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(54) **Title**: REPELLENT COMPOSITIONS AND GENETIC APPROACHES FOR CONTROLLING HUANGLONGBING

(57) **Abstract**: The invention provides a method for controlling Huanglongbing (HLB) disease of citrus plants through expressing genes encoding synthases for sesquiterpenes such as β- caryophyllene, and α-copaene, and combinations thereof, in citrus plants. Methods of controlling HLB comprising applying at least one purified sesquiterpene, which repels *Diaphohna citri* and/or *Tryoza erytrae* psyllid insects, so as to control the HLB disease of citrus plants, are also disclosed.

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REPELLENT COMPOSITIONS AND GENETIC APPROACHES FOR CONTROLLING HUANGLONGBING

FIELD OF INVENTION

The invention relates to repellent compositions comprising sesquiterpenes to control Huanglongbing (HLB) in citrus plants. Methods for controlling HLB through genetic modification of citrus plants are also disclosed.

BACKGROUND OF THE INVENTION

Huanglongbing (HLB), also known as citrus vein phloem degeneration (CVPD), citrus greening disease, yellow shoot disease (translated from Chinese huang-lunpin), likubin in Taiwan (translated from Chinese as Immediate Withering Disease), leaf mottle yellows in the Philippines, and citrus dieback in India, is probably the worst disease of citrus caused by a vectored pathogen. The causative agent is a motile bacterium, *Candidatus* Liberibacter spp., which is transmitted by Asian citrus psyllids (Sternorrhyncha: Psyllidae), also known as *Diaphorina citri* or, in Africa, by *Trioza erytreae*, the African citrus psyllid.

Distribution of HLB is primarily in tropical and subtropical Asia. It has been reported in all citrus-growing regions in Asia. The disease has affected crops in China, Taiwan, India, Sri Lanka, Malaysia, Indonesia, Myanmar, the Philippines, Pakistan, Thailand, the Ryukyu Islands, Nepal, Réunion, Mauritius, and Afghanistan. Areas outside Asia such as Saudi Arabia, Brazil and Florida in the U.S. have also reported the disease.

Although existing insecticidal and repellent compositions may be useful in controlling HLB, the safety of these compositions has been questioned as many of these compositions are excessively toxic to other organisms in the ecosystem. In addition, many of these compositions are extraordinarily long-lived, and persist within the environment to which they are applied almost indefinitely. Moreover, many insect species have evolved resistance to many of the known insecticidal and repellent compositions. Thus, a need exists for a relatively non-toxic, shorter-lived, effective repellent composition, such as a biological repellent composition.

It is long known in Vietnam that guava grown in proximity to or intercropped with citrus has a protectant or repellant effect against the Asian citrus psyllid. Guavas are plants in the myrtle family (*Myrtaceae*) genus *Psidium*, which contains about 100 species of tropical shrubs and small trees. The most frequently-encountered species, and the one often simply referred to as "the guava", is the Apple Guava (*Psidium guajava*).

The protective effect of interplanting guava and citrus is likely due to volatiles produced from the guava leaves because the protective effect is present year round. Although fifty-seven components including 27 terpenes (or sesquiterpenes) along with 14 alcohols and 4 esters have been identified in guava leaf oil using GC-MS obtained from a hydrodistillation of guava leaves, the exact mechanism underlying the protective effect of guava is not known. A recent report (Rouseff et al., Sulfur Volatiles in Guava (*Psidium guajava* L.) Leaves: Possible Defense Mechanism, J. Agric. Food Chem, 56:8905-10, 2008) suggests that sulfur volatiles such as dimethyl disulfide are responsible for the repulsive effect of guava, not the major volatiles such as β-caryophyllene since the latter is also present in citrus. However, sulfur volatiles contained in guava leaves are emitted at tiny levels and only for a period of about 30 minutes after wounding, indicating that these compounds have nothing or little to do with what is observed in citrus-guava intercroppings in Vietnam. On the other hand, β-caryophyllene is present in undamaged citrus leaves at concentrations generally below 0.2 % of total volatiles, while it represents usually more than 50% of total volatiles emitted by guava leaves.

There remains a need, for controlling HLB in citrus plants, and the present invention provides a novel solution to this problem.

SUMMARY OF THE INVENTION

It has been found that sesquiterpenes such as β-caryophyllene and α-copaene produced from the guava leaves repel *Diaphorina citri* and/or *Tryoza erytrae* psyllid insects. Thus, the invention satisfies a need of the industry for controlling HLB in citrus plants by providing a biological repellent composition comprising sesquiterpenes. Methods for controlling HLB through genetic modification of citrus plants to over-express genes coding sesquiterpene synthases are also provided.

Thus, the invention relates in general to a method for controlling HLB in citrus plants, which comprises expressing at least one gene encoding a polypeptide having sesquiterpene synthase activity in citrus plants to produce additional sesquiterpenes in order to repel *Diaphorina citri* and/or *Tryoza erytrae* psyllid insects so as to control HLB, wherein the sesquiterpene over-accumulated is β-caryophyllene, α-copaene, or combinations thereof. In this method, the at least one gene has β-caryophyllene synthase and/or α-copaene synthase activity.

In certain embodiments, the expression of the at least one gene is driven by its own promoter and terminator regions. In other preferred embodiments, the expression of the at least one gene is driven by a heterologous regulator region providing strong constitutive, tissue-specific or inducible expression. More preferably, the heterologous regulator region provides strong tissue-specific expression in the cytosol, chloroplasts or mitochondria.

In additional embodiments, the present method further comprises expressing a gene encoding a farnesyl pyrophosphase synthase to enhance the accumulation of the sesquiterpene produced by the polypeptide having sesquiterpene synthase activity.

The invention also relates to a method for controlling Huanglongbing (HLB) disease of citrus plants comprising applying at least one purified sesquiterpene, which repels *Diaphorina citri* and/or *Tryoza erytrae* psyllid insects, so as to control the HLB disease of citrus plants, wherein the at least one sesquiterpene is selected from the group consisting of β-caryophyllene and α-copaene, or combinations thereof. In this method, the at least one purified sesquiterpene is purified from an organism selected from the group consisting of plants, bacteria and yeasts. In a preferred embodiment, the at least one purified sesquiterpene is purified from the guava plant, preferably, the leave extracts of the guava plant.

In some preferred embodiments, the at least one sesquiterpene is applied to the citrus plants through slow delivery systems as those already used by entomologists to deliver pheromones.

BRIEF DESCRIPTION OF THE FIGURES

FIGs. 1A and B show the nucleotide sequences of SEQ ID NOs:1 and 3, and protein sequences of SEQ ID NOs:2 and 4.

FIG. 2 shows volatile terpenes emitted (upper chromatogram) and extracted (lower chromatogram) from guava leaves.

FIG. 3 shows representative gas chromatographic separation of volatiles emitted from the leaves of different citrus genotypes.

FIG. 4 shows representative gas chromatographic separation of volatiles emitted from guava leaves at different developmental stages: A, flush; B, young; C, mature; D, old.

FIG. 5A shows representative total ion chromatogram of sesquiterpene products of SEQ ID NO:2 . Assays were conducted with crude protein extracts from in vitro expression with farnesyl diphosphate as substrate. Peak 2 corresponds to an internal standard. FIG. 5**B**

shows comparision of TIC fragmentation pattern of background-corrected peak 1 and spectra of β-caryophyllene obtained from libraries.

FIG. 6 shows representative percentage and number of selections of *Diaphorina citri* to citrus volatiles ± guava odor (A) and to guava volatiles versus clean air (B) in two arms and four arms olfactometers, respectively.

FIG. 7 shows alignments of the amino acid sequences of beta-caryophyllene synthase QHS1 from Artemisia annua (Accession No. AAL79181), beta-caryophyllene synthase from Mikania micrantha (Accession No. ACN67535), beta-caryophyllene synthase from Cucumis sativus (Accession No. AAU05952), beta-caryophyllene/alpha-humulene synthase from Arabidopsis thaliana (Accession No. AAO85539), (E)-beta-caryophyllene synthase from Zea mays (Accession No. ABY79206) and (E)-beta-caryophyllene/beta-elemene synthase from Oryza sativa (Accession No. ABJ16553). Highlighted in black and highlighted in gray are respectively identical and similar residues in these sequences.

FIGs. 8A and **8B** show alignments of the amino acid sequences of beta-caryophyllene synthase QHS1 from Artemisia annua (Accession No. AAL79181), beta-caryophyllene synthase from Mikania micrantha (Accession No. ACN67535), beta-caryophyllene synthase from Cucumis sativus (Accession No. AAU05952), and beta-caryophyllene/alpha-humulene synthase from Arabidopsis thaliana (Accession No. AAO85539) (**A**), and alignments of the amino acid sequences of (E)-beta-caryophyllene synthase from Zea mays (Accession No. ABY79206) and (E)-beta-caryophyllene/beta-elemene synthase from Oryza sativa (Accession No. ABJ16553) (**B**). Highlighted in black and highlighted in gray are respectively identical and similar residues in these sequences.

FIG. 9 shows alignments of the amino acid sequences of beta-caryophyllene synthase QHS1 from Artemisia annua (Accession No. AAL79181), beta-caryophyllene synthase from Mikania micrantha (Accession No. ACN67535), beta-caryophyllene synthase from Cucumis sativus (Accession No. AAU05952), (E)-beta-farnesene synthase from Citrus junos (Accession No. AAK54279), terpene synthase from Citrus junos (Accession No. AAG01339), putative terpene synthase from Citrus x paradisi (Accession No. AAM00426), and beta-caryophyllene/alpha-humulene synthase from Arabidopsis thaliana (Accession No. AAO85539). Highlighted in black and highlighted in gray are respectively identical and similar residues in these sequences. Primers designed for amplifying beta-caryophyllene synthase genes are underlined.

FIG. 10 shows partial DNA sequence corresponding to citrus sesquiterpene synthase (SEQ ID NO:3) and primers designed for rapid amplification of its 5′- and 3′- ends employing RACE methodology.

FIG. 11 shows alignments of the nucleotide and amino acid sequences of farnesyl pyrophosphate synthase 1 (FPPS1) from Arabidopsis thaliana (Accession No. X75789). Underlined are sequences used to design primers for amplifying a full-length cDNA encoding a functional FPP synthase gene.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to methods for controlling HLB based on the observation that sesquiterpenes produced by the guava leaves repel the HLB vectors, *Diaphorina citri* and/or *Tryoza erytrae* psyllid insects.

A terpene is an unsaturated hydrocarbon based on an isoprene unit (C_5H_8), which may be acyclic or cyclic. A sesquiterpene is a terpene based on a C_{15} structure.

The guava, like other aromatic plants or essential-oil-plants, accumulates large amounts of sesquiterpenes in their leaves. Typically, sesquiterpenes such as β-caryophyllene and α-copaene represents 50-60% of the total amount of volatiles emitted by guava leaves. In guava plants, the sesquiterpenes are often synthesized and accumulated in specialized anatomical structures, glandular trichomes or secretory cavities, localized on the leaves and stems surface. The sesquiterpenes accumulated in the plants can be extracted by different means such as steam distillation that produces the so-called essential oil containing the concentrated sesquiterpenes.

In certain embodiments of the invention, as a method to control HLB, at least one sesquiterpene extracted from guava plants, preferably β-caryophyllene, is applied to the citrus plants through spray or slow delivery systems. Slow delivery systems have been already used by entomologists to deliver pheromones. In such systems, pure β-caryophyllene is disposed in a PVC resin that preserves and releases the chemical compound. This resin is applied directly to citrus trees in the orchards. It has the property of releasing the compound over a period of 3-4 months.

The price and availability of the plant natural extracts are dependent on the abundance, the oil yield and the geographical origin of the plants. Because of the complexity of their structure, production of individual sesquiterpene molecules by chemical synthesis is often limited by the cost of the process and may not always be chemically or financially feasible.

Therefore, a biochemical route for the production of sesquiterpene molecules would be of great interest. The engineering of a biochemical route for the production of sesquiterpene molecules requires a clear understanding of the biosynthesis of sesquiterpenes and the isolation of the genes encoding enzymes involved in specific biosynthetic steps.

The biosynthesis of sesquiterpenes in plants has been extensively studied. The common five-carbon precursor to all terpenes is isopentenyl pyrophosphate (IPP). Most of the enzymes catalyzing the steps leading to IPP have been cloned and characterized. Two distinct pathways for IPP biosynthesis coexist in the plants. The mevalonate pathway is found in the cytosol and endoplasmic reticulum and the non-mevalonate pathway (or deoxyxylulose 5-phosphate (DXP) pathway) is found in the plastids. In the next step IPP is repetitively condensed by prenyl transferases to form the acyclic prenyl pyrophosphate terpene precursors for the sesquiterpenes, farnesyl-pyrophosphate (FPP). These precursors serve as substrate for the sesquiterpene synthases, which catalyze complex multiple step cyclizations. Thus, the first committed step of β-caryophyllene biosynthesis is the cyclization of the universal sesquiterpene precursor FPP by a sesquiterpene synthase. A set of genes encoding β-caryophyllene synthases has been cloned from plants. These synthases are usually involved in the production of different sesquiterpenes. For example, a single protein encoded by a β-caryophyllene synthase gene from Arabidopsis is capable of converting FPP into the sesquiterpene products (-)-α-copaene, α-humulene, and (-)-(E)-β-caryophyllene (Chen et al., Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers, Plant Cell 15: 481-494 (2003); Tholl et al., Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from *Arabidopsis* flowers, The Plant J. 42: 757-771 (2005)). A β-caryophyllene synthase from maize produces δ-elemene, α-humulene, and (-)-(E)-β-caryophyllene (Kollner et al. A maize (*E*)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties, The Plant Cell 20:482-494 (2008)). β-caryophyllene synthases have been also isolated from *Artemisia annua* (Cai et al., A cDNA clone for β-caryophyllene synthase from *Artemisia annua*, Phytochem 61:523-529 (2002)), cucumber (Mercke et al., Combined transcript and metabolite analysis reveals genes involved in spider mite induced volatile formation in cucumber plants, Plant Phys. 135: 2012-2024), rice (Cheng et al., The rice (*E*)-β-caryophyllene synthase (OsTPS3) accounts for the major inducible volatile sesquiterpenes, Phytochem 68: 1632-1641 (2007)) and oregano (Degenhardt et al., Restoring a maize root

signal that attracts insect-killing nematodes to control a major pest, PNAS 106:13213-218 (2009)).

Chimeric genes encoding sesquiterpene synthases have been used to genetically transform plants with the aim to attract/repel pollinators (Kessler et al., Field experiments with transformed plants reveal the sense of floral scents, Science 321: 1200-1202 (2008)), to attract pest-killing insects (Kappers et al., Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis, Science 309: 2070-2072 (2005); Schnee et al., The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores, PNAS 103: 1129-1134 (2006)) and nematodes (Degenhardt et al., Restoring a maize root signal that attracts insect-killing nematodes to control a major pest, PNAS 106:13213-218 (2009)), and directly to kill insect pests (Wu et al., Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants, Nature Biotechnology, 24: 1441-1447 (2006)), indicating that transgenic strategies addressed to increase the accumulation of such sesquiterpenes, including β-caryophyllene, could be an ecologically sound alternative, that alone or in combination with a reduced use of pesticides, would prevent damages caused by pest-transmitted devastating diseases.

One embodiment of the present invention relates to the isolation of nucleic acids encoding for sesquiterpene synthases from citrus plants, citrus-related plants belonging to the Rutaceae family, guava, guava-related plants from the Myrtaceae family, or any other living organism. As used herein, a sesquiterpene synthase is any enzyme that catalyzes the synthesis of a sesquiterpene. One can determine whether a polypeptide encoded by a nucleic acid of the invention has sesquiterpene synthase activity by the enzyme characterization assay described in the examples herein.

In an embodiment of the nucleic acid of the invention, the nucleic acid is chosen from (a) a nucleic acid comprising the nucleotide sequence substantially as set out in SEQ ID NO:1; or (b) a nucleic acid encoding the polypeptide substantially set out in SEQ ID NO:2, wherein the polypeptide encoded by said nucleic acid has sesquiterpene synthase activity.

In another embodiment of the nucleic acid of the invention, the nucleic acid is chosen from (a) a nucleic acid comprising the nucleotide sequence substantially as set out in SEQ ID NO:3; or (b) a nucleic acid encoding the polypeptide substantially set out in SEQ ID NO:4, wherein the polypeptide encoded by said nucleic acid has sesquiterpene synthase activity.

Preferably, a nucleic acid and/or polypeptide of the invention is isolated from citrus plants. In an embodiment, the nucleic acid is isolated from guava plants.

Preferably, the nucleic acid according to the invention comprises SEQ ID NO:1.

In a particular embodiment, the invention relates to certain isolated nucleotide sequences including those that are substantially free from contaminating endogenous material. The terms "nucleic acid" or "nucleic acid molecule" refer to a deoxyribonucleotide or ribonucleotide polymer in either single-or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides. A "nucleotide sequence" also refers to a polynucleotide molecule or oligonucleotide molecule in the form of a separate fragment or as a component of a larger nucleic acid. Some of the nucleic acid molecules of the invention are derived from DNA or RNA isolated at least once in substantially pure form and in a quantity or concentration enabling identification, manipulation, and recovery of its component nucleotide sequence by standard biochemical methods. Examples of such methods, including methods for PCR protocols that may be used herein, are disclosed in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989), Current Protocols in Molecular Biology edited by F. A. Ausubel et al., John Wiley and Sons, Inc. (1987), and Innis, M. et al., eds., PCR Protocols: A Guide to Methods and Applications, Academic Press (1990).

As described herein, the nucleic acid molecules of the invention include DNA in both single-stranded and double-stranded form, as well as the RNA complement thereof. DNA includes, for example, cDNA, genomic DNA, chemically synthesized DNA, DNA amplified by PCR, and combinations thereof. Genomic DNA, including translated, non-translated and control regions, may be isolated by conventional techniques, e.g., using any one of the cDNAs of the invention, or suitable fragments thereof, as a probe, to identify a piece of genomic DNA which can then be cloned using methods commonly known in the art. In general, nucleic acid molecules within the scope of the invention include sequences that hybridize to sequences of the invention at temperatures 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, or 30 °C below the melting temperature of the DNA duplex of sequences of the invention, including any range of conditions subsumed within these ranges.

In another embodiment, the nucleic acids of the invention comprises a sequence substantially as set out in SEQ ID NO:1 or SEQ ID NO:3. In one embodiment, the nucleic acids are at least 70%, at least 85%, at least 90%, or at least 95% identical to nucleotides SEQ ID NO:1 or SEQ ID NO:3, and each nucleic acid encodes a protein that has sesquiterpene synthase activity, as demonstrated, for example, in the enzyme assay described in the

examples, with increased stability and efficacy as compared with that of the polypeptide encoded by SEQ ID NO:1 or SEQ ID NO:3. In one embodiment, the nucleic acid comprises the nucleotide sequence SEQ ID NO:1. In another embodiment, the nucleic acid comprises the nucleotide sequence SEQ ID NO:3.

In yet another embodiment, the nucleic acid comprises a contiguous stretch of at least 50, 100, 250, 500, or 750 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:3. Such contiguous fragments of these nucleotides may also contain at least one mutation so long as the mutant sequence retains the functionality of the original sequence and the capacity to hybridize to these nucleotides under low or high stringency conditions, such as for example, moderate or high stringency conditions. Such a fragment can be derived, for example, from nucleotide (nt) 200 to nt 1700, from nt 800 to nt 1700, from nt 1000 to nt 1700, from nt 200 to nt 1000, from nt 200 to nt 800, from nt 400 to nt 1600, or from nt 400 to nt 1000 of SEQ ID NO:1 or SEQ ID NO:3.

As described above, polypeptides encoded by the nucleic acids of the invention are encompassed by the invention. The isolated nucleic acids of the invention may be selected from a nucleic acid encoding the polypeptide substantially set out in SEQ ID NO:2 or SEQ ID NO:4. In one embodiment, the polypeptides are at least 70 %, at least 85 %, at least 90 %, or at least 95 % identical to SEQ ID NO:2 or SEQ ID NO:4, and the polypeptides have sesquiterpene synthase activity, as demonstrated, for example, in the enzyme assay described below.

Due to the degeneracy of the genetic code wherein more than one codon can encode the same amino acid, multiple DNA sequences can code for the same polypeptide. Such variant DNA sequences can result from genetic drift or artificial manipulation (e.g., occurring during PCR amplification or as the product of deliberate mutagenesis of a native sequence). The present invention thus encompasses any nucleic acid derived from SEQ ID NO:1 or SEQ ID NO:3 capable of encoding a polypeptide substantially set out in SEQ ID NO:2 or SEQ ID NO:4.

Deliberate mutagenesis of a native sequence can be carried out using numerous techniques well known in the art. For example, oligonucleotide-directed site-specific mutagenesis procedures can be employed, particularly where it is desired to mutate a gene such that predetermined restriction nucleotides or codons are altered by substitution, deletion or insertion. Exemplary methods of making such alterations are disclosed by Walder et al. (Gene 42:133, 1986); Bauer et al. (Gene 37:73, 1985); Craik (BioTechniques, Jan. 12-19,

1985); Smith et al. (Genetic Engineering: Principles and Methods, Plenum Press, 1981); Kunkel (Proc. Natl. Acad. Sci. USA 82:488, 1985); Kunkel et al. (Methods in Enzymol. 154:367, 1987); and U.S. Pat. Nos. 4,518,584 and 4,737,462.

In one embodiment, the invention provides for isolated polypeptides. As used herein, the term "polypeptides" refers to a genus of polypeptide or peptide fragments that encompass the amino acid sequences identified herein, as well as smaller fragments. Alternatively, a polypeptide may be defined in terms of its antigenic relatedness to any peptide encoded by the nucleic acid sequences of the invention. Thus, in one embodiment, a polypeptide within the scope of the invention is defined as an amino acid sequence comprising a linear or 3-dimensional epitope shared with any peptide encoded by the nucleic acid sequences of the invention. Alternatively, a polypeptide within the scope of the invention is recognized by an antibody that specifically recognizes any peptide encoded by the nucleic acid sequences of the invention. Antibodies are defined to be specifically binding if they bind polypeptides of the invention with a K_a of greater than or equal to about $10^7\ M^{-1}$, such as greater than or equal to $10^8\ M^{-1}$.

A polypeptide "variant" as referred to herein means a polypeptide substantially homologous to a native polypeptide, but which has an amino acid sequence different from that encoded by any of the nucleic acid sequences of the invention because of one or more deletions, insertions or substitutions.

Variants can comprise conservatively substituted sequences, meaning that a given amino acid residue is replaced by a residue having similar physiochemical characteristics. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. See Zubay, Biochemistry, Addison-Wesley Pub. Co., (1983). The effects of such substitutions can be calculated using substitution score matrices such a PAM-120, PAM-200, and PAM-250 as discussed in Altschul, (J. Mol. Biol. 219:555-65, 1991). Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known.

Naturally-occurring peptide variants are also encompassed by the invention. Examples of such variants are proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the polypeptides described herein. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in

different types of host cells, due to proteolytic removal of one or more terminal amino acids from the polypeptides encoded by the sequences of the invention.

Variants of the sesquiterpene synthases of the invention may be used to attain desired enhanced or reduced enzymatic activity, modified regiochemistry or stereochemistry, or altered substrate utilization or product distribution. Furthermore, variants may be prepared to have at least one modified property, for example an increased affinity for the substrate, an improved specificity for the production of one or more desired compounds, a different product distribution, a different enzymatic activity, an increase of the velocity of the enzyme reaction, a higher activity or stability in a specific environment (pH, temperature, solvent, etc), or an improved expression level in a desired expression system. A variant or site direct mutant may be made by any method known in the art. As stated above, the invention provides recombinant and non-recombinant, isolated and purified polypeptides, such as from guava or citrus plants. Variants and derivatives of native polypeptides can be obtained by isolating naturally-occurring variants, or the nucleotide sequence of variants, of other or same plant lines or species, or by artificially programming mutations of nucleotide sequences coding for native guava or citrus polypeptides. Alterations of the native amino acid sequence can be accomplished by any of a number of conventional methods.

Accordingly, the present invention provides a method for preparing a variant functional sesquiterpene synthase, the method comprising the steps of (a) selecting any of nucleic acids from the group consisting of SEQ ID NO: 1 or 3, (b) altering the nucleic acid sequence to obtain a population of mutant nucleic acids, and, (c) transforming host cells with the mutant nucleic acid to express polypeptides, and, (d) screening the polypeptides for a functional polypeptide having at least one modified property. The modified property may be any desired property, for example the properties mentioned above. The alteration of the selected nucleic acid may be performed by random mutagenesis, site-specific mutagenesis or DNA shuffling, for example. The alteration may be at least one point mutation, deletion or insertion. For example, polypeptides having an amino acid sequence encoded by a nucleic acid obtained from shuffling techniques, involving at least any of SEQ ID NO: 1 or 3, are also encompassed by the present invention. The steps of the method according to this embodiment of the invention, such as screening the polypeptides for a functional polypeptide, are known to the skilled person who will routinely adapt known protocols to the specific modified property that is desired.

For example, mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion. Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene wherein predetermined codons can be altered by substitution, deletion or insertion. The present invention also encompasses nucleic acids obtained from altering a nucleic acid of the present invention, for example in order to obtain a variant polypeptide.

There are several methods known in the art for the creation of transgenic plants. These include, but are not limited to: electroporation of plant protoplasts, liposome-mediated transformation, *Agrobacterium*-mediated transformation, polyethylene-glycol-mediated transformation, microinjection of plant cells, and transformation using viruses. In one embodiment, direct gene transfer by particle bombardment is utilized. In another embodiment, *Agrobacterium*-mediated transformation is utilized.

Agrobacterium tumefaciens-mediated transformation is the most common method used to transform citrus plants. It uses a disarmed *A. tumefaciens* strain carrying the gene(s) of interest in its genome as a vector to transform citrus cells or tissues by cocultivation for a few days.

Genetic transformation is used naturally by *Agrobacterium* species as a system to insert fragments of their DNA in infected plant cells. Expression of the genes transferred by the bacteria in plant cells leads to the elicitation of crown gall tumors in plants usually at wound sites. In *A. tumefaciens* the transferred DNA is called T-DNA and it resides in a megaplasmid called Ti (from Tumor-Inducing) plasmid.

Disarmed Ti plasmids can be engineered by removing T-DNA genes involved in tumor formation. Then, heterologous genes with the appropriate regulatory regions can be inserted into a new chimeric T-DNA region that once incorporated in *Agrobacterium* cells carrying a disarmed Ti plasmid can be used to integrate and express the foreign genes in plant cells. From transformed plant cells it is possible to regenerate whole transgenic plants by using standard tissue culture systems.

Direct gene transfer by particle bombardment provides another example for transforming plant tissue. In this technique a particle, or microprojectile, coated with DNA is shot through the physical barriers of the cell. Particle bombardment can be used to introduce

DNA into any target tissue that is penetrable by DNA coated particles, but for stable transformation, it is imperative that regenerable cells be used. Typically, the particles are made of gold or tungsten. The particles are coated with DNA using either $CaCl_2$ or ethanol precipitation methods which are commonly known in the art.

DNA coated particles are shot out of a particle gun. A suitable particle gun can be purchased from Bio-Rad Laboratories (Hercules, Calif.). Particle penetration is controlled by varying parameters such as the intensity of the explosive burst, the size of the particles, or the distance particles must travel to reach the target tissue.

The DNA used for coating the particles may comprise an expression cassette suitable for driving the expression of the gene of interest that will comprise a promoter operably linked to the gene of interest.

Methods for performing direct gene transfer by particle bombardment are disclosed in U.S. Pat. No. 5,990,387 to Tomes et al. In one embodiment, transfected DNA is integrated into a chromosome of a non-human organism such that a stable recombinant systems results. Any chromosomal integration method known in the art may be used in the practice of the invention, including but not limited to, recombinase-mediated cassette exchange (RMCE), viral site specific chromosomal insertion, adenovirus, and pronuclear injection.

Other than in the operating example, or where otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should be construed in light of the number of significant digits and ordinary rounding approaches.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements. The following examples are intended to illustrate the invention without limiting the scope as a result. The percentages are given on a weight basis.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice of the present invention, exemplary methods and materials are described for illustrative purposes. All publications mentioned in this application are incorporated by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

Additionally, the publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates, which may need to be independently confirmed.

EXAMPLES

The following examples are intended to illustrate the preferred embodiments of the invention without limiting the scope as a result.

Material

Citrus plants used in the present examples were obtained from The Citrus Germplasm Bank (Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain). Guava seeds were obtained from local growers from Vietnam and Brazil and were grown in a green house at the IVIA. Other available sources of Guava mature plants were Politechnical University and Botanical Garden from Valencia (Spain).

Example 1

Analysis of Leaf Volatile Content and Emission from Citrus and Guava Plants

Volatile content and emission was studied in leaves from different genotypes (*Psidium guajava*, *Citrus sinensis*, *Citrus aurantifolia* and *Citrus clementina*). Analyses were conducted using leaves at different developmental stages, collected at different hours of the day and in different seasons. Each sample was analyzed at least four times (two biological replicates plus two technical replicates).

To determine volatile content, collected leaf tissue was immediately frozen in liquid nitrogen and stored at -80 °C until extraction. Freeze ground material (200 mg) was weighted

in screw-cap Pyrex tubes and immediately 3 mL of cold pentane was added. Samples were homogenized on ice for 30 s with a Yellowline homogenizer (model DI 25). The suspension was vortexed for 15 s and 3 mL of Milli-Q water were added, further vortexed for 30 s and centrifuged at 1800 g for 10 min at 4 °C. The organic phase was recovered with a Pasteur pipette and the aqueous phase re-extracted two times more with 3 mL of pentane. An aliquot of 2 μL of the pooled organic phases was directly injected into the GC-MS for volatile analysis (see below).

As headspace analysis gives a more realistic picture of the volatile profile emitted by plants and detected by insects that respond to plant volatiles, static headspace sampling with a solid phase microextraction (SPME) device was performed. Leaf samples were enclosed in 50 mL screw-cap Pyrex tubes carrying a septum on the top and containing 1 mL of milli-Q water for avoiding leaf hydric stress. SPME fiber (100 μm poly(dimethyl) siloxane, Supelco, Bellefonte, PA) was exposed, at a controlled temperature of 22 °C, between 1 and 4 hours. Immediately afterwards, fiber was transferred to GC injector (220 °C) and thermal desorption was prolonged to 4 mins.

Results showed that main compounds identified through volatile content were also the major volatiles emitted by leaves (for a representative example, see Fig. 2). As described in the literature, more than 80% of total volatiles contained and emitted by citrus leaves were monoterpenes, with linaool being the most abundant one in all the genotypes and sampling dates analysed (Fig. 3). In these samples, β-caryophyllene was not detected or detected at very low levels and not in all the replicates. In guava leaves, sesquiterpenes were the predominant volatiles and, in all the samples analysed, β-caryophyllene constituted at least 50% of total compounds (Fig. 4).

Example 2

GC-MS Analysis

A Thermo Trace GC Ultra coupled to a Thermo DSQ mass spectrometer with electron ionization mode (EI) at 70 eV was used. The ion source and the transfer line were at 200 and 260 °C, respectively. Volatile compounds were separated on a HP-INNOWax (Agilent J&C Columns) column (30 m x 0.25 mm i.d. x 0.25 μm film). The column temperatures were programmed from 40 °C for 5 min, raised to 150 °C at 5 °Cmin^{-1}, then raised to 250 °C at 20 °Cmin^{-1} and held 2 min at 250 °C. The injector temperature was 220 °C. Helium was the carrier gas at 1.5 mL min^{-1} in the splitless mode. Electron impact mass spectra were recorded

in the 30-400 amu range with a scanning speed of 0.5 $scans^{-1}$. Compounds were identified by matching of the acquired mass spectra with those stored in reference libraries (NIST, MAINLIB, REPLIBT) or from authentic standard compounds when available.

Example 3

Response of *D. citri* to Guava Volatiles

A series of experiments were designed in order to investigate the effect of guava leaf volatiles on *D. citri* behaviour. *D. citri* adults of mixed sex were obtained from continuously reared cultures on *C. limonia* seedlings mantained in Fundecitrus (Araraquara, Brazil) at 25 ± 1 °C, 70 ± 10 relative humidity and a L14:D10 photoperiod.

A Y-tube olfactometer with one 12.5 cm long entrance arm and two 21.0 cm long test arms (0.6 cm inner diameter) was used for behavioural assays. Charcoal cleaned air (granulated 4-8 mm. Applichem GmbH, Darmstadt, Germany) was pumped (0.4 L min^{-1}) through two glass jars of 2 L containing the volatile sources, consisting of air or guava seedlings. 40 psyllids were assayed in each experiment, and each experiment was conducted at least per triplicate. Position of psyllids was determined after 10 min and those which had passed 10.0 cm after branching were recorded as 'responsive'. Results showed that guava volatiles repelled or immobilized psyllids or limited citrus attractivenes (Fig. 6A). Further studies were conducted employing a four-arm olfactometer. Charcoal cleaned air was humidified by passage through a glass cylinder filled with water and separated into four flows (0.4 L min^{-1}) each of which was directed to one of the four olfactometer fields. The air flowing into the test field of the olfactometer passed through a glass cylinder (1 L) containing the samples or clean air. 40 psyllids were assayed in each experiment, and each experiment was conducted at least per triplicate. Results from guava versus clean air clearly indicated that guava volatiles had a repellent effect (Fig. 6B).

Effect of pure β-caryophyllene (puriss >98.5 %; Sigma-Aldrich, Germany) and α-copaene (puriss >90.0 %; Sigma-Aldrich, Germany) on *D. citri* was also evaluated employing a four-arm olfactometer. In each observation, a psyllid was placed in the middle of the olfactometer permeated by air coming from each of its four stretched-out arms. In two of the arms clean air was pumped, while in the remaining arms air was pumped through glass vials containing 10 µL of pure compounds. Each observation lasted five min, and the first and final choices of every individual psyllid were recorded. At least 29 psyllids were employed for studying behavioral response to each compound. In the preliminary test with clean air, no

significant differences were obtained between the arms, indicating that all of them were equivalent and did not introduce any bias during choice tests. Assays conducted with β-caryophyllene and α-copaene showed a higher level of psyllids entering the unscented arm, envisaging the repellent effect of these compounds (Table 1).

Table 1

	β-caryophyllene	Clean air	χ^2	*p*	n
First choice	42.3%	57.7%	1.23	0.4054	29(26)
Final choice	32.0%	68.0 %*	6.48	**0.0237**	29(25)
	α-copaene	**Clean air**	χ^2	***P***	**n**
First choice	42.0%	58.0%	0.76	0.4862	34(33)
Final choice	33.0%	67.0%**	7.33	**0.0138**	34(33)

Table 1: Representative results from Y-tube olfactometric assays. Percentage of individuals that chose each compound in four-arm olfactometer trials examining responses of psyllids to treatment odor sources versus a clean air control. n: total sample size (with number of individuals that made a choice in parentheses). Data were compared using chi-square test (*$p < 0.05$; ** $p < 0.01$).

These results have been reproduced when lower doses of the pure compounds were used in olfactometric assays.

Example 4

Isolation of Total RNA

Leaves were collected from Citrus and Guava plants, immediately frozen in liquid nitrogen and grounded using a mortar and pestle. Total RNA was extracted using the Qiagen RNeasy Mini. As described by Keszei et al. (Phytochem. 71: 844-852. 2010) the extraction buffer was modified by adding 2 % PVP (Sigma-Aldrich) and 120 mg/mL of sodium-isoascorbate (to saturation) (Sigma-Aldrich) to inhibit the oxidative conjugation of phenolics to the RNA. Typically, an average of 100 µg total RNA was obtained from 200 mg of grounded tissue. The concentration of RNA was estimated from the OD at 260 nm. The

integrity of the RNA was evaluated on an agarose gel by verifying the integrity of the ribosomic RNA bands.

Example 5

Reverse Transcription (RT) and PCR amplification

Synthesis of cDNAs was performed with 1 μg of total RNA. RT was carried out in the presence of 500 ng of oligo-dT and 200 units of SuperScript II Reverse transcriptase (Gibco BRL, Germany). Samples were incubated 5 min at 65 °C, 50 min at 42 °C and 15 min at 70 °C. For PCR amplification primers containing start and stop codons of SEQ ID NO:1 and SEQ ID NO:3 were designed (Table 2).

Table 2

	Primer	Primer sequence (5'→ 3')	Orientation
SEQ ID NO:5	B27F	CACCATGTCCGCTCAAGTTC	S
SEQ ID NO:6	B28R	TCAGATGGTAACAGGGTCTC	AS
SEQ ID NO:7	B27F	GCATGAGGGATGTTAAGAG	S
SEQ ID NO:8	B28R	CTGTTTTCTTTGAAGACTAGGC	AS

S, Sense; AS, Antisense.

Table 2: primers employed for amplifying cDNAs corresponding to sequences SEQ ID NO:5 (B25F) and SEQ ID NO:6 (B26R), SEQ ID NO:7 (B27F) and SEQ ID NO:8 (B28R). The start and stop codons are underlined.

The thermal cycler conditions were: 5 min at 95 °C; 35 cycles of 30 sec at 94 °C, 30 sec at 56 °C and 150 sec at 72 °C; and finally 10 min at 72 °C. The sizes of the PCR products were evaluated on a 1% agarose gel. The bands corresponding to the expected size were excised from the gel, purified using the QIAquick™ Gel Extraction Kit (Qiagen) and cloned in the pTZ57R/T (Fermentas GMBH). Inserted DNA fragments were then subject to DNA sequencing and the sequence compared against the GenBank non-redundant protein database (NCBI) using the BLASTX algorithm (Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403-410).

Example 6

Construction of Expression Plasmids for Sesquiterpene Synthase Activity Assay

Each expression cassette prepared comprises a transcription initiation or transcriptional control region (s) (e.g. a promoter), the coding region for the protein of interest, and a transcriptional termination region. In order to avoid possible toxicity of the sesquiterpene compound when it is accumulated in a microbial host, an inducible promoter was selected. For functional expression of the sesquiterpene synthases, the cDNAs were sub-cloned in the pF3K WG (BYDV) Flexi® Vector from Promega, designed for in vitro expression of proteins. This vector carries the lethal barnase gene, which is replaced by the DNA fragment of interest and acts as a positive selection for successful ligation of the insert, and a kanamycin resistance gene for selection of bacterial colonies. Additionally, it allows the directional cloning of PCR products in SgfI and PmeI restriction sites. In this plasmid the cDNA is placed downstream of the SP6 promoter.

Inserts were amplified by PCR using an amino-terminal PCR oligonucleotide including the start codon and SgfI site and a carboxy-terminal PCR primer including the stop codon and PmeI site (Table 3). In the forward primer six in-frame histidine codons where added in order to create a N-terminal tagged protein. The amplified cDNAs were purified, and ligated into pF3K WG (Promega) plasmid according to the manufacturer protocol. Constructs were verified by digestion and DNA sequencing.

All amplifications of cDNA for expression were performed using the Pfu DNA polymerase (Promega), in a final volume of 50 μL containing 5 μ L of Pfu DNA polymerase 10X buffer, 200 μM each dNTP, 0.4 μM each forward and reverse primer, 2.9 units Pfu DNA polymerase and 5 μL of 1μ L of cDNA (prepared as described above). The thermal cycling conditions were as follows: 2 min at 95°C; 25 cycles of 30 sec at 95 °C, 30 sec at 52 °C and 4 min at 72 °C; and 10 min at 72°C. The PCR products were purified on an agarose gel and eluted using the QIAquick® Gel Extraction Kit (Qiagen).

Table 3

Primer	Primer sequence (5'→ 3')	Orientation
SQS5F	*GCGATCGCC*ATG**CATCACCATCACCATCAC**GGATCCGCTCAAGTTCTAGC (SEQ ID NO:9)	S
SQS5R	*GTTTAAAC*TCAGATGGTAACAGGGTCTCT (SEQ ID NO:10)	AS

Table 3: primers employed for cloning SEQ ID NO: 1 in pF3KW Flexi Vector (Promega) corresponding to sequence SEQ ID NO:9 (SQS5F) and SEQ ID NO:10 (SQS5R). S, Sense; AS, Antisense. Restriction sites are marked in itallics. Nucleotides encoding HHHHHH are bold-lettered. The start and stop codons are underlined.

Example 7

Sesquiterpene Synthase Expression and Enzyme Assays

In a standard protein expression experiment, the expression plasmids containing the sesquiterpene synthase cDNA as well as the empty plasmid (negative control) were transformed into XL1-Blue *E. coli* cells (Stratagene). Single colonies of transformed *E. coli* were used to inoculate 5 ml LB medium. Liquid cultures of the bacteria harboring the expression construct or the empty plasmid were grown at 37 °C to an OD_{600} of 0.8. Plasmid DNA was isolated from bacteria using QIAprep spin miniprep kit (Qiagen) following manufacturer instructions. 2 μg of purified plasmid DNA were employed for in vitro transcription/translation in wheat germ extract cell free expression system from Promega (TnT® SP6 High-yield wheat germ protein expression system) in a final volume of 50 μL. Following incubation at 25 °C for 2 hours protein production was confirmed analyzing an aliquot of 1 μL by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting following standard procedures. Nitrocellulose membranes were probed with monoclonal Anti His G HRP antibody from Invitrogen.

The enzymatic assays were performed in 15 mL Teflon sealed glass tubes using 49 μL of protein extract in a final volume of 1 mL reaction buffer (25 mM potassium phosphate buffer pH 6.8; 10 mM MgCl2; 1mM MnSO4; 1 mM DTT) supplemented with 100 μM FPP (Sigma). The medium was overlaid with 1 ml pentane to trap volatile products and the tubes incubated over-night at 30 °C. The pentane phase, containing the sesquiterpene, was recovered and the medium extracted with a second volume of pentane. 2 μL of the combined pentane

fractions were analyzed by Gas Chromatography as described above. In this way, caryophyllene synthase activity was attributed to sequence SEQ ID NO:2 (Fig. 5).

Example 8

Isolating Partial Sequences Corresponding to Sesquiterpene Synthases Using RT-PCR

The deduced amino-acid sequences of plant β-caryophyllene synthases were aligned to identify conserved regions and design plant β-caryophyllene synthase-specific oligonucleotides (Fig. 7). Aminoacid sequences from plant sesquiterpene synthases with demonstrated β-caryophyllene synthase activity were obtained from the NCBI database. Homology analysis showed low (around 21%) protein identity between known plant β-caryophyllene synthases. However, higher homology was found if protein sequences were separated in two groups (Fig. 8). The first group (Fig. 8A) contained the sequences of the β-caryophyllene synthases from dicotyledonous plants, as *Arabidopsis* (Chen et al., Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers, Plant Cell 15: 481-494 (2003); Tholl et al. Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from *Arabidopsis* flowers, The Plant J. 42: 757-771 (2005)), cucumber (Mercke et al Combined transcript and metabolite analysis reveals genes involved in spider mite induced volatile formation in cucumber plants. Plant Phys. 135: 2012-2024), *Mikania Micrantha* (Wang et al. Cloning, expression and wounding induction of β-caryophyllene synthase gene from *Mikania micrantha* H.B.K. and allelopathic potential of β-caryophyllene, Alleophaty J. 24:35-44 (2009)) and *Artemisia annua* (Cai et al. A cDNA clone for β-caryophyllene synthase from *Artemisia annua*. Phytochem 61:523-529 (2002)). The second group (Fig. 8B) contained sequences of β-caryophyllene synthases from monocotyledonous plants as maize (Kollner et al. A maize (*E*)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most american maize varieties, The Plant Cell 20:482-494 (2008)), and rice (Cheng et al. The rice (*E*)-β-caryophyllene synthase (OsTPS3) accounts for the major inducible volatile sesquiterpenes. Phytochem 68: 1632-1641 (2007). β-caryophyllene synthases from monocotyledonous presented an overall identity of 47%, *M. Micrantha* and *Artemisia annua* β-caryophyllene synthases showed 58 % homology, while that from *Arabidopsis* and cucumber showed low identity (around 17%) with the rest of amino acid sequences. In order to gain insight into citrus sesquiterpene synthases, amino acid sequences from the NCBI database were aligned with β-caryophyllene synthases from

dicotyledonous plants (Fig. 9). Sequences employed for analysis were (E)-β-farnesene synthase from *Citrus junos* (AAK54279), putative terpene synthase from *Citrus junos* (AAG01339), putative terpene synthase from *Citrus paradisi* (AAM00426), and valencene synthase from *Citrus sinensis* (AAQ04608). Metal ion-binding motif (DDxxD) and RRx_8W domain, both characteristic of plant sesquiterpene synthases (both mono- and dicotiledonous) were identified in all the peptidic sequences. In addition, other conserved amino acids were identified, localized mostly in the central region of the sequences. Based on these conserved regions among plants, degenerated primers from β-caryophyllene synthases and citrus sesquiterpene synthases were designed (underlined in Fig. 9) in order to isolate β-caryophyllene synthase from guava plants. Detailed sequence of these primers is provided in Table 4. The highest sequence homology was found in the central part of the sequences. Three regions containing sufficiently conserved amino acids were selected and degenerated oligonucleotides specific for these regions were designed (i.e. four forward (CSF1A, CSF1B, CSF2, CSF3) and three reverse primers (CSR3, CSR2, CSR1) were deduced) (Table 4). Partial sequences were amplified, cloned and sequenced following standard procedures. On the basis of these sequences, new primers were designed and amplification of 5′ and 3′ ends was performed employing the 5′/3′ RACE Kit (Roche, Mannheim, Germany) following the instructions of the manufacturer.

Table 4

	Primer	Primer sequence (5'→ 3′)	Orientation
SEQ ID NO:11	CSF1A	TKGGKGTRKCKTATCAYTTTGA	S
SEQ ID NO:12	CSF1B	GGAGTRKCMTAYCATTTTGAA	S
SEQ ID NO:13	CSF2	GGSATGTTAAGTTTGTAYGARGC	S
SEQ ID NO:14	CSF3	GATGAYACWTWTGACGCKTAYGG	S
SEQ ID NO:15	CSR3	CCRTAMGCGTCAWTWGTRTCATC	AS
SEQ ID NO:16	CSR2	TCCTCATTCATATCTTTCCA	AS
SEQ ID NO:17	CSR1	CTATCTCTTGCAAAAGG	AS

S, Sense; AS, Antisense. K: G or T; R: A or G; M: C or A; W: T or A; Y: T or C.

Table 4: CSF1A, CSF1B, CSF2, CSF3 and CSR1, CSR2 and CSR3 are primers designed based on conserved motifs in order to amplify partial sequences corresponding to

sesquiterpene synthases. FPPF and FPPR, are primers designed for amplifying FPP synthase from *Arabidopsis thaliana.*

Example 9

Isolation Of Full-Length Sequences Corresponding To Sesquiterpene Synthase genes. 3'- and 5'-RACE

Synthesis of first-strand cDNAs from leaf tissues and PCR amplifications were performed as described above. Combinations of sense and antisense primers detailed in Table 3 were used for PCR. PCR products were ligated into pTZ57R/T (Fermentas GMBH) and sequenced. Sequence information allowed rapid amplification of cDNA ends by RACE-PCR strategy, using the 5′/3′ RACE Kit (Roche, Mannheim, Germany) according to the manufacturer instructions.

For example, CSF1B and CSR1 amplified a band of 621 bp with high homology to sesquiterpene synthase genes from NCBI database. On basis of this sequence, new specific primers were designed for 5′-end (B62R and B63R) and 3′-end (B61F) amplification (Fig. 10). For 5′-end amplification, first strand cDNA was synthesized using the specific primer CSR1 (Table 4). A homopolymeric A-tail was added to the 3′end of purified cDNA and then it was PCR amplified using the gene specific primer B62R and specific oligo-dT anchor primer. A band of about 800 bp was amplified, gel-purified QIAquick Gel Extraction Kit (Qiagen) and further amplified by a second PCR using the nested gene specific primer B63R and the PCR anchor primer. The amplification product (700 bp) was purified from agarose gel, ligated into pTZ57R/T (Fermentas GMBH) and sequenced. For 3′- end amplification, cDNA was synthesized using oligo dT-anchor primer, and PCR amplification was performed with PCR anchor primer and the gene specific primer B61F. The amplification product (900 bp) was purified from agarose gel, ligated into pTZ57R/T (Fermentas GMBH) and sequenced.

Obtained sequences were first compared against the GenBank non-redundant protein database (NCBI) using the BLASTX algorithm (Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403-410) and then compared against the initial DNA sequence to ensure that significant DNA sequence overlap was obtained.

Example 10

Cloning of FPPS and Transit Peptides

With the aim of further increasing sesquiterpene content and emission in transgenic citrus plants, a FPP synthase gene cassette was introduced in binary plasmids for plant transformation (see below). A similar strategy was adopted by Wu et al. (Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nature Biotech 24: 1441-1447(2006)) who employed transgenic co-expression of FPP synthase and amorphadiene synthase in plastids for successful, high-level synthesis of amorphadiene in transgenic tobacco (*Nicotiana tabacum*) plants. To date, no FPP synthase has been isolated from citrus fruits, although one nucleotide sequence from CFGP (Citrus Functional Genomic Project) database, namely acL9351contig1, presents high identity (78%; 87% homology) with a FPP synthase from *Arabidopsis* (Acc. N° X75789). In order to select a FPP synthase gene, a search in public NCBI database was performed. FPP synthase protein is highly conserved among dicotyledonous plants. For example, FPP synthase proteins from such unrelated species as *Arabidopsis* (Acc. N° Q09152), *Malus domestica* (Acc. N° AAM08927), *Vitis vinifera* (Acc. N° AAX76910), *Gossypum hirsutum* (Acc. N° CAA72793) and *Hevea brasilensis* (Acc. N° AAM98379) showed an overall identity of 79% (89% homology). Identity of FPP synthase proteins from mono and dicotyledonous plants is also high. For example, FPPS from *Zea mays* (Acc. N° AC634051) is 71% identical (85% similar) to that of *Arabidopsis*. Finally, the high degree of similarity between FPPS from plants and FPPS from other kingdoms, i.e. *Saccharomyces cerevisae* FPPS (Acc. N° EDN63217) is 66% homologous to that of *Arabidopsis*, suggest a good conservation of this protein along evolution. Thus, any FPPS from a dicotyledonous plant was though to be suitable for increasing FPP production in citrus plants. As that from *Arabidopsis* had been well characterized and its activity demonstrated (Cunillera et al. *Arabidopsis thaliana* contains two differentially expressed farnesyl diphosphate synthase genes. J. Biol. Chem 221: 7774-7780 (1996)), primers were designed based on AtFPS1 sequence (Acc. No. X75789) in order to amplify a full length cDNA encoding a functional FPP synthase (Fig. 11). Total RNA was extracted from *Arabidosis thaliana* (ecotype Columbia) leaves using the Qiagen RNeasy Mini Kit following the manufacturer instructions and cDNA was synthesized as described above. Amplification of cDNA was performed employing Pfu DNA polymerase (Promega) and the same reaction mix as described above. The thermal cycling conditions were: 2 min at 95 °C, 30 cycles of 30 sec and 95 °C, 30 sec at 50 °C and 1.5 min at 72 °C, and 10 min at 72 °C. PCR

product was purified from agarose gel with the QIAquick®-Gel extraction Kit (Qiagen), ligated into pTZ57R/T (Fermentas GMBH) and its identity confirmed by sequencing.

Besides this, shifting sesquiterpene biosynthesis from cytosol to plastids or mitochondria can improve results of metabolic engineering. First, this can avoid possible detrimental effects of sesquiterpene accumulation in cytosol and, second, avoiding competition for cytosolic pool of FFP by introduced sesquiterpene synthase and endogenous FPP-utilizing enzymes can provide a substantial substrate pool for this engineered pathway without compromising plant growth. This strategy has been successfully employed before by Wu et al. (Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nature Biotech 24: 1441-1447(2006)) who performed transgenic co-expression of FFP synthase and amorphadiene synthase in plastids for successful, high-level synthesis of amorphadiene in transgenic tobacco (*Nicotiana tabacum*) plants. Switching the subcellular localization of the introduced sesquiterpene synthase to the mitochondrion has been also previously employed by Kappers et al. (Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. Science 309: 2070-2072 (2005)), who reported that FPP is readily available in this organele for sesquiterpene biosynthesis.

The transit peptide (TP) of the small sub-unit of Rubisco (SSU) from *Pissum sativum* (Acc. No. X00806) was synthesised as three overlapping oligonucleotide fragments and subsequently PCR-amplified introducing NdeI and ClaI restriction sites in its 5′- and 3′- ends, respectively. The PCR product was ligated in pTZ57R/T (Fermentas GMBH) generating pTZ-TPssu plasmid and sequenced. The CoxIV sequence (from *Saccahromyces cerevisae*, Acc. No. X01048) was synthesized as two complementary oligonucleotide fragments and subsequently PCR-amplified introducing NdeI and ClaI restriction sites in its 5′- and 3′- ends, respectively. The PCR product was ligated in pTZ57R/T (Fermentas GMBH) generating pTZ-mtTP plasmid and sequenced.

Example 11

Expression Vector Construction

For generating the expression vectors, plasmids pMOG 180 (Mogen International, ampicillin resistance) and pROK binary vector (kanamycin resistance, Invitrogen), both carrying the constitutive CaMV 35S promoter and Nopaline synthase (NOS) terminator sequences were employed. The T-DNA from pROK also carried the Neomycin

Phosphotransferase II (NPTII) gene under the control of the NOS promoter and terminator sequences.

Cloned sesquiterpene synthases and FPPS1 were PCR-amplified from pTZ57R/T (Fermentas GMBH) adding convenient restriction sites for ligation of transit peptides (NdeI, ClaI) and in pMOG vector (BamHI), ligated again in pTZ57R/T (Fermentas GMBH) and sequenced, generating plasmids pTZ-CsCS+rest, pTZ-PgCS+rest, pTZ-CsCoS+rest, pTZ-PgCoS+rest and pTZ-FPPS+rest. All these plasmids and those carrying transit peptides (pTZ-TPssu and pTZ-mtTP, see example above) were 10-fold over digested with NdeI and ClaI restriction enzymes from Takara (Shuzo, Co. Ltd). Digested fragments of adecuate size were excised from agarose gel and purified using QIAquick®-Gel extraction Kit (Qiagen) following manufacturer instructions. After plasmids dephosphorilation with Antartic Phosphatase (New England Biolabs), ligation of fragments corresponding to both transit peptides was performed with T4-Ligase (Invitrogen), according to the manufacturer instructions. Correct orientation and maintenance of ORFs from inserts was checked by sequencing and digestion. The resulting plasmids harboring TPssu for plastid import were denominated as pTZ-TPssu-CsCS, pTZ-TPssu-PgCS, pTZ-TPssu-CsCoS, pTZ-TPssu-PgCoS and pTZ-TPssu-FPPS, while those with mitochondrial import signal were named pTZ-mtTP-CsCS, pTZ- mtTP-PgCS, pTZ- mtTP-CsCoS, pTZ- mtTP-PgCoS and pTZ- mtTP-FPPS. Plasmids with FPPS, harboring or not transit peptide, were 10-fold over digested with BamHI and ligated between CaMV 35S promoter and Nopaline synthase (NOS) terminator of pMOG 180 vector. Resulting plasmids were named pMOG-FPPS, pMOG-TPssu-FPPS and pMOG-mtTP-FPPS. Correct orientation and maintenance of ORFs from inserts was checked by sequencing and digestion. Cassettes from these three plasmids were PCR amplified with specific primers for CaMV 35S promoter and NOS terminator containing AseI restriction sites for subcloning purposes. PCR products were over-digested with AseI and ligated in pROK vector, generating pROK-FPPS, pROK-TPssu-FPPS and pROK-mtTP-FPPS vectors. ORFs from sesquiterpene synthase genes were amplified from pTZ-CsCS , pTZ-TPssu-CsCS, pTZ-mtTPCsCS, pTZ- CsCoS, pTZ-TPssu- CsCoS, pTZ-mtTP- CsCoS, pTZ-PgCS , pTZ-TPssu-PgCS, pTZ-mtTP-PgCS, pTZ-PgCoS, pTZ-TPssu-PgCoS and pTZ-mtTP-PgCoS with specific primers for pTZ57R/T plasmid containing XbaI restriction site.

Amplified products were gel-purified, over digested with XbaI (New England Biolabs) and ligated into expression vectors pROK, pROK-FPPS, pROK-TPssu-FPPS and pROK-mtTP-FPPS. Correct orientation and maintenance of ORFs from inserts was checked by

sequencing and digestion. Resulting plasmids expressing sesquiterpene synthase peptides directed to cytosol were named pROK-CsCs, pROK-CsCoS, pROK-PgCS, pROK-PgCoS. Resulting plasmids expressing FPPS plus a sesquiterpene synthase peptide, both directed to cytosol, were named pROK-FPPS-CsCs, pROK-FPPS -CsCoS, pROK-FPPS -PgCS, pROK-FPPS -PgCoS. Plasmids expressing sesquiterpene synthase peptides directed to chloroplast were named pROK-TPssu-CsCs, pROK-TPssu -CsCoS, pROK-TPssu -PgCS, pROK-TPssu - PgCoS, while those expressing sesquiterpene synthase peptides directed to mitochondrion were named pROK-mtTP-CsCs, pROK- mtTP-CsCoS, pROK- mtTP-PgCS, pROK- mtTP-PgCoS. Resulting plasmids expressing FPPS plus a sesquiterpene synthase peptide, both directed to chloroplast, were named pROK- TPssu -FPPS- TPssu -CsCs, pROK- TPssu -FPPS - TPssu -CsCoS, pROK -TPssu -FPPS -TPssu -PgCS, pROK- TPssu -FPPS - TPssu -PgCoS. Resulting plasmids expressing FPPS plus a sesquiterpene synthase peptide, both directed to mitochondrion, were named pROK- mtTP-FPPS- mtTP-CsCs, pROK- mtTP-FPPS- mtTP-CsCoS, pROK - mtTP-FPPS - mtTP-PgCS, pROK- mtTP- -FPPS - mtTP-PgCoS.

Each construct was incorporated by electroporation into *A. tumefaciens* competent cells. NPTII was used as selectable marker gene as its expression provided to plant cells resistance to aminoglycoside antibiotics, as kanamycin, neomycin, geneticin, and others.

Example 12

Transformation of Citrus Plants With a Sesquiterpene Synthase Gene from Guava or Citrus

The sweet orange (*Citrus sinensis* L. Osb.) varieties Valencia and Pera were transformed with a β-caryophyllene synthase gene or a α-copaene syntase gene from sweet orange or from *Psidium guajava,* under the control of the constitutive CaMV 35S promoter and Nopaline synthase (NOS) terminator.

The strain EHA 105 of *A. tumefaciens* was used in this example for transformation of mature citrus explants. Bacteria were cultured overnight in an orbital shaker at 28 °C and 200 rpm in Luria Broth (LB) medium containing the proper antibiotics to grow the binary systems. Bacterial cells were pelleted at 3500 rpm for 10 min, resuspended and diluted to 4×10^7 or 4×10^8 cells/ml in liquid inoculation medium, which consisted of MS salt solution, 0.2 mg/L thiamine hydrochloride, 1 mg/L pyridoxine hydrochloride, 1mg/L nicotinic acid, and 3% (w/v) sucrose, pH 5.7.

For the transformation of sweet orange mature tissues, buds were collected from trees maintained in a greenhouse and were grafted onto seedlings of a vigorous rootstock under

glasshouse conditions (18-27 °C), and newly elongated shoots were used as starting material. Stem pieces (20 cm in length) were stripped of their leaves and thorns disinfected for 10 min in a 2% (v/v) sodium hypochlorite solution and rinsed three times with sterile distilled water. Internodal stem segments (about 1 cm long) were cut transversely and incubated for 15 min in 10-cm-diameter plates containing 15 ml of the bacterial suspension in inoculation medium by gentle shaking. The infected explants were blotted dry on sterile filter paper and placed horizontally on plates with the cocultivation medium for a 3-day co-cultivation period. Inoculation medium consisted of 4.3 g/L MS salts, 10 mL/L vitamin stock solution, 30 g/L sucrose, pH 5.7. 10. Co-cultivation medium consisted of inoculation medium plus 2 mg/L 2,4-D, 2 mg/L IAA, 1 mg/L 2,i-P, 8 g/L agar, pH 5.7.

After co-cultivation, the explants were blotted dry with sterile filter paper and transferred to shoot regeneration medium (SRM), which consisted of MS salts, 0.2 mg/L thiamine hydrochloride, 1mg/L pyridoxine hydrochloride, 1 mg/L nicotinic acid, 3% (w/v) sucrose, 1% (w/v) agar, pH 5.7, plus 100 mg/L kanamycin for the selection of transgenic shoots, and 250 mg/L vancomycin and 500 mg/L cefotaxime to control bacterial growth. This medium was supplemented with 3 mg/L BAP. Cultures were maintained in the dark for 4 weeks at 26 ◦C and then were transferred to 16- h photoperiod, 45 μEm−2 s−1 illumination, and 26 ◦C.

Shoots regenerating from explants cultured in the kanamycin-containing selection medium were excised from the explants and cut in two pieces. The basal portion was PCR-assayed and, if the reaction was positive for the gene of interest, the apical part was grafted in vitro onto a nontransgenic decapitated in vitro-grown seedling. About 3–4 weeks after shoot-tip grafting, plantlets were again grafted on a vigorous seedling rootstock in a greenhouse at 18 to 27°C.

Methods for transforming mature citrus tissues are disclosed in U.S. Patent No. 6,103,955 to Peña et al. The transformation resulted in plants that produced high amounts of β-caryophyllene or α-copaene, constitutively. Three independently transformed lines were selected, based on that trait. Behavioral bioassays in olfactometers revealed that transgenic citrus plants did not attract but repelled citrus psyllids.

Example 13

Production of β-caryophyllene and α-copaene and its use to repel *Diaphorina citri* in the field through a slow-delivery system

β-caryophyllene is a widespread compound in the vegetable kingdom, i.e. it is present in many oleoresins of the majority of conifers of the *Pinaceae* family. However, this sesquiterpene is typically produced in low abundance in the host organism. The oil of the clove tree Eugenia caryophyllata (*Syzygium aromaticum*) contains β-caryophyllene in considerable amount and serves as a preparative source for the isolation of this compound. α-copaene is found as a minor component in the essential oils of leaves from various plant species, such as guava, while is abundant in roots and seeds of copaiba (*Copaifera officinalis* (Jacq) L.) and angelica (*Angelica archangelica* L.) (Jacobson et al. Optical isomers of α-copaene derived from several plant sources. J. Agric. Food Chem 35 (5): 798-800 (1987)).

Nowadays, despite using modern techniques, isolation of these sesquiterpenes from plant sources suffers from low yields and high consumption of natural resources (i.e. Quispe-Condori et al. Obtaining β-caryophyllene from *Cordia verbenacea* de Candolle by supercritical fluid extraction. J. of Supercritical Fluids 46:27-32 (2008), Jacobson et al. Optical isomers of α-copaene derived from several plant sources. J. Agric. Food Chem 35 (5): 798-800 (1987)). Furthermore, β-caryophyllene and α-copaene concentration in plants depends on factors difficult to control, such as weather conditions and plant diseases. On the other hand, although chemical synthesis of β-caryophyllene had been accomplished decades ago (Corey et al. Total synthesis of d,l-caryophyllene and d,l-iscaryophyllene. J. Am. Chem. Soc. 86:485-492 (1964)) it is still difficult to scale for industrial production.

Thus, this invention also provides methods for biosynthesis and metabolic engineering of β-caryophyllene and α-copaene with the goal of developing cost effective methods for stable production at large scale and with consistent quality.

An attractive alternative strategy is to engineer metabolic pathways for production of β-caryophyllene in a diverse host. Even a cell, which cannot synthesize sesquiterpenes, contains farnesyl pyrophosphate if it synthesizes steroids or terpenes. Since every cell contains at least either steroids or terpenes, theoretically almost all hosts are capable of synthesizing sesquiterpenes using the DNA sequences of the present invention as far as a suitable host-vector system is available. Host-vector systems are known, for example, for plants such as *Nicotiana tabacura*, *Petunia hybrida* and the like, microorganisms such as bacteria, for example *Escherichia coli*, *Zymornonas mobilis* and the like, yeasts, for example *Saccharomyces cerevisiae* and the like, and fungus (i.e. ascomycetes and basidiomycetes species).

Engineering of β-caryophyllene production in a heterologous host may require availability of its precursor (FPP) in sufficient amount in order to avoid competition for FPP pool by exogenous and endogenous FPP-utilizing enzymes that could result in detrimental effects. To that end different approaches would be efficient: (i) switching enzyme cellular compartmentation from cytosol to mitochondria or plastid employing known peptide transit signals (i.e. Kappers et al. Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. Science 309: 2070-2072 (2005)), (ii) co-expressing β-caryophyllene synthase and FPP synthase genes (i.e. Wu et al. Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nature Biotechnology 24: 1441-1447 (2006)), (iii) engineering the host with a heterologous MVA/MEP pathway (i.e. Yu et al. Molecular cloning and functional characterization of α-humulene synthase, a possible key enzyme of zerumbone biosynthesis in shampoo ginger (*Zingiber zerumbet* Smith). Planta 227:1291-1299 (2008)), (iv) increasing expression of HMGR, a putative rate-limiting step in the MVA pathway, (v) optimizing the flux through the MEP pathway, i.e. upregulation of DXR in transgenic peppermint plants resulted in a 50% increase of essential oil yield (Mahmoud and Croteau. Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. Proc. Natl. Acad. Sci. U. S. A. 98: 8915–8920 (2001)). Combinations of these approaches, i.e., coexpression of β-caryophyllene synthase and FPP synthase genes while diverting subcellular compartment of both enzymes could also be employed. In addition, optimization of the β-caryophyllene synthase gene to reflect host codon usage bias may increase β-caryophyllene production in some organisms.

In order to express the DNA sequences of the present invention in a host, it is necessary to insert the genes into a vector to introduce it into the host. Any of various known vectors can be used, such as pBIN19, pC2200, pROK or the like for plant cells (*Nicotiana tabacura, Petunia hybrida*); pUC19, pET101 or the like for *E. coli*; and *YEp13* or the like for yeast.

Furthermore, it is necessary to transcribe the DNA sequences of the present invention into mRNA in the host. For this purpose, a variety of promoters such as CaMV35S, NOS, TR1', TR2' (for plants); lac, Tc^r, CAT, trp (for *E. coli*); Tc^r, CAT (for *Zymomonas mobilis*); ADH1, GAL7, PGK, TRP1 (for yeast) and the like can be used in the present invention. In the case of prokaryotic hosts, it is necessary to place a ribosome-binding site (SD sequence in *E. coli*) several nucleotides-upstream from the initiation codon (ATG).

The transformation of the host with the vector thus obtained can be conducted by any appropriate method, which is conventionally used in the fields of genetic manipulation or cell biology. Appropriate publications or reviews can be referenced; for example, for the transformation of microorganisms, T. Maniatis, E. F. Fritsch and J. Sambrook: "Molecular Cloning A Laboratory Manual", Cold Spring Harbor Laboratory (1982).

Culturing conditions of the transformants are essentially the same as those commonly used for the nontransformed host. Yielding of the microbial cultures could be improved if β-caryophyllene is removed from bioreactors, i.e. using ion exchange resins or pervaporation, thus avoiding possible inhibitory effects on growing. β-caryophyllene can be recovered by different methods well known for those skilled in the art, for example, pervaporation of microbial cultures or leaves distillation.

Pure β-caryophyllene is disposed in a PVC resin that preserves and releases the chemical compound. This resin is applied directly to citrus trees in the orchards. It has the property of releasing the compound over a period of 3-4 months. PVC-resin dispensers for release rate studies are prepared by mixing 40% by weight vinyl chloride/vinyl acetate emulsion copolymer (Vinnol E5/65C, Wacker Chemicals Ltd., UK) with a 1:1 mixture of the plasticizers Cereclor (S45, ICI Ltd., UK) and di-(2-ethylhexyl)-phthalate (DEHP, Sigma Aldrich Ltd., UK). The chemical compounds of interest are include with antioxidant and UV screener Waxoline Black (WB, ICI Ltd., UK) that are added to the prepolymer as required, typically 1% each by weight. The prepolymer are mixed and degassed on a rotary evaporator for 1 h under vacuum, poured into a mould composed of two glass plates with suitable spacers (0.1 cm) and cured by heating to 150 °C for 15 min. The resulting PVC sheets are removed from the moulds and cut into 1 cm squares.

The embodiments and examples illustrated and discussed in this specification are intended only to teach those skilled in the art the best way known to the inventors to make and use the invention. The above-described embodiments of the invention may be modified or varied, without departing from the invention, as easily appreciated by those skilled in the art in light of the above teachings. Accordingly, all expedient modifications readily attainable by one of ordinary skill in the art from the disclosure set forth herein, or by routine experimentation therefrom, are deemed to be within the spirit and scope of the invention as defined by the appended claims.

THE CLAIMS

What is claimed is:

1. A method for controlling HLB in citrus plants comprising expressing at least one gene encoding a polypeptide having sesquiterpene synthase activity in citrus plants to produce additional sesquiterpenes in order to repel *Diaphorina citri* and/or *Tryoza erytrae* psyllid insects so as to control HLB, wherein the sesquiterpene over-accumulated is β-caryophyllene, α-copaene, or combinations thereof.

2. The method of claim 1, wherein the polypeptide encoded by the at least one gene has β-caryophyllene synthase and/or α-copaene synthase activity.

3. The method of claim 1, wherein the at least one gene has one or more mutations as compared with its wildtype form, wherein the polypeptide encoded by the at least one gene retains the sesquiterpene synthase activity, and wherein the polypeptide encoded by the at least one gene has increased stability and efficacy.

4. The method of claim 1, wherein the expression of the at least one gene is driven by constitutive promoter and terminator regions.

5. The method of claim 1, wherein the expression of the at least one gene is driven by a heterologous regulator region providing strong constitutive, tissue-specific or inducible expression.

6. The method of claim 5, wherein the heterologous regulator region provides strong expression in the cytosol, chloroplasts or mitochondria.

7. The method of claim 1, which further comprises expressing a gene encoding a farnesyl pyrophosphase synthase to enhance the accumulation of the sesquiterpene produced by the a polypeptide having sesquiterpene synthase activity.

8. A method for controlling Huanglongbing (HLB) disease of citrus plants comprising applying at least one purified sesquiterpene, which repels *Diaphorina citri* and/or

Tryoza erytrae psyllid insects, so as to control the HLB disease of citrus plants, wherein the at least one sesquiterpene is selected from the group consisting of β-caryophyllene and α-copaene, or combinations thereof.

9. The method of claim 8, wherein the at least one purified sesquiterpene is purified from an organism selected from the group consisting of plants, bacteria and yeasts, or its genetically modified counterparts able to overproduce these sesquiterpene compounds.

10. The method of claim 9, wherein the at least one purified sesquiterpene is purified from the guava plant.

11. The method of claim 10, wherein the at least one purified sesquiterpene is purified from leaf, fruit or stem extracts of the guava plant.

12. The method of claim 8, wherein the at least one sesquiterpene is applied to the citrus plants through slow delivery systems.

INTERNATIONAL SEARCH REPORT

International application N°
PCT/BR2010/000353

A. CLASSIFICATION OF SUBJECT MATTER

IPC (2010.01): A01N 65/28

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)

IPC (2010.01): A01N

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

b351 (DIALOG), epodoc(EPOQUE), Dgene (STN) and USgene (STN).

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

medline (EPOQUE) and SINPI.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claims N°
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P,X	US 2010/0166896 A1(STERLING INTERNATIONAL INC. [US]) 1 July 2010 (2010-07-01), see the whole document.	1-7
X	WO 2006111924 A3 (CLARK ANTHONY [FR]) 26 April 2007 (2007-04-26), see the whole document.	1-7
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

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"T" later document published after the internationa 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 obvius to a person skilled in the art

"&" document member ot the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 January 2011	080211

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INTERNATIONAL SEARCH REPORT

International application N°
PCT/BR2010/000353

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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