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SYSTEM AND METHODS FOR THE BIOCONTROL OF PLANT PATHOGENS

This application claims the benefit of and priority to U.S. Provisional Application No. 62/430,671, filed December 6, 2016. The entire specification and figures of the above-referenced application are hereby incorporated, in their entirety by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

The field of the present invention relates generally to plant molecular biology and plant biotechnology. More specifically it relates to constructs and methods to suppress the expression of targeted genes or to down-regulate targeted genes. The inventive technology further relates to the biocontrol of plant pathogens, pests and/or herbivores. Specifically, the invention may relate to novel techniques, systems, and methods for the biocontrol of disease-transmitting plant pathogens through the delivery of inhibitory RNA molecules through genetically modified bacterium.

BACKGROUND OF THE INVENTION

Domestication of agricultural crops, estimated at 2,500+ species globally, has involved artificial selection of desirable traits that enhance yield and quality of the harvested product. While breeding for agronomic targets in high input environments has successfully increased global crop productivity, it has tended to produce modern crop varieties with relatively low levels of diversity. This reduced genetic diversity could limit the availability of varieties adapted for crop production under non-optimal conditions. Plant defensive traits can be lacking or expressed weakly in domesticated plants as a consequence of selection for other desirable traits. This poses a particular challenge for improving the sustainability of crop production as it suggests that modern varieties would perform poorly in low input systems with restricted pesticide use. While crop productivity has increased over the past century, combined global crop losses due to weeds, pests and diseases can be up to 40% (Oerke and Dehne, 2004). Across all vegetation systems, foliage, sap and root feeding herbivores remove >20% of net plant

productivity (Agrawal, 2011). These losses occur despite increased pesticide use over recent decades (Oerke and Dehne, 2004), highlighting the need to develop sustainable approaches for pathogen and pest control with less reliance on chemical inputs.

Disease control is often achieved and/or enhanced by the use of plants that have been bred for good resistance to many diseases, and by plant cultivation approaches such as crop rotation, use of pathogen-free seed, appropriate planting date and plant density, control of field moisture, and pesticide use. Across large regions and many crop species, it is estimated that diseases typically reduce plant yields by 10% every year in more developed settings, but yield loss to diseases that often exceeds 20% in less developed settings. While traditional cultivation techniques have produced significant gains in agricultural output, such techniques cannot address all of the threats to plant populations. Such threats being especially acute with respect to cash and food stuff crops.

With these trends in mind, a growing concern involves the fact that plant pathogens are a direct threat to the quality and abundance of food, feed, and fiber produced by growers around the world. Different approaches may be used to prevent, mitigate or control plant diseases. Beyond traditional agronomic and horticultural practices, growers are often forced to rely on chemical fertilizers and pesticides. Such inputs to agriculture have contributed significantly to the spectacular improvements in crop productivity and quality over the past 100 years. However, the environmental pollution caused by excessive use and misuse of agrochemicals, as well as fear-mongering by some opponents of pesticides, has led to considerable changes in peoples' attitudes towards the use of pesticides in agriculture. Today, there are strict regulations on chemical pesticide use, and there is political pressure to remove reliable and effective chemicals from the market. Additionally, the spread of plant diseases in natural ecosystems may preclude successful application of chemicals, because of the scale to which such applications might have to be applied.

One way to address the use and/or overuse of pesticides and herbicides is through the development of transgenic plants. Transgenic plant DNA is modified using genetic engineering techniques. The aim is to introduce a new trait to the plant which does not occur naturally in the species. A transgenic plant may contain a gene or genes that have been artificially inserted. The inserted gene sequence is known as the transgene, it may come from an unrelated plant or from a completely different species. Example of such transgenic

plants may include the insertion of genes that code for proteins such as anti-viral protein complexes, pesticides, or metabolic pathway enzymes that may convert precursors such as beta-carotene into vitamin A. The purpose of inserting a combination of genes in a plant is to make it as useful and productive as possible. However, such transgenic plant systems also have several scientific and practical drawbacks.

In one example, transgenic plants have been developed to control specific insect/pests through the expression of Bt-based toxins. Expression of Bt insecticidal proteins which help in the permeabilization of gut epithelial cell's membrane in susceptible insects have proven initially effective. However, this approach is limited for some specific crops to manage some specific pests, and there is also a threat that some insects can develop resistance against Bt. Additional limitations on transgenic plant systems may include the fact that they are difficult and time-consuming to select and generate. For example, the development of a transgenic strain may be limited by the ability to successfully reproduce seeds or allow the natural outgrowth and spread of the transgenic plant species in any given environment. In addition, significant social pressures have arisen in opposition to the use of transgenic plants. Movements in Western and European countries have sought to prevent the use of transgenic plants in food stuffs. Additionally, new markets for organic and non-transgenic products have concurrently arisen in the last decade which allows non-transgenic plants to be sold at a market premium. Finally, many transgenic plants are controlled by large agricultural conglomerates making supply of seeds for transgenic plants limited, and thereby inhibiting their widespread use in many third-world countries, as one example.

In an effort to address some of the limitations, plant-based microorganisms, such as endophytes and the like, may be utilized as delivery vectors for beneficial genes, as well as genetic inhibition of undesirable genes, such as those that cause plant diseases. Plants may harbor a number of beneficial bacteria intracellularly as well as on their surfaces, including roots, leaves, and stem tissues. Endophytic and ectopic bacteria that live in association with plants include those in the following subphyla: Acidobacteria, Actinobacteria, Alphaproteobacteria, Armatimonadetes, Bacteriodes, Betaproteobacteria, Deltaproteobacteria, Firmicutes, Grammaproteobacteria, TM7, Bacillus, Escherichia among others.

Many of these bacteria can be quickly cultured *in vitro* and can be genetically engineered to express foreign RNA molecules that may be made available to the plant by trans-kingdom

delivery systems that are currently poorly characterized (Baulcombe, 2015; Kim et al., 2014; Arguel et al., 2012). For example, it has been demonstrated that tomato pathogenic fungi can deliver sRNAs to plants and that the fungal sRNAs can be mobilized throughout the plant through the vasculature tissue (Baulcombe, 2015; Weiberg and Jin, 2015). Trans-kingdom
5 delivery of siRNA from transgenic plants has also been successfully demonstrated and used to control nematodes that ingest the siRNA (Bakheti et al., 2005; Knip et al., 2014).

In addition, trans-kingdom delivery of siRNA and dsRNA produced in cytoplasm and chloroplasts of microalgae, respectively, that is targeted to inactivate an essential gene, HKT, involved in tryptophan metabolism in mosquitoes, has been shown to suppress 3-
10 hydroxykynurenine transaminase ("HKT") expression and lead to elevated mosquito mortality (Kumar et al., 2015). In contrast to eukaryotes, however, bacteria do not have a Dicer/RISC complex capable of generating siRNAs from dsRNA precursors. However, as noted above, such systems are still poorly understood and inefficient in both their application and commercial viability. Thus, it is not surprising that bacterial delivery of inhibitory RNA molecules to plants
15 to control pathogens, pests and herbivores that feed on plants has not been effectively demonstrated. For example, some have attempted to apply dsRNA or sRNA directly on plants to control gene expression but at great cost to produce the RNA and with limited lifetime for the RNA due to degradation. (See e.g., Nature Plants 3, Article number: 16207 (2017) doi:10.1038/nplants.2016.207) To the contrary, the novel system described here results in the
20 continuous production of dsRNA or sRNA at no apparent cost as the inhibitory RNA are made by genetically modified bacteria.

The foregoing problems regarding the biocontrol of diseases endemic in plant and/or herbivore populations through effective bacterial delivery of inhibitory RNA molecules may represent a long-felt need for an effective -- and economical -- solution to the same. While
25 implementing elements may have been available, actual attempts to meet this need may have been lacking to some degree. This may have been due to a failure of those having ordinary skill in the art to fully appreciate or understand the nature of the problems and challenges involved.

As a result of this lack of understanding, attempts to meet these long-felt needs may have failed to effectively solve one or more of the problems or challenges here identified. These
30 attempts may even have led away from the technical directions taken by the present inventive technology and may even result in the achievements of the present inventive technology being

considered to some degree an unexpected result of the approach taken by some in the field. As will be discussed in more detail below, the current inventive technology overcomes the limitations of traditional plant pathogen control systems, while meeting the objectives of a truly effective vector biocontrol strategy.

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BRIEF SUMMARY OF THE INVENTION

Generally, the inventive technology relates to novel strategies for the control of plant pathogens and pests including plant pathogens, plant viruses, fungal pathogens, insect pests and the like. Specifically, the invention may comprise novel techniques, systems, and methods for the biocontrol of select plant pathogens and herbivores.

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In some embodiments, the invention relates to a novel trans-kingdom delivery of inhibitory RNAs homologous to the viral genome efficiently down-regulates or eliminates viral replication and translation of viral proteins.

One preferred embodiment may include a novel trans-kingdom delivery of hairpin RNA / dsRNA molecules targeting pathogen coding RNAs for degradation, resulting in the reduction of the encoded protein accumulation levels. This novel trans-kingdom delivery may be accomplished through infection of the plant by genetically modified endophyte bacteria carrying a nucleotide construct encoding the regulatory dsRNA homologous to pathogen coding or regulatory RNA sequences.

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Another preferred embodiment may include a novel trans-kingdom delivery of hairpin RNA targeting viral pathogen encoded proteins which results in the reduction of viral protein accumulation levels. This novel trans-kingdom delivery may be accomplished through infection of the plant by genetically modified endophyte bacteria carrying a nucleotide construct encoding a hairpin RNA that is homologous to the target pathogen protein.

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Another preferred embodiment may include a novel trans-kingdom delivery of hairpin RNA targeting fungal pathogen encoded proteins which results in the reduction of fungal protein accumulation levels. This novel trans-kingdom delivery may be accomplished through infection of the plant by genetically modified endophyte bacteria carrying a nucleotide construct encoding a hairpin RNA that is homologous to the target pathogen protein.

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One preferred embodiment may include a novel trans-kingdom delivery of hairpin RNA targeting pest encoded proteins which results in the reduction of viral protein accumulation

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levels. This novel trans-kingdom delivery may be accomplished through infection of the plant by genetically modified endophyte bacteria carrying a nucleotide construct encoding a hairpin RNA that is homologous, or directed to the target pathogen protein.

In one preferred embodiment, the invention may include innovative systems and strategies to control plant-borne disease agents using a novel, cross-kingdom mechanism to incapacitate, potentially kill and/or prevent replication of plant pathogens by introducing genetically engineered microorganisms that may target inactivation of unique targeted genes in the pathogen involved in reproduction, pathogenicity, and/or general metabolism using inhibitory RNA molecules such as hpRNA, dsRNA, shRNA, siRNA or microRNAs, whose expression may lead to the turnover of the targeted mRNA for the gene of interest. Examples of unique targeted genes of interest include viral coat proteins, fungal cell wall genes, insect exoskeleton component genes and species-specific unique mRNA targets for metabolic genes in fungi and insects.

Additional embodiments include the expression of inhibitory RNA molecules, such as hpRNA, dsRNA, shRNA, siRNA and micro RNAs, in engineered endophytic bacteria that may be found in plant roots, stems, leaves and reproductive organs. In this embodiment, the inventive technology may include various cross-kingdom mechanisms for the knock-down of essential or other targeted plant pathogen and/or herbivore genes. In certain embodiments, this may be accomplished through the introduction of engineered microorganisms into plants that express specific inhibitory RNA molecules that may down-regulate targeted plant, pathogen and/or herbivore genes needed or essential to pathogenicity or reproduction and the like. Additional embodiments of the inventive technology encompass genetic constructs, such as plasmids and the like, having various promoter and other genetic elements to allow targeted levels of expression of specific inhibitory RNA molecules, and other proteins, in endophytic bacteria, plant and/or herbivore systems.

According to one aspect, the present invention provides a method of down-regulating a pathogen gene(s) by sequence homology targeting in a plant cell and a nucleic acid construct for use in this method, as well as an inhibitory RNA polynucleotide, such as a hpRNA or annealed dsRNA, for use in the nucleic acid construct. The method comprises introducing into the cell a nucleic acid construct capable of producing inhibitory RNA and expressing the nucleic acid construct for a time sufficient to produce siRNAs (small interfering RNAs) or microRNA

(miRNA), wherein the siRNA/miRNA inhibits expression of the target pathogen gene or sequence. miRNA constructs comprise a polynucleotide encoding a modified RNA precursor capable of forming a double-stranded RNA (dsRNA) or a hairpin (hpRNA), wherein the modified RNA precursor comprises a modified miRNA and a sequence complementary to the modified miRNA, wherein the modified miRNA is a miRNA modified to be (i) fully or partially complementary to the target sequence. As is well known in the art, the pre-miRNA forms a hairpin which in some cases the double-stranded region may be very short, e.g., not exceeding 21-25 bp in length. The nucleic acid construct may further comprise a promoter operably linked to the polynucleotide.

In some embodiment, as described in more detail below, the cell may be a plant cell, either monocot or dicot, including, but not limited to, corn, wheat, rice, barley, oats, sorghum, millet, sunflower, safflower, cotton, soy, canola, alfalfa, Arabidopsis. Some embodiments may include plants described herein can also be specific dicot crops, such as apple, grape, citrus, pear, peach, plum, orange, lemon, lime, grapefruit, pomegranate, olive, peanut, tobacco, etc. Also, the plants can be horticultural plants such as rose, marigold, primrose, dogwood, pansy, geranium, etc. In other embodiments, the plants are tobacco, such as *N. benthamiana*, *N. tabacum*. Additional examples are provided below. The promoter may be a pathogen-inducible promoter or other inducible promoters. The binding of the modified miRNA to the target RNA leads to cleavage of the target RNA. The target sequence of a target RNA may be a non-coding untranslated region of a gene, a coding sequence, an intron or a splice site.

According to another aspect, the present invention provides an isolated polynucleotide encoding a modified plant miRNA or siRNA precursor, the modified precursor is capable of forming a dsRNA or a hairpin and comprises a modified miRNA and a sequence complementary to the modified miRNA, wherein the modified miRNA is an miRNA modified to be (i) fully or partially complementary to the target sequence. Expression of the polynucleotide produces an miRNA precursor which is processed in a host cell to provide a mature miRNA which inhibits expression of the target sequence. The inhibitory RNA polynucleotide(s) may formed from a nucleic acid construct, such as a plasmid, that may be delivered to the plant through a genetically modified micro-organism, such as an endophytic bacteria, or a neutralized pathogenic bacteria. The nucleic acid construct may further comprise a promoter operably linked to the polynucleotide. The promoter may be a pathogen-inducible promoter or other inducible

promoter. The binding of the modified miRNA to the target RNA leads to cleavage of the target RNA. The target sequence of a target RNA may be a non-coding untranslated region of a gene, a coding sequence, a non-coding sequence or a splice site.

According to another aspect, the present invention provides an inhibitory RNA nucleic acid construct for suppressing a multiple number of target sequences. The nucleic acid construct comprises at least two and up to 45 or more polynucleotides, each of which encodes an miRNA precursor capable of forming a dsRNA or a hairpin. Each miRNA is substantially complementary to a target or is modified to be complementary to a target as described herein. In some embodiments, each of the polynucleotides encoding precursor miRNAs in the construct is individually placed under control of a single promoter. In some embodiments, the multiple polynucleotides encoding precursor miRNAs are operably linked together such that they can be placed under the control of a single promoter. The promoter may be operably linked to the construct of multiple miRNAs or it may be operably linked to a single promoter. The promoter may be a pathogen-inducible promoter or other inducible promoter. In some embodiments, the multiple polynucleotides are linked one to another so as to form a single transcript when expressed. Expression of the polynucleotides in the nucleic acid construct produces multiple miRNA precursors which are processed in a host cell to provide multiple mature miRNAs, each of which inhibits expression of a target sequence. In one embodiment, the binding of each of the mature miRNA to each of the target RNA leads to cleavage of each of the target RNA. The target sequence of a target RNA may be a non-coding untranslated region of a gene, a coding sequence, non-coding untranslated region of a gene, a non-coding sequence or a splice site. The inhibitory RNA polynucleotide(s) may be formed from a nucleic acid construct, such as a plasmid, that may be delivered to the plant through a genetically modified micro-organism, such as an endophytic bacteria, or a neutralized pathogenic bacteria.

According to another aspect, the invention provides methods and compositions useful for delivering inhibitory RNA molecules to a plant cell through genetically modified bacteria. Such genetically modified bacteria may include genetic modifications to efficiently and stably produce inhibitory RNA molecules, such as dsRNA and hpRNA. In some embodiment, such genetically modified bacteria may have been modified to have reduced, or no RNAaseIII activity, which may degrade dsRNA present in a bacteria. In some embodiments, such genetically modified

bacteria may have been modified to include a knock-out of the RNAase III, or express a mutant-type RNAase III having reduced or no enzymatic activity.

In certain embodiment, the compositions selectively suppress the target gene expression by encoding an inhibitory RNA having substantial complementarity to a region of the target
5 sequence. The miRNA / siRNA is provided in a nucleic acid construct which, when transcribed into RNA, is predicted to form a hairpin structure which is processed by the cell to generate the mature siRNAs / miRNAs , which then suppresses expression of the target gene or sequence as generally described below.

In additional embodiments, a nucleic acid construct is provided to encode the inhibitory
10 RNA for any specific target pathogen gene. This may be encoded on, for example, a plasmid that may be expressed by a genetically modified bacteria capable of colonizing in or around a plant cell and expressing and transmitting the inhibitory RNA to the plant cell for miRNA processing, resulting in suppression of the target pathogen gene as generally described herein. Any inhibitory RNA can be inserted into the construct, such that the encoded inhibitory RNA selectively targets
15 and suppresses the target pathogen gene.

In additional embodiments, a method for suppressing a target sequence is provided. The method employs the constructs above, in which an inhibitory RNA molecule is designed to a region of the target sequence, and inserted into a construct, such as a bacterial plasmid. Upon introduction into a cell through an endophytic bacteria for example, the miRNA produced within
20 the bacteria is produced and transmitted to the plant cell and suppresses expression of the targeted sequence as generally described herein. In some embodiments, the genetically modified bacteria may exhibit, whether through selection or genetic manipulation hypervesiculation. In a certain embodiment, this hypervesiculation may aid in the efficient transport of inhibitory RNA molecules produced by the bacterial plasmid into the surrounding cell, or cell surface, or other
25 cellular plant structure.

In a certain embodiment, the target gene or sequence can be an endogenous plant gene, or a heterologous gene. The target gene may also be a gene from a plant pathogen, such as a pathogenic bacteria or virus, nematode, herbivore, insect, or mold or fungus. A plant, cell, and seed comprising the construct and/or the miRNA is provided. Typically, the cell will be a cell
30 from a plant, but other eukaryotic cells are also contemplated, including but not limited to yeast, insect, nematode, or animal cells. Plant cells include cells from monocots and dicots. The

invention also provides plants and seeds comprising the construct and/or the miRNA. Viruses and prokaryotic cells comprising the construct are also provided.

In some embodiments, the target gene or sequence, (the terms being generally interchangeable) is selected from a plant pathogen. Plants or cells comprising a inhibitory RNA molecules directed to the target gene or of the pathogen are expected to have decreased sensitivity and/or increased resistance to the pathogen. In some embodiments, the inhibitory RNA molecule is encoded by a nucleic acid construct, such as a bacterial plasmid being expressed in an endophytic bacteria further and comprising an operably linked promoter. In some embodiments, the promoter is a pathogen-inducible promoter.

One aim of the current invention includes development of bacterial dsRNA stabilization and delivery systems to block targeted gene expression and viral replication in plants. As shown understood, dsRNA production and delivery to plants is a multi-component process involving dsRNA biogenesis, export, stabilization, import into the host and regulation of inactivation of pathogen replication.

One aim of the current invention includes systems and methods for enhancing transfer of dsRNA from bacteria to plant host cells. In certain embodiments, this may include the use of bacteria strains and helper genes that enhance dsRNA production, stabilization, export and/or delivery. For example, certain classes of bacteria have developed a variety of molecular secretion systems to enable various molecules including toxins and proteins to gain access to the host organism. Six different bacterial secretion systems have been described below. These six secretion systems have common as well as unique modes of action. The Type II and Type V secretion systems are two-step processes in which proteins are transported first through the inner membrane (IM) and then through the outer membrane (OM) of the bacterium. For secretion via the Type I, Type III, and Type IV secretion systems, the material is transferred directly into the extracellular milieu or into the host cell. The Type III system is specific for the transport of factors by pathogenic bacteria and involves a direct injection into the host cell. The distinct Type VI secretion system enables bacteria to secrete a large complex group of proteins and lipids into the extracellular milieu in extra-cellular vesicles formed from the bacterial outer membrane. These vesicles then fuse with the host cell to deliver their cargo.

Objects, advantages and novel features, and further scope of applicability of the present invention will be set forth in part in the detailed description to follow, taken in conjunction with

the accompanying drawing, and in part will become apparent to those skilled in the art upon examination of the following, or may be learned by practice of the invention. The objects and advantages of the invention may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Map of the hpRNA vector pAD-WRKY-GHY1.

Figure 2: Map of the hpRNA vector pAD-ADH-GHY2.

Figure 3: Map of the hpRNA vector pOXB-WRKY-GHY3.

Figure 4: Map of the hpRNA pOXB-ADH-GHY4.

Figure 5: Map of the hpRNA pAD-WRKY-GHY5.

Figure 6: Map of the hpRNA pAD-ADH-GHY6.

Figure 7: Map of the hpRNA pAD-WRKY-GHY7.

Figure 8: PCR analysis to verify the successful construction of RNaseIII mutants.

Figure 9: Identification of *B. cereus* 53522 strain. Lane M: 1kb DNA ladder. Lane 1: PminiT-16s rRNA was digested with XhoI. Lane 2: PminiT-23s rRNA was digested with XhoI. Lane 3: PminiT-motB was digested with XhoI. Lane 4: PminiT-endo-glucanase was digested with XhoI. Lane 5: PminiT-rncA was digested with XhoI.

Figure 10: Symptoms of infiltration in *N. tabacum* after 7 dpi. (At: *Agrobacterium tumefaciens*; TMV: pJL-TRBO-G; MJM109: M-JM109-GHY2 (RNaseIII mutant, no antibiotic); GHY5: pAD-WRKY-GHY5; GHY6: pAD-ADH-GHY6; GHY3: pOXB-WRKY-GHY3; GHY4: pOXB-ADH-GHY4)

Figure 11a: Symptoms and GFP signal detected under UV illumination of health and infiltrated leaves of *N. tabacum*.

Figure 11b: TMV-GFP signal of *N. tabacum* under UV illumination at 7dpi.

Figure 11c: TMV-GFP signal of *N. tabacum* treated by M-JM109-GHY2/pAD-WRKY-GHY1 under UV illumination at 7dpi.

Figure 12: Time-course images demonstrating the efficiency of hpRNA inhibiting the spread of TMV-encoded GFP signal in tobacco plant.

Figure 13: Tobacco infiltration with *Bacillus subtilis* CCB422/pAD-WRKY-GHY5 in *N. tabacum*.

Figure 14: Hypervesiculating *E. coli* strain HT27 with hpRNA in inhibiting the spreading of At / TMV signal in a whole leaf infiltration in *N. benthamiana*.

Figure 15: Time-course demonstration of the efficiency of hpRNA mediated GFP signal suppression in model plant organism.

Figure 16: Symptoms of siRNA migration limit the spreading of TMV-GFP through leaf structure at 7 dpi.

Figure 17: MJM109-GHY2/pAD-WRKY-GHY5 derived siRNA limited the spreading of TMV-GFP signal in tobacco plant model.

Figure 18a-b: siRNA limiting the spreading of TMV-GFP signal in model tobacco plant.

Figure 19a-c: Colonies of Bc53522/pAD43-25 and Bc53522/pAD-WRKY-GHY5 under 4X magnification observed with natural light and with exposure to fluorescein isothiocyanate (FITC). A) demonstrates a colony of Bc53522/pAD43-25 viewed under nature light at 4X magnification; B) demonstrates a colony of Bc53522/pAD43-25 with exposure to FITC a 4X magnification showing a green signal; C) demonstrates a colony of Bc53522/pAD-WRKY-GHY5 viewed under nature light at 4X magnification; D) demonstrates a colony of Bc53522/pAD-WRKY-GHY5 with exposure to FITC a 4X magnification showing no signal.

Figure 20a-c: Colonization of *B. cereus* 53522 in an exemplary tobacco plant. A) Inoculated *B. cereus* to tobacco showed no harmful symptom to plants. B) GFP signal under UV. C) GFP signal of different areas of plant under fluorescence stereo microscope.

Figure 21: Trans-kingdom delivery of hairpin RNA targeting viral encoded GFP results in the reduction of viral protein accumulation levels. A) *Bacillus subtilis* CCB422 mediated trans-kingdom delivery of hpRNA successfully triggers host's RNAi response leading to significant reduction Viral GFP accumulation in *N. tabacum* plants. B) Quantification of GFP protein levels in TMV-GFP infected and TMV-GFP + pAD-WRKY-GHY5 (CCB422) infiltrated samples. GFP signal normalized to RBCL. TMV-GFP replicate #1 used as reference. C) Viral GFP protein is undetectable in TMV samples co-infiltrated with HT27 encoding hpRNA trigger. GFP3c signal from immuno-detection. RBCL as loading control (Ponceau stain).

Figure 22: qPCR to monitor the TMV-GFP mRNA suppression by HT27/pAD-WRKY-GHY5. qPCR data on GFP mRNA suppression in plants infected with bacteria expressing the hpRNA versus controls (TMV-GFP alone, TMV-GFP plus bacteria without hpRNA).

Tables and sequence listings also are provided herein and are part of the specification.

MODE(S) FOR CARRYING OUT THE INVENTION(S)

The present invention includes a variety of aspects, which may be combined in different ways. The following descriptions are provided to list elements and describe some of the embodiments of the present invention. These elements are listed with initial embodiments, however it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and preferred embodiments should not be construed to limit the present invention to only the explicitly described systems, techniques, and applications. Further, this description should be understood to support and encompass descriptions and claims of all the various embodiments, systems, techniques, methods, devices, and applications with any number of the disclosed elements, with each element alone, and with any and all various permutations and combinations of all elements in this or any subsequent application.

Disclosed herein are methods and compositions for genetic control of plant pathogens, pest infestations, and herbivores all generally being collectively described as plant pathogen(s).

Methods for identifying one or more gene(s) essential to the lifecycle of a plant pathogen, pest and/or herbivore for use as a target gene for enhanced siRNA-mediated interference are also provided. DNA plasmid vectors encoding inhibitory RNA molecules may be designed to suppress one or more target gene(s) essential for growth, survival, development, and/or reproduction of a plant pathogen. Genetically modified endophytic bacteria, or other microorganisms, that may be engineered to efficiently infect, produce, and deliver such inhibitory RNA molecules is also described in the present invention.

In some embodiments, the present invention provides methods for post-transcriptional repression of expression or inhibition of a target gene via nucleic acid molecules that are complementary to a coding or non-coding sequence of the target gene in a plant pathogen. In these and further embodiments, a pest or herbivore may ingest one or more dsRNA, siRNA, miRNA, and/or hpRNA molecules transcribed from all or a portion of a nucleic acid molecule that is complementary to a coding or non-coding sequence of a target gene, thereby providing a plant-protective effect.

One embodiment of the present inventive technology may include systems and methods for introducing inhibitory RNA molecules into a target plant cell through infection by genetically engineered microorganisms. In one embodiment, the invention may provide for genetically engineered microorganisms such as bacteria, fungi or even viruses that may express one or more inhibitory RNA molecules within plant cells. In certain embodiments such inhibitory RNA molecules may initiate biological process in which the inhibitory RNA molecules inhibit or knock-down gene expression, typically by causing the destruction of specific targeted mRNA molecules within the cell. Additional embodiments may introduce inhibitory RNA molecules into plant systems that inhibit genes necessary for plant pathogenesis. Such embodiments may include introduction of inhibitory RNA molecules into plant systems that may target viral coat proteins, fungal cell wall genes, unique, species-specific gene sequences targeting suppression of genes involved in the replication and production of DNA and RNA, insect exoskeleton component genes, species-specific metabolic genes and herbivores.

Additional preferred embodiments may include improved delivery systems for inhibitory RNA molecules, for example through the use of stabilizing factors, such as stabilizing proteins and the like. Another preferred embodiment of the inventive technology may include improved systems to facilitate the transmission of inhibitory RNA molecules within the plant cell, either by

co-delivery of smRNA or dsRNA precursor stabilizing proteins or hpRNA / dsRNA intra-cellular transport proteins. In one embodiment, specific RNA motifs may be incorporated into dsRNA that may facilitate its transmission through, for example the plant phloem, or direct smRNA loading to a specific effector complex. Yet further embodiments may include
5 genetically modified microorganisms that may include genetic constructs that may further co-express certain protein having processing enzymatic activity.

Other similar embodiments may include the introduction of microorganisms into plant systems that may express, or even over-express various genes that may enhance mobilization of inhibitory RNA molecules and/or genes that may activate secondary downstream host genes that
10 may target pathogenic pathways.

One preferred embodiment of the present invention may be to provide leaf and root endophytic and ectophytic bacteria that may further be genetically engineered to express inhibitory RNA molecules, such as dsRNA, shRNA, hpRNA (that may, in some embodiments, contain an intron from a targeted organism located at the hairpin loop of the dsRNA), siRNA,
15 and microRNAs that are homologous to the pathogen genome, and again target critical or essential genes responsible for growth, reproduction, metabolism or pathogenicity. These inhibitory RNA molecules may inactivate and/or knock-down expression of these essential targeted genes in plant viral, fungal, and insect pathogens and pests through generation of ~21-22 nucleotide siRNAs mediated by the host Dicer/RISC complex. This process may generally be
20 referred to as RNA interference or RNAi.

As used herein, RNA interference (RNAi) is a biological mechanism which leads to post transcriptional gene silencing (PTGS) triggered by double-stranded RNA (dsRNA) molecules, for example provided by hpRNA, to prevent the expression of specific genes. For example, in one preferred embodiment, RNA interference may be accomplished as short hpRNA molecules
25 may be imported directly into the cytoplasm, anneal together to form a dsRNA, and then cleaved to short fragments by the Dicer enzyme. This enzyme Dicer may processes the dsRNA into ~21-22-nucleotide fragment with a 2-nucleotide overhang at the 3' end, small interfering RNAs (siRNAs). The antisense strand of siRNA become specific to endonuclease-protein complex, RNA-induced silencing complex (RISC), which then targets the homologous RNA and degrades
30 it at specific site that results in the knock-down of protein expression.

Endophytic bacteria that may transmit hpRNA, dsRNA, shRNA, siRNA and microRNA species to plants include those in the subphyla: *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Armatimonadetes*, *Bacteriodes*, *Betaproteobacteria*, *Deltaproteobacteria*, *Firmicutes*, *Grammaproteobacteria*, *TM7*, *Bacillus*, *Escherichia* and the like. In one preferred embodiment, endophytic, entophytic, or any bacteria that may infect or otherwise colonize a plant or live in its surface leaf, stem or root, may be transformed with artificially created genetic constructs, such as plasmids that may generate the inhibitory RNA molecules. Such plasmids may be constructed to be transferrable to other bacteria through conjugation which may allow for widespread environmental inoculation in some instances, as well as vertical transmission among offspring of the plant and any pest or herbivore that ingests the plant matter as an example.

In this embodiment, such constructs may include transcription regulation portions, such as promoters, terminators, co-activators and co-repressors and similar control elements that may be regulated in prokaryotic, as well as eukaryotic systems. Such systems may allow for control of the type, timing, and amount of inhibitory RNA molecules expressed within the systems. Additional embodiments may include genetic constructs that may be induced through outside factors, such as the presence of a specific protein or compound within the plant cell, such as stress-related proteins generated in response to plant pathogens or even the proteins and other precursor compounds generated by plant pathogens and the like.

In another preferred embodiment, the present inventive technology may include systems and methods whereby genetically transformed leaf and root endophytic and ectophytic bacteria that generate one or more inhibitory RNA molecules may be delivered to targeted plant phyla and/or species where the inhibitory RNA molecules may inactivate and/or knock-down expression of target viral, fungal as well as insect pathogens and herbivores. In certain embodiments, a selection of microorganisms, such as bacteria that are known to be specific to one phyla or even a certain species may be utilized. In another embodiment, transcriptional activation and promotion of inhibitory RNA molecules may be dependent on the presence of certain factors that may be specific to one phyla, or even a certain plant or herbivore species. Additional embodiments may include increased virulence factors, such as the expression or inhibition of surface protein and importation factors for specific plant phyla or even specific plant species, as well as the possible inhibition of bacterial factors, such as surface or other by-

product proteins that may be recognized by, for example, intracellular host receptors that may cause the host plant cell to mount an immune-type defense.

In another preferred embodiment, the inventive technology may include systems and methods for the trans-kingdom delivery of pathogen as well as species-specific inhibitory RNA molecules that may further be stabilized by one or more proteins or protein complexes. In certain 5 embodiments, such stabilizing proteins may include the P19 protein selected from plant RNA virus, tombusvirus which may contribute to the suppression of a plant's RNA silencing response. In certain embodiments, for example the P19 protein may recognize and bind siRNA, typically as a dimer. In the manner, the P19 protein may sequester the siRNA, whether exogenous or 10 endogenous, and as a consequence prevent its loading into Argonaute proteins and formation of RISC(RNA-induced silencing complex).

In another preferred embodiment, the inventive technology may include the incorporation of RNA mobilization motifs into inhibitory RNA molecules, such as dsRNA, to facilitate transmission of the bacterial synthesized RNA through the plant phloem and may be co- 15 expressed in the bacteria with the RNA species targeting inactivation of pathogen and herbivore essential genes. For example, plasmodesmata and phloem form a simplistic network that mediates direct cell-cell communication and transport throughout a plant. Selected endogenous RNAs, viral RNAs, avoid traffic between specific cells or organs via this network. As noted above, in one preferred embodiment, inhibitory RNA molecules may be generated to contain 20 sequence/structural motifs that interact with cellular factors to facilitate trafficking across cellular boundaries.

In certain embodiments multiple inhibitory RNA molecules, expressing different sequence/structural motifs may be concurrently expressed. In this embodiment, the differing sequence/structural motifs may allow recognition of specific motifs allowing multi-direction 25 trafficking of inhibitory RNA molecules across multiple specific cellular boundaries and/or cell types. Such motifs may further be configured to exhibit specific activity, or inactivity within certain phyla and/or species.

Yet another embodiment of the inventive technology may include development of novel bacterial strains that have decreased RNase III expression, or inactivated RNase III function or 30 activity, generally referred to as suppresses activity. This decrease or inactivation in RNAase III

expression and/or activity may inhibit or decrease RNase III-mediated processing of dsRNA species into smaller RNA species.

Additional embodiments may include novel bacterial strains that overexpress RNase III such that processing of dsRNA species to smaller RNA species is enhanced. The inventive
5 technology may also include development of systems and methods to bacterially express dsRNAs encoding viral siRNA to trigger secondary host (plant) RNA-dependent RNA Polymerase-dependent expression of secondary siRNAs to target viral and fungal inactivation. Each of the aforementioned systems may be embodied in genetic constructs that may include transcription regulation elements such as promoters, terminators, co-activators and co-repressors
10 and other control elements that may be regulated in prokaryotic as well as eukaryotic systems. Such systems may allow for control of the type, timing and amount of, for example RNAase III or other proteins, expressed within the system. Additional embodiments may include genetic constructs that may be induced through outside factors, such as the presence of a specific protein or compound within the plant cell, such as stress-related proteins generated in response to plant
15 pathogens or even proteins and other precursor compounds generated by plant pathogens and the like.

Another embodiment of the inventive technology may include systems and methods to facilitate the overexpression of plant host, exogenous or genetically-modified strains of bacteria having genes known to enhance siRNA mobilization. For example, as shown in Tables 5-8 and
20 below, overexpression of certain proteins identified in column one below may enhance siRNA mobilization throughout the subject plant or other system.

In one preferred embodiment, additional embodiments of the inventive technology may include a novel system of trans-kingdom delivery and expression of hpRNAs homologous to a pathogen's genome/gene and efficiently down-regulates or eliminates viral replication and
25 translation of essential proteins necessary for pathogen growth, metabolism, or pathogenicity etc.

Additional embodiments of the inventive technology may include a novel system of trans-kingdom delivery and expression of inhibitory RNA molecules, such as dsRNA, shRNA, siRNA and micro RNAs, in engineered endophytic bacteria that may be found in plant roots, stems, leaves and reproductive organs. In this embodiment, the inventive technology may
30 include various cross-kingdom mechanisms for the knock-down of targeted genes that may, for example, allow for enhanced cold, heat, salt, drought, and abiotic stress tolerance as well as

integrating novel traits in various plant species including insect/pest/pathogen resistance and enhanced nutritional output, to name a few.

In another preferred embodiment, RNase III mutants of endophytic bacteria harboring plasmids encoding hairpin dsRNAs targeting suppression of pathogen proteins will suppress said
5 proteins in plants infected with said pathogen expressing a pathogen genome encoded pathogen gene.

One preferred embodiment includes an endophytic bacterial delivery of dsRNA, processing to siRNA in plants and suppressing one or more pathogen encoded genes. In another preferred embodiment, an endophytic bacterial delivery of dsRNA, may include RNase III
10 mutants of bacteria having no, or suppressed RNase III, harboring plasmids encoding hairpin dsRNAs, targeting suppression of pathogen proteins that may suppress said proteins in plants infected with said pathogen.

One embodiment of the present invention provides for a method of enhancing a plant's resistance to plant pathogens, such as viral and fungal diseases by introducing one or more
15 genetically modified bacteria that may naturally colonize said target plant, encoding or interfering RNA molecules described herein such that the plant containing the heterologous DNA produces the interfering RNA molecule, such as hpRNA, and the interfering RNA molecule kills target plant pathogen, or suppresses expression of one or more of the target plant's pathogenic or critical genes.

Another embodiment provides for a method of enhancing a plant's resistance to viral
20 diseases wherein the plant's resistance to the target viral diseases is greater than said wild-type plant's resistance to the target viral diseases. Another embodiment provides for a method of enhancing a plant's resistance to pests, wherein the plant's resistance to the target pest is greater than said wild-type plant's resistance to the target pest. Another embodiment provides for a
25 method of enhancing a plant's resistance to herbivores, wherein the plant's resistance to the target herbivore is greater than said wild-type plant's resistance to the target herbivore.

Another embodiment demonstrated by the current inventors, may include the generation of pathogen resistant plant. In this preferred embodiment, targeting a pathogen's essential RNA transcripts by the host's RNAi machinery may result in essential pathogen RNA degradation and
30 hence failure to translate pathogen's essential mRNAs into proteins. In this embodiment, boosting of the plant host's RNAi defenses, by a novel trans-kingdom delivery of an inhibitory

RNA molecule, such as a hairpin RNA homologous to the pathogen encoded protein, may result in the loss or decrease in the pathogen's encoded protein accumulation by triggering smRNA biogenesis in the plant host, ultimately conferring resistance to pathogen infection.

Another embodiment demonstrated by the current inventors, may include the generation of a viral pathogen resistant plant. In this preferred embodiment, a plant may be infected with endophyte bacteria producing an hpRNA homologous to an essential viral encoded protein. These hpRNA transcripts may be processed by the host's RNAi machinery and result in essential viral RNA degradation and hence failure to translate the viral pathogen's essential mRNAs into proteins. In this embodiment, boosting of the plant host's RNAi defenses, by a novel trans-kingdom delivery of an inhibitory RNA molecule, such as a hairpin RNA homologous to the viral encoded protein, may result in the loss or decrease in viral encoded accumulation by triggering smRNA biogenesis in the plant host, ultimately conferring resistance to pathogen infection.

In one embodiment, the invention may include the use of *Bacillus cereus* as an endophytic bacteria expressing plasmids encoding hpRNAs targeting the suppression of pathogen proteins expressed in a plant cell. In one embodiment, this *Bacillus cereus* strain may be genetically modified to be RNase III deficient, where deficient may mean decreased RNase III expression, or inactivated RNase III function. In a preferred embodiment, this strain may include the strain 53522 as described herein.

In one embodiment, the invention may include the use of *Bacillus subtilis* as an endophytic bacteria expressing plasmids encoding hpRNAs targeting the suppression of pathogen proteins expressed in a plant cell. In one embodiment, this *Bacillus subtilis* strain may be genetically modified to be RNase III deficient, where deficient may mean decreased RNase III expression, or inactivated RNase III function. In a preferred embodiment, this strain may include the strain CCB422 as described herein.

In one embodiment, the invention may include the use of *E. coli* as an endophytic bacteria expressing plasmids encoding hpRNAs targeting the suppression of pathogen proteins expressed in a plant cell. In one embodiment, this strain may be genetically modified to be RNase deficient. In one embodiment, this strain may be naturally, or genetically modified to express a hypervesiculation genotype/phenotype. In a preferred embodiment, this strain may include the strain HT27 as described herein.

In one embodiment, the invention may include the use of *a Bascillis* as an endophytic bacteria expressing plasmids encoding hpRNAs targeting the suppression of pathogen proteins expressed in a plant cell. In one embodiment, this strain may be genetically modified to be RNase deficient. In one embodiment, this strain may be naturally, or genetically modified to express a hypervesiculation genotype/phenotype. In a preferred embodiment, this strain may include the strain HT27 as described herein.

In certain other embodiments, the hpRNA generated by one or more of these endophytic bacteria strains may migrate throughout the plant, leaf, root or stem from an original site of infection or introduction. In certain other embodiments, the hpRNA generated by one or more of these endophytic bacteria strains may migrate throughout the plant, leaf, root or stem from an original site of infection or introduction, as well as prevent the migration of plant pathogen migration/signal.

Further still, another embodiment comprises a composition comprising a genetically modified bacteria configured to colonize a target plant and deliver one or more inhibitory RNA molecules to inhibit one or more plant pathogens essential genes, as well as provide increased pathogen resistance, such method as disclosed herein as a topical treatment of plants at risk for infection or for plant that are already infected with a target pathogen.

In certain embodiments, this may include the use of bacteria strains and helper genes that enhance dsRNA production, stabilization, export and/or delivery into plant cells or locations where plant pathogens are located. As noted above, bacteria have developed a number of secretion systems including:

Type I secretion systems (T1SSs), exemplified by the haemolysin secretion system in *Escherichia coli*, are simple, tripartite systems facilitating the passage of proteins of various sizes across the cell envelope of Gram-negative bacteria. They consist of an ATP-binding cassette (ABC) transporter or a proton-antiporter, an adaptor protein that bridges the inner membrane (IM) and outer membrane (OM), and an outer membrane pore. They secrete substrates in a single step without a stable periplasmic intermediate. T1SSs are involved in the secretion of cytotoxins belonging to the RTX (repeats-in-toxin) protein family, cell surface layer proteins, proteases, lipases, bacteriocins, and haem-acquisition proteins.

Type II secretion systems (T2SSs) are multicomponent machines that use a two-step mechanism for translocation. During the first step, the precursor effector protein is translocated

through the inner membrane by the Sec translocon or the Tat pathway. Once in the periplasm, the effector protein is translocated by the T2SS through the outer membrane. The T2SS translocon consists of 12–16 protein component that are found in both bacterial membranes, the cytoplasm and the periplasm. The T2SS shows an evolutionary relationship with the type IV pilus assembly machinery.

Type III secretion systems (T3SSs), also called injectisomes, mediate a single-step secretion mechanism and are used by many plant and animal pathogens, including *Salmonella* spp., *Shigella* spp., *Yersinia* spp., enteropathogenic and enterohaemorrhagic *Escherichia coli* and *Pseudomonas aeruginosa*. The T3SS is illustrated by the *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* system, which uses the invasion (Inv) and Prg proteins (see lower figure). T3SSs deliver effector proteins into the eukaryotic host cell cytoplasm in a Sec-independent manner. T3SSs are genetically, structurally and functionally related to bacterial flagella. They are composed of more than 20 different proteins, which form a large supramolecular structure crossing the bacterial cell envelope.

Type IV secretion systems are versatile systems that are found in Gram-negative and Gram-positive bacteria and that secrete a wide range of substrates, from single proteins to protein–protein and protein–DNA complexes. These systems are exemplified by the *Agrobacterium tumefaciens* VirB/D system.

Type V secretion systems (T5SSs) include autotransporters and two-partner secretion systems. T5SSs translocate substrates in two steps. Autotransporter proteins, such as NalP from *Neisseria meningitidis*, are multidomain proteins that are secreted as precursor proteins across the inner membrane in a Sec-dependent process. Subsequently, the translocator domain of the protein inserts into the outer membrane and facilitates surface localization of the passenger domain. In two-partner secretion systems, a separate translocator protein (TpsB) mediates the secretion of the effector protein (TpsA) through the outer membrane. Over 700 proteins with functions that include auto-aggregation, adherence, invasion, cytotoxicity, serum resistance, cell-to-cell spread and proteolysis use these two secretion systems to cross both inner and outer membranes during a simple two-step process.

Type VI secretion systems (T6SSs) are recently discovered secretion systems that are found in several pathogens such as *P. aeruginosa*, enteroaggregative *E. coli*, *S. Typhimurium*,

Vibrio cholerae and *Yersinia pestis*. T6SSs are multi-component systems that could be composed of 12 to 25 subunits.

One embodiment of the present invention may include exploiting bacterial secretion systems to enhance delivery of dsRNA to the plant host. In one preferred embodiment, the invention may include a system for enhancing bacterial Type IV secretion systems to deliver dsRNA to plant cells. For example, the plant pathogen *Agrobacterium tumefaciens* uses the type IV secretion system to deliver a VirD2-single stranded DNA complex as well as the virulence proteins VirD5, VirE2, VirE3, and VirF into host cells. One embodiment may include a fusion of dsRNA binding proteins to the Vir proteins could facilitate dsRNA delivery to insect cells.

In one embodiment, the invention may include a system for enhancing bacterial Type VI secretion systems for delivery of dsRNA to cells. In this preferred embodiment, overexpression of the bacterial gene *yfgL* may enhance phosphatidyl glycerol (membrane lipid) synthesis and vesicle production and budding. In another embodiment, mutation of the *hns* gene, a global regulatory factor that regulates many virulence factors, may cause an increase in *E. coli* vesicle production. In another embodiment, *Salmonella* and *P. aeruginosa* mutants missing the LPS O-antigen side chain also show increased vesicle formation.

In one embodiment, the invention may include a system for Enhancing dsRNA stabilization and mobilization into the plant cell. Some embodiments may include, but not be limited to:

- Co-expression, or over expression the nucleotidyltransferase (*MUT2*) is essential for mobilization of RNA between cells
- MicroRNAs derived from dsRNA having a motif that is bound by sumoylated hnRNPA2B1 have been found to be enriched in vesicles secreted from T cells,
- RNAs with a miR-1289 binding site and a sequence motif (CUGCC presented in a stem-loop structure),
- MicroRNAs with non-templated 3' uridylation
- endogenous RNAs in cells can modulate the sorting of miRNAs into extracellular vesicles.
- Co-expression, or over expression the Ago2 protein
- Four *C. elegans* proteins with relatively well characterized roles in the import of RNA into cells were identified (Winston et al., 2002). Additional alleles of the same genes were identified in two other screens - the fed (feeding RNAi defective) screen (Timmons

et al., 2003) and the rsd (RNAi spreading defective) screen.

- Co-expression of the bromovirus P19 protein to facilitate stabilization and delivery of shRNA to cell
- Expression of fusion proteins between secretion effector proteins and dsRNA binding protein, e.g. Argonaut (Ago) protein involved in dsRNA processing, to facilitate stabilization and delivery of shRNAs into host cells.
- Co-expression, or over expression any gene identified in Tables 5-8 below.
- Employed, as part of the invention described herein, any of the strain identified in Table 7 below.

Further still, another embodiment comprises a composition comprising a genetically modified bacteria configured to colonize a target plant and deliver one or more inhibitory RNA molecules to inhibit one or more plant pathogen essential genes, as well as provide increased pathogen resistance, such method as disclosed herein as an aerial treatment of plants at risk for infection or for plants that are already infected with a target pathogen. In a preferred embodiment, this aerial treatment may include an aerosol, a liquid or desiccated bacteria or spore application.

In an embodiment, the plant is a monocot, or a cell of a monocot plant. For example, the monocot plant is in the gramineae and cereal groups. Non-limiting exemplary monocot species include grains, tropical fruits and flowers, bananas, maize, rice, barley, duckweed, gladiolus, sugar cane, pineapples, dates, onions, rice, sorghum, turfgrass and wheat.

In another embodiment, the plant is a dicot, or a cell of a monocot plant. For example, the dicot plant is selected from the group consisting of Anacardiaceae (e.g., cashews, pistachios), Asteraceae (e.g., asters and all the other composite flowers), Brassicaceae (e.g., cabbage, turnip, and other mustards), Cactaceae (e.g., cacti), Cucurbitaceae (e.g., watermelon, squashes), Euphorbiaceae (e.g., cassaya (manioc)), Fabaceae (e.g., beans and all the other legumes), Fagaceae (e.g., oaks), Geraniales (e.g., geraniums), Juglandaceae (e.g., pecans), Linaceae (e.g., flax), Malvaceae (e.g., cotton), Oleaceae (e.g., olives, ashes, lilacs), Rosaceae (e.g., roses, apples, peaches, strawberries, almonds), Rubiaceae (e.g., coffee), Rutaceae (e.g., oranges and other citrus fruits), Solanaceae (e.g., potatoes, tomatoes, tobacco), Theaceae (e.g., tea), and Vitaceae (e.g., grapes).

The term “plant” or “target plant” includes any plant sustainable to a pathogen. It further includes whole plants, plant organs, progeny of whole plants or plant organs, embryos, somatic embryos, embryo-like structures, protocorms, protocorm-like bodies (PLBs), and suspensions of plant cells. Plant organs comprise, e.g., shoot vegetative organs/structures (e.g., leaves, stems and tubers), roots, flowers and floral organs/structures (e.g., bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit (the mature ovary), plant tissue (e.g., vascular tissue, ground tissue, and the like) and cells (e.g., guard cells, egg cells, trichomes and the like). The class of plants that can be used in the method of the invention is generally as broad as the class of higher and lower plants amenable to the molecular biology and plant breeding techniques described herein, specifically angiosperms (monocotyledonous (monocots) and dicotyledonous (dicots) plants including eudicots. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous. The genetically altered plants described herein can be monocot crops, such as, sorghum, maize, wheat, rice, barley, oats, rye, millet, and triticale. The invention may also include Cannabaceae and other Cannabis strains, such as *C. sativa* generally.

Examples of additional plants species of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus*, *B. rapa*, *B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley; vegetables, ornamentals, and conifers. Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.),

and members of the genus *Cucumis*, such as cucumbers (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnations (*Dianthus caryophyllus*), poinsettias (*Euphorbia pulcherrima*), and chrysanthemums. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*), Douglas-fir (*Pseudotsuga menziesii*), Western hemlock (*Tsuga canadensis*), Sitka spruce (*Picea glauca*), redwood (*Sequoia sempervirens*), true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*), and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). In specific embodiments, plants of the present invention are crop plants (for example, as noted above, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.).

A “plant pathogen” or “pathogen” refers to an organism (bacteria, virus, protist, algae or fungi) that infects plants or plant components. Examples include molds, fungi and rot that typically use spores to infect plants or plant components (e.g fruits, vegetables, grains, stems, roots). A “plant pathogen” also includes all genes necessary for the pathogenicity or pathogenic effects in the plant, or that by their suppression or elimination, such effects are reduced or eliminated.

In one preferred embodiment, the present invention may be applied to one or more of the following non-limiting group of plant viruses, including pathogen gene targets, generally referred to as gene targets, or essential genes, which would be recognized and available to those of ordinary skill in the art without undue experimentation:

Plant Pathogens	hosts	Pathogenic genes	References
Virus diseases			
<i>Tobacco mosaic virus</i> (TMV)	Tobacco, tomato, and other solanaceous plants. It can infect well over 350 different species of plants. The typical symptoms are necrosis, mosaic,	Replicase gene, movement protein, coat protein	(Scholthof, 2004)

	mottling, stunting, leaf curling, and yellowing of plant tissues, etc.		
<i>Tomato spotted wilt virus</i> (TSWV)	Over 1,000 species in over 85 families, including many vegetables, peanut, and tobacco, are susceptible to TSWV. The Solanaceae and Compositae families contain the largest numbers of susceptible plant species. TSWV also replicates in its insect vector, thrips (Thysanoptera: Thripidae)	glycoproteins (GPs), NSm protein, NSs protein, virus RNA genomic segment L, M, and S.	(Adkins, 2000)
<i>Tomato yellow leaf curl virus</i> (TYLCV)	Plants are stunted or dwarfed, can infect about 50 different plant species	V1, V2, C1, C2, C3, and C4. V1 protein	(Czosnek, 2007)
<i>Cucumber mosaic virus</i> (CMV)	Over 1,200 species in over 100 families of monocots and dicots, including many vegetables, ornamentals and woody and semi-woody plants	1a, 2a and 2b protein, 3a and coat protein	(Palukaitis & García-Arenal, 2003)
<i>Potato virus Y</i> (PVY)	PVY mostly infects plants in the family Solanaceae. The Solanaceous plants include economically important ones like potato (<i>Solanum tuberosum</i>), as well as tomato, tomatillo, green pepper, chili pepper, eggplant, petunia and many weeds, such as the nightshades. Easily	P1, HC-Pro, P3, 6k1, CI, 6k2, NIa, Nib, and CP	(Jakab et al., 1997)

	spread by aphids		
<i>Cauliflower mosaic virus (CaMV)</i>	CaMV infects mostly plants of the Brassicaceae family (such as cauliflower and turnip) but some CaMV strains (D4 and W260) are also able to infect Solanaceae species of the genera <i>Datura</i> and <i>Nicotiana</i> .	Movement protein, two aphid transmission factors (P2 and P3), the precursor of the capsid proteins (P4), and polyprotein precursor of proteinase, P5, and P6 protein.	(Hoh et al., 2010)
<i>African cassava mosaic virus (ACMV)</i>	Mosaic, leaf distortion and stunting	AV1, AV2, AC1, AC2, AC3, AC4, BC1, BV1	(Bock & Harrison, 1985, Fauquet & Fargette, 1990)
<i>Plum pox virus (PPV)</i>	<i>Prunus</i> species is widespread in most stone fruit-producing countries. Symptoms include chlorotic and necrotic ring patterns or blotches	P1, HC-Pro, P3, 6k1, CI, 6k2, NIa, Nib, and CP	(Cambra et al., 2006, Ilardi & Tavazza, 2015)
<i>Brome mosaic virus (BMV)</i>	BMV is cosmopolitan and found virtually wherever wheat is grown.	1a, 2a, movement protein, coat protein	(Miller et al., 1985, Ahlquist & Janda, 1984)
<i>Potato virus X (PVX)</i>	PVX is found mainly in potatoes and is only transmitted mechanically, most infections are transmitted by farm machinery, can also be transmitted by vectors such as grasshoppers or biting insects	Replicase, TGB1, TGB2, TGB3, and coat protein	(Kaniewski et al., 1990, Kutnjak et al., 2014)

Additional plant pathogens may include: *Citrus tristeza virus*, *Barley yellow dwarf virus*, *Potato leafroll virus* and *Tomato and bushy stunt virus*.

In one preferred embodiment, the present invention may be applied to one or more of the
5 following non-limiting group of plant fungal pathogens, including pathogen gene targets,

generally referred to as gene targets, or essential genes, which would be recognized and available to those of ordinary skill in the art without undue experimentation:

Plant Pathogens	hosts	Pathogenic genes	References
<i>Magnaporthe oryzae</i>	Rice (<i>Oryza sativa</i>).	<i>MoABC1</i> , <i>MoMAC1</i> and <i>MoPMK1</i> , pathogenicity-related genes, effectors Iug6, Iug9 and Iug18, secreted proteins: MoMpg1, MoEmp1, MoMhp1, MoMsp1, MC69, and Slp1, etc.	(Zhu et al., 2017, Dong et al., 2015)
<i>Botrytis cinerea</i>	It can infect over 200 plant species, causing grey mould, evident on the surface as grey fluffy mycelium. Worldwide, it causes annual losses of \$10 billion to \$100 billion.	pathogenicity-related genes <i>Bcpgl</i> and <i>BMP1</i>	(Williamson et al., 2007, Have et al., 1998, Zheng et al., 2000)
<i>Puccinia</i> spp.	Wheat and barley, common barberry (and some additional <i>Berberis</i> , <i>Mahoberberis</i> , and <i>Mahonia</i> spp.)	pathogenicity-related genes MAPK, cyclophilin, and calcineurin regulatory subunit, and secreted proteins etc.	(Stakman & Levine, 1944, Chen, 2005, Rampitsch et al., 2006, Panwar et al., 2013, Cantu et al., 2013)
<i>Fusarium graminearum</i>	Wheat (<i>Triticum aestivum</i>), Durum Wheat (<i>Triticum durum</i>), Barley (<i>Hordeum vulgare</i>) and Oat (<i>Avena sativa</i>). <i>F. graminearum</i> parasitize s roots, stems, leaves, and reproductive tissues of many species of cereals and grasses.	MAP1, and MAP kinase gpmk1 etc.	(Goswami & Kistler, 2004, Urban et al., 2003, Jenczmionka et al., 2003)
<i>Fusarium oxysporum</i>	It can infect many plants including potato, sugarcane, garden bean, cowpea, Prickly pear, cultivated zinnia, pansy, Assam rattlebox, Baby's	MAP kinase, pg1, pathogenicity-related genes, secreted proteins, etc.	(Di Pietro et al., 2001, Michielsse & Rep, 2009, Di Pietro & Roncero,

	breath, and <i>Musa</i> sp.		1998)
<i>Blumeria graminis</i>	Causing powdery mildew on grasses, including cereals.	effector gene Avra10, pathogenicity-related genes, secreted proteins, etc.	(Nowara et al., 2010, Bindschedler et al., 2016)
<i>Mycosphaerella graminicola</i>	causing septoria leaf blotch, in most years is the second most important disease of wheat in the United States	pathogenicity-related genes, <i>MgSlt2</i> , ABC Transporter Genes <i>MgAtr1</i> and <i>MgAtr2</i> , secreted proteins, etc.	(Goodwin et al., 2011, Brading et al., 2002, Mehrabi et al., 2006, Zwiers & De Waard, 2000)
<i>Colletotrichum</i> spp.	black spot disease in the common bean plant	<i>CMK1</i> , and <i>clk1</i> , pathogenesis-related protein 10, secreted proteins, etc.	(Cannon et al., 2012, Takano et al., 2000, Dufresne et al., 1998, Lo et al., 1999)
<i>Ustilago maydis</i>	causing smut on maize and teosinte	pathogenicity-related genes, <i>Kpp2</i> , <i>ukc1</i> , secreted proteins, etc.	(Kämper et al., 2006, Müller et al., 1999, Dürrenberger & Kronstad, 1999)
<i>Melampsora lini</i>	flax rust	pathogenicity-related genes, secreted proteins, etc.	(Flor, 1956, Lawrence et al., 1981, Nemri et al., 2014)

Any gene being expressed in a cell (preferably a plant cell) can be targeted. A gene that is expressed in the cell is one that is transcribed to yield RNA (e.g., miRNA) and, optionally, a protein. The target gene can be an endogenous gene or an exogenous or foreign gene (i.e., a transgene or a pathogen gene). For example, a transgene that is present in the genome of a cell as a result of genomic integration of the viral delivery construct can be regulated using inhibitory RNA according to the invention. The foreign gene can be integrated into the host genome (preferably the chromosomal DNA), or it may be present on an extra-chromosomal genetic

construct such as a plasmid or a cosmid. For example, the target gene may be present in the genome of the cell into which the interfering RNA is introduced through the novel trans-kingdom method described herein, or similarly in the genome of a pathogen, such as a virus, a bacterium, a fungus or a protozoan, which is capable of infecting such organism or cell.

5 Preferably the target gene is an endogenous gene of the cell or a heterologous gene relative to the genome of the cell, such as a pathogen gene. Preferably, the gene of a pathogen is from a pathogen capable of infecting an eukaryotic organism. Most preferably, said pathogen is selected from the group of virus, bacteria, fungi and nematodes as noted above. By expressing the inhibitory RNA of the invention in plants, not only plant genes can function as target genes
10 for gene silencing, but also genes of organisms which infect plants or eat plants (as food or feed). Thus the target gene can also be a gene of an animal or plant pathogen. The target gene is preferably selected from the group consisting of genes in a plant or of a plant infecting pathogen. Preferably, the expression of the target gene (as measured by the expressed RNA or protein) is reduced, inhibited or attenuated by at least 10%, preferably at least 30% or 40%, preferably at
15 least 50% or 60%, more preferably at least 80%, most preferably at least 90% or 95% or 100%.

 The levels of target products such as transcripts or proteins may be decreased throughout an organism such as a plant, pest or herbivore, or such decrease in target products may be localized in one or more specific organs or tissues of the organism. For example, the levels of products may be decreased in one or more of the tissues and organs of a plant including without
20 limitation: roots, tubers, stems, leaves, stalks, fruit, berries, nuts, bark, pods, seeds and flowers. A preferred organ is a seed of a plant. A broad variety of target genes can be modulated by using the method of the invention, including genes in a plant but also genes or plant infecting or eating pathogens, animals, or even human. Preferably, the target gene is selected from the group consisting of plant endogenous, transgenes, or genes from a plant infecting pathogen. More
25 preferably the plant infecting pathogen is selected from the group consisting of viruses, fungi, bacteria, insects, and nematodes.

 In the case of pathogens, the target or essential gene may, for example, be a house-keeping or other gene, which is essential for viability or proliferation of the pathogen. The attenuation or silencing of the target gene may have various effects (also depending on the nature
30 of the target gene). Preferably, silencing or attenuating said target gene results in loss or reduction or the pathogen's harmful effects, i.e., pathogenicity, or an agronomic trait. Said

agronomic trait may preferably be selected from the group consisting of disease resistance, herbicide resistance, resistance against biotic or abiotic stress, and improved nutritional value. In this context, the target gene may, for example, be preferably selected from the group consisting of genes involved in the synthesis and/or degradation of proteins, peptides, fatty acids, lipids, waxes, oils, starches, sugars, carbohydrates, flavors, odors, toxins, carotenoids, hormones, polymers, flavinoids, storage proteins, phenolic acids, alkaloids, lignins, tannins, celluloses, glycoproteins, and glycolipids. All these sequences are well known to the person skilled in the art and can be easily obtained from DNA data bases by those of ordinary skill in the art (e.g., GenBank).

In certain embodiments, the novel trans-kingdom delivery of inhibitory RNA molecules, namely the methods and means of the invention may be especially suited for obtaining pathogen (e.g., virus or nematode) resistance, in eukaryotic cells or organisms, particularly in plant cells and plants. It is expected that the inhibitory RNA molecules (or the dsRNA molecules derived therefrom) produced by transcription in a host organism (e.g., a plant), can spread systemically throughout the organism. Thus it is possible to reduce the phenotypic expression of a nucleic acid in cells of a non-transgenic scion of a plant grafted onto a transgenic stock comprising the genes of the invention (or vice versa) a method which may be important in horticulture, viticulture or in fruit production. A resistance to plant pathogens such as arachnids, fungi, insects, nematodes, protozoans, viruses, bacteria and diseases can be achieved by reducing the gene expression of genes which are essential for the growth, survival, certain developmental stages (for example pupation), or the multiplication of a certain pathogen. A suitable reduction can bring about a complete inhibition of the above steps, but also a delay of one or more steps. This may include plant genes which, for example, allow the pathogen to enter, but may also be pathogen-homologous genes. Preferably, the inhibitory RNA (such as hpRNA or the dsRNA derived therefrom) is directed against genes of the pathogen. For example, plants can be treated with suitable formulations of above mentioned agents, for example sprayed or dusted; the plants themselves, however, may also comprise the agents in the form of a transgenic organism and pass them on to the pathogens, for example in the form of a stomach poison. Various essential genes of a variety of pathogens are known to those of ordinary skill in the art (for example for nematode resistance: WO 93/10251, WO 94/17194).

Another aspect of methods of the novel trans-kingdom delivery of inhibitory RNA molecules described here provides a method where the target gene for suppression encodes a protein in a plant pathogen (e.g., an insect or nematode). In this aspect, a method comprises introducing into the genome of a pathogen-targeted plant a nucleic acid construct comprising
5 DNA, such as a plasmid, which is transcribed into a inhibitory RNA, such as a hpRNA, that forms at least one dsRNA molecule which is effective for reducing expression of a target gene within the pathogen when the pathogen (e.g., insect or nematode) ingests or infects cells from said plant. In a preferred embodiment, the gene suppression is fatal to the pathogen. Most preferred as a pathogen are fungal pathogens, to the extent not already listed elsewhere, such as
10 *Phytophthora infestans*, *Fusarium nivale*, *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium oxysporum*, *Blumeria graminis*, *Magnaporthe grisea*, *Sclerotinia sclerotium*, *Septoria nodorum*, *Septoria tritici*, *Alternaria brassicae*, *Phoma lingam*, and nematodes such as *Globodera rostochiensis*, *G. pallida*, *Heterodera schachtii*, *Heterodera avenae*, *Ditylenchus dipsaci*, *Anguina tritici* and *Meloidogyne hapla*.

Resistance to pathogenic viruses can be obtained for example by reducing the expression
15 of a viral coat protein, a viral replicase, a viral protease, a structural protein, a toxin and the like. A large number of plant viruses, and suitable target genes are known to those of ordinary skill in the art. The methods and compositions of the present invention are especially useful to obtain nematode resistant plants (for target genes see e.g., WO 92/21757, WO 93/10251, WO
20 94/17194).

Also provided by the invention is a method for obtaining pathogen resistant organisms, particularly plants, comprising the steps of providing cells of the organism with an inhibitory RNA molecule of the invention, said inhibitory RNA molecule capable to provide in an eukaryotic cell an at least partially double-stranded RNA molecule, said inhibitory RNA
25 molecule comprising a) at least one first ribonucleotide sequence that is substantially identical to at least a part of a target nucleotide sequence of at least one gene of a pathogen, b) at least one second ribonucleotide sequence which is substantially complementary to said first nucleotide sequence and is capable to hybridize to said first nucleotide sequence to form a double-stranded RNA structure, and c) at least one third ribonucleotide sequence located between said first and
30 said second ribonucleotide sequence comprising at least one removable RNA element, which can be removed by the RNA processing mechanism of an eukaryotic cell without subsequently

covalently joining the resulting sequences comprising said first and said second ribonucleotide sequence, respectively. Preferably, said first ribonucleotide sequence has between 65 and 100% sequence identity, preferably between 75 and 100%, more preferably between 85 and 100%, most preferably between 95 and 100%, with at least part of the nucleotide sequence of the genome of a pathogen. More preferably the pathogen is selected from the group of virus, bacteria, fungi, and nematodes.

Because this invention involves production of genetically modified bacteria and involves recombinant DNA techniques, the following definitions are provided to assist in describing this invention. The terms "isolated," "purified," or "biologically pure" as used herein, refer to material that is substantially or essentially free from components that normally accompany the material in its native state or when the material is produced. 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 or particular bacteria that are the predominant species present in a preparation is substantially purified. In an exemplary embodiment, the term "purified" denotes that a nucleic acid or protein that 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%.

The term "contact" (with an plant): As used herein, the term "contact with" or "uptake by" an organism (e.g., a plant or pest or herbivore), with regard to a nucleic acid molecule, includes internalization of the nucleic acid molecule into the organism, for example and without limitation: ingestion of the molecule by the organism (e.g., by feeding); contacting the organism with a composition comprising the nucleic acid molecule; soaking of organisms with a solution comprising the nucleic acid molecule; injecting the organism with a composition comprising the nucleic acid molecule; and spraying the organism with an aerosol composition comprising the nucleic acid molecule.

The term "expression," as used herein, or "expression of a coding sequence" (for example, a gene or a transgene) refers to the process by which the coded information of a nucleic acid transcriptional unit (including, e.g., genomic DNA or cDNA) is converted into an operational, non-operational, or structural part of a cell, often including the synthesis of a

protein. Gene expression can be influenced by external signals; for example, exposure of a cell, tissue, or organism to an agent that increases or decreases gene expression. Expression of a gene can also be regulated anywhere in the pathway from DNA to RNA to protein. Regulation of gene expression occurs, for example, through controls acting on transcription, translation, RNA transport and processing, degradation of intermediary molecules such as mRNA, or through activation, inactivation, compartmentalization, or degradation of specific protein molecules after they have been made, or by combinations thereof. Gene expression can be measured at the RNA level or the protein level by any method known in the art, including, without limitation, Northern blot, RT-PCR, Western blot, or in vitro, in situ, or in vivo protein activity assay(s).

The term "nucleic acid" or "nucleic acid molecules" include single- and double-stranded forms of DNA; single-stranded forms of RNA; and double-stranded forms of RNA (dsRNA). The term "nucleotide sequence" or "nucleic acid sequence" refers to both the sense and antisense strands of a nucleic acid as either individual single strands or in the duplex. The term "ribonucleic acid" (RNA) is inclusive of iRNA (inhibitory RNA), dsRNA (double stranded RNA), siRNA (small interfering RNA), mRNA (messenger RNA), miRNA (micro-RNA), hpRNA (hairpin RNA), tRNA (transfer RNA), whether charged or discharged with a corresponding acylated amino acid), and cRNA (complementary RNA). The term "deoxyribonucleic acid" (DNA) is inclusive of cDNA, genomic DNA, and DNA-RNA hybrids. The terms "nucleic acid segment" and "nucleotide sequence segment," or more generally "segment," will be understood by those in the art as a functional term that includes both genomic sequences, ribosomal RNA sequences, transfer RNA sequences, messenger RNA sequences, operon sequences, and smaller engineered nucleotide sequences that encoded or may be adapted to encode, peptides, polypeptides, or proteins.

As used herein "hairpin RNA" (hpRNA) refers to any self-annealing double-stranded RNA molecule. In its simplest representation, a hairpin RNA consists of a double stranded stem made up by the annealing RNA strands, connected by a single stranded RNA loop, and is also referred to as a, "pan-handle RNA." However, the term "hairpin RNA" is also intended to encompass more complicated secondary RNA structures comprising self-annealing double stranded RNA sequences, but also internal bulges and loops. The specific secondary structure adapted will be determined by the free energy of the RNA molecule, and can be predicted for

different situations using appropriate software such as FOLDRNA (Zuker and Stiegler (1981) Nucleic Acids Res 9(1):133-48; Zuker, M. (1989) Methods En- zymol. 180:262-288).

In still other embodiments of the invention, inhibition of the expression of one or more plant pathogen gene products by RNAi may be obtained by hairpin RNA (hpRNA) interference or intron-containing hairpin RNA (ihpRNA) interference. For hpRNA interference, the expression cassette is designed to express an RNA molecule that hybridizes with itself to form a hairpin structure that comprises a single-stranded loop region and a base-paired stem. The base-paired stem region comprises a sense sequence corresponding to all or part of the endogenous messenger RNA encoding the gene product whose expression is to be inhibited, in this case, a cytochrome P450 polypeptide described herein, and an antisense sequence that is fully or partially complementary to the sense sequence. Alternatively, the base-paired stem region may correspond to a portion of a promoter sequence controlling expression of the gene encoding the target polypeptide to be inhibited. Thus, the base-paired stem region of the molecule generally determines the specificity of the RNA interference. hpRNA molecules are highly efficient at inhibiting the expression of endogenous genes, and the RNA interference they induce is inherited by subsequent generations of plants. See, for example, Chuang and Meyerowitz (2000) Proc. Natl. Acad. Sci. USA 97:4985-4990; Stoutjesdijk et al. (2002) Plant Physiol. 129:1723-1731; and Waterhouse and Helliwell (2003) Nat. Rev. Genet. 4:29-38. Methods for using hpRNA interference to inhibit or silence the expression of genes are described, for example, in Chuang and Meyerowitz (2000) Proc. Natl. Acad. Sci. USA 97:4985-4990; Stoutjesdijk et al. (2002) Plant Physiol. 129:1723-1731; Waterhouse and Helliwell (2003) Nat. Rev. Genet. 4:29-38; Pandolfini et al. BMC Biotechnology 3:7, and U.S. Patent Publication No. 20030175965; each of which is herein incorporated by reference. A transient assay for the efficiency of hpRNA constructs to silence gene expression in vivo has been described by Panstruga et al. (2003) Mol. Biol. Rep. 30:135-140, herein incorporated by reference.

For ihpRNA, the interfering molecules have the same general structure as for hpRNA, but the RNA molecule additionally comprises an intron that is capable of being spliced in the cell in which the ihpRNA is expressed. The use of an intron minimizes the size of the loop in the hairpin RNA molecule following splicing, and this increases the efficiency of interference. See, for example, Smith et al. (2000) Nature 407:319-320. In fact, Smith et al. show 100% suppression of endogenous gene expression using ihpRNA-mediated interference. Methods for

using ihpRNA interference to inhibit the expression of endogenous plant genes are described, for example, in Smith et al. (2000) *Nature* 407:319-320; Wesley et al. (2001) *Plant J* 27:581-590; Wang and Waterhouse (2001) *Curr. Opin. Plant Biol.* 5:146-150; Waterhouse and Helliwell (2003) *Nat. Rev. Genet.* 4:29-38; Helliwell and Waterhouse (2003) *Methods* 30:289-295, and
5 U.S. Patent Publication No. 20030180945, each of which is herein incorporated by reference.

As used herein, an oligonucleotide is a short nucleic acid polymer. Oligonucleotides may be formed by cleavage of longer nucleic acid segments, or by polymerizing individual nucleotide precursors. Automated synthesizers allow the synthesis of oligonucleotides up to several hundred base pairs in length. Because oligonucleotides may bind to a complementary nucleotide
10 sequence, they may be used as probes for detecting DNA or RNA. Oligonucleotides composed of DNA (oligodeoxyribonucleotides) may be used in PCR, a technique for the amplification of DNA and RNA (reverse transcribed into a cDNA) sequences. In PCR, the oligonucleotide is typically referred to as a "primer," which allows a DNA polymerase to extend the oligonucleotide and replicate the complementary strand.

The term "gene" or "sequence" refers to a coding region operably joined to appropriate regulatory sequences capable of regulating the expression of the gene product (e.g., a polypeptide or a functional RNA) in some manner. A gene includes untranslated regulatory regions of DNA (e.g., promoters, enhancers, repressors, etc.) preceding (up-stream) and following (down-stream) the coding region (open reading frame, ORF) as well as, where
20 applicable, intervening sequences (i.e., introns) between individual coding regions (i.e., exons). The term "structural gene" as used herein is intended to mean a DNA sequence that is transcribed into mRNA which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

A nucleic acid molecule may include either or both naturally occurring and modified
25 nucleotides linked together by naturally occurring and/or non-naturally occurring nucleotide linkages. Nucleic acid molecules may be modified chemically or biochemically, or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those of skill in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications (e.g., uncharged
30 linkages: for example, methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.; charged linkages: for example, phosphorothioates, phosphorodithioates, etc.; pendent

moieties: for example, peptides; intercalators: for example, acridine, psoralen, etc.; chelators; alkylators; and modified linkages: for example, alpha anomeric nucleic acids, etc.). The term “nucleic acid molecule” also includes any topological conformation, including single-stranded, double-stranded, partially duplexed, triplexed, hair-pinned, circular, and padlocked conformations.

As used herein with respect to DNA, the term “coding sequence,” “structural nucleotide sequence,” or “structural nucleic acid molecule” refers to a nucleotide sequence that is ultimately translated into a polypeptide, via transcription and mRNA, when placed under the control of appropriate regulatory sequences. With respect to RNA, the term “coding sequence” refers to a nucleotide sequence that is translated into a peptide, polypeptide, or protein. The boundaries of a coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. Coding sequences include, but are not limited to: genomic DNA; cDNA; EST; and recombinant nucleotide sequences.

As used herein, the term “genome” refers to chromosomal DNA found within the nucleus of a cell, and also refers to organelle DNA found within subcellular components of the cell. The term “genome” as it applies to bacteria refers to both the chromosome and plasmids within the bacterial cell. In some embodiments of the invention, a DNA molecule may be introduced into a bacterium such that the DNA molecule is integrated into the genome of the bacterium. In these and further embodiments, the DNA molecule may be either chromosomally-integrated or located as or in a stable plasmid.

The term “sequence identity” or “identity,” as used herein in the context of two nucleic acid or polypeptide sequences, refers to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

As used herein, the term “percentage of sequence identity” may refer to the value determined by comparing two optimally aligned sequences (e.g., nucleic acid sequences) over a comparison window, wherein the portion of the sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleotide or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window, and

multiplying the result by 100 to yield the percentage of sequence identity. A sequence that is identical at every position in comparison to a reference sequence is said to be 100% identical to the reference sequence, and vice-versa.

Methods for aligning sequences for comparison are well-known in the art. Various programs and alignment algorithms are described in, for example: Smith and Waterman (1981) Adv. Appl. Math. 2:482; Needleman and Wunsch (1970) J. Mol. Biol. 48:443; Pearson and Lipman (1988) Proc. Natl. Acad. Sci. U.S.A. 85:2444; Higgins and Sharp (1988) Gene 73:237-44; Higgins and Sharp (1989) CABIOS 5:151-3; Corpet et al. (1988) Nucleic Acids Res. 16:10881-90; Huang et al. (1992) Comp. Appl. Biosci. 8:155-65; Pearson et al. (1994) Methods Mol. Biol. 24:307-31; Tatiana et al. (1999) FEMS Microbiol. Lett. 174:247-50. A detailed consideration of sequence alignment methods and homology calculations can be found in, e.g., Altschul et al. (1990) J. Mol. Biol. 215:403-10. The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST™; Altschul et al. (1990)) is available from several sources, including the National Center for Biotechnology Information (Bethesda, Md.), and on the internet, for use in connection with several sequence analysis programs. A description of how to determine sequence identity using this program is available on the internet under the “help” section for BLAST™. For comparisons of nucleic acid sequences, the “Blast 2 sequences” function of the BLAST™ (Blastn) program may be employed using the default BLOSUM62 matrix set to default parameters. Nucleic acid sequences with even greater similarity to the reference sequences will show increasing percentage identity when assessed by this method.

As used herein, the terms “hybridizable” and “complementary” are terms that indicate a sufficient degree of complementarity such that stable and specific binding occurs between the nucleic acid molecule and a target nucleic acid molecule. Hybridization between two nucleic acid molecules involves the formation of an anti-parallel alignment between the nucleic acid sequences of the two nucleic acid molecules. The two molecules are then able to form hydrogen bonds with corresponding bases on the opposite strand to form a duplex molecule that, if it is sufficiently stable, is detectable using methods well known in the art. A nucleic acid molecule need not be 100% complementary to its target sequence to be specifically hybridizable. However, the amount of sequence complementarity that must exist for hybridization to be specific is a function of the hybridization conditions used, and may be between 50%-100%.

As used herein, the term “homologous” with regard to a contiguous nucleic acid sequence, refers to contiguous nucleotide sequences that hybridize under appropriate conditions to the reference nucleic acid sequence. For example, homologous sequences may have from about 70%-100, or more generally 80% to 100% sequence identity, such as about 81%; about 82%; about 83%; about 84%; about 85%; about 86%; about 87%; about 88%; about 89%; about 90%; about 91%; about 92%; about 93%; about 94% about 95%; about 96%; about 97%; about 98%; about 98.5%; about 99%; about 99.5%; and about 100%. The property of substantial homology is closely related to specific hybridization. For example, a nucleic acid molecule is specifically hybridizable when there is a sufficient degree of complementarity to avoid non-specific binding of the nucleic acid to non-target sequences under conditions where specific binding is desired, for example, under stringent hybridization conditions.

The term, “operably linked,” when used in reference to a regulatory sequence and a coding sequence, means that the regulatory sequence affects the expression of the linked coding sequence. “Regulatory sequences,” or “control elements,” refer to nucleotide sequences that influence the timing and level/amount of transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters; translation leader sequences; introns; enhancers; stem-loop structures; repressor binding sequences; termination sequences; polyadenylation recognition sequences; etc. Particular regulatory sequences may be located upstream and/or downstream of a coding sequence operably linked thereto. Also, particular regulatory sequences operably linked to a coding sequence may be located on the associated complementary strand of a double-stranded nucleic acid molecule.

As used herein, the term “promoter” refers to a region of DNA that may be upstream from the start of transcription, and that may be involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A promoter may be operably linked to a coding sequence for expression in a cell, or a promoter may be operably linked to a nucleotide sequence encoding a signal sequence which may be operably linked to a coding sequence for expression in a cell. A “plant promoter” may be a promoter capable of initiating transcription in plant cells. Examples of promoters under developmental control include promoters that preferentially initiate transcription in certain tissues, such as leaves, roots, seeds, fibers, xylem vessels, tracheids, or sclerenchyma. Such promoters are referred to as “tissue-preferred.” Promoters which initiate transcription only in certain tissues are referred to as “tissue-specific.”

A “cell type-specific” promoter primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. An “inducible” promoter may be a promoter which may be under environmental control. Examples of environmental conditions that may initiate transcription by inducible promoters include anaerobic conditions and the presence
5 of light. Tissue-specific, tissue-preferred, cell type specific, and inducible promoters constitute the class of “non-constitutive” promoters. A “constitutive” promoter is a promoter which may be active under most environmental conditions or in most cell or tissue types.

Any inducible promoter can be used in some embodiments of the invention. See Ward et al. (1993) Plant Mol. Biol. 22:361-366. With an inducible promoter, the rate of transcription
10 increases in response to an inducing agent. Exemplary inducible promoters include, but are not limited to: Promoters from the ACEI system that responds to copper; In2 gene from maize that responds to benzenesulfonamide herbicide safeners; Tet repressor from Tn10; and the inducible promoter from a steroid hormone gene, the transcriptional activity of which may be induced by a glucocorticosteroid hormone are general examples (Schena et al. (1991) Proc. Natl. Acad. Sci.
15 USA 88:0421).

As used herein, the term “transformation” or “genetically modified” refers to the transfer of one or more nucleic acid molecule(s) into a cell. A microorganism is “transformed” or “genetically modified” by a nucleic acid molecule transduced into the bacteria when the nucleic acid molecule becomes stably replicated by the bacteria. As used herein, the term
20 “transformation” or “genetically modified” encompasses all techniques by which a nucleic acid molecule can be introduced into, such as a bacteria.

The term “vector” refers to some means by which DNA, RNA, a protein, or polypeptide can be introduced into a host. The polynucleotides, protein, and polypeptide which are to be introduced into a host can be therapeutic or prophylactic in nature; can encode or be an antigen;
25 can be regulatory in nature, etc. There are various types of vectors including virus, plasmid, bacteriophages, cosmids, and bacteria.

An “expression vector” is nucleic acid capable of replicating in a selected host cell or organism. An expression vector can replicate as an autonomous structure, or alternatively can integrate, in whole or in part, into the host cell chromosomes or the nucleic acids of an organelle,
30 or it is used as a shuttle for delivering foreign DNA to cells, and thus replicate along with the host cell genome. Thus, an expression vector are polynucleotides capable of replicating in a

selected host cell, organelle, or organism, e.g., a plasmid, virus, artificial chromosome, nucleic acid fragment, and for which certain genes on the expression vector (including genes of interest) are transcribed and translated into a polypeptide or protein within the cell, organelle or organism; or any suitable construct known in the art, which comprises an "expression cassette." In contrast, as described in the examples herein, a "cassette" is a polynucleotide containing a section of an expression vector of this invention. The use of the cassettes assists in the assembly of the expression vectors. An expression vector is a replicon, such as plasmid, phage, virus, chimeric virus, or cosmid, and which contains the desired polynucleotide sequence operably linked to the expression control sequence(s).

A polynucleotide sequence is operably linked to an expression control sequence(s) (e.g., a promoter and, optionally, an enhancer) when the expression control sequence controls and regulates the transcription and/or translation of that polynucleotide sequence.

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), the complementary (or complement) sequence, and the reverse complement sequence, 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)). Because of the degeneracy of nucleic acid codons, one can use various different polynucleotides to encode identical polypeptides. Table 1a, infra, contains information about which nucleic acid codons encode which amino acids.

TABLE 1a Amino acid Nucleic acid codons

Amino Acid	Nucleic Acid Codons
Ala/A	GCT, GCC, GCA, GCG
Arg/R	CGT, CGC, CGA, CGG, AGA, AGG
Asn/N	AAT, AAC
Asp/D	GAT, GAC

Cys/C	TGT, TGC
Gln/Q	CAA, CAG
Glu/E	GAA, GAG
Gly/G	GGT, GGC, GGA, GGG
His/H	CAT, CAC
Ile/I	ATT, ATC, ATA
Leu/L	TTA, TTG, CTT, CTC, CTA, CTG
Lys/K	AAA, AAG
Met/M	ATG
Phe/F	TTT, TTC
Pro/P	CCT, CCC, CCA, CCG
Ser/S	TCT, TCC, TCA, TCG, AGT, AGC
Thr/T	ACT, ACC, ACA, ACG
Trp/W	TGG
Tyr/Y	TAT, TAC
Val/V	GTT, GTC, GTA, GTG

Oligonucleotides and polynucleotides that are not commercially available can be chemically synthesized e.g., according to the solid phase phosphoramidite triester method first described by Beaucage and Caruthers, Tetrahedron Letts. 22:1859-1862 (1981), or using an automated synthesizer, as described in Van Devanter et al., Nucleic Acids Res. 12:6159- 6168 (1984). Other methods for synthesizing oligonucleotides and polynucleotides are known in the art. 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).

The term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, organism, 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 may express genes that are not found within the native (nonrecombinant or wild-type) form of the cell or express native genes that are otherwise abnormally expressed--over-expressed, under expressed or not expressed at all.

The terms "approximately" and "about" refer to a quantity, level, value or amount that varies by as much as 30%, or in another embodiment by as much as 20%, and in a third embodiment by as much as 10% to a reference quantity, level, value or amount. As used herein, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a bacterium" includes both a single bacterium and a plurality of bacteria.

As used here "suppression" or "silencing" or "inhibition" are used interchangeably to denote the down-regulation of the expression of the product of a target sequence relative to its normal expression level in a wild type organism. Suppression includes expression that is decreased by about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% relative to the wild type expression level. An "effective amount" is an amount of inhibitory RNA sufficient to result in suppression or inhibition of a plant pathogen. .

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or is synthetically designed, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. A heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention. By "host cell" is meant a cell which contains an introduced nucleic acid construct and supports the replication and/or expression of the construct. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as fungi, yeast, insect, amphibian, nematode, or mammalian cells. Alternatively, the host cells are monocotyledonous or dicotyledonous plant cells. An example of a monocotyledonous host cell is a maize host cell.

Polynucleotide sequences may have substantial identity, substantial homology, or substantial complementarity to the selected region of the target gene. As used herein "substantial identity" and "substantial homology" indicate sequences that have sequence identity or homology to each other. Generally, sequences that are substantially identical or substantially homologous will have about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity wherein the percent sequence identity is based on the entire sequence and is determined by GAP alignment using existing default parameters (GCG, GAP version 10, Accelrys, San Diego, CA). GAP uses the algorithm of Needleman and Wunsch

((1970) J Mol Biol 48:443-453) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of sequence gaps. Sequences which have 100% identity are identical. "Substantial complementarity" refers to sequences that are complementary to each other, and are able to base pair with each other. In describing
 5 complementary sequences, if all the nucleotides in the first sequence will base pair to the second sequence, these sequences are fully complementary.

EXAMPLES

Example 1: Demonstrates construction of bacterial plasmids encoding hairpin (hp) RNA constructs encoding a foldback RNA targeting a green fluorescent protein (GFP).

10 As demonstrated by the present inventors, hpRNAs encoding plasmid vectors were constructed using NEBuilder® HiFi DNA Assembly Cloning Kit from New England BioLabs Inc. Here, seven hpRNA vectors were constructed to target the green fluorescent protein (GFP) encoding mRNA expressed from pJL-TRBO-G (Lindbo 2007) as an exemplary embodiment.

The maps for these plasmids are shown in the following figures 1=7: pAD-WRKY-
 15 GHY1 (Fig.1), pAD-ADH-GHY2 (Fig.2), pOXB-WRKY-GHY3 (Fig.3), pOXB-ADH-GHY4 (Fig.4), pAD-WRKY-GHY5 (Fig.5), pAD-ADH-GHY6 (Fig.6). One hpRNA designed using TMV movement protein: pAD-WRKY-GHY7 (Fig.7). were constructed to target both movement protein and GFP from PjL-TRBO-G Bold bases are overlapped sequences. Italicized bases are inserted sequences.

20 Among the above seven hpRNAs, five shuttle vectors pAD-WRKY-GHY1, pAD-ADH-GHY2, pAD-WRKY-GHY5, pAD-ADH-GHY6, and pAD-WRKY-GHY7 were constructed by the present inventors by ligating the GFP sense fragment, an intervening intron, and the GFP antisense fragment into the backbone plasmid pAD43-25 (Dunn and Handelsman 1999) digested with *Xba*I+*Nsi*I. Two vectors pOXB-WRKY-GHY3 and pOXB-ADH-GHY4 were constructed
 25 using the backbone plasmid of pSF-OXB19 (Oxford Genetics Biology Engineered) digested with *Xba*I.

Exemplary primers used to construct the hpRNA insert in the plasmids and for DNA sequencing are shown in Table 1 below. All the maps of hpRNAs were made using SnapGene™ software.

30 Example 2: Demonstrates construction of bacterial plasmids encoding an intervening intron between the sense and antisense strands of the GFP gene target.

One intron was from the *Arabidopsis thaliana* putative WRKY-type DNA-binding protein, 287bp in length, and cloning from plasmid pJawohl3-RNAi (Accession no. AF404854) (Table 9: SEQ ID NO. 26).

Another intron was generated by the current inventors from *Zea mays* alcohol dehydrogenase intron Adh1, 566bp in length, and cloning from plasmid pMCG161 (Accession no. AY572837). (Table 9: SEQ ID NO. 27-29)

Example 3: Demonstrates construction and sequence listing of exemplary hpRNAs.

In this embodiment, exemplary hpRNAs were constructed comprising SEQ ID NO. 30-36 presented in Table 10 below.

Example 4: Demonstrates construction of RNaseIII mutant bacterial strain for the stable expression of dsRNA constructs.

In this embodiment, the present inventors used exemplary *E. coli* strain JM109(DE3) to construct an RNaseIII mutant (Genotype: *endA1, recA1, gyrA96, thi, hsdR17* (r_k^- , m_k^+), *relA1, supE44, λ^- , $\Delta(lac-proAB)$, [F', *traD36, proAB, lacI^qZAM15*], IDE3. Promega, USA). Two *E. coli* RNaseIII mutants were made by using Red-mediated homologous recombination system expressed from plasmid pSIJ8 (Jensen et al. 2015).*

The design of RNaseIII mutant was performed using the DNA sequence for the *rnc* gene of *Escherichia coli* str. K-12 substr. MG1655 (NCBI Reference Sequence: NC_000913.3). The primers RNaseIII50-5 and RNaseIII50-3 were used for amplifying the kanamycin resistance gene with 50-bp homologous sequences with the *rnc* genes. The targeting PCR fragments with Kan^R were amplified by using Q5[®] High-Fidelity DNA Polymerase. The plasmid pKD4 was used as template (Yin et al. 2009). The targeting PCR fragments with KanR were ligated to Pmini T2.0 vector to obtain Pmini4T plasmid and then Pmini-4T was used as template to amplify the targeting fragments by using phosphorothioated primers Ec_phos_50-5 and Ec_phos_50-3. The mutants were generated according to Jensen *et al* (Jensen et al. 2015). The mutant containing Kanamycin resistance marker was labelled as M-JM109-GHY1. The mutant eliminating Kanamycin resistance was labelled as M-JM109-GHY2 and may be used to deliver hpRNA to plants. Primers were listed in Table 2. PCR amplification analysis to verify the mutants is demonstrated in Fig.1.

Example 5: Demonstrates PCR analysis to confirm construction of RNaseIII mutant bacterial strain for the stable expression of hpRNA constructs.

As shown in Figure 8A-C, the present inventors conducted a PCR analysis to verify the successful construction of *E. coli* RNaseIII mutants. Again, as demonstrated in Figure 8A, PCR analysis was generated to verify mutants M-JM109-GHY1. Lanes 1-16: Colony PCR performed with M-JM109-GHY1 with the primers JD-5 and JD-3, expected fragment size 1,936 bp; and the wide-type is 879 bp. Picking up the possible correct mutants 4, 13, 14, 15, and 16 and inoculated them in LB (Kanamycin, 50 µg/ml) media and cultivated overnight for further identification. Mutants 13 and 15 grew well. These two mutants were used for further identification by the present inventors.

As shown in Figure 8B, further identification the purified mutants 13 and 15 was conducted. Two colonies were analyzed. Lanes 1-3: the first colony mutant was analyzed with primers JD-5 and JD-3, JD-5 and Cat-3, and Cat-5 and JD-3, respectively; lanes 4-6: the second colony mutant were analyzed with primers JD-5 and JD-3, JD-5 and Cat-3, and Cat-5 and JD-3, respectively; lane 7: The wild-type JM109(DE3) was amplified with JD-5 and JD-3. Correct mutant size PCR products were 1936 bp (amplified with primers JD-5 and JD-3), 1664 bp (amplified with primers JD-5 and Cat-3), and 1787 bp (amplified with primers Cat-5 and JD-3). All the DNA sequencing results were correct. The correct mutant containing Kanamycin resistant marker was labelled as M-JM109-GHY1.

As shown in Figure C, PCR performed on Kanamycin resistance gene eliminated mutant with the primers JD-5 and JD-3, expected fragment size 543 bp. Lanes 1-8: Eight individual mutants amplified with primers JD-5 and JD-3. The correct mutant without Kanamycin resistant marker was labelled as M-JM109-GHY2. (References Lanes = M₁: 100 bp DNA ladder; lane M₂: 1 Kb DNA ladder.) All the PCR products were sent for sequencing and all the sequencing results were confirmed and reproduced below in Table 11. (SEQ ID NO. 37-39)

Example 6: Demonstrates the use of a genetically modified *E. coli* RNaseIII mutants containing plasmids encoding hpRNA molecules to infiltrate plant leaves, express and deliver dsRNA or hpRNAs to the plant to be processed into siRNAs to inactivate TMV encoded GFP as an exemplary embodiment of bacterial dsRNA-mediated silencing of a viral encoded gene.

Here the present inventors used two *E. coli* RNase III mutants (one *Bacillus subtilis* CCB422 RNaseIII mutant) containing the indicated plasmids to infiltrate leaves, express and deliver dsRNA or hpRNAs to the plants to be processed into siRNAs to inactivate Tobacco

Mosaic Virus (TMV) encoded GFP, thereby demonstrating bacterial dsRNA-mediated silencing of a viral encoded gene.

The present inventors conducted tobacco infiltration assays with bacteria containing plasmids encoding hpRNA targeting the suppression of the GFP gene were performed according to Lindbo (Lindbo 2007). Symptoms were observed and GFP signal was detected under UV illumination after 5, 7, or 9 days post-infiltration (dpi). Two tobacco species: *Nicotiana tabacum* and *Nicotiana benthamiana* were used in this embodiment. For example, as an exemplary embodiment, in Fig. 11A, infiltration symptoms and GFP signal detected under UV illumination of healthy and infiltrated leaves of *N. tabacum* is demonstrated.

The present inventors further demonstrated TMV encoded GFP was expressed from vector pJL-TRBO-G (Lindbo 2007). The vector was transformed into *Agrobacterium tumefaciens* (At) and co-infiltrated with bacterial RNaseIII mutants containing different hpRNAs. The bacterial concentration was measured using a spectrometer and all the bacterial concentrations were adjusted to OD₆₀₀ between 0.9950 ~ 1.0050. The present inventors further demonstrate that the hpRNA inhibited the TMV-GFP signal, and confirmed by co-infiltration of the bacterial containing hpRNA with *Agrobacterium* GV3101 containing vector pJL-TRBO-G. The RNaseIII mutants and hpRNA used are listed in Table 3.

As shown in FIGS. 10-11B, present inventors demonstrated that both MJM109 and HT27, each expressing different hpRNAs, show inhibition to the spreading of TMV-encoded GFP signal. The shuttle hpRNA vectors were shown by the inventors to produce better inhibition of GFP expression. MJM109 showed a better result compared to HT27 in this exemplary embodiment.

As further shown in FIG. 11C, the present inventors have shown that both MJM109 and HT27 with different hpRNAs show different inhibition to the spreading of TMV-encoded GFP signal. The present inventors demonstrated that shuttle hpRNA vectors produce better inhibition of GFP expression. For example, MJM109 shows increased inhibition of TMV-GFP signal compared to HT27. The present inventors demonstrated that all the hpRNA can produce dsRNA structures that can generate RNAi effects in plants, which is consistent with disease symptoms show in Fig11B and elsewhere.

Legend FIG. 10:

- At: *Agrobacterium tumefaciens*; TMV: pJL-TRBO-G; MJM109: M-JM109-GHY2 (RNaseIII mutant, no antibiotic); GHY5: pAD-WRKY-GHY5; GHY6: pAD-ADH-GHY6; GHY3: pOXB-WRKY-GHY3; and GHY4: pOXB-ADH-GHY4.

Legend FIG. 11:

- 5 - At: *Agrobacterium tumefaciens*; TMV, TMVexpressing GFP: pJL-TRBO-G (TMV green fluorescence signal); MJM109: M-JM109-GHY2 (RNaseIII mutant, no antibiotic); HT27, RNaseIII mutant, Tetracycline resistance; GHY5: pAD-WRKY-GHY5; GHY6: pAD-ADH-GHY6; GHY3: pOXB-WRKY-GHY3; GHY4: pOXB-ADH-GHY4.
- A and G: At/TMV;
- 10 - B: At/TMV+MJM109;
- H: At/TMV+HT27;
- C: At/TMV+MJM109/GHY5;
- D: At/TMV+MJM109/GHY6;
- E: At/TMV+MJM109/GHY3;
- 15 - F: At/TMV+MJM109/GHY4;
- I: At/TMV+HT27/GHY5;
- J: At/TMV+HT27/GHY6;
- K: At/TMV+HT27/GHY3; and
- L: At/TMV+HT27/GHY4.

20 Example 7: Demonstrates that hpRNA inhibits the spread of TMV-encoded GFP signal in model tobacco plant.

As demonstrated in FIGS. 12, the present inventors demonstrated that after At/TMV infection, tobacco plants were inoculated with hpRNAs over a course of 6 day. The present inventors demonstrated that within three days that the expression of hpRNAs could inhibit the spreading of the TMV-encoded GFP signal.

Legend FIG. 12:

- A: hpRNAs inoculated 1 day later;
- B: hpRNAs inoculated 2 days later;
- C: hpRNAs inoculated 3 days later;
- 30 - D: hpRNAs inoculated 4 days later;
- E: hpRNAs inoculated 5 days later; and

- F: hpRNAs inoculated 6 days later.

Example 8: Demonstrates tobacco plant infiltration with *Bacillus subtilis* CCB422/pAD-WRKY-GHY5 in *N. tabacum*

As demonstrated in FIG. 13, the present inventors demonstrated that a genetically modified *B. subtilis* expressing hpRNA could successfully infiltrate and inhibit the spreading of At/TMV signal in *N. tabacum*. In this embodiment, the present inventors demonstrated that CCB422 with hpRNA can significantly inhibit the spreading of At/TMV signal.

Legend FIG. 13:

- At: *Agrobacterium tumefaciens*; TMV: pJL-TRBO-G (TMV green fluorescence signal); CCB422 (*Bacillus subtilis* RNaseIII mutant, Spectinomycin and Kanamycin resistance); GHY5: pAD-WRKY-GHY5, and each treatment repeated three times. Red arrows indicated infiltrated areas.
- A: Blank Negative Control
- B: At/TMV (TMV expressing GFP)
- C: CCB422 (bacteria without plasmid only)
- D: CCB422/GHY5 (bacteria with plasmid but no TMV)
- E: TMV+CCB422/GHY5 (TMV and hpRNA)

Example 9: Hypervesiculating bacteria strains show increased ability to inhibit the spreading of At/TMV signal in *N. benthamiana*.

As demonstrated in FIG. 14, the present inventors demonstrated that a genetically modified *B. subtilis* expressing hpRNA could successfully infiltrate and inhibit the spreading of At/TMV signal in *N. benthamiana*. In this embodiment, the present inventors demonstrated that an hypervesiculating *E. coli* strain HT27 with hpRNA can significantly inhibit the spreading of At/TMV signal. In this embodiment, the present inventors demonstrated that, hypervesiculating strains may increase the ability of interfering RNAs, like hpRNA, inhibit target pathogen gene expression.

Legend FIG. 14:

- Right panel: upper, three repeats of At/TMV treatment; lower, three repeats of TMV+HT27/GHY5

- At: *Agrobacterium tumefaciens*; TMV: pJL-TRBO-G (TMV green fluorescence signal); HT27, RNaseIII mutant, Tetracycline resistance; GHY5: pAD-WRKY-GHY5, and each treatment repeated three times.
- A: Blank Negative Control
- 5 - B: At/TMV
- C: HT27
- D: HT27/GHY5
- E: TMV+HT27/GHY5

Example 10: Time-course demonstration of the efficiency of hpRNA mediated GFP signal suppression in model plant organism.

The present inventors demonstrated the hpRNA mediated GFP signal suppression in a tobacco plant of over a prescribed time-course. Here, the present inventors a tobacco plant with inoculated hpRNA: MJM109-GHY2/pAD-WRKY-GHY5, then inoculated the same plant with At/TMV in different dates. As generally shown in FIG. 15, after hpRNA inoculation, the model tobacco plant was protected from the modified TMV virus infection up to at least 6 dpi. The present inventors further demonstrated in figures 16-17, that hpRNA migrated to different parts of the infiltration area within a leaf 7 dpi. For example, in figure 16 - 17, TMV-GFP was shown to be surrounding by hpRNA at 7 dpi. This demonstrates the long-term and continuing nature of the inoculation and suppression of the target viral gene.

Legend FIG. 15:

- A: At/TMV inoculated 1 day later
- B: At/TMV inoculated 2 days later
- C: At/TMV inoculated 3 days later
- D: At/TMV inoculated 4 days later
- 25 - E: At/TMV inoculated 5 days later
- F: At/TMV inoculated 6 days later

Example 11: hpRNA can effectively produce siRNAs in tobacco and limit the spreading of At/TMV signal in *N. tabacum*

The present inventors demonstrated that hpRNA can effectively produce siRNAs in plants and limit the spreading, and expression of At/TMV signal. In this embodiment, hpRNA derived from MJM109-GHY2/pAD-WRKY-GHY5 infiltrated *N. tabacum* and processed into

siRNA as generally described above. As shown in figure 18, MJM109-GHY2/pAD-WRKY-GHY5 derived siRNA limited the spreading of TMV-GFP signal.

Legend FIG. 17:

- Dark arrows indicate At/TMV infiltration treatment.
- 5 - White arrows indicate hpRNA (MJM109-GHY2/pAD-WRKY-GHY5) infiltration treatment.
- At: *Agrobacterium tumefaciens*;
- TMV: pJL-TRBO-G (TMV green fluorescence signal)

Example 12: siRNA can effectively limit the spreading of TMV-GFP signal in *N. tabacum*.

The present inventors demonstrated that hpRNA can effectively produce siRNAs in plants and limit the spreading, and expression of TMV-GFP signal. In this embodiment, MJM109-GHY2/pAD-WRKY-GHY7 derived siRNA limited the spreading of TMV-GFP signal. (Fig. 18A). In addition, the present inventors demonstrated that, CCB422/pAD-WRKY-GHY5
15 derived siRNA also limited the spreading of TMV-GFP signal. (Fig. 18B). In this embodiment, the present inventors demonstrated that hpRNA may induce more siRNAs production for RNAi in *N. tabacum* in a dose-dependent manner and limit the spreading of TMV-GFP signal.

Legend FIG. 18A-B:

- Dark arrows indicate At/TMV infiltration treatment.
- 20 - White arrows indicate hpRNA (MJM109-GHY2/pAD-WRKY-GHY5) infiltration treatment.
- Fig. 18A: At: *Agrobacterium tumefaciens*;
- Fig. 18B: TMV: pJL-TRBO-G (TMV green fluorescence signal)

Example 13: Demonstrates *Bacillus cereus* identification and colonization.

25 As an exemplary embodiment, the present inventors demonstrate the endophytic bacteria *Bacillus cereus*, to express a shuttle vector pAD43-25 encoding green fluorescent protein can colonize the leaves of tobacco.

In this exemplary embodiment, *B. cereus* 53522 was obtained from ATCC and primers used for identification were listed in Table 4. The correct PCR fragments were ligated to the
30 vector of PminiT 2.0 vector and then digested with *XhoI* to inspect the correct sizes and then identify the correct clones by DNA sequence analysis to confirm the identity of the *B. cereus*

strain (Fig.9). Results of *B. cereus* 53522 (UW85) sequencing are provided in Table 12: 16S rRNA: 1514 bp; 23S rRNA: 855 bp; motB: 574 bp; endo-b-1,4-glucanase genes: 659 bp, mrc gene: 738 bp; SCAP: no PCR bands.

Example 14: Transformation and expression of hpRNA in *Bacillus cereus* as well as inoculation and colonization into model plant organism.

The shuttle vector pAD43-25 and hpRNA vector of pAD-WRKY-GHY5 was transformed into *Bacillus cereus* 53522 strain by the present inventors, and then inoculated into an exemplary tobacco plant to confirm the expression of GFP signal under the constitutive promoter P_{gfp}. This signal is demonstrated by the present inventors in Figure 19, and the colonization of the transformed *B. cereus* in the model tobacco plant is further demonstrated in Figure 20.

Example 15: Reduction in viral encoded GFP protein accumulation / infection severity mediated by hairpin RNA endophyte delivery.

As noted above, targeting of TMV-GFP RNA transcript by the host's RNAi machinery is expected to result in viral RNA degradation and hence failure to translate viral mRNAs into proteins. Boosting of the host's RNAi defenses, by trans-kingdom delivery of a hairpin RNA homologous to the viral encoded GFP, is therefore predicted to result in loss or decrease in viral encoded GFP accumulation by triggering siRNA biogenesis in the *N. tabacum* host, ultimately conferring resistance to viral infection.

To evaluate this hypothesis, 2x 0.4mm leaf discs were collected from infiltrated areas (5dpi) of *N. tabacum* plants infected with TMV-GFP, or co-infiltrated with TMV-GFP and bacterial strains carrying the pAD-WRKY-GHY5 construct – encoding the viral GFP homologous hairpin RNA, as well as experimental controls (see figure 21). Total protein extraction was performed with 100µl of protein extraction buffer and insoluble material pelleted for 15min; 16,000g, 4°C according to Baumberger and Baulcombe (2005). Samples were resolved by PAGE and blotted to a nitrocellulose membrane by semi-dry transfer. Membranes were stained with Ponceau S prior to western blotting to allow quantification of Rubisco largest subunit (RBCL). Viral GFP was detected by incubation with anti-GFP antibody (Millipore 3F8.2; 1: 10,000) followed by incubation with goat anti-mouse HRP secondary antibody (Agrisera, 1: 20,000). Signal detection and acquisition was performed with ECL western detection kit according to manufacturer's instructions (GE) and ChemiDoc MP imaging system

(BioRad) respectively. GFP signal quantification was performed with Image Lab software (BioRad).

As demonstrated by the present inventors in figure 21, co-infiltration of TMV-GFP virus with the endophyte *Bacillus subtilis* CCB422 (Durand et al. 2012) (figure 21a,b) encoding the GFP-hpRNA resulted in a decrease in GFP accumulation in infected plants when compared to infiltration of TMV-GFP alone. Significantly, a replicate experiment using *E. coli* HT27 as vehicle for trans-kingdom delivery (figure 21c) failed to detect accumulation of any viral encoded GFP when co-infiltration was performed, strongly contrasting with the observed GFP accumulation in unchallenged TMV infections. Our results indicate that trans-kingdom delivery of hpRNAs homologous to the viral genome efficiently downregulates or eliminates viral replication and translation of viral proteins.

Example 16: qPCR to monitor the TMV-GFP mRNA suppression by HT27/pAD-WRKY-GHY5.

L25 ribosomal protein gene mRNA levels were used as the *N. tabacum* reference gene, and primers targeting amplification of TMV-GFP RNA were used in a quantitative PCR experiment (qPCR) for analysis of relative RNA levels in different biological treatments infected with TMV-GFP, TMV-GFP plus bacteria alone, and TMV plus bacteria expressing hpRNA-GFP. Total RNA was extracted from different treated tobacco leaves by using *mirVana*TM miRNA Isolation kit. The cDNA was synthesized using the EcoDryTM Premix kit. QRT-PCR was performed using iTaqTM Universal SYBR® Green Supermix. The results are generally shown in Fig. 22 and Table 6.

The present inventors demonstrated a reduction in TMV-GFP due to the presence of the bacteria (~ 43%). The present inventors demonstrated bacteria expressing hpDNA-GFP had nearly a 90% reduction in GFP RNA levels consistent with enhanced siRNA-mediated interference in TMV-GFP RNA levels.

TABLES

Table 1: Primers used for the construction of hpRNAs. Bold bases are overlapped sequences. Italicized bases are inserted sequences. (SEQ ID NO. 1-25)

Primers	Sequences (5' → 3')	SEQ ID No.
pAD-JL-F1	GGAA ~~AACTGTATGTATT~~ ~~TGATCCTTGCCCGAAGGTT~~ ATGTACAGG	SEQ ID No. 1
pJL-WRKY-R1	GCAGAGGAGGAGAAAGGGCAGATTGTGTCGACA	SEQ ID No. 2
WRKY-F1	CAATCTGCCC ~~TTTTCTCCTCCTCTGCTAACGTAAG~~	SEQ ID No. 3
WRKY-R1	CAATCTGCCC ~~TTTTCTGTGGTTGGAGAAGCTAG~~	SEQ ID No. 4
pJL-WRKY-F1	CTCCAACCACAGAAAGGGCAGATTGTGTCGACA	SEQ ID No. 5
pAD-JL-R1	AAGTTAAGGGATGCAGTTTATGCATGCCCCGAAGGTT ATGTACAGG	SEQ ID No. 6
pJL-adh-R2	TTGCACTTGATCAAAGGGCAGATTGTGTCGACA	SEQ ID No. 7
pJL-adh-F2	GTGCAGCTGCGGAAAGGGCAGATTGTGTCGACA	SEQ ID No. 8
adh-F1	CAATCTGCCC ~~TTTGATCAAGTGCAAAGGTCGCTTG~~	SEQ ID No. 9
adh-R1	CAATCTGCCC ~~TTTCCGCAGCTGCACGGGTCC~~	SEQ ID No. 10
OXB19-JL-F1	ACCGCGATATCTACCTCGAGGTTTGGCCCGAAGGTT ATGTACAGG	SEQ ID No. 11
OXB19-JL-R1	AGTCAGTGCAGGAGGAGACAAC ~~TTTGGCCCGAAGGT~~ TATGTACAGG	SEQ ID No. 12
pAD-JL-F2	GGAA ~~AACTGTATGTATT~~ ~~TGATCCTCTAGATTAAAGA~~ AGGAGATATACATTTGCCCGAAGGTTATGTACAGG	SEQ ID No. 13
TMVMP-F1	GGAA ~~AACTGTATGTATT~~ ~~TGATCCTCTAGATTAAAGA~~ AGGAGATATACATTTCTCGGATCTTACTACACAGCAGC	SEQ ID No. 14
TMVMP-R1	TAGCAGAGGAGGAGTTCCCTTTGCGGACATCAC	SEQ ID No. 15
TMVMP-WRKY-F1	CCGCAAAGGGAACTCCTCCTCTGCTAACGTAAGCC	SEQ ID No. 16
TMVMP-WRKY-R1	CCGCAAAGGGAACTGTGGTTGGAGAAGCTAGAACC	SEQ ID No. 17
TMVMP-F2	CTCCAACCACAGTCCCTTTGCGGACATCACTCT	SEQ ID No. 18
TMVMP-R2	AAGTTAAGGGATGCAGTTTATGCATCTCGGATCTTA CTACACAGCAGC	SEQ ID No. 19
Identification/Sequencing primers		
pAD-JD-F1	GCGTGCAAACGCATGAATATC	SEQ ID No. 20
PAD-JD-R1	AGGGCCTCGTGATACGCCT	SEQ ID No. 21
WRKY-JD-F1	ATCGTGATCGGAAGTGATAAAG	SEQ ID No. 22
OXB-JD-F1	GCTTCCGAGCTCTCGAATTC	SEQ ID No. 23
adh-JD-F1	CGATGAACAGTGCCGCAG	SEQ ID No. 24
adh-JD-F2	TGAGTGGCCCTGTTCTCG	SEQ ID No. 25

Table 2. Primers used in construction of M-JM109-GY1 and M-JM109-GHY2. Underlined bases are 50 bp homologous sequences to the *E.coli rnc* gene. The italicized bases are primers used for amplification of the Kanamycin resistance marker. *Denoting the phosphorothioated primers. SEQ. ID No. 45 through 52, numbered sequentially from top to bottom of middle column.

Primers	Primer sequence (5'→3') ^a	Specific use
Ec_RNas eIII50-5	<u>ATGAACCCCATCGTAATTAATCGG</u> <u>CTTCAACGGAAGCTGGGCTACACT</u> <i>TTAGCGATTGTGTAGGCTGGAG</i>	Target primer, underlined sequence is 50bp homologous sequence
Ec_RNas eIII50-3	<u>CTGATCGTGCGCTTCGCCACGTAC</u> <u>CTGGACTACCAGATAAGTCGGCAG</u> <i>CGTTAACGGCTGACATGGGAATTAG</i>	Target primer, underlined sequence is 50bp homologous sequence
Ec_phos_ 50-5	A*T*G*A*ACCCCATCGTAATTAAT CGGC	phosphorothioated primers
Ec_phos_ 50-3	C*T*G*A*TCGTGCGCTTCGC	phosphorothioated primers
JD-5	ACCGGTAAACTGAAACTGCA	Mutant screening
JD-3	TGGAGATTTTCTGCCCCAG	Mutant screening
Cat-5	AGCGATTGTGTAGGCTGGAGCT	mutant screening
Cat-3	TTAACGGCTGACATGGGAATTAGC	mutant screening

Table 3. *E. coli* RNaseIII mutants and associated plasmids encoding hpRNAs, plus *Agrobacterium* utilized in tobacco plant infiltration assays

Plasmid/RNaseIII mutants	Purposes	Specificity	References
pAD-WRKY-GHY1	hpRNA to target GFP from TMV-GFP	Pupp (uracil phosphoribosyl transferase) promoter, shuttle vector, low-copy number, containing <i>Arabidopsis</i> WRKY transcription factor 33 intron	This study
pAD-ADH-GHY2	hpRNA to target GFP from TMV-GFP	Pupp ((uracil phosphoribosyl transferase) promoter, shuttle vector, low-copy number, containing maize adh1 intron	This study
pOXB-WRKY-GHY3	hpRNA to target GFP from TMV-GFP	LacUV5 promoter, pUC high copy number vector for <i>E.coli</i> , containing <i>Arabidopsis</i> WRKY transcription factor 33 intron	This study
pOXB-ADH-GHY4	hpRNA to target GFP from TMV-GFP	LacUV5 promoter, pUC high copy number vector for <i>E.coli</i> , containing maize adh1 intron	This study
pAD-WRKY-GHY5	hpRNA to target GFP from TMV-GFP	Pupp (uracil phosphoribosyl transferase) promoter, shuttle vector, low-copy number, containing <i>Arabidopsis</i> WRKY transcription factor 33 intron	This study
pAD-ADH-GHY6	hpRNA to target GFP from TMV-GFP	Pupp ((uracil phosphoribosyl transferase) promoter, shuttle vector, low-copy number, containing maize adh1 intron	This study
pAD-WRKY-GHY7	hpRNA to target GFP from TMV-GFP	Pupp ((uracil phosphoribosyl transferase) promoter, shuttle vector, low-copy number, containing <i>Arabidopsis</i> WRKY transcription factor 33 intron	This study
pJL-TRBO-G	Used to express TMV encoded GFP signal	Kanamycin resistance	(Lindbo 2007)
M-JM109-GHY2	RNaseIII mutant, genotype: endA1, recA1, gyrA96, thi, hsdR17 (rk ⁻ , mk ⁺), relA1, supE44, λ ⁻ , Δ(lac-proAB), [F', traD36, proAB, lacIqZAM15], IDE3.	No antibiotic resistance	This study
HT27	RNaseIII mutant, as a "helper" hyper-vesiculating phenotype which we predicted would facilitate packaging and transfer of dsRNA to the host (hypervesiculating amino acid auxotrophic mutant), genotype: his ⁻ , ilv ⁻ , pglA8	Tetracycline resistance	(Takiff and Chen 1989)
CCB422	RNaseIII mutant	Kanamycin and spectinomycin resistance	(Durand et al. 2012)
<i>Bacillus cereus</i> 53522	Endophytic in plant, RNaseIII wide-type	no antibiotic resistance, used to check the colonization of bacteria in plants and express dsRNA	(Lozano et al. 2016; Raffel et al. 1996)
<i>Agrobacterium tumefaciens</i> GV3101	was used to infiltration tobacco	Rifampin resistance	(Lindbo 2007)

Table 4 Primers used for identification and verification of *Bacillus* spp. SEQ. ID No. 53 through 62 numbered from top to bottom or middle column.

Genes	Upper primers	Size (bp)	Purposes
16S rRNA	8F: AGAGTTTGATCCTGGCTCAG	~1500	Non-coding 16S rRNA gene
	1492R: CGGTTACCTTGTACGACTT		
23S rDNA	BAC11: AGAGTGCCTAATAGCTCAC	880	Non-coding 23S rDNA
	BAC19: CGGTCTAGAACTTACCGACAAGG		
<i>motB</i>	BCFomp1: ATCGCCTCGTTGGATGACGA	575	encoding flagellar motor protein; a unique conserved sequence from <i>B.</i>
	BCRomp1: CTGCATATCCTACCGCAGCTA		
endo-b-1,4-glucanase	EN1F: CCAGTAGCCAAGAATGGCCAGC	1311	Encoding the endoglucanase gene
genes	EN1R: GGAATAATCGCCGCTTTGTGC		
SCAR	PAGA1: ATGAGCCCTGTGATCAGGAAGATCG	270	Sequence-characterized amplified regions
	PAGB1: ACGATGAGCCTTCTCAGCAAATGCG		

5

Table 5: Additional mobilization Genes

Reduced RNA silencing spread	Enhanced RNA silencing spread	Do not affect PDS and/or SUL silencing spread
<i>ago1</i> ^a	<i>ago4</i> ^{b,c,d}	<i>ago4</i> ^{a,d}
<i>clsyl</i> ^b	<i>dcl3</i> ^{b,d}	<i>dcl2</i> ^a
<i>dcl1</i> ^a		<i>dcl3</i> ^{a,d}
<i>dcl4</i> ^{b,e}		<i>drb4</i> ^a
<i>fca</i> ^f		<i>hst1</i> ^g
<i>fpa</i> ^f		<i>hyl1</i> ^a
<i>fy</i> ^h		<i>rdr1</i> ^a
<i>jmj14</i> ^c		<i>rdr6</i> ⁱ
<i>hen1</i> ^a		<i>sde3</i> ⁱ
<i>hpr1</i> ^j		<i>sde5</i> ^{j,k}
<i>nripd1</i> ^{a,b}		
<i>nripd2a</i> ^b		
<i>rdr2</i> ^{a,b}		
<i>tex1</i> ^l		

Table 6: Primers used for qRT-PCR in Figure 22.

Primer names	Sequences (5-3)	Purpose and references
L25F1	CCCCTCACCACAGAGTCTGC	<i>N. tabacum</i> reference gene, (Schmidt & Delaney, 2010)
L25R1	AAGGGTGTGTGTTGTCCTCAATCTT	
TMV-GFP-F2	GATGACGGGAAC TACAAGACG	Amplification TMV-GFP, designed in this study
TMV-GFP-R2	GTTTGTGTCCGAGAATGTTTCC	

Table 7: Helper gene strains that enhance dsRNA delivery.

Bacterial Strains	Helper Activity
HT115	<i>E. coli</i> RNase III deficient strain suitable for production of dsRNA. Used in many studies for trans-kingdom delivery of dsRNA
HT27	<i>E. coli</i> RNase III deficient strain suitable for production of dsRNA. It also has two auxotrophies (histidine and isoleucine). Auxotrophy has been shown to enhance nanotube formation with other bacteria. These nanotubes facilitate transfer of biomaterial (proteins, nucleic acids)
JC8031	<i>E. coli</i> with enhanced hyper-vesiculation activity

Table 8: Helper genes that enhance dsRNA delivery.

Helper Gene	Activity
VrrA	Small non-coding RNA that upon binding to OmpA mRNA increases bacterial hyper-vesiculation activity
SID1	dsRNA-specific transporter that facilitates systemic RNAi
SID2	Membrane protein that is required for uptake of ingested dsRNA
AGO1, AGO2 and AGO7	Member of Argonaute family that is specific to provide host defense against RNA viruses

YmdB	Bacterial regulatory protein that suppresses RNase III cleavage
YmdB	Bacterial regulatory protein that suppresses RNase III cleavage
dsRNA-binding proteins: Staufen and RDE-4	Staufen and RDE-4 proteins can bind dsRNA. Co-expression of these proteins and dsRNA in bacteria can enhance the lifetime of the produced dsRNA and facilitate their transport into cells
HlyA	Use of Hemolysin A transport signal to enhance secretion of dsRNA-binding proteins
Sec- and Tat-secretory signal peptides	Use of Sec and Tat signaling peptides to enhance secretion of dsRNA-binding proteins
Cell-penetrating peptides (CPPs): Tat and Antennapedia	Use of CPPs such as Tat and Antennapedia or similar peptides to enhance uptake of dsRNA-binding proteins in cells . Can be combined with secretory peptides
ACC deaminase	1-aminocyclopropane-1-carboxylate (ACC) deaminase, enzyme responsible for degradation of plant ethylene precursor and hence reduction of plant ethylene levels
Phloem RNA transporters; PP2-A1 and PSRP1	involved in mobilization to and transport of mRNA and smRNAs in the phloem
DRB1 and DRB4	bind dsRNA miRNA precursors and siRNA precursors, respectively, and are required for recruitment of downstream RNA processing enzymes.
HEN1	Protein involved in smRNA stabilization via methylation of 3'-end of smRNA duplexes
STV1	ribosomal protein involved in miRNA biogenesis, required for localization of pri-miRNA precursors to nuclear dicing bodies

SEQUENCE LISTINGS

TABLE 9. Sequence Listing for Introns Sequences

5

>WRKY_intron-287 (SEQ ID NO. 26)

CTCCTCCTCTGCTAACGTAAGCCTCTCTGTTTTTTTTCTCTGTTTCTTTTGAATGAATCCAAT
 TAGTGATGATAATCTGTGTTTGATGTATCATTGATTTAACATCTTGACAATGAATCGTGATCG
 GAAGTGATAAAGTTATGGGTCAACGGTTTCAAAGAGAGAGAAAAGACTTTTAGAGTCAACTC
 10 TCGACTCTTTCTTAATTATGTTATTGCTATTTGTCTCTTTTCTTGAAGTCTGAACAATTCTTGG
 GATTGTTTTGCAGGTTCTAGCTTCTCCAACCACAG

>adh1_intron-566: (SEQ ID NO. 27)

GATCAAGTGCAAAGGTCCGCCTTGTTTCTCCTCTGTCTCTTGATCTGACTAATCTTGGTTTTAT
 15 GATTTCGTTGAGTAATTTTGGGGAAAGCTAGCTTCGTCCACAGTTTTTTTTTCGATGAACAGTG
 CCGCAGTGGCGCTGATCTTGATGCTATCCTGCAATCGTGGTGAACCTTATGTCTTTTATATCC
 TTCACTACCATGAAAAGACTAGCTAGTAATCTTTCTCGATGTAACATCGTCCAGCACTGCTAT
 TACCGTGTGGTCCATCCGACAGTCTGGCTGAACACATCATAACGATATTGAGCAAAGATCGAT
 CTATCTTCCCTGTTCTTTAATGAAAGACGTCATTTTCATCAGTATGATCTAAGAATGTTGCAA
 20 CTTGCAAGGAGGCGTTTCTTTCTTTGAATTTAACTAACTCGTTGAGTGGCCCTGTTTCTCGGA
 CGTAAGGCCTTTGCTGCTCCACACATGTCCATTTCGAATTTTACCGTGTTTAGCAAGGGCGAA
 AAGTTTGCATCTTGATGATTTAGCTTGACTATGCGATTGCTTTCCCTGGACCCGTGCAGCTGCG
 G

>TMV-GFP-dsRNA-360 (SEQ ID NO. 28)

TGCCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACTACAAGAC
 GCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTG
 ATTTTAAAGAAGATGGAACATTCTCGGACACAACTCGAGTACAACCTATAACTCACACAAT
 GTATACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGCCACA
 30 ACATTGAAGATGGATCCGTTCACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGAT
 GGCCCTGTCTTTTACCAGACAACCATTACCTGTGACACAATCTGCCCTT

>TMV-MP-dsRNA-360 (SEQ ID NO. 29)

TCTCGGATCTTACTACACAGCAGCTGCAAAGAAAAGATTTTCAGTTCAAGGTCGTTCCCAATT
 35 ATGCTATAACCACCCAGGACGCGATGAAAAACGTCTGGCAAGTTTTAGTTAATATTAGAAAT
 GTGAAGATGTCAGCGGGTTTCTGTCCGCTTTCTCTGGAGTTTGTGTGCGGTGTGTATTGTTTAT
 AGAAATAATATAAAAATTAGGTTTGGAGAGAGAAGATTACAAACGTGAGAGACGGAGGGCCCA
 TGGAACCTTACAGAAGAAGTCGTTGATGAGTTCATGGAAGATGTCCCTATGTCGATCAGGCTT
 GCAAAGTTTCGATCTCGAACCGGAAAAAAGAGTGATGTCCGCAAAGGGAA

40

TABLE 10: Sequence Listing for Exemplary hpRNAs.

>pAD-WRKY-GHY1 (SEQ ID NO. 30)

GAATTCGAGCTCGGTACCCGGGGATCGCGTGAAAGAAATTTTCGGCGCAGAGCACGTAAAT
 GTTCAACCACACTCTGGTGACAAGCGAACATGGCAGTATACTTCACGATTTTAGAGCAAGG
 CGATACAGTACTTGGTATGAATTTATCTCATGGTGGTCACTTAACACACGGAAGCCCTGTTA
 ACTTCAGTGGAGTACAATATAATTTTCGTAGAATATGGCGTGGATGCTGACTCTCACCGTATT
 AATTACGATGATGTATTAGCAAAAAGCGAAAGAACATAAACCAAAATTAATCGTTGCAGGTG

CAAGTGCATACCCTCGTGTTATCGATTTC AAGCGATTCCGTGAGATTGCAGATGAAGTGGGC
GCTTATTTAATGGTTGATATGGCACATATCGCTGGTTTAGTAGCTGCTGGTTTACATCCAAAT
CCAGTACCACATGCACATTTTCGTTACAACGACAACACATAAAACGTTACGTGGCCCCGCGTGG
TGGTATGATTTTATGTGAAGAGCAATTTGCAAAACAAATTGATAAATCAATCTTCCCTGGTA
5 TTCAAGGTGGTCCACTTATGCACGTAATCGCTGCAAAAGCTGTTGCGTTTGGTGAAGCACTT
CAAGATGATTTCAAAACATATGCACAAAATATCATTAAACAATGCCAACCCTTAGCTGAAG
GTCTTCAAAAAGAAGGACTTACACTTGTCTTGGCGGAACAGACAATCACTTAATCTTGATT
GATGTTTCGTAACCTTAGAAAATCACAGGTAAAGTAGCAGAGCACGTATTAGATGAAGTTGGTAT
TACAGTGAACAAAAATACAATTCATTTGAAACAGCAAGCCCATTTGTAACAAGTGGTGTAC
10 GTATCGGTACAGCAGCTGTAACATCTCGTGGTTTTCGTTTGTAGAAGAAATGGATGAAATTGCG
TCACTTATTGCTTATACATTAATAAATCATGAAAATGAAGCTGCATTAGAAGAAGTACGTAA
GCGTGTAGAAGCGTTAACTAGCAAATTTCCAATGTATCCAAATCTATAATAGATTGAAGAAG
ACTGCCGAGACTTAATTGTTTTGGCGGTCTTTTTGTGGACATATATTATTTTTAAAGTATGT
ATACAAATGATGAATAAATTTTGGCGATATAATGAAGGATACAGCTCCCATAAATTGGTAAAG
15 ATACTAGATAGATTCATCGTAAATCATGATTTTGCCAAATTTGCCCTTGAATAATTAGTAGCG
TTTTCTTTACAATCGTAAATAGTGTAATAAAGCGTGCAACGCATGAATATCATCTAAAGGA
GAGATTCACATGGGAAAACTGTATGTATTTGATCCTTGCCCGAAGGTTATGTACAGGAACGC
ACTATATCTTTCAAAGATGACGGGAACCTACAAGACGCGTGCTGAAGTCAAGTTTGAAGGTG
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20 GGACACAAACTCGAGTACAATACTCACACAATGTATACATCACGGCAGACAAACAAA
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GCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCA
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TCTGTTTCTTTTGAATGAATCCAATTAGTGATGATAATCTGTGTTTGATGTATCATTGATTT
25 AACATCTTGACAATGAATCGTGATCGGAAGTGATAAAGTTATGGGTCAACGGTTTCAAAGA
GAGAGAAAGACTTTTAGAGTCAACTCTCGACTCTTTCTTAATTATGTTATTGCTATTTGTCTC
TTTTCTTGAAGTCTGAACAATTCTTGGGATTGTTTTGCAGGTTCTAGCTTCTCCAACACAGA
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 CGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCCGGTGTAGGTGCTTCGCTCCAA
 35 GCTGGGCTGTGTGCACGAACCCCCCGTTACGCCCCGACCGCTGCGCCTTATCCGGTAACTATC
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 50 AAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTGAGAAGTAAGTTGGCCGCAGTGTTATC
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 CTTGCCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATC
 ATTGGAAAACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTC
 GATGTAACCCACTCGTGACCCCACTGATCTTCAGCATCTTTTACTTTACCCAGCGTTTCTGG
 5 GTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAAGGGAATAAGGGCGACACGGAAATG
 TTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTCTCATG
 AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCGCGCACATTTCC
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 GCGGTATCACGAGGCCCTTTCGTCTTCAA

>pAD-ADH-GHY2 (SEQ ID NO. 31)

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 ACTTCAGTGGAGTACAATATAATTTCTGATAGAAATATGGCGTGGATGCTGACTCTCACCGTATT
 AATTACGATGATGTATTAGCAAAAAGCGAAAGAACATAAACCAAAATTAATCGTTGCAGGTG
 CAAGTGCATACCCTCGTGTTATCGATTTCAAGCGATTCCGTGAGATTGCAGATGAAGTGGGC
 GCTTATTTAATGGTTGATATGGCACATATCGCTGGTTTAGTAGCTGCTGGTTTACATCCAAAT
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 30 GCGTGTAGAAGCGTTAACTAGCAAATTTCCAATGTATCCAAATCTATAATAGATTGAAGAAG
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 GATGTAACATCGTCCAGCACTGCTATTACCGTGTGGTCCATCCGACAGTCTGGCTGAACACA
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 TTTTACCGTGTTTAGCAAGGGCGAAAAGTTTGCATCTTGATGATTTAGCTTGACTATGCGATT
 GCTTTCTGACCCGTCAGCTGCGGAAAGGGCAGATTGTGTGACAGGTAATGGTTGTCTG
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 CTTCTTTAAAATCAATACCTTTTAACTCGATACGATTAACAAGGGTATCACCTTCAAACCTTGA
 CTTTCAGCACGCGTCTTGTAGTTCCCGTCATCTTTGAAAGATATAGTGCCTTCTGTACATAAC
 CTTTCGGGCATGCATAAACTGCATCCCTTAACTTGTCTTTCGTGTGCCTATTTTTTGTGAATCGC
 5 TAAGAAACCATTATTATCATGACATTAACCTATAAAAAATAGGCGTATCACGAGGCCCTTTCG
 TCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCA
 CAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGT
 TGGCGGGTGTCCGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACC
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 10 TCTGCTTTTTTTATTTGATTATGTAATTTTTTAATGCCAGGATGCCAATAAGCCATAACCTCAA
 ATGCACCATTTGCAACCTCGTCATCTTCTTCTCAATCTTGACCAGATCGCCGTCTCTCCGCAT
 CACCAAGATTCAGCTCTTTATGTATCTCCTTCAAAATGCCACCGTATCCAATTAACCTTCGAG
 CTGCCAACGCATCATCCAAGTAAAGCACCGTGTTTCAGATTGTCTTCAGTCACCTTATTACCGC
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>pOXB-WRKY-GHY3 (SEQ ID NO. 32)

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10 >pOXB-ADH-GHY4 (SEQ ID NO. 33)

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>pAD-WRKY-GHY5 (SEQ ID NO. 34)

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>pAD-ADH-GHY6 (SEQ ID NO. 35)

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>pAD-WRKY-GHY7 (SEQ ID NO. 36)

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CATAGTAAGGTGCCCCAATCTTACTCTCTAACGCTTCGTAATGCTCCGCCATGAGGTTTCGTCC
45 GTCTCTTTTTCCCTTCCAATCCCGCTTTTTACCTGTTGCGGTTTTATCTTCGAGGATGCTATA
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TATTTTGATTTCGACACCTTAATAATAGCAGAAGGAGTTTTTACCTGTCAAAGAACCATCAAA

CCCTTGATACACAAGGCTTTGACCTAATTTTGAAAAATGATGTTGTTTCTATATAGTATCAAG
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TAAAGTGGGATATTTTAAATATATATTTATGTTACAGTAATATTGACTTTTAAAAAAGGAT
5 TGATTCTAATGAAGAAAGCAGACAAGTAAGCCTCCTAAATTCACTTTAGATAAAAAATTTAGG
AGGCATATCAAATGAACCTTTAATAAAATTGATTTAGACAATTGGAAGAGAAAAGAGATATT
TAATCATTATTTGAACCAACAAACGACTTTTAGTATAACCACAGAAATTGATATTAGTGTTTT
ATACCGAAACATAAAACAAGAAGGATATAAATTTTACCCTGCATTTATTTTCTTAGTGACAA
GGGTGATAAACTCAAATACAGCTTTTAGAACTGGTTACAATAGCGACGGAGAGTTAGGTTAT
10 TGGGATAAGTTAGAGCCACTTTATACAATTTTGTATGGTGTATCTAAAACATTCTCTGGTATT
TGGACTCCTGTAAAGAATGACTTCAAAGAGTTTTATGATTTATACCTTTCTGATGTAGAGAA
ATATAATGGTTCGGGGAAATTGTTTTCCCAAAACACCTATACCTGAAAATGCTTTTTCTCTTTC
TATTATTCCATGGACTTCATTTACTGGGTTTAACTTAAATATCAATAATAATAGTAATTACCT
TCTACCCATTATTACAGCAGGAAAATTCATTAATAAAGGTAATTCAATATATTTACCGCTATC
15 TTTACAGGTACATCATTCTGTTTGTGATGGTTATCATGCAGGATTGTTTATGAACTCTATTCA
GGAATTGTCAGATAGGCCTAATGACTGGCTTTTATAATATGAGATAATGCCGACTGTACTTT
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GCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTT
GTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGG
20 GTGTCCGGGGCGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGATACTGGCTTAACTA
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TGCGTAAGGAGAAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCG
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25 ACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCAC
AAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGT
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CCGCTTTCTCCCTTCGGGAAGCGTGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTT
CGGTGTAGGTCGTTCCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTACGCCCGACCGC
30 TGCGCCTTATCCGTAACCTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACT
GGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTC
TTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCT
GAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCT
GGTAGCGGTGGTTTTTTTGTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGA
35 AGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGGA
TTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAATAATGAAGTT
TAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGT
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TAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAG
40 ACCCAGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCG
CAGAAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTA
GAGTAAGTAGTTCCGCCAGTTAATAGTTTGCAGAACGTTGTTGCCATTGCTGCAGGCATCGTG
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ACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTGAG
45 AAGTAAGTTGGCCGAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGT
CATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAAT
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 TTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAG
 GGAATAAGGGCGACACGGAAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAG
 5 CATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTAGAAAAATAAAC
 AAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATT
 ATCATGACATTAACTATAAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAA

TABLE 11: Sequence Listing for RNaseIII mutants

>Escherichia_coli_str._K-12_substr. MG1655_rnc gene (SEQ ID NO. 37)

ATGAACCCCATCGTAATTAATCGGCTTCAACGGAAGCTGGGCTACACTTTTAATCATCAGGA
 ACTGTTGCAGCAGGCATTAATCATCGTAGTGCCAGCAGTAAACATAACGAGCGTTTAGAAT
 15 TTTTAGGCGACTCTATTCTGAGCTACGTTATCGCCAATGCGCTTTATCACCGTTTCCCTCGTG
 TGGATGAAGGCGATATGAGCCGGATGCGCGCCACGCTGGTCCGTGGCAATACGCTGGCGGA
 ACTGGCGCGCAATTTGAGTTAGGCGAGTGCTTACGTTTAGGGCCAGGTGAACTTAAAAGCG
 GTGGATTTCTGTCGTGAGTCAATTCTCGCCGACACCGTCAAGCATTAAATTGGTGGCGTATTC
 CTCGACAGTGATATTCAAACCGTCGAGAAATTAATCCTCAACTGGTATCAAACCTCGTTTGA
 20 CGAAATTAGCCCAGGCGATAAAACAAAAAGATCCGAAAACGCGCTTGCAAGAATATTTGCAG
 GGTCGCCATCTGCCGCTGCCGACTTATCTGGTAGTCCAGGTACGTGGCGAAGCGCACGATCA
 GGAATTTACTATCCACTGCCAGGTGAGCGGCCTGAGTGAACCGGTGGTTGGCACAGGTTCAA
 GCCGTCGTAAGGCTGAGCAGGCTGCCGCCGAACAGGCGTTGAAAAAACTGGAGCTGGAATG
 A

>M-JM109-GHY1-Kanymycin-resistant-mutant-1936bp (SEQ ID NO. 38)

ACCGGTAAACTGAAACTGCAGCGAAGCAGTTAGCAGAACCATGTATATCAGGTCTGTTTCGT
 GTGCTGAATTGTTGACGCATTTATTTATTGGTATCGCATGAACCCCATCGTAATTAATCGGCT
 30 TCAACGGAAGCTGGGCTACACTTTAGCGATTGTGTAGGCTGGAGCTGCTTCGAAGTTCCTAT
 ACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCAAGATCCCCTCACGCTGCCGCAAGC
 ACTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAAC
 GGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGC
 AAAGAGAAAGCAGGTAGCTTGCACTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTA
 35 TGGACAGCAAGCGAACCAGGAAATGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCT
 GCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCT
 GATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTT
 CTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGC
 TCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGA
 40 CCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACG
 ACGGGCGTTCCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCT
 ATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTAT
 CCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGAC
 CACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATC
 45 AGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCCGCCAGGCTCAA
 GCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATA

TCATGGTGGAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGAC
 CGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGC
 TGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGCAGCGCATCGCCTTCTATCG
 CTTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCC
 5 CAACCTGCCATCACGAGATTTTCGATTCCACCGCCGCCCTTCTATGAAAGGTTGGGCTTCGGAA
 TCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTC
 GCCCACCCAGCTTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGG
 AATAGGAACTAAGGAGGATATTCATATGGACCATGGCTAATTCCCATGTCAGCCGTAAACGC
 TGCCGACTTATCTGGTAGTCCAGGTACGTGGCGAAGCGCACGATCAGGAATTTACTATCCAC
 10 TGCCAGGTCAGCGGCCTGAGTGAACCGGTGGTTGGCACAGGTTCAAGCCGTCGTAAGGCTG
 AGCAGGCTGCCGCCGAACAGGCGTTGAAAAAACTGGAGCTGGAATGAGCATCGATAAAAAGT
 TACTGCGGATTTATTGCCATCGTCGGACGTCCGAACGTTGGCAAATCCACATTGTTGAACAA
 ACTGCTGGGGCAGAAAATCTCCA

15 >M-JM109-GHY1-mutant-543bp (SEQ ID NO. 39)

ACCGGTAAACTGAAACTGCAGCGAAGCAGTTAGCAGAACCATGTATATCAGGTCTGTTTCGT
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 TCAACGGAAGCTGGGCTACACTTTAGCGATTGTGTAGGCTGGAGCTGCTTCGAAGTTCCTAT
 20 ACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTAAGGAGGATATTCATATGGACCATGG
 CTAATTCCCATGTCAGCCGTAAACGCTGCCGACTTATCTGGTAGTCCAGGTACGTGGCGAAG
 CGCACGATCAGGAATTTACTATCCACTGCCAGGTCAGCGGCCTGAGTGAACCGGTGGTTGGC
 ACAGGTTCAAGCCGTCGTAAGGCTGAGCAGGCTGCCGCCGAACAGGCGTTGAAAAAACTGG
 AGCTGGAATGAGCATCGATAAAAAGTTACTGCGGATTTATTGCCATCGTCGGACGTCCGAACG
 25 TTGGCAAATCCACATTGTTGAACAAACTGCTGGGGCAGAAAATCTCCA

TABLE 12: Sequence Listing *B. cereus* 53522 (UW85) sequencing

30 >Bc53522-16SrRNA-complete-1514 (SEQ ID NO. 40)

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCG
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 CCTGCCCATAAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAA
 CTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCAT
 35 TAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG
 ATCGGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
 TTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTC
 GTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTAC
 CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
 40 GTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGC
 CCACGGCTCAACCGTGGAGGGTCAATTGGAACTGGGAGACTTGAGTGCAAGAGGAAAGT
 GGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAAGTGGCGAAGGCG
 ACTTTCTGGTCTGTAACCTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATA
 CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTG
 45 CTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAG
 GAATTGACGGGGGCCCCGACAAAGCGGTGGAGCATGTGGTTTAATTGCAAGCAACGCGAAGA
 ACCTTACCAGGTCTTGACATCCTCTGAAAACCTAGAGATAGGGCTTCTCCTTCGGGAGCAG
 AGTGACAGGTGGTGCATGGTTGTCGTACGCTCGTGCTGAGATGTTGGGTAAAGTCCCGCA

ACGAGCGCAACCCCTTGATCTTAGTTGCCATCATTAAAGTTGGGCACTCTAAGGTGACTGCCGG
 TGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCCTTATGACCTGGGCTA
 CACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCAT
 AAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGT
 5 AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
 CCACGAGAGTTTGTAAACACCCGAAGTCGGTGGGGTAACCTTTTGGAGCCAGCCGCCTAAGG
 TGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAACCG

>BC53522-23S-rRNA-complete-855 (SEQ ID NO. 41)

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 GTGAAGTCAGACCGGAAGGACTGGTGGAGCGCTTAGAAGTGAGAATGCCGGTATGAGTAGC
 GAAAGACGGGTGAGAATCCCGTCCACCGAATGCCTAAGGTTTCCTGAGGAAGGCTCGTCCG
 15 CTCAGGGTTAGTCAGGACCTAAGCCGAGGCCGACAGGCGTAGGCGATGGACAACAGGTTGA
 TATTCCTGTACCACCTCTTTATCGTTTGAGCAATGGAGGGACGCAGAAGGATAGAAGAAGCG
 TGCGATTGGTTGTGCACGTCCAAGCAGTTAGGCTGATAAGTAGGCAAATCCGCTTATCGTGA
 AGGCTGAGCTGTGATGGGGAAGCTCCTTATGGAGCGAAGTCTTTGATTCCCCGCTGCCAAGA
 AAAGCTTCTAGCGAGATAAAAGGTGCCTGTACCGCAAACCGACACAGGTAGGCGAGGAGAG
 20 AATCCTAAGGTGTGCGAGAGAACTCTGGTTAAGGAACTCGGCAAAATGACCCCGTAACTTC
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 AAAAACACAGCTCTCTGCGAAGCCGTAAGGCGAAGTATAGGGGGTGACACCTGCCCGGTGC
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>BC53522-motB-574 (SEQ ID NO. 42)

TCGCCTCGTTGGATGACGACTTTTACAGATTTAACGATGTTATTATTAACCTTTCTTCGTATTA
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 30 TACGGGGCAAGTAGATGCAAAAGTAATGGAAAATACAATAACCAGATATTTACATGAAAAA
 AATGATGAAAAAATGATCTCAAAAAAGAGAATGGATGAATTATATAAGAAGTTGAAAAGCGT
 ATGTAGATAATAACGGTATTAGTCAAGTGAATGTATATCGAGAGGATACAGGGGTAAAGCGT
 CGTTATAGTAGATAATTTAATATTTGATACAGGCGATGCGAACGTTAAGCCCGAAGCGAAAG
 GGATAATAAGTCAATTAGTTGGATTTTTTCAATCCGTACCTAACCCAATTGTTGTAGAGGGA
 35 CATACAGATAGTAGACCTATTCATAACGAGAAATTCCTTCTAATTGGGAGTTATCTTCAGC
 ACGAGCGGCAAATATGATTCACCATTTAATTGAAGTGTATAATGTGGATGATAAAAGGCTAG
 CTGCGGTAGGATATGCAG

>Bc53522-endoglucanase-complete-659 (SEQ ID NO. 43)

CCAGTAGCCAAGAATGGCCAGCAACCAGCAAGTGGATTCCAACCGCCTGATTTTCATTAGTTC
 TGACATTTCTTTTGTACTGCTTTTGTCTTTCAAGGTCTTTGCTTACATCACCGTACTTTTCT
 TTAGCTTCTGCAGTTCAGGTTGCATTTTCTTCATTTTCGCTTGACTGCGATATTGCGAAACAG
 CTAATGGAATCATTGCTGAACGAATAACGAGCGTCATAATAATGATGGCAATCCCAAAGCT
 45 AGCTCCAGGTATATGATGAGCGACAAATTGAATCATAAACGAGATTGGATATACAAAATAA
 TGATCCCAAATCCAGTACTATGTGCATCAATTGGGGCTGCATTACTGCAACCAGATAAAAAC
 AAAAACAAATAATAATGATAAACTAACGAGCACAGCTCGGTATGATTTTAACATGTTTCATT
 CTCCAATGTTTCGTAATTATTTAGCGAAACATTGGTGTATACGGACCGACCTCGTCATTCTCT

TCACCTTGATTCTTGTCTGCGTACAGAACGAATCATTCCATATTTATAAAAAATAGAAAATAG
CGGTACATTTCCCGCACTATCTTCACATGTATCAACTAGCGCAAAAGCTCTTTGTCTACCGCT
TGATGCTTGTTGACGCACAAAGCGGCGATTATTCC

5 >**Bacillus-cereus-53522-correct-rnc-738 (SEQ ID NO. 44)**

ATGCCGTACCGAAAATATAGAGAAAAAAAATACGAAACAAAATATCGTGAAGCATTAAAG
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CATTTCATCGTATGTGAATGAGCATCGAAAAAAACCGCATGAAGATAATGAGCGTCTTGAATT
10 TCTTGGAGATGCAGTATTGGAACCTTACTGTATCGCAGTATCTGTTTCAAAAATATCCGACAA
TGAGCGAAGGAGAGTTAACAAAACCTACGTGCAGCTATTGTATGTGAGCCATCTCTTGTTCGT
TTTGCGAACGAATTGTCATTTGGTAGCCTTGTTTTATTAGGAAAAGGTGAAGAAATGACAGG
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15 AGGGTGCTTTTTCTCATGTGATGGATTATAAGAGTCAGTTACAAGAATTGATTCAGCGTGAT
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GAAGCAGAGCAACAAGCTGCTGCAGAAGCATTGAAAAAATTAAAAGAACAACCTATAA

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CLAIMS

What is claimed is:

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1. A system for the bio-control of a plant pathogens comprising the steps of:

- generating a genetically modified endophyte bacteria having suppressed RNaseIII activity;
- transforming said genetically modified endophyte bacteria with a nucleic acid construct comprising a polynucleotide encoding at least one small inhibitory ribonucleic acid (siRNA) capable of forming a double-stranded RNA (dsRNA) or a hairpin (hpRNA) directed to at least one target sequence in a plant pathogen;
- introducing said genetically modified endophyte bacteria to a plant; and
- expressing said nucleic acid construct for a time sufficient to induce an RNA interference (RNAi) mechanism in said plant, wherein said RNAi mechanism causes the downregulation of said at least one target sequence in a plant pathogen.

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2. The method of claim 1, wherein said step of introducing said genetically modified endophyte bacteria to a plant comprises a step selected from the group consisting of: through one or more soaking, spraying, injecting, aerosolized disbursement, environmental aerosolized disbursement, environmental aerosolized disbursement in water sources, lyophilized, freeze-dried, microencapsulated, desiccated, in an aqueous carrier, in a solution, brushing, dressing, dripping, and coating.

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3. The method of claim 2, wherein said plant is a monocot and/or a dicot.

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4. The method of claim 3, wherein said plant is selected from the group consisting of: grains, corn, wheat, rice, barley, oats, sorghum, millet, sunflower, safflower, cotton, soy, canola, alfalfa, Arabidopsis, cannabis, potato, Brassica, peanut, tobacco, tropical fruits and flowers, banana, duckweed, gladiolus, sugar cane, pineapples, dates, onions, pineapple, cashews, pistachios, flowers, ornamentals, conifers, deciduous, grapes, citrus, roses, apples, peaches, strawberries,

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almonds, coffee, oaks, beans, legumes, watermelon, squashes, cabbage, turnip, mustard, cacti, pecans, flax, sweet potato, soybean, coconut, avocado, beets, cantaloupe and vegetables.

5 5. The method of claim 4, wherein said plant pathogen comprises a plant pathogen selected from the group consisting of: a plant virus; a plant viroid; a fungus; a pest, a herbivore, and a mold.

6. The method of claim 5, wherein said plant virus is selected from the group consisting of: Tobacco mosaic virus (TMV); Tomato spotted wilt virus (TSWV); Tomato yellow leaf curl virus (TYLCV); Cucumber mosaic virus (CMV); Potato virus Y (PVY); Cauliflower mosaic virus
10 (CaMV); African cassava mosaic virus (ACMV); Plum pox virus (PPV); Brome mosaic virus (BMV); Potato virus X (PVX); Citrus tristeza virus, Barley yellow dwarf virus, Potato leafroll virus and Tomato and bushy stunt virus.

7. The method of claim 6, wherein said target sequence is selected from the group consisting of a
15 sequence of a critical region of a virus, a conserved sequence of a family of viruses and a conserved sequence among members of different viral families.

8. The method of claim 6, wherein said target sequence which is suppressed is selected from the group consisting of: replicase gene, movement protein, coat protein in said Tobacco mosaic
20 virus; glycoproteins (GPs), NSm protein, NSs protein, virus RNA genomic segment L, M, S in said Tomato spotted wilt virus; V1, V2, C1, C2, C3, C4, V1 protein in said Tomato yellow leaf curl virus; 1a, 2a2b protein, 3a, coat protein in said Cucumber mosaic virus; P1, HC-Pro, P3, 6k1, CI, 6k2, NIa, Nib, CP in said Potato virus Y; Movement protein, two aphid transmission factors (P2 and/or P3), the precursor of the capsid proteins (P4), polyprotein precursor of
25 proteinase, P5, P6 protein in said Cauliflower mosaic virus; AV1, AV2, AC1, AC2, AC3, AC4, BC1, BV1 in said African cassava mosaic virus; P1, HC-Pro, P3, 6k1, CI, 6k2, NIa, Nib, CP in said Plum pox virus; 1a, 2a, movement protein, coat protein in said Brome mosaic virus; replicase, TGB1, TGB2, TGB3, coat protein in said Potato virus X.

30 8. The method of claim 4, wherein said fungus is selected from the group consisting of: *Magnaporthe oryzae*; *Botrytis cinerea*; *Puccinia* spp.; *Fusarium graminearum*; *Fusarium*

oxysporum; *Blumeria graminis*; *Mycosphaerella graminicola*; *Colletotrichum* spp.; *Ustilago maydis*; *Melampsora lini*, *Phakopsora pachyrhizi* and *Rhizoctonia solani*.

9. The method of claim 8, wherein said target sequence is selected from the group consisting of a
5 sequence of a critical region of a fungus essential gene, a conserved sequence of a family of essential genes and a conserved essential genes among members of different viral families.

10. The method of claim 1, 7 and 9, wherein said target sequence is selected from the group
10 consisting of: a non-coding region of RNA, a coding region of RNA; the target sequence containing a splice site of RNA.

11. The method of claim 1, wherein the nucleic acid construct is a plasmid.

12. The method of claim 11, wherein said plasmid is modified to include the target sequence
15 from the group of plasmids consisting of: pAD-WRKY-GHY1; pAD-ADH-GHY2; pOXB-WRKY-GHY3; pOXB-ADH-GHY4; pAD-WRKY-GHY5; hpRNA pAD-ADH-GHY6; and hpRNA pAD-WRKY-GHY7.

13. The method of claim 1, wherein said siRNA configured to be capable of forming a double-
20 stranded RNA (dsRNA) or a hairpin (hpRNA) directed to at least one target sequence in a plant pathogen is operably linked to a promoter.

14. The method of claim 13, wherein said promoter comprises a promoter selected from the
25 group consisting of: a non-constitutive promoter; a inducible promoter, a pathogen-inducible promoter; a tissue-preferred; a tissue-specific promoter, a plant-specific promoter, or a constitutive promoter.

15. The method of claim 1, wherein said genetically modified endophyte bacteria having
30 suppressed RNaseIII is selected from the group consisting of: Acidobacteria, Actinobacteria, Alphaproteobacteria, Armatimonadetes, Bacteriodes, Betaproteobacteria, Deltaproteobacteria, Firmicutes, Grammaproteobacteria, TM7, Bacillus, Escherichia.

16. The method of claim 15, wherein said genetically modified endophyte bacteria is M-JM109-GHY2.

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17. The method of claim 15, wherein said genetically modified endophyte bacteria is *Bacillus subtilis* CCB422.

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18. The method of claim 15, wherein said genetically modified endophyte bacteria is *E. coli* HT27, or HT115.

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19. The method of claims 1 and 18, wherein said genetically modified endophyte bacteria having suppressed RNaseIII comprises a genetically modified endophyte bacteria having hyper-vesiculation activity, JC8031, and/or and a genetically modified endophyte bacteria having one or more auxotrophies sufficient to enhance nanotube formation with other bacteria.

20. The method of claim 1, wherein said siRNA migrates throughout the plant.

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21. The method of claim 1, wherein said step of expressing said nucleic acid construct for a time sufficient to induce an RNA interference (RNAi) mechanism in said plant, wherein said RNAi mechanism causes the downregulation of said at least one target sequence in a plant pathogen comprises the step of continually expressing said nucleic acid construct and thereby initiating a sustained RNAi mechanism in said plant, wherein said sustained RNAi mechanism causes the plant to have a greater than wildtype resistance to said plant pathogen infection.

25

22. The method of claim 1, and further comprising the step of co-expressing at least one helper gene.

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23. The method of claim 1, and further comprising the step of co-expressing at least one additional siRNA molecule directed to at least one different target sequence in a plant pathogen.

24. The method of claim 22, wherein said helper gene is selected from the group consisting of: YrrA; SID1; SID2; AGO1; AGO2; AGO7; YmdB; YmdB; Staufen; RDE-4; HlyA; Sec- and Tat-secretory signal peptides; one or more cell-penetrating peptides (CPPs); Tat; Antennapedia ; ACC deaminase; one or more phloem RNA transporters; PP2-A1; PSRP1; DRB1; DRB4; HEN1
5 and STV1.

25. The method of claim 23, wherein said co-expressed helper genes are expressed from said nucleic acid construct and/or a secondary nucleic acid construct transformed in said genetically modified endophyte bacteria.

26. The method of claim 5, wherein said plant comprises a pathogen infected plant.

27. The method of claim 1, wherein said plant comprises a plant that has not been infected with said plant pathogen.

28. The method of claim 9, wherein said one target sequence of said fungal plant pathogen is selected from the group consisting of: MoABC1, MoMAC1 and MoPMK1, Iug6, Iug9 and Iug18, MoMpg1, MoEmp1, MoMhp1, MoMsp1, MC69, and Slp1 in said *Magnaporthe oryzae*; Bcp1 and BMP1 in said *Botrytis cinerea*; APK, cyclophilin, and calcineurin regulatory subunit, and secreted proteins in said *Puccinia* spp.; MAP1, and MAP kinase gpmk1 in said *Fusarium graminearum*; MAP kinase, pg1, pathogenicity-related genes, secreted proteins in said *Fusarium oxysporum*; effector gene Avra10, pathogenicity-related genes, secreted proteins in said *Blumeria graminis*; pathogenicity-related genes, MgSlt2, ABC Transporter Genes MgAtr1 and MgAtr2, secreted proteins in said *Mycosphaerella graminicola* CMK1, and clk1, pathogenesis-related protein 10, secreted proteins in said *Colletotrichum* spp.; pathogenicity-related genes, Kpp2, ukc1, secreted proteins in said *Ustilago maydi*; and pathogenicity-related genes, secreted proteins in said *Melampsora lini*.

29. The method of claim 1, wherein said small inhibitory ribonucleic acid (siRNA) is configured to downregulate at least one essential gene of a plant pathogen comprises a small inhibitory

ribonucleic acid (siRNA) configured to have one or more specific RNA motifs may be incorporated into dsRNA that may facilitate its transmission through the plant phloem.

30. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant comprising the steps:

- generating a genetically modified endophyte bacteria having suppressed RNaseIII activity;
- transforming said genetically modified endophyte bacteria with a nucleic acid construct comprising a polynucleotide encoding at least one small inhibitory ribonucleic acid (siRNA) configured to downregulate at least one essential gene of a plant pathogen;
- introducing said genetically modified endophyte bacteria to a plant; and
- expressing said nucleic acid construct for a time sufficient to induce an RNA interference (RNAi) mechanism in said plant, wherein said RNAi mechanism causes the downregulation of said at least one essential gene of said plant pathogen.

31. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 30 wherein said siRNA comprises one or more hpRNAs fully or partially complementary to the target sequence of at least one essential gene of said plant pathogen.

32. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 31 wherein said step of introducing said genetically modified endophyte bacteria to a plant comprises a step selected from the group consisting of: through one or more soaking, spraying, injecting, aerosolized disbursement, environmental aerosolized disbursement, environmental aerosolized disbursement in water sources, lyophilized, freeze-dried, microencapsulated, desiccated, in an aqueous carrier, in a solution, brushing, dressing, dripping, and coating.

33. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 31 wherein said plant is a monocot and/or a dicot.

34. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 33 wherein said plant is selected from the group consisting of: grains, corn, wheat, rice, barley, oats, sorghum, millet, sunflower, safflower, cotton, soy, canola, alfalfa, Arabidopsis, cannabis, potato, Brassica, peanut, tobacco, tropical fruits and flowers, banana, duckweed, gladiolus, sugar cane, pineapples, dates, onions, pineapple, cashews, pistachios, flowers, 5 ornamentals, conifers, deciduous, grapes, citrus, roses, apples, peaches, strawberries, almonds, coffee, oaks, beans, legumes, watermelon, squashes, cabbage, turnip, mustard, cacti, pecans, flax, sweet potato, soybean, coconut, avocado, beets, cantaloupe and vegetables.
- 10 35. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 34 said plant pathogen comprises a plant pathogen selected from the group consisting of: a plant virus; a plant viroid; a fungus; a pest, a herbivore, and a mold.
- 15 36. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 35 wherein said plant virus is selected from the group consisting of: Tobacco mosaic virus (TMV); Tomato spotted wilt virus (TSWV); Tomato yellow leaf curl virus (TYLCV); Cucumber mosaic virus (CMV); Potato virus Y (PVY); Cauliflower mosaic virus (CaMV); African cassava mosaic virus (ACMV); Plum pox virus (PPV); Brome mosaic virus (BMV); Potato virus X (PVX); Citrus tristeza virus, Barley yellow dwarf virus, Potato leafroll virus and 20 Tomato and bushy stunt virus.
- 25 37. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 36 wherein said at least one essential gene of said viral plant pathogen is selected from the group consisting of a sequence of a critical region of a virus, a conserved sequence of a family of viruses and a conserved sequence among members of different viral families.
- 30 38. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 37 wherein said essential gene of an viral plant pathogen is selected from the group consisting of: replicase gene, movement protein, coat protein in said tobacco mosaic virus; glycoproteins (GPs), NSm protein, NSs protein, virus RNA genomic segment L, M, S in said Tomato spotted wilt virus; V1, V2, C1, C2, C3, C4, V1 protein in said Tomato yellow leaf curl

virus; 1a, 2a2b protein, 3a, coat protein in said Cucumber mosaic virus; P1, HC-Pro, P3, 6k1, CI, 6k2, NIa, Nib, CP in said Potato virus Y; Movement protein, two aphid transmission factors (P2 and/or P3), the precursor of the capsid proteins (P4), polyprotein precursor of proteinase, P5, P6 protein in said Cauliflower mosaic virus; AV1, AV2, AC1, AC2, AC3, AC4, BC1, BV1 in said African cassava mosaic virus; P1, HC-Pro, P3, 6k1, CI, 6k2, NIa, Nib, CP in said Plum pox virus; 1a, 2a, movement protein, coat protein in said Brome mosaic virus; Replicase, TGB1, TGB2, TGB3, coat protein in said Potato virus X.

39. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 35 wherein said fungus is selected from the group consisting of: *Magnaporthe oryzae*; *Botrytis cinerea*; *Puccinia* spp.; *Fusarium graminearum*; *Fusarium oxysporum*; *Blumeria graminis*; *Mycosphaerella graminicola*; *Colletotrichum* spp.; *Ustilago maydis*; *Melampsora lini*, *Phakopsora pachyrhizi* and *Rhizoctonia solani*.

40. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 39 wherein said at least one essential gene of said fungal plant pathogen is selected from the group consisting of a sequence of a critical region of a fungal essential gene, a conserved sequence of a family of fungal genes and a conserved sequence among members of different fungal essential gene families.

41. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 40 wherein said at least one essential gene of said fungal plant pathogen is selected from the group consisting of: MoABC1, MoMAC1 and MoPMK1, pathogenicity-related genes, effectors Iug6, Iug9 and Iug18, MoMpg1, MoEmp1, MoMhp1, MoMsp1, MC69, and Slp1 in said *Magnaporthe oryzae*; Bcp1 and BMP1 in said *Botrytis cinerea*; APK, cyclophilin, and calcineurin regulatory subunit, and secreted proteins in said *Puccinia* spp.; MAP1, and MAP kinase gpmk1 in said *Fusarium graminearum*; MAP kinase, pg1, pathogenicity-related genes, secreted proteins in said *Fusarium oxysporum*; effector gene Avra10, pathogenicity-related genes, secreted proteins in said *Blumeria graminis*; pathogenicity-related genes, MgSlt2, ABC Transporter Genes MgAtr1 and MgAtr2, secreted proteins in said *Mycosphaerella graminicola* CMK1, and clk1, pathogenesis-related protein 10, secreted proteins in said *Colletotrichum* spp.;

pathogenicity-related genes, Kpp2, ukc1, secreted proteins in said *Ustilago maydi*; and pathogenicity-related genes, secreted proteins in said *Melampsora lini*.

42. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described
5 in claim 30 wherein said nucleic acid construct is a plasmid.

43. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described
in claim 42 wherein said plasmid is modified to include the target sequence from the group of
plasmids consisting of: pAD-WRKY-GHY1; pAD-ADH-GHY2; pOXB-WRKY-GHY3; pOXB-
10 ADH-GHY4; pAD-WRKY-GHY5; hpRNA pAD-ADH-GHY6; and hpRNA pAD-WRKY-
GHY7.

44. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described
in claim 42 wherein said nucleic acid construct comprises a polynucleotide encoding at least one
15 small inhibitory ribonucleic acid (siRNA) configured to downregulate at least one essential gene
of a plant pathogen is linked to a promoter.

45. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described
in claim 44 wherein said promoter comprises a promoter selected from the group consisting of: a
20 non-constitutive promoter; a inducible promoter, a pathogen-inducible promoter; a tissue-
preferred; a tissue-specific promoter, a plant-specific promoter, or a constitutive promoter.

46. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described
in claim 30 wherein said genetically modified endophyte bacteria having suppressed RNaseIII is
25 selected from the group consisting of: Acidobacteria, Actinobacteria, Alphaproteobacteria,
Armatimonadetes, Bacteriodes, Betaproteobacteria, Deltaproteobacteria, Firmicutes,
Grammaproteobacteria, TM7, Bacillus, Escherichia.

47. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described
30 in claim 46 wherein said genetically modified endophyte bacteria is M-JM109-GHY2.

48. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 46 wherein said genetically modified endophyte bacteria is *Bacillus subtilis* CCB422.

49. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 46 wherein said genetically modified endophyte bacteria is *E. coli* HT27, or HT115.

50. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claims 30 and 49 wherein said genetically modified endophyte bacteria having suppressed RNaseIII comprises a genetically modified endophyte bacteria having hyper-vesiculation activity, JC8031 and/or and a genetically modified endophyte bacteria having one or more auxotrophies sufficient to enhance nanotube formation with other bacteria.

51. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 30 wherein said siRNA migrates throughout the plant.

52. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 30 wherein said step of expressing said nucleic acid construct for a time sufficient to induce an RNA interference (RNAi) mechanism in said plant, wherein said RNAi mechanism causes the downregulation of said at least one essential gene of said plant pathogen comprises the step of continually expressing said nucleic acid construct and thereby initiating a sustained RNAi mechanism in said plant, wherein said sustained RNAi mechanism causes the plant to have a greater than wildtype resistance to said plant pathogen infection.

52. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 30 and further comprising the step of co-expressing at least one helper gene.

53. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 52 wherein said helper gene is selected from the group consisting of: VrrA; SID1; SID2; AGO1; AGO2; AGO7; YmdB; YmdB; Staufen; RDE-4; HlyA; Sec- and Tat-secretory signal peptides; one or more cell-penetrating peptides (CPPs); Tat; Antennapedia ; ACC deaminase; one or more phloem RNA transporters; PP2-A1; PSRP1; DRB1; DRB4; HEN1 and STV1.

54. A trans-kingdom system of delivering of an inhibitory RNA molecule to a plant as described in claim 30 wherein said small inhibitory ribonucleic acid (siRNA) is configured to downregulate at least one essential gene of a plant pathogen comprises a small inhibitory
5 ribonucleic acid (siRNA) configured to have one or more specific RNA motifs may be incorporated into dsRNA that may facilitate its transmission through the plant phoelem.

55. A method of suppressing a pathogen in in a plant comprising the steps of:

- generating a genetically modified endophyte bacteria having suppressed RNaseIII
10 activity; and
- transforming said genetically modified endophyte bacteria with a nucleic acid construct comprising a polynucleotide encoding at least one small inhibitory ribonucleic acid (siRNA) configured to downregulate expression of said at least one target gene in a plant pathogen through the induction of an RNA interference (RNAi) mechanism in the plant;
- 15 - introducing said genetically modified endophyte bacteria to a plant.

56. A method of assessing the efficacy or effectiveness of a histone deacetylase (HDAC) inhibitor therapy treatment being administered to a cancer subject, the method comprising comparing:

- 20 - the expression level of a bio marker measured in a first sample obtained from the subject at a time t_0 , wherein the marker is biomarker selected from the group identified in Table 4;
- the level of the biomarker in a second sample obtained from the subject at time t_1 ; and,
- wherein a change in the level of the biomarker in the second sample relative to the first
25 sample is an indication that the histone deacetylase (HDAC) inhibitor treatment is effective for treating cancer in the subject.

57. The method of claim 56, wherein the comparing is repeated over a range of times.

30 58. A method of assessing the effectiveness of RNA-mediated pathogen suppression in in a plant, the method comprising comparing:

- determine the expression level of a plant pathogen target gene measured in a first sample obtained from the plant at a time t_0 ,
- generating a genetically modified endophyte bacteria having suppressed RNaseIII activity wherein said genetically modified endophyte bacteria with a nucleic acid construct comprising a polynucleotide encoding at least one small inhibitory ribonucleic acid (siRNA) capable of forming a double-stranded RNA (dsRNA) or a hairpin (hpRNA) directed to at least one target gene in a plant pathogen;
- introducing said genetically modified endophyte bacteria to a plant at t_1 ;
- allowing of expressing said nucleic acid construct to induced an RNA interference (RNAi) mechanism in said plant, wherein said RNAi mechanism causes the downregulation of said at least one target sequence in a plant pathogen; and
- determine the expression level of said plant pathogen target gene measured in a first sample obtained from the plant at a time t_2 .

57. The method of claim 58, wherein the comparing is repeated over a range of times.

58. A method of assessing the efficacy RNA-mediated pathogen suppression in in a plant, the method comprising comparing:

- determine the level of a plant pathogen measured in a first sample obtained from the plant at a time t_0 ,
- generating a genetically modified endophyte bacteria having suppressed RNaseIII activity wherein said genetically modified endophyte bacteria with a nucleic acid construct comprising a polynucleotide encoding at least one small inhibitory ribonucleic acid (siRNA) capable of forming a double-stranded RNA (dsRNA) or a hairpin (hpRNA) directed to at least one essential target gene in a plant pathogen;
- introducing said genetically modified endophyte bacteria to a plant at t_1 ;
- allowing of expressing said nucleic acid construct to induced an RNA interference (RNAi) mechanism in said plant, wherein said RNAi mechanism causes the downregulation of said at least one target sequence in a plant pathogen; and
- determine the level of said plant pathogen measured in a first sample obtained from the plant at a time t_2 .

59. The method of claim 58, wherein the comparing is repeated over a range of times.

60. A method of treating a pathogen-infected plant comprising the steps of:

- introducing an effective amount of aa genetically modified endophyte bacteria having suppressed RNaseIII activity wherein said genetically modified endophyte bacteria with a nucleic acid construct comprising a polynucleotide encoding at least one small inhibitory ribonucleic acid (siRNA) capable of forming a double-stranded RNA (dsRNA) or a hairpin (hpRNA) directed to at least one target sequence in a plant pathogen.

60. A method of generating pest resistant plant comprising the steps of:

- generating a genetically modified endophyte bacteria having suppressed RNaseIII activity;
- transforming said genetically modified endophyte bacteria with a nucleic acid construct comprising a polynucleotide encoding at least one small inhibitory ribonucleic acid (siRNA) capable of forming a double-stranded RNA (dsRNA) or a hairpin (hpRNA) directed to at least one essential gene in a pest or herbivore;
- introducing said genetically modified endophyte bacteria to a plant; and
- expressing said nucleic acid construct for a time sufficient to induce an RNA interference (RNAi) mechanism in said plant, wherein said RNAi mechanism causes the accumulation of dsRNA of hpRNA directed to at least one essential gene in a pest or herbivore.
- having the plant consumed by the pest and/or herbivore such that dsRNA of hpRNA directed to at least one essential gene in a pest or herbivore induced an RNA interference (RNAi) mechanism resulting in suppression in at least one essential gene in a pest and/or herbivore in said pest and/or herbivore.

61. The method of claim 60, wherein said genetically modified endophyte bacteria can colonize a pest gut.

62. The method of claim 60, wherein said siRNA comprises one or more hpRNAs fully or partially complementary to the target sequence of at least one exoskeleton gene of an insect.

63. The method of claim 62, wherein said at least one essential gene of said viral pest or herbivore is selected from the group consisting of a sequence of a critical region of a pest or herbivore, a conserved sequence of a family of pest or herbivore, and a conserved sequence among members of different pest or herbivore families.

64. The method of claim 63, wherein said genetically modified endophyte bacteria having suppressed RNaseIII activity is a *Bacillus*, and/or *Escherichia*.

64. The method of claim 64, wherein said genetically modified endophyte bacteria is selected from the group consisting of: M-JM109-GHY2; *Bacillus subtilis* CCB422, HT27, HT115, JC8031.

65. The method of claim 64, and further comprising the step of co-expressing at least one helper gene.

66. The method of claim 65, wherein said helper gene is selected from the group consisting of: VrrA; SID1; SID2; AGO1; AGO2; AGO7; YmdB; YmdB; Staufen; RDE-4; HlyA; Sec- and Tat-secretory signal peptides; one or more cell-penetrating peptides (CPPs); Tat; Antennapedia ; ACC deaminase; one or more phloem RNA transporters; PP2-A1; PSRP1; DRB1; DRB4; HEN1 and STV1.

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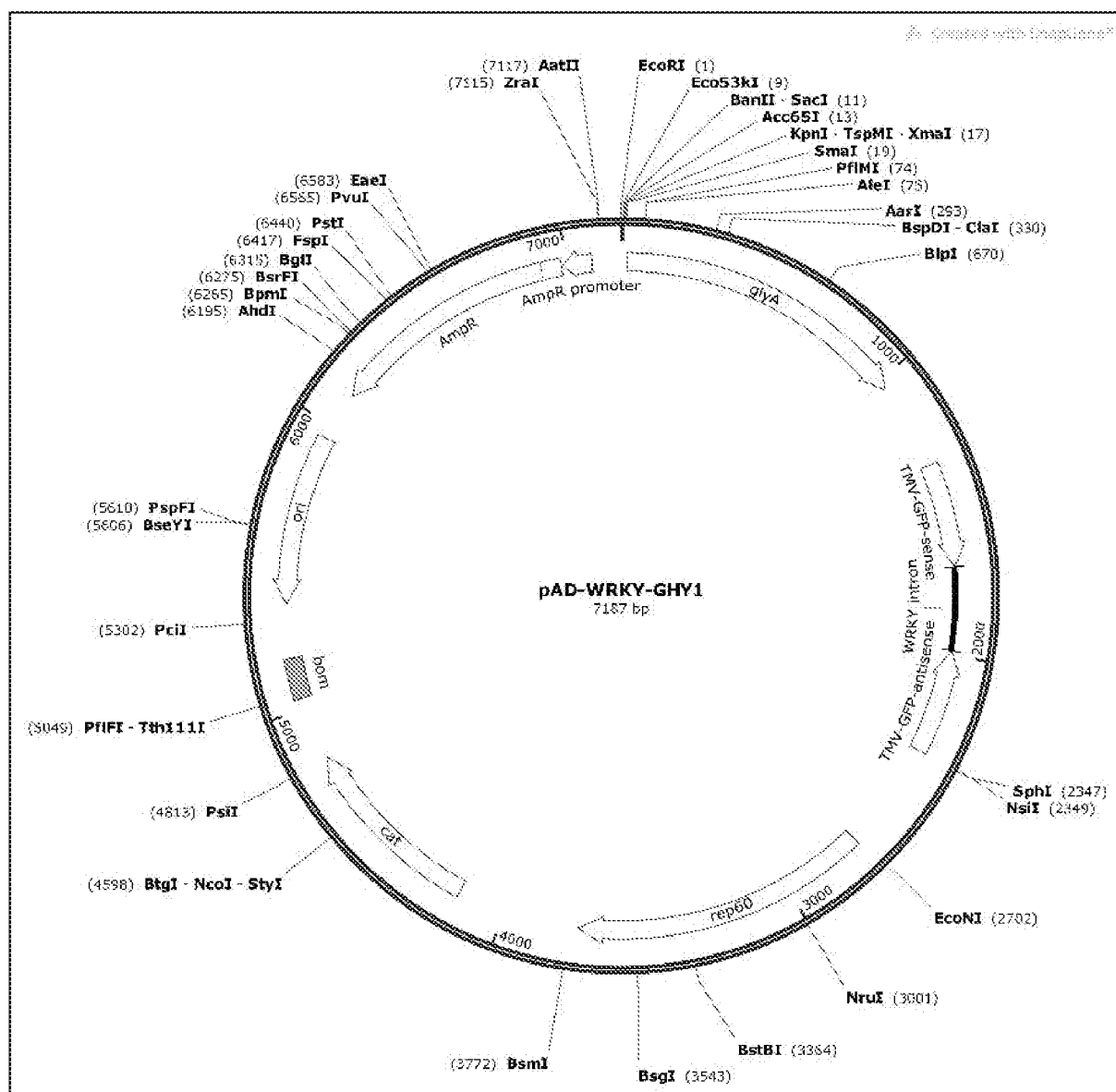


Fig. 1

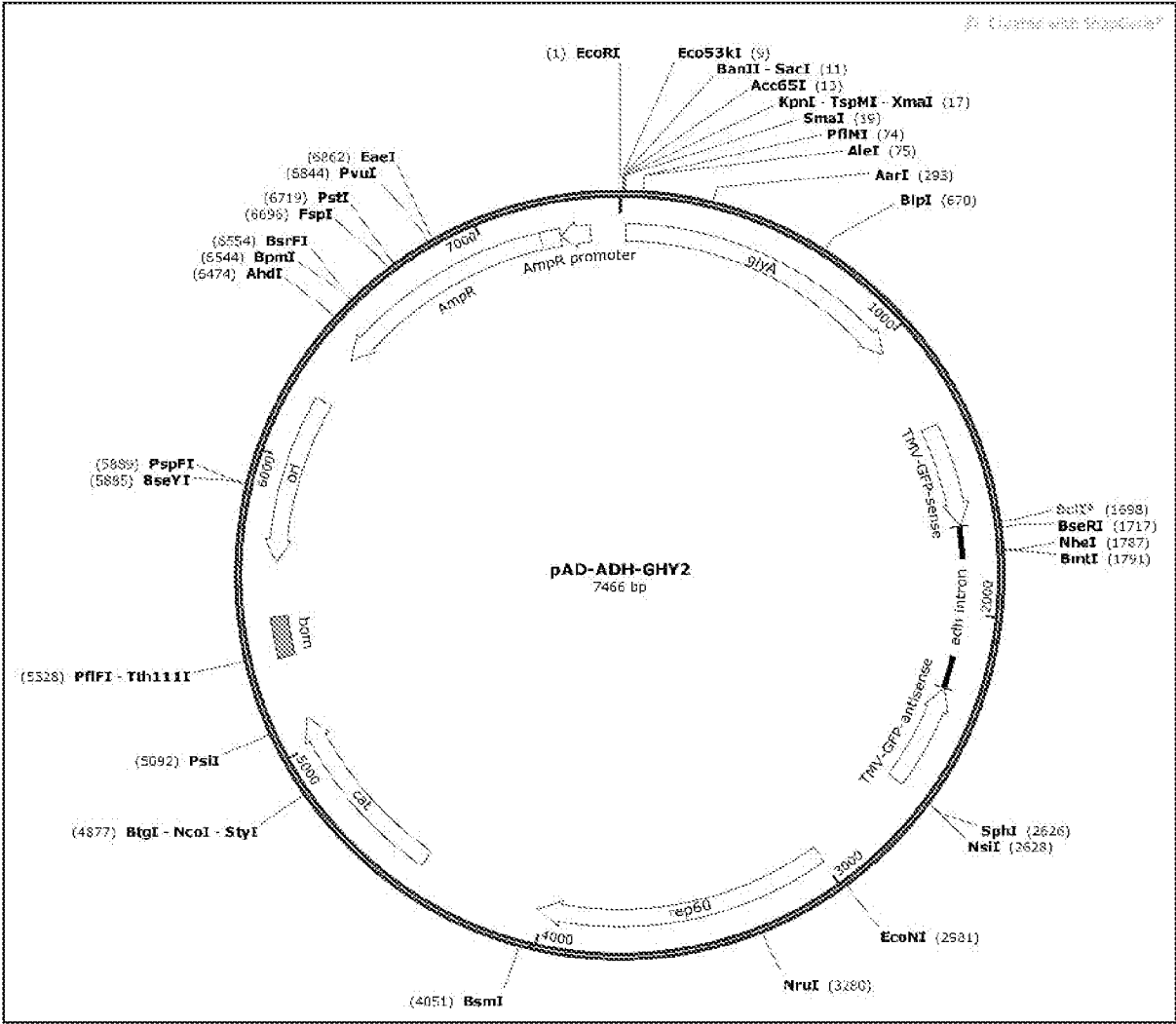


Fig. 2

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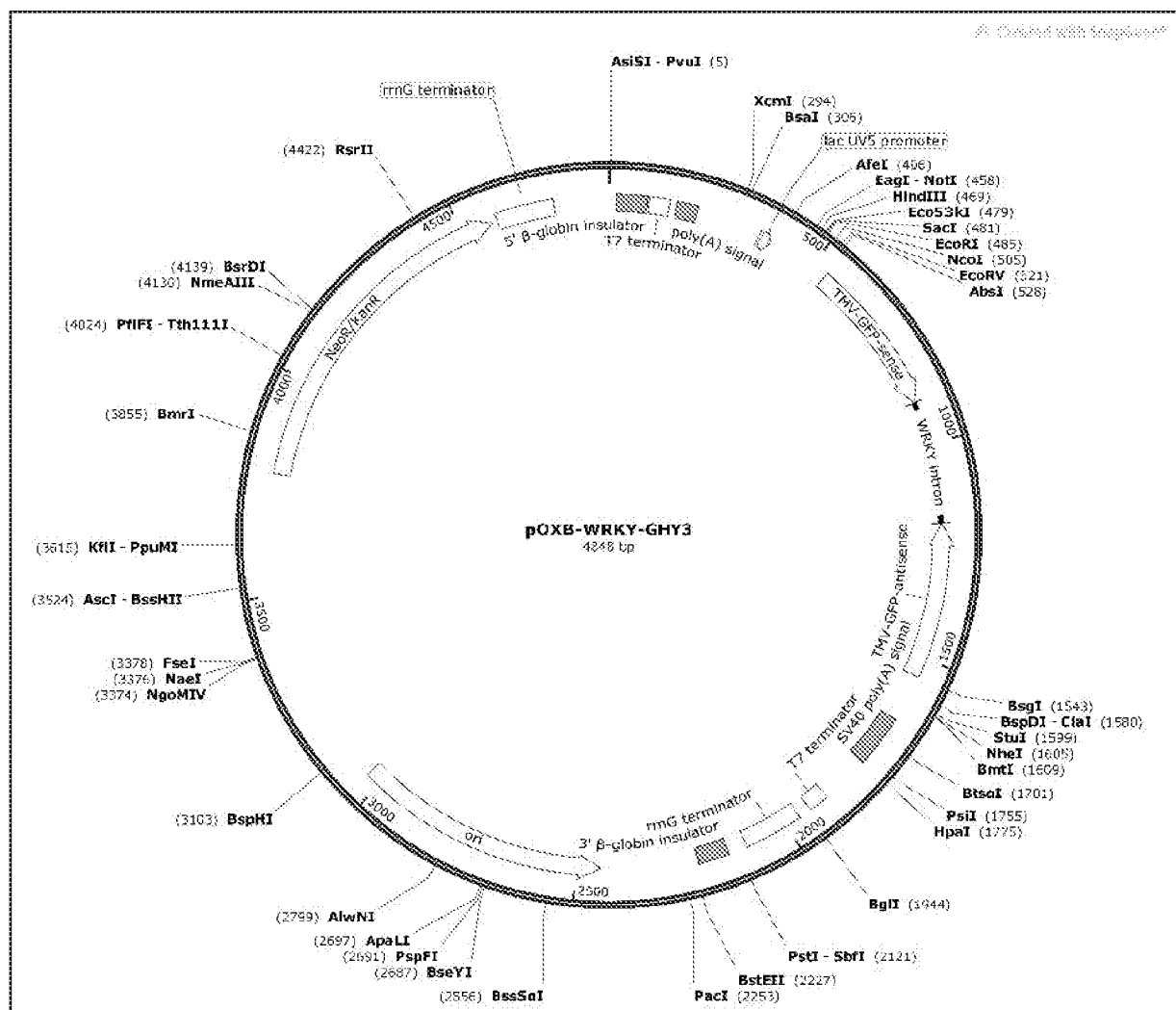


Fig. 3

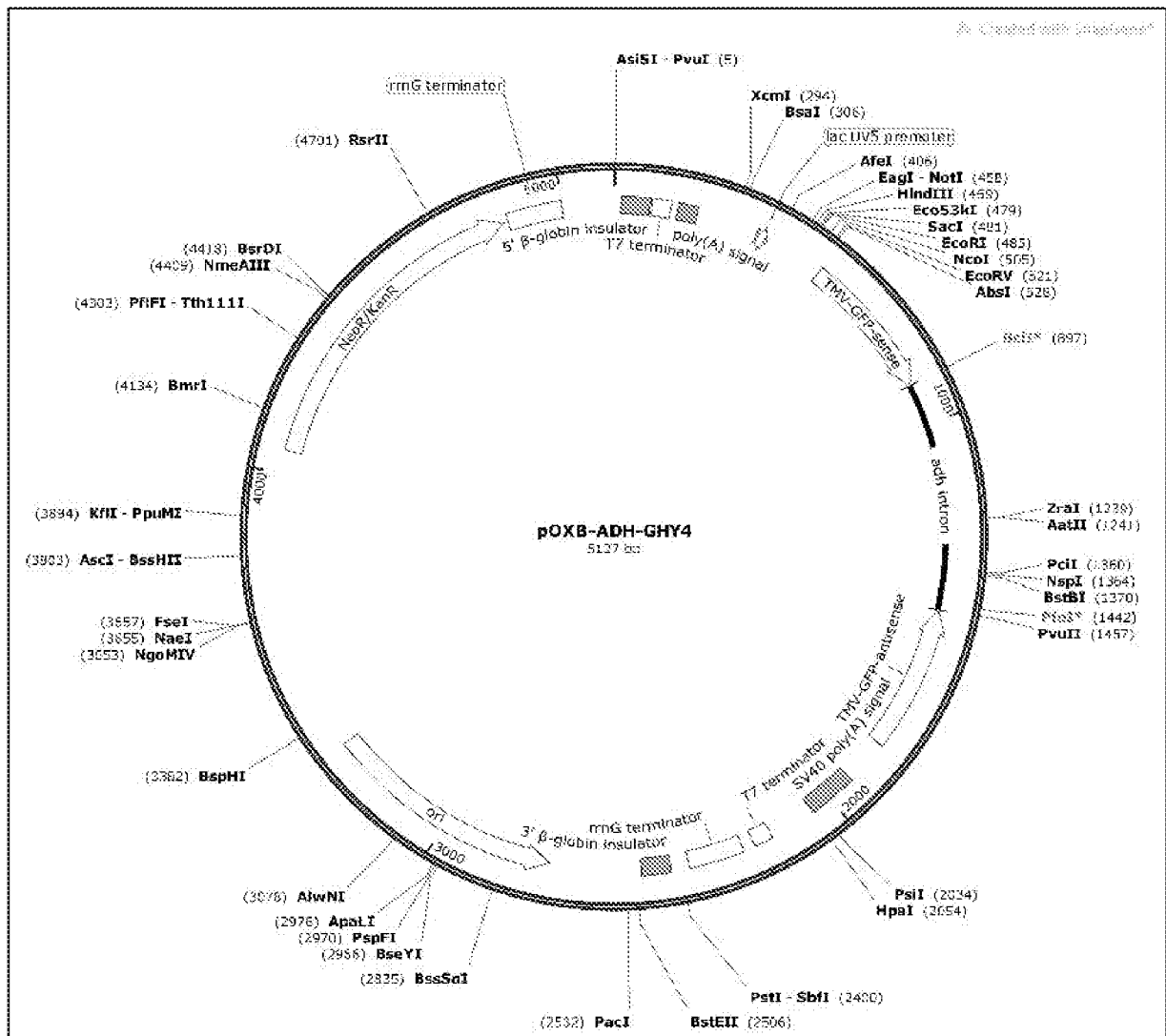


Fig.4

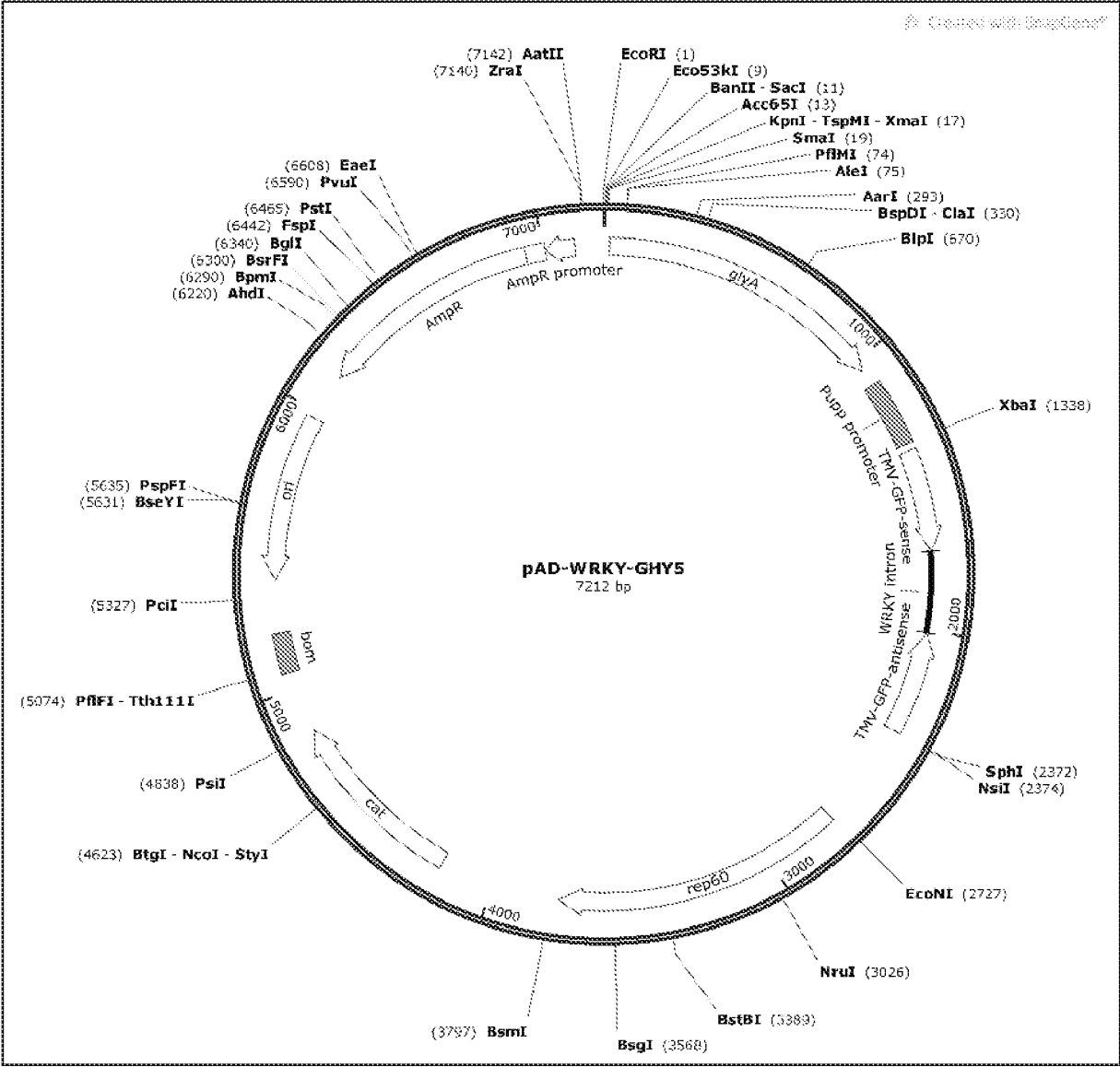


Fig. 5

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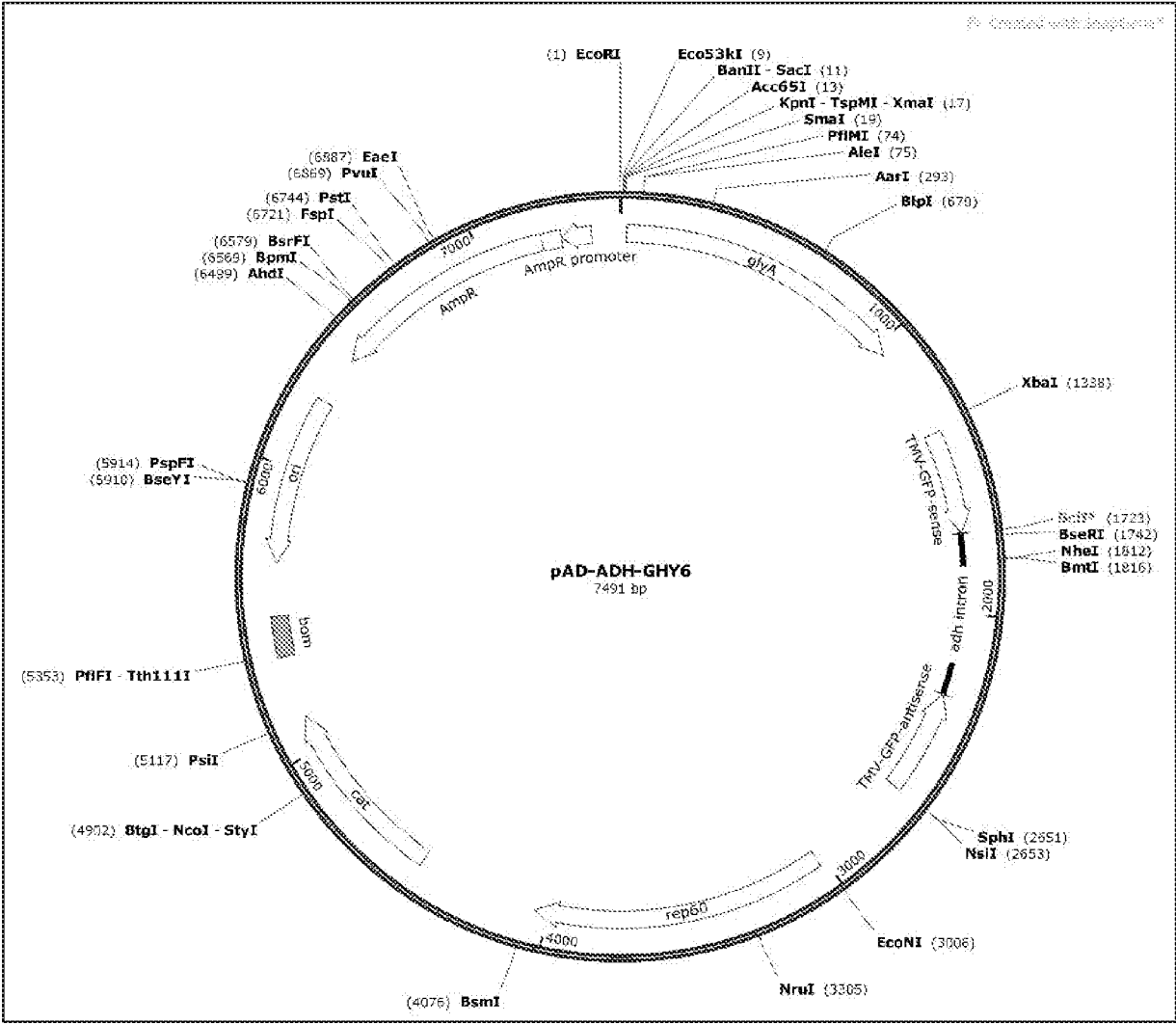


Fig.6

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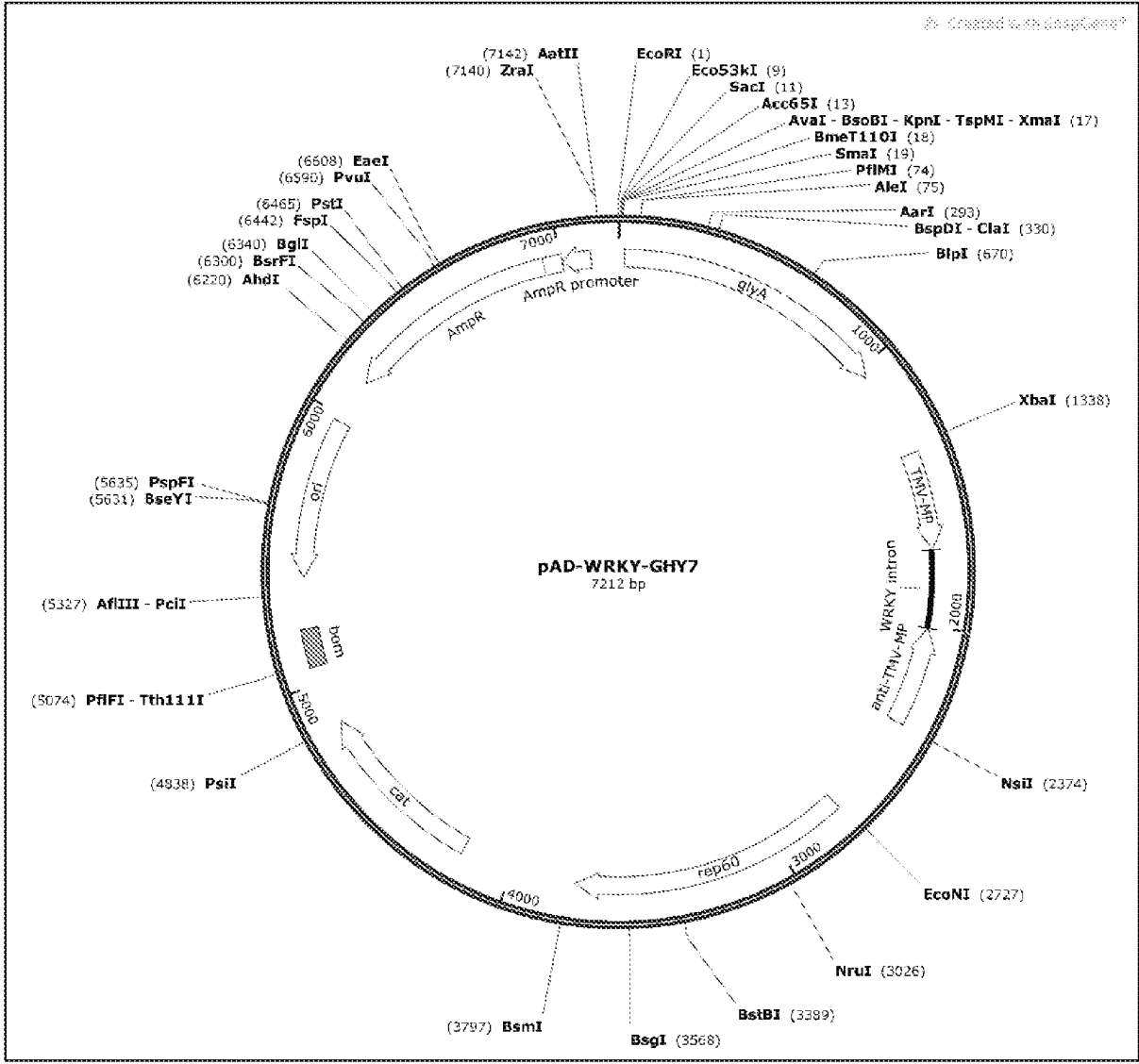


Fig. 7

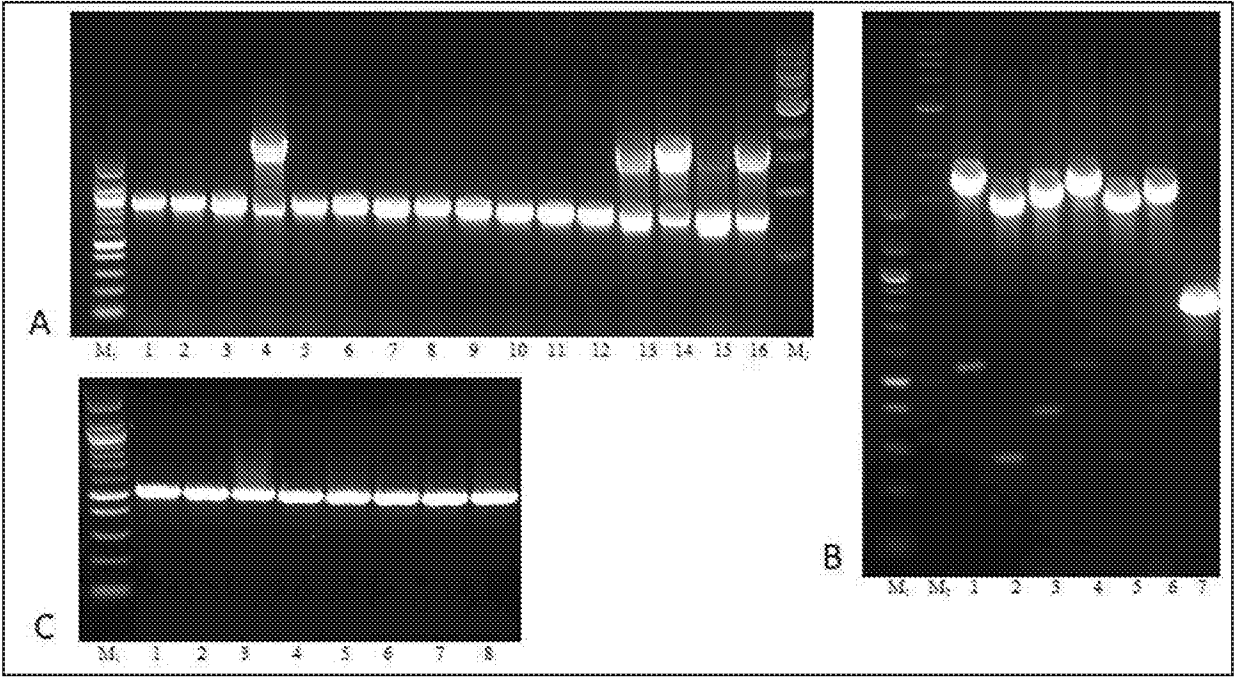


Fig. 8

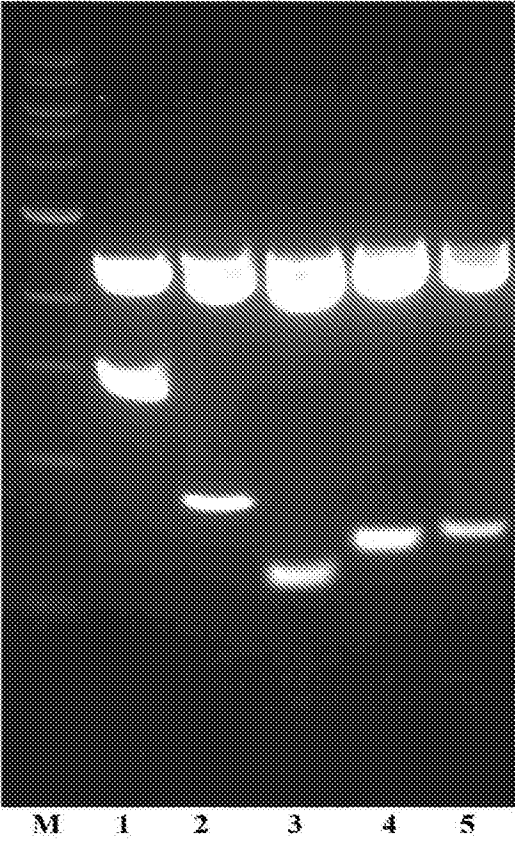


Fig. 9

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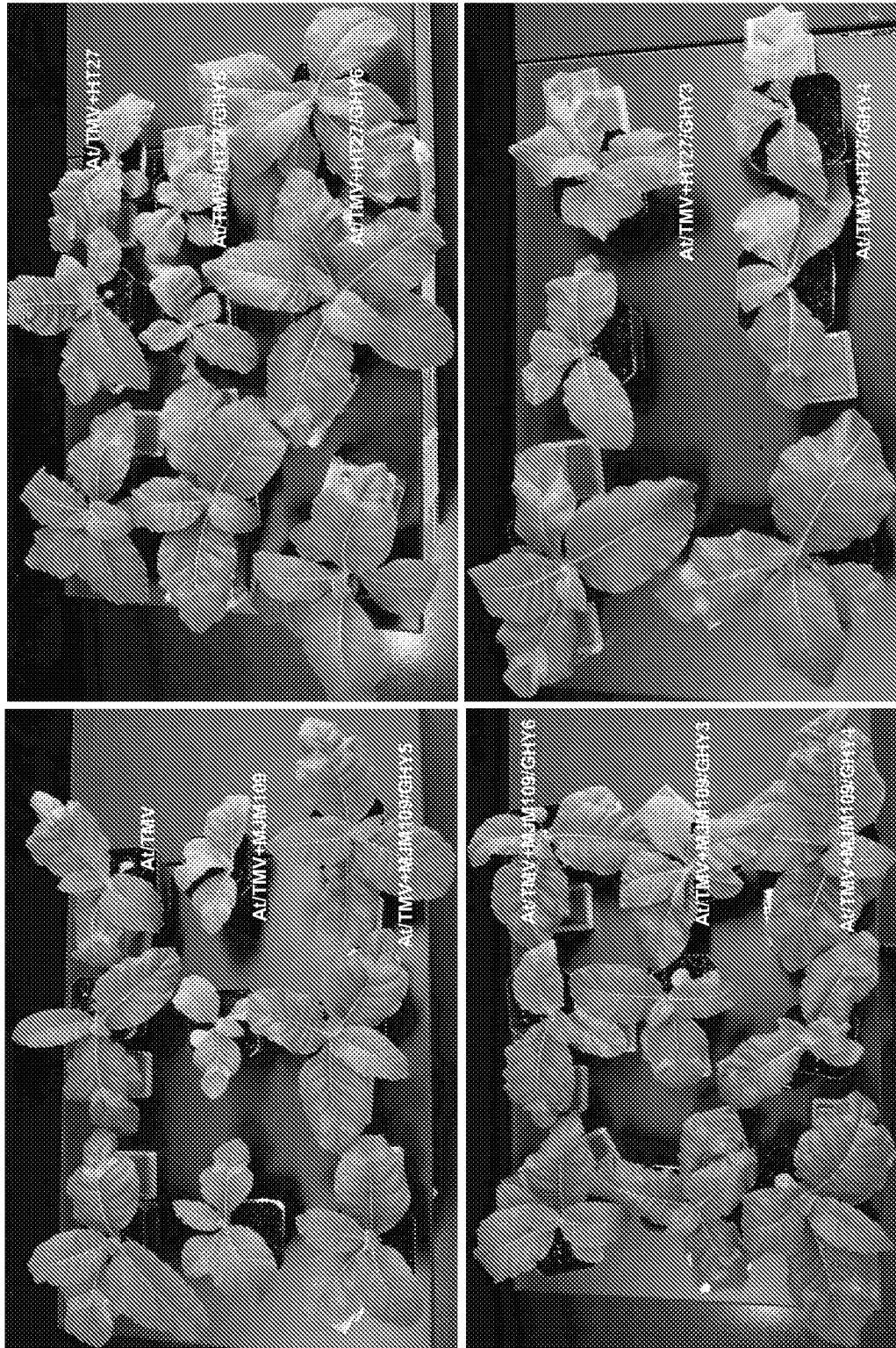


Fig. 10

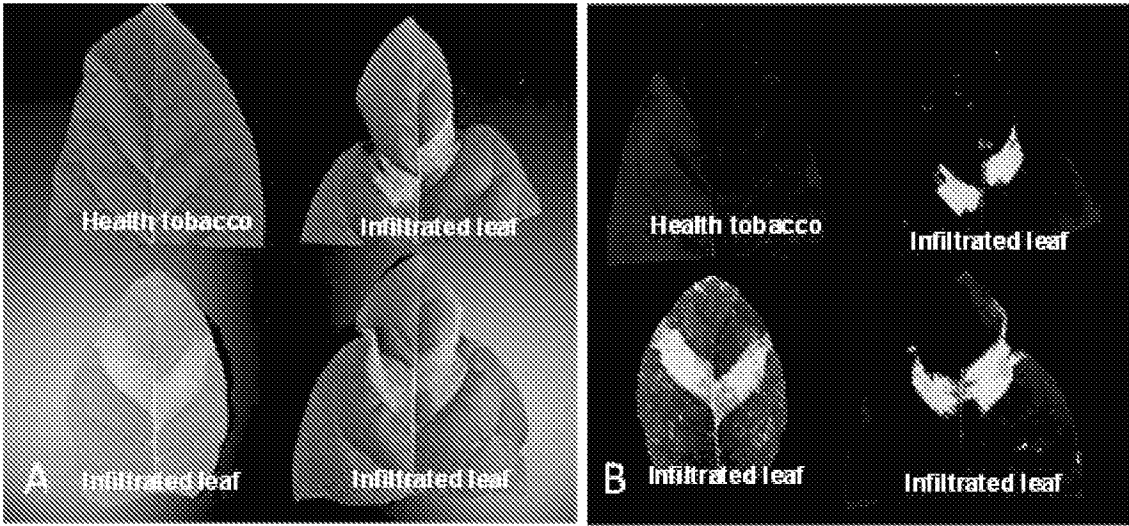


Fig. 11A

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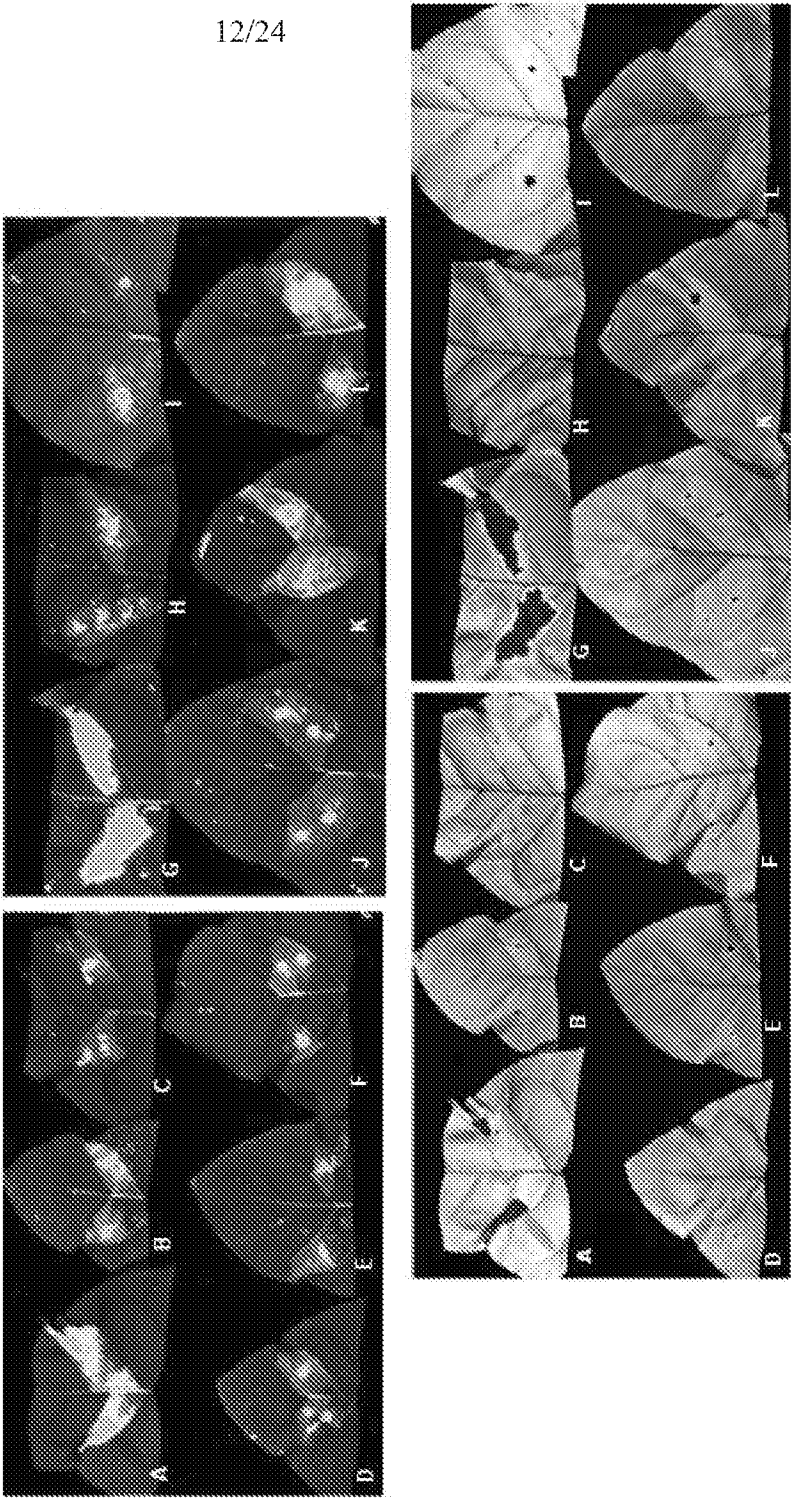


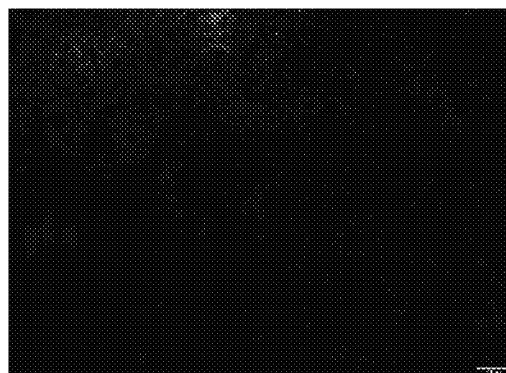
Fig. 11B

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M-JM109-GHY2 (RNaseIII mutant) + pAD-WRKY-GHY1 (hpRNA)

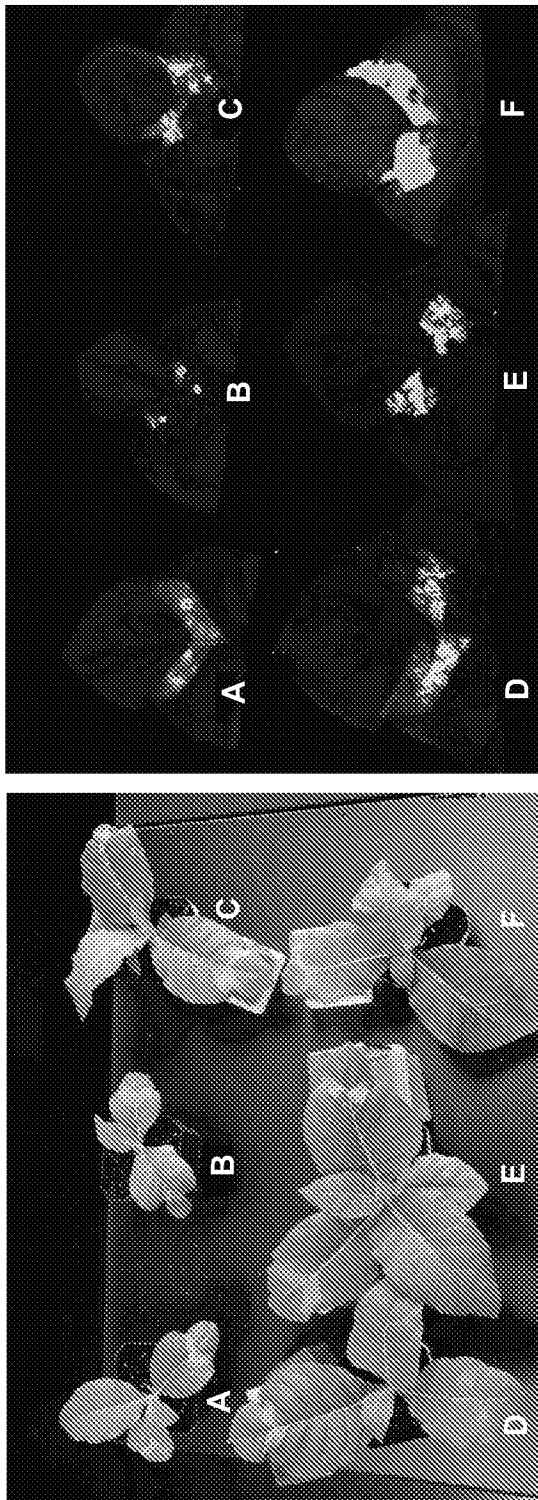


TMV-GFP



TMV-GFP+hpRNA

Fig. 11C



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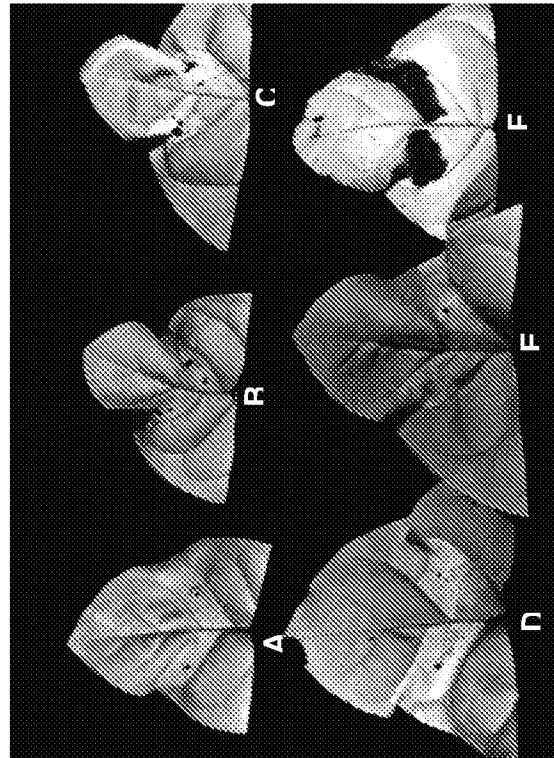


Fig. 12

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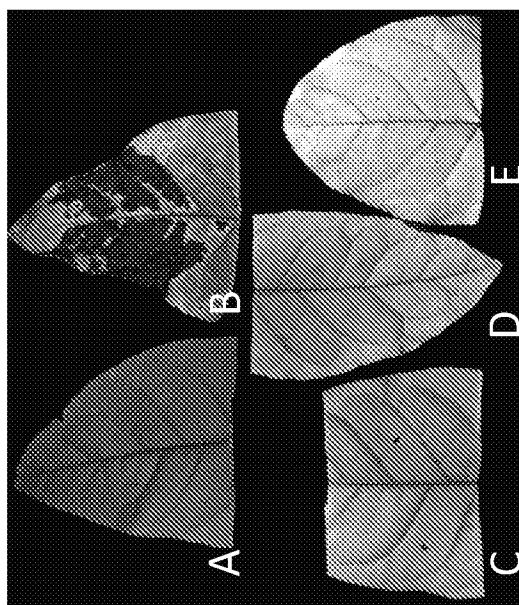
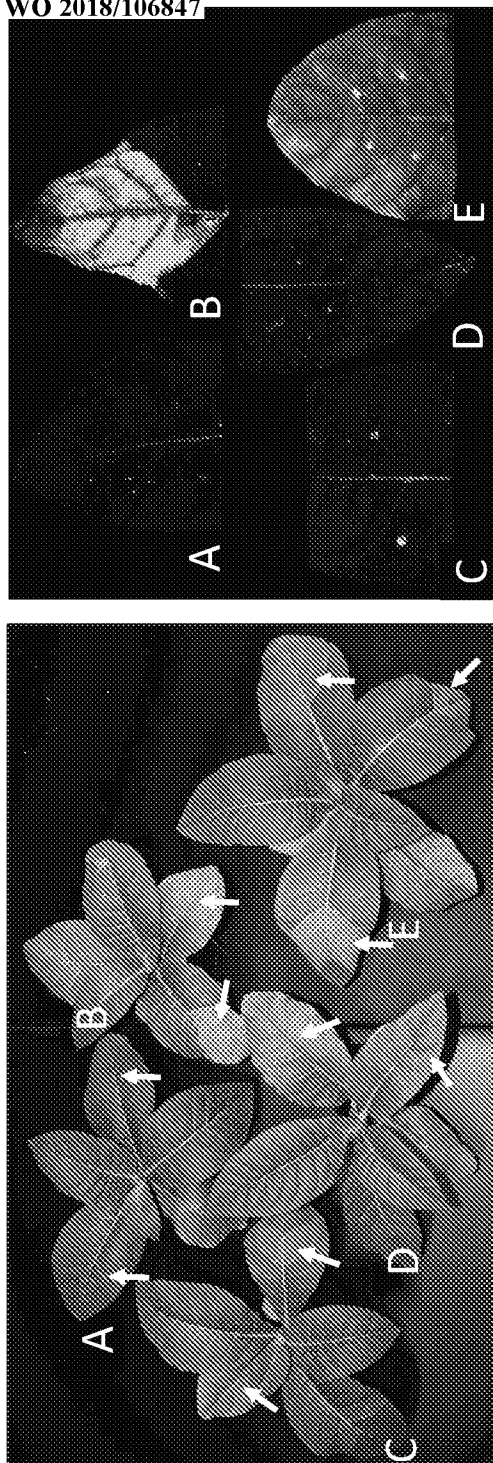


Fig. 13

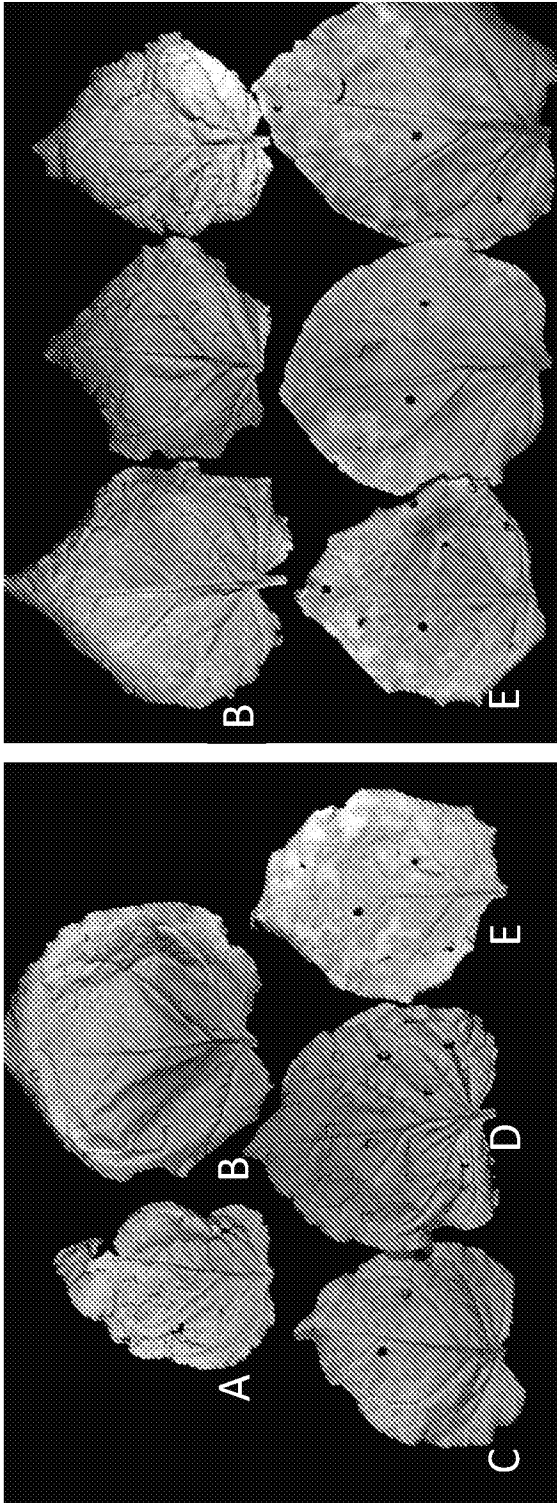
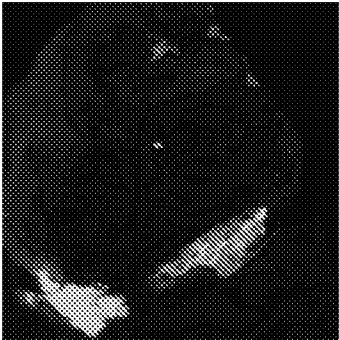
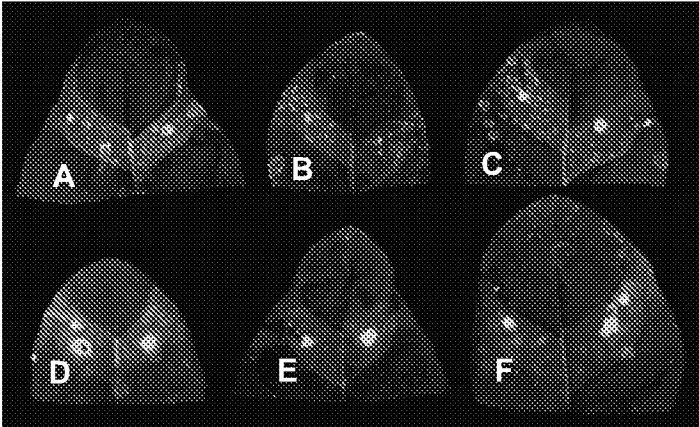


Fig. 14



Postive control:



Positive control:

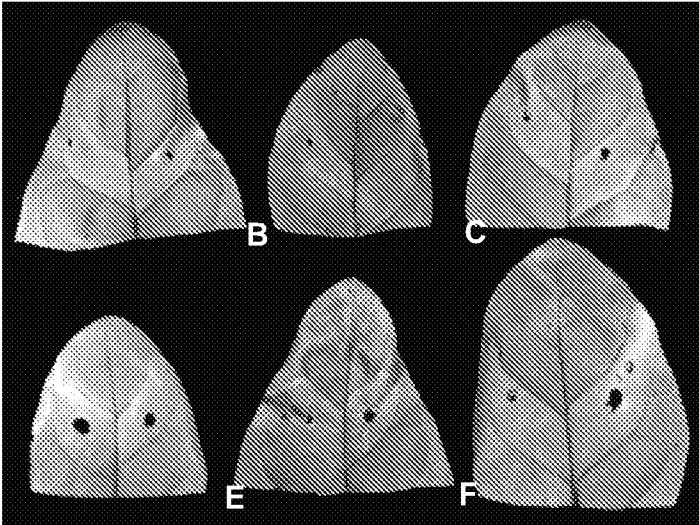


Fig. 15

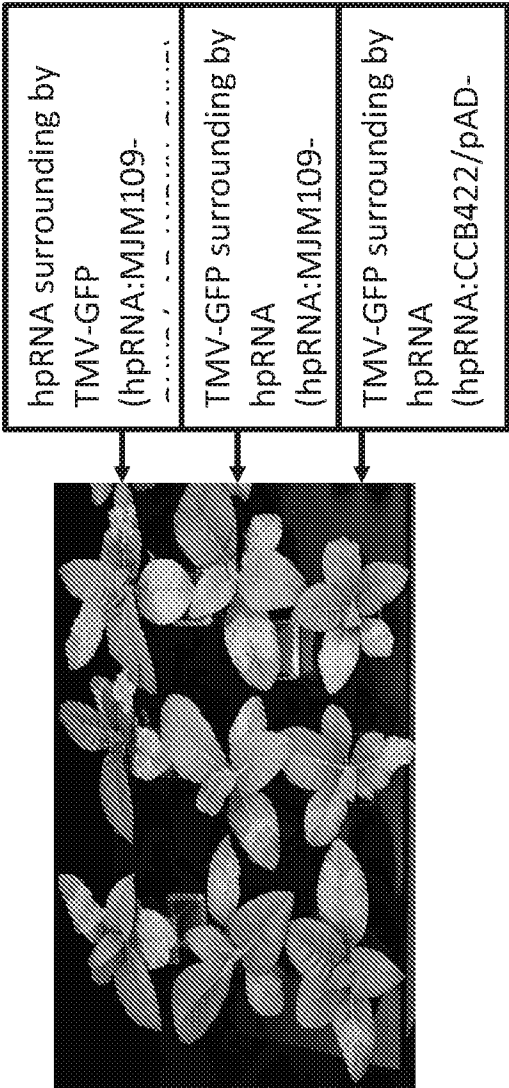


Fig. 16

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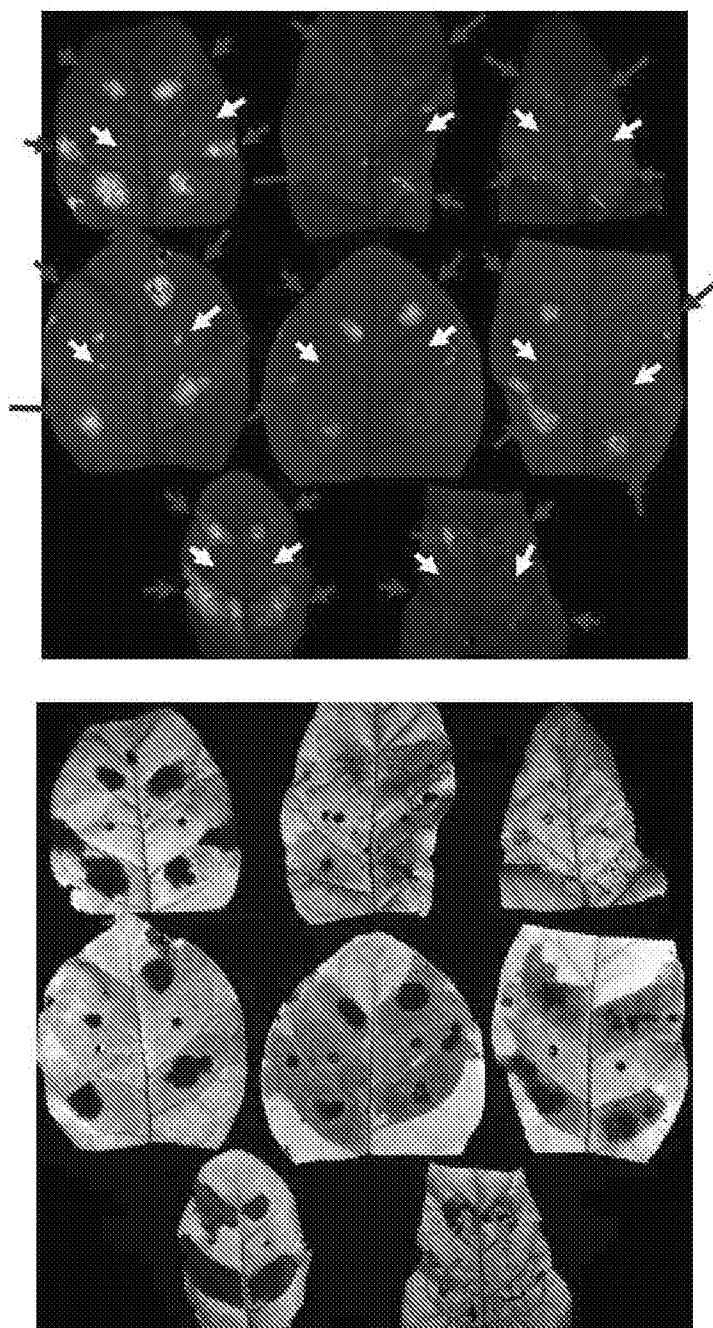


Fig. 17

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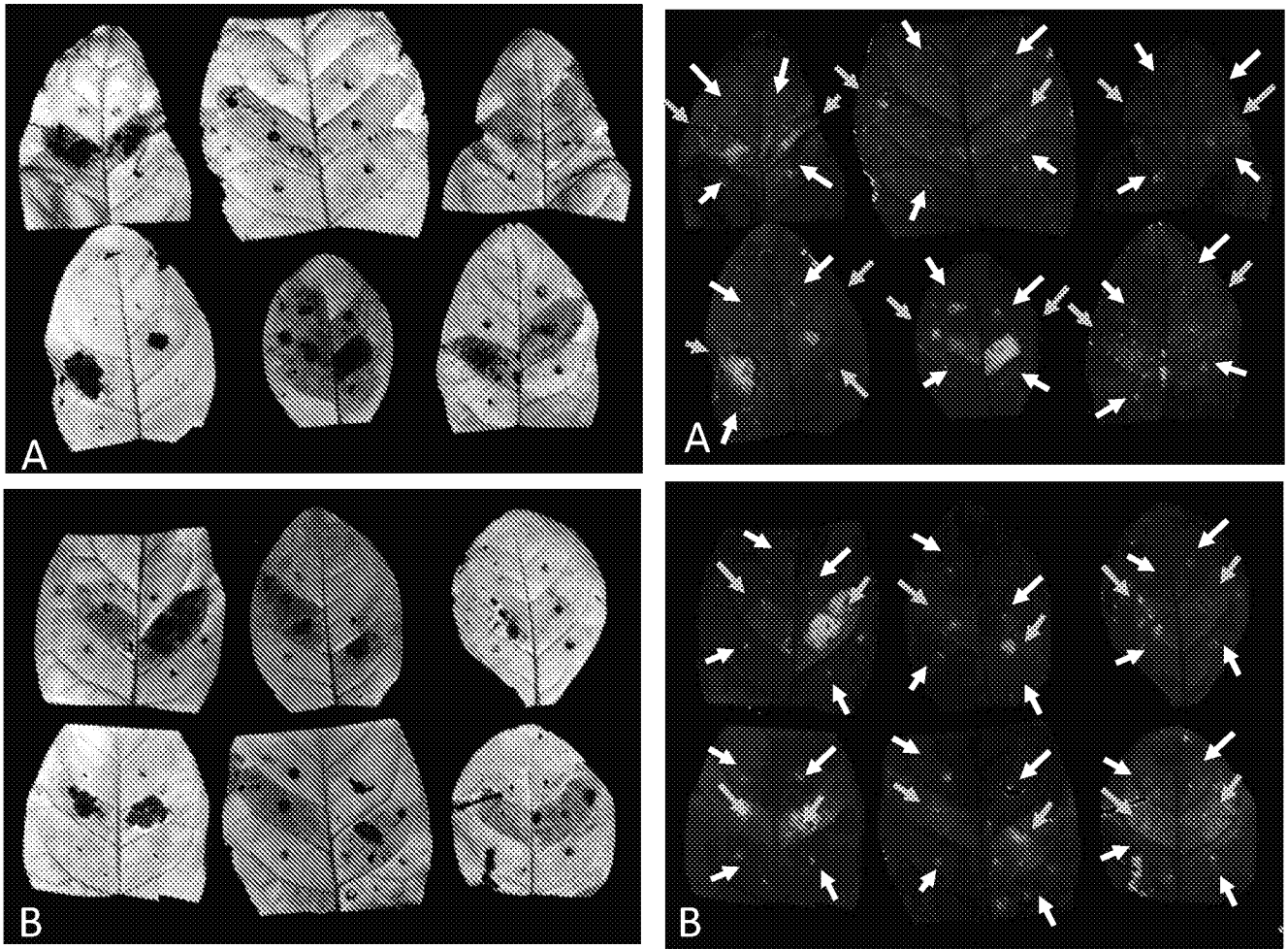


Fig. 18

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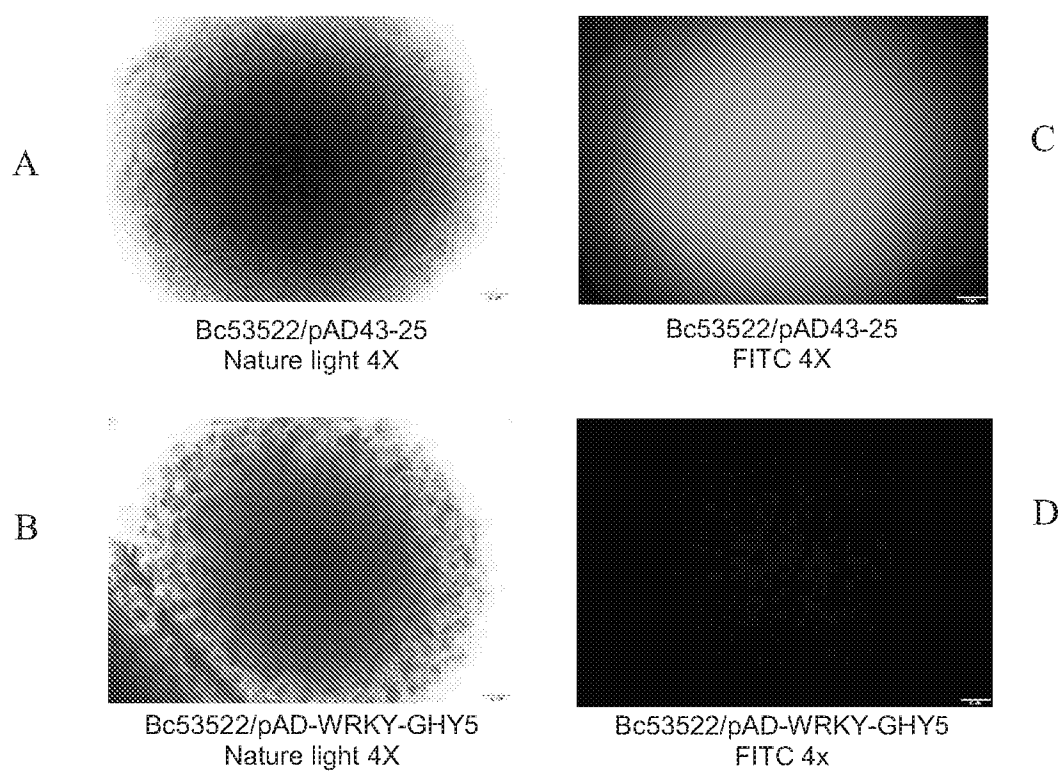


Fig. 19

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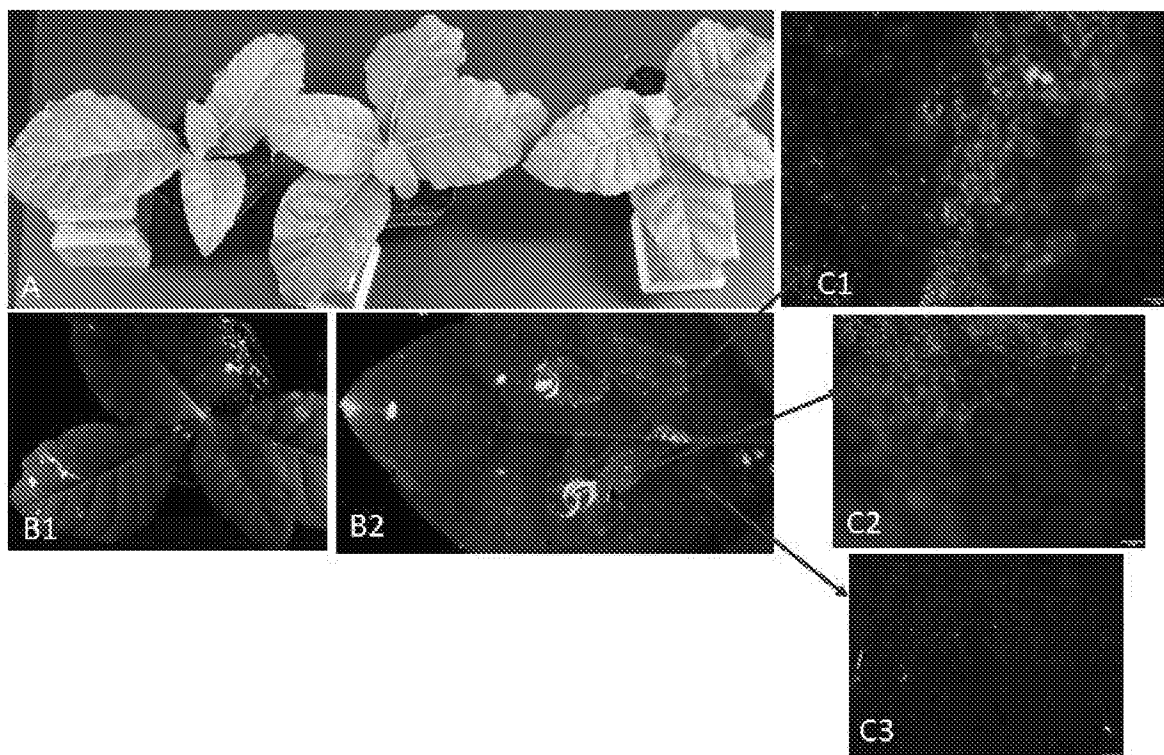


Fig. 20

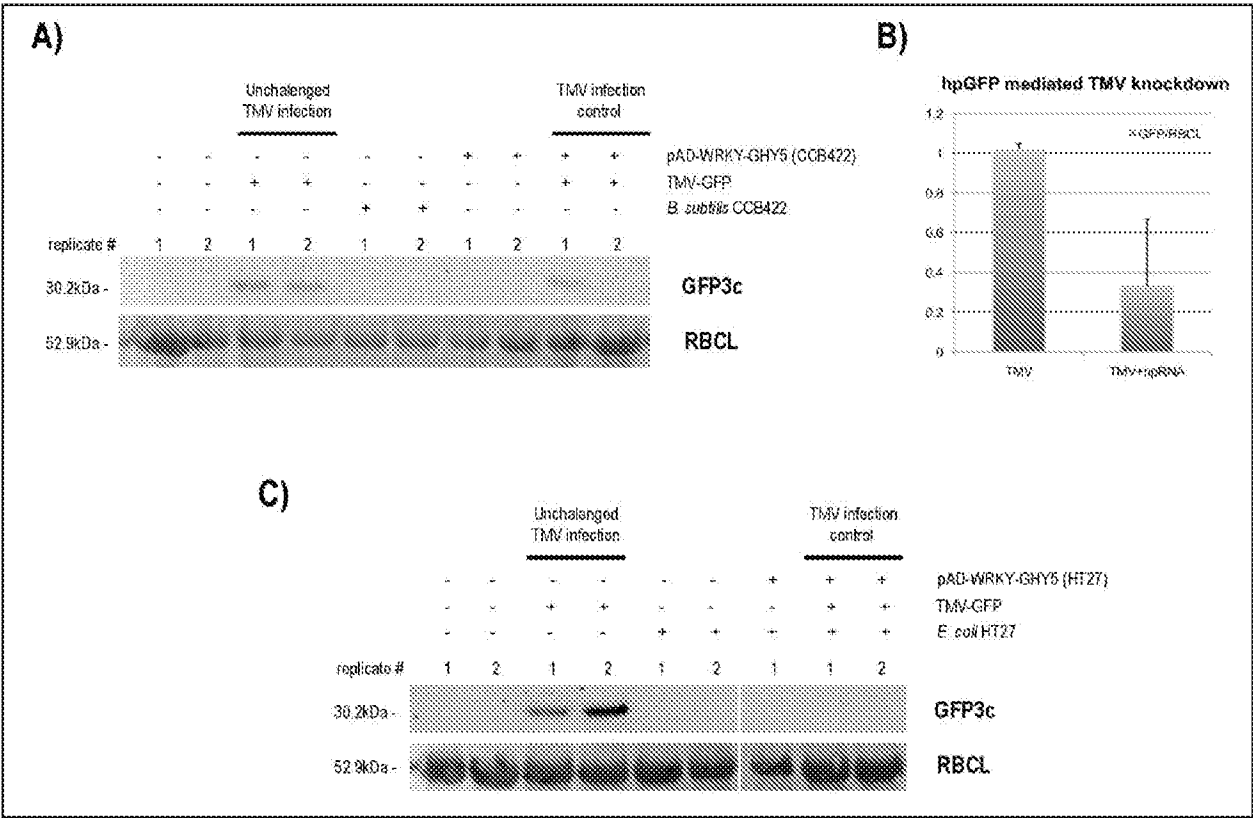


Fig. 21

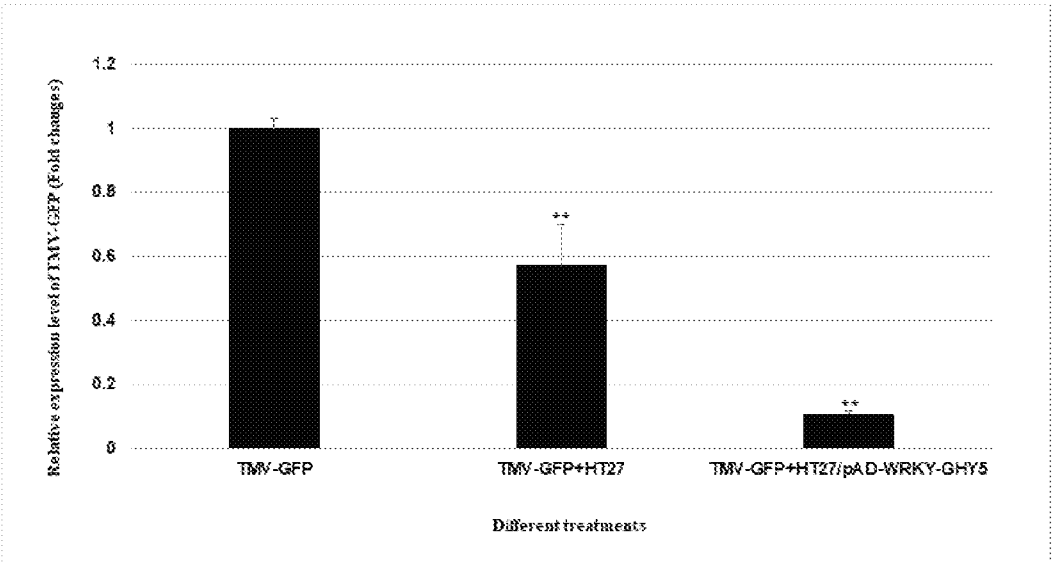


Fig. 22

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/064977

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01H 5/00; A01N 63/02; C12N 15/1 (2018.01)

CPC - A01N 25/00; A01N 63/00; C12N 2310/14 (2018.02)

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)

See Search History document

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

USPC - 435/320.1; 514/44A; 536/24.5 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/0337302 A1 (SYNGENTA PARTICIPATIONS AG) 26 November 2015 (26.11.2015) entire document	1-7, 8a, 8b, 9-11, 13-15, 17, 18, 20-42, 44-46, 48, 49, 51, 52a, 52b, 53-55, 57b, 58a, 58b, 59, 60a, 60b, 61-63, 64a, 64b
Y	TENLLADO et al. "Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections," BMC Biotechnology, 20 March 2003 (20.03.2003), Vol. 3, Pgs. 1-11. entire document	1-7, 8a, 8b, 9-11, 13-15, 17, 18, 20-42, 44-46, 48, 49, 51, 52a, 52b, 53-55, 57b, 58a, 58b, 59, 60a, 60b, 61-63, 64a, 64b
Y	HU et al. "Down-regulation of Fusarium oxysporum endogenous genes by Host-Delivered RNA interference enhances disease resistance," Front. Chem., 20 January 2015 (20.01.2015), Vol. 3, Article 1, Pgs. 1-10. entire document	8b, 9, 28, 39-41
Y	GILET et al. "Small stable RNA maturation and turnover in Bacillus subtilis." Mol Microbiol, 01 January 2015 (01.01.2015), Vol. 95, Pgs. 270-282. entire document	17, 48
Y	US 2016/0108096 A1 (SPOGEN BIOTECH INC.) 21 April 2016 (21.04.2016) entire document	20, 51
Y	WO 2016/096923 A1 (PARIS SCIENCES ET LETTRES - QUARTIER LATIN et al) 23 June 2016 (23.06.2016) entire document	22, 24, 25, 52b, 53



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 another 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

14 April 2018

Date of mailing of the international search report

30 APR 2018

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, VA 22313-1450

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Blaine R. Copenheaver

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/064977

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 19, 50
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/064977

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HAYWOOD et al. "Phloem long-distance trafficking of GIBBERELLIC ACID-INSENSITIVE RNA regulates leaf development," The Plant Journal, 01 April 2005 (01.04.2005), Vol. 42, Pgs. 49-68. entire document	29, 54
Y	CASH et al. "Symbiotic Bacteria Direct Expression of an Intestinal Bactericidal Lectin," Science, 25 August 2006 (25.08.2006), Vol. 313, Pg. 1126-1130. entire document	61
Y	US 2012/0164205 A1 (BAUM et al) 28 June 2012 (28.06.2012) entire document	62, 63, 64a, 64b
A	CHANG et al. "The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition," Proc Natl Acad Sci USA, 11 February 2014 (11.02.2014), Vol. 111, Pgs. 2247-2252. entire document	1-7, 8a, 8b, 9-18, 20-49, 51, 52a, 52b, 53-56, 57a, 57b, 58a, 58b, 59, 60a, 60b, 61-63, 64a, 64b, 65, 66
A	US 2009/0304594 A1 (FANTIN et al) 10 December 2009 (10.12.2009) entire document	1-7, 8a, 8b, 9-18, 20-49, 51, 52a, 52b, 53-56, 57a, 57b, 58a, 58b, 59, 60a, 60b, 61-63, 64a, 64b, 65, 66

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/064977

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 sequence listing:

- a. ☒ forming part of the international application as filed:
☒ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*. 1(a)).
☐ on paper or in the form of an image file (Rule 13*ter*. 1(b) and Administrative Instructions, Section 713).
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 forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 53-62 were searched.