptible plant is a member selected from the group consisting of sugar beet (*Beta vulgaris*), tomato (*solanum lycopersicum*), bean (*Phaseolus vulgaris*), and pepper (*Capsicum* spp.). In another exemplary embodiment, the susceptible plant is sugar beet. In another exemplary embodiment, the susceptible plant is tomato.

[0010] In another exemplary aspect, the disclosure provides a method for inducing immunity to Beet Curly Top Virus (BCTV) and related curtovirus species in a susceptible plant, thereby protecting the susceptible plant from infection with BCTV and related curtovirus species, the method comprising: (i) transfecting the susceptible plant with a suspension of *Agrobacterium tumefaciens* to provide a vaccinated plant, wherein the *Agrobacterium tumefaciens* comprises a plant vaccine that comprises an expression vector comprising an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of nucleic acid sequences having at least about 90% sequence identity to SEQ ID NO: 10, at least about 90% sequence identity SEQ ID NO:14 and at least about sequence identity 90% SEQ ID NO:17, operably linked to a promoter sequence, wherein expression of the isolated BCTV vaccine nucleic acid sequence in the vaccinated plant induces transient silencing of the BCTV and related curtovirus species, thereby protecting the susceptible plant from infection with BCTV and related curtovirus species. In an exemplary embodiment, the isolated BCTV vaccine nucleic acid sequence is a member selected from the group consisting of nucleic acid sequences having at least about 95% sequence identity to SEQ ID NO:10, at least about 95% sequence identity to SEQ ID NO:14 and at least about 95% sequence identity to

SEQ ID NO:17, operably linked to a promoter sequence. In another exemplary embodiment, the isolated BCTV vaccine nucleic acid sequence is a member selected from the group consisting of the group consisting of nucleic acid sequences having at least about 99% sequence identity to SEQ ID NO:10, at least about 99% sequence identity to SEQ ID NO:14 and at least about 99% sequence identity to SEQ ID NO:17, operably linked to a promoter sequence. In another exemplary embodiment, the isolated BCTV vaccine nucleic acid sequence is a member selected from the group consisting of SEQ ID NO:10, SEQ ID NO:14 and SEQ ID NO:17. In another exemplary embodiment, the isolated BCTV vaccine nucleic acid sequence forms a hairpin structure, wherein the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure consists essentially of smaller BCTV vaccine nucleic acid sequences that are arranged sequentially to form the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure, wherein the smaller BCTV vaccine nucleic acid sequences are arranged so that a first sequence having at least about 99% sequence identity to SEQ ID NO:10 is placed next to a second sequence consisting of an intron, and the intron is placed next to a third sequence having at least about 99% sequence identity to SEQ ID NO:11, wherein the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure is operably linked to a promoter sequence. In another exemplary embodiment, the transfection is transient transfection. In another exemplary embodiment, transient transfection is carried out by direct injection of the suspension of *Agrobacterium tumefaciens* into a stem of the susceptible plant. In another exemplary embodiment, the transient transfection is carried out by airbrushing the suspension of *Agrobacterium tumefaciens* into one or more leaves of the susceptible plant. In another exemplary embodiment, the method induces immunity to Beet Curly Top Viruses that are members selected from the group consisting of beet severe curly top virus (BSCTV) and beet mild curly top virus (BMCTV). In another exemplary embodiment, the susceptible plant is a member selected from the group consisting of sugar beet (*Beta vulgaris*), tomato (*solanum lycopersicum*), bean (*Phaseolus vulgaris*), and pepper (*Capsicum spp.*). In another exemplary embodiment, the susceptible plant is sugar beet. In another exemplary embodiment, the susceptible plant is tomato.

[0011] Other features, objects and advantages of the invention will be apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1. Illustrates the genomic organization of *Beet severe curly top virus* (BSCTV) and is generally representative of the genomes of other curtoviruses, including BMCTV, BCTV, and others.

[0013] FIG. 2 MFold analysis of hairpin shaped small folded RNA produced by pFGC5941-CurtovirusHP (Mfold web server for nucleic acid folding and hybridization prediction Nucl. Acids Res. (2003) 31(13): 3406-3415).

[0014] FIG. 3 Comparison of curly top symptom severity when plants were inoculated at 8, 14 and 24 days post-treatment with silencing construct pFGC5941-curtovirusHP, illustrating near complete control of BSCTV when inoculated with BSCTV 24 days after treatment.

[0015] FIG. 4 Effectiveness of pFGC5941-curtovirusHP treatment for control of Beet severe curly top virus when inoculated with virus at 20 days following treatment using agroinoculation with BSCTV. Results represent composite of two separate replications of 20 treated plants each.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0016] The term "plant" as used herein refers to whole plants, plant bodies, plant organs (*e.g.*, leaves, stems, flowers, roots, etc.), seeds, plant tissues, plant cells and progeny of same. In an exemplary embodiment, a plant cell includes callus. In another exemplary embodiment, a plant organ includes a root, a leaf, a flower and/or the like. The term "plant" refers to the broad class of higher plants amenable to transformation techniques. The term "plant" also includes monocotyledonous and dicotyledonous plants, plants of any variety of ploidy levels, including polyploid, diploid, haploid and hemizygous.

[0017] Some exemplary plants include, but are not limited, to sugar beet (*Beta vulgaris*), bean (*Phaseolus vulgaris*), pepper (*Capsicum* ssp), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), members of the genus *Cucurbita e.g.*, Hubbard squash (*C. Hubbard*), Butternut squash (*C. moschata*), Zucchini (*C. pepo*), Crookneck squash (*C. crookneck*), *C. argyrosperma*, *C. argyrosperma* ssp *sororia*, *C. digitata*, *C. ecuadorensis*, *C. foetidissima*, *C. lundelliana*, and *C. martinezii*, and

members of the genus *Cucumis* such as cucumber (*Cucumis sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamental plants *e.g.*, azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherima*), cabbage, *Arabidopsis*, etc.

[0018] The term “transgenic plant” as used herein refers to a plant comprising at least one heterologous nucleic acid sequence that was introduced into the plant, at some point in its lineage, by genetic engineering techniques. In an exemplary embodiment, a transgenic plant is a plant that is the progeny or descendant of a plant that is transformed with at least one heterologous nucleic acid sequence and which comprises heterologous nucleic acid. Thus, the term “transgenic plant” refers to plants which are the direct result of transformation with a heterologous nucleic acid or transgene, and the progeny and descendants of transformed plants which comprise the introduced heterologous nucleic acid or transgene.

[0019] The expression “isolated BCTV vaccine nucleic acid” “isolated BCTV vaccine nucleic acid sequence” or “isolated BCTV vaccine sequence” as used herein, refers to isolated BCTV vaccine nucleic acids which comprise a nucleotide sequence identical to or substantially identical to SEQ ID NO:10, SEQ ID NO:14 or SEQ ID NO:17 and which are able to induce immunity to beet curly top virus (BCTV) in plants.

[0020] An exemplary isolated BCTV vaccine nucleic acid consists essentially of a nucleotide sequence as shown in SEQ ID NO:10 or segment or fragment thereof. In one exemplary embodiment, an “isolated BCTV vaccine nucleic acid sequence” refers to an isolated nucleotide sequence identical to or substantially identical to SEQ ID NO:14 or segment or fragment thereof or an isolated nucleic acid identical to or substantially identical to SEQ ID NO:17 or segment or fragment thereof, and which are able to induce immunity to beet curly top virus (BCTV) and related curtoviruses in susceptible plants. Thus, an exemplary isolated BCTV vaccine sequence is SEQ ID NO:10. Another exemplary isolated BCTV vaccine sequence is SEQ ID NO:14. Still another exemplary isolated sequence is illustrated as SEQ ID NO:17. Typically, isolated BCTV vaccine sequences are derived from specific nucleic acid segments derived from BCTV genome as disclosed hereinbelow. However, isolated BCTV vaccine sequences can be isolated from any source and/or can be synthetically made, by methods known on the art (*see*

e.g., US Patent 5,942,609) as long as they are substantially identical to isolated BCTV vaccine nucleic acid sequences as disclosed herein. Methods for determining nucleotide sequence identity and “substantial identity” are described below. However, in general, two nucleic acid sequences are considered to be substantially identical when the two molecules or their complements hybridize to each other under stringent hybridization conditions, as described below.

[0021] The expression “isolated BCTV nucleic acid” or “isolated BCTV sequence” or other grammatically equivalent expressions as used herein, refer to isolated BCTV nucleic acids derived from any known BCTV genomic sequence e.g., derived from *Beet severe curly top virus* (BSCTV; GenBank Accession Number NC_004754) *Beet mild curly top virus* (BMCTV; NC_004753) *Beet curly top virus* (BCTV; NC_001412) . Thus, in an exemplary embodiment, a BCTV vaccine sequence comprises segments or fragments of isolated BCTV nucleic acids.

[0022] The terms “isolated,” “purified,” or “biologically pure” as used herein, refer to material that is substantially or essentially free from components that normally accompany it as found in its native state. In an exemplary embodiment, purity and homogeneity are determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A nucleic acid that is the predominant species present in a preparation is substantially purified. In one exemplary embodiment, an isolated BCTV vaccine nucleic acid is separated from open reading frames and/or other nucleic acid sequences that flank the isolated BCTV vaccine nucleic acid in its native state. In an exemplary embodiment, the term “purified” denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Typically, isolated nucleic acids or proteins have a level of purity expressed as a range. The lower end of the range of purity for the component is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

[0023] The term “nucleic acid” as used herein, refers to a polymer of ribonucleotides or deoxyribonucleotides. Typically, “nucleic acid” polymers occur in either single- or double-stranded form, but are also known to form structures comprising three or more strands. The term “nucleic acid” includes naturally occurring nucleic acid polymers as well as nucleic acids comprising known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally

occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Exemplary analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

[0024] Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (*see e.g.*, Batzer *et al.*, Nucleic Acid Res. 19:5081 (1991); Ohtsuka *et al.*, J. Biol. Chem. 260:2605-2608 (1985); and Rossolini *et al.*, Mol. Cell. Probes 8:91-98 (1994)).

[0025] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers as well as amino acid polymers in which one or more amino acid residues is an artificial chemical mimetic of a corresponding naturally occurring amino acid.

[0026] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α -carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0027] Amino acids are referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by

their commonly accepted single-letter codes.

[0028] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0029] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art (*see, e.g.*, Creighton, Proteins (1984)). Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles.

[0030] The following eight groups illustrate some exemplary amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);

- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
- 7) Serine (S), Threonine (T); and
- 8) Cysteine (C), Methionine (M)

[0031] Macromolecular structures such as polypeptide structures are described in terms of various levels of organization. For a general discussion of this organization, *see, e.g.,* Alberts *et al.*, Molecular Biology of the Cell (3rd ed., 1994) and Cantor and Schimmel, Biophysical Chemistry Part I: The Conformation of Biological Macromolecules (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 50 to 350 amino acids long. Typical domains are made up of sections of lesser organization such as stretches of β -sheet and α -helices. "Tertiary structure" refers to the complete three dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the noncovalent association of independent tertiary units. Anisotropic terms are also known as energy terms.

[0032] The term "label" as used herein, refers to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. Exemplary labels include ³²P, fluorescent dyes, electron-dense reagents, enzymes (*e.g.,* as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available.

[0033] As used herein a "nucleic acid probe or oligonucleotide" refers to a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (*i.e.,* A, G, C, or T) or modified bases (*e.g.,* 7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, for example, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence

depending upon the stringency of the hybridization conditions. In one exemplary embodiment, probes are directly labeled as with isotopes, chromophores, lumiphores, chromogens etc. In other exemplary embodiments probes are indirectly labeled *e.g.*, with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

[0034] Thus, the term "labeled nucleic acid probe or oligonucleotide" as used herein refers to a probe that is bound, either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

[0035] The term "primer" as used herein, refers to short nucleic acids, typically DNA oligonucleotides of at least about 15 nucleotides in length. In an exemplary embodiment, primers are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand. Annealed primers are then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, *e.g.*, by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

[0036] PCR primer pairs are typically derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5 ©1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with its length. Thus, for example, a primer comprising 20 consecutive nucleotides of an isolated BCTV vaccine sequence will anneal to a related target sequence with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in an exemplary embodiment, greater specificity of a nucleic acid primer or probe, is attained with probes and primers selected to comprise 20, 25, 30, 35, 40, 50 or more consecutive nucleotides of a selected sequence.

[0037] Nucleic acid probes and primers are readily prepared based on the nucleic acid sequences disclosed herein. Methods for preparing and using probes and primers and for labeling and guidance in the choice of labels appropriate for various purposes are discussed, *e.g.*, in Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* 2nd ed.

1989, Cold Spring Harbor Laboratory; and *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., 1994, John Wiley & Sons). The term "recombinant" when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, over expressed, under expressed or not expressed at all.

[0038] The term "promoter" or "promoter complex" or "promoter sequence" as used herein refers to an array of nucleic acid expression control sequences that direct transcription of a nucleic acid. As used herein, a "promoter" or "promoter complex" or "promoter sequence" comprises necessary nucleic acid sequences near the start site of transcription, such as, *e.g.*, a polymerase II type promoter, a TATA element etc to "control" transcription of an operably linked nucleic acid. In some exemplary embodiments, a "promoter complex" or "promoter sequence" also includes distal enhancer or repressor elements, which can be, but are not necessarily located as much as several thousand base pairs from the start site of transcription. In other exemplary embodiments, "promoter" or "promoter complex" or "promoter sequence" includes sequences that facilitate transcription of an operably linked heterologous nucleic acid and/or expression of the final protein or nucleic acid product of the heterologous nucleic acid *e.g.*, BCTV vaccine sequences as disclosed herein.

[0039] The term "capable of hybridizing under stringent hybridization conditions" as used herein, refers to annealing a first nucleic acid to a second nucleic acid under stringent hybridization conditions (defined below). In an exemplary embodiment, the first nucleic acid is a test sample, and the second nucleic acid is the sense or antisense strand of an isolated BCTV nucleic acid. Hybridization of the first and second nucleic acids is conducted under standard stringent conditions, *e.g.*, high temperature and/or low salt content, which tend to disfavor hybridization of dissimilar nucleotide sequences.

[0040] The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as *e.g.*, an isolated curtovirus nucleic acid, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs expression *e.g.*, transcription, of the nucleic acid

corresponding to the second sequence. In an exemplary embodiment, a promoter *e.g.*, an isolated curtovirus nucleic acid, that is “operably linked” to a heterologous nucleic acid is located upstream of and in-frame with the heterologous nucleic acid.

[0041] The term "heterologous" 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 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).

[0042] An "expression cassette" as used herein, refers to a nucleic acid construct, typically generated recombinantly or synthetically, which comprises a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. In an exemplary embodiment, an expression cassette comprises a heterologous nucleic acid to be transcribed *e.g.*, a BCTV vaccine nucleic acid, operably linked to a promoter.

[0043] Typically, an “expression cassette” is part of an “expression vector”. The term "vector" as used herein, refers to nucleic acid capable of replicating in a selected host cell or organism. A vector can replicate as an autonomous structure, or alternatively can integrate into the host cell chromosomes and thus replicate along with the host cell genome. Thus, an “expression vector” is a nucleic acid capable of replicating in a selected host cell or organism *e.g.*, a plasmid, virus, artificial chromosome, nucleic acid fragment, or any suitable construct known in the art, which comprises an “expression cassette”.

[0044] The term “transformation” or “transfection” are used interchangeably herein and encompasses any and all techniques by which a nucleic acid molecule might be introduced into a cell, including but not limited to, transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, Agrobacterium infection, and particle gun acceleration.

[0045] The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence

comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length isolated BCTV vaccine sequence or gene sequence given in a sequence listing, or may comprise a complete isolated BCTV vaccine sequence or gene sequence.

[0046] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, 85% identity, 90% identity, 99%, or 100% identity), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using a sequence comparison algorithm or by manual alignment and visual inspection.

[0047] The phrase "substantially identical", in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at least about 85% identity, at least about 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. In an exemplary embodiment, the substantial identity exists over a region of the sequences that is at least about 50 residues in length. In another exemplary embodiment, the substantial identity exists over a region of the sequences that is at least about 100 residues in length. In still another exemplary embodiment, the substantial identity exists over a region of the sequences that is at least about 150 residues or more, in length. In one exemplary embodiment, the sequences are substantially identical over the entire length of nucleic acid or protein sequence.

[0048] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0049] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of

from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection (see, *e.g.*, *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

[0050] An exemplary algorithm for sequence comparison is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, *e.g.*, version 7.0 (Devereaux *et al.*, *Nuc. Acids Res.* 12:387-395 (1984)).

[0051] Another example of algorithm that is suitable for determining percent

sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, Nuc. Acids Res. 25:3389-3402 (1977) and Altschul *et al.*, J. Mol. Biol. 215:403-410 (1990), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

[0052] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin & Altschul, Proc. Nat'l. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is

less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0053] An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

[0054] The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (*e.g.*, total cellular or library DNA or RNA). In general, two nucleic acid sequences are said to be "substantially identical" when the two molecules or their complements selectively or specifically hybridize to each other under stringent conditions.

[0055] The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10 °C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30

°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For high stringency hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary high stringency or stringent hybridization conditions include: 50% formamide, 5xSSC and 1% SDS incubated at 42°C or 5xSSC and 1% SDS incubated at 65 °C, with a wash in 0.2xSSC and 0.1% SDS at 65°C. However, other high stringency hybridization conditions known in the art can be used.

[0056] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides that they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary “moderately stringent hybridization conditions” include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 1xSSC at 45 °C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

I. Introduction:

[0057] Beet curly top virus (BCTV) is the most widespread geminivirus in the United States. It is endemic in the West, causing economic damage to a wide variety of crops. In the USA, the Curtovirus group consists essentially of seven viruses – Beet curly top virus (BCTV), Beet mild curly top virus (BMCTV), Beet severe curly top virus (BSCTV), Pepper curly top virus (PCTV), Pepper yellow dwarf virus (PYDV), Spinach curly top virus (SCTV) and Horseradish curly top virus (HrCTV). When more than one of these viruses infects a plant, they can readily exchange genes and recombine, so variation in their incidence is common, and a considerable degree of sequence variation can exist among viruses within the genus curtovirus and within the individual curtovirus species listed above.

[0058] Beet leafhoppers (*Circulifer tenellus*) are the primary vectors that transmit BCTV and other curtovirus species from plant-to-plant in North America. Other leafhopper species can transmit curtoviruses in other parts of the world. Leafhoppers

typically survive throughout the year in weeds and field crops. When weeds dry out and susceptible crops e.g., sugarbeets, spinach, are planted, leafhoppers migrate from drying vegetation and weeds to irrigated agricultural fields to feed on young green tissue of the susceptible crops. Beet acquire the virus while feeding on the phloem of infected plants. The virus is ingested and moves from the digestive system to salivary glands. When the leafhoppers feed on a non-infected plant, the virus is injected with saliva into the plant. The virus is transmitted in a persistent manner, which means that leafhoppers are able to transmit the virus for the rest of their lives. However, the virus does not replicate in the leafhopper and is not passed on to the offspring (*see e.g.*, Soto and Gilbertson, (2003) *Phytopathology* 93: 478- 484).

[0059] Although direct feeding by beet leafhopper causes relatively minor crop damage, Beet curly top virus is extremely destructive disease of sugarbeet as well as other crops (*e.g.*, tomatoes). Indeed, the leaves of plants infected with this virus are dwarfed, crinkled, and rolled upward and inward. Veins are roughened and often swollen. Roots become distorted, often with a proliferation of hair roots (not to be confused with Rhizomania). Phloem tissue often becomes necrotic and appears as dark rings in cross sections or dark streaks in longitudinal sections of the root. Thus, BCTV is a devastating crop pest.

[0060] To combat BCTV farm managers have employed insecticides and pesticides and weed control. Unfortunately, since transmission of BCTV requires only a brief feeding interval by leafhoppers, insecticides are not entirely effective at blocking transmission and spread of the virus. Furthermore, pesticide use on food crops is undesirable and many farm managers are making efforts to reduce pesticide usage. Thus, what is needed in the art are new and effective methods for controlling BCTV.

[0061] Numerous efforts at developing traditional host plant resistance have been attempted in several crops including bean, sugarbeet, and tomato. In sugarbeet, host plant resistance involves several genes and is difficult to move among breeding lines. Furthermore, the seed industry has been unable to separate curly top resistance from a propensity for lower than desired yields. Therefore, locations in which growers are forced to grow sugarbeets with host plant resistance (sugarbeet genes that reduce severity of curly top) cannot achieve the yields even with resistant beets that are possible in areas where curly top is not a problem. Some sources of natural resistance were developed in tomato several years ago, however, those sources were also not deemed

suitable for commercialization by the tomato seed industry.

[0062] One serious obstacle to commercial development of resistance to curly top is the cost of developing a genetically modified crop. The limited number of genetically modified crops on the market required a long and extensive approval process and required far more testing and legal expenses than would be necessary for host plant resistance.

[0063] Therefore, in an exemplary embodiment, the invention provides a plant vaccine that protects crops from infection with BCTV and related curtoviruses which does not permanently alter the genetic makeup of the plant. The vaccine is administered to susceptible plants by treating said plants with a suspension of *Agrobacterium tumefaciens* containing a construct that induces transient silencing of the primary BCTV (*e.g.*, Beet severe curly top virus (BSCTV), Beet mild curly top virus (BMCTV), and Beet curly top virus (BCTV)) affecting agriculture in the United States. In an exemplary embodiment, plants are treated as seedlings with a genetic construct that induces specific elimination of virus RNA.

[0064] In exemplary embodiments, plants are transiently transfected by direct injection into the stem, or by spraying a suspension of *A. tumefaciens* containing the genetic construct necessary to induce silencing onto the leaves of the plant treated plants. Transiently transfected plants are subsequently rinsed to wash away the delivery agent (*A. tumefaciens*), and transplanted to the field. Unlike traditional biotechnology approaches, treated plants are not permanently modified genetically.

[0065] Without being bound by theory, it is believed that administration of the vaccine stimulates the plants own “immune system” to reject the BCTV and prevent infection of the whole plant, thereby controlling BCTV. The “immune system” employs virus-induced gene silencing (VIGS), which is triggered in plants upon infection by viruses. VIGS causes selective, specific degradation of viral genome sequences, as well as any additional sequences inserted into it (*see e.g.*, Voinnet, et al. EMBO J. 2002 September 2; 21(17): 4671–4679). VIGS can initiate even in the first cell the virus infects, preventing whole plant infection, and can be transmitted systemically throughout the plant. Although gene silencing is known primarily in the context of RNA viruses, silencing also can be effective for control of DNA viruses (*see e.g.*, Vanitharani et al., PNAS (2003) 100(16): 9632; Yang, et al. 2004: Phytopathology 94: 490-496; Abhary et al., 2006 Arch. Virol. 151: 2349-2363).

[0066] Although gene silencing is known primarily in the context of RNA viruses, a limited number of examples have shown that silencing also can be effective for control of DNA viruses (see e.g., Vanitharani et al., PNAS (2003) *supra*; Yang, et al. 2004: *supra*; Abhary et al., 2006 *supra*). There are numerous examples of RNA viruses for which gene silencing based control was effectively demonstrated as a means of control. This is not the case with DNA viruses, which like higher organisms, use RNA only for expression of virus encoded proteins, and in limited cases (retroviruses and pararetroviruses) for virus replication. Furthermore, there is considerable genetic variability within the family Geminiviridae, which has resulted in separation of the family in to multiple genera based on genetic and biological variation among viruses. Demonstration of a method to induce gene silencing for one genus cannot be guaranteed to work on others, in part due to structural and functional differences among the diverse viruses within the family (Fauquet et al. 2008. Geminivirus strain demarcation and nomenclature. Arch Virol 153: 783–821.), as well as the limited studies that have been conducted on gene silencing for control of DNA viruses in plants in general.

[0067] Curtoviruses, like other members of the family Geminiviridae, exist almost exclusively as DNA. The virus genome is single-stranded DNA, and the virus replicates by a unique method known as rolling circle replication, using viral DNA as the template for replication (Stenger et al., 1991; PNAS 88: 8029-8033). The only point at which RNA is produced is for expression of virus encoded proteins, which are necessary for normal virus function in the host plant cell, as well as for acquisition and transmission by leafhopper vectors (reviewed in Geminiviruses (Geminiviridae); Encyclopedia of Virology, 2nd Ed., Granoff & Webster, Eds., Academic Press, San Diego, CA. 1999. pp597-606). Consequently, choosing to use RNA silencing for control of curtoviruses was a bit of a risk. Very few studies have examined silencing-based control of curtoviruses, and even fewer have examined this at the whole plant level as was done here.

[0068] Studies examining gene silencing as a means of control for viruses in the family *Geminiviridae* usually focus on the ability to induce silencing in individual cells (see e.g., Vanitharani et al., (2003) PNAS 100: 9632-9636). Some examples have extended this resistance to whole plants, with the primary examples of this in the family *Geminiviridae* occurring for whitefly-transmitted viruses in the genus *Begomovirus*, particularly *Tomato yellow leaf curl virus* (TYLCV) and African cassava mosaic virus

(ACMV) for which a few laboratories have explored biotechnology-based gene silencing as a means of control (*see e.g.*, Yang, et al. 2004: *Phytopathology* 94: 490-496; Abhary et al., 2006 *Arch. Virol.* 151: 2349-2363; Zhang et al., 2005: *Plant Biotech. J.* 3: 385-397). Although studies suggest this method may be able to reduce TYLCV levels in tomato there is insufficient information to assume related viruses within the genus *Begomovirus* can be controlled with similar approaches, and virtually no information for more distantly related viruses in the genus *Curtovirus*. Furthermore, some studies have shown an ability of resistance based on a single portion of the TYLCV genome to be overcome by the virus which was to be controlled (*see e.g.*, Noris et al., 2004; *J. Gen. virol.* 85: 1745-1749). TYLCV, family *Geminiviridae*, genus *Begomovirus*, is genetically distinct from *Beet curly top virus* and related species (Family *Geminiviridae*, Genus *Curtovirus*), as the genomes of TYLCV exhibit some differences (reviewed in *Geminiviruses (Geminiviridae)*; *Encyclopedia of Virology*, 2nd Ed., Granoff & Webster, Eds., Academic Press, San Diego, CA. 1999. pp597-606). No other studies have been published showing effective reduction of curtovirus concentration in plants and decreased symptom development using a gene silencing approach.

[0069] The economic significance of curly top disease to the tomato and sugar beet industries in the United States, lack of effective and/or environmentally acceptable insecticide-based control of the leafhopper vector, lack of commercially acceptable natural resistance in these crops, as well as significant impact on numerous other crops warranted aggressive efforts to determine if gene silencing-based control could effectively reduce severity of curly top by preventing infection by BCTV and other curtoviruses.

[0070] In another exemplary embodiment, stably transfected plants are prepared by dipping of seedlings in a suspension for plant transformation. There are three delivery methods: stem injection, spraying leaves and dipping of seedlings in a suspension.

II. Isolating Curtovirus nucleic acids and Constructing Expression Vectors

A. General Recombinant DNA Methods

[0071] This invention utilizes routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning--A Laboratory Manual* (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989; Kriegler, *Gene Transfer and*

Expression: A Laboratory Manual (1990); and Current Protocols in Molecular Biology (Ausubel *et al.*, eds., 1994)). Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology maybe found in *e.g.*, Benjamin Lewin, Genes V, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

[0072] For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). Estimates are typically derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

[0073] Oligonucleotides that are not commercially available can be chemically synthesized *e.g.*, according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, Tetrahedron Letts. 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et al.*, Nucleic Acids Res. 12:6159-6168 (1984). Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Reanier, J. Chrom. 255:137-149 (1983).

[0074] The sequence of the cloned genes and synthetic oligonucleotides can be verified after cloning using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, Gene 16:21-26 (1981).

B. Methods for the Isolation of Nucleic Acids Comprising Isolated BCTV Vaccine Sequences

[0075] Isolated BCTV vaccine nucleic acids can be isolated using any of a variety of methods known to those of skill in the art which may be used for isolation of nucleic acids. For example, BCTV vaccine sequences are prepared from isolated BCTV nucleic acids. BCTV nucleic acids can be isolated from purified from curtovirus-infected plant tissue using any of several available total nucleic acid purification methods commonly known to those with skill in the art (*see e.g.*, Dellaporta, S., Wood, J., and Hicks, J.B.

1983. A plant DNA miniprep: Version II. Plant Molecular Biology Reporter 1: 19-21.1983).

[0076] In one exemplary embodiment BCTV nucleic acids used for the preparation of isolated BCTV vaccine sequences are isolated from plant tissue infected with *Beet severe curly top virus* (BSCTV; GenBank Accession Number NC_004754). In another exemplary embodiment BCTV nucleic acids used for the preparation of isolated BCTV vaccine sequences are isolated from plant tissue infected with *Beet mild curly top virus* (BMCTV; NC_004753). In still another exemplary embodiment BCTV nucleic acids are used for the preparation of isolated BCTV vaccine sequences are isolated from plant tissue infected with *Beet curly top virus* (BCTV; NC_001412).

[0077] In another exemplary embodiment, the nucleic acid sequences comprising isolated BCTV nucleic acid sequences and related nucleic acid sequences are cloned from genomic DNA libraries using amplification techniques and labeled oligonucleotide primers.

[0078] Isolated BCTV vaccine sequences typically comprise sequences that are identical to, or show substantial sequence identity (as defined above) to SEQ ID NO:10 (Construct #1 sense) and consist of a nucleotide sequence according to SEQ ID NO:14 (Construct #2), SEQ ID NO:17 (construct #3) or segment or fragment thereof. Thus, in exemplary embodiments, an isolated BCTV vaccine sequence is a member selected from the group consisting of SEQ ID NO:10, SEQ ID NO:14, and SEQ ID NO:17, variants thereof, and fragments thereof. In another exemplary embodiment, variants of isolated BCTV vaccine nucleic acids have at least about 80% sequence identity, at least about 85% sequence identity or at least about 90%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, sequence identity to SEQ ID NO:10, SEQ ID NO:14, or SEQ ID NO:17 and thus consist essentially of a nucleotide sequence according to SEQ ID NO:10, SEQ ID NO:14, or SEQ ID NO:17 or segments or fragments thereof.

[0079] Thus, isolated BCTV nucleic acid sequences typically hybridize to SEQ ID NO:10, SEQ ID NO:14 or SEQ ID NO:17 under stringent hybridization conditions.

[0080] Other methods known to those of skill in the art can also be used to isolate DNA fragments comprising isolated BCTV nucleic acids for the preparation of BCTV vaccine sequences. *See e.g.*, Sambrook, *et al.* for a description of other techniques for the isolation of DNAs related to DNA molecules of known sequence and the construction of artificial DNA sequences.

[0081] Once a putative isolated BCTV vaccine sequence is prepared from isolated BCTV nucleic acid sequences, it can be tested for virus control activity, *e.g.*, tested for the ability to confer immunity to BCTV. Methods for testing the activity of BCTV in plant cells are known in the art (*see e.g.*, Wintermantel, W.M. and Kaffka, S.R. 2006. Plant Disease 90: 657-662; Chen et al., 2010; Chen, L.F., Vivoda, E., and Gilbertson, R.L. 2010. Arch Virol. 2011 156(4): 547-555; Strausbaugh, C.A., Wintermantel, W.M., Gillen, A.M., and Eujayl, I.A. 2008. Curly top survey in the western United States. Phytopathology 98: 1212-1217.)

[0082] Briefly, susceptible plants are inoculated with vaccines protective against BCTV and related curtoviruses as disclosed herein. To determine if vaccination was successful and has therefore conferred immunity/resistance to BCTV, PCR with BCTV-specific primers is conducted by methods known in the art (*see e.g.*, Strausbaugh et al., (2008) *supra*). Thus, immunity is indicated by a lack of development of disease symptoms and confirmation of lack of infection by an inability to detect virus nucleic acid using PCR based detection, or the inability to detect virus protein using serological testing methods such as ELISA, when control (untreated) plants develop infection at a high rate (85% or higher).

[0083] Thus, isolated BCTV vaccine sequences that hybridize to SEQ ID NO:10, SEQ ID NO:14, or SEQ ID NO:17 under stringent hybridization conditions are tested for their ability to confer immunity to BCTV and related curtoviruses in susceptible plants *e.g.*, beets, tomatoes, by methods known in the art.

Sequence Features of Isolated BCTV Vaccine Sequences

[0084] The full length isolated *The Beet severe curly top virus* (BSCTV) genome is known in the art and is available as Accession No. NC_004754 from the National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA. See also Drake C. Stenger, (1994) Mol. Plant Microbe Interact. 7 (1), 154-157. The genomes of other curtoviruses capable of inducing curly top disease exhibit similar genomic organization (e.g. Stanley et al., 1986: EMBO J. 5: 1761-1767).

[0085] FIG. 1 illustrates the genomic organization of *Beet severe curly top virus* (BSCTV) and is generally representative of the genomes of other curtoviruses, including BMCTV, BCTV, and others.

[0086] Features that contribute to an effective curtovirus (e.g., BCTV) vaccine include the design of BCTV nucleic acid sequences into structures and arrangements that facilitate gene silencing. This includes sequences having a relatively high level of identity (89% or greater) with the target viruses, which includes BSCTV, BMCTV, BCTV, and other members of the genus, Curtovirus. Silencing constructs were designed to share at least about 89% identity/similarity with BSCTV, BMCTV, BCTV. In exemplary embodiments, BCTV nucleic acid sequences are cloned from the genomes of BMCTV and BSCTV, however, sequences cloned from other curtoviruses may also share such levels of identity in these select regions of the viral genome as well and thus, would have the same effect. Regions included for control include nucleic acids encompassing the C1/C4 protein encoding region, clockwise through the origin of replication (FIG. 1).

[0087] Changes to the sequence that reduce genomic similarity to below 89% compared to these genomes might prevent effectiveness against these viruses.

[0088] In addition to sequence similarity, which is a primary determinant for effectiveness of silencing with vaccine constructs 2 and 3, the design of the hairpin structure used in vaccine construct #1 is also relevant. In vaccine construct #1, three segments of the BSCTV genome, including regions encoding adjacent ends of proteins C1 and C2, C2 and C3, and V1 and V2 (FIG. 1) were assembled sequentially to form a linear (+) sense sequence, followed by an intron, then the inverted (-) sense sequence, allowing formation of a hairpin structure *in vivo* (FIG. 2). This functional, synthetic structure is responsible for induction of gene silencing. Alteration of any of the three

segments in either DNA strand (sense or antisense), to render identity to BSCTV, BMCTV or BCTV lower than 89% could potentially interfere with gene silencing. However, rearrangement of any of these sequences would likely still result in the silencing effect due to the nature of gene silencing and the role of small RNAs (*see e.g.*, Voinnet O (2001) *Trends Genet*, **17**, 449–459).

C. Construction of Vectors Comprising isolated BCTV Vaccine Sequences

[0089] Once an isolated BCTV vaccine sequence been prepared/isolated, various methods may be used to construct expression cassettes, vectors and other DNA constructs. Expression cassettes comprising isolated BCTV vaccine sequence can be constructed in a variety of ways. The skilled artisan is well aware of the genetic elements that must be present on an expression construct/vector in order to successfully transform, select and propagate the expression construct in host cells. Techniques for manipulation of nucleic acids encoding isolated BCTV vaccine sequences such as subcloning nucleic acid sequences into expression vectors, labeling probes, DNA hybridization, and the like are described generally in Sambrook, *et al.*, *supra*.

[0090] In an exemplary embodiment, an isolated BCTV vaccine sequence is cloned into an expression vector via suitable restriction endonuclease sites such that a promoter is upstream of and in-frame with the BCTV vaccine sequence.

[0091] DNA constructs comprising an isolated BCTV vaccine nucleic acid operably linked to a promoter sequence can be inserted into a variety of vectors (*e.g.*, pFGC5941 available as accession number Vector:1004952070 from The Arabidopsis Information Resource (TAIR) located at the Carnegie Institution for Science Department of Plant Biology, Stanford, California.). Typically, the vector chosen is an expression vector that is useful in the transformation of plants and/or plant cells. The expression vector may be a plasmid, virus, cosmid, artificial chromosome, nucleic acid fragment, or the like. Such vectors can be constructed by the use of recombinant DNA techniques well known to those of skill in the art. The expression vector comprising an isolated BCTV vaccine sequence may then be transfected either stably or transiently into the susceptible host plants. Successfully transfected plants are identified based the development of immunity to BCTV infection.

[0092] A number of recombinant vectors are available to those of skill in the art for use in the stable transfection of plant cells or for the establishment of transgenic plants

(see *e.g.*, Weissbach and Weissbach, (1989) *Methods for Plant Molecular Biology*, Academic Press; Gelvin *et al.*, (1990) *Plant Molecular Biology Manual*; *Genetic Engineering of Plants, an Agricultural Perspective*, A. Cashmore, Ed.; Plenum: NY, 1983; pp 29 38; Coruzzi, G. *et al.*, The Journal of Biological Chemistry, 258:1399 (1983); and Dunsmuir, P. *et al.*, Journal of Molecular and Applied Genetics, 2:285 (1983). As is known in the art, the choice of a vector is influenced by the method that will be used to transform host plants, and appropriate vectors are readily chosen by one of skill in the art. In an exemplary embodiment, known vectors are used to create expression constructs comprising isolated BCTV vaccine sequences.

D. Plant Hosts, Plant Transformation and Plant Selection and Regeneration Techniques

[0093] DNA constructs comprising an isolated BCTV vaccine sequence operably linked to a heterologous promoter sequence can be used to transfect plant cells and produce plants with desired phenotypic characteristics *e.g.*, immunity to BCTV.

[0094] Exemplary plants for transformation with expression constructs comprising isolated BCTV vaccine sequences include, but are not limited to; to sugar beet (*Beta vulgaris*), bean (*Phaseolus vulgaris*), pepper (*Capsicum ssp*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), members of the genus *Cucurbita e.g.*, Hubbard squash (*C. Hubbard*), Butternut squash (*C. moschata*), Zucchini (*C. pepo*), members of the genus *Cucumis* such as cucumber (*Cucumis sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamental plants *e.g.*, azalea (*Rhododendron spp.*), hydrangea (*Macrophylla hydrangea*), arabidopsis (*Arabidopsis thaliana*), etc.

[0095] Transformation and regeneration of dicotyledonous plant cells is well known in the art, *see e.g.*, Weising *et al.* Ann. Rev. Genet. 22:421-477 (1988); U.S. Patent 5,679,558; *Agrobacterium Protocols* Kevan M. A. Gartland *ed.*(1995) Humana Press Inc. and Wang, M., *et al.* (1998) Acta Hort. (ISHS) 461:401-408. The choice of method varies with the type of plant to be transformed, the particular application and/or the desired result. The appropriate transformation technique is readily chosen by the skilled practitioner.

[0096] Exemplary transformation/transfection methods available to those skilled in the art include, but are not limited to: direct uptake of foreign DNA constructs (see *e.g.*,

EP 295959); techniques of electroporation (see *e.g.*, Fromm *et al.*, (1986) Nature (London) 319:791) high-velocity ballistic bombardment with metal particles coated with the nucleic acid constructs (see *e.g.*, Kline *et al.*, Nature (London) 327:70 (1987), and U.S. Pat. No. 4,945,050); methods to transform foreign genes into commercially important crops, such as rapeseed (see De Block *et al.*, Plant Physiol. 91:694 701 (1989)), sunflower (Everett *et al.*, Bio/Technology 5:1201 (1987)), soybean (McCabe *et al.*, Bio/Technology 6:923 (1988); Hinchey *et al.*, Bio/Technology 6:915 (1988); Chee *et al.*, Plant Physiol. 91:1212 1218 (1989); Christou *et al.*, Proc. Natl. Acad. Sci USA 86:7500 7504 (1989); EP 301749), rice (Hiei *et al.*, Plant J. 6:271 282 (1994)), corn (Gordon-Kamm *et al.*, Plant Cell 2:603 618 (1990); Fromm *et al.*, Biotechnology 8:833 839 (1990)), and Hevea (Yeang, H. Y., *et al.*, In, Engineering Crop Plants for Industrial End Uses. Shewry, P. R., Napier, J. A., David, P. J., Eds.; Portland: London, 1998; pp 55 64). Other known methods are disclosed in *e.g.*, U.S. Patents 5,597,945; 5,589,615; 5,750,871; 5,268,526; 5,262,316; and 5,569,831.

[0097] Another exemplary method includes: transformation with DNA employing *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transforming agent, electroporation, particle acceleration, etc. (see, *e.g.*, EP 295959 and EP 138341). In one exemplary embodiment, Ti-derived vectors are used to transform a wide variety of higher plants, including dicotyledonous plants, such as *e.g.*, potato, soybean, cotton, rape, tobacco, and rice (see *e.g.*, Pacciofti *et al.*, Bio/Technology 3:241 (1985); Byrne *et al.*, Plant Cell, Tissue and Organ Culture 8:3 (1987); Sukhapinda *et al.*, Plant Mol. Biol. 8:209 216 (1987); Lorz *et al.*, Mol. Gen. Genet. 199:178 (1985); Potrykus, (1985) *supra*; Park *et al.*, J. Plant Biol. 38(4):365 71 (1995); and Hiei *et al.*, Plant J. 6:271 282 (1994)).

[0098] *Agrobacterium tumefaciens*-mediated transformation techniques are well described in the scientific literature. See, *e.g.*, Horsch *et al.* Science (1984) 233:496-498, and Fraley *et al.* (1983) Proc. Natl. Acad. Sci. USA 80:4803. Typically, a plant cell, an explant, a meristem or a seed is infected with *Agrobacterium tumefaciens* transformed with the expression vector/construct which comprises an isolated BCTV vaccine sequence. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots, roots, and develop further into plants. The nucleic acid segments can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of *Agrobacterium tumefaciens*. The Ti plasmid is transmitted to plant cells upon infection by *Agrobacterium tumefaciens*, and is stably integrated into the plant genome

(Horsch et al., (1984) "Inheritance of Functional Foreign Genes in Plants," Science, 233:496-498; Fraley et al., (1983) Proc. Nat'l. Acad. Sci. U.S.A. 80:4803.

[0099] Plants and plant cells can be transfected/transformed either stably or transiently. Plant cells stably transformed by *Agrobacterium* and whole plants regenerated from the transformed cells can also be transformed so as to produce transformed whole plants which contain the transferred expression vector/construct which comprises an isolated BCTV vaccine sequence.

[00100] Transformed plant cells which are stably constructed by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the desired transformed phenotype. Such 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. Plant regeneration from cultured protoplasts is described in Evans *et al.*, Protoplasts Isolation and Culture, Handbook of Plant Cell Culture, pp. 124-176, MacMillan Publishing Company, New York, 1983; and Binding, Regeneration of Plants, Plant Protoplasts, pp. 21-73, CRC Press, Boca Raton, 1985, all of which are incorporated herein by reference. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee et al. Ann. Rev. of Plant Phys. 38:467-486 (1987).

[00101] One of skill will recognize that, after an expression cassette comprising isolated BCTV nucleic acid sequences is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

[00102] In some exemplary embodiments, susceptible plant hosts are transiently transfected with an expression vector/construct which comprises an isolated BCTV vaccine sequence.

[00103] In one exemplary embodiment, an expression vector/construct comprising an isolated BCTV vaccine sequence is transfected into a susceptible host plant by direct injection (see e.g., Plant Journal, Ryu et al., 2004, which is incorporated herein by reference). In one exemplary embodiment, an expression vector/construct comprising an isolated BCTV vaccine sequence is transfected into a susceptible host plant by Airbrush spray method (see e.g., Dinesh-Kumar, Cold Spring Harbor protocol 2009, which is incorporated herein by reference).

[00104] The following examples are offered to illustrate, but not to limit the invention.

EXAMPLES

Example 1:

[00105] The following Example illustrates preparation of DNA constructs comprising BCTV vaccine nucleic acids.

Constructs for Induction of Gene Silencing

Vaccine Construct #1: pFGC5941-CurtovirusHP

[00106] This construct, pFGC5941-CurtovirusHP, produces a hairpin configuration of nucleic acid, an RNA structure known to induce gene silencing against many types of RNAs. The hairpin in pFGC5941-BSCTV comprises three separate regions of the *Beet severe curly top virus* (BSCTV) genome (see arrows indicating genomic locations in BSCTV diagram FIG.1) combined to form a hairpin-shaped construct. The construct was designed by combining portions of these three genomic regions adjacent to one another in two constructs as disclosed hereinbelow. SEQ ID NO:10 is in the sense or coding orientation, whereas SEQ ID NO:11 is a complementary sequence in the antisense orientation. pFGC5941-CurtovirusHP was constructed based on methods known in the art for a distantly related member of the *Geminiviridae* (see e.g., Abhary et al., 2006 Arch. Virol. 151: 2349-2363).

[00107] Briefly, three regions of the BSCTV genome (312 nt of the C1/C2 coding region, 250 nucleotides (nt) of the C2/C3 coding region, and 212 nt of V1/V2 coding region) were amplified by PCR and cloned separately in both sense and antisense directions into the binary vector, pFGC5941. pFGC5941 contains a CHSA (Chalcone Synthase A) intron, resulting in production of a hairpin structure once both sense and antisense regions are incorporated on either side of the CHSA intron. pFGC5941 is available as accession number Vector:1004952070 from The Arabidopsis Information Resource (TAIR) located at the Carnegie Institution for Science Department of Plant Biology, Stanford, California. A diagram of the construct as folded nucleic acid is shown in FIG.2.

[00108] Vaccine construct 1 was prepared by first cloning the individual sequences of the three above named genomic regions individually into the TOPO[®] TA vector (Life Technologies). The sequence of each region is shown below.

[00109] C1/C2 SUBCLONE: Primers 1863F (SEQ ID NO:1): ATATGGATCCATTTAAATTTACAYGGYAGWTTGARCTTGC and 2174R (SEQ ID NO:2): ATATAAGCTTGTTTTTRTATAACGTCATTGATGAC were used in PCR to amplify and clone a portion of the genes encoding the C1/C2 proteins of BCTV

[00110] Restriction sites for cloning BamHI and SmaI are shown in bold and italicized font, respectively on 1863F primer. HINDIII is shown in bold, italicized font on 2174R primer. The complete sequence of the resulting C1/C2 subclone is shown below (SEQ ID NO:3):

ATATGGATCCATTTAAATTTACATGGCAGATTGAGCTTGCGAGGACGCTTCT
TGATTGTTATCAAAGAGAGGACTTGTGAGTTTGGCGAAGACTGAATTTTGTA
ATGTCCAGGACCTAAGGGCTTCATTTTCTGATTTATTGAGGAAGTCCTGGTA
AGAGCTGCCTTCGCCTGGATTGCATAATATAATACTGGGAATACCACCTTTA
ATGACACGTGGTTTTCCATACTTTAAGTTTGTCTGCCACTCTCTTTGTGCGCC
TATAAGGTGCTTCCAATGCTTCATCTTTAAGTAATTTGGATCTACGTCATCAA
TGACGTTATATAGAACAAGCCCTATAT

[00111] C2/C3 SUBCLONE: Primers 1584F
ATAT***CTCGAGCT***YTCAAAAAACGGTCYCCAG (SEQ ID NO:4): and 1832R (SEQ ID NO:5): TTAAAAGCTTATCASGGATTTTCGCACRGAR were used to clone portions of the genes encoding the C2/C3 proteins of BSCTV. Primer 1584F sequence (underlined) begins at nt 1584 with a four nt generic leader, followed by a XhoI site

(bold italic) ahead of BCTV primer sequence. Primer 1832R contains a four nt generic leader followed by an HindIII site and BCTV sequence. The complete sequence of the resulting C2/C3 subclone is shown below (SEQ ID NO:6):

ATATCTCGAGCTTTCAAAAAACGGTCCCCAGTTAATGTCCTGTGTGCTCCAGT
GATCGTCAAATCTATCCAGCACTTGTGAAGATTCAAGTATTTGCGGAGGTTG
TGGTTGAATCTTATCTGGACTTTTCAGTTGATAAACTGGCCCCGAACGGAAGA
AGTCCTGGAGTTTGAGGTACAATGGATTGGGTACCAAGTCCACGGGTATGGA
ATTCGTCGCTTGTTCAGCGTGATGGGTTCCCTCCGTGCGAAAAATCCCTGAAA
AGCTTTTAA

[00112] V1/V2 SUBCLONE: Primers 700F and 911R were used to clone a portion of the genes encoding the V1/V2 proteins of BSCTV. Primer sequence matching BSCTV sequence is underlined. Primer 700F (SEQ ID NO:7):

TCATTCTAGAGGCGCGCCTGAATCCTCAAAGTGCGTGGC contains XbaI and
AscI restriction sites and a four nt generic leader, and primer 911R (SEQ ID NO: 8):

ATTTCTCGAGTCTCTGACTATACCAATACCCTGG contains an XhoI site and a
four nt generic leader.

[00113] The complete sequence of the resulting V1/V2 subclone is shown below
(SEQ ID NO:9):

TCATTCTAGAGGCGCGCCTGAATCCTCAAAGTGCGTGCCGAAGAAGAGGAG
GACTACTACGATTTTCGAGGAAATACCAATGGAGGAGACCTGTGACAAAAA
CAGGACTCTGAAGTTAAAGATGTATGATGATATGTTGGGTGCTGGTGGTATA
GGATCTACCATTAGTAATAATGGTATGATTACTATGTTGAATAATTATGTCCA
GGGTATTGGTGATAGTCAGAGACTCGAGAAAT

[00114] C1/C2 and C2/C3 subclone sequences were assembled using restriction
enzyme sites designed into primers shown above into the TOPO[®] TA plasmid vector to
form Subclone C1/C2/C3. The insert in Subclone C1/C2/C3 was subsequently excised
and ligated back into Subclone V1/V2 in the TOPO[®] TA vector between the BamHI and
XhoI sites, ahead of the V1/V2 insert to form the combined construct, Subclone
C1/C2/C3/V1/V2. The complete insert in Subclone C1/C2/C3/V1/V2 was excised using
XbaI and BamHI enzymes, and ligated in sense orientation into the binary vector,
pFGC5941 to create Subclone C1/C2/C3/V1/V2senseHP. The complete insert in
Subclone C1/C2/C3/V1/V2 was separately excised using SwaI and AscI and ligated in

the antisense orientation into Subclone C1/C2/C3/V1/V2senseHP to create the final construct, pFGC5941BSCTV hairpin. Restriction sites are underlined to assist in the location of segments from each subclone. The final folded structure of the pFGC5941 BSCTV hairpin construct is shown below (FIG. 2).

[00115] BCTV C1/C2/C3/V1/V2 Sense Direction (in pFGC5941 vector) (SEQ ID NO:10):

GGCGCGCCTGAATCCTAAAGTGCGTGGCCGAAGAAGAGGAGGACTACTACG
ATTTTCGAGGAAATACCAATGGAGGAGACCTGTGACAAAAACAGGACTCTG
AAGTTAAAGATGTATGATGATATGTTGGGTGCTGGTGGTATAGGATCTACCA
TTAGTAATAATGGTATGATTACTATGTTGAATAATTATGTCCAGGGTATTGGT
GATAGTCAGAGACTCGAGCTTTCAAAAAACGGTCCCCAGTTAATGTCCTGTG
TGCTCCAGTGATCGTCAAATCTATCCAGCACTTGTGAAGATTCAAGTATTTGC
GGAGGTTGTGGTTGAATCTTATCTGGACTTTCAGTTGATAAACTGGCCCGGA
ACGGAAGAAGTCCTGGAGTTTGAGGTACAATGGATTGGGTACCAAGTCCAC
GGGTATGGAATTCGCCCTTATATAGGGCTTGTTCTATATAACGTCATTGATGA
CGTAGATCCAAATTACTTAAAGATGAAGCATTGGAAGCACCTTATAGGCGCA
CAAAGAGAGTGGCAGACAACTTAAAGTATGGAAAACCACGTGTCATTAAA
GGTGGTATTCCCAGTATTATATTATGCAATCCAGGCGAAGGCAGCTCTTACC
AGGACTTCCTCAATAAATCAGAAAATGAAGCCCTTAGGTCCTGGACATTACA
AAATTCAGTCTTCGCCAACTCACAAGTCCTCTCTTTGATAACAATCAAGAA
GCGTCCTCGCAGCTCAATCTGCCATGTAAATTTAAAT

[00116] BCTV C1/C2/C3/V1/V2 Antisense Direction (in pFGC5941 vector) (SEQ ID NO:11):

GATCCATTTAAATTTACATGGCAGATTGAGCTTGCGAGGACGCTTCTTGATT
 GTTATCAAAGAGAGGACTTGTGAGTTTGGCGAAGACTGAATTTTGTAATGTC
 CAGGACCTAAGGGCTTCATTTTCTGATTTATTGAGGAAGTCCTGGTAAGAGC
 TGCCTTCGCCTGGATTGCATAATATAATACTGGGAATACCACCTTTAATGAC
 ACGTGGTTTTCCATACTTTAAGTTTGTCTGCCACTCTCTTTGTGCGCCTATAA
 GGTGCTTCCAATGCTTCATCTTTAAGTAATTTGGATCTACGTCATCAATGACG
 TTATATAGAACAAGCCCTATATAAGGGCGAATTCCATACCCGTGGACTTGGT
 ACCCAATCCATTGTACCTCAAACCTCCAGGACTTCTTCCGTTCCGGGCCAGTTT
 ATCAACTGAAAGTCCAGATAAGATTCAACCACAACCTCCGCAAATACTTGAA
 TCTTCACAAGTGCTGGATAGATTTGACGATCACTGGAGCACACAGGACATTA
 ACTGGGGACCGTTTTTTTGAAAGCTCGAGTCTCTGACTATCACCAATACCCTG
 GACATAATTATTCAACATAGTAATCATACCATTATTACTAATGGTAGATCCT
 ATACCACCAGCACCCAACATATCATCATACATCTTTAACTTCAGAGTCCTGTT
 TTTTGTACAGGTCTCCTCCATTGGTATTTTCCTCGAAATCGTAGTAGTCCTCC
 TCTTCTTCGGCCACGCACTTTAGGATTCAGGCGCGCC

[00117] Vaccine Construct #2: Briefly, pTRV-CFH1 involved cloning a region of BSCTV into a virus-based gene expression vector comprising of a modified Tobacco rattle virus RNA2 (pTRV2) clone inserted into an Agrobacterium vector (*see e.g.*, Dinesh-Kumar et al., Plant J. (2002) Sep;31(6):777-86). A 415 nt section of the region encoding the C4 protein within the Rep gene (C1), corresponding to nt 2538 – nt 25, and encompassing the origin of replication of BSCTV was amplified by PCR and inserted into the multiple cloning site (MCS) between BamHI & EcoRI in the pTRV2 vector (*see e.g.*, Dinesh-Kumar et al., 2002, *supra*) which uses a double 35S promoter to induce Virus Induced Gene Silencing (VIGS), to form pTRV-CFH1. pTRV-CFH1 requires addition of pTRV1, which encodes the TRV RNA1 (pTRV1) genome necessary for function *in planta*. pTRV1 is also expressed in an Agrobacterium vector (*see e.g.*, Dinesh-Kumar et al., Plant J. (2002) Sep;31(6):777-86). Both pTRV1 and pTRV-CFH1 are agroinoculated together to deliver the TRV to the plant, carrying the silencing inducer construct.

[00118] Primers used to amplify BSCTV insert for vaccine construct #2: Primer 2538F GCATATGGATCCTGCAGCATCATTAGCCGTCTG, (SEQ ID NO:12) which includes a six nt leader sequence and a BamHI restriction site. Primer 25R CCGAATCCGGATTTGAAGAGAGCCCGATTC, (SEQ ID NO:13) which includes a 2 nt leader sequence and an EcoRI restriction site.

[00119] Sequence of Vaccine Construct 2 silencing construct encompassing nt 2538 – nt 25 of genome of BSCTV (NC_004754).

TGCAGCATCATTAGCCGTCTGTTGACCTCCGCGTGCAGATCTTCCATCGACCT
GAAATTCACCCCAGTCGATGTGATCTCCGTCCTTTGAGACGTAGGACTTGAC
GTCGGAACCTGGATTTAGCTCCCTGAATATTGCAGTGGAATTGTTTGCTGGTA
CTTCGATGTTGCAGATCGAAGTAACGGGCATTACGGATCTGGACTTTTCCTTC
GAATTGAATAAGGGCATGCAGATGTGGTTCCCCATTTTCATGTAATTCTCTGC
AGATGCGAATATATTTTTTATTCGAAGGTGTATTTATAGCGAGGAGCTGTTCT
AAGGCGTCTTCTTTGGTTACTGAACATTGAGGGTATGTAAGGAAAAAATTTT
TGGCTTTTTTGTA AAAAGGCATATTGAATCGGGCTCTCTTCAAATCC (SEQ ID
NO:14)

[00120] **Vaccine Construct #3:** pTRV-CFH/WOR. This construct was designed from the 3' terminal 312 nt of the C1 region, which encodes the curtovirus replication protein. Assembly involved PCR amplification of the region from nt 1863 – 2174 near the 3' end of the C1 gene of BSCTV (NC_004754) and the corresponding sequence with *Beet mild curly top virus* (BMCTV; Accession No. NC_004753) and cloning into the NcoI & BamHI sites of pTRV2 (*see e.g.*, Dinesh-Kumar et al., 2002, *supra*) using PCR amplification of the viral genomic segment and primers including restriction enzyme sites utilized for cloning into pTRV2. Primer sequences are Primer VC3_1863F GATCGGATTCTTACAYGGYAGWTTGARCTTGC (SEQ ID NO: 15), which includes a 4 nt leader sequence and a BamHI restriction site, and Primer VC_2173R GATCCCATGGGTTYTRTATAACGTCATTGATGAC (SEQ ID NO: 16), which includes a 4 nt leader sequence and an NcoI restriction site.

Vaccine Construct #3: Sequence of BSCTV sequence Vaccine Construct in pTRV-CFH/WOR, encompassing nucleotides 1863-2174 BSCTV, but also corresponding to same region of BMCTV and related to other viruses within the genus. pTRV-CFH/WOR (SEQ ID NO:17):

TTACAAGGAAGTTTGATCTTGCGAGGACGCTTCTTGATTGTTATCAAAGAGAG
GACTTGTGAGTTTGGCGAAGACTGAATTTTGTAATGTCCAGGACCTAAGGGCT
TCATTTTCTGATTTATTGAGGAAGTCCTGGTAAGAGCTGCCTTCGCCTGGATT
GCATAATATAATACTGGGAATACCACCTTTAATGACACGTGGTTTTCCATACT
TAAAGTTTGTCTGCCACTCTCTTTGTGCGCCTATAAGGTGCTTCCAATGCTTCA
TCTTTAAGTAATTGGGATCTACGTCATCAATGACGTTATACAGAAC

[00121] All three constructs for induction of gene silencing were transformed into *Agrobacterium tumefaciens* strain AGL1 for delivery to plants. *Agrobacterium tumefaciens* strain AGL1 is known in the art (*see e.g.*, Lazo GR, et al (1991) Biotechnology (NY) Oct;9(10):963-967).

Example 2:

[00122] The following Example illustrates delivery of gene silencing inducer constructs using *A. tumefaciens* strain AGL1 and various delivery systems into *Nicotiana benthamiana*, and tomato, *Solanum lycopersicum*, with the DNA construct disclosed in Example 1. Delivery method #1: Direct injection into plants is known in the art (*see e.g.*, Ryu et al., (2004) The Plant Journal 40:322-331). Vaccines 2 and 3 (See Example 1) were delivered using the TRV vector and consisted of TRV RNA1 and the gene silencing inducer construct inserted into a TRV RNA2 vector (Dinesh-Kumar, et al, 2002, *supra*) mixed in a 1:1 ratio. pTRV1 and either pTRV-CFH1 (vaccine construct 2) or pTRV-CFH/WOR (vaccine construct 3), described above, which are inserted into pTRV2, the cloned version of TRV RNA2. The *Agrobacterium* carrying each construct in TRV was grown separately to log phase at 30°C, chilled, centrifuged at low speed, and the cells were resuspended in a buffer consisting of 10mM MES, 10mM MgCl₂ and 400 uM acetosyringone to an absorbance of 1.0 at OD600, after which the cells were grown an additional 4 hours at 30°C. Approximately 100µL was injected directly into the stem of each of the test plants or infiltrated into two leaves using a needleless syringe.

[00123] Constructs were also delivered to plants via Delivery method #2: Airbrush.

Airbrush is known in the art (*see e.g.*, Dinesh-Kumar, Cold Spring Harbor protocol 2009). The Agrobacterium carrying each construct was grown separately was grown to log phase at 30°C, chilled, centrifuged at low speed, and the cells were resuspended in a buffer consisting of 10 mM MES, 10 mM MgCl₂ and 400 uM acetosyringone to an absorbance of 1.5-2.0 at OD600, after which the cells were grown an additional 4 hours at 30°C. Loaded agrobacterium suspension into an artist's airbrush (Paasche VL) and sprayed the ventral side of test plants at 80 psi from approximately 8 inches distance for about 3-5 seconds. Approximately 1 mL solution was used per plant.

[00124] No carriers were used for delivery of constructs other than the MES/MgCl₂/Acetosyringone buffer solution described above.

[00125] Lifetime of immunity was not conclusively determined due to laboratory testing limitations, but was effective until age related resistance develops as seedlings mature. Virus inoculations were conducted at up to 45 days post treatment to determine if resistance remained effective however, plants at that age are so large that the positive controls no longer develop disease symptoms, and virus may or may not accumulate significantly in such plants (referred to as age-related resistance). Essentially the treatment method is protecting the young plants during their most susceptible period. Once plants obtain substantial size and age, they seem to develop age related resistance, or at least require higher levels of inoculum than was possible in the lab to develop infection.

[00126] Maximum gene silencing based suppression of virus occurs after 18-20 days post inoculation, based on levels of virus detected by qPCR. Plants were assayed for evaluation of resistance following treatment either with injection or spray, by inoculation with curtoviruses BSCTV and/or BMCTV, visually scoring plants for symptom development at 3 weeks post-inoculation, and extracting total nucleic acid from non-inoculated leaves and testing by PCR using standard primer sequences for detection of BSCTV and BMCTV as described in Strausbaugh, C.A., Wintermantel, W.M., Gillen, A.M., and Eujayl, I.A. 2008. Curly top survey in the western United States. *Phytopathology* 98: 1212-1217.

Example 3:

[00127] The following Example illustrates results of an exemplary method for detection of immunity to BCTV infection by induced gene silencing.

[00128] Testing of plants for infection by BSCTV and BMCTV was performed using both natural transmission of virus by beet leafhopper (*C. tenellus*) (*see e.g.*, Wintermantel, W.M. and Kaffka, S.R. 2006. Plant Disease 90: 657-662.) and using *Agrobacterium tumefaciens* to deliver virus to the plant (*see e.g.*, Chen, L.F., Vivoda, E., and Gilbertson, R.L. 2010. Arch Virol. 2011 156(4): 547–555). All experiments were performed in growth chambers under standard condition, 16 hour days, 25°C. Confirmation of infection of test plants was performed using PCR with BSCTV and BMCTV-specific primers by methods known in the art (*see e.g.*, Strausbaugh, C.A., et al (2008) *supra*).

[00129] Table 1. Results of testing resistance against curtoviruses using virus induced gene silencing.

Curly Top Virus Silencing in Tomato and Benth		
Construct	Tomato	Benth
TRV-CFH1 (Trial 1) ¹	13/14	3/3
TRV-CFH1 (Trial 2) ¹	15/15	NA
Agrovectov-BCTV Hairpin (Trial 1) ¹	6/6	4/6 ²
Agrovectov-BCTV Hairpin (Trial 2) ¹	18/20	NA
Agrovectov-BCTV Hairpin (Trial 3) ¹	17/20 (9/20)*	NA

¹Testing by ELISA (direct method)

²of the 2 that were positive, the abs readings were 2-4X lower than the positive control benth plants

³Results of Visual Inspection for BCTV Symptoms

*The positive control plants for Trial 3 were negative by ELISA, therefore the test was rerun.

The retest results are in parantheses where 4/5 positive control plants were positive.

[00130] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

WHAT IS CLAIMED IS:

1. A plant vaccine composition for inducing immunity to Beet Curly Top Virus (BCTV) and related curtovirus species in a susceptible plant, the plant vaccine comprising: an expression vector comprising an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of nucleic acid sequences having at least about 90% sequence identity to SEQ ID NO: 10, at least about 90% sequence identity SEQ ID NO:14 and at least about sequence identity 90% SEQ ID NO:17, operably linked to a promoter sequence.
2. The plant vaccine composition of claim 1, wherein the expression vector comprises an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of nucleic acid sequences having at least about 95% sequence identity to SEQ ID NO:10, at least about 95% sequence identity to SEQ ID NO:14 and at least about 95% sequence identity to SEQ ID NO:17, operably linked to a promoter sequence.
3. The plant vaccine composition of claim 1, wherein the expression vector comprises an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of nucleic acid sequences having at least about 99% sequence identity to SEQ ID NO:10, at least about 99% sequence identity to SEQ ID NO:14 and at least about 99% sequence identity to SEQ ID NO:17, operably linked to a promoter sequence.
4. The plant vaccine composition of claim 1, wherein the expression vector comprises an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of SEQ ID NO:10, SEQ ID NO:14 and SEQ ID NO:17, operably linked to a promoter sequence.
5. The plant vaccine composition of claim 1, wherein the expression vector comprises an isolated BCTV vaccine nucleic acid sequence that forms a

hairpin structure, wherein the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure consists essentially of smaller BCTV vaccine nucleic acid sequences that are arranged sequentially to form the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure,

wherein

the smaller BCTV vaccine nucleic acid sequences are arranged so that a first sequence having at least about 99% sequence identity to SEQ ID NO:10 is placed next to a second sequence consisting of an intron, and the intron is placed next to a third sequence having at least about 99% sequence identity to SEQ ID NO:11,

wherein

the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure is operably linked to a promoter sequence.

6. The plant vaccine composition of claim 1, wherein the vaccine induces immunity to Beet Curly Top Viruses that are members selected from the group consisting of beet severe curly top virus (BSCTV) and beet mild curly top virus (BMCTV).
7. The plant vaccine composition of claim 1, wherein the susceptible plant is a member selected from the group consisting of sugar beet (*Beta vulgaris*), tomato (*solanum lycopersicum*), bean (*Phaseolus vulgaris*), and pepper (*Capsicum spp.*).
8. The plant vaccine composition of claim 7, wherein the susceptible plant is sugar beet.
9. The plant vaccine composition of claim 7, wherein the susceptible plant is tomato.
10. A method for inducing immunity to Beet Curly Top Virus (BCTV) and related curtovirus species in a susceptible plant, thereby protecting the susceptible

plant from infection with BCTV and related curtovirus species, the method comprising:

(i) transfecting the susceptible plant with a suspension of

Agrobacterium tumefaciens to provide a vaccinated plant,

wherein

the *Agrobacterium tumefaciens* comprises a plant vaccine that comprises an expression vector comprising an isolated BCTV vaccine nucleic acid sequence that is a member selected from the group consisting of nucleic acid sequences having at least about 90% sequence identity to SEQ ID NO: 10, at least about 90% sequence identity SEQ ID NO:14 and at least about sequence identity 90% SEQ ID NO:17, operably linked to a promoter sequence,

wherein

expression of the isolated BCTV vaccine nucleic acid sequence in the vaccinated plant induces transient silencing of the BCTV and related curtovirus species,

thereby

protecting the susceptible plant from infection with BCTV and related curtovirus species.

11. The method of claim 10, wherein the isolated BCTV vaccine nucleic acid sequence is a member selected from the group consisting of nucleic acid sequences having at least about 95% sequence identity to SEQ ID NO:10, at least about 95% sequence identity to SEQ ID NO:14 and at least about 95% sequence identity to SEQ ID NO:17, operably linked to a promoter sequence.

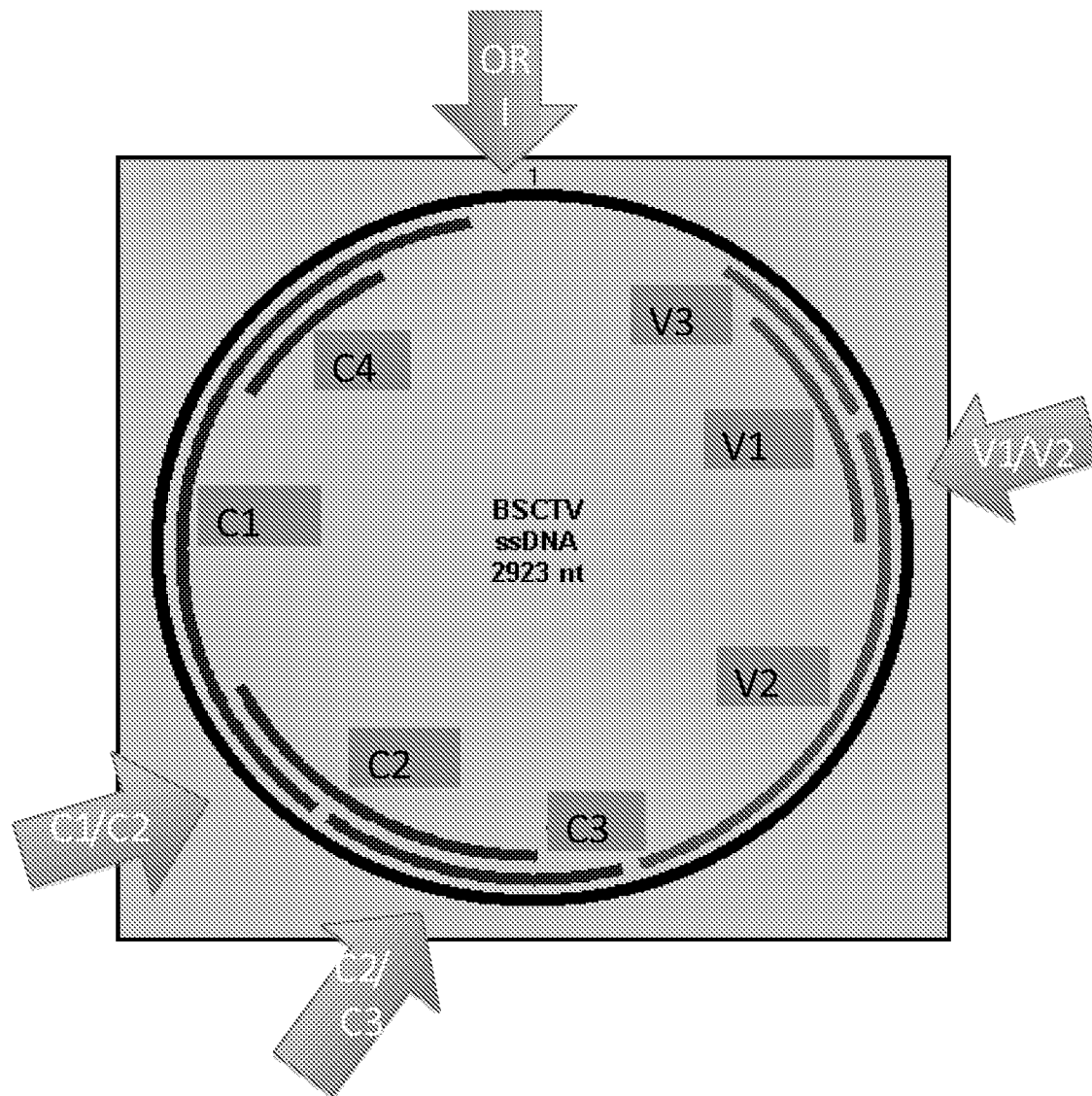
12. The method of claim 10, wherein the isolated BCTV vaccine nucleic acid sequence is a member selected from the group consisting of the group consisting of nucleic acid sequences having at least about 99% sequence identity to SEQ ID NO:10, at least about 99% sequence identity to SEQ ID NO:14 and at least about 99% sequence identity to SEQ ID NO:17, operably linked to a promoter sequence.

13. The method of claim 10, wherein the isolated BCTV vaccine nucleic acid sequence is a member selected from the group consisting of SEQ ID NO:10, SEQ ID NO:14 and SEQ ID NO:17.
14. The method of claim 10, wherein the isolated BCTV vaccine nucleic acid sequence forms a hairpin structure, wherein the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure consists essentially of smaller BCTV vaccine nucleic acid sequences that are arranged sequentially to form the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure,
wherein
the smaller BCTV vaccine nucleic acid sequences are arranged so that a first sequence having at least about 99% sequence identity to SEQ ID NO:10 is placed next to a second sequence consisting of an intron, and the intron is placed next to a third sequence having at least about 99% sequence identity to SEQ ID NO:11,
wherein
the isolated BCTV vaccine nucleic acid sequence that forms a hairpin structure is operably linked to a promoter sequence.
15. The method of claim 10, wherein the transfection is transient transfection.
16. The method of claim 15, wherein transient transfection is carried out by direct injection of the suspension of *Agrobacterium tumefaciens* into a stem of the susceptible plant.
17. The method of claim 15, wherein transient transfection is carried out by airbrushing the suspension of *Agrobacterium tumefaciens* into one or more leaves of the susceptible plant.
18. The method of claim 10, wherein the method induces immunity to Beet Curly Top Viruses that are members selected from the group consisting of beet severe curly top virus (BSCTV) and beet mild curly top virus (BMCTV).

19. The method of claim 10, wherein the susceptible plant is a member selected from the group consisting of sugar beet (*Beta vulgaris*), tomato (*solanum lycopersicum*), bean (*Phaseolus vulgaris*), and pepper (*Capsicum* spp.).
20. The method of claim 19, wherein the susceptible plant is sugar beet.
21. The method of claim 19, wherein the susceptible plant is tomato.

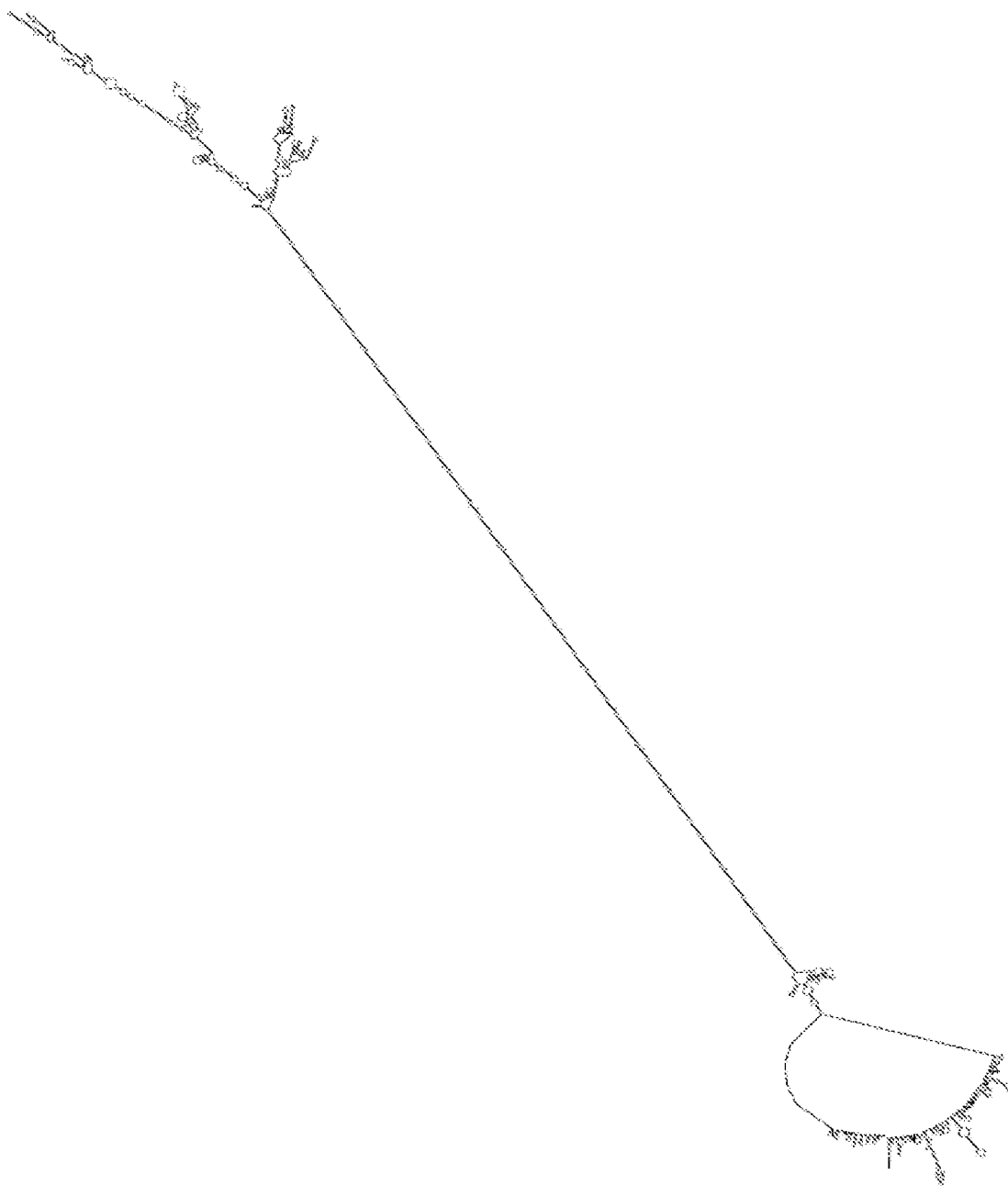
1/3

FIG. 1



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FIG. 2



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FIG. 3

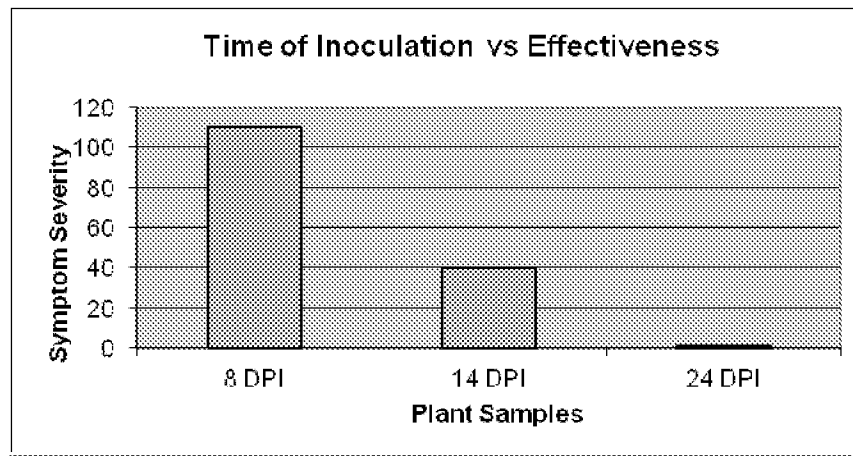
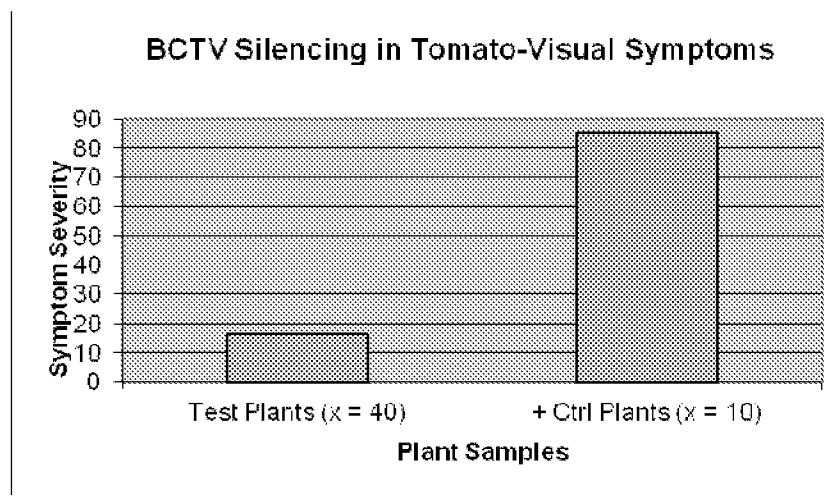


FIG. 4



A. CLASSIFICATION OF SUBJECT MATTER**C12N 15/83(2006.01)i, C12N 15/33(2006.01)i, A01N 63/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/83; C12N 15/82; A01H 5/00; A01H 1/00; C12N 5/04; C12N 15/33; A01N 63/00

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & keywords: Beet Curly Top Virus (BCTV), plant vaccine, vector, infection

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2006-0206959 A1 (TAVAZZA, M. et al.) 14 September 2006 See abstract; paragraphs [0104]–[0106]; table 4; claims 51–55, 58, 60 and 68.	1–4, 6–13, 15–21
A		5, 14
Y	NCBI, GenBank Accession No. U02311.1, `Beet severe curly top virus - Cfh, complete genome`, 29 March 2004 See the whole document.	1–4, 6–13, 15–21
A		5, 14
A	US 2002-0138867 A1 (HANLEY-BOWDOIN, L. et al.) 26 September 2002 See abstract; claims 1, 7–9, 15, 27, 37 and 42–43.	1–21
A	MARILLONNET, S. et al., `Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants`, Nature Biotechnology, June 2005, Vol. 23, No. 6, pp. 718–723. See abstract; figures 1–3.	1–21
A	STRAUSBAUGH, C. A. et al., `Curly top survey in the Western United States`, Phytopathology, November 2008, Vol. 98, No. 11, pp. 1212–1217. See abstract; figure 1.	1–21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 January 2014 (20.01.2014)

Date of mailing of the international search report

21 January 2014 (21.01.2014)

Name and mailing address of the ISA/KR


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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/062293

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

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

a. a sequence listing filed or furnished

☐

on paper

☒

in electronic form

b. time of filing or furnishing

☒

contained in the international application as filed

☐

filed together with the international application in electronic form

☐

furnished subsequently to this Authority for the purposes of search

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/062293

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 00-54573 A9	25/10/2001