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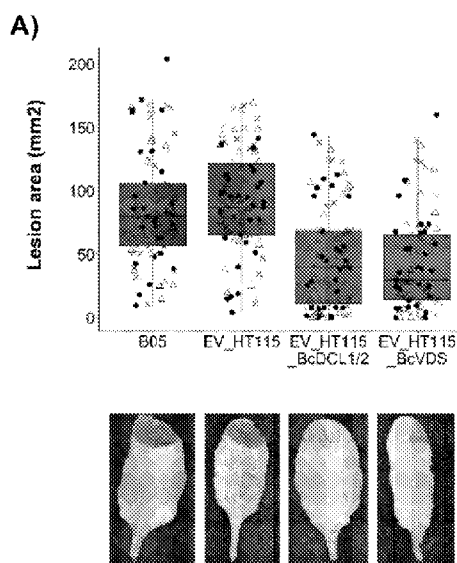
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(54) Title: NUCLEIC ACID MOLECULES DELIVERED VIA EXTRACELLULAR VESICLES TO PATHOGENS FOR CROP PROTECTION



(57) Abstract: In one aspect, the present application discloses compositions of extracellular vesicles (EVs) comprising heterologous effector RNAs, such as antifungal RNAs. In certain embodiments, the effector RNAs comprise dsRNA, while in other embodiments the effector RNAs comprise mRNA. In certain embodiments, the EVs are plant EVs, microbial EVs (e.g., bacterial EVs or fungal EVs), or artificial EVs. In particular embodiments, the EVs are plant EVs and the effector RNA is mRNA. In other particular embodiments, the EVs are microbial EVs and the effector RNA is dsRNA. In other aspects, the application discloses microbes that produce RNA-loaded EVs; methods of treating and/or preventing fungal diseases by applying such microbes and/or RNA-loaded EVs to plants, plant parts, and/or soil; and methods of producing such RNA-loaded EVs. In certain examples, the antifungal RNAs may target DCL1/2 genes, the VDS genes, and/or the tetraspanin (PLS1) genes of a fungal pathogen such as *B. cinerea*, *Verticillium dahliae*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Fusarium graminearum*, *Aspergillus niger* or even oomycete pathogens, such as *Phytophthora infestans*.



**NUCLEIC ACID MOLECULES DELIVERED VIA EXTRACELLULAR VESICLES TO
PATHOGENS FOR CROP PROTECTION**

Government License Right:

5 This invention was made with government support under contract/grant number R35GM136379 awarded by the National Institutes of Health (NIH); contract/grant number 2020731 awarded by the National Science Foundation (NSF); and contract/grant number 2021-67013-34258 awarded by the U.S. Department of Agriculture (USDA). The government has certain rights in the invention.

Cross-Reference to Related Application:

10 This application claims priority to U.S. Provisional Patent Application No. 63/610,326, filed on December 14, 2023, entitled “Nucleic Acid Molecules Delivered Via Extracellular Vesicles To Fungal Pathogens for Crop Protection,” and U.S. Provisional Patent Application
15 No. 63/632,811, filed on April 11, 2024, entitled “Delivery of Fungal-Gene Targeting RNAs Using Microbial Extracellular Vesicles,” the entire contents of which are incorporated by reference herein.

Reference to Sequence Listing Submitted Electronically:

20 This application contains a sequence listing, which is submitted electronically as a XML formatted sequence listing with a file name “Sequence_Listing_UC2024_728_PCT.xml”, creation date of December 13, 2024, and having a size of 177,525 bytes. The sequence listing submitted electronically is part of the specification and is herein incorporated by reference in its entirety.

Field of Invention:

25 This application pertains to the fields of agriculture and plant pathology, particularly delivery of RNAs, such as mRNAs and/or dsRNAs, using extracellular vesicles (EVs), such as plant, microbial, and/or artificial EVs, to plant pathogens, such as fungal pathogens.

30 **Background of the Invention:**

Fungal pathogens are a threat to global food security and can cause crop yield losses of up to 20% along with additional postharvest product losses of up to 10%. Currently, resistant strains of fungi to every major fungicide used in agriculture have been identified. In order to continue to safeguard global food security, novel strategies for combatting fungal pathogens must be developed.

Host-microbe interactions represent a molecular battleground involving exchanges of diverse classes of biomolecules (1–3). For example, small RNAs (sRNAs) are a class of short non-coding RNAs that can induce silencing of target genes with sequence complementarity (4). Recent discoveries show that some microbes deliver sRNAs into host cells and hijack host Argonaute (AGO) proteins to silence host genes for successful infection, a process named “cross-kingdom RNAi” (5–9). During the co-evolutionary arms race between hosts and microbes, hosts also transfer sRNAs into interacting microbes to silence virulence related genes in pathogens (1,10–12).

Certain recent advances in crop protection rely on RNAs to target fungal pathogens. For example, in Spray-Induced Gene Silencing (SIGS), antifungal RNAs are applied to plant material through spray application. SIGS techniques utilize RNAi technology, which allows for the versatile design of antifungal RNAs that are species specific and target multiple genes simultaneously. SIGS has been successfully utilized to control a wide variety of fungal pathogens, insects, and viruses. A major drawback to SIGS approaches is the instability of RNA in the environment, which can be rapidly broken down by RNases and also can degrade rapidly when exposed to rainfall, high humidity, and UV light. Further, many fungal pathogens are soil-borne, and RNAs are rapidly broken down in the soil. Thus, current SIGS methods require periodic re-application of RNA to ensure long-lasting protection. These RNAs are also often chemically synthesized, which can be cost-prohibitive, limiting practical application of SIGS.

In contrast to sRNA and double-stranded RNA (dsRNA), messenger RNA (mRNA) conveys genetic information within cells that is usually translated into proteins to fulfil its biological function. Intercellular and systemic mRNA trafficking within an organism has been reported in animals and plants (18–21).

Extracellular vesicles (EVs) are a diverse group of cell-derived membranous structures that are released into the extracellular environment. EVs can contain a cargo of various biomolecules, including proteins, lipids, nucleic acids (such as RNA and DNA), and metabolites

(13). They serve as important mediators of intercellular communication by transferring biological molecules, thereby influencing various physiological and pathological processes in diverse organisms (14).

In animals, EVs are important for intercellular and systemic sRNA and mRNA trafficking within an organism (18,22–24). In animals, EVs have gained significant attention in the scientific and medical communities due to their potential as diagnostic and therapeutic tools (14). In plants, EVs play an important role in protecting sRNA during trafficking from hosts to interacting microbes, to the detriment of pathogen infection (10,15). Strikingly, fungal pathogen *Botrytis cinerea*, which causes grey mold disease on more than 1400 plant species (16), uses similar strategy as its plant host to also deploy EVs to protect and transport sRNA effectors into host cells for cross-kingdom RNAi (17). Recently, the fungal pathogen of maize, *Ustilago maydis*, was shown to secrete EVs containing mRNAs which may participate in plant-pathogen interactions (25).

There remains a need in the art for improved strategies for protection of plants from pathogens, such as fungal pathogens, including improved methods that utilize RNAs, such as antifungal RNAs.

Summary of the Invention:

In one embodiment, the present application describes the use of extracellular vesicles (EVs) comprising heterologous RNAs that are effective for treatment and/or prevention of pathogen infection of plants, such as antifungal RNAs for treatment and/or prevention of fungal pathogens, as well as compositions used for the same. Heterologous RNAs that are effective for treatment and/or prevention of pathogen infection of plants are generally referred herein as “effector RNAs” and EVs comprising such RNAs are generally referred to herein as “RNA-loaded EVs.”

In certain examples, effector RNAs are effective for treatment and/or prevention of one or more fungal pathogens, which may be referred to as “antifungal RNAs,” while RNA-loaded EVs comprising such RNAs may be referred to as “antifungal EVs.” In other examples, effector RNAs are effective for treatment and/or prevention of other eukaryotic pathogens that have RNAi machinery, such as oomycete pathogens.

In certain examples, the effector RNAs comprise messenger mRNA. In other examples, the effector RNAs comprise RNAs useful for RNA interference (RNAi), such as double-stranded RNAs (dsRNAs), short-interfering RNAs (siRNAs), or hairpin RNAs (hpRNAs).

5 In certain examples, the effector RNAs allow targeting of multiple genes at the same time, such as one or more effector RNAs capable of targeting genes in multiple distinct pathogens and/or one or more effector RNAs capable of targeting multiple genes in the same pathogen.

In certain examples, RNA-loaded EVs are derived from plants ("plant EVs") or microbes ("microbial EVs"), such as bacteria ("bacterial EVs") or fungi ("fungal EVs"). In other examples, RNA-loaded EVs are synthetically/artificially produced ("artificial EVs").

10

In other embodiments, the present application describes microbes that produce RNA-loaded microbial EVs, such as antifungal EVs. In further embodiments, the present application describes methods of applying to plant material and/or the surrounding media RNA-loaded EVs, such as RNA-loaded microbial EVs, and/or microbes that produce them. In still further
15 embodiments, the present application comprises methods of producing RNA-loaded EVs, such as antifungal microbial EVs.

In one aspect, this application pertains to a composition comprising a microbial EV isolated from a microbe, said microbial EV containing one or more effector RNAs, such as one or more antifungal RNAs, wherein said one or more effector RNAs are heterologous to the microbe (e.g.,
20 an effector RNA derived from one species or type of microbe in an EV derived from a different species or type of microbe).

In another aspect, this application pertains to a composition comprising a microbial EV containing one or more effector RNAs, such as one or more antifungal RNAs, wherein the microbial EV was produced by a first microbe and then isolated from said first microbe, wherein
25 the one or more effector RNAs were chemically synthesized and then isolated or produced by a second microbe and then isolated from said second microbe, and wherein the isolated one or more effector RNAs were then loaded into the isolated microbial EV. In certain examples, the first microbe is a first bacterium, the microbial EV is a bacterial EV, and the bacterial EV is isolated from the first bacterium; the second microbe is a second bacterium and the one or more effector
30 RNAs are isolated from the second bacterium; and the isolated one or more effector RNAs are loaded into the bacterial EV. In other examples, the first microbe is a bacterium, the microbial EV

is a bacterial EV, and the bacterial EV is isolated from the bacterium; the second microbe is a fungus and the one or more effector RNAs are isolated from the fungus; and the isolated one or more effector RNAs are loaded into the bacterial EV. In further examples, the first microbe is a fungus, the microbial EV is a fungal EV, and the fungal EV is isolated from the fungus; the second
5 microbe is a bacterium and the one or more effector RNAs are isolated from the bacterium; and the isolated one or more effector RNAs are loaded into the fungal EV. In still further examples, the first microbe is a first fungus, the microbial EV is a fungal EV, and the fungal EV is isolated from the first fungus; the second microbe is a second fungus and the one or more effector RNAs are isolated from the second fungus; and the isolated one or more effector RNAs are loaded into
10 the fungal EV.

In a further aspect, this application pertains to a microbe for production of EVs containing one or more effector RNAs, said microbe comprising and expressing one or more heterologous nucleic acid sequences encoding the one or more effector RNAs.

In yet another aspect, the present application pertains to a method of treating or preventing
15 fungal disease of a plant, the method comprising applying to the plant, one or more portions of the plant, or soil in the vicinity of the roots of the plant a plurality of engineered microbes; wherein said engineered microbes comprise and express one or more heterologous nucleic acid sequences encoding one or more effector RNAs; wherein the plurality of microbes produces microbial extracellular vesicles (EVs) containing the one or more effector RNAs.

In a further aspect, the present application pertains to a method of treating or preventing
20 fungal diseases or other eukaryotic pathogen diseases of a plant, the method comprising applying to the plant, one or more portions of the plant, or soil in the vicinity of the roots of the plant a plurality of microbial extracellular vesicles (EVs), wherein the plurality of microbial EVs were isolated from a plurality of microbes, wherein the plurality of microbial EVs contain one or more
25 effector RNAs, wherein said one or more effector RNAs are heterologous to the plurality of microbes.

In a still further aspect, the present application pertains to a method of producing a microbial extracellular vesicle (EV) comprising an effector RNA, the method comprising growing
30 a plurality of engineered microbes, wherein the engineered microbes comprise and express one or more heterologous nucleic acid sequences encoding one or more effector RNAs, and wherein the

engineered microbes produce microbial EVs comprising the one or more heterologous effector RNAs; and isolating from the growth media a plurality of EVs.

In an additional aspect, the present application pertains to a method of producing a microbial extracellular vesicle (EV) comprising one or more effector RNAs, the method comprising growing a plurality of microbes in growth media, wherein the microbes produce a plurality of microbial EVs; isolating the plurality of microbial EVs from the growth media; and introducing into the isolated microbial EVs one or more heterologous effector RNAs.

In certain embodiments the effector RNA comprises a double-stranded RNA. In certain other embodiments the effector RNA comprises a mRNA. In some embodiments, the one or more effector RNAs target one or more fungal pathogens selected from the group consisting of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus niger*. In certain particular embodiments, the antifungal RNA targets *Botrytis cinerea*. In other particular embodiments, the antifungal RNA targets *Verticillium dahlia*. In additional embodiments, the antifungal RNA targets one or more oomycete pathogens, such as *Phytophthora infestans*.

In certain embodiments the antifungal RNA targets the DCL1/2 genes (dicer genes). In certain other embodiments the antifungal RNA targets one or more of the VDS genes (the vacuolar protein sorting 51 (VPS51) gene, the dynactin (DCTN1) gene, the suppressor of actin (SAC1) gene), and/or the tetraspanin gene Punchless 1 (PLS1) of a fungal pathogen. In additional embodiments the antifungal RNA targets both the DCL1/2 genes and the VDS genes. In certain embodiments, the one or more antifungal RNAs can comprise one or more nucleic acid sequences at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12.

In certain embodiments the microbe generating the EVs and/or the microbe producing the effector RNAs may be a bacterium, and the bacterium may include *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*. In other embodiments the microbe generating the EVs and/or the microbe producing the effector RNAs may be a fungus, and the fungus may include *Trichoderma virens* or a mycorrhizal fungus.

In certain embodiments, one or more heterologous nucleic acid sequences encoding the one or more effector RNAs is introduced into the microbe (*e.g.*, bacterium or fungus). The one or

more heterologous nucleic acid sequences may be introduced by any suitable means, and such means will be well known to a person of ordinary skill in the art. In certain examples, the one or more heterologous nucleic acid sequences encoding the one or more effector RNAs may be introduced by introducing a bacterial plasmid, bacterial artificial chromosome, or yeast artificial chromosome. In certain examples, the bacterial plasmid may be any of the following: a T777T
5 plasmid that is introduced into the HT115 strain of *Escherichia coli*; a pDG148-Stu plasmid that is introduced into the BG322 strain of *Bacillus subtilis*; a pJOE7771.1 plasmid that is introduced into the CMA702 strain of *Pseudomonas putida*.

In other examples, the one or more heterologous nucleic acid sequences encoding the one
10 or more effector RNAs may be introduced by inserting the heterologous nucleic acids into the genome of the microbe. This can be accomplished by any suitable means, and such means will be well known to a person of ordinary skill in the art. In particular examples, the one or more heterologous nucleic acid sequences encoding one or more effector RNAs may be inserted into the genome using CRISPR/Cas9 genome editing. In other examples, the one or more heterologous
15 nucleic acid sequences encoding one or more antifungal RNAs may be inserted into the genome through homologous recombination.

In certain examples, the one or more effector RNAs are produced from one or more heterologous nucleic acid sequences introduced into a microbe (*e.g.*, bacterium or fungus) and are then isolated from the growth media and microbes. In other embodiments the one or more
20 effector RNAs are chemically synthesized. In some embodiments the effector RNA that is chemically synthesized is loaded into an extracellular vesicle isolated from a bacterium. In some other embodiments the effector RNA that is chemically synthesized is loaded into an extracellular vesicle isolated from a fungus.

In certain examples, the microbes or EVs described herein are applied to the plant itself, or
25 one or more parts thereof. In particular examples, application may be to the plant's leaves, stems, flowers, roots, and/or fruit. In other examples, the microbes or EVs described herein are applied to the soil in the vicinity of the roots of the plant.

Brief Description of the Drawings:

30 **FIG. 1A:** Representative schematic of a plasmid used to overexpress dsRNA that targets pathogen genes. Different dsRNA sequences can be easily inserted using the StuI/NotI cut sites.

FIG. 1B: Growth curve of *B. subtilis* strains containing either an empty plasmid (BG322), plasmids expressing the DCL1/2 construct (DCL and DCL_const.), or a plasmid expressing dsYFP (YFP). No significant growth effect is observed between the DCL1/2 and BG322 strains.

FIG. 2A and FIG. 2B: BEVs were isolated from *B. subtilis* cultures and characterized using nanoparticle tracking analysis and microscopy. Microbial EVs are exosome-like in nature and similar in size and morphology to plant extracellular vesicles.

FIG. 2C: RT-PCR was used to confirm that the desired dsRNA is present in BEVs isolated from *B. subtilis* cultures.

FIGS. 3 and 4: The *Arabidopsis* endogenous mRNAs present in isolated EVs from *B. cinerea* infected tissues were identified and categorized to determine those that encode genes associated with biotic stress or defense responses (**FIG. 3**) as well as those that can be found in mitochondrial proteomes (**FIG. 4**).

FIG. 5A: Full length plant transcripts were detected by RT-PCR in EVs isolated from Mock-treated and *B. cinerea*-infected Col-0 leaves. Total RNAs from Mock, Total Infected leaves were used as controls. DNA size markers are in base pairs (bp).

FIG. 5B: Full-length host transcripts were detected by RT-PCR after Micrococcal Nuclease and Proteinase K digestion, indicating that they are inside the EVs. Treatments are as indicated (+). DNA size markers are in base pairs (bp).

FIG. 5C: Full-length plant transcripts were detected in TET8-positive exosomes by RT-PCR. EVs were isolated from *Arabidopsis* leaves infected by *B. cinerea*. TET8-positive exosomes were obtained by immunocapture with TET8-specific antibody from isolated EVs of P100 fraction. IgG non-specific antibody was used as a control. The same transcripts were only weakly detected in a tet8/tet9 double mutant line. OEP6, PRO5 and GRF10 were used as negative controls. Bc-Actin was used as a pathogen control gene, which was only detected in infected Col-0 leaves. DNA size markers are in base pairs (bp).

FIG. 6A: Quantification of tagged transcripts in EVs isolated from *N. benthamiana*. The data are presented as mean $\pm$ s.d., n = 6 optical slices. Ordinary one-way ANOVA using Dunnett's

multiple comparisons test was conducted to identify statistically significant differences. Small black circles represent individual values.

FIG. 6B: Quantification of protein TET8-mCherry in EVs isolated from *N. benthamiana* in (A). The data are presented as mean $\pm$ s.d., n = 6 optical slices.

5 **FIG. 6C:** Quantification of tagged transcripts in EVs isolated from *Arabidopsis* transgenic lines. The data are presented as mean $\pm$ s.d., n = 6 optical slices.

FIG. 6D: Quantification of total EVs isolated from *Arabidopsis* transgenic lines. The data are presented as mean $\pm$ s.d., n = 6 optical slices.

10 **FIG. 7A:** Full-length transcripts were detected by RT-PCR in *B. cinerea* cells isolated from infected Col-0 (Infected), but not in cultured *B. cinerea* mixed with uninfected leaves (Mixed control), which was subjected to the same procedure. Plant transcripts were largely reduced in *B. cinerea* cells purified from the infected tet8/tet9 double mutant line compared with those from infected Col-0. OEP6, GRF10 and PRO5 were used as plant endogenous controls and Bc-Actin and Bc-Tubulin as pathogen controls. DNA size markers are in base pair (bp).

15 **FIG. 7B:** The *Arabidopsis* SAG21-3WJ-4xBro and APS1-3WJ-4xBro tagged transcripts were detected within *B. cinerea* hyphae after co-incubation, whereas OEP6-3WJ-4xBro was not. EVs purified from *Arabidopsis* expressing SAG21-3WJ-4xBro, APS1-3WJ-4xBro or OEP6-3WJ-4xBro were incubated with in vitro cultured *B. cinerea* conidia for 4 h. Tagged transcripts were detected by RT-PCR after Triton-X100 treatment and wash to remove EVs in the mixed solution.
20 DNA size markers are in base pair (bp)

FIG. 7C: Quantification of plant tagged transcripts in *Botrytis* hyphae. Ordinary one-way ANOVA using Dunnett's multiple comparisons test was conducted to identify statistically significant differences. The data are presented as mean $\pm$ s.d., n = 6 optical slices. Small black circles (D, F) represent individual values.

25 **FIG. 7D:** Quantification of tagged transcripts in *Botrytis* cells isolated from infected *Arabidopsis* transgenic lines. The data are presented as mean $\pm$ s.d., n = 6 optical slices.

FIG. 7E: RT-PCR shows that tagged transcripts translocated from the plants into interacting fungal cells were detected in purified Bc-cells. *B. cinerea* cells were isolated from

infected transgenic *A. thaliana* lines expressing SAG21-3WJ-4xBro, APS1-3WJ-4xBro, or OEP6-3WJ-4xBro (control).

FIG. 8A: Full-length plant transcripts were detected in TRAP-isolated fungal ribosome fraction after 36 h infection (Infection) by *B. cinerea* expressing ribosomal subunit BcRPL23-YFP, but were not detected in in vitro cultured *B. cinerea* transgenic BcRPL23-YFP hyphae mixed with Col-0 (Control) (upper panels). Immunoblot shows that TRAP specifically pulls down BcRPL23-YFP from infected Col-0 tissue using α -GFP antibody beads. OEP6, GRF10 and PRO5 were used as plant control genes, Bc-Actin as a pathogen control gene. DNA size markers are in base pair (bp). Protein size markers are in kilodaltons (KD).

FIG. 8B: RT-PCR shows that the transferred *Arabidopsis* mRNAs were associated with *B. cinerea* polysomes. The transferred plant mRNAs were shifted from the polysome fractions to the monosome fractions upon puromycin treatment. Ten fractions of equal volume were collected from top to bottom of 15% to 55% sucrose gradients. Treatment of puromycin or not is as indicated (+ or -).

FIG. 8C: Western blot analysis shows SAG21-YFP and APS1-YFP proteins were not detectable in the extracellular fractions, including apoplastic wash fluids (AWF), the P100 EV fraction (EVs), or the supernatant of the P100 fraction (S). As a positive control, Annexin1(ANN1)-YFP tagged protein was secreted into AFs and present in EVs. The abundantly secreted pathogen-related protein1 (PR1), absent in EVs, was used as a secretion control. TET8 native protein was used as a marker for EV containing fractions. S=Supernatant after 100,000 x g centrifugation.

FIG. 8D: RT-PCR shows that the full-length YFP-tagged wild type transcripts SAG21 and APS1, as well as the YFP-tagged mutated transcripts mSAG21 and mAPS1 were detected in fungal cells incubated with EVs for 24 h.

FIG. 8E: Quantification of translated proteins from transferred plant YFP-tagged transcripts in fungal hyphae. T-test was conducted to identify statistically significant differences. The data are presented as mean $\pm$ s.d., n = 6 optical slices. Small black circles represent individual values.

FIG. 8F: Only the transferred wild type SAG21 and APS1 mRNAs but not the mutated versions were translated into tagged proteins in fungal cells. Samples were subjected to

immunoblot using α -GFP as primary antibody. Size markers are indicated in KD, and protein loading is represented by Ponceau staining (PS).

FIG. 9A and 9B: *B. cinerea* transformants expressing *Arabidopsis* SAG21-YFP or APS1-YFP under a constitutive promotor show reduced virulence compared with transformants expressing the mutated transcript mSAG21-YFP or mAPS1-YFP or control YFP. Relative lesion sizes were measured at 60 h post-infection. The data are presented as mean $\pm$ s.d., n=10 leaves from at least three replicates. Ordinary one-way ANOVA using Dunnett's multiple comparisons test was conducted to identify statistically significant differences. Small black circles represent individual values.

FIG. 9C and 9D: Enhanced susceptibility to *B. cinerea* was observed in T-DNA insertion knockout line *sag21* (SALK_099663) and APS1 T-DNA insertion knockout line (*aps1*, SALK_046518). Complemented transgenic *sag21* line expressing SAG21-YFP driven by its native promotor shows no significant difference in susceptibility with Col-0, and complemented transgenic *aps1* line expressing APS1-YFP driven by its native promotor shows no significant difference in susceptibility with Col-0. Relative lesion sizes were measured at 60 h post-infection. The data are presented as mean $\pm$ s.d., n=10 leaves from at least three replicates. Ordinary one-way ANOVA using Dunnett's multiple comparisons test was conducted to identify statistically significant differences. Small black circles represent individual values.

FIG. 10A: RT-PCR of BEVs isolated from BG322-VDS-3wj cultures under different enzymatic treatments. The presence of a band corresponding to VDS-3wj after nuclease and triton treatment supports the role of BEVs in protecting mRNA cargo.

FIG. 10B: BEVs isolated from BG322-VDS-3wj cultures were purified using density gradient ultracentrifugation and RT-PCR performed on each fraction. VDS-3wj is enriched in densities from 1.13-1.21 g/cm³, which corresponds to the fractions with BEV enrichment.

FIG. 11A and FIG. 11B: Lesion size area produced by *B. cinerea* after 3 dpi was reduced in the plants that were treated by BEVs isolated from HT115-modified bacteria that are able to produce dsRNA against *B. cinerea* target DCL1/2 and VDS genes. Plants treated with just BEVs did not exhibit reduced infection, indicating that delivery of dsRNA targeting DCL1/2 or VDS genes is necessary for pathogen inhibition.

FIG. 11C: Relative gene expression of BcDCL2 and BcVPS51 in *B. cinerea* after 12 hours of co-incubation with BEVs isolated from BG322-modified bacteria producing dsRNA targeting

BcDCL1/2 or BcVDS. Approximately 50% reduction in relative gene expression is observed in both cases.

FIG. 12A: Disease produced in *A. thaliana* by *V. dahliae* at 21 dpi, when soil was treated by the bacterial solution 2 hours before plants were transplanted and 7 days after the inoculation. Relative fungal biomass was calculated by the expression of actin genes in *V. dahliae* and *A. thaliana*. Overhead pictures of bacterial treated *A. thaliana* plants after 3 weeks of inoculation with *V. dahliae* JR2 are displayed.

FIG. 12B: Canopy area of tomato plants inoculated with *V. dahliae* in bacterial treated soil at 21 dpi. Overhead picture of inoculated tomato MoneyMaker plants in bacterial treated soil at 21 dpi are displayed.

FIG. 12C: Relative gene expression of VdDCL1/2 in *V. dahliae* after 6, 12, and 24 hrs of co-incubation with *B. subtilis* that produces the DCL1/2 dsRNA construct.

FIG. 13A and FIG. 13B: *A. thaliana* leaves were pre-treated by spray with HT115 (A) or BG322 (B) bacterial suspensions, then inoculated with *B. cinerea* spores. Lesion size areas were measured with the help of a digital calibrator at 3 dpi. Treatment with HT115_BcDCL1/2 as well as BG322_BcDCL1/2 showed a statistical reduction of lesion size area as determined by a one-way ANOVA followed by Tukey's HSD test (** $p < 0.01$). Additionally, representative pictures of fungal lesions on *A. thaliana* leaves at 3 days post inoculation are shown.

Brief Description of the Sequences:

SEQ ID NO:1	<i>Botrytis cinerea</i> (Bc)-DCL1 full length gene sequence
SEQ ID NO:2	Bc-DCL1 cDNA corresponding to RNAi fragment
SEQ ID NO:3	Bc-DCL2 full length gene sequence
SEQ ID NO:4	Bc-DCL2 cDNA corresponding to RNAi fragment
SEQ ID NO:5	Bc-VPS51 full length gene sequence
SEQ ID NO:6	Bc-SAC1 full length gene sequence
SEQ ID NO:7	Bc-DCTN1 full length gene sequence
SEQ ID NO:8	Bc-PLS1 full length gene sequence
SEQ ID NO:9	<i>Verticillium dahliae</i> (Vd)-DCL1 full length gene sequence
SEQ ID NO:10	Vd-DCL1 cDNA corresponding to RNAi fragment

	SEQ ID NO:11	Vd-DCL2 full length gene sequence
	SEQ ID NO:12	Vd-DCL2 cDNA corresponding to RNAi fragment
	SEQ ID NO:13	Vd-DCTN1 full length gene sequence
	SEQ ID NO:14	Vd-SAC1 full length gene sequence
5	SEQ ID NO:15	Vd-VPS51 homologous gene full length sequence
	SEQ ID NO:16	Vd-PLS1 full length gene sequence
	SEQ ID NO:17	<i>Sclerotinia sclerotiorum</i> (Ss)-DCL1 full length gene sequence
	SEQ ID NO:18	Ss-DCL2 full length gene sequence
	SEQ ID NO:19	Ss-VPS51 full length gene sequence
10	SEQ ID NO:20	Ss-SAC1 full length gene sequence
	SEQ ID NO:21	Ss-DCTN1 full length gene sequence
	SEQ ID NO:22	Ss-PLS1 full length gene sequence
	SEQ ID NO:23	<i>Fusarium oxysporum</i> (Fo)-DCL1 full length gene sequence
	SEQ ID NO:24	Fo-DCL2 full length gene sequence
15	SEQ ID NO:25	Fo-VPS51 full length gene sequence
	SEQ ID NO:26	Fo-DCTN1 full length gene sequence
	SEQ ID NO:27	Fo-SAC1 full length gene sequence
	SEQ ID NO:28	Fo-PLS1 full length gene sequence
	SEQ ID NO:29	<i>Fusarium graminearum</i> (Fg)-DCL1 full length gene sequence
20	SEQ ID NO:30	Fg-DCL2 full length gene sequence
	SEQ ID NO:31	Fg-VPS51 full length gene sequence
	SEQ ID NO:32	Fg-DCTN1 full length gene sequence
	SEQ ID NO:33	Fg-SAC1 full length gene sequence
	SEQ ID NO:34	Fg-PLS1 full length gene sequence
25	SEQ ID NO:35	<i>Aspergillus niger</i> (An)-DCL1 full length gene sequence
	SEQ ID NO:36	An-DCL2 full length gene sequence
	SEQ ID NO:37	An-VPS51 homologous gene full length sequence
	SEQ ID NO:38	An-DCTN1 homologous gene full length sequence

	SEQ ID NO:39	An-SAC1 full length gene sequence
	SEQ ID NO:40	pDG148-Stu plasmid sequence
	SEQ ID NO:41	pJOE7771.1 plasmid sequence
	SEQ ID NO:42	<i>Phytophthora infestans</i> -PiDCL1 full length gene sequence
5	SEQ ID NO:43	<i>Phytophthora infestans</i> - Pihmp1 full length gene
	SEQ ID NO:44	<i>Phytophthora infestans</i> - PiGPB1 full length gene
	SEQ ID NO:45	Forward primer for amplifying <i>A. thaliana</i> SAG21 gene
	SEQ ID NO:46	Reverse primer for amplifying <i>A. thaliana</i> SAG21 gene
	SEQ ID NO:47	Forward primer for amplifying <i>A. thaliana</i> APS1 gene
10	SEQ ID NO:48	Reverse primer for amplifying <i>A. thaliana</i> APS1 gene
	SEQ ID NO:49	Forward primer for amplifying <i>A. thaliana</i> OEP6 gene
	SEQ ID NO:50	Reverse primer for amplifying <i>A. thaliana</i> OEP6 gene

Detailed Description of the Invention:

15 Provided herein are RNA-loaded EVs for the delivery of effector RNAs to protect and treat plants from pathogens, such as fungal pathogens. These RNA-loaded EVs can be produced from plants or various microbes, including bacterial and fungal species, or can be artificially synthesized. The effector RNAs can be chemically synthesized or derived from inserting genetic information into a bacteria or fungi. The effector RNA can be loaded into the EV by the bacteria
20 or fungi, or the EV and effector RNA can be separately isolated and the effector RNA can be loaded into the EV. The effector RNA can comprise any suitable RNA, such as a double-stranded RNA or mRNA. These RNA-loaded EVs can be used in Spray-Induced Gene Silencing (SIGS) approaches to protect crops and post-harvest plant material from pathogens, such as fungal pathogens. Once loaded with effector RNAs, the EVs or the microbes producing the EVs can be
25 sprayed onto plants or the soil to confer protection against the target pathogens.

 The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description. Such description is intended to be illustrative and not limiting with respect to the scope of the present invention. It will be

understood that other embodiments may be practiced with some variations without departing from the spirit or scope of the subject invention.

I. Definitions

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

As used in this application and the appended claims, the singular forms “a,” “an” and “the” include plural references unless the context clearly dictates otherwise. For example, reference to a component is intended also to include composition of a plurality of components. References to a composition containing “a” constituent is intended to include other constituents in addition to the one named.

The use of the alternative (*e.g.*, “or”) should be understood to mean either one, both, or any combination thereof of the alternatives. The term “and/or” should be understood to mean either one, or both of the alternatives.

By “comprising” or “containing” or “including” is meant that at least the named compound, element, particle, or method step is present in the composition or article or method, but does not exclude the presence of other compounds, materials, particles, method steps, even if the other such compounds, material, particles, method steps have the same function as what is named. Additionally, the term “comprises” is intended to include embodiments where the method, apparatus, composition, etc., consists essentially of and/or consists of the listed steps, components, etc. Similarly, the term “consists essentially of” is intended to include embodiments where the method, apparatus, composition, etc., consists of the listed steps, components, etc.

As used herein, the term “about” indicates a close range around a numerical value when used to modify that specific value. If “X” were the value, for example, “about X” would indicate a value from 0.75X to 1.25X, 0.8X to 1.2X, or 0.9X to 1.1X, *e.g.*, a value from 0.95X to 1.05X, or a value from 0.98X to 1.02X, or a value from 0.99X to 1.01X. Any reference to “about X” specifically indicates at least the values X, 0.9X, 0.91X, 0.92X, 0.93X, 0.94X, 0.95X, 0.96X, 0.97X, 0.98X, 0.99X, 1.01X, 1.02X, 1.03X, 1.04X, 1.05X, 1.06X, 1.07X, 1.08X, 1.09X, and 1.1X, and values within this range.

Reference throughout this specification to “one embodiment,” “an embodiment,” “a particular embodiment,” “a related embodiment,” “a certain embodiment,” “an additional embodiment,” or “a further embodiment” or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one
5 embodiment. Thus, the appearances of the foregoing phrases in various places throughout this application are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments. It is also understood that the positive recitation of a feature in one embodiment, serves as a basis for excluding the feature in a particular embodiment.

10 As used herein, a “heterologous” product (*e.g.*, a nucleic acid sequence or RNA) refers to a product that is not naturally occurring in the relevant organism (*e.g.*, the bacterium or fungus), but which instead has been artificially added to or produced in the relevant organism. For example, a nucleic acid sequence is heterologous to a bacteria if that bacteria, as it occurs in nature, would not possess that nucleic acid sequence.

15 As used herein, “expression” of a nucleic acid sequence refers to the conversion of the information contained in the nucleic acid sequence, such as DNA in a bacterial plasmid, an artificial chromosome, or the genome, into a transcriptional product (*e.g.*, mRNA, antisense RNA, or any other type of RNA).

20 As used herein, “treatment” or “treating” includes any beneficial or desirable effect on the effects or pathology of a disease and may include even minimal reductions in one or more measurable markers of the disease being treated. Treatment can optionally involve delaying of the progression of the disease. “Treatment” does not necessarily indicate complete eradication of the disease or associated effects thereof.

25 As used herein, “prevent” and similar words, such as “prevention,” “prevented,” “preventing,” etc., indicate an approach for preventing, inhibiting, or reducing the likelihood of the occurrence or recurrence of a disease. It also refers to delaying the onset or recurrence of a disease or delaying the occurrence or recurrence of the effects of a disease. As used herein, “prevention” and similar words also includes reducing the intensity, effect, and/or burden of a disease prior to onset or recurrence of the disease.

As used herein, a “pathogen” of a plant refers to an organism that produces pathogenic effects on or in that plant and includes, but is not limited to, viruses, bacteria, nematodes, fungi, or insects (*see, e.g., Agrios, Plant Pathology* (Academic Press, San Diego, Calif. (1988))).

The term “plant” includes whole plants, shoot vegetative organs and/or structures (*e.g.,* 5 leaves, stems and tubers), roots, flowers and floral organs (*e.g.,* bracts, sepals, petals, stamens, carpels, anthers), ovules (including egg and central cells), seed (including zygote, embryo, endosperm, and seed coat), fruit (*e.g.,* the mature ovary), seedlings, plant tissue (*e.g.,* vascular tissue, ground tissue, and the like), cells (*e.g.,* guard cells, egg cells, trichomes and the like), and progeny of same. A particular plant may be, for example, an angiosperm (a monocotyledonous or 10 dicotyledonous plant), a gymnosperm, a fern, or a multicellular alga. Plants may be of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid, and hemizygous.

As used herein, the term “vesicle” encompasses any compartment enclosed by a lipid structure such as a lipid monolayer or a lipid bilayer. The vesicles may be, for example, liposomes, lipid micelles, and non-micellar lipid particles. The term “extracellular vesicle” or “EV” refers to 15 a vesicle that exists outside of a cell. EVs may be naturally produced, for example by a plant, bacterium, or fungus.

As used herein, the term “DCL 1/2 genes” refers to the Dicer-like 1 and Dicer-like 2 genes in fungal pathogens including but not limited to *Botrytis cinerea*.

As used herein, the term “VDS genes” refers the vacuolar protein sorting 51 (VPS51) gene, 20 the dynactin (DCTN1) gene, or the suppressor of actin (SAC1) gene) of fungal pathogens including but not limited to *Botrytis cinerea*.

To facilitate an understanding of the principles and features of the various embodiments of the disclosure, various illustrative embodiments are explained herein. Although exemplary embodiments of the disclosure are explained in detail, it is to be understood that other embodiments 25 are contemplated. Accordingly, it is not intended that the disclosure is limited in its scope to the details of construction and arrangement of components set forth in the description or examples. The disclosure is capable of other embodiments and of being practiced or carried out in various ways.

II. Extracellular Vesicles

Provided herein are compositions comprising an EV containing one or more effector RNAs for delivery of the one or more effector RNAs to plants to prevent and/or treat pathogens, such as antifungal RNAs to treat prevent and/or treat fungal pathogens. The EV can help shield the effector RNA from degradation (*e.g.*, nuclease degradation, UV degradation) and can be easily taken up by the target pathogen, such as a fungal pathogen. EVs can be, for example, plant EVs, microbial EVs, or artificial EVs.

In some embodiments, the EVs are derived from a plant (“plant EVs”) or plant cell, such as *Arabidopsis*. Plant EVs can be loaded with one or more effector RNAs by any suitable means.

In certain examples, the effector RNAs can be produced in a plant or plant cell that produces the EV, such that the EV is released from a plant cell pre-loaded with effector RNAs. In such cases, effector RNAs can be heterologous to the plant cell. The heterologous effector RNAs can be introduced into the plant cell by any suitable means, including through recombinant DNA and/or genetic manipulation (DNA editing, genetic modification) methods that are well known to persons of ordinary skill in the art. In other examples, plant EVs are purified and then are loaded with one or more effector RNAs produced from a different source, such as a different plant, a microbe, or synthetically. Such effector RNAs can be introduced into the plant EV by any suitable means, as would be well known to a person of ordinary skill in the art.

In other embodiments, the EVs are microbial EVs produced by microbes, including fungi and bacteria. Microbial EVs can be exosome-like in nature and similar in size and morphology to plant extracellular vesicles. In various embodiments the microbe that produces the EVs loads the vesicle with heterologous effector RNA. In other embodiments, the effector RNA is loaded into the microbial EV after it is produced by the microbe.

In some embodiments bacterial EVs are produced by *Escherichia coli* (HT115), *Bacillus subtilis* (BG322), and *Pseudomonas putida* (CMA702). In other embodiments other bacterial species, which promote plant growth, may also be used, such as *Bacillus spp.* including *B. licheniformis*, *B. cereus*, and *B. pumilus*; *Burkholderia cepacia*; *Pseudomonas spp.* such as *P. chlororaphis* and *P. fluorescens*; *Serratia spp.* such as *S. marcescens* and *S. plymuthica*; *Streptomyces spp.* such as *S. lividans* and *S. lydicus*; and various *Rhizobia spp.* that have already been accepted as commercial biofertilizers. In other embodiments fungal EVs are produced. In certain examples, the fungal EVs may be produced by plant-beneficial/biocontrol fungal species,

such as *Trichoderma virens*, plant-colonizing fungi, such as mycorrhizal fungi, or other soil fungi. The bacterial and fungal RNA-loaded EVs contain the desired effector RNA, such as mRNA or dsRNA targeting pathogen virulence related genes.

In some embodiments genetically tractable microbes with known plant beneficial properties are used to produce the effector RNA and deliver this RNA to pathogens through RNA-loaded EVs. In some embodiments these RNA-loaded EVs can be isolated and applied directly to the plant material and/or soil by spraying. In other embodiments the microbes that produce the EVs can be applied to the plant material and or soil by spraying.

III. Effector RNAs

In some embodiments, the one or more effector RNAs contained in the EV comprise double-stranded RNA. In other embodiments, the one or more effector RNAs contained in the EV comprise mRNA. In certain embodiments, the target pathogen is a fungus and the effector RNA is an antifungal RNA.

The effector RNAs can exert their effects on the target pathogens by any known means. In certain embodiments, the effector RNAs work through gene silencing. In certain examples, the effector RNA are species specific and can target multiple genes in the target pathogens and silence those genes. This can be accomplished, for example, through RNA interference (“RNAi”). RNAi is the phenomenon in which, when a double-stranded RNA having a sequence identical or similar to that of the target gene is introduced into a cell, the expressions of both the inserted exogenous gene and target endogenous gene are suppressed. The double-stranded RNA may be formed from two separate complementary RNAs or may be a single RNA molecule that comprises internally complementary sequences that form a double-stranded RNA region. RNAi is also known to be effective in plants in reducing levels of RNA expressed by target gene of interest (*see, e.g.,* Chuang, C. F. & Meyerowitz, E. M., *Proc. Natl. Acad. Sci. USA* 97: 4985 (2000); Waterhouse *et al.*, *Proc. Natl. Acad. Sci. USA* 95:13959-13964 (1998); Tabara *et al.* *Science* 282:430-431 (1998); Matthew, *Comp Funct. Genom.* 5: 240–244 (2004); Lu, *et al.*, *Nucleic Acids Research* 32(21):e171 (2004)).

In certain embodiments, the microbe in which the effector RNA is produced exhibits reduced RNaseIII activity, which promotes the production of the effector RNA molecules.

Antifungal RNAs can be used to target any suitable gene in a fungal pathogen that, when targeted, results in reduction of that fungal pathogen (*e.g.*, killing the fungus or reduced reproductive time). In some examples, the targeted genes include one or more fungal genes selected from DCL 1 gene, DCL 2 gene (collectively referred to as DCL1/2 genes; for example, SEQ ID NOs: 1, 3, 9, 11, 15, 16, 21, 22, 27, 28), vacuolar protein sorting 51 (VPS51) gene (for example, SEQ ID NOs: 5, 17, 23, 29), dynactin (DCTN1) gene (for example, SEQ IN NOs: 7, 13, 19, 24, 31), or suppressor of actin (SAC1) gene (for example, SEQ ID NOs: 6, 14, 18, 26, 32) (collectively referred to as VDS genes). SEQ ID NOs: 2, 4, 10, and 12 provide examples of cDNAs of antifungal RNAs effective in RNAi against DCL1 and 2 genes of *Botrytis cinerea* and *Verticillium dahlia*. In particular examples, the one or more antifungal RNAs comprise one or more nucleic acid sequences at least about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12. However, these are only examples of suitable target sequences that can be used. A person of ordinary skill in the art would be able to design appropriate target sequences based on their particular needs.

In other examples, the effector RNA is an mRNA that is translated into a protein in the pathogen, which protein impacts the pathogen cell, such as by killing the cell, stopping or decreasing cell division, or stopping or decreasing necessary cellular machinery and/or processes. In certain embodiments, an effector RNA can be an mRNA that targets morphology and/or functioning of the pathogen's mitochondria. Mitochondria are essential organelles in most eukaryotes that produce energy in the form of ATP to enable many cellular processes. In certain embodiments, the effector RNAs comprise an mRNA encoding the plant SAG21 (Senescence-associated gene 21) protein and/or APS1 (ATP sulfurylase 1) protein, such as an mRNA homologous to the *Arabidopsis thaliana* SAG21 or APS1 gene or an mRNA encoding a protein homologous to the *Arabidopsis thaliana* SAG21 or APS1 protein. However, these are only examples of suitable mRNAs that can be used. A person of ordinary skill in the art would be able to design appropriate target sequences based on their particular needs.

Production of the desired effector RNA within a microbe can be accomplished by any suitable means, and such means would be well known to a person of ordinary skill in the art. For example, one or more heterologous nucleic acid sequences encoding one or more effector RNAs can be introduced into the desired microbe as a separate genetic element, for example through

introduction of bacterial plasmids or bacterial artificial chromosomes (into bacteria) or yeast artificial chromosomes (into fungi) that carry the one or more heterologous nucleic acid sequences. In other examples, the one or more heterologous nucleic acid sequences can be introduced into the microbe's genome. This can be accomplished by any suitable means known in the art, including use of a CRISPR-Cas genome editing system, ZFNs, TALENs, and/or homologous recombination. Microbes containing the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs can express those effector RNAs and, in some instances, load those effector RNAs into the microbial EVs that the microbe produces.

In some embodiments, effector RNA constructs targeting pathogen virulence related genes are designed for bacterial expression through modification of bacterial plasmids and introduction of such plasmids into bacteria. In some embodiments these plasmids are the T777T plasmid (*e.g.*, introduced into HT115), pDG148-Stu plasmid (*e.g.*, introduced into HT115 or BG322; SEQ ID NO: 33), and pJOE7771.1 plasmid (*e.g.*, introduced into CMA702; SEQ ID NO: 34). In further embodiments, the bacteria into which the plasmids are introduced are *E. coli* HT115, *B. subtilis* BG322, or *P. putida* CMA702. HT115 is an *Escherichia coli* strain that was derived from the W3110 strain, which is a commonly used wild-type of the non-pathogenic *E. coli* K-12 strain. (Caenorhabditis Genetics Center. University of Minnesota). BG322 is a *Bacillus subtilis* strain that comes from the BG1 wild type whose *rncS* (RNaseIII) sequence is absent. (David Bechhofer. Mount Sinai School of Medicine of New York University). CMA702 is a *Pseudomonas putida* strain in which the *rnc* gene (RNaseIII) has been truncated. This strain is derived from the KT2440 strain, which is a commonly used and safe derivative of the *P. putida* mt-2 strain. (Sandra C Viegas, Universidade Nova de Lisboa, Portugal).

IV. Plant Cells and Microbes That Produce Extracellular Vesicles Containing Effector RNA

In another aspect, the present application relates to plant or microbe cells that produce EVs comprising one or more effector RNAs. In certain embodiments, the application describes an engineered plant cell or microbe for production of EVs containing one or more effector RNAs, said cell comprising and expressing one or more heterologous nucleic acid sequences encoding the one or more effector RNAs.

Any suitable plant or microbe can be used. In certain embodiments, the microbe is a fungus and produces fungal extracellular vesicles, for example a *Trichoderma virens* or a mycorrhizal fungus. In other embodiments, the microbe is a bacterium and produces bacterial extracellular vesicles, for example, an *Escherichia coli*, *Bacillus subtilis*, or *Pseudomonas putida* bacterium.

The plant or microbe can be engineered by any suitable means to produce one or more effector RNAs. In certain examples, one or more heterologous nucleic acid sequences encoding one or more effector RNAs can be introduced into the desired cell as a separate genetic element, for example through introduction of bacterial plasmids or bacterial artificial chromosomes (into bacteria) or yeast artificial chromosomes (into fungi) that carry the one or more heterologous nucleic acid sequences. In other examples, the one or more heterologous nucleic acid sequences can be introduced into the cell's genome. This can be accomplished by any suitable means known in the art, including use of a CRISPR-Cas genome editing system, ZFNs, TALENs, and/or homologous recombination. Cells containing the one or more heterologous nucleic acid sequences encoding the one or more effector RNAs can express those effector RNAs and, in some instances, load those effector RNAs into the EVs that the cell produces.

In certain embodiments, the cell has been engineered to express one or more effector RNAs that target one or more genes of a pathogen, such as one or more genes of a fungal pathogen. In certain examples, the one or more antifungal RNAs can target one or more of fungal pathogens *B. cinerea* and *Verticillium*.

In certain examples, the one or more targeted genes are selected from the group consisting of: DCL 1 gene, DCL 2 gene, vacuolar protein sorting 51 (VPS51) gene, dynactin (DCTN1) gene, suppressor of actin (SAC1) gene, and the tetraspanin gene Punchless 1 (PLS1). The antifungal RNAs produced by the microbe can be, for example, dsRNA or mRNA. In particular examples, the one or more antifungal RNAs comprise one or more nucleic acid sequences at least about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12.

V. Methods for Producing Extracellular Vesicles

Also provided herein are methods for producing EVs that contain the desired antifungal RNAs. In certain embodiments, the method comprises growing a plurality of engineered cells, such as engineered microbes, wherein the engineered cells comprise and express one or more heterologous nucleic acid sequences encoding one or more effector RNAs, and wherein the engineered cells produce EVs comprising the one or more heterologous effector RNAs; and isolating from the growth media a plurality of EVs. In other embodiments, the method comprises growing a plurality of cells in growth media, wherein the cells produce a plurality of EVs; isolating the plurality of EVs from the growth media; and introducing into the isolated EVs one or more heterologous effector RNAs. In other embodiments, the EVs are produced by genetically engineering a plant to produce the desired RNA-loaded EVs and harvesting/isolating the RNA-loaded EVs from the engineered plant.

In some embodiments one or more effector RNAs are loaded into the EV by the cell that produces the EV, such as an engineered microbe. In such examples, the microbes producing the RNA-loaded EVs can be grown in a suitable growth medium, as would be well known to a person of ordinary skill in the art, and then the RNA-loaded EVs can be isolated from the growth medium and microbes by any suitable means, as would be well known to a person of ordinary skill in the art. Such isolation methods can include, for example, centrifugation methods.

In additional embodiments, the microbe may not load the effector RNA into the EVs. In such instances, the microbial EVs can be isolated from the growth medium and microbes, as discussed above, and then can be loaded with one or more effector RNAs that have been generated from another source. In certain examples, such effector RNAs can be chemically synthesized. In other examples, such effector RNAs can be produced in a separate microbe (bacterium or fungus) as otherwise described above. The microbes producing the EVs and effector RNAs can be of the same or different strain or species, and can even be the same or different types of microbes. For example, the effector RNA can be produced in first bacteria and the EVs produced in a second bacteria; the effector RNA can be produced in a first fungus and the microbial EVs produced in a second fungus, the effector RNA can be produced in a bacteria and the EVs can be produced in a fungus; or the effector RNA can be produced in a fungus and the microbial EVs can be produced in a bacteria.

VI. Methods for Treating or Preventing Fungal Disease in Plants

Also provided herein are methods for treating or preventing disease in plants by applying to plant material or adjacent soil any of the compositions comprising effector RNA as described in the present application. In some embodiments, the target disease will be caused by a fungal pathogen and the effector RNA will comprise an antifungal RNA. In some embodiments, RNA-

5 loaded EVs comprising one or more effector RNAs are applied directly to the plant or the soil adjacent to the plant or its roots. In other embodiments, microbes that produce RNA-loaded EVs are applied directly to the plant or soil.

In certain examples, the RNA-loaded EVs and/or microbes are applied to the plant or one or more portions thereof, such as one or more of the leaves, stem, flowers, roots, and fruit of the

10 plant. In other examples, RNA-loaded EVs and/or microbes are applied to the soil adjacent to the plant or roots, such as the soil that encompasses the plant's roots. In further examples, RNA-loaded EVs and or microbes are applied to both the plant (or parts thereof) and the soil. In particular examples, RNA-loaded EVs are applied to the plant and microbes that produce RNA-

loaded EVs are applied to the adjacent soil.

15 Application of the RNA-loaded EVs and/or microbes can be by any suitable means. In certain embodiments, the RNA-loaded EVs and/or microbes are applied by spraying a liquid containing the RNA-loaded EVs and/or microbes, for example a liquid suspension. Compositions comprising RNA-loaded EVs may be applied to plants manually or in automated fashion. A crop sprayer or other such agricultural application machine may be used. A crop

20 spray may contain a tank carried on a chassis, for trailing behind a tractor or for use as a self-propelled unit having an integral cab and engine. The machine may further include an extending boom which provides a transverse line of uniformly spaced spray nozzles connected by pipes to the tank. During operation the application machine may be moved across fields of crops to apply the RNA-loaded EV composition in a controlled manner.

25 The plant to which the RNA-loaded EVs and/or microbes are applied may be of any type, species, or variety in which control of fungal disease is desired. In some embodiments, the plant is an ornamental plant. In some embodiments, the plant is a fruit- or vegetable-producing plant. In certain examples, the plant may be a species from the genera *Allium*, *Asparagus*, *Atropa*, *Avena*, *Brassica*, *Citrus*, *Citrullus*, *Capsicum*, *Cucumis*, *Cucurbita*, *Daucus*, *Fragaria*, *Glycine*,

30 *Gossypium*, *Helianthus*, *Heterocallis*, *Hordeum*, *Hyoscyamus*, *Lactuca*, *Linum*, *Lolium*, *Lycopersicon*, *Malus*, *Manihot*, *Majorana*, *Medicago*, *Nicotiana*, *Oryza*, *Panicum*, *Pannisetum*,

Persea, *Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Rosa*, *Secale*, *Senecio*, *Sinapis*, *Solanum*, *Solanaceae*, *Sorghum*, *Trigonella*, *Triticum*, *Vitis*, *Vigna*, and *Zea*. In some embodiments, the plant is a vining plant, *e.g.*, a species from the genus *Vitis*. In some embodiments, the plant is an ornamental plant, *e.g.*, a species from the genus *Rosa*. In some embodiments, the plant is a monocot. In some embodiments, the plant is a dicot.

VII. Examples:

Example 1 - Bacteria can be engineered to express and deliver RNA and extracellular vesicles to fungal pathogens.

We demonstrated the feasibility of using engineered microbes to deliver antifungal RNAs in microbial EVs using three model bacterial species: *Escherichia coli*, a gram-negative bacterium, *Bacillus subtilis*, a gram-positive (plant-beneficial) bacterium, and *Pseudomonas putida*, a gram-negative (plant-beneficial) bacterium. Specifically, we genetically engineered *Escherichia coli* strain HT115, *Bacillus subtilis* strain BG322, and *Pseudomonas putida* strain CMA702 to produce and deliver the two fungal pathogen targeting RNA constructs that target that DCL1/2 and VDS genes of the target fungi. All chosen bacterial strains exhibit reduced RNaseIII activity, which promotes the production of inhibitory dsRNA molecules (for example, DCL1/2 and VDS). These bacterial strains also have been previously shown to produce bacterial extracellular vesicles (EVs).

Two dsRNA constructs targeting the DCL1/2 and VDS genes of *Botrytis cinerea* were designed for bacterial expression through modification of the T777T plasmid (HT115 expression), pDG148-Stu plasmid (HT115 and BG322 expression), and pJOE7771.1 plasmid (CMA702 expression). A representative schematic of one of the dsRNA overexpression plasmids (pDG148) is shown in **FIG. 1A**. Examples of heterologous nucleic acids useful for RNAi targeting of DCL1/2 in *Botrytis cinerea* are set forth in SEQ ID NOS: 2 and 4.

The effect of overexpressing the dsRNA constructs targeting DCL1/2 genes has on the bacterial growth of *B. subtilis* was evaluated by measuring the optical density at 600 nm in solution over 8 hours. **FIG. 1B** shows the growth curve of the BG322 strain of *B. subtilis* containing either an empty plasmid (BG322), plasmids expressing the DCL1/2 construct (DCL and DCL_const.), or a plasmid expressing dsYFP (YFP) (yellow fluorescent protein). No significant growth effect is observed between the DCL1/2 construct and BG322 strains.

CLSM analysis of *B. cinerea* spores after incubation with *B. subtilis* expressing OpuAC-YFP (EV marker) was conducted to determine whether fungal pathogens can take up the bacterial EVs produced by these microbes, such as the BG322 strain of *B. subtilis*. The results demonstrated a strong uptake by spores of *Botrytis cinerea*, as determined by detection of the fluorescent signal of the YFP-labeled bacterial EVs.

CLSM analysis of *B. cinerea* spores after incubation with *B. subtilis* expressing mRNA-3WJ (fluorescent RNA aptamer reporter) was conducted. (Figure 1D). A fluorescent signal was detected indicating that *B. cinerea* can take up mRNA-3WJ from *B. subtilis*, which demonstrates mRNA can be trafficked cross-kingdom from bacteria to a fungal pathogen.

Example 2 - Bacterial extracellular vesicles (BEVs) can be isolated and used to deliver RNA to fungal pathogens.

Bacterial EVs (BEVs) were isolated from *B. subtilis* cultures and characterized using nanoparticle tracking analysis. The concentration (particles/ml) was measured versus the size (nm) of the BEVs. (FIG. 2A). The BEVs were imaged using transmission electron microscopy. (FIG. 2B). The nanoparticle tracking analysis and the imaging from the transmission electron microscopy revealed that the BEVs are exosome-like in nature and similar in size and morphology to plant extracellular vesicles.

Whether the BEVs containing the desired dsRNA construct targeting pathogen virulence-related genes produced by *Escherichia coli* (HT115), *Bacillus subtilis* (BG322), and *Pseudomonas putida* (CMA702) can be taken up by *B. cinerea* was assessed using fluorescent labeling. BEVs were labeled with YFP and added to *B. cinerea* germlings and pictures were taken using confocal laser microscopy after three hours of incubation. Fluorescence signals were visible in the cells indicating the BEVs were taken up by *B. cinerea*.

The *B. cinerea* cells were then treated with Triton X-100 to disrupt EVs not taken up by the fungal cells. RT-PCR was performed to confirm that the dsRNA modified to target fungal pathogen genes was present in the BEVs that were taken up by the *B. cinerea*, which confirmed the presence of the dsRNA. (FIG. 2C).

This demonstrates that the different microbial strains tested all produce extracellular vesicles (BEVs) containing the dsRNA and can secrete these RNA-loaded vesicles to deliver dsRNA to fungal pathogens. We have found that BEVs can be produced from all three bacterial

strains mentioned above and that these BEVs can be taken up by *B. cinerea* and contain the desired dsRNA construct targeting pathogen virulence-related genes.

Example 3 – Plant mRNAs can be found in EVs, taken up by fungal pathogens, and translated in fungal pathogens.

To determine the feasibility of using mRNAs as effector RNAs in RNA-loaded EVs, we examined whether plant EVs can contain mRNAs and, if so, whether those mRNAs can be taken up and translated within fungal pathogens.

Plant EVs carry mRNAs

To investigate whether plant mRNAs are associated with EVs during natural fungal infection, we conducted mRNA profiling analysis on purified plant EVs (P100 fraction: ultracentrifugation at 100,000 x g) from leaf apoplastic wash fluid collected early (16 h) in the interaction between *Botrytis cinerea* and *Arabidopsis* leaves and from uninfected leaves (mock) as described in Huang *et al* (26). The time point of 16 h post *B. cinerea* infection (hpi) is recognized to be during the early biotrophic phase of infection before any host cell death occurs (27). The quality of the apoplastic wash fluids (AWF) from infected plant leaves was evaluated before 100,000 x g ultracentrifugation by western blot analysis to measure the potential contamination of chloroplasts, mitochondria, and their fragments from cell leakage and death.

The chloroplast membrane protein Tic40 and mitochondrial inner membrane protein Tim17 were not detected in the AWF fraction. The quality of EVs was further monitored by Transmission Electron Microscopy (TEM) and nanoparticle tracking analysis.

Using 100 normalized Reads Per Kilobase of transcript per Million mapped reads (RPKM) in each biological repeat as a cutoff, a total of 567 *Arabidopsis* transcripts were identified in the EV samples from 16 hpi samples (EV_infected) and nearly 30% of them were induced after infection as compared with the EVs isolated from uninfected leaves (**FIGS. 3 and 4**). Gene ontology (GO) analysis revealed that 228 out of the 567 (40.2%) EV-associated *Arabidopsis* mRNAs encode genes associated with biotic stress or defense responses with clear enrichment of genes involved in detoxification, response to reactive oxygen species, defense response to fungus, hormone metabolic process, secondary metabolic process and immune response, etc. (**FIG. 3**), whereas biotic stress or defense responses associated genes only

represent 11% of total genes in the *Arabidopsis* genome (30). Notably, the protein products of 167 EV-associated mRNAs (29% of 567 genes) could be found in mitochondrial proteomes (**FIG. 4**) according to the SUBcellular location database for *Arabidopsis* proteins (SUBA4, <http://suba.live>). This represents an almost 4-fold enrichment compared to the percent of mitochondria-localized protein genes in the entire *Arabidopsis* genome (7.4%) (31). RNA-Seq analysis on total mRNAs from *Botrytis*-infected *Arabidopsis* leaves was performed for comparative analysis (data not shown). The profiles of EV-associated mRNAs were distinct from the total mRNA profiles; for example, considering the 100 most abundant *Arabidopsis* mRNAs in each dataset in the libraries generated from infected plants, only 33 were shared (data not shown). This suggests that transcript abundance in leaf cells does not directly explain transcript abundance in EVs.

We experimentally validated a selection of EV-mRNA candidates of various lengths that have potential roles in plant defense or stress responses, in addition to their developmental roles of some genes. The full-length mRNA transcripts (open reading frame) of 15 candidates were detected in the purified EV P100 fraction (**FIG. 5A**). Transcripts abundant in total mRNA and absent in the EV dataset, Outer Envelope Protein 6 (OEP6), General Regulatory Factor 10 (GRF10), and Profilin 5 (PRO5) were used as negative controls (**FIG. 5A**). We chose four *Arabidopsis* transcripts for further characterization from the EV dataset that are induced during infection and, in addition to their known functions in uninfected plants, could thus play a role in plant immunity: Senescence-associated gene 21 (SAG21) (32), ATP sulfurylase 1 (APS1) (33,34), Peroxiredoxin IIC (PRXIIC) (35) and Hevein-like (HEL) (36). The full-length transcripts of these genes were still detected in purified EVs after micrococcal nuclease and proteinase K digestion unless the vesicles were first ruptured with Triton X-100 (**FIG. 5B**), demonstrating that these mRNAs are indeed contained within the vesicles rather than bound to the outer surface or associated with independent protein aggregates.

Plants produce different classes of EVs based on their biogenesis pathways and specific protein markers (37,38). We previously showed that Tetraspanin (TET)-positive EVs (considered as plant exosomes), especially TET8- and TET9-positive EVs, are mainly responsible for sRNA transport from plants to fungal pathogens. The *tet8* mutant shows fewer EVs under TEM (10,39), and has impaired immune responses against fungal infection (10). To determine whether plant mRNAs are transported by TET8-positive EVs, we examined the levels

of selected EV-mRNAs in immuno-captured TET8-positive exosome fractions purified using a TET8-specific antibody (15,26). Specificity of the immuno-isolation was verified using an independent control antibody (IgG). Full-length transcripts of SAG21, APS1, PRXIIC and HEL were detected in the immuno-captured TET8-exosome fractions, but not in the IgG control (**FIG. 5C**). Moreover, the transcripts of these genes were barely detectable in EVs from the tet8/tet9 double (tet8 knockout, tet9 knockdown) mutant (10) compared to EVs prepared from wild type *Arabidopsis* (**FIG. 5C**). Together, these results confirm that plant exosomes also carry mRNAs in addition to sRNAs.

10 Plant mRNAs are observed in EVs

To visualize the mRNAs in host EVs, we applied an improved RNA reporter system using a fluorescent RNA aptamer, Three-Way Junction-4 x Broccoli (3WJ-4xBro), which was optimized for RNA imaging in plant cells (40,41). Here, we tagged full-length SAG21, APS1, PRXIIC and HEL transcripts with 3WJ-4xBro aptamer, which allowed the tagged mRNA transcripts to be directly observed in TET8-positive EVs when co-expressed with TET8-mCherry in *N. benthamiana* cells, whereas the negative control OEP6-3WJ-4xBro was not detectable in EVs (**FIG. 6A and 6B**). The full-length SAG21-, APS1-, PRXIIC- and HEL-3WJ-4xBro fusion transcripts were also detected in purified EVs from *N. benthamiana* (data not shown).

For further in-depth functional analysis, we selected two genes, SAG21 and APS1. Both SAG21 and APS1 can be targeted to mitochondria (31), and APS1 possesses a dual-targeting signal directing it to both mitochondria and chloroplasts (42). SAG21 is induced by the infection of *B. cinerea* and numerous bacterial, fungal, and oomycete pathogens, pathogen elicitors, oxidative stress, and by plant defense hormones salicylic acid, methyl jasmonate, and ethylene (32,43). Transgenic plants overexpressing SAG21 exhibit less susceptibility to the infection of *B. cinerea* and bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (32). APS1, which is also induced by *B. cinerea*, oomycete pathogen *Phytophthora infestans*, and oxidative stress (43). APS1 participates in the biosynthesis of essential metabolites, including glucosinolates, which are toxic to fungal cells (33,34,44). However, there is no direct evidence that APS1 participates in defense response to *B. cinerea* infection. We generated stable transgenic *Arabidopsis* plants expressing full-length SAG21-3WJ-4xBro, APS1-3WJ-4xBro, or control OEP6-3WJ-4xBro, and these transgenic lines did not show any obvious developmental

difference from the wild type. Quantitative RT-PCR and confocal microscopy revealed that all fusion transcripts were expressed and detected in plant cells. As expected, only SAG21-3WJ-4xBro and APS1-3WJ-4xBro transcripts, but not OEP6-3WJ-4xBro, were observed in the purified EV P100 fraction from uninfected plants (Figure 2D-F). The full-length transcripts of tagged SAG21 and APS1 were also detected (**FIG. 6C and 6D**). These findings provide direct evidence that plant EVs can carry specific mRNAs.

Plant mRNAs are transported into fungal cells

To determine whether these EV-associated plant mRNAs can be delivered into interacting fungal cells during infection, we isolated pure *B. cinerea* cells from infected *Arabidopsis* leaves using a sequential protoplasting strategy (10,45). Cultured *B. cinerea* mixed with uninfected leaves was subjected to the same procedure as a negative control to exclude potential contamination during the experimental procedure. The full-length EV-associated plant mRNAs (SAG2, APS1, PRXIIC and HEL) were detected in *B. cinerea* cells isolated from infected leaves (**FIG. 7A**), indicating that these mRNAs are taken up by fungal cells. In contrast, transcripts not associated with EVs (OEP6, PRO5, GRF10) were not detected in fungal cells, as anticipated (**FIG. 7A**). None of the plant transcripts that were detected in fungal cells after infection were detected in the negative control of cultured *B. cinerea* cells mixed with uninfected *Arabidopsis* leaves right before the fungal cell isolation (**FIG. 7A**). This result shows that full-length EV-associated plant mRNAs are indeed transferred into fungal cells during infection. We also examined the levels of SAG21, APS1, PRXIIC and HEL in *B. cinerea* cells isolated from infected tet8/tet9 leaves. Significantly less EV-associated transcripts were detected in *B. cinerea* cells isolated from tet8/tet9 than Col-0 (**FIG. 7A**), indicating that plant exosomes play an important role in the plant mRNAs becoming associated with fungal cells.

To further test the involvement of plant EVs in cross-kingdom mRNA trafficking into fungal cells, *Arabidopsis* EVs were isolated from the transgenic lines expressing 3WJ-4xBro tagged SAG21, APS1 or OEP6, and incubated with in vitro cultured *B. cinerea* conidia for 4 hours (h). After incubation, the full-length transcripts of SAG21-3WJ-4xBro and APS1-3WJ-4xBro, but not OEP6-3WJ-4xBro, were detected in purified *B. cinerea* cells (**FIG. 7B**). The fluorescence of SAG21-3WJ-4xBro and APS1-3WJ-4xBro transcripts, but not OEP6-3WJ-4xBro, was observed in fungal hyphae after incubation with EVs prepared from uninfected

transgenic plants expressing 3WJ-4xBro-tagged transcripts (**FIG. 7C**). These results support our finding that plant mRNAs are transported by EVs into fungal cells.

To test whether we can also observe plant mRNA transcripts inside fungal cells during natural infection, *B. cinerea* cells were isolated from infected 3WJ-4xBro-tagged transgenic *Arabidopsis*. As expected, we found fungal cells showing fluorescence of SAG21-3WJ-4xBro or APS1-3WJ-4xBro and detected the full-length tagged transcripts of SAG21 and APS1 in fungal cells (**FIG. 7D and E**). The tagged OEP6 transcript from the negative control OEP6-3WJ-4xBro was not detected (**FIG. 7D and E**). These results confirmed that plant mRNAs are indeed entering fungal cells during natural infection.

Plant mRNAs can be translated in fungal cells

As most mRNAs are translated into proteins to perform biological functions, we asked whether these transferred plant mRNAs can be translated into proteins in fungal cells. We adopted the Translating Ribosome Affinity Purification followed by RNA-seq (TRAP-seq) method, which is an effective way to identify actively translated mRNAs (46,47). We generated a *B. cinerea* transformant strain expressing Yellow Fluorescent Protein (YFP)-tagged *B. cinerea* Ribosome Protein Large subunit 23 (BcRPL23-YFP), a subunit presents at the surface of the Ribosome complex (48). This strain exhibits similar growth and infection phenotype as the wild type strain, and allows pulling down all the mRNAs associated with fungal ribosomes. TRAP-seq analysis was performed to isolate and sequence *B. cinerea* ribosome-associated mRNAs from BcRPL23-YFP cells during *Arabidopsis* infection (TRAP_infected). Cultured hyphae of the (*B. cinerea*) transformant mixed with uninfected *Arabidopsis* Col-0 leaves was used as a control (TRAP_mix) to exclude potential contamination during the experimental procedure. A total of 320 plant protein-coding mRNAs were associated with fungal ribosomes in all three biological replicates with at least 50 RPKM (considering only reads mapping to *Arabidopsis*) and >3-fold change (TRAP_infected /mix) as a cutoff. Consistent with the proposed mechanism of EV-mediated transfer, 63% (201) of 320 TRAP-associated genes overlapped with the mRNAs identified in EVs. GO analysis revealed that defense response-related genes were highly enriched in the Botrytis BcRPL23 TRAP gene list. Strikingly, 128 out of the 201 genes (64%) present in both TRAP- and EV-associated gene lists are biotic stress- or defense response-related. Again, 72 of the TRAP-associated genes (23%) encode proteins that are present in mitochondrial

proteomes (31), 48 of them (67%) overlap with transcripts encoding mitochondria-targeted proteins in EVs. It is worth noting that abundant plastid genome-derived mRNAs and nuclear photosynthesis-associated mRNAs are not enriched in the TRAP dataset, indicating that there is no significant contamination with abundant host cellular transcripts. The full-length transcripts of plant EV-associated mRNAs SAG21, APS1, PRXIIC and HEL were detected by RT-PCR after BcRPL23-YFP pull-down from infected samples but not from the control samples in which in vitro cultured *B. cinerea* hyphae were mixed with uninfected *Arabidopsis* (**FIG. 8A**). This is consistent with the hypothesis that these plant mRNAs can be transferred to Botrytis and become associated with fungal ribosomes.

It remained a possibility that mRNAs were bound to mono-ribosomes and are not actively being translated into proteins. To investigate whether mRNAs were associated with actively translating polysomes, *B. cinerea* cells were isolated from infected *Arabidopsis* leaves, and the cell extracts were then fractionated by sucrose gradient centrifugation to separate the polysomes from monosomes and ribosomal subunits. We focused on SAG21 and APS1, and RT-PCR results showed that these transcripts were highly enriched in fractions containing polysomes, consistent with the *B. cinerea* endogenous control transcript Bc_Actin (**FIG. 8B**). To further confirm that SAG21 and APS1 accumulated in actively translating polysomes, the fungal cell lysates were treated with the translation inhibitor Puromycin, which can specifically disrupt polysomes (49,50), before being loaded onto sucrose gradients. Like Bc-Actin, both SAG21 and APS1 plant mRNAs shifted to the more slowly sedimenting monosomal fractions of the gradient (**FIG. 8B**). This result indicates that transferred plant mRNAs are associated with actively translating fungal polysomes, and thus are translated into proteins within fungal cells.

To determine whether we can detect the proteins translated from the transferred mRNAs in the fungal cells, we generated *Arabidopsis* transgenic plants expressing SAG21-YFP, APS1-YFP or mutated (m)SAG21-YFP, mAPS1-YFP which could not be translated. A premature stop codon was introduced by a single nucleotide insertion (for mSAG21) or replacement (for mAPS1) to minimize the change in the mRNA secondary or tertiary structures, which could be required for EV-loading and trafficking. Initially, we tested whether the SAG21-YFP and APS1-YFP proteins are secreted. Immunoblotting showed that both fusion proteins were not detectable in the apoplastic fluid, the P100 EV fraction, or the supernatant of the P100 fraction (**FIG. 8C**). Moreover, SAG21-YFP or APS1-YFP fusion proteins were not detected in TET8-positive EVs

when co-expressed with TET8-mCherry in *N. benthamiana* cells, whereas the positive control Annexin 1 (ANN1)-YFP fusion was detectable in EVs. This result demonstrates that the SAG21-YFP and APS1-YFP fusion proteins are not secreted or exported via EVs. Therefore, the presence of SAG21-YFP and APS1-YFP in fungal cells is due to translation of plant mRNAs by
5 fungal ribosomes.

To determine that transferred plant mRNAs are indeed translated in fungal cells, we isolated EVs from uninfected *Arabidopsis* transgenic lines expressing SAG21-YFP, APS1-YFP. The purified plant EVs, which did not show any detectable fusion proteins, were incubated with cultured *B. cinerea* conidia for 24 h. While all the transcripts of SAG21-YFP, APS1-YFP,
10 mSAG21-YFP and mAPS1-YFP were detected in the fungal cells after incubation with isolated EVs for 24 h (**FIG. 8D**), only the fluorescent proteins SAG21-YFP and APS1-YFP from corresponding transgenic *Arabidopsis* lines, but not the mutated versions, were observed in fungal cells incubated with EVs after 24 h (**FIG. 8E and 8F**). This provides strong evidence that these proteins were translated from the transferred mRNAs in the fungal cells.

Plant mRNAs in fungal cells could reduce infection

To assess if transferred plant mRNAs could act in fungal cells to reduce infection, we generated *B. cinerea* transformants that ectopically express SAG21-YFP, APS1-YFP, or mSAG21-YFP, mAPS1-YFP or free YFP as controls. Both SAG21-YFP and APS1-YFP strains
20 displayed reduced fungal growth as compared with mSAG21-YFP, mAPS1-YFP or free YFP strains (**FIG. 9A and 9B**). Both transcripts and proteins were detected in the corresponding SAG21-YFP and APS1-YFP strains, but only the transcript, not the protein, could be detected in the corresponding mSAG21-YFP or mAPS1-YFP strains (not shown). The transformants expressing SAG21-YFP or APS1-YFP showed significantly reduced infection on *Arabidopsis*
25 compared to the control free YFP strain, whereas the mSAG21-YFP or mAPS1-YFP strain did not show significant differences in terms of infection compared with the free YFP strain (**FIG. 9A and 9B**). These results confirm that these transferred mRNAs can limit pathogen infection following translation into proteins. Furthermore, both SAG21-YFP and APS1-YFP proteins partially localized to *Botrytis* mitochondria and resulted in similar morphological changes, with
30 enlarged separated mitochondria and disrupted mitochondrial network (not shown). The free YFP control localized in the cytoplasm and did not alter mitochondrial morphology or network.

The morphology change of the mitochondria likely disrupts mitochondrial function and perturbs the subcellular network formed between fungal mitochondria.

Finally, we also identified T-DNA insertion knock-out *Arabidopsis* mutants for SAG21 and APS1 and subjected these to *B. cinerea* infection. The mutant lines showed increased susceptibility to *B. cinerea* infection compared to wild-type plants (**FIG. 9C and 9D**), whereas no significant difference was observed between the complemented transgenic lines and wild-type plants (**FIG. 9C and 9D**). These data suggest that SAG21 and APS1 contribute to reduced plant susceptibility to fungal infection.

Collectively, these studies demonstrated to the inventors that mRNAs could have possible use as effector RNAs in RNA-loaded EVs.

Example 4 - Bacterial extracellular vesicles (BEVs) can be loaded with different RNA species, including mRNAs.

Tests were done to demonstrate how BEVs can be loaded with different RNA species, including mRNAs, for delivery to plant pathogens. BEVs were isolated from BG322-VDS-3wj *B. subtilis* cultures and visualized using confocal scanning laser microscopy. BEVs were also stained with FM4-64, which stains membrane-derived vesicles. Labeling of the VDS RNA with a fluorophore demonstrated colocalization. Colocalization was not observed in the BEVs isolated from bacterial strains that did not produce the labeled VDS RNA.

The ability of BEVs to encapsulate and protect VDS RNA was also demonstrated by subjecting BEVs containing VDS-3wj to enzymatic treatment and performing RT-PCR. The presence of the band corresponding to VDS-3wj after nuclease and triton treatment supports the role of BEVs in protecting mRNA cargo (**FIG. 10A**).

To further confirm that the RNA was loaded into the BEVs, BEVs isolated from BG322-VDS-3wj cultures were purified using density gradient ultracentrifugation and RT-PCR was performed on each fraction. A strong band corresponding to VDS-3wj was present in the density fractions 1.13-1.21 g/cm³. These fractions correlate with the fractions that were enriched in BEVs, demonstrating that the RNA is loaded into the BEVs (**FIG. 10B**).

Together, these results demonstrate how BEVs can be loaded with mRNAs in addition to dsRNAs for plant protection against fungal pathogens.

Example 5 – RNA-Loaded EVs Can Provide Protection Against Fungal Pathogens and Silence Fungal Virulence-Related Genes.

Plant material (*Arabidopsis thaliana* leaves) was treated with BEVs isolated from *B. subtilis* (gram-positive) or *E. coli* (gram-negative) overexpressing the DCL1/2 or VDS constructs to demonstrate how isolated BEVs containing the dsRNA of interest can be used directly to provide protection against fungal pathogens. Lesion size area produced by *B. cinerea* after 3 dpi was reduced in the plants that were treated by BEVs isolated from HT115-modified strains of *E. coli*, wherein the bacteria was able to produce dsRNA against *B. cinerea* target genes, DCL1/2 and VDS. Plants treated with just BEVs (mock treatment) did not exhibit reduced infection, indicating that DCL1/2 or VDS delivery is necessary for pathogen inhibition (**FIG. 11A**). Lesion size area produced by *B. cinerea* after 3 dpi was reduced in the plants that were treated by BEVs isolated from BG322-modified strains of *B. subtilis*, wherein the bacteria was able to produce dsRNA against *B. cinerea* target genes, DCL1/2 and VDS. Plants treated with just BEVs (mock treatment) did not exhibit reduced infection, indicating that DCL1/2 or VDS delivery is necessary for pathogen inhibition (**FIG. 11B**). These results are confirmed by a clear reduction in target gene expression (approximately 50-70% reduction in relative gene expression is observed for *Bc-DCL1*, *Bc-DCL2*, *Bc-VPS51*, *Bc-DCTN1*, and *Bc-SAC1*) after 12 hours of co-incubation with BEVs isolated from BG322-modified bacteria producing dsRNA targeting BcDCL1/2 or BcVDS, respectively as compared to the mock controls (BcYFP) (**FIG. 11C**).

Example 6 - Application of Microbes Producing RNA-Loaded EVs Provides Protection Against the Root Pathogen, *Verticillium dahliae*

The ability of these engineered bacteria to provide protection against soilborne pathogens including *Verticillium dahliae* was also analyzed using the model plant *A. thaliana* and the agriculturally-relevant crop, tomato. Prior to *V. dahliae* inoculation, the soil was treated with a solution of engineered bacteria 2 hours before the plants were transplanted and again 7 days after the inoculation. The relative fungal biomass was then calculated using the expression of actin genes in *V. dahliae* and *A. thaliana* or tomato 21 days post inoculation. Overhead pictures of the bacteria-treated *A. thaliana* plants were also taken. Application of bacteria that are expressing the dsRNA construct, VdDCL1/2, resulted in reduced fungal biomass compared to the unmodified bacterial and mock treatment controls (**FIG. 12A**).

Similar results were observed regarding using engineered bacteria for tomato protection against *V. dahliae*. The canopy area of inoculated tomato plants in bacterial treated soil at 21 dpi was measured and overhead pictures were taken. Treatment of the soil with the bacteria expressing the dsRNA construct, VdDCL1/2, resulted in greater canopy sizes compared to the unmodified and mock treatment bacterial controls (**FIG. 12B**).

Relative gene expression of *Vd-DCL1* and *Vd-DCL2* in *V. dahliae* after 6, 12, and 24 hrs of co-incubation with *B. subtilis* modified to produce a DCL1/2 dsRNA construct was measured. (**FIG. 13C**). The results show that the RNAi effect from the dsRNA-producing *B. subtilis* increased over time, as evidenced by the significant and maintained decrease in fungal gene expression after 6, 12, and 24 hours of co-incubation (**FIG. 12C**). These results demonstrate that engineered microbes can be used to combat soil-borne pathogens like *V. dahliae* even 21 days post infection.

Example 7 - Spray Application of Microbes Producing RNA-Loaded EVs Provides Protection Against *B. cinerea*.

The dsRNA-producing bacteria was directly applied to *Arabidopsis thaliana* leaves to analyze the protection provided against *B. cinerea*. *A. thaliana* leaves were pre-treated by spray with HT115, a strain of *E. coli*, (**FIG. 13A**) or BG322, a strain of *B. subtilis* (**FIG. 13B**), bacterial suspensions, then inoculated with *B. cinerea* spores. Lesion size areas were measured with the help of a digital calibrator at 3 dpi. Treatment with HT115_BcDCL1/2 as well as BG322_BcDCL1/2, bacteria with dsRNA targeting DCL1/2 genes in *B. cinerea*, showed a statistical reduction of lesion size area as determined by a one-way ANOVA followed by Tukey's HSD test (** $p < 0.01$) (**FIG 13A and 13B**). Representative pictures of fungal lesions on *A. thaliana* leaves at 3 days post inoculation were taken (**FIG 13A and 13B**). This corroborated with relative gene expression data of *Bc-DCL1* and *Bc-DCL2* in *V. dahliae* after 12, 24, and 36 hrs of co-incubation with *B. subtilis* modified to produce a DCL1/2 dsRNA construct was measured (not shown). The results show that the RNAi effect from the dsRNA-producing *B. subtilis* increased over time, as evidenced by the significant and maintained decrease in fungal gene expression after 12, 24, and 36 hours of co-incubation.

Example 8 – Applicability to other microbial species

Although the above examples primarily showcase the results from one Gram-positive model bacterial species, *B. subtilis*, we have also obtained similar results using two other Gram-negative model species, *E. coli* and *P. putida*. These results demonstrate how this technology can be easily extended to plant-growth promoting bacterial species such as *Bacillus spp.* including *B. licheniformis*, *B. cereus*, and *B. pumilus*, *Burkholderia cepacia*, *Pseudomonas spp.* such as *P. chlororaphis* and *P. fluorescens*, *Serratia spp.* such as *S. marcescens* and *S. plymuthica*, *Streptomyces spp.* such as *S. lividans* and *S. lydicus*, and various *Rhizobia spp.* that have already been accepted as commercial biofertilizers. This technology can also be applied to plant-beneficial fungal species that are known biocontrol agents or other plant-colonizing fungal species. As such, this would offer a dual approach of controlling fungal infection through dsRNA delivery and the inherent biocontrol pathways of species such as *Trichoderma virens*.

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CLAIMS

What is claimed is:

1. A composition comprising a microbial extracellular vesicle (EV) isolated from a microbe, said microbial EV containing one or more antifungal RNAs, wherein said one or more antifungal RNAs are heterologous to the microbe.
2. The composition of claim 1, wherein the one or more antifungal RNAs comprise a double-stranded RNA or mRNA.
3. The composition of claim 1 or 2, wherein the one or more antifungal RNAs target one or more genes of a fungal pathogen, wherein the one or more targeted genes are selected from the group consisting of: DCL 1 gene, DCL 2 gene, vacuolar protein sorting 51 (VPS51) gene, dynactin (DCTN1) gene, suppressor of actin (SAC1) gene, and the tetraspanin gene Punctless 1 (PLS1).
4. The composition of claim 3, wherein the one or more antifungal RNAs comprise one or more nucleic acid sequences at least about 75%, about 80%, about 85%, about 86%, about 87%, about 88%, about 89%, 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12.
5. The composition of any one of claims 1-4, wherein the microbe is a bacterium, optionally selected from the group consisting of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*.
6. The composition of any one of claims 1-4, wherein the microbe is a fungus, optionally selected from the group consisting of *Trichoderma virens* or a mycorrhizal fungus.
7. The composition of claim 5, wherein the microbial EV is a bacterial EV produced by the bacterium and the one or more antifungal RNAs are generated by the bacterium.

8. The composition of claim 7, wherein the one or more antifungal RNAs are derived from the introduction into the bacterium of a bacterial plasmid or bacterial artificial chromosome comprising one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs.

9. The composition of claim 8, wherein the bacterial plasmid and bacterium are selected from the group consisting of:

- a. a genetically modified T777T plasmid introduced into HT115 strain of *Escherichia coli*;
- b. a genetically modified pDG148-Stu plasmid introduced into BG322 strain of *Bacillus subtilis*; and
- c. a genetically modified pJOE7771.1 plasmid introduced into CMA702 strain of *Pseudomonas putida*.

10. The composition of claim 7, wherein the one or more antifungal RNAs are derived from one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs that have been inserted into the genome of the bacterium.

11. The composition of claim 10, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs are inserted into the genome of the bacterium using CRISPR/Cas9 genome-editing.

12. The composition of claim 10, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs are inserted into the genome of the bacterium through homologous recombination.

13. The composition of claim 6, wherein the microbial EV is a fungal EV produced by the fungus and the one or more antifungal RNAs are generated by the fungus.

14. The composition of claim 13, wherein the antifungal RNA is derived from the introduction into the fungus of a yeast artificial chromosome comprising one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs.
15. The composition of claim 13, wherein the one or more antifungal RNAs are derived from one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs that have been inserted into the genome of the fungus.
16. The composition of claim 15, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs are inserted into the genome of the fungus using CRISPR/Cas9 genome-editing.
17. The composition of claim 15, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs are inserted into the genome of the fungus through homologous recombination.
18. The composition of any one of claims 1-17, wherein the one or more antifungal RNAs target one or more fungal pathogens selected from the group consisting of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus niger* as well as potential oomycete pathogens, such as *Phytophthora infestans*.
19. A composition comprising a microbial extracellular vesicle (EV) containing one or more antifungal RNAs, wherein the microbial EV was produced by a first microbe and then isolated from said first microbe, wherein the one or more antifungal RNAs were chemically synthesized and then isolated or produced by a second microbe and then isolated from said second microbe, and wherein the isolated one or more antifungal RNAs were then loaded into the isolated microbial EV.
20. The composition of claim 19, wherein the one or more antifungal RNAs comprise a double-stranded RNA or mRNA.

21. The composition of claim 19 or 20, wherein the one or more antifungal RNAs target one or more genes of a fungal pathogen, wherein the one or more targeted genes are selected from the group consisting of: DCL 1 gene, DCL 2 gene, vacuolar protein sorting 51 (VPS51) gene, dynactin (DCTN1) gene, suppressor of actin (SAC1) gene, and the tetraspanin gene Punchless 1 (PLS1).

22. The composition of claim 21, wherein the one or more antifungal RNAs comprise one or more nucleic acid sequences at least about 75%, about 80%, 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 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12.

23. The composition of any one of claims 19-22, wherein the first microbe is a bacterium and the microbial EV is a bacterial EV, optionally wherein the bacterium is selected from the group consisting of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*.

24. The composition of any one of claims 19-22, wherein the first microbe is a fungus and the microbial extracellular vesicle is a fungal EV, optionally wherein the fungus is selected from the group consisting of *Trichoderma virens* or a mycorrhizal fungus.

25. The composition of any of claims 19-24, wherein the one or more antifungal RNAs are derived from the introduction into the second microbe of a plasmid or artificial chromosome comprising one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs.

26. The composition of claim 25, wherein the second microbe is a bacterium and the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs are introduced as a bacterial plasmid.

27. The composition of claim 26, wherein the bacterial plasmid and bacterium are selected from the group consisting of:

- a. a genetically modified T777T plasmid introduced into HT115 strain of *Escherichia coli*;
- b. a genetically modified pDG148-Stu plasmid introduced into BG322 strain of *Bacillus subtilis*; and
- c. a genetically modified pJOE7771.1 plasmid introduced into CMA702 strain of *Pseudomonas putida*.

28. The composition of claim 25, wherein the one or more antifungal RNAs are derived from one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs that have been inserted into the genome of the second microbe.

29. The composition of claim 28, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs are inserted into the genome of the second microbe using CRISPR/Cas9 genome-editing.

30. The composition of claim 28, wherein the heterologous nucleic acid sequence encoding the antifungal RNA is inserted into the genome of the second microbe through homologous recombination.

31. The composition of any one of claims 19-22, wherein the first microbe is a first bacterium, the microbial EV is a bacterial EV, and the bacterial EV is isolated from the first bacterium; wherein the second microbe is a second bacterium and the one or more antifungal RNAs are isolated from the second bacterium; and wherein the isolated one or more antifungal RNAs are loaded into the bacterial EV.

32. The composition of any one of claims 19-22, wherein the first microbe is a bacterium, the microbial EV is a bacterial EV, and the bacterial EV is isolated from the bacterium; wherein the second microbe is a fungus and the one or more antifungal RNAs are isolated from the fungus; and wherein the isolated one or more antifungal RNAs are loaded into the bacterial EV.

33. The composition of any one of claims 19-22, wherein the first microbe is a fungus, the microbial EV is a fungal EV, and the fungal EV is isolated from the fungus; wherein the second microbe is a bacterium and the one or more antifungal RNAs are isolated from the bacterium; and wherein the isolated one or more antifungal RNAs are loaded into the fungal EV.

34. The composition of any one of claims 19-22, wherein the first microbe is a first fungus, the microbial EV is a fungal EV, and the fungal EV is isolated from the first fungus; wherein the second microbe is a second fungus and the one or more antifungal RNAs are isolated from the second fungus; and wherein the isolated one or more antifungal RNAs are loaded into the fungal EV.

35. The composition of any one of claims 19-22, wherein the antifungal RNA is chemically synthesized.

36. The composition of any one of claims 19-35, wherein the one or more antifungal RNAs target one or more of fungal pathogens selected from the group consisting of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus niger* as well as potential oomycete pathogens, such as *Phytophthora infestans*.

37. A microbe for production of extracellular vesicles (EVs) containing one or more antifungal RNAs, said microbe comprising and expressing one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs.

38. The microbe of claim 37, wherein the one or more antifungal RNAs comprise a double-stranded RNA or mRNA.

39. The microbe of claim 37 or 38, wherein the one or more antifungal RNAs target one or more genes of a fungal pathogen, wherein the one or more targeted genes are selected from the group consisting of: DCL 1 gene, DCL 2 gene, vacuolar protein sorting 51 (VPS51) gene, dynactin (DCTN1) gene, or suppressor of actin (SAC1) gene.

40. The microbe of claim 39, wherein the one or more antifungal RNAs comprise one or more nucleic acid sequences at least about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12.
41. The microbe of claim 37, wherein the microbe is a bacterium, optionally selected from the group consisting of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*.
42. The microbe of claim 37, wherein the microbe is a fungus, optionally selected from the group consisting of *Trichoderma virens* or a mycorrhizal fungus.
43. The microbe of claim 40, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been introduced into the bacterium on a bacterial plasmid or bacterial artificial chromosome.
44. The microbe of claim 43, wherein the bacterial plasmid and bacterium are selected from the group consisting of:
- a. a genetically modified T777T plasmid introduced into HT115 strain of *Escherichia coli*;
 - b. a genetically modified pDG148-Stu plasmid introduced into BG322 strain of *Bacillus subtilis*; and
 - c. a genetically modified pJOE7771.1 plasmid introduced into CMA702 strain of *Pseudomonas putida*.
45. The microbe of claim 43, wherein the one or more heterologous nucleic acid sequences encoding the antifungal RNA have been inserted into the genome of the bacterium.
46. The microbe of claim 45, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacterium using CRISPR/Cas9 genome-editing.

47. The microbe of claim 45, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacterium through homologous recombination.

48. The microbe of claim 42, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been introduced into the fungus on a yeast artificial chromosome.

49. The microbe of claim 42, wherein the one or more heterologous nucleic acid sequences encoding the antifungal RNA have been inserted into the genome of the fungus.

50. The microbe of claim 49, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the fungus using CRISPR/Cas9 genome-editing.

51. The microbe of claim 49, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the fungus through homologous recombination.

52. The microbe of any one of claims 37-51, wherein the one or more antifungal RNAs target one or more of fungal pathogens selected from the group consisting of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus niger* as well as potential oomycete pathogens, such as *Phytophthora infestans*.

53. A method of treating or preventing fungal disease of a plant, the method comprising applying to the plant, one or more portions of the plant, or soil in the vicinity of the roots of the plant a plurality of engineered microbes; wherein said engineered microbes comprise and express one or more heterologous nucleic acid sequences encoding one or more antifungal RNAs; wherein the plurality of microbes produces microbial extracellular vesicles (EVs) containing the one or more antifungal RNAs.

54. The method of claim 53, wherein the one or more antifungal RNAs target one or more genes of a fungal pathogen, wherein the one or more targeted genes are selected from the group consisting of: DCL 1 gene, DCL 2 gene, vacuolar protein sorting 51 (VPS51) gene, dynactin (DCTN1) gene, suppressor of actin (SAC1) gene, and the tetraspanin gene Punchless 1 (PLS1).

55. The method of claim 54, wherein the one or more antifungal RNAs comprise one or more nucleic acid sequences at least about 75%, about 80%, 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 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12.

56. The method of any one of claims 53-55, wherein the application of the plurality of engineered microbes is to the soil in the vicinity of the roots of the plant.

57. The method of any one of claims 53-55, wherein the application of the plurality of engineered microbes is to one or more of the leaves, stem, flowers, roots, and fruit of the plant.

58. The method as in any one of claims 53-57, wherein the plurality of engineered microbes comprises bacteria, optionally selected from the group consisting of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*.

59. The method as in any one of claims 53-57, wherein the plurality of microbes comprises fungi, optionally selected from the group consisting of *Trichoderma virens* or a mycorrhizal fungus.

60. The method of claim 58, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been introduced into the bacterium on a bacterial plasmid or bacterial artificial chromosome.

61. The method of claim 60, wherein the bacterial plasmid and bacterium are selected from the group consisting of:

- a. a genetically modified T777T plasmid introduced into HT115 strain of *Escherichia coli*;
- b. a genetically modified pDG148-Stu plasmid introduced into BG322 strain of *Bacillus subtilis*; and
- c. a genetically modified pJOE7771.1 plasmid introduced into CMA702 strain of *Pseudomonas putida*.

62. The method of claim 58, wherein the one or more heterologous nucleic acid sequences encoding the antifungal RNA have been inserted into the genome of the bacteria.

63. The method of claim 62, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacteria using CRISPR/Cas9 genome-editing.

64. The method of claim 62, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacterium through homologous recombination.

65. The method of claim 59, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been introduced into the fungi on a yeast artificial chromosome.

66. The method of claim 59, wherein the one or more heterologous nucleic acid sequences encoding the antifungal RNA have been inserted into the genome of the fungi.

67. The composition of claim 66, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the fungus using CRISPR/Cas9 genome-editing.

68. The composition of claim 66, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the fungus through homologous recombination.

69. The composition of any one of claims 53-68, wherein the one or more antifungal RNAs target one or more of fungal pathogens selected from the group consisting of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus niger* as well as potential oomycete pathogens, such as *Phytophthora infestans*.

70. A method of treating or preventing fungal disease of a plant, the method comprising applying to the plant, one or more portions of the plant, or soil in the vicinity of the roots of the plant a plurality of microbial extracellular vesicles (EVs), wherein the plurality of microbial EVs were isolated from a plurality of microbes, wherein the plurality of microbial EVs contain one or more antifungal RNAs, wherein said one or more antifungal RNAs are heterologous to the plurality of microbes.

71. The method of claim 70, wherein the one or more antifungal RNAs target one or more genes of a fungal pathogen, wherein the one or more targeted genes are selected from the group consisting of: DCL 1 gene, DCL 2 gene, vacuolar protein sorting 51 (VPS51) gene, dynactin (DCTN1) gene, suppressor of actin (SAC1) gene, and the tetraspanin gene Punchless 1 (PLS1).

72. The method of claim 71, wherein the one or more antifungal RNAs comprise one or more nucleic acid sequences at least about 75%, about 80%, 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 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12.

73. The method of any one of claims 70-72, wherein the application of the plurality of microbial EVs is to the soil in the vicinity of the roots of the plant.

74. The method of any one of claims 70-72, wherein the application of the plurality of microbial EVs is to one or more of the leaves, stem, flowers, roots, and fruit of the plant.
75. The method as in any one of claims 70-74, wherein plurality of microbes comprise bacteria, optionally selected from the group consisting of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*.
76. The method as in any one of claims 70-74, wherein plurality of microbes comprise fungi, optionally selected from the group consisting of *Trichoderma virens* or a mycorrhizal fungus.
77. The method of any one of claims 70-76, wherein the one or more antifungal RNAs are derived from the introduction into a bacterium of a bacterial plasmid or bacterial artificial chromosome comprising one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs.
78. The method of claim 77, wherein the bacterial plasmid and bacterium are selected from the group consisting of:
- a genetically modified T777T plasmid introduced into HT115 strain of *Escherichia coli*;
 - a genetically modified pDG148-Stu plasmid introduced into BG322 strain of *Bacillus subtilis*; and
 - a genetically modified pJOE7771.1 plasmid introduced into CMA702 strain of *Pseudomonas putida*.
79. The method of any one of claims 70-76, wherein the one or more antifungal RNAs are derived from one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs that have been inserted into the genome of a bacterium.
80. The method of claim 79, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacterium using CRISPR/Cas9 genome-editing.

81. The method of claim 79, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacterium through homologous recombination.

82. The method of any one of claims 70-76, wherein the one or more antifungal RNAs are derived from the introduction into a fungus of a yeast artificial chromosome comprising one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs.

83. The method of claim 82, wherein the one or more antifungal RNAs are derived from one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs that have been inserted into the genome of the fungus.

84. The method of claim 83, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the fungus using CRISPR/Cas9 genome-editing.

85. The method of claim 83, wherein the one or more heterologous nucleic acid sequence encoding the one or more antifungal RNAs have been inserted into the genome of the fungus through homologous recombination.

86. The method of any one of claims 70-76, wherein the one or more antifungal RNAs have been isolated from a first bacteria and loaded into an EV isolated from a second bacteria.

87. The method of any one of claims 70-76, wherein the one or more antifungal RNAs have been isolated from a bacteria and loaded into an EV isolated from a fungus.

88. The method of any one of claims 70-76, wherein the one or more antifungal RNAs have been isolated from a fungus and loaded into an EV isolated from a bacteria.

89. The method of any one of claims 70-76, wherein the one or more antifungal RNAs have been isolated from a first fungus and loaded into an EV isolated from a second fungus.

90. The method of any one of claims 70-76, wherein the one or more antifungal RNAs have been chemically synthesized and loaded into the microbial EVs.

91. The method of any one of claims 70-80, wherein the one or more antifungal RNAs target one or more of fungal pathogens selected from the group consisting of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus niger* as well as potential oomycete pathogens, such as *Phytophthora infestans*.

92. A method of producing a microbial extracellular vesicle (EV) comprising an antifungal RNA, the method comprising:

growing a plurality of engineered microbes, wherein the engineered microbes comprise and express one or more heterologous nucleic acid sequences encoding one or more antifungal RNAs, and wherein the engineered microbes produce microbial EVs comprising the one or more heterologous antifungal RNAs;

isolating from the growth media a plurality of EVs.

93. The method of claim 92, wherein the one or more antifungal RNAs comprise a double-stranded RNA or mRNA.

94. The method of claim 92 or 93, wherein the one or more heterologous antifungal RNAs target one or more genes of a fungal pathogen, wherein the one or more targeted genes are selected from the group consisting of: DCL 1 gene, DCL 2 gene, vacuolar protein sorting 51 (VPS51) gene, dynactin (DCTN1) gene, suppressor of actin (SAC1) gene, and the tetraspanin gene Punchless 1 (PLS1).

95. The method of claim 94, wherein the one or more antifungal RNAs comprise one or more nucleic acid sequences at least about 75%, about 80%, 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 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12.

96. The method of any one of claims 92-95, wherein the plurality of engineered microbes comprise bacteria, optionally selected from the group consisting of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*.

97. The method of any one of claims 92-95, wherein the plurality of engineered microbes comprise fungi, optionally selected from the group consisting of *Trichoderma virens* or a mycorrhizal fungus.

98. The method of claim 96, wherein the one or more heterologous nucleic acid sequences encoding one or more antifungal RNAs have been introduced into the bacteria on a bacterial plasmid or bacterial artificial chromosome.

99. The method of claim 98, wherein the bacterial plasmid and bacterium are selected from the group consisting of:

- a. a genetically modified T777T plasmid introduced into HT115 strain of *Escherichia coli*;
- b. a genetically modified pDG148-Stu plasmid introduced into BG322 strain of *Bacillus subtilis*; and
- c. a genetically modified pJOE7771.1 plasmid introduced into CMA702 strain of *Pseudomonas putida*.

100. The method of claim 96, wherein the one or more heterologous nucleic acid sequences encoding one or more antifungal RNAs have been inserted into the genome of the bacteria.

101. The method of claim 100, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacteria using CRISPR/Cas9 genome-editing.

102. The method of claim 100, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacteria through homologous recombination.

103. The method of claim 97, wherein the one or more heterologous nucleic acid sequences encoding one or more antifungal RNAs have been introduced into the fungi on a yeast artificial chromosome.

104. The method of claim 97, wherein the one or more heterologous nucleic acid sequences encoding one or more antifungal RNAs have been inserted into the genome of the fungi.

105. The method of claim 104, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the fungus using CRISPR/Cas9 genome-editing.

106. The method of claim 104, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the fungus through homologous recombination.

107. The method of any one of claims 92-106, wherein the one or more antifungal RNAs target one or more of fungal pathogens selected from the group consisting of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus niger* as well as potential oomycete pathogens, such as *Phytophthora infestans*.

108. A method of producing a microbial extracellular vesicle (EV) comprising one or more antifungal RNAs, the method comprising:

- growing a plurality of microbes in growth media, wherein the microbes produce a plurality of microbial EVs;
- isolating the plurality of microbial EVs from the growth media; and
- introducing into the isolated microbial EVs one or more heterologous antifungal RNAs.

109. The method of claim 108, wherein the plurality of microbes are bacteria, optionally selected from the group consisting of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*.

110. The method of claim 108, wherein the plurality of microbes are fungi, optionally selected from the group consisting of *Trichoderma virens* or a mycorrhizal fungus.

111. The method of any one of claims 108-110, wherein the one or more heterologous antifungal RNAs comprise a double-stranded RNA or mRNA.

112. The method of any one of claims 108-111, wherein the one or more heterologous antifungal RNAs target one or more genes of a fungal pathogen, wherein the one or more targeted genes are selected from the group consisting of: DCL 1 gene, DCL 2 gene, vacuolar protein sorting 51 (VPS51) gene, dynactin (DCTN1) gene, suppressor of actin (SAC1) gene, and tetraspanin gene Punchless 1 (PLS1).

113. The method of claim 112, wherein the one or more antifungal RNAs comprise one or more nucleic acid sequences at least about 75%..., about 80%, 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 99%, or completely identical to a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 10, and 12.

114. The method of any one of claims 108-123, wherein the one or more heterologous antifungal RNAs are chemically synthesized or are purified from an engineered microbe, wherein the engineered microbe comprises one or more heterologous nucleic acid sequences encoding the one or more heterologous antifungal RNAs.

115. The method of claim 114, wherein the one or more heterologous antifungal RNAs are purified from an engineered microbe, wherein said engineered microbe is a bacteria, wherein one or more heterologous nucleic acid sequences encoding the one or more heterologous antifungal

RNAs have been introduced into said bacteria on a bacterial plasmid or bacterial artificial chromosome, wherein said bacteria expresses the one or more heterologous antifungal RNAs.

116. The method of claim 115, wherein the bacterial plasmid and bacterium are selected from the group consisting of:

- a. a genetically modified T777T plasmid introduced into HT115 strain of *Escherichia coli*;
- b. a genetically modified pDG148-Stu plasmid introduced into BG322 strain of *Bacillus subtilis*; and
- c. a genetically modified pJOE7771.1 plasmid introduced into CMA702 strain of *Pseudomonas putida*.

117. The method of claim 114, wherein the one or more heterologous antifungal RNAs are purified from an engineered microbe, wherein said engineered microbe is a bacteria, wherein one or more heterologous nucleic acid sequences encoding the one or more heterologous antifungal RNAs have been inserted into the genome of said bacteria, wherein said bacteria expresses the one or more heterologous antifungal RNAs.

118. The method of claim 117, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacteria using CRISPR/Cas9 genome-editing.

119. The method of claim 117, wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the bacteria through homologous recombination.

120. The method of claim 114, wherein the one or more heterologous antifungal RNAs are purified from an engineered microbe, wherein said engineered microbe is a fungus, wherein one or more heterologous nucleic acid sequences encoding the one or more heterologous antifungal RNAs have been introduced into said fungus on a yeast artificial chromosome, wherein said fungus expresses the one or more heterologous antifungal RNAs.

121. The method of claim 114, wherein the one or more heterologous antifungal RNAs are purified from an engineered microbe, wherein said engineered microbe is a fungus, wherein one or more heterologous nucleic acid sequences encoding the one or more heterologous antifungal RNAs have been inserted into the genome of said fungus, wherein said fungus expresses the one or more heterologous antifungal RNAs.

122. The method of claim 121, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the fungus using CRISPR/Cas9 genome-editing.

123. The method of claim 120, wherein, the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs have been inserted into the genome of the fungus through homologous recombination.

124. The method of any one of claims 108-123, wherein the one or more antifungal RNAs target one or more of fungal pathogens selected from the group consisting of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus niger* as well as potential oomycete pathogens, such as *Phytophthora infestans*.

125. A composition comprising an extracellular vesicle (EV), said EV being derived from an organism selected from a bacteria, a fungus, or a plant, said EV containing one or more antifungal mRNAs, wherein said one or more antifungal mRNAs are heterologous to the organism from which the EV is derived.

126. The composition of claim 125, wherein the organism is a bacteria, wherein said bacteria is optionally selected from the group consisting of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*.

127. The composition of claim 125, wherein the organism is a fungus, wherein said fungus is optionally selected from the group consisting of *Trichoderma virens* or a mycorrhizal fungus.

128. The composition of claim 125, wherein the organism is a plant.

129. The composition of any one of claims 125-128, wherein the one or more antifungal mRNAs are derived from one or more heterologous nucleic acid sequences encoding the one or more antifungal mRNAs that have been inserted into the genome of the organism, optionally wherein the one or more heterologous nucleic acid sequences encoding the one or more antifungal RNAs are inserted into the genome of the organism using CRISPR/Cas9 genome-editing and/or homologous recombination.

130. The composition of any one of claims 125-128, wherein the one or more antifungal mRNAs are chemically synthesized or produced by a microbe, isolated, and then loaded into the EV.

131. The composition of any one of claims 125-129, wherein the one or more antifungal mRNAs target one or more fungal pathogens selected from the group consisting of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Aspergillus niger* as well as potential oomycete pathogens, such as *Phytophthora infestans*.

132. A method of treating or preventing fungal disease of a plant, the method comprising applying to the plant, one or more portions of the plant, or soil in the vicinity of the roots of the plant a plurality of EVs as described in any one of claims 125-131.

FIG. 1

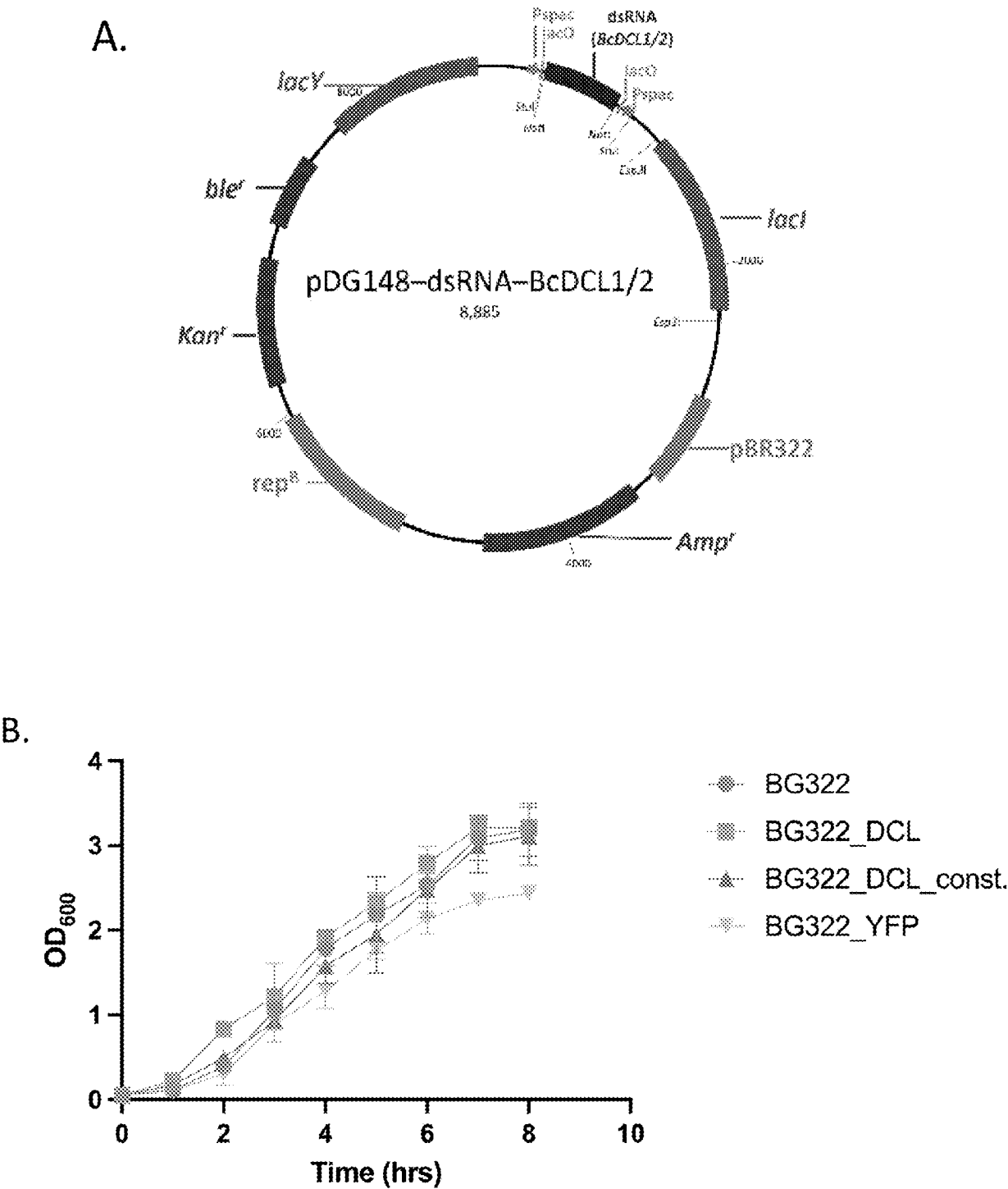
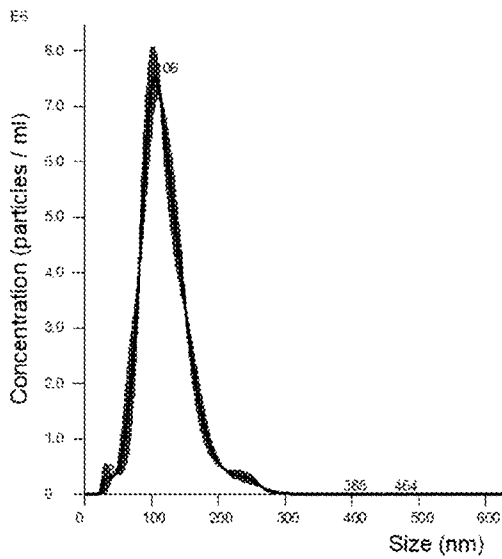
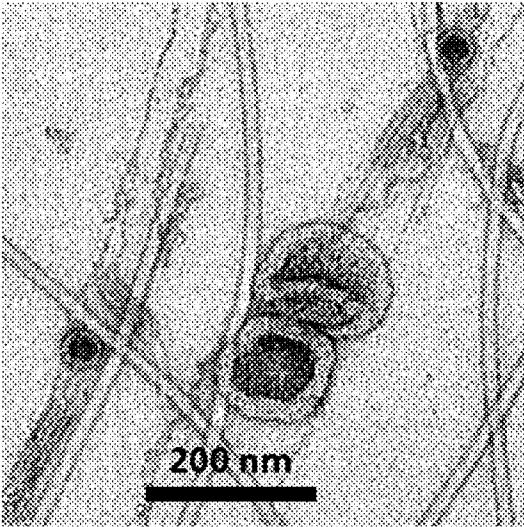


FIG. 2

A.



B.



C.

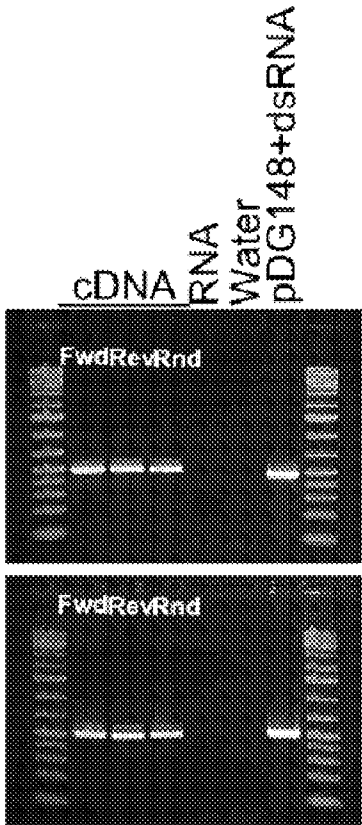


FIG. 3

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AT1G02930	GSTF6	AT2G36530	ENO2	AT4G16890	SNC1
AT1G05010	ACO4	AT2G38470	WRKY33	AT4G17040	CLPR4
AT1G07000	ATEXO70B2	AT2G38800	NA	AT4G20260	PCAP1
AT1G07890	APX1	AT2G39730	RCA	AT4G20830	NA
AT1G08830	CSD 1.00	AT2G40000	HSPRO2	AT4G21960	PER42
AT1G09070	SRC2	AT2G40140	CZF1	AT4G22690	CYP706A1
AT1G09970	LRR XI-23	AT2G41090	CML10	AT4G23810	WRKY53
AT1G11260	STP1	AT2G41410	CML35	AT4G24570	PUMP4
AT1G11650	RBP45B	AT2G41430	ERD15	AT4G25030	NA
AT1G14870	PCR2	AT2G43970	LARP6B	AT4G25100	FSD1
AT1G14880	PCR1	AT2G44490	BGLU26	AT4G25620	NA
AT1G17420	LOX3	AT2G46330	AGP16	AT4G26090	RPS2
AT1G18080	RACK1A	AT3G01290	HIR3	AT4G26850	VTC2
AT1G18300	NUDT4	AT3G02470	SAMDC1	AT4G27280	KRP1
AT1G18570	MYB51	AT3G02840	NA	AT4G27652	NA
AT1G19020	NA	AT3G04120	GAPC1	AT4G29780	NA
AT1G19380	NA	AT3G04720	HEL	AT4G30270	XTH24
AT1G20620	CAT3	AT3G07390	AIR12	AT4G30530	GGP1
AT1G23710	NA	AT3G09390	MT2A	AT4G31500	CYP83B1
AT1G26630	ELF5A-2	AT3G10930	NA	AT4G32020	NA
AT1G27100	NA	AT3G14050	RSH2	AT4G32150	VAMP711
AT1G27730	ZAT10	AT3G14210	ESM1	AT4G32940	GAMMA-VPE
AT1G28370	ERF11	AT3G14415	NA	AT4G34390	XLG2
AT1G32928	NA	AT3G14420	GLO1	AT4G34410	ERF109
AT1G35580	CINV1	AT3G15210	ERF4	AT4G34710	ADC2
AT1G37130	NIA2	AT3G15356	LEC	AT4G35750	NA
AT1G45145	TRX5	AT3G15730	PLDALPHA1	AT4G35770	STR15
AT1G47128	RD21A	AT3G16530	NA	AT4G36040	ATJ11
AT1G51400	NA	AT3G16770	RAP2-3	AT4G36500	NA
AT1G52740	HTA9	AT3G17390	METK4	AT4G36550	PUB5
AT1G54100	ALDH7B4	AT3G17800	NA	AT4G38540	MO2
AT1G55450	NA	AT3G18250	NA	AT4G38550	NA
AT1G59870	ABCG36	AT3G18740	RPL30C	AT4G38800	MTN1
AT1G59910	FH7	AT3G18780	ACT2	AT4G38970	FBA2
AT1G61250	SCAMP3	AT3G19010	NA	AT4G39090	RD19A
AT1G61890	DTX37	AT3G19030	NA	AT4G39950	CYP79B2
AT1G63720	NA	AT3G20770	EIN3	AT5G02500	MED37E
AT1G65930	CICDH	AT3G22231	PCC1	AT5G06320	NHL3
AT1G65970	PRXIIC	AT3G22600	NA	AT5G09810	ACT7
AT1G65980	PRXIIB	AT3G22890	APS1	AT5G10450	GRF6
AT1G66090	NA	AT3G26830	CYP71B15	AT5G17920	MS1
AT1G72520	LOX4	AT3G28940	AIG2B	AT5G19240	NA
AT1G73260	KTI1	AT3G44260	CAF1-9	AT5G20010	RAN1
AT1G73540	NUDT21	AT3G44300	NIT2	AT5G20230	BCB
AT1G75380	BBD 1.00	AT3G44720	ADT4	AT5G20250	DIN10
AT1G75750	GASA1	AT3G45640	MPK3	AT5G22250	CAF1-11
AT1G76180	ERD14	AT3G47250	NA	AT5G25250	FLOT1
AT1G76490	HMG1	AT3G48000	ALDH2B4	AT5G26030	FC1
AT1G76600	NA	AT3G48140	NA	AT5G26920	CBP60G
AT1G78380	GSTU19	AT3G48680	GAMMACAL2	AT5G27420	ATL31
AT1G78460	NA	AT3G49120	PER34	AT5G35735	NA
AT1G80840	WRKY40	AT3G50500	SPK-2-2	AT5G41740	NA
AT2G01670	NUDT17	AT3G50930	HSR4	AT5G41750	NA
AT2G05070	LHCB2.2	AT3G50950	RPP13L4	AT5G44430	PDF1.2C
AT2G05100	LHCB2.1	AT3G51920	CML9	AT5G45110	NPR 3.00
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AT2G06050	OPR3	AT3G55430	NA	AT5G52750	HIPP13
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AT2G18280	TULP2	AT3G55980	SZF1	AT5G54770	THI1
AT2G18690	NA	AT3G56410	NA	AT5G54810	TSB1
AT2G20960	pEARLI4	AT3G57450	NA	AT5G57560	XTH22
AT2G23320	WRKY15	AT3G57520	RFS2	AT5G58430	EXO70B1
AT2G23810	TET8	AT4G01850	SAM2	AT5G59550	NA
AT2G24600	NA	AT4G02380	SAG21	AT5G59820	ZAT12
AT2G25450	GSL-OH	AT4G02520	GSTF2	AT5G60360	AALP
AT2G26190	IQM4	AT4G05150	NA	AT5G61210	SNAP33
AT2G26560	PLP2	AT4G09320	NDK1	AT5G61410	RPE

FIG. 4

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AT1G03130	PSAD2	AT3G08940	LHCB4.2	AT4G32260	NA
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AT1G06680	PSBP1	AT3G09840	CDC48A	AT4G37830	NA
AT1G08830	CSD 1.00	AT3G11940	RPS5B	AT4G38970	FBA2
AT1G09640	NA	AT3G13920	EIF4A1	AT4G40040	HTR8
AT1G11910	APA1	AT3G14415	NA	AT5G01530	LHCB4.1
AT1G13440	GAPC2	AT3G14420	GLO1	AT5G02500	MED37E
AT1G14320	RPL10A	AT3G15356	LEC	AT5G03630	MDAR2
AT1G15500	AATP2	AT3G15730	PLDALPHA1	AT5G06320	NHL3
AT1G15820	LHCB6	AT3G17390	METK4	AT5G08670	NA
AT1G17290	ALAAAT1	AT3G18780	ACT2	AT5G09810	ACT7
AT1G20620	CAT3	AT3G22235	NA	AT5G10860	CBSX3
AT1G23310	GGAT1	AT3G22890	APS1	AT5G11670	NADP-ME2
AT1G29910	LHCB1.1	AT3G24503	ALDH2C4	AT5G13630	CHLH
AT1G29930	LHCB1.3	AT3G25520	ATL5	AT5G14780	FDH1
AT1G31330	PSAF	AT3G26520	TIP1-2	AT5G17920	MS1
AT1G32060	PRK	AT3G26650	GAPA1	AT5G20010	RAN1
AT1G32928	NA	AT3G44300	NIT2	AT5G20250	DIN10
AT1G37130	NIA2	AT3G44310	NIT1	AT5G23900	RPL13D
AT1G55450	NA	AT3G47470	LHCA4	AT5G38410	NA
AT1G55670	PSAG	AT3G48000	ALDH2B4	AT5G43940	HOT5
AT1G59870	ABCG36	AT3G48140	NA	AT5G44340	TUBB4
AT1G61520	LHCA3	AT3G48680	GAMMACAL2	AT5G46110	APE2
AT1G67090	RBCS-1A	AT3G49120	PER34	AT5G50920	CLPC1
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FIG. 5

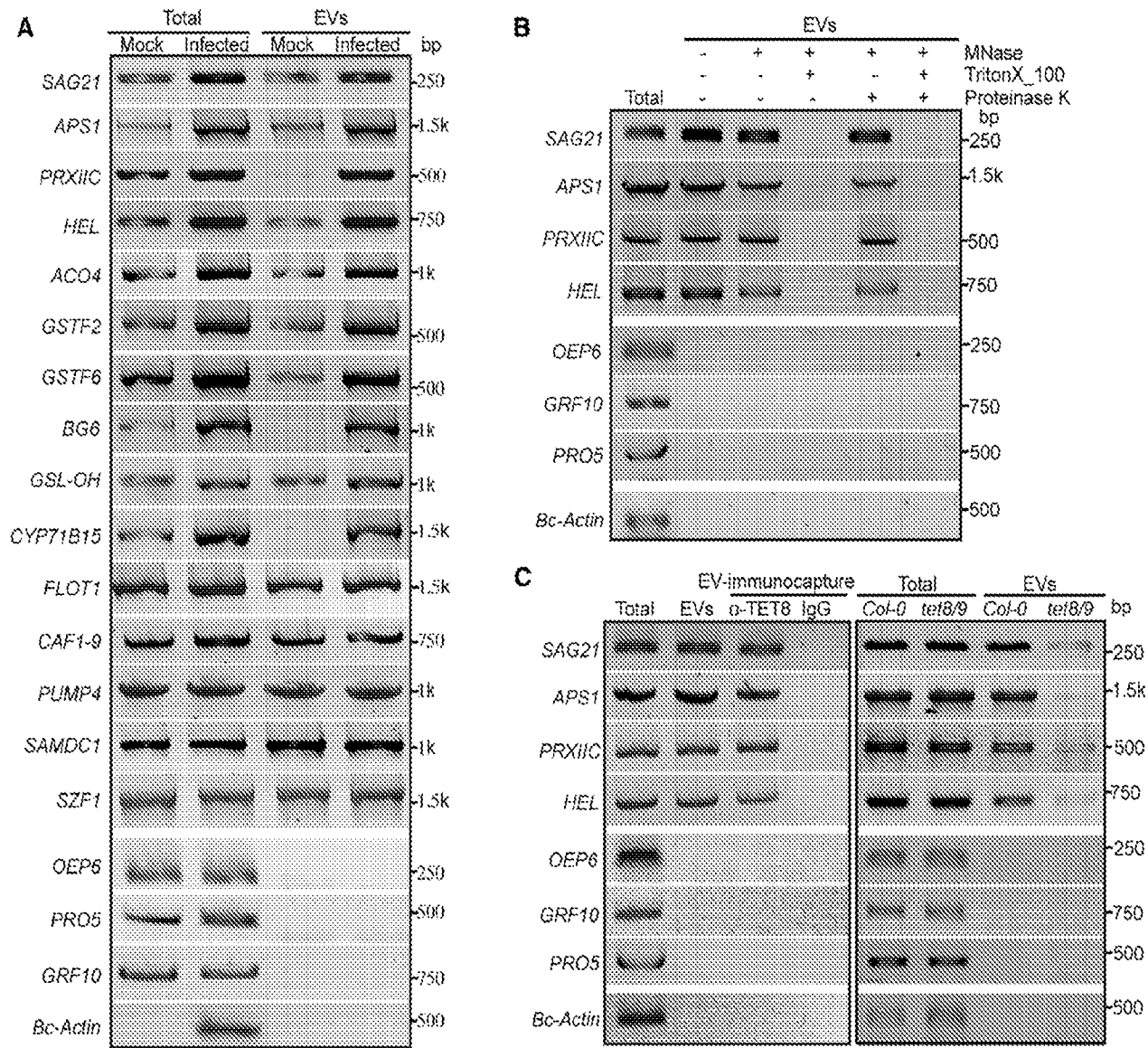
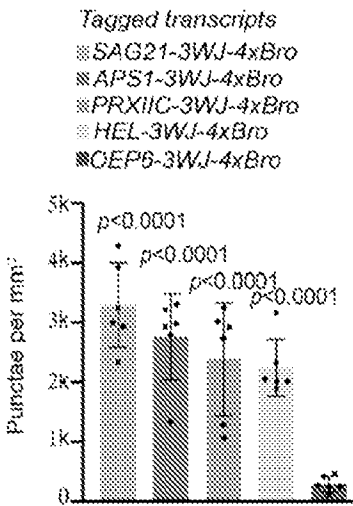
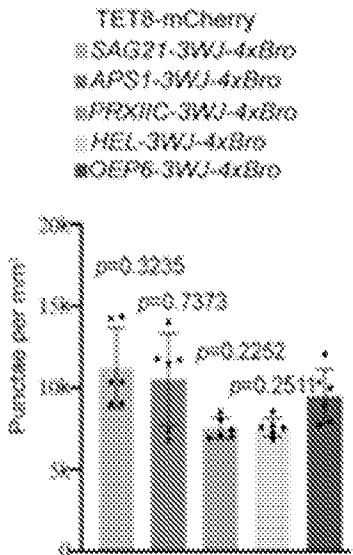


FIG. 6

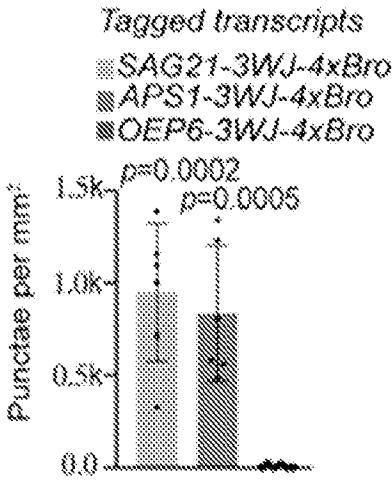
A.



B.



C.



D.

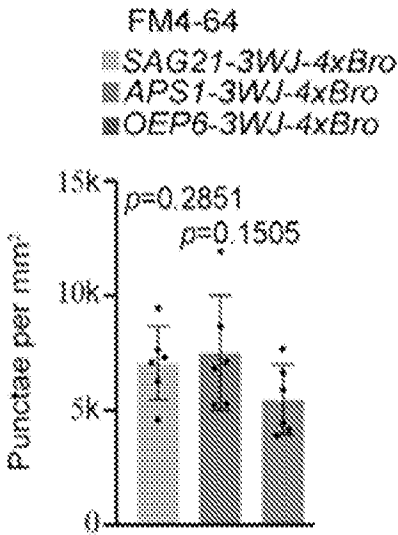


FIG. 7

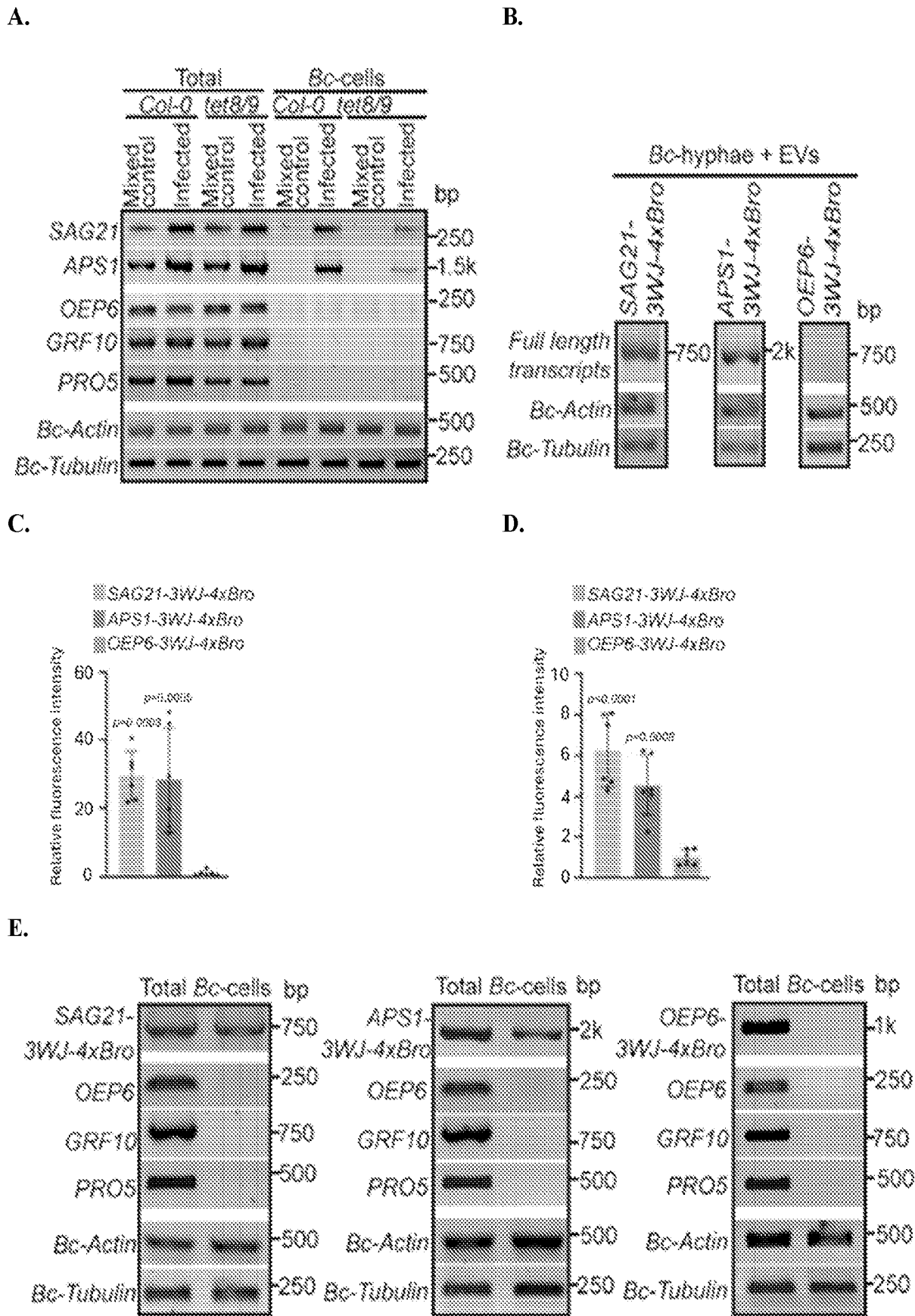
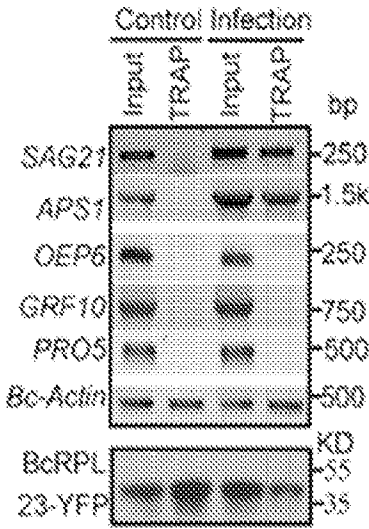
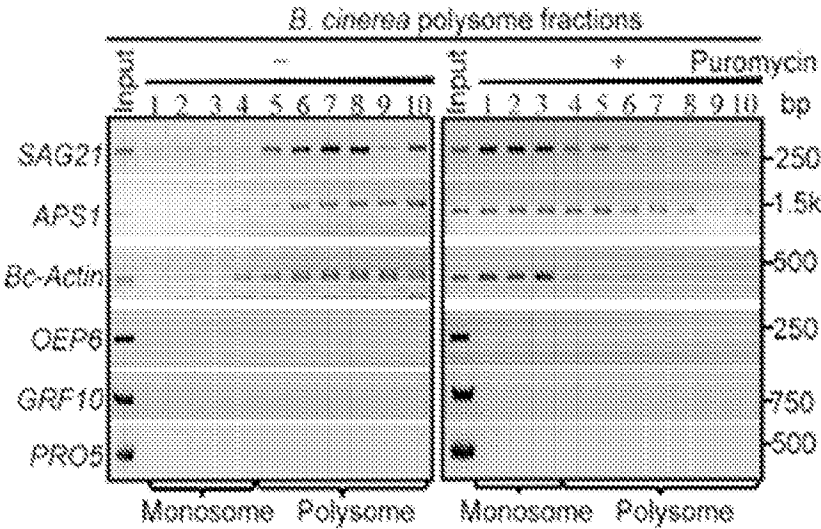


FIG. 8

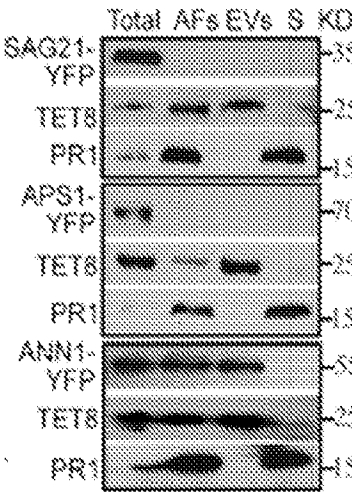
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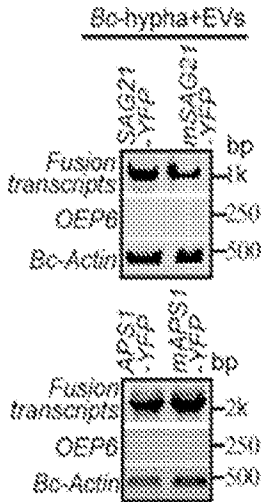
B.



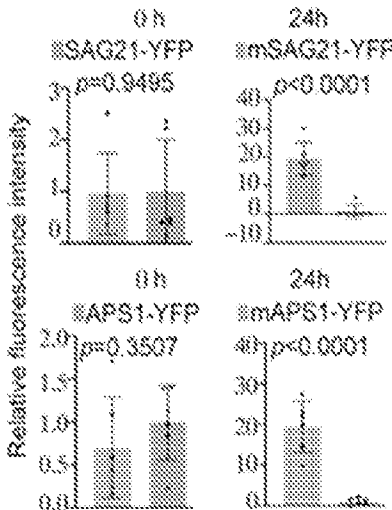
C.



D.



E.



F.

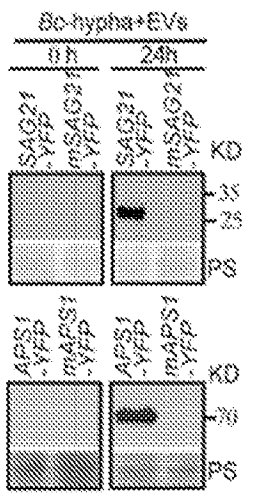


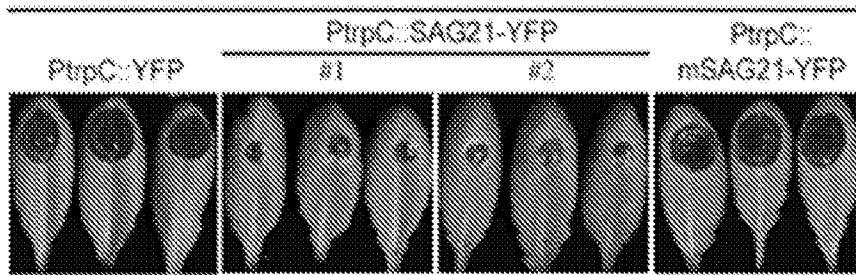
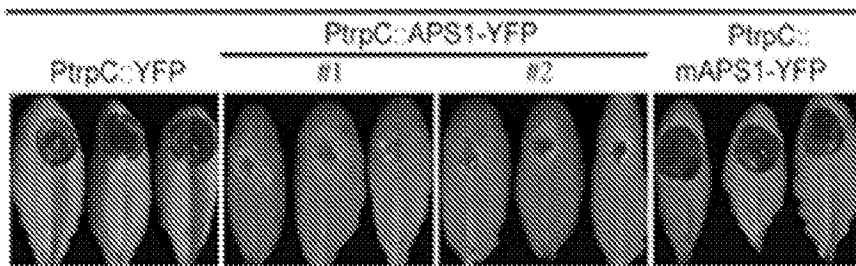
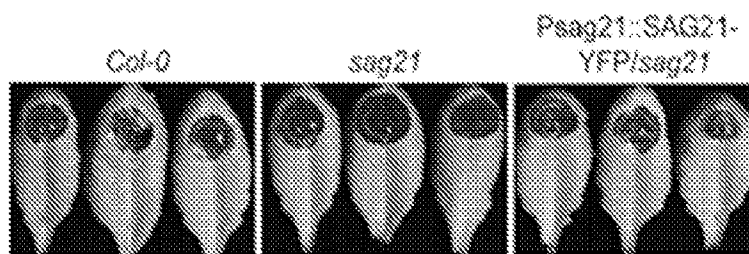
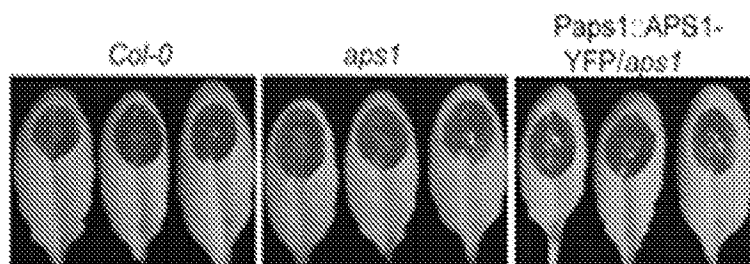
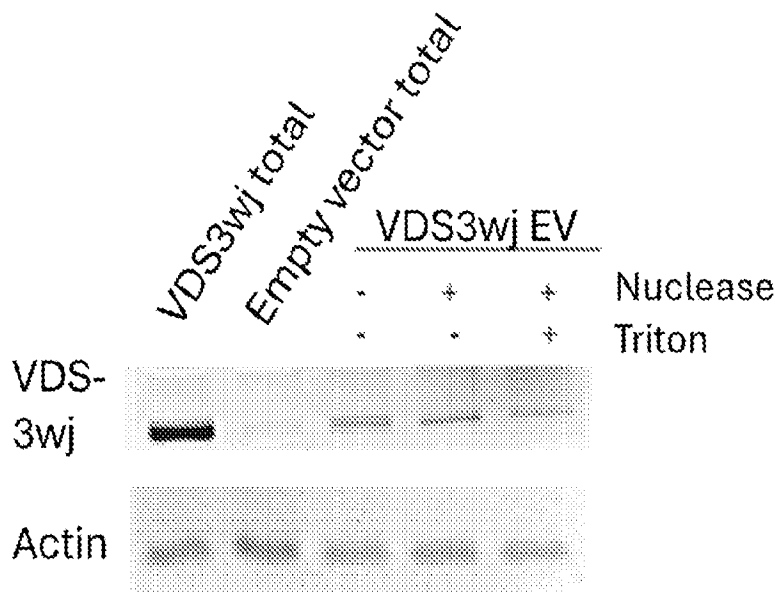
FIG. 9**A.****B.****C.****D.**

FIG. 10

A.



B.

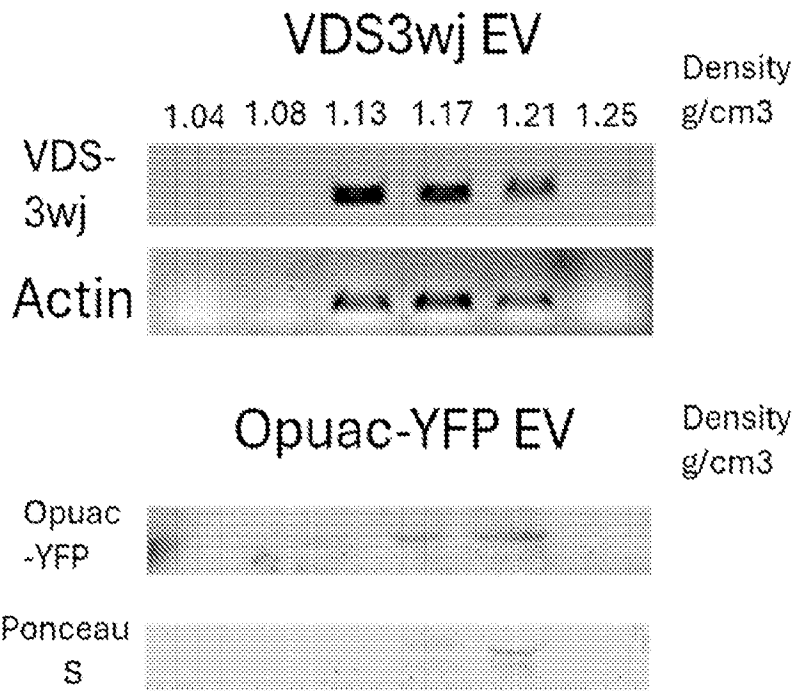


FIG. 11

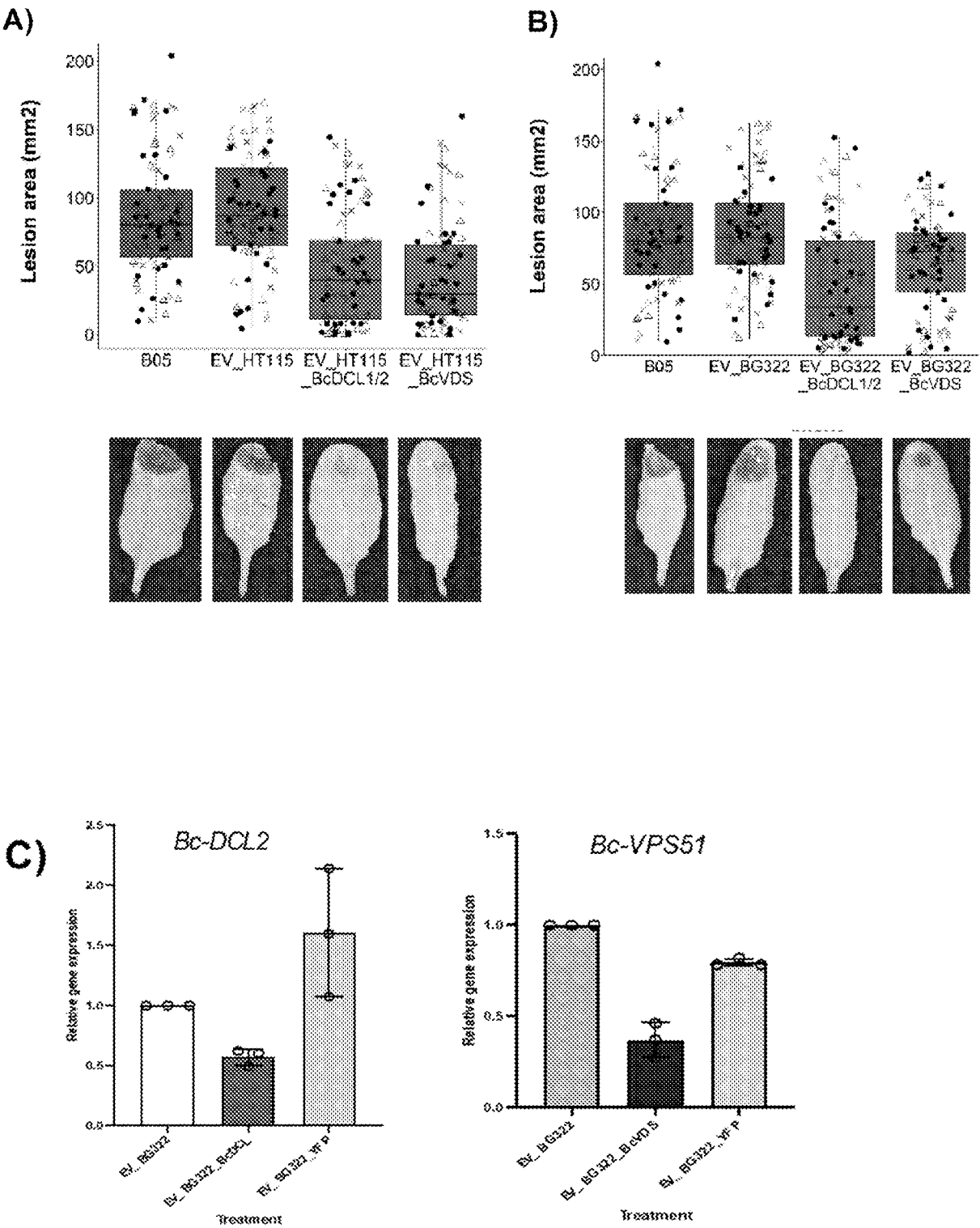


FIG. 12

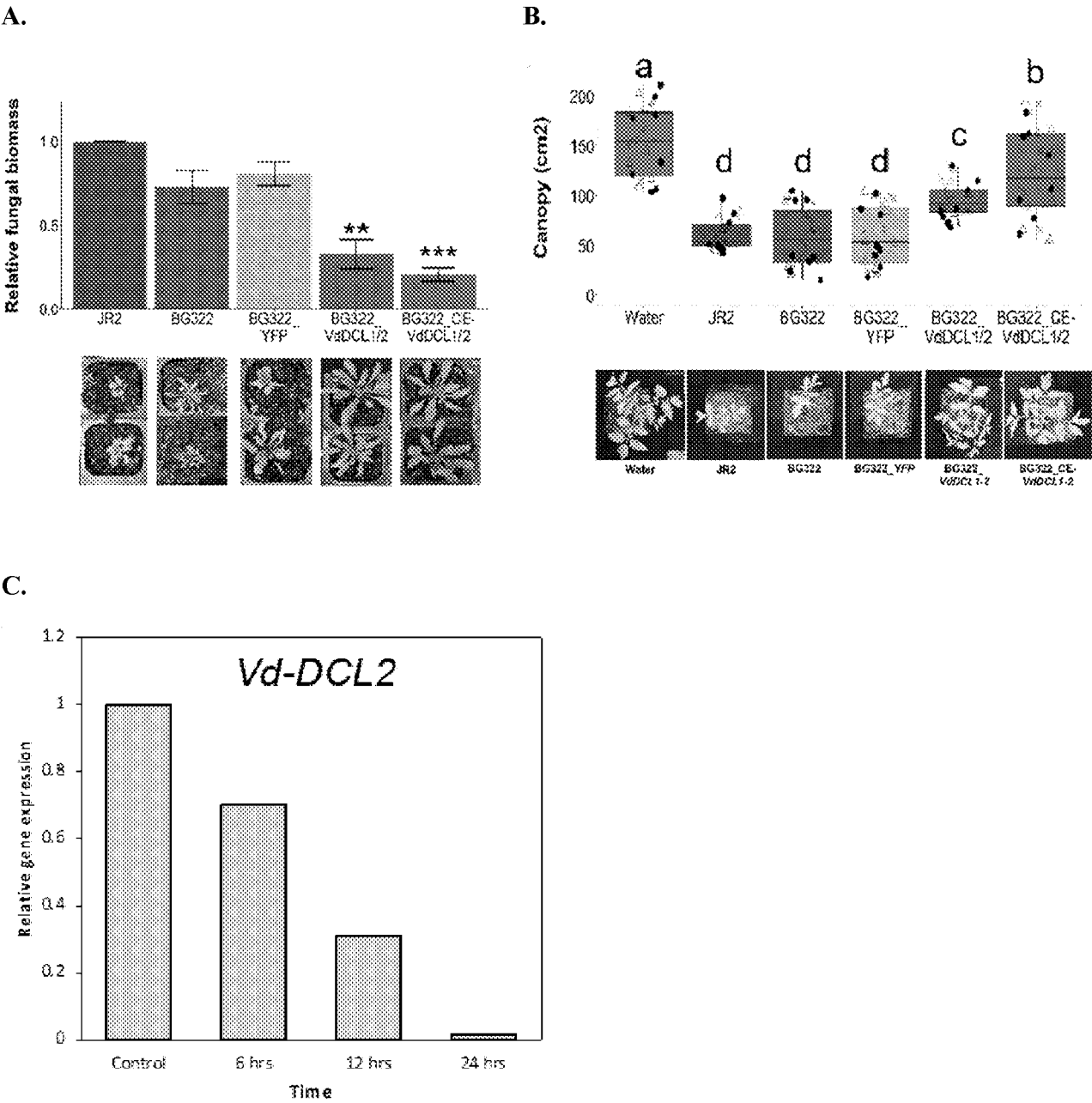
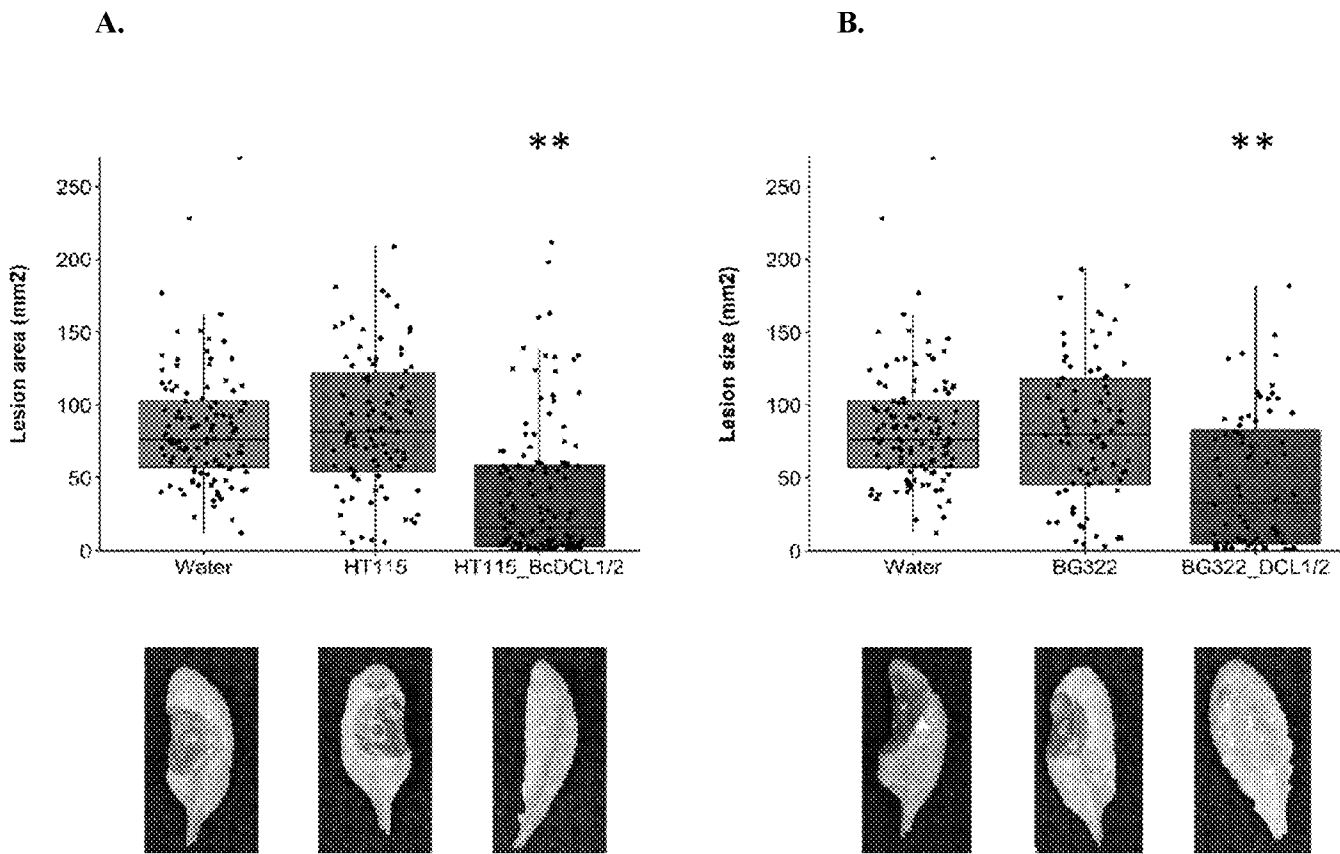


FIG. 13



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/060257

A. CLASSIFICATION OF SUBJECT MATTERIPC: **C12N 15/82** (2025.01); **C12N 15/113** (2025.01); **A01P 3/00** (2025.01); **C12N 1/00** (2025.01)CPC: **C12N 15/8218**; **C12N 15/8282**; **C12N 15/113**; **A01P 3/00**; **C12N 1/00**; **C12N 2310/14**

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

See Search History Document

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.
X	WO 2022/053689 A2 (IMMUNRISE et al.) 17 March 2022 (17.03.2022) abstract; pg 4, ln 14-16, 19-22; pg 6, ln 3-4; pg 19, ln 15; pg 24, ln 10-12; pg 25, ln 6; pg 56, ln 25-27; pg 57, ln 1; pg 79, ln 24-26; pg 90, ln 11-17; pg 91, ln 21-23; pg 99, ln 19-24; claim 2	1-3, 125, 128, 129/(125, 128)
Y	abstract; pg 4, ln 14-16, 19-22; pg 6, ln 3-4; pg 19, ln 15; pg 24, ln 10-12; pg 25, ln 6; pg 56, ln 25-27; pg 57, ln 1; pg 79, ln 24-26; pg 90, ln 11-17; pg 91, ln 21-23; pg 99, ln 19-24; claim 2	4, 19-22, 126-127, 129/(126-127), 130
Y	US 2016/0032314 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 04 February 2016 (04.02.2016) abstract; para [0008], [0152]	4, 22
Y	WO 2023/183794 A2 (MERCURY BIO, INC.) 28 September 2023 (28.09.2023) abstract; pg 10, ln 12-13; pg 25, ln 23-24; claim 1	19-22, 127, (128-129)/(127), 130
Y	US 2020/0071713 A1 (PEBBLE LABS USA INC.) 05 March 2020 (05.03.2020) abstract; para [0033], [0099]	126, 129/(126), 130/(126)

☐ 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

“D” document cited by the applicant in the international application

“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

10 April 2025 (10.04.2025)

Date of mailing of the international search report

16 April 2025 (16.04.2025)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/060257

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.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/060257

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.: **5-18, 23-36, 58-69, 75-91, 96-107, 112-124, 131-132**
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:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I+: Claims 1-4, 19-22, and 125-130, directed to a composition comprising a microbial extracellular vesicle (EV) isolated from a microbe. The composition will be searched to the extent that the composition encompasses an antifungal RNA targeting DCL1, SEQ ID NO: 2. The first named invention was determined based on first claim antifungal target (claims 1, 3-4). This first named invention has been selected based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines. It is believed that claims 1-4, 19-22, and 125-130, encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass a composition comprising a microbial extracellular vesicle (EV) isolated from a microbe comprising an antifungal RNA targeting DCL1, SEQ ID NO: 2. Additional compositions comprising additional antifungal RNAs will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected compositions. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a composition comprising a microbial extracellular vesicle (EV) isolated from a microbe comprising an antifungal RNA targeting DCL2, SEQ ID NO: 4 (claims 1-4, 19-22, 125-130).

Group II+: Claims 37-52, directed to a microbe for production of extracellular vesicles. Group II+ will be searched upon payment of additional fees. The microbe may be searched for an additional fee and election as such, for example, to the extent that the microbe encompasses an antifungal RNA targeting DCL1, SEQ ID NO: 2 (claims 37-52). Additional microbes will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected microbes. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be a microbe for production of extracellular vesicles comprising an antifungal RNA targeting DCL2, SEQ ID NO: 4 (claims 37-52).

Group III+: Claims 53-57 and 70-74, directed to a method of treating or preventing fungal disease of a plant. Group III+ will be searched upon payment of additional fees. The method may be searched for an additional fee and election as such, for example, to the extent that the method encompasses an antifungal RNA targeting DCL1, SEQ ID NO: 2 (claims 53-57, 70-74). Additional methods will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected methods. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be a method of treating or

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

preventing fungal disease of a plant comprising an antifungal RNA targeting DCL2, SEQ ID NO: 4 (claims 53-57, 70-74).

Group IV+: Claims 92-95 and 108-111, directed to a method of producing a microbial extracellular vesicle. Group IV+ will be searched upon payment of additional fees. The method may be searched for an additional fee and election as such, for example, to the extent that method encompasses an antifungal RNA targeting DCL1, SEQ ID NO: 2 (claims 92-95, 108-111). Additional methods will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected methods. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be a method of producing a microbial extracellular vesicle comprising an antifungal RNA targeting DCL2, SEQ ID NO: 4 (claims 92-95, 108-111).

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.: **1-4, 19-22, and 125-130, limited to an antifungal RNA targeting DCL1, SEQ ID NO: 2**

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.