(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 May 2026 (07.05.2026)

WIPO | PCT

(10) International Publication Number
WO 2026/094085 A1

(51) International Patent Classification:
A01N 25/28 (2006.01) *A01N 65/00* (2009.01)
A01N 63/40 (2020.01)

(21) International Application Number:
PCT/IT2024/000029

(22) International Filing Date:
04 November 2024 (04.11.2024)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant: **DIAMANTE SOCIETÀ BENEFIT S.R.L.** [IT/IT]; Viale del Lavoro 33, 37135 Verona (IT).

(72) Inventors: **AVESANI, Linda**; c/o Diamante Società Benefit S.r.l., Viale del Lavoro 33, 37135 VERONA (IT). **ZAMPIERI, Roberta**; c/o Diamante Società Benefit S.r.l., Viale del Lavoro 33, 37135 VERONA (IT). **GARONZI, Valentina**; c/o Diamante Società Benefit S.r.l., Viale del Lavoro 33, 37135 VERONA (IT). **DANZI, Davide**; c/o Diamante Società Benefit S.r.l., Viale del Lavoro 33, 37135 VERONA (IT). **VANDELLE, Elodie Geneviève Germaine**; c/o Diamante Società Benefit S.r.l., Viale del Lavoro 33, 37135 VERONA (IT).

(74) Agent: **PREDAZZI, Valentina**; c/o Società Italiana Brevetti S.p.A., Piazza di Pietra 39, 00186 Roma (IT).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ,

(54) Title: COMPOSITION OF MATTER FOR INDUCING DEFENSE OR GROWTH IN A PLANT AND METHODS OF USE AND MANUFACTURE THEREOF

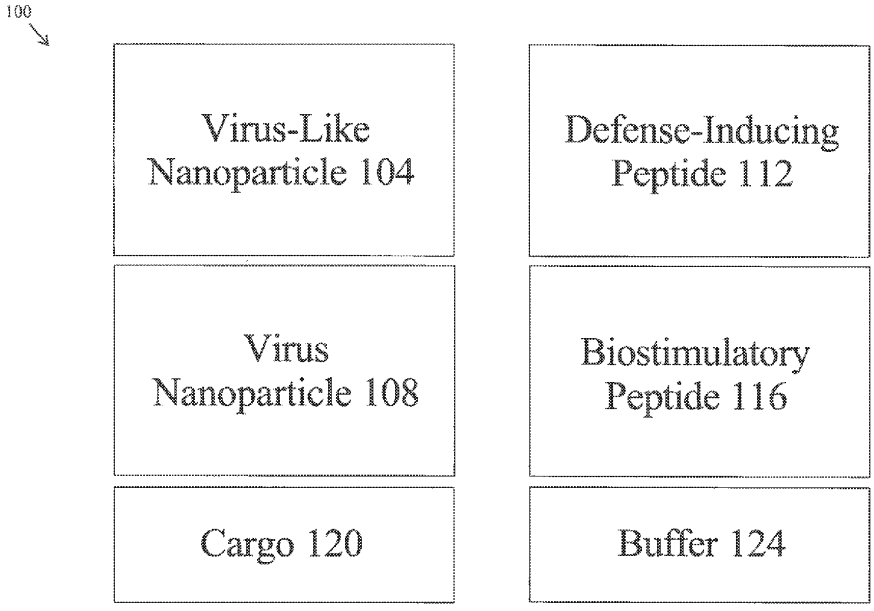

FIG. 1

(57) Abstract: Abstract of the Disclosure: A method of manufacturing a composition for inducing defense in a plant includes infecting a plant with a virus to produce a virus-like nanoparticle, sampling symptomatic leaves from the plant, homogenizing the virus-like nanoparticle, incubating the virus-like nanoparticle, centrifuging the virus-like nanoparticle, and filtrating the virus-like nanoparticle.

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DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

COMPOSITION OF MATTER FOR INDUCING DEFENSE OR GROWTH IN A PLANT AND METHODS OF USE AND MANUFACTURE THEREOF

FIELD OF THE INVENTION

[0001] The present invention generally relates to the field of plant biology and agricultural technology. In particular, the present invention is directed to a composition of matter for inducing defense or growth in a plant and methods of use and manufacture thereof.

REFERENCE TO SEQUENCE LISTING

[0002] This specification includes a sequence listing submitted herewith, which includes the file entitled 1266-005USP1.xml having the following size: 9,426 bytes which was created October 23, 2024, the contents of which are incorporated by reference herein.

BACKGROUND

[0003] Plant protection products such as without limitation pesticides and fertilizers are widely used in agriculture to reduce losses and maintain high product quality. However, the large use of these products represents a source of persistent pollutants which pose many concerns about human and environmental health. Products for crop protection and nutrition systems that are both efficient and safe to use remain elusive.

SUMMARY OF THE DISCLOSURE

[0004] In an aspect, a composition for inducing defense or growth in a plant is described. The composition includes a virus-like particle engineered to express at least a defense-inducing peptide and a buffer. The plant may include *Brassicaceae*, *Arabidopsis* including *Arabidopsis thaliana*, *Solanaceae*, *Solanum including Solanum lycopersicum*, *Rosaceae*, *Fragaria* including *Fragaria x ananassa*, and *Poaceae*. The at least a defense-inducing peptide may include flg22, cape1, or systemin, among others.

[0005] In another aspect, a composition for treating a condition is described. The composition includes a virus-like particle engineered to express at least a peptide at its surface. The composition further includes a cargo encapsulated within the virus-like particle, wherein the cargo includes at least an active substance. The composition further includes a buffer.

[0006] In another aspect, a method of manufacturing a composition for inducing defense or growth in a plant is described. The method includes infecting a plant with a virus to produce a virus-like nanoparticle. The method further includes sampling symptomatic leaves from the plant. The method further includes homogenizing the virus-like nanoparticle. The method further includes incubating the virus-like nanoparticle. The method further includes centrifuging the virus-like nanoparticle. The method further includes filtrating the virus-like nanoparticle.

[0007] In another aspect, another method of manufacturing a composition for inducing defense or growth in a plant is described. The method includes agroinfiltrating a plant with a virus to produce a virus-like nanoparticle. The method further includes sampling symptomatic leaves of the plant. The method further includes homogenizing the virus-like nanoparticle. The method further includes centrifuging the virus-like nanoparticle. The method further includes filtrating the virus-like nanoparticle.

[0008] These and other aspects and features of nonlimiting embodiments of the present invention will become apparent to those skilled in the art upon review of the following description of specific nonlimiting embodiments of the invention in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] For the purpose of illustrating the invention, the drawings show aspects of one or more embodiments of the invention. However, it should be understood that the present invention is not limited to the precise arrangements and instrumentalities shown in the drawings, wherein:

FIG. 1 is a schematic illustration of an exemplary embodiment of a composition for inducing defense or growth in a plant;

FIG. 2 is a schematic illustration of an exemplary embodiments of a virus-like particle;

FIG. 3 is a schematic illustration of a *Cowpea mosaic virus* (CPMV);

FIG. 4 is a flow diagram illustrating an exemplary embodiment of a method of manufacturing the composition for inducing defense or growth in a plant;

FIG. 5 is a flow diagram illustrating an exemplary embodiment of another method of manufacturing the composition for inducing defense or growth in a plant;

FIG. 6 includes experimental data where AMP3 synthetic peptide displayed a strong antimicrobial activity against all plant pathogens tested; values leading to complete inhibition were 8 μg/mL for both Psa (panel A) and Pst (panel B), while against *A. tumefaciens* EHA105, the value was as low as 1,4 μg/mL (panel C);

FIG. 7 includes experimental data where AMP3-DE synthetic peptide displayed a strongly reduced antimicrobial activity compared to AMP3; while it was able to inhibit the growth of Psa (see panel A) at 11 μg/mL, it was completely harmless against both Pst (see panel B) and EHA105 (see panel C);

FIG. 8 includes experimental data where AMP3 and AMP3-DE displayed strong antifungal activity against *B. cinerea*; even when the AMP3 dosage was reduced to 2%, the antifungal activity was reduced to only 3%; the same approach with AMP3-DE lead to a reduction by 24%; values represent the average from measurements of three independent replicates;

FIG. 9 includes experimental data where AMP2 was mostly ineffective in growth inhibition against all bacterial strain tested; this result further confirms AMP2 as an antifungal peptide;

FIG. 10 includes experimental data where antibacterial activity of AMP2-DE reproduces the behavior AMP2, suggesting that the addition of foreign amino acids does not impact the antibacterial activity of AMP2;

FIG. 11 includes experimental data where AMP2-DE displayed an antifungal activity similar to the unmodified AMP2 peptide; both were able to inhibit the growth of *B. cinerea* to the same extent under the same concentration; values represent the average of measurements from three independent replicates;

FIG. 12 includes experimental data describing the expression of eCPMV-AMP1 and eCPMV-AMP2DE particles at 6 dpi; eCPMV- AMP1 shows a significantly reduced expression compared to eCPMV-AMP2DE; in both cases, the small CP was not detected; electrophoresis was performed under non-reducing conditions;

FIG. 13 includes experimental data where protein extraction and electrophoresis under strong reducing conditions allow for visualization of the SCP of eCPMV-AMP2DE and eCPMV-AMP1, suggesting that these subunits tend to form aggregates in the absence of a reducing agent; different lanes for the same construct represent two different rounds of leaf collection.; the presence of the VP60 precursor protein indicates that not all the eCPMV subunits have been processed;

FIG. 14 includes experimental data describing (panel A) a successful trial of purification with eCPMV-AMP2DE, but particles were recovered in a very low amount; (panel B) an example of purification trial with eCPMV-AMP1 and eCPMV-AMP2DE showing that PEG precipitation wasn't always reproducible;

FIG. 15 includes experimental data showing that the purification of eCPMV-AMP2DE (left panel) and of eCPMV-AMP1 (right panel) by chromatographic techniques was not possible due to the interaction between particles and the anionic exchange resin; a small portion of eCPMV-AMP2DE particles were recovered in the flow through (lane 3), while most of them flow in the column washing steps (lanes 4-5) and underwent a degradation process; eCPMV-AMP1 particles showed the same behavior as eCPMV-AMP2DE, but their interaction was stronger with the AEC resins, since they were recovered only after the elution step with the counter ion (lane 6); lanes 1 and 2 are representative of the first two steps of the purification process, while lane 7 indicates the sample before the loading into the SEC column;

FIG. 16 includes experimental data showing the effect of modifications to AMP3 on their expression on the eCPMV surface; all the modifications were able to slightly increase the expression of the

recombinant particle, suggesting that, in the genetic engineering of eCPMV, both the pI and the length of the peptide should be taken into consideration; the occurrence of the small CP was not observed regardless of the reducing conditions or the presence of Cys residues;

FIG. 17 includes experimental data showing that the infiltration of the MG132 proteasome inhibitor did not affect the expression of recombinant eCPMV-AMP3 particles, suggesting that the low expression is not due to a degradation process;

FIGS. 18A-B include schemes showing strategies of chemical conjugation targeting LYS residues on the surface of eCPMV; (panel As) direct conjugation of the flurophore AlexaFluor555-NHS which directly attaches to exposed LYS; (panel Bs) mechanism of Staudinger ligation; in the scheme, R should be considered as the CPMV while P as the peptide or the fluorescent molecule; briefly, LYS are targeted by NHS mediated chemistry with a double functional linker carrying both a NHS and a phosphine functional groups; after conjugation to lysines, the target molecule (P) reacts specifically with the phosphine reactive group; the Staudinger ligation method may be preferred due to its higher specificity and efficiency, especially for peptide-protein conjugation; the average yield of conjugated fluorophores is 27.6 for NHS and 25 for the Staudinger ligation;

FIG. 19 includes data showing the conjugation of AlexaFluor 488 to exposed lysines on eCPMV surface; the outcome of the conjugation is revealed by exposing the gel to UV-light before staining with InstantBlue™ (Expedeon); (1) CPMV-AlexaFluor 2 μg, (2) eCPMV-AlexaFluor 4 μg, and (3) eCPMV 4 μg;

FIG. 20 includes data showing the conjugation of AlexaFluor 488 to exposed lys on eCPMV surface; the outcome of the conjugation is revealed by exposing the gel to UV-light before staining with InstantBlue™ (Expedeon); UC stands for the unconjugated sample, while F stands for the excesses molecular fold (moles of peptide: moles of eCPMV asymmetric unit) used in the bioconjugation reaction;

FIG. 21 includes data from native agarose elctrophoresis of eCPMV conjugated chemically with 1. phosphine linker, 2. AlexaFluor488, 3. unconjugated eCPMV, 4. AMP3 with 5-fold molar excess of peptide, 5. AMP3 with 3 molar fold excess of peptide (see panel A); native agarose electrophoresis of eCPMV conjugated with AMP3: 1. control, 2-6. chemical conjugation with increasing molar excess (1-5) of peptide (see panel B); both in panel A and panel B, a precipitation of particles in the wells was observed, suggesting that once conjugated, eCPMV-AMP3 particles are not soluble;

FIG. 22A includes data from chemical conjugation by the Staudinger ligation reaction with a control peptide (VIP); eCPMV samples were assayed by an anti-VIP antibody; the presence of a signal corresponding to eCPMV subunits indicates that the conjugation occurred in both LCP and SCP;

FIG. 22B includes data from an evaluation regarding the presence of eCPMV particles in crude extract by western blot: 1. crude extracts filtered by miracloth, 2. extracts filter-sterilized through 0.20-μm filters which have subsequently been used in the experiment, 3. pellet recovered from the first centrifuge step;

FIG. 23 includes data from an evaluation of the fungal growth in the presence of crude extract expressing eCPMV-AMPs particles, both undiluted and diluted 1:10; results shown are the average of three independent experiments; no difference between the treatment was observed and ANOVA analysis failed in distinguishing between means; 48/24, 72/24 and 72/48 refer to the ration between different timing of measurements, which were taken starting 24 hours after the preparation of the plate;

FIG. 24 includes data from ultrafiltration of eCPMV-AMPs particles by centrifuge filters devices; crude extracts were concentrated by 1000 kDa MWCO filters, and both upper and lower fractions were loaded in SDS-PAGE and assayed by western blotting; 1. crude leaf extract, 2. centrifuged extract, 3. lower fraction (< 1000 kDa), 4. upper fraction (> 1000 kDa);

FIG. 25 includes data from expression analysis of recombinant eCPMV-ITPs particles; electrophoresis was performed under high denaturant condition to allow the visualization of small CPs, which show their expected increase in size;

FIG. 26 includes data from purification of eCPMV-ITP1 by combined chromatography techniques; the size increase of the small coat protein of eCPMV-ITP1 is clearly visible in all collected fractions;

FIG. 27 includes data from purification of eCPMV-ITP2; lanes after the positive control identify different recovered SEC fractions;

FIG. 28 includes data from eCPMV-ITP2 and eCPMV-ITP3 by PEG precipitation coupled with centrifuge-based separation. Lanes: 1. centrifuge supernatant, 2. pellet recovered after PEG removal, 3. ultracentrifuge supernatant, 4. ultracentrifuge pellet;

FIG. 29 includes data from purification of eCPMV-ITP2 and eCPMV-ITP3 by combined chromatography techniques; lanes: 1. crude extract after miracloth filtration, 2. centrifuge supernatant, 3. flow through of DEAE AEC, 4. (ITP3) elution from AEC, (ITP2) flow through, 5. AEC eCPMV-ITP2 elution;

FIG. 30 includes data from particle analysis by dynamic light scattering; (panel A) comparison between eCPMV-WT (red) and eCPMV-ITP1 (green) particles highlighting their similarity; (panel B) aggregation of eCPMV-WT; and (panel C) aggregation of eCPMV-ITP1 over the time; this phenomenon was initially observed at 24 h when particles are stored under RT or 4 °C; at later time points; aggregation did not increase significantly;

FIG. 31 includes TEM images showing eCPMV-WT (left panel) and eCPMV-ITP1 (right panel) particles; all particles showed the same shape and size;

FIG. 32 includes data showing that native agarose gel electrophoresis of eCPMV and eCPMV-ITP1 allow for visualization of entire capsids as broad bands into the gel after Coomassie staining; eCPMV occurs with two migration forms, due to removal of the C-terminus of the small CP leading to the fast form; particles retaining this sequence appear as the slow form in the upper part of the gel;

FIGS. 33A-B include data pertaining to an Apoaequorin based calcium assay performed in *Arabidopsis thaliana* Col-0 background plantlets; (A) Calibration of the assay based on the dose of the ITP1 peptide; (B) calcium assay with eCPMV and eCPMV-ITP1 nanoparticle treatment; five replicas have been performed for each condition;

FIG. 34 includes data showing that eCPMV-ITP2 treatment did not induce any variation in intracellular Ca^{2+} concentration; the figure shows the results of the first 20 minutes;

FIG. 35 includes data from visualization of *A. thaliana* protoplasts under bright field microscope with 40× magnification;

FIG. 36 includes data describing penetration into plant leaves; plant tissues were homogenized and then extract were assayed against an anti-CPMV antibody; numbers indicate the time of treatment (1 h, 6 h, and 24 h) while A and B indicate that the sample has been rinsed with water or not, respectively;

FIG. 37 includes data from a GUS staining assay on *A. thaliana* Col-0 pJAZ2:GUS transgenic plants; treatment was carried out for 24 h before staining; tissues were allowed to clarify completely in a 70% ethanol solution before image acquisition;

FIG. 38 includes data from GUS staining quantification by measurement of the saturated pixel using ImageJ; asterisks indicate the statistical significance after a t test comparison; *($p < 0.05$) ** ($p < 0.01$);

FIG. 39 includes data from analysis of ITP1-induced gene 1 h after nanoparticle treatment; values represent the average among three biological replicates; asterisks indicate the statistical significance after a t-test against the mock treated samples; * ($p < 0,1$), ** ($p < 0.05$), *** ($p < 0.01$);

FIG. 40 includes data from analysis of ITP1-responsive genes 1 h post treatment; here two different systems of treatment administration were tested: contact by dispensing the nanoparticles or the controls directly into the wells or by plants vacuum infiltration; values represent the average among three biological replicates; asterisks indicate the statistical significance after a t-test against the mock treated samples; * ($p < 0,1$), ** ($p < 0.05$), *** ($p < 0.01$);

FIG. 41 includes data from analysis of genes related to the principal defense hormone pathways; values represent the average among three biological replicates; asterisks indicate the statistical significance after a t-test against the mock treated samples; * ($p < 0,1$), ** ($p < 0.05$), *** ($p < 0.01$);

FIG. 42 includes data from analysis of ITP1-responsive genes after 24 h of treatment. The induction of these genes at 24 h suggests the occurrence of a delayed response to eCPMV-ITP1. Values represent the average among three biological replicates; asterisks indicate the statistical significance after a t-test against the mock treated samples; * ($p < 0,1$), ** ($p < 0.05$), *** ($p < 0.01$);

FIG. 43 includes data from analysis of ITP1-responsive genes after 6 h of treatment; the lack of induction of these genes, with the exception of ITP1 and ISG3, confirm the transient induction at 1 h post treatment; values represent the average among three biological replicates; asterisks indicate the statistical significance after a t-test against the mock treated samples; * ($p < 0,1$), ** ($p < 0.05$), *** ($p < 0.01$);

FIG. 44 includes data describing protection ability of eCPMV and eCPMV-ITP particles against *Pseudomonas syringae* pv. Tomato DC3000 inoculation; 5-8 two weeks old plants of *A. thaliana* were inoculated with Pst DC3000 after 24 or 48 h of treatment with eCPMV particles; bacterial growth was evaluated 3 days post inoculation; Silwett L-77 was added to the treatment to a final concentration of 0.02% to increase leaf wettability;

FIG. 45 includes data showing that eCPMV-WT and eCPMV-ITP1 particles start to exploit protection 24 h after treatment; when the treatment was performed 48 h before bacteria inoculation, complete protection was achieved from Pst DC3000 colonization;

FIG. 46 includes data showing that eCPMV and eCPMV-ITP1 could not provide protection from Pst DC3000 when it is vacuum infiltrated into plant leaves; the lack of protection hint at the possibility that particles play a role in hampering the bacterial colonization, preventing their penetration into plant leaves;

FIG. 47 shows a view of the interior cavity of eCPMV; the five-fold axis, which is the joining point of the small coat proteins, leaves a small opening indicated in the middle;

FIG. 48 includes data from native agarose electrophoresis showing the difference in migration rate between unloaded (UL) and loaded particles;

FIG. 49A includes data from native agarose electrophoresis showing the effect of the removal of the C-term on the migration rate of eCPMV particles; the presence of the C-term sequence slows down the migration of eCPMV in the agarose gel;

FIG. 49B includes data from dynamic light scattering of eCPMV before and after the cleavage reaction with chymotrypsin; particles size is not affected by the removal of the C-term; eCPMV forms naturally with and without this sequence;

FIG. 50 includes data from native agarose electrophoresis showing different trials of eCPMV loading; different batches of eCPMV purification gave different loading efficiencies; some molecules were loaded into eCPMV while others did not permeate the surface of the capsid;

FIG. 51 includes data describing resveratrol release from loaded eCPMV particles as an antifungal particle against *B. cinerea*; the release of resveratrol could not be evaluated as a growth inhibition since it strongly contributes to the absorbance itself; therefore, the release from eCPMV could be evaluated as an increase of absorbance over time, regardless of fungal growth; and

FIG. 52 includes data from evaluation of the release of kanamycin from eCPMV; the release of the molecule has been evaluated for its antibacterial activity against *P. syringae* pv. *actinidiae*; the growth of the bacteria was inhibited in two different media KB (panel A) and HIM (panel B), showing the ability of eCPMV to exploit its cargo delivery under different pH conditions.

The drawings are not necessarily to scale and may be illustrated by phantom lines, diagrammatic representations and fragmentary views. In certain instances, details that are not necessary for an understanding of the embodiments or that render other details difficult to perceive may have been omitted.

DETAILED DESCRIPTION

[0010] At a high level, aspects of the present disclosure are directed to a composition for inducing a defense or growth in a plant and the method of manufacture thereof. The composition includes a virus-like particle engineered to express at least a defense-inducing peptide and a buffer.

[0011] Aspects of the present disclosure can be used to protect plants from pathogens and/or stimulate the growth thereof without creating negative environmental impact. Exemplary embodiments illustrating aspects of the present disclosure are described below in the context of several specific examples.

[0012] To facilitate the understanding of this invention, a number of terms are defined below and throughout the disclosure. Unless otherwise defined, 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. The terminology herein is used to describe specific embodiments of the invention, but their usage does not limit the invention, except as outlined in the claims.

[0013] It is to be understood that any aspect and/or element of any embodiment of the method(s) described herein or otherwise may be combined in any way to form additional embodiments of the method(s) all of which are within the scope of the method(s).

[0014] Where a process is described herein, those of ordinary skill in the art will appreciate that the process may operate without any user intervention. In another embodiment, the process includes some human intervention (e.g., a step is performed by or with the assistance of a human).

[0015] For the purposes of this disclosure, including the claims, the phrase "at least some" means "one or more" and includes the case of only one. Thus, e.g., the phrase "at least some ABCs" means "one or more ABCs" and includes the case of only one ABC.

[0016] For the purposes of this disclosure, including the claims, the term "at least one" should be understood as meaning "one or more" and therefore includes both embodiments that include one or multiple components. Furthermore, dependent claims that refer to independent claims that describe features with "at least one" have the same meaning, both when the feature is referred to as "the" and "the at least one".

[0017] For the purposes of this disclosure, the term "portion" means some or all. Therefore, for example, "A portion of X" may include some of "X" or all of "X". In the context of a conversation, the term "portion" means some or all of the conversation.

[0018] For the purposes of this disclosure, including the claims, the phrase "using" means "using at least" and is not exclusive. Thus, e.g., the phrase "using X" means "using at least X". Unless specifically stated by use of the word "only", the phrase "using X" does not mean "using only X".

[0019] For the purposes of this disclosure, including the claims, the phrase "based on" means "based in part on" or "based, at least in part, on" and is not exclusive. Thus, e.g., the phrase "based on factor X" means "based in part on factor X" or "based, at least in part, on factor X". Unless specifically stated by use of the word "only", the phrase "based on X" does not mean "based only on X".

[0020] In general, for the purposes of this disclosure, including the claims, unless the word "only" is specifically used in a phrase, it should not be read into that phrase.

[0021] For the purposes of this disclosure, including the claims, the phrase "distinct" means "at least partially distinct". Unless specifically stated, distinct does not mean fully distinct. Thus, e.g., the phrase "X is distinct from Y" means that "X is at least partially distinct from Y" and does not mean that "X is fully distinct from Y". Thus, for the purposes of this disclosure, including the claims, the phrase "X is distinct from Y" means that X differs from Y in at least some way.

[0022] It should be appreciated that the words "first", "second", and so on, in the description and claims, are used to distinguish or identify, and not to show a serial or numerical limitation.

[0023] Similarly, letter labels (e.g., "(A)", "(B)", "(C)", and so on, or "(a)", "(b)", and so on) and/or numbers (e.g., "(i)", "(ii)", and so on) are used to assist in readability and to help distinguish or identify, and are not intended to be otherwise limiting or to impose or imply any serial or numerical limitations or orderings. Similarly, words such as "particular", "specific", "certain", and "given", in the description and claims, if used, are to distinguish or identify, and are not intended to be otherwise limiting.

[0024] For the purposes of this disclosure, including the claims, the terms "multiple" and "plurality" mean "two or more," and include the case of "two". Thus, e.g., the phrase "multiple ABCs" means "two or more ABCs" and includes "two ABCs". Similarly, e.g., the phrase "multiple PQRs" means "two or more PQRs" and includes "two PQRs".

[0025] The present invention also covers the exact terms, features, values, and ranges, etc., in case these terms, features, values, and ranges, etc., are used in conjunction with terms such as "about", "around", "generally", "substantially", "essentially", "at least", etc. Thus, e.g., "about 3" or "approximately 3" shall also cover exactly 3, and "substantially constant" shall also cover exactly constant.

[0026] For the purposes of this disclosure, unless stated otherwise, the terms "about" or "approximately" refer to a value that is within 10% above or below the value being described.

[0027] For the purposes of this disclosure, including the claims, singular forms of terms are to be construed as also including the plural form and vice versa, unless the context indicates otherwise. Thus, it should be noted that for the purposes of this disclosure, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. In other words, terms such as "a", "an", and "the" are not intended to refer to only a singular entity but include the general class of which a specific example may be used for illustration.

[0028] Throughout the description and claims, the terms "comprise", "including", "having", "contain", and their variations should be understood as meaning "including but not limited to" and are not intended to exclude other components unless specifically so stated.

[0029] For the purposes of this disclosure, the terms "administration" or "administering" refer to a method of giving a dosage of a compound or pharmaceutical composition to a subject. A composition described herein may be administered to a subject by any one of a variety of manners or a combination of varieties of manners. For example, a composition may be administered orally,

nasally, intraperitoneally, or parenterally, by intravenous, intramuscular, topical, or subcutaneous routes, or by injection into tissue.

[0030] For the purposes of this disclosure, an "effective amount" or "therapeutically effective amount" is the amount of a composition of this disclosure which, when administered to a subject, is sufficient to effect treatment of a disease or condition in the subject. The amount of a composition of this disclosure which constitutes a "therapeutically effective amount" may vary depending on the composition, the condition and its severity, the manner of administration, and the age of the subject to be treated.

[0031] For the purposes of this disclosure, the terms "treat", "treating", or "treatment" refer to administration of a compound or pharmaceutical composition for a therapeutic purpose. To "treat a disorder" or use for "therapeutic treatment" refers to administering treatment to a patient already suffering from a disease to ameliorate the disease or one or more symptoms thereof to improve the patient's condition (e.g., by reducing one or more symptoms of a neurological disorder). The term "therapeutic" includes the effect of mitigating deleterious clinical effects of certain processes (i.e., consequences of the process, rather than the symptoms of processes). As nonlimiting examples, a treatment may include (i) preventing a disease or condition from occurring in a subject, in particular, when such subject is predisposed to the condition but has not yet been diagnosed as having it; (ii) inhibiting a disease or condition, i.e., arresting its development; (iii) relieving a disease or condition, i.e., causing regression of the disease or condition; or (iv) relieving the symptoms resulting from a disease or condition, i.e., relieving pain without addressing the underlying disease or condition.

[0032] It will be appreciated that variations to the embodiments of the invention can be made while still falling within the scope of the invention. Alternative features serving the same, equivalent, or similar purpose can replace features disclosed in the specification, unless stated otherwise. Thus, unless stated otherwise, each feature disclosed represents one example of a generic series of equivalent or similar features.

[0033] Use of exemplary language, such as "for instance", "such as", "for example" ("e.g.,"), and the like, is merely intended to better illustrate the invention and does not indicate a limitation on the scope of the invention unless specifically so claimed.

[0034] While the invention has been described in connection with what is presently considered to be the most practical and embodiments thereof are further described in the examples below, it is to be understood that the invention is not to be limited to the disclosed embodiment, but on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

[0035] The following description sets forth various examples along with specific details to provide a thorough understanding of claimed subject matter. It will be understood by those skilled in the art, however, that claimed subject matter may be practiced without one or more of the specific details disclosed herein. Further, in some circumstances, well-known methods, procedures, systems, and/or components have not been described in detail in order to avoid unnecessarily obscuring claimed subject matter. The illustrative embodiments described in the detailed description and claims are not meant to be limiting. Other embodiments may be utilized, and other changes may be made, without departing from the spirit or scope of the subject matter presented here. It will be readily understood that the aspects of the present disclosure, as generally described herein, can be arranged, substituted, combined, and designed in a wide variety of different configurations, all of which are explicitly contemplated and make part of this disclosure.

[0036] Modern agriculture relies mainly on the monoculture approach. This approach proved to increase the yield but at the same time establishes the perfect environment for the spread and evolution of pathogens. As a result, pathogens outbreaks can be highly detrimental due to the increasing adaptation of pathogens to the same variety of cultivated crops. From an evolutionary point of view, crops are often facing an extremely disadvantageous challenge in the field, which is well described by the "Red Queen" paradox. This principle, from a plant pathology perspective, states that crops (hosts) are endlessly evolving to gain reproductive advantage, but their efforts are depleted by the continuously evolving threats (pathogens, pests and weeds) in an evolving environment. leading to an increasingly worthless effort and eventually a failure in reaching that advantage. In other words, everything is moving faster and faster, but nothing changes. From this point of view, crop resistance should be strategically driven for consideration of long-lasting resistance. Most agronomic strategies to prevent pathogen outbreaks may rely on the employment of new crop varieties (i.e., a genetic approach) and large amounts of agrochemical products (a chemical approach). Agrochemicals are very effective in reducing pathogen populations, which ultimately protects a production from significant losses, but this effect has been repetitively shown to be transient. Indeed, the problem of pests and pathogens resistance against chemical substances is a phenomenon observed already at the beginning of the XIX century and continuously increasing nowadays. Large pathogen populations and frequent outbreaks favor the evolution of pathogens developing pesticides resistance even towards traditional copper-based products, which have been always considered unlikely to be overcome.

[0037] The genetic approach also considers the replacement of susceptible varieties to resistant alternatives, either by traditional crossing or by genetic engineering. Although genetically modified

organisms (GMOs) proved to be highly efficient in protecting crops from pests, their employment is hampered by an ongoing debate regarding the safety issues and regulation of GMOs, at least in the European Union. Strategies to develop highly resistant crops aim to provide durable resistance by either eliminating susceptibility genes by implementing the pyramiding concept, wherein resistance is treated as an outcome of multiple recognition spectra and environmental optima into a single background. Although this strategy holds promise, the cost for the development of a GM crop is often incredibly high.

[0038] In addition, agriculture is far from being efficient, and some of it is due to the high dispersal of both fertilizers and pesticides. Indeed, 10-75% of fertilizers are typically lost, while less than 10% of pesticides are capable of reaching their targets.

[0039] Plant protection plays a major role in the development of sustainable agriculture and agricultural biotechnology, which is at the center of global food security. For the purposes of this disclosure, a pesticide is a chemical composition whose goal is to inhibit pests, pathogens, and/or weeds. A pesticide may include any type of agrochemical capable of fulfilling this goal, as recognized by a person of ordinary skill in the art, upon reviewing the entirety of this disclosure. Pesticides and their formulations often raise concerns regarding their target specificity, efficacy, and environmental safety, etc. Indeed, their extensive usage with questionable dispersion methods is often the object of continuous concerns and scrutiny.

[0040] Innovation in agronomy, particularly in the development of alternative plant protection products to replace or complement current solutions, is therefore considered of primary importance for achieving the sustainability goals in agriculture under the Common Agricultural Policy (CAP). In addition, the development of tools for the release of active ingredients from plant protection products is often considered equally important. These aspects may facilitate the creation of tools that can be adapted to producers' needs while safeguarding the toxicological profile of the agrarian ecosystem and human health.

[0041] Nanotechnology approached agriculture at the beginning of this century to address the inefficiencies therein by targeting sustainability goals in different aspects: economic, environmental, and social, among others. For the purposes of this disclosure, nanotechnology is a type of technology based on nanomaterials. For the purposes of this disclosure, a nanomaterial is a natural, incidental, or manufactured material containing particles, either in an unbound, freestanding state or as an aggregate or agglomerate, where 50% or more of the particles have one or more external dimensions in the size range from 1 nanometer to 100 nanometers. At a nanoscale level, nanomaterials are expected to be more efficient due to their higher surface to volume ratio, bioavailability, persistence,

and reactivity, although their mechanisms of action are not yet completely understood. Applications of nanomaterials in agriculture include without limitation remote sensing for assessment of physiological conditions of crops, nanodevices configured to respond to different external stimuli, manipulation of a microbiome, and efficient allocation and use of resources and irrigation to enable a smart release of chemical compounds, among others. The use of nanomaterials in agriculture may also be referred to as precision agriculture and is expected to transform the agricultural landscape. However, while this approach holds promise, it should be noted that agriculture is a more complex system than the medical field where nanomaterials have achieved wide-spread success. Indeed, the heterogeneity of climatic conditions, soil, crop, wildlife, and environment in general makes it more challenging to develop a product that addresses all possible side effects. Moreover, due to the low-profit margin nature of this industry, products must be sufficiently economical with an increased efficiency with respect to what is traditionally employed. The high potential of nanomaterials is generally shown at a laboratory scale, but often lack the information pertaining to the efficacy in the field due to the high cost associated thereto.

[0042] Given the need to develop alternative, effective, and safe crop protection and nutrition systems for sustainable agriculture capable of delivering high-quality products, the invention described herein is directed towards the development of an innovative form of pathogen biocontrol and/or biostimulants. The invention is implemented using protein nanoparticles coupled with fully biodegradable biomolecules. These biodegradable biomolecules are based on peptides capable of inducing plant defense or stimulating plant growth. Protein nanoparticles functionalized with such biodegradable biomolecules may be produced on a medium scale using plants as "bioreactors" for eco-sustainable and inexpensive production. The invention may be applied to small fruits including without limitation strawberries and tomatoes, whose cultivation is economically relevant and requires high quality standards.

[0043] Referring now to FIG. 1, an exemplary embodiment of a composition 100 for inducing defense or growth in a plant illustrated. In one or more embodiments, composition 100 may include a virus-like particle 104. For the purposes of this disclosure, a "virus-like particle (VLP or eVLP)" is a nanoparticle that structurally resembles a virus but is devoid of genetic material at its center. In one or more embodiments, composition 100 may include a virus nanoparticle 108. For the purposes of this disclosure, a "virus nanoparticle" is a proteinaceous and often infectious nanoscale structure that is capable of delivering its nucleic acid efficiently into a host cell, enabling production of new viruses therein. In other words, the presence or absence of genetic material is the difference that distinguishes between virus-like particles (VLPs or eVLPs) and viral nanoparticles (VNPs). "Virus"

and "virus nanoparticle" may be used interchangeably throughout this disclosure. Similarly, "plant virus" and "virus-like particle" may be used interchangeably as well.

[0044] With continued reference to FIG. 1, viruses are a source of naturally occurring nanomaterials. Viruses are characterized by a broad diversity of shapes, sizes and surface chemistries. Viral structures are also known for their stability and resistance against extreme chemical-physical conditions, such as without limitation UV-light, extreme temperatures, and gastrointestinal fluids, among others. Plant viruses are generally considered safe for both humans and mammals, since it is unlikely for a plant virus to replicate in a mammalian cell. Plant viruses may be used to stimulate an immune response and may be considered candidates as vaccine adjuvants or for cancer therapy. Recent advances on high-resolution imaging techniques and bioinformatics facilitate the development of nanotools based on viral structures, but the dynamics of how a capsid assembles and what residues are important are still under investigation and far from being completely understood for most viruses. Particularly, some viruses are able to assemble into complete capsids without retaining any genetic material therein, which makes them completely un-infectious, hence biologically safe.

[0045] A virus includes a capsid. For the purposes of this disclosure, a "capsid" is a protein shell exposed at the exterior of a virus that possesses a specific geometric pattern. The terminologies "capsid" and "viral capsid" may be used interchangeably throughout this disclosure. Viral capsids can be regarded as naturally occurring nanoparticles that are optimized to carry, protect, and deliver a cargo to specific targets. Viral capsids have uniform size and shape that is unlikely to be reproduced by a chemist and/or via a synthetic approach. The genetic information encoding the viral capsids with such high accuracy is stored in the viral genome. As a nonlimiting example, capsid may possess an icosahedral shape. For the purposes of this disclosure, an "icosahedron" is a geometric shape with 20 sides, each composed of an equilateral triangle. As another nonlimiting example, capsid may include a filamentous structure. For the purposes of this disclosure, a "filamentous structure" is an elongated, thread-like formation that makes up a capsid of certain viruses. As another nonlimiting example, capsid may include a rod-shaped structure. As another nonlimiting example, capsid may include a helical structure. For the purposes of this disclosure, a "helical structure" is a type of structure characterized by a cylindrical, elongated shape with a helical symmetry. This structure may be formed by the regular, repeating arrangement of protein subunits around a viral nucleic acid, providing protection, structural integrity, and aiding in the infectivity of a virus. As another nonlimiting example, capsid may include a spherical structure. Additionally,

and/or alternatively, capsid may adopt any geometry not disclosed herein yet deemed possible by a person of ordinary skill in the art upon reviewing the entirety of this disclosure.

[0046] With continued reference to FIG. 1, NPs, VLPs, and/or eVLPs may be prepared using genetic engineering methods described in further detail below, such as without limitation by infecting a plant host using a plasmid. For the purposes of this disclosure, a "plasmid" is a circular, double-stranded DNA molecule distinct from a cell's chromosomal DNA and capable of autonomous replication. A plasmid may be used as a vector for insertion, expression, and propagation of foreign genes within a host organism. Such vectors may include specific sequences for an origin of replication, selectable markers, and cloning sites, enabling manipulation and study of genetic material for applications in research, biotechnology, and therapeutic development.

[0047] With continued reference to FIG. 1, the surface chemistry of VNPs, VLPs, and/or eVLPs may be modified using bioconjugation. Specifically, additional moieties may be attached to VNPs, VLPs, and/or eVLPs through their exposed functional groups. Such functional groups may include without limitation an azide group, a carboxyl group, a phenol group, a mercaptan group, an amino group, an alkynyl group, and/or the like. In some cases, this step may be implemented using click chemistry, biorthogonal chemistry, or the like, as recognized by a person of ordinary skill in the art, upon reviewing the entirety of this disclosure.

[0048] With continued reference to FIG. 1, in one or more embodiments, virus-like particle 104 and/or virus nanoparticle 108 may include at least a unit or subunit of a viral protein. For the purposes of this disclosure, a "viral protein" is a protein that constitutes or is produced by a virus. Since plant viruses are unable to replicate in mammals, viral proteins may be a safer option for medical applications in human hosts. Nanoparticles created from subunits of viral proteins may be genetically engineered to express, on their external surface, a peptide capable of inducing defense or growth in a plant, as described in further detail in this disclosure.

[0049] With continued reference to FIG. 1, in one or more embodiments, viral protein may be produced through a process of molecular farming. For the purposes of this disclosure, "molecular farming" is a process of producing pharmaceutically important and commercially valuable proteins in plants. Once extracted, viral proteins may be used to create virus-like particle 104. Virus-like particle 104 and/or viral protein may be sourced from a variety of plant hosts. Suitable host plants for such purpose may include a *Nicotiana benthamiana* plant, a *Nicotiana tabacum* plant, a *Solanum lycopersicum* or *Lycopersicon esculentum* plant, a *Cycorium intybus* plant, a *Brassica oleracea* var. *capitata* plant, a *Beta vulgaris* var *cicla* plant, a *Ocimum basilicum* plant, a red beet plant, a spinach plant, or the like. For the purposes of this disclosure, a "*Nicotiana benthamiana* plant" is a close

relative of tobacco and a species of Nicotiana indigenous to Australia. It may be used for farming monoclonal antibodies and other recombinant proteins. For the purposes of this disclosure, a "*Nicotiana tabacum plant*", commonly known as cultivated tobacco, is a plant species that belongs to the *Nicotiana* genus in the *Solanaceae* family. It is a widely grown herbaceous plant primarily used to produce tobacco products. This species is characterized by large, broad leaves and is cultivated in various climates worldwide. In some cases, a Nicotiana tabacum plant may serve as a model organism and a host for genetic engineering, enabling the expression and study of recombinant proteins, vaccines, and other biologically significant compounds. For the purposes of this disclosure, a "*Solanum lycopersicum* plant" or "*Lycopersicon esculentum* plant", commonly known as the tomato plant, is a plant species that belongs to the *Solanaceae* family and characterized by its production of edible, fleshy fruits. *Solanum lycopersicum* is noted for its agricultural significance and its utility in genetic engineering and plant breeding. This species may serve as a model organism for studying plant genetics, disease resistance, and metabolic pathways. It may also be utilized in biotechnological applications for the production of recombinant proteins, novel traits, and improved cultivars through genetic modification techniques. For the purposes of this disclosure, a "*Cycorium intybus* plant" is a hardy plant widely used in folklore medicine to treat various ailments ranging from wounds to diabetes. It is believed to have antimicrobial, anthelmintic, antimalarial, hepatoprotective, antidiabetic, gastroprotective, anti-inflammatory, analgesic, antioxidant, tumor-inhibitory, and antiallergic activities across several different cultures. For the purposes of this disclosure, a "*Brassica oleracea* var. *capitata* plant" is a is a biennial plant grown as an annual for its dense-leaved heads and characterized by a short stem and a rosette of green, purple, or white leaves that form a tight, globular, and compact head. It is known as wild cabbage in its uncultivated form. Some cultivated forms of *Brassica oleracea* var. *capitata* plant include cabbage, broccoli, cauliflower, kale, brussels sprouts, collard greens, savoy cabbage, kohlrabi and gai lan. It is native to costal southern and western Europe. For the purposes of this disclosure, a "*Beta vulgaris* var *cicla* plant" is a plant that is more commonly known as chard or spinach beet. It originates from the Mediterranean and has some medicinal properties, mainly in boosting the immune system and lowering blood pressure. For the purposes of this disclosure, an "*Ocimum basilicum* plant" is a member of the *Lamiaceae* (mint) family and is more commonly referred to as basil. For the purposes of this disclosure, a "red beet plant" is a biennial plant grown as an annual for its edible root and leafy greens and characterized by its swollen root, which is typically deep red or purple in color. It is a promising candidate for some medicinal uses. As a nonlimiting example, the phytochemicals present in red beet may provide protection against diseases including cancer and cardiovascular

diseases. For the purposes of this disclosure, a "spinach plant" is a leafy green that belongs to the amaranth family and is closely related to beets and quinoa.

[0050] With continued reference to FIG. 1, in one or more embodiments, virus-like particle 104 and/or virus nanoparticle 108 may be related to or derived from *Cowpea mosaic virus* (CPMV). For the purposes of this disclosure, a "*Cowpea mosaic virus* (CPMV)" or "*Sunn-hemp mosaic virus*" is a *picorna*-like, non-enveloped virus that belongs to the order *Picornavirales*, the family *Secoviridae*, and the genus *Comoviridae*. Infection of a susceptible cowpea leaf may cause a "mosaic" pattern in the leaf and result in high virus yields. CMPV may also naturally infect the black-eyed pea plant *Vigna unguiculata*. CMPV is a bipartite virus that encapsidates a genome containing two separate RNA segments: RNA-1 (6 kb) and RNA-2 (3.5 kb). The capsid of CPMV shows three quasi-equivalent conformers of a T = 3 icosahedral lattice. The RNA-2 segment contains the sequence encoding the VP60 polyprotein, the precursor of the Small (S) and Large (L) coat proteins, which are separated by a proteolytical cleave by the 24 K proteinase encoded by the RNA-1. A CPMV capsid contains 60 copies of an asymmetric unit, wherein each asymmetric unit includes the small (S) and the large (L) coat protein organized in three β-barrel domains. The structure of CPMV is well-characterized to an atomic resolution. CPMV may be thermostable and readily isolated from plants. There are many stable mutants of CPMV already prepared that allow specific modification of their capsid surfaces. In some cases, CPMV may include an icosahedral capsid, as described above. The Cowpea mosaic virus (CPMV) has been one of the most extensively studied viruses since its first description and has been exploited for many different applications due to its feasibility to be handled and modified, which makes CPMV one of the most suitable viral scaffolds for further development.

[0051] With continued reference to FIG. 1, the assembly model for both CPMV and eCPMV has been recently proposed, and it has been proven that the C-terminus plays a central role in the formation of the capsid without genomic RNA. This hypothesis has been recently demonstrated by multiple amino acid substitutions in the C-term sequence and by C-term deletion mutants. The biotechnological approach in the creation of deconstructed viral vectors (i.e., the isolation of the sequences encoding the VP60 polyprotein and the 24 K proteinase) provided for their expression as highly efficient vectors. This approach resulted in the generation of the pEAQ-HT vectors in 2013 that have been further improved to achieve improved expression and translational efficiencies. Moreover, through *Agrobacterium*-mediated transformation of plants, it is possible to produce eCPMV particles in *Nicotiana benthamiana* plants with a high yield, up to 1 mg/g of fresh tissue. For comparison, a typical yield is around 0.1-0.2 mg/g.

[0052] With continued reference to FIG. 1, the very first applications of CPMV in the medical field date back to the 90s when CPMV emerged as a potential nanoscale tool for the display of immunogenic peptides as a chimeric nanoparticle. The initial enthusiasm then fades following the revelation of safety issues due to the presence of viral RNA within the nanoparticles. However, the development of eVLP systems partly addressed this issue. From the discovery that eCPMV can bind to vimentin filament in cancer cells, the era of eCPMV began wherein eCMPV was used as an adjuvant in cancer therapy for *in situ* vaccine treatment. Moreover, a substantial number of pieces of evidence showed that both CPMV VNP and eVLPs are inherently safe for mammals and are rapidly cleared after intravenous injection without any side effect even after high dosage (up to 100 mg/ kg of body weight). The possibility for chemical modification on the virus capsid, such as without limitation bioconjugation with a fluorophore, has been widely exploited to study the properties of CPMV during its interaction with human cells. As a nonlimiting example, CPMV labelled with AlexaFluor 647 or Oregon Green 488 combined with or without PEG2000 led to the discovery that the capsid can bind to vimentin; this interaction is no longer present when PEG2000 is present, which suggested a specific recognition mechanism. Moreover, CPVM can pass through the membrane of mammalian cells before releasing its genetic material, but it is not able to complete its replication cycle afterwards. More recent discoveries highlight an intriguing role for both VNP and eVLPs in cancer treatment. Particularly, eCPMV nanoparticles can induce a protective barrier for tumor development in mice by activating an immune response within the tumor microenvironment instead of by directly killing the tumor itself. CPMV and eCPMV similarly stimulate the immune system by the activation of TLR receptors which recognize the repetitive units in the capsid, but only CPMV provides further stimulation due to the presence of the genetic material therein. All viral capsids include repetitive units and some of these can be recognized as pathogen-associated molecular patterns (PAMPs) by mammal cells to trigger an immune response. Indeed, the CPMV capsid may be recognized by TLR2/TLR4 receptors and its RNA by TLR5/TLR7 receptors, either in mouse spleen cells or in human peripheral mononuclear blood cells. Therefore, to achieve proper therapeutic effects, both eCPMV and CPMV could be employed in combination with other antitumoral compounds to strengthen the immune response. Compared to other adjuvants like Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA), CPMV offers a promising alternative due to its possibility; chemical modifications may be introduced in a manner similar to peptide conjugation, which make it a bifunctional ingredient that is both an adjuvant and an epitope display platform. This is of particular interest due to the low production cost of CPMV *in planta* and due to the development of precise and site-specific conjugation reactions. However, the

cost of chemical conjugation or other modifications may increase the overall cost for producing recombinant CPMV VNPs or eVLPs and at the same time decrease the yield of the functionalized nanoparticles. As an alternative, peptide display could be achieved by a molecular farming approach, as described above, although this approach could be sometimes cumbersome depending on the peptide sequence inserted. However, the development of a CPMV-based therapy by chemical conjugation could be economically advantageous for the medical field but not for an agronomical application, for which the genetic approach is likely more suitable.

[0053] With continued reference to FIG. 1, the availability of deconstructed clones raised the possibility of genetic engineering of both CPMV and eCPMV, i.e., the possibility to present on the exterior surface of the capsid a peptide or an entire protein. Indeed, on eCPMV, there are a few amenable sites for modifications, but the βB-βC loop on the small (S) coat protein is generally preferred due to its higher solvent exposition. Accordingly, the first approach has been successful and encouraging, and the first example of a potential vaccine candidate completely produced in plants was demonstrated. It is worth noting that these first trials were made with VNPs and not with eVLPs. However, recently, the feasibility of this approach has been supported experimentally in the medical field with eCPMV. Despite these recent advances, no applications in agriculture have been attempted so far, and the genetic engineering of eCPMV for peptide display remains controversial. Indeed, more recent efforts focus on the chemical modification of eCPMV for both medical and agricultural applications instead. This invention is focused on the development of CPMV as a virus-like particle (VLP), which hereinafter may simply be referred to as eCPMV.

[0054] With continued reference to FIG. 1, in one or more embodiments, virus-like particle 104 and/or virus nanoparticle 108 may be configured to express at least a defense-inducing peptide 112. For the purposes of this disclosure, a "defense-inducing peptide" is a short chain of amino acids designed to induce a defensive response in a plant. In some cases, defense-inducing peptide 112 may also be referred to as an elicitor. Nonlimiting examples of defense-inducing peptide 112 include flg22, cape1, systemin, and/or the like. In one or more embodiments, virus-like particle 104 and/or virus nanoparticle 108 may be configured to express at least a biostimulatory peptide 116. For the purposes of this disclosure, a "biostimulatory peptide" or "BP" is a short chain of amino acids designed to stimulate the growth of a plant. In one or more embodiments, defense-inducing peptide 112 and/or biostimulatory peptide 116 may include an ion transport peptide. In some cases, defense-inducing peptide 112 and/or biostimulatory peptide 116 may be referred to as a bioactive peptide. In some cases, inducing peptide 112 and/or biostimulatory peptide 116 may be configured for a programmed release from virus-like particle 104 and/or virus nanoparticle 108. Additional details

will be provided below in this disclosure. Recognition of peptides derived from microbes or the plant itself may activate plant defense responses that protect them against a wide range of pathogens. In addition, natural peptides may function as biostimulants with a capability of improving plant growth and resilience even against abiotic stresses. However, despite their efficiency under controlled conditions, peptides directly applied to plants may not be stable and are often prone to degradation. Moreover, the use of peptides may be limited by their low bioavailability, prohibitive cost, and/or the environmental impact of the materials used for their synthesis. There is therefore a need to combine bioactive peptides with novel delivery techniques by exploiting nanotechnology, such as without limitation by using virus-like particle 104 and/or virus nanoparticle 108. These (nano)particles are protein structures with a normal viral architecture but sometimes without genetic material inside and may be engineered to expose peptides of interest on their surface.

[0055] With continued reference to FIG. 1, in one or more embodiments, virus-like particle 104 and/or virus nanoparticle 108 may include a cargo 120. In other words, virus-like particle 104 and/or virus nanoparticle 108 may function as a nanocarrier. For the purposes of this disclosure, a "cargo" is a chemical composition that is capable of being encapsulated in a hollow structure and delivered to a target location within a living organism. Cargo 120 may include any cargo deemed suitable and/or relevant by a person of ordinary skill in the art, upon reviewing the entirety of this disclosure. Nonlimiting examples of cargo 120 may include pharmaceutical compounds such as kanamycin-sulfate, DAPI, dicumarol, quercetin, luteolin, resveratrol, folpet, and/or the like, as described in further detail below in this disclosure.

[0056] With continued reference to FIG. 1, in one or more embodiments, composition 100 may further include a buffer 124. Buffer may stabilize virus-like particle 104/virus nanoparticle 108 and maintain a pH for increased stability and functionality. For the purposes of this disclosure, a "buffer" is a solution or mixture that contains at least a pair of weak acid, HA, and its conjugate base, A^-, (i.e., the weak acid minus one proton) in a molar ratio between 10:1 and 1:10, wherein the solution maintains a stable pH close to the pK_a (i.e., the negative log of the acid dissociation constant, K_a) of the weak acid, against addition of acidic or basic chemical species. For simplicity, a buffer containing a pair of conjugate base and acid may be written as A^-/HA. Additional examples will be provided below. The pH of a buffer solution may be calculated using the Henderson Hasselbalch equation:

$$pH = pK_a + \log\left(\frac{[A^-]}{[HA]}\right)$$

[0057] With continued reference to FIG. 1, a buffer may include any type of buffer deemed suitable by a person of ordinary skill in the art upon reviewing the entirety of this disclosure. As another nonlimiting example, a buffer may include an acetate buffer (i.e., CH_3COONa/CH_3COOH). As another nonlimiting example, a buffer may include a borate buffer (i.e., $Na_2B_4O_7 \cdot 10H_2O/H_3BO_3$). As another nonlimiting example, a buffer may include a bicarbonate buffer (i.e., $NaHCO_3/H_2CO_3$ or $Na_2CO_3/NaHCO_3$, depending on the desired pH). As another nonlimiting example, a buffer may include a cacodylate buffer (i.e., $NaC_2H_6AsO_2/HC_2H_6AsO_2$). As another nonlimiting example, a buffer may include a Good's buffer. For the purposes of this disclosure, "Good's buffers" are a group of more than 20 conjugate acid/base pairs selected and described by Norman Good and colleagues for biochemical and biological research during 1966–1980. For simplicity, only the conjugate acid may be shown for each conjugate acid/base pair. Good's buffers include MES ($C_6H_{13}NO_4S$), ACES ($C_4H_9NO_4S$), PIPES ($C_8H_{18}N_2O_6S_2$), MOPS ($C_7H_{15}NO_4S$), TES ($C_6H_{15}NO_6S$), HEPES ($C_8H_{18}N_2O_4S$), Tricine ($C_6H_{13}NO_5$), TRIS ($C_4H_{11}NO_3$), Bicine ($C_6H_{13}NO_4$), TAPS ($C_7H_{17}NO_6S$), CHES ($C_8H_{17}NO_3S$), CAPS ($C_9H_{19}NO_3S$), AMPSO ($C_9H_{19}NO_4S$), Gly-Gly ($C_4H_8N_2O_3$), ADA ($C_4H_7NO_4$), BES ($C_6H_{15}NO_5S$), MOPSO ($C_7H_{15}NO_5S$), EPPS ($C_9H_{20}N_2O_4S$), HEPPS ($C_{11}H_{24}N_2O_4S$), CAPSO ($C_9H_{19}NO_4S$), HEPPSO ($C_9H_{20}N_2O_5S$), CABS ($C_{10}H_{19}NO_3S$), ACESO ($C_4H_9NO_5S$), TES-Na ($C_6H_{14}NO_6SNa$), BICINE-Na ($C_6H_{12}NO_4Na$), TRICINE-Na ($C_6H_{12}NO_5Na$), MES-Na ($C_6H_{12}NO_4SNa$), HEPES-Na ($C_8H_{17}N_2O_4SNa$), MOPS-Na ($C_7H_{14}NO_4SNa$), and PIPES-Na ($C_8H_{17}N_2O_6S_2Na$). As a nonlimiting example, buffer may include a phosphate buffer (i.e., NaH_2PO_4/H_3PO_4, Na_2HPO_4/NaH_2PO_4, or Na_3HPO_4/ Na_2HPO_4, depending on the desired pH). As another nonlimiting example, buffer may include a phosphate-buffered saline (PBS) solution, a commonly used buffer in biological research and pharmaceutical formulations that typically contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. A person of ordinary skill in the art, upon reviewing the entirety of this disclosure, will be able to recognize suitable buffers for composition 100.

[0058] With continued reference to FIG. 1, composition 100 may be used to treat a condition and/or alleviate one or more symptoms thereof. Such conditions may include without limitation a bacterial, fungal, and/or viral infection. In some cases, composition 100 may be used to treat one or more conditions pertaining to humans or animals. Accordingly, composition 100 may be applied in any suitable form of dosage and/or delivery, including without limitation oral dosage and intravenous dosage. For the purposes of this disclosure, an "oral dosage" is an ingestion of a composition through the mouth. Oral dosage of composition 100 may include use of pills, syrup, tablets, thin film, liquid solution, powder, solid crystals, natural or herbal plants, seeds, or food,

pastes, or the like. For the purposes of this disclosure, an "intravenous dosage" is an administration of a composition using injection. Composition 100 may be given intravenously in any suitable manner, including as a bolus and/or as an infusion. Alternatively, other injection methods such as without limitation intramuscular injection, intraperitoneal injection, subcutaneous injection, and/or transcutaneous injection may be used.

[0059] With continued reference to FIG. 1, in some other cases, composition 100 may be used to treat one or more conditions pertaining to a plant. Such conditions may include without limitation powdery mildew, a fungal disease that produces white powdery spots on leaves and stems, affecting crops such as wheat, grapes, and cucumbers; blight, a class of diseases causing rapid tissue death, including late blight in potatoes and tomatoes caused by *Phytophthora infestans*; rust, a fungal infection caused by *Puccinia* species that creates rust-colored spots on plants such as without limitation wheat, beans, and coffee; leaf spot, a class of bacterial or fungal infections causing discolored spots on leaves, including without limitation *Septoria* leaf spot in tomatoes and *Cercospora* leaf spot in sugar beets; root rot, a disease often caused by fungi such as without limitation *Pythium*, *Rhizoctonia*, and *Fusarium* that leads to decayed roots in crops such as without limitation soybeans and cotton; fusarium wilt, a soil-borne fungal disease caused by *Fusarium oxysporum* that affects crops such as without limitation tomatoes, bananas, and cotton; downy mildew, a disease caused by fungi-like organisms (*Peronospora* species) that leads to fluffy growth on the underside of leaves, commonly affecting cucumbers, grapes, and lettuce; anthracnose, a disease caused by *Colletotrichum* species that leads to dark lesions on leaves, stems, and fruits, impacting beans, corn, and peppers, among others; clubroot, a disease of *brassicas* (e.g., cabbage, broccoli) caused by *Plasmodiophora brassicae* that leads to swollen and deformed roots; bacterial wilt, a disease caused by *Ralstonia solanacearum* that impacts crops such as without limitation tomatoes and potatoes by affecting their the vascular system; black spot, a fungal disease that causes black spots on leaves and commonly affects roses and other ornamental plants; *verticillium* wilt, a soil-borne fungal disease caused by *Verticillium dahlia* that leads to wilting and yellowing in crops like tomatoes, eggplants, and potatoes; canker, an infection caused by bacterial or fungal pathogens that leads to sunken, dead areas on tree bark, often affecting apple, citrus, and oak trees; scab, a fungal disease caused by *Venturia inaequalis* (apple scab) and *Streptomyces scabies* (potato scab) that produces rough, scabby lesions on fruits and tubers, commonly affecting apples and potatoes; mosaic virus, a viral infection that causes mosaic-like patterns on leaves, affecting crops such as without limitation tobacco, tomatoes, cucumbers, and beans. Accordingly, composition 100 may be applied using suitable means, such as without limitation spraying including electrostatic spraying,

dusting, soil injection/soil drenching, seed treatment, fumigation, granular application, aerial application, chemigation, trunk injection, and/or wipe-on application, among others. Composition 100 may be applied to any region of a plant, such as without limitation the leaves, flower, fruit, stem, root, or the like. Composition 100 may be applied to any type of plant, such as without plants that may be used as food sources (e.g., crops), horticulture plants, ornamental plants, and/or the like. Plants that may be treated with composition 100 include without limitation botanical families, genera, or species such as *Brassicaceae* or *Cruciferae*, *Arabidopsis* including *Arabidopsis thaliana*, *Solanaceae*, *Solanum* including *Solanum lycopersicum*, *Rosaceae*, *Fragaria* including *Fragaria x ananassa*, and *Poaceae*, among others.

[0060] Referring now to FIG. 2, an exemplary embodiment 200 of virus-like particle 104 is illustrated. Embodiment 200 may be a structural representation of CPMV. In one or more embodiments, virus-like particle 104 may include a large coat protein subunit 204. As a nonlimiting example, for CPMV, virus-like particle 104 may include 60 copies of large coat protein subunit 204, consistent with details described above. In one or more embodiments, virus-like particle 104 may include a small coat protein subunit 208. As a nonlimiting example, virus-like particle 104 may include 60 copies of small coat protein subunit 208. In one or more embodiments, virus-like particle 104 may include at least a defense-inducing peptide 112. In some cases, defense-inducing peptide 112 may be embedded within coat protein. In some cases, defense-inducing peptide 112 may be located outside of coat protein. In some cases, defense-inducing peptide 112 may be exposed on the surface of large coat protein subunit 204. In some cases, defense-inducing peptide 112 may be located inside of virus-like particle 104. In some cases, defense-inducing peptide 112 may be placed inside of coat protein. The embodiments described herein may also apply to one or more biostimulatory peptides 116 instead of or in addition to defense-inducing peptide 112.

[0061] With continued reference to FIG. 2, in one or more embodiments, virus-like particle 104 may include an icosahedral structure. In one or more embodiments, virus-like particle 104 may include a filamentous structure. In one or more embodiments, virus-like particle 104 may include a rod-shaped structure. In one or more embodiments, virus-like particle 104 may include a helical structure. In one or more embodiments, virus-like particle 104 may include a spherical structure. In one or more embodiments, virus-like particle 104 may be homogeneous or uniform in size. In one or more embodiments, virus-like particle 104 may be homogeneous or uniform in shape. Additionally, and/or alternatively, virus-like particle 104 may adopt any geometry not disclosed herein yet deemed possible by a person of ordinary skill in the art upon reviewing the entirety of this disclosure. The

same embodiments disclosed with respect to FIG. 2, embodiment 200, may apply to virus nanoparticle 108 instead of virus-like particle 104.

[0062] Referring now to FIG. 3, an exemplary structure 300 of CPMV is illustrated, consistent with details described elsewhere in this disclosure.

[0063] Referring now to FIG. 4, a flow diagram illustrating an exemplary embodiment of method 400 for manufacturing virus-like particle 104 is illustrated. It is worth noting that method 400 may not be limited to virus-like particle 104 but may instead be applicable to a wide variety of virus-based nanostructures, such as without limitation virus nanoparticle 108. Virus-like particle 104 may include any type of virus-like particle described in this disclosure without limitation. At step 405, method 400 includes infecting a plant with a virus to produce virus-like particle 104. For the purposes of this disclosure, "infection" is a process of delivering viral genes into a host, wherein the viral genes are capable of replication to produce new copies of the corresponding virus. Infection allows a plant to produce virus-like particle 104. In one or more embodiments, infection may be performed by spontaneous infiltration. For the purposes of this disclosure, "spontaneous infiltration" is a type of infiltration that is associated with a negative Gibbs free energy change and occurs naturally without energy input or intervention. For the purposes of this disclosure, "infiltration" is a process through which one or more substances penetrate or permeate from the surface of a plant into its tissues. As a nonlimiting example, spontaneous infiltration may include spraying infectious viral genes onto a plant, spraying viral solution obtained from previously infected leaves, immersion of plants into a solution obtained from previously infected leaves and the like. As another nonlimiting example, infection may be done by infiltration of viral genes into a plant. As another nonlimiting example, infection may be done by infiltration of viral solution obtained from previously infected leaves. In one or more embodiments, infection may be performed by forced infiltration. For the purposes of this disclosure, "forced infiltration" is a type of infiltration that requires a force, a pressure, an energy input, or a similar form of intervention to be applied. As a nonlimiting example, forced infiltration may include syringe infiltration, vacuum infiltration, and the like. As another nonlimiting example, vacuum infiltration may include vacuum infiltration using a vacuum pump, vacuum infiltration using a syringe, and the like. A person of ordinary skill in the art, upon reviewing the entirety of this disclosure, will be aware of various infiltration techniques that may be applied to generate virus-like particle 104 as described in this disclosure.

[0064] With continued reference to FIG. 4, at step 410, method 400 further includes sampling symptomatic leaves from the plant. For the purposes of this disclosure, "sampling" is an action of taking samples from the leaves of plant to inspect whether they have produced virus-like particles

104. This may happen after a certain time period subsequent to a successful infection step, as described above. Samples may be taken from leaves that are symptomatic. For the purposes of this disclosure, "symptomatic" is a descriptor that describes one or more characteristics of an object that appear different due to viral infection. As a nonlimiting example, it may take three to six days for a CPMV-based nanoparticle to form and accumulate in a plant. As a result, leaves may appear symptomatic in several possible ways, such as without limitation, light and dark green patches or irregular mottling, a stunted, curled, or puckered appearance, and veins of leaves appearing lighter than normal or banded with dark green or yellow. Once virus-like particles 104 are detected, they may be extracted following steps described below.

[0065] With continued reference to FIG. 4, at step 415, method 400 further includes homogenizing the virus-like particle 104. For the purposes of this disclosure, "homogenizing" is a process of blending elements into a uniform mixture with a consistent or substantially consistent composition across its entirety. In one or more embodiments, a homogenization process may include combining a tissue of plant containing virus-like particles 104 with an extraction buffer solution. For the purposes of this disclosure, an "extraction buffer solution" is an aqueous solution capable of breaking open cells and releasing elements therein. In one or more embodiments, extraction buffer solution may include salts to regulate its acidity. In the case of the TBSV, a sodium acetate solution may be used as extraction buffer solution.

[0066] With continued reference to FIG. 4, at step 420, method 400 further includes incubating virus-like particle 104. A homogenous mixture of plant leaves and extraction buffer is incubated in ice for a period of time. For the purposes of this disclosure, "incubating" is a process of subjecting an item to a hot or cold temperature for a certain period of time, until a certain goal is accomplished.

[0067] With continued reference to FIG. 4, in some cases, at step 425, method 400 may further include centrifuging virus-like particle 104. For the purposes of this disclosure, "centrifuging" is a process of separating multiple components in a mixture based on their difference in density by applying a centrifugal force to the mixture using a centrifuge device. For the purposes of this disclosure, a "centrifuge device" is a device comprising a rotating element attached to a stationary axis and configured to a spin sample under a high rotational speed in order to achieve separation between various elements therein. The amount of time to apply for this centrifuging step may vary from one type of virus-like particle 104 to another. In some cases, this step may be repeated multiple times to achieve an improved yield. As a nonlimiting example, for TBSV, centrifuging step may be repeated two or three times.

[0068] With continued reference to FIG. 4, at step 430, method 400 further includes filtrating virus-like particle 104. In some cases, filtrating virus-like particle 104 may include filtrating the virus-like particle 104 using tangential flow filtration (TFF), nanofiltration (NF), and/or gel-filtration chromatography, among other similar separation/purification techniques. For the purposes of this disclosure, tangential flow filtration (TFF), also known as cross-flow filtration, is a separation technique utilized to filter and concentrate biomolecules in solution. In TFF, a feed solution may flow tangentially across the surface of a filter membrane, while an applied pressure forces some of the fluid through the membrane as filtrate (permeate). A tangential motion may help reduce membrane fouling and allow continuous filtration. TFF is commonly employed in bioprocessing for concentrating proteins, clarifying cell lysates, and purifying biopharmaceuticals, providing an efficient method for separating components based on size and molecular weight. For the purposes of this disclosure, "nanofiltration (NF)" is a membrane filtration process that separates particles and solutes in the nanometer range, typically between 1 and 10 nanometers. NF may utilize a semi-permeable membrane to selectively allow certain molecules, such as monovalent ions and small organic molecules, to pass through while rejecting larger molecules, multivalent ions, and contaminants. NF may operate under moderate pressure and is commonly employed in water treatment, pharmaceutical purification, and food processing, providing a means for removing impurities, softening water, and concentrating valuable substances with high efficiency. For the purposes of this disclosure, "gel-filtration chromatography" is a type of separation and purification technique based on a differing ability of chemical species to retain in pores of a gel-filtration medium. Gel-filtration chromatography is also known as size-exclusion chromatography. A column used for gel-filtration chromatography may be packed with fine, porous beads composed of dextran polymers, agarose, polyacrylamide, and/or the like. The pore sizes of these beads are used to estimate the dimensions of macromolecules and separate them accordingly. Generally, chemical species of smaller sizes tend to retain in gel-filtration medium for a longer period of time (i.e., separates from a column later), whereas chemical species of larger sizes tend to retain in gel-filtration medium for a shorter period of time (i.e., separates from a column earlier). The main application of gel-filtration chromatography is the fractionation of proteins and other water-soluble polymers. Gel-filtration chromatography may be contrasted with gel permeation chromatography, which is a similar separation and purification technique often used to analyze the molecular weight distribution of organic-soluble polymers.

[0069] Referring now to FIG. 5, a flow diagram illustrating another exemplary embodiment of method 400 for manufacturing virus-like particle 104 is illustrated. It is worth noting that method

500 may not be limited to virus-like particle 104 but may be applicable to a wide variety of virus-based nanostructures instead, such as without limitation virus nanoparticle 108, consistent with details described above. Virus-like particle 104 may include any type of virus-like particle described in this disclosure without limitation. At step 505, method 500 includes agroinfiltrating a plant with a virus to produce virus-like particle 104. For the purposes of this disclosure, "agroinfiltration" is a method used in plant biology to induce transient expression of genes in a plant in order to produce a desired protein. As a nonlimiting example, agroinfiltration may be used for CPMV and/or for TBSV. As a nonlimiting example, to perform agroinfiltration, *Agrobacterium tumefaciens* may be directly injected into a plant leaf or brought into association with plant cells immobilized on a porous support. Subsequently, bacteria may transfer a desired gene into plant cells via transfer of T-DNA. Agroinfiltration may be beneficial when compared to more traditional plant transformations due to its speed and convenience, although yields of the recombinant protein may generally also be higher and more consistent. Additionally, and/or alternatively, other production processes may also be used to produce virus-like particle 104. Once a plant has undergone such production processes, it may produce virus-like particles 104 to be used in composition 100.

[0070] With continued reference to FIG. 5, at step 510, method 500 further includes sampling leaves of the plant. This step may be performed utilizing any process of sampling consistent with details described above.

[0071] With continued reference to FIG. 5, at step 515, method 500 further includes homogenizing the virus-like particle. This step may be performed utilizing any process of homogenization as explained above. As a nonlimiting example, for CPMV, a phosphate buffer or a PBS buffer may be used, consistent with details described above.

[0072] With continued reference to FIG. 5, at step 520, method 500 further includes centrifuging the virus-like particle 104. This step may be performed utilizing any process of centrifugation as described above. It is worth noting that production of CPMV-based nanoparticles may not necessarily involve an incubation step described above for method 400.

[0073] With continued reference to FIG. 5, at step 525, method 500 includes filtrating virus-like particle 104. This step may be performed utilizing any process of filtration as described above.

[0074] EXAMPLE 1: INNOVATIVE SOLUTIONS FOR STRAWBERRY CROP GROWTH AND PROTECTION BASED ON AN INNOVATIVE DELIVERY SYSTEM USING PROTEIN NANOPARTICLES

[0075] The invention described herein may proceed through several stages:

[0076] 1. Obtaining protein nanoparticles based on viral capsids for induction of a plant's innate defense system;

[0077] 2. Obtaining protein nanoparticles based on viral capsids for biostimulation of plant growth and resilience to abiotic stress;

[0078] 3. Evaluating the efficacy of these protein nanoparticles as defense inducers or biostimulants in model plants and plants of agricultural interest;

[0079] 4. Verifying the ability of these protein nanoparticles to penetrate leaf tissues and trigger expected responses, detecting eCPMV on leaves, and analyzing gene expression.

[0080] 5. Producing an innovative peptide delivery system that is harmless to the environment including without limitation non-target organisms, e.g., plants, insects, and/or microfauna.

[0081] **CPMV-ion transport peptide (ITPs) nanoparticle production**

[0082] Particle expression tests of eCPMV-ITPs were conducted with promising results and satisfactory yield.

[0083] Production and purification of eCPMV-ITPs viral nanoparticles in plants

[0084] Selected elicitor peptides have the ability to stimulate the plant defense system in plants afferent to agronomically relevant botanical families such as without limitation *Cruciferae*, *Solanaceae*, and *Poaceae*. These peptides may be able to activate plant defense responses as a result of binding to a receptor located on the plant cell membrane. The plant to which these peptides may be applied include, for example and without limitation, species of the *Rosaceae* family, which includes not only strawberry but also other species such as raspberry, blackberry, and apple. The activity of the chosen peptides (flg22, cape1, systemin) are capable of protecting plants from pathogen infection. Regarding biostimulatory peptides, bioactive peptides may be identified among the most representative of a protein hydrolysate of animal origin.

[0085] Suitable vectors for the production of engineered viral nanoparticles for the exposure of peptides with elicitor activity against the innate defense system of plants will be identified by a person of ordinary skill in the art, upon reviewing the entirety of this disclosure.

[0086] A viral nanoparticle purification protocol may be used. This purification protocol may be based on a generic protocol for isolation of eCPMV viral nanoparticles from plant materials. Optimization of this purification protocol, therefore, starts from an advanced stage of system development that may need further optimization specific to each exposed peptide.

[0087] The first step of characterizing the viral nanoparticles may include assessing the purity of the preparation using polyacrylamide gel electrophoresis and/or agarose gel electrophoresis. The second step may include estimating the hydrodynamic radius of the nanoparticles by dynamic light

scattering (DLS). The last step may include visualizing the viral nanoparticles by electron microscopy to confirm their shape and size. The yield of the preparation may be expressed as mg/g of purified eCPMV-ITPs per gram of sampled fresh tissue.

[0088] Design, expression, production and purification of CPMV-BP nanoparticles

[0089] Suitable conditions for the exposure of the biostimulatory peptide on the viral capsid surface of eCPMV may be evaluated. This design phase involves an *in silico* study of modified capsid proteins in order to evaluate the outward exposure of a peptide of interest so as to increase its bioavailability. The expression level of eCPMV-BP nanoparticles in plants and optimization of the purification protocol may be evaluated. eCPMV-BP nanoparticles may be characterized following the procedures described above.

[0090] Overall, the yield of viral nanoparticles may be expressed in mg/g of purified eCPMV-BP per gram of sampled fresh tissue. eCPMV-ITP nanoparticles may be purified. The vector for the expression of eCPMV-BP nanoparticles may be obtained. eCPMV-BPs nanoparticles may be purified. The stability of eCPMV-ITP and eCPMV-BP nanoparticles may be evaluated.

[0091] Production of eCPMV nanoparticles exposing the elicitor peptide with a system for controlled removal of the peptide from the viral structure

[0092] Programmed release of the elicitor peptide may occur via proteolytic cleavage mediated by enzymes present in the apoplast or specifically released upon perception of a pathogen. Nonlimiting examples of such proteolytic cleavage may be found in several classes of proteases such as cystein-, serin-, and asparagin-proteases, among others. An initial feasibility of the system may be established through commercially available proteases whose recognition site is known, and once the "proof of concept" is obtained, strawberry-specific proteases can be proceeded with. More specifically, peptide release can be assessed following incubation of the viral nanoparticles in the apoplastic extract of a plant of interest and visualized as a weight difference of the viral nanoparticle following agarose and/or polyacrylamide gel electrophoresis. Peptide release may be verified accordingly on gel or using a functional approach.

[0093] Evaluation of alternative viral platforms for bioactive peptide exposure

[0094] Expression of elicitor or biostimulatory peptide on novel viral nanoparticles that self-assemble in plants even in the absence of viral genetic material

[0095] Existing constructs for the production of VLPs based on the structure of AltMV and TBSV may be tested. Following cloning the sequence encoding the elicitor or biostimulatory peptide in such systems, these new constructs may be tested for the production of such viral nanoparticles in plant systems. The resulting particles may then be characterized to evaluate the presence of the

peptide and their production yield in plant systems. Purified nanoparticles (derived from other viruses) that expose one or more elicitors or biostimulatory peptides may be obtained.

[0096] Evaluation of the efficacy of eCPMV nanoparticles as elicitors of the plant defense system

[0097] Treatment of model plants (*Arabidopsis thaliana* or *A. thaliana*) and plants of interest (strawberry or the like; families including without limitation *Rosaceae*; genera including without limitation *Fragaria*; species including without limitation *Fragaria x ananassa*) with viral nanoparticles bearing the elicitors or biostimulatory peptides

[0098] The plants may be grown *in vitro* in order to eliminate external contamination that could potentially affect the experimental conditions and consequently the reliability of the experiment. Seedlings of *A. thaliana* and strawberry may be evaluated together or in succession. The decision to add *A. thaliana* as a model plant among the species of interest is due to the considerable availability of information associated thereto, which, in contrast to what is available on strawberry, may facilitate the early stages of setting up the experimental conditions. The functionality of viral nanoparticles as defense inducers may be assessed based on the activation of molecular mechanisms of plant defense by monitoring the expression level of defense genes specifically regulated by the peptides of interest (by real-time qPCR). For all chosen peptides, the signal transduction pathway that follows their perception by the plant cell may be recognized by a person of ordinary skill in the art, upon reviewing the entirety of this disclosure. Therefore, in some cases, identification of genes specifically induced by viral nanoparticle treatments may be performed based on data available in the scientific literature. Specifically, the selected genes may include without limitation the following genes that are known to respond to the peptides of interest, namely *FRK1. PDF1.2, WRKY29, PR5, ERF1.*

[0099] Evaluation of the ability of viral nanoparticles to protect against infection with biotrophic and necrotrophic pathogens under controlled conditions, in *Arabidopsis thaliana* (model plant for setting conditions) and strawberry plants

[0100] The ability of plants to be protected by viral nanoparticles may be evaluated. The main pathogens of strawberry in addition to the model pathosystem *A. thaliana* include without limitation *Pseudomonas syringae* pv. tomato DC3000 and *Botrytis cinerea* (*B. cinerea*). For the purposes of this disclosure, "*Pseudomonas syringae* pv. tomato DC3000" or "Pst DC3000" is a plant pathogen that causes bacterial speck in tomatoes and can also infect other plants. For the purposes of this disclosure, "*Botrytis cinerea*" is a fungal pathogen known for causing gray mold in a wide range of plants. In both cases, plants may be grown under sterile conditions *in vitro*, treated with viral

nanoparticles, and infected according to methods recognized by a person of ordinary skill in the art, upon reviewing the entirety of this disclosure. These protection assays may also be performed on strawberry fruit, specifically against *B. cinerea*, a pathogen that causes significant post-harvest losses. To determine the level of protection, a disease index and a severity index, i.e., the percentage of symptomatic samples (leaves or fruit) out of the total and the severity of symptoms caused by the pathogen, may be evaluated. For experiments on strawberry, the ability to protect against infection by *Xanthomonas fragariae* agent of angular leaf spot (ALS) may also be evaluated. Treatments with viral nanoparticles on various plants and/or against the various pathogens may be performed before infection for protective effect or after infection for curative effect.

[0101] Evaluation of the ability of functionalized nanoparticles to protect strawberry against biotrophic (*X. fragariae*) and necrotrophic (*B. cinerea*) pathogens under controlled conditions

[0102] Following the results obtained from steps above, the same assays may be applied to plants grown in a controlled environment but no longer under sterile conditions. This further transfer of experimental conditions may allow evaluation of an effective plant protection capacity under conditions similar to those of strawberry cultivation. Expression of defense genes in *A. thaliana* and strawberry may be characterized following treatment with eCPMV-ITPs. Accordingly, plant protection activity following treatment with eCPMV-ITPs may be reported. The disease and severity indices of strawberry plants grown under controlled conditions and inoculated with different pathogens in the presence or absence of eCPMV-ITPs may be assessed.

[0103] **Evaluation of the biostimulatory capacity of viral eCPMV-BP nanoparticles**

[0104] The biostimulatory effect of eCPMV-BP viral particles

[0105] The biostimulatory effect of eCPMV-BP viral particles may be tested on strawberry plants raised in hydroponic culture through characterization of growth parameters. For the purposes of this disclosure, "hydroponic culture", also known as hydroponics, is a method of growing plants in a nutrient-rich water solution without soil. The plants are grown in an artificial environment, and the nutrient solution is continuously circulated and monitored. In particular, any effects on biomass increase and root system development will be determined using WinRhizoTM software. The performance of the viral particles exposing the peptide will be compared with that of the peptide itself and the starting biostimulant product (protein hydrolysate of animal origin). The amount of biomass produced by strawberry plants grown in hydroponics and treated with eCPMV-BP may be evaluated.

[0106] Scientific methodologies implemented in this example include without limitation:

[0107] **Production of bioactive nanoparticles:** cloning of inserts into vectors for in-plant expression, transformation of *Agrobacterium tumefaciens*, agro-infiltration of *Nicotiana benthamiana*

[0108] **Nanoparticle expression in plant:** protein extraction from agro-infiltrated plant tissues, immuno-detection of protein nanoparticles on extracted samples

[0109] **Purification of functionalized nanoparticles:** ion exchange chromatography, size exclusion chromatography, ultracentrifugation

[0110] **Characterization of purified functionalized nanoparticles:** hydrodynamic beam analysis by dynamic light scattering (DLS), shape and size analysis by transmission electron microscopy, purification purity analysis by polyacrylamide gel electrophoresis

[0111] **Evaluation of programmed peptide release from nanoparticles:** *in vitro* enzymatic cutting and/or *in vitro* proteolytic cutting by plant and/or bacterial extracts

[0112] **Induction of plant defense system:** total RNA extraction, mRNA retro-transcription, gene expression analysis by real-time RT-qPCR

[0113] **Ability to protect plants from pathogen infection:** infections under controlled conditions and evaluation of disease and severity indices

[0114] **Evaluation of biostimulatory effect:** evaluation of biostimulatory effect through determination of morpho-physiological parameters of hydroponically grown plants

[0115]

[0116] EXAMPLE 2: GENETIC ENGINEERING OF eCPMV NANOPARTICLES FOR ANTIMICROBIAL ACTIVITY

[0117] Introduction

[0118] Antimicrobial peptides: exploiting a natural defense mechanism

[0119] Antimicrobial peptides (AMPs) constitute the first line of defense of many living organisms against invading pathogens. The term antimicrobial is currently falling in disuse since these peptides exploit different mechanisms to protect their host, therefore, in some cases, cationic host defense peptide (CHDC) is now the preferred term instead. The first description of AMPs dates back to the 1960s, when it was discovered that the skin of amphibians has the ability of protecting itself. This field gained more attention in the late 1990s. Interestingly, CHDC shares the same features regardless of whether it is sourced from an animal or a plant. Accordingly, its mechanism of action relies mainly on the interaction with the cell membrane of a microbe, leading to its disintegration. Therefore, it is not expected that microbes would easily develop resistance against these peptides, which makes CHDC a very promising alternative to common antibiotics.

[0120] Bioactive peptides are among the smallest members of the plant proteome, playing the most diverse roles in the life cycle of plants. They are involved in plant growth, development, reproduction, stress responses and, interestingly, are considered to play a central role in the establishment of plant symbiotic interactions. The origin of bioactive peptides may include a broad number of possibilities. Interestingly, small peptides, i.e., peptides that are less than 20-amino acid long, may originate from small open reading frames (ORFs) as well as from primary microRNA (pri-miRNA). This variability shows the different evolutionary mechanisms developed by plants to survive and adapt to different environmental conditions and to promptly respond to external clues. Antimicrobial peptides may come from a non-functional precursor and may be distinguished between Cys-rich and non-Cys-rich peptides, which generally are not subjected to post-translational modifications (PTMs). The rise of antimicrobial peptides as an alternative to common antibiotics started in the late 90s consequently to the alarming emergence of antibiotic resistance. Indeed, both the veterinary and the medical fields are struggling to find alternatives to common antibiotics for infectious disease treatment, since antibiotic-resistant bacteria are isolated from patients and livestock at an increasing frequency. The same issue exists in agriculture. Therefore, there is an urgent need to find valuable alternatives. Pesticide resistance, from an evolutionary point of view, can emerge by a *de novo* mutation, which is expected to occur in the open field, especially when target-specific resistance is applied (i.e., the pesticide is directed against a single molecular target). However, mutations leading to resistance are unlikely to occur when they are not associated with an overall greater fitness advantage for the microorganism. Thus, it is assumed that resistance traits emerging from mutations conferring only a partial resistance are more likely to occur compared to those conferring a complete resistance. Then, the speed of resistance emergence may depend on the number of alleles, the size of the population, and the selection pressure. Consequently, the search for new pesticides is now focused on "natural" compounds, many which are continuously being discovered. The vast variety of plant secondary metabolites is an example of an interesting source of environmentally friendly agrochemicals, but they can be difficult to isolate due to the close similarities among compounds of the same class. Moreover, the process could be difficult to scale up, and generally, these compounds have a short half-life and must be stored in very controlled conditions, which makes them less competitive than common chemically synthesized products. Plants are an interesting source of antimicrobial peptides since they can be exploited by a molecular farming approach to produce foreign proteins in large quantities and relatively inexpensively with respect to other production platforms like mammalian cells and yeasts.

[0121] Mechanism of action of antimicrobial peptides

[0122] The high and wide efficacy of AMPs is mainly ascribable to their positive charge at physiological pH, which enhances their interaction with negatively charged cell membranes. Another common property of AMPs is the presence of a significant portion of hydrophobic residues, which facilitates the interaction with the fatty acyl chains. The interaction results in membrane permeabilization of the target cell leading to a disruption of the electrochemical gradient, membrane disruption, a leakage of ions and other cellular components, and eventually to cell death. However, other mechanisms of action have been proposed. An alternative mechanism suggests that the action of AMPs may occur after the interaction of AMPs with specific cell membrane components, which then triggers cell death through mechanisms different from membrane permeabilization. Lipid binding may be the first step of antimicrobial activity and may occur with a substantial degree of specificity. Indeed, the nature and the abundance of lipids within the cell membrane may vary among microbes and may be responsible for the specificity of action. Among plant AMPs, defensins may exploit a huge variety of mechanisms for antimicrobial activity. These mechanisms may include an inhibition of protein synthesis and blockage of ion channels, which go beyond the membrane disruption. It is assumed that AMPs accumulate in the proximity of cell membranes and then trigger cell death, in a process that may be dependent on both timing and AMP concentration. Therefore, the broad antimicrobial activity of AMPs may not be considered as widespread among different species and genera of microorganisms, since it relies on specific components of the cell membrane. An AMP can be highly efficient on a target microorganism while being harmless to very close species. Such property may be useful for plant protection applications, as the choice of AMPs may drive the development of new strategies showing broad- or narrow- range effects, depending on the target.

[0123] Defensins, ubiquitous plant small AMPs

[0124] Plant AMPs are part of the plant immune system and are generally encoded by pathogenesis-related (PR) genes. Based on their tertiary structure, the presence of cysteine residues forming disulfide bridges, and their overall charge and length, plant AMPs have been divided into different families. The structure plays a major role in the classification of plant AMPs, which are generally divided into 4 families, namely α, β, αβ and non αβ. The first two families are characterized by the presence of α-helices or β-sheets stabilized by disulfide bridges. The third family displays both secondary structures, as in the case of defensins, while the fourth family does not exhibit any of α or β structure. Other classifications, which are based on structure-related antimicrobial activity, distinguish AMPs into different families, such as without limitation thionins (PR-13), cyclottides, snakins, lipid-transfer proteins (LTP, PR-14), heveins, knottins, and defensins (PR-12). Defensins are considered suitable candidates for the invention described herein, as they are

among the most studied plant AMPs and are active against both bacteria and fungi at low micromolar concentrations. Moreover, despite their broad antimicrobial activity, it has been reported that some members of this family are harmless to human and plant cells. Interestingly, some plant defensins have been thoroughly investigated to identify of key features of their mechanisms of action, which seem mostly structure related. It is assumed that defensins may accumulate in the proximity of the cell membrane as monomers then interact with each other leading to pore formation into the membrane for internalization. For example, Psd1 from *Pisum sativum* may enter fungal cell membrane through interaction with glucosylceramide sphingolipids and target the nucleus, causing cell cycle arrest and fungal cell death. Other defensins like MsDef1 from *Medicago sativa* and HsAFP1 from *Heuchera sanguinea* may enter fungal cell membrane leading to an activation of different pathways involved in cell wall integrity, ion homeostasis, and production of reactive oxygen species (ROS) and resulting in cell death. While plant defensins are mostly known for their antifungal activity but the antibacterial activity of some of plant defensins was highlighted recently. This highlights the huge number of possibilities to develop new candidates for pathogen inhibition, opening many opportunities for a biotechnological application. More interestingly, defensins, and almost all AMPs, carry the γ-core motif, which includes a loop between the two antiparallel β2 and β3 sheets further stabilized by one to four disulfide bridges. This sequence is considered responsible for antimicrobial activity in most cases and for the interaction with the cell membrane. Furthermore, there is convincing experimental evidence showing the ability of MtDef4 γ-core motif alone in inhibiting the mycelial growth of *Fusarium graminearum*. Interestingly, a γ-core swap between MtDef4 and *Medicago sativa* defensin 1 (MsDef1) provided the latter the same ability to penetrate fungal cells. The biotechnological application of plant defensins started with a generation of transgenic plants expressing Raphanus sativus antifungal protein 2 (RsAFP2), which gained much attention and has been similarly employed with other crops and showed the ability to protect them from fungal colonization. Besides genetic engineering of crops, which is still limited and hindered by the EU legislation, AMPs can be delivered to crops by presenting on virus scaffolds. Indeed, while AMPs are generally produced in low amounts in plant tissues their purification may be a limiting factor, the genetic engineering of viruses may address these issues. Despite that the genetic engineering of eCPMV has a few limitations in terms of length and pI of the inserted peptide, it is possible to exploit these peptides in which the antimicrobial activity is carried mainly or solely by a core motif. Moreover, eCPMV nanoparticles can be recovered at high yield (up to 1 mg/g of fresh tissue) and the purification method does not involve expensive processes. Therefore, a combination

of the potency of AMP activity with the high stability and other advantages of the nanoscale size from the virus scaffold might result in a new concept of nanopesticides.

[0125] Materials and Methods

[0126] Plant material

[0127] *Nicotiana benthamiana* plants have been grown in soil pots under controlled conditions for 4-5 weeks (22-24 °C, 120 µmol photons $m^{-2}s^{-1}$, 24 °C, 70% humidity, 10/14 h day/night).

[0128] Synthetic antimicrobial peptides

[0129] Synthetic antimicrobial peptides (AMP2, AMP2DE, AMP3 and AMP3DE) were synthesized by Biomatik (Biomatik Corporation, Ontario, Canada) with a high degree of purity (> 95%). Synthetic peptides were solubilized in pure DMSO and stored at -20 °C.

[0130] Preparation of expression vectors

[0131] Peptide sequences were codon-optimized for expression in *N. benthamiana*. To allow for the specific insertion of peptides between Ala22 and Pro23 of the βB-βC loop of the eCPMV small coat protein, synthetic sense and antisense oligos (Eurofins genomics) carrying the complementary sequence to AtaI/HindIII restriction enzymes restriction site were annealed *in vitro*. Both oligos were mixed in equal amounts to a final concentration of 100 ng/µL in Milli-Q water, then samples were denatured by heating the sample to 95 °C for 5 minutes and cooling it down at room temperature. The pEAQ-HT-VP60 vector was double digested with AatI and HindIII restriction enzymes according to manufacturer instructions (ThermoFisher Scientific). DNA electrophoresis in 0.7% w/v agarose gel in TAE buffer (40 mM Tris-acetate, 1mM EDA pH 8.0) was performed to separate the plasmid digested from the undigested form. MidoriGreen DNA stain (NipponGenetics GmbH) was used to visualize DNA fragments after electrophoresis under UV-light. The desired DNA fragment was recovered from the agarose gel and purified using the Wizard SV gel and PCR clean-up system (Promega) following manufacturer instructions. The T4 DNA ligase (Promega) was used to insert the fragment into the pEAQ-HT- VP60 vector. Typically, 100 ng of purified plasmid were used in the ligation reaction. The reaction was incubated overnight at 4°C, then *Escherichia coli* (*E. coli*) TOP10 competent cells were transformed by heat shock using 2 µL of the ligation reaction.

[0132] *E. coli* TOP10 cells were thawed in ice, then the ligation reaction was added by gentle mixing, and the competent cells were incubated in ice for 30 minutes. Heat shock was performed at 42 °C for 46 seconds without shaking, then tubes were immediately chilled in ice. 400 µL of LB medium (10 g/L Tryptone, 10 g/L NaCl, 5 g/L Yeast Extract, pH 7.5) was added to cells and subsequently incubated at 37 °C for 1 h with constant shaking. The cell suspension was spread onto a solid selective medium and incubated overnight at 37 °C. Colonies grown on the selective medium

were subjected to PCR to confirm the integration of the plasmid. Plasmid DNA was isolated from positive colonies and sequenced (Eurofins genomics GmbH) to confirm the correctness of the inserted DNA fragments.

[0133] Colony PCR

[0134] Bacterial colonies were dissolved in Milli-Q water and subjected to a heat treatment at 95 °C for 7 minutes. Cell's debris were pelleted by centrifugation at 10000×g for 1 minute at room temperature, and 2 µL of the supernatant were used as the template in the PCR reaction. The DreamTaq DNA polymerase (Thermo Fisher Scientific) was used to amplify the DNA sequence encompassing the βB-βC loop of the CPMV small coat protein (left primer: CTAATCCGGGTATTGATGG, right primer: CAGATTTCCAAGCAGCAGTA). DNA fragments were separated by electrophoresis in 2% agarose gel.

[0135] Plasmid DNA isolation

[0136] Plasmid DNA extraction was carried out using the E.Z.N.A ® Plasmid Mini Kit (OMEGA Bio-tek) following manufactured instructions.

[0137] *Agrobacterium tumefaciens* LBA4404 strain transformation

[0138] *Agrobacterium tumefaciens* (*A. tumefaciens*) LBA4404 overnight grown cultures were centrifuged at 4000×g for 5 minutes at 4 °C. The collected cells were washed three times by gentle resuspension in ice-cold 10% glycerol followed by centrifugation. Washed cells were finally suspended in a small volume (approximately 80 µL) of ice-cold 10% glycerol. 50-100 ng of plasmid DNA was added to the cell suspension and gently mixed. The mixture was then transferred into an electroporation cuvette. Electroporation was performed at 2.5 kV, then cells were recovered with liquid YEB medium and incubated at 28 °C for 2 h. The cell suspension was then spread into a selective agar medium and plates were incubated for approximately 48-72 h (until an appearance of colonies was detected) at 28 °C.

[0139] Production and purification of eCPMV and eCPMV-AMPs nanoparticles

[0140] *A. tumefaciens* LBA4404 strain carrying the parent or recombinant pEAQ-HT-VP60-AMP or pEAQ-HT-24K cultures were grown in liquid YEB medium for 24 h at 28 °C with constant shaking (200 rpm). Then cells were collected by centrifugation (20 minutes at 4000 ×g at room temperature) and resuspended in MMA buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$, 200 µM acetosyringone) solution to reach an OD600 (optical density measured at 600 nm) of 0.8. Suspensions were incubated for 2-3 h, then each suspension carrying a different pEAQ-HT-VP60-AMP vector was mixed with an equal amount of LBA4404 carrying the pEAQ-HT 24k vector and manually infiltrated in 5-6 weeks old *Nicotiana benthamiana* plants. Leaves were sampled 6-8 days

post infiltration and immediately frozen in liquid nitrogen for storage at -80 °C until purification. Briefly, to purify eCPMV nanoparticles, plant leaves were ground in liquid nitrogen, then sodium phosphate buffer 0,1 M pH 7.0 (NaP buffer) was added with a 1:3 ratio (weight/volume). Polyvinvyl-polypyrrolidone (PVPP) was added to reach a final concentration of 2%, and the suspension was thoroughly vortexed and filtrated through 3 layers of miracloth membrane (Merck Millipore). The filtrated solution was centrifuged for 20 minutes at 13000×g at 4 °C. Polyethylene glycol 6000 (PEG6000) and sodium chloride (NaCl) were added to the recovered supernatant to reach a final concentration of 4% and 0.2 M respectively. The suspension was stirred overnight in a cold room at 4 °C. Precipitated virus nanoparticles were recovered by centrifuging the mixture for 20 minutes at 13000×g at 4°C. The pellet was resuspended in NaP buffer (10 mM, pH 7.0) by vortexing the mixture to reach a concentration of 0.5 mg/mL. The suspension was clarified by centrifugation at 27000×g for 20 minutes at 4 °C, and the supernatant was subjected to ultracentrifugation at 118700 ×g for 150 minutes at 4 °C. The pellet was resuspended in NaP (10 mM, pH 7.0). Finally, the solution was centrifuged at 10000×g at room temperature for 15 minutes to remove all remaining plant contaminants.

[0141] eCPMV-AMPs nanoparticle purification by size exclusion chromatography

[0142] Frozen infiltrated leaves of *N. benthamiana* were homogenized in liquid nitrogen using a mortar and a pestle. Then sodium phosphate 0,1 M pH 7.0 supplemented with polyvinyl-polypirrolidone (PVPP) at 2% and protease inhibitors (Complete Protease inhibitor Cocktail, Roche) were added to the powder with a ratio of 1:4 (w/v). The mixture was vortexed for 1 minute at room temperature and then chilled in ice. This process was repeated until complete dissolution of the powder. The mixture was allowed to clarify through 3 layers of miracloth and then was centrifuged at 30000 ×g for 60 minutes at 4 °C to precipitate all remaining plant debris. The supernatant was recovered and fractionated against DEAE Sephadex A-50 columns equilibrated with NaP buffer (pH 7.0). Sodium chloride was added to the flow-through to reach a final concentration of 0.2 M, and the mixture was ultrafiltrated using 100 kDa molecular weight cutoff (MWCO) centrifugal filters (Amicon® Ultra Centrifugal Filters, Merk Millipore). The concentrated extract was further centrifuged at 10000×g for 10 minutes at 4 °C to remove insoluble particles and loaded into a Sephacryl HiPrep HR-500 column to allow for fractionation according to the protein size. Size exclusion chromatography was performed in 0.1 M sodium phosphate buffer (pH 7.0) supplemented with 0.15 M NaCl under a constant flow rate of 0.8 mL/min. Fractions collected from approximately 50 to 80 minutes after sample injection were analyzed by SDS-PAGE followed by silver staining to assess the presence of particle subunits. Pure fractions were mixed and concentrated by ultrafiltration

against 100 kDa MWCO centrifugal units. eCPMV concentration was determined by measuring the absorbance at 280 nanometers at a Nanodrop One spectrofluorometer (Thermo Fischer Scientific). An extinction coefficient of 1.28 $mLmg^{-1}cm^{-1}$ was used for eCPMV.

[0143] Total soluble protein extraction

[0144] For normal purposes, total soluble proteins were extracted from plant leaves homogenized in liquid nitrogen by an addition of sodium phosphate buffer (0.1 M pH 7.0) at a ratio of 1:3 (w/v). Otherwise, proteins were extracted under reducing conditions.

[0145] SDS-PAGE

[0146] Polyacrylamide gel electrophoresis was routinely applied for the analysis of the purity of protein extracts. Total soluble proteins samples, as well as purified eCPMV, were mixed with a loading buffer containing dithiothreitol (DTT) to a final concentration of 50 mM. In the case of purified particles, 5-10 ug were loaded into the gel. Run was performed as follows: 10 minutes at 100 V then 80 minutes at 130V. After rinsing with milli-Q water, gels were stained for 20 minutes in Coomassie R-250 staining solution (25% isoprophyl alcohol, 10% acetic acid, 0.05% commassie R-250) and destained in 10% acetic acid until complete removal of background.

[0147] Western blot

[0148] Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Amersham™) for 1 h at 100 V at 4 °C. The membrane was saturated for 2 h in phosphate buffered saline (PBS) supplemented 5% skim milk with 0.05% tween 20 before leaving the membrane in an overnight incubation at 4 °C with anti-CPMV polyclonal antibody. The membrane was washed three times in PBS solution containing 0,05% Tween® 20 and incubated with the secondary antibody conjugated with the HRP enzyme for 90 minutes at room temperature. Excess secondary antibody was removed by rinsing the membrane three times in PBS solution. Signals were revealed with Amersham™ ECL Select™ detection reagents. Images were acquired by Chemidoc Biorad.

[0149] Silver staining

[0150] After SDS-PAGE, gels were rinsed with distilled water and placed in a cold fixing solution (50% Milli-Q water, 40% ethanol, 10% acetic acid) for one h with gentle agitation. Gels were then washed for at least 30 minutes with Milli-Q water with several changes of water and sensitized for 1 minute in 0.02% sodium thiosulfate solution. After rinsing with Milli-Q water, the gels were placed in the cold silver nitrate solution (0.1 % silver nitrate, 0.02% formaldehyde). Gels were rinsed three times with Milli-Q water and placed in a new staining tray and developed with a solution containing 3% sodium carbonate and 0.05% formaldehyde. The reaction was stopped with 5% acetic acid, then the gels were stored in 1% acetic acid.

[0151] *N. benthamiana* leaf extracts preparation for antifungal assay

[0152] *N. benthamiana* leaves were collected at 6 days post agroinfiltration and pestled to a fine powder with mortar and pestle in liquid nitrogen. Sodium phosphate buffer (0.1 M, pH 7.0) supplemented with 2% PVPP was added to the leaf homogenate in a ratio of 1:3 (w/v), and the suspension was thoroughly vortexed and kept in ice. Leaf extracts were filtered through 3 layers of miracloth membrane to remove large plant debris and then centrifuged at 13000×g at 4 °C for 20 minutes. The supernatant was filter sterilized by 0.20-µm sterile centrifuge filters. An aliquot of each extract was used to assess the presence of eCPMV-AMP nanoparticles by western blotting.

[0153] *Botrytis cinerea* growth inhibition in microtiter plates

[0154] Mature conidia of *Botrytis cinerea* BMM were collected from fully sporulated cultures grown on tomato agar plates. Conidia suspension in MilliQ water and 0.05% tween® 20 was filtered through three layers of miracloth membrane (MerckMillipore) to remove large mycelium debris, diluted to a final concentration of 10^6 conidia/mL in PDB, and dispensed in the well of a transparent 96-well plate. The optical density at 420 nanometers was measured in a Tecan Infinite PRO 200, and sixteen points of measure per well were taken as the mycelium develops unevenly into the wells. Plates were moved into an incubator with a constant temperature of 22 °C and in the dark, and the growth of mycelium was evaluated at 24, 48, 72 and 96 h. For growth inhibition experiments with *N. benthamiana* leaf extracts containing eCPMV nanoparticles, 20 µL of plant extract was added to each well after 24 h. Leaves agroinfiltrated with the *A. tumefaciens* LBA4404 strain carrying the pEAQ-HT-24K vector was used as the negative control, while amphotericin B served as a positive control at 20 µg/mL. The optical density was measured both before and after the addition of extracts to the plate. The evaluation of fungal growth inhibition with synthetic AMPs was carried out in the same manner by adding 20 µL of the peptide solution to each well 24 h after the dispensation of conidia suspension in a 96-well plate. Five independent biological replicates and three technical replicates for each biological replica were performed.

[0155] Bacterial growth inhibition in microtiter plates

[0156] *Pseudomonas syringae* pv. *actinidiae* and *P. syringae* pv. tomato DC3000 were grown in liquid KB medium (peptone 20 g/L, glycerol 1 %, $MgSO_4$ 5 mM, K_2HPO_4 8.6 mM, pH 7.2), *A. tumefaciens* LBA4404 strain was grown in a liquid YEB medium for 24 h at 28 °C with constant shaking at 200 rpm. Bacterial cells were collected by centrifugation at 4000×g for 5 minutes at room temperature. The pellet was resuspended in fresh KB or YEB according to the bacterial species, and suspensions were diluted to a final OD600 of 0.1 with the same medium. 180 µL of bacterial suspension was dispensed into each well of a 96-well plate and 20 µL of the synthetic peptide

solution was added. Bacterial growth was monitored for 24 h based on the increase of optical density at 600 nanometers in a Tecan Infinite PRO 200 plate reader. Three biological replicates were performed including at least three technical replicates for each experiment.

[0157] Chemical conjugation to lysine

[0158] Chemical conjugation of eCPMV particles through exposed lysine residues was performed in collaboration with the Plant Virus Biotechnology group (P.I. Fernando Ponz Ascaso) at the Centro De Biotecnología y Genómica de Plantas (CBGP, Campus de Montegancedo, Pozuelo de Alarcón, Madrid). The chemical conjugation followed two different approaches: direct conjugation to exposed lysine residues and indirect conjugation by "Staudinger Ligation". The first approach directly targets the exposed lysine residues using an N-Hydroxysuccinimide group (NHS) due to its high reactivity with the primary amino groups (-NH_2) on the N-terminus of polypeptides or in the side chain of lysine residues within protein sequences. Thus, eCPMV nanoparticles at 2 mg/mL in NaP buffer (0.1 M pH 7.0) were mixed with 20-, 50-, and 100-fold molar excess of AlexaFluor 555 NHS ester (Thermofisher Scientific), and the reaction was incubated overnight at room temperature in the dark. The excess of the fluorophore was removed by ultracentrifugation at 150000×g for 2 h at 4 °C. Pelleted conjugated nanoparticles were resuspended in NaP buffer (0.1 M pH 7.0), and the degree of eCPMV labelling was estimated by spectrophotometric measurements using a NanoDrop Spectrophotometer using specific settings for AlexaFluor555 fluorophore.

[0159] The Staudinger Ligation approach considers indirect conjugation strategies using the bioorthogonal reactants: phosphine and azide groups. First, exposed lysine residues on eCPMV surface were targeted with an NHS-phosphine bifunctional linker. eCPMV nanoparticles at 2 mg/mL in NaP buffer (0.1 M pH 7.0) were mixed with a 10-fold molar excess of the NHS-phosphine linker, which was subsequently removed by ultracentrifugation as previously described for AlexaFluor 555 NHS ester. eCPMV nanoparticles were recovered in a small volume of NaP buffer (0.1 M pH 7.0) and diluted to a concentration of 1 mg/mL. Then AlexaFluor488-azide (ThermoFisher Scientific) was added with a 20-fold molar excess, and the reaction was incubated overnight in the dark at room temperature under constant agitation. AMP3-azide (Caslo ApS, Denmark) was added with 1- to 5-fold molar excesses, and the reaction was incubated overnight at room temperature. The same procedure was followed with the vasoactive intestinal peptide VIP-azide (Caslo ApS, Denmark). The excess of azide-conjugated compounds was removed by several rounds of centrifugation with 10 kDa MWCO centrifugal filters (AmiconUltra, MerkMillipore).

[0160] Results

[0161] Selection of antimicrobial peptides

[0162] AMPs were selected mainly among those of plant origin after an investigation of the available literature and databases. Different criteria were included for the selection. First, they should have a strong reported antimicrobial activity. Then, peptides with a minimal identified portion carrying the antimicrobial activity were preferred. Indeed, it is widely accepted that the antimicrobial activity of AMPs is often carried by a small part of the whole peptide, while other portions confer mechanical strength and, in some cases, target specificity. Most of the literature about natural AMPs refers to the entire peptides. The information about the effect of single and multiple residue substitutions on the antimicrobial activity, toxicity towards mammal cells and the chemical-physical parameters is also limited. Moreover, the prediction of antimicrobial activity of peptides can be performed *in silico* using free, available tools, which may sometimes be included in AMP databases e.g., CAMPR3. However, such approaches should be considered more as an indication about the possibility of a peptide to show an antimicrobial activity and could serve to exclude those which fail to be recognized as potentially active. Indeed, real activity could be influenced by the experimental conditions, since AMPs efficacy relies mostly on the chemo-physical properties of the peptide which changes according to the environment. Therefore, experimental evidence is the most valuable information about the activity of the antimicrobial peptide against a specific target. Their ability to function as synthetic peptides was also taken into consideration. Indeed, peptides can undergo post-translational modifications (PTMs), which may be necessary for peptide stability and activity, but such PTM events may occur on peptides exposed on the surface of eCPMV. Third, according to peptide requirements for exposure on CPMV surface, AMP stretches shorter than 40 residues and with a pI < 9 were preferred. Alternatively, AMPs in which antimicrobial activity is known to be carried out by a small stretch may be preferred, such as the γ-core motif of some plant defensins. Finally, hemolytic activity, together with an absence of toxicity against mammalian cells, was considered to fit with the safety issues described for the development of sustainable alternative strategies in agriculture. Additional details are included in Table 1 below.

Table 1. Antimicrobial peptides and their reported antimicrobial properties

Name	Source	pI	MW	Antimicrobial activity
AMP1 (AMP1)	Plant	8.67	2216.58	Antifungal
AMP2	Plant	10.45	1959.32	Antifungal
AMP2-DE (AMP2)	Plant	8.57	2318.61	Antifungal
AMP3 (AMP3)	Animal	9.93	2268.74	Antifungal, antibacterial
AMP3-DE	Animal	8.92	2713.13	Antifungal, antibacterial

AMP3-C3AC16A	Animal	10.9	2204.65	Antifungal, antibacterial
AMP3-CDT	Animal	11.84	1854.21	Antifungal, antibacterial

[0163] Based on these criteria, two peptides were selected from plant sources (AMP1 and AMP2). It is worth mentioning that, to meet the criterium of pI, AMP2 was slightly modified to reduce its pI to below 9 by adding two amino acids, namely Asp and Glu (AMP2-DE). Moreover, due to its outstanding antimicrobial activity and the demonstration of its efficiency against phytopathogenic bacteria when expressed *in planta*, a third peptide has been selected, though from animal origin, namely AMP3. Two supplementary versions of this peptide were then included, in which Cys residues, which are involved in the formation of a disulfide bond, have been removed (AMP3-CDT) or substituted with Ala residues (AMP- C3AC16A), to avoid the hemolytic activity of AMP3.

[0164] Evaluation of the antimicrobial activity of AMP2 and AMP3 synthetic peptides

[0165] The antimicrobial activity of AMP2 and AMP3 peptides, alongside their modified analogues, was evaluated against bacterial and fungal pathogens. *Pseudomonas syringae* pv. *actinidiae* (Psa), *Pseudomonas syringae* pv. tomato (Pst), and *Agrobacterium tumefaciens* strain EHA105 have been treated with synthetic peptides in liquid-rich medium (KB for *P. syringae* and YEB for *A. tumefaciens*), and their growth was monitored for 24 h. No information was available about the effect of such AMPs against these bacterial pathogens, therefore, the tested concentrations aimed to display inhibition of the bacterial growth, following a dose reduction achieving the complete growth inhibition. AMP3 showed a marked antimicrobial activity, especially against *A. tumefaciens*, leading to a complete growth inhibition at 1.4 μg/mL, while the effective concentration for both Psa and Pst was close to 8 μg/mL (see FIG. 6, embodiment 600). Even if AMP3-DE showed antimicrobial activity, its efficacy was highly lower compared to the unmodified AMP3 peptide (see FIG. 7, embodiment 700). Indeed, at the highest concentration used for each strain, *A. tumefaciens* was completely unaffected by the treatment. Pst was still able to grow, showing only a delay compared with the control. Psa was not completely inhibited and reached almost 50% of the bacterial density respect to the untreated control at 24 h. While no antimicrobial activity was observed in agar plate, the same pattern was observed in liquid medium against the necrotrophic fungus *Botrytis cinerea*, for which, in the same manner, the modified peptides partially lost the ability to inhibit the fungal growth efficiently, although the difference was not as evident as with bacterial pathogens (see FIG. 8, embodiment 800). Of note, no dose-effect on growth inhibition was observed with AMP3 against *B. cinerea*, meaning that this peptide could be active at very low

concentrations respect to the ones tested. By contrast, this phenomenon occurred for AMP3-DE, suggesting that its efficacy relies on higher concentrations and is already affected at high doses when compared to AMP3. Indeed, a lower dosage of AMP3 could likely result in a reduced antimicrobial effect, and accordingly, AMP3-DE did not display the same efficacy of AMP3, suggesting that it is effective at higher doses. The different behavior between the two peptides suggests that the antimicrobial activity deeply relies on the chemical-physical properties of the peptide and that the addition of negatively charged amino acids may significantly modify such properties, leading to a reduction in antimicrobial efficiency.

[0166] AMP2 may function as an antifungal peptide and, accordingly, it didn't show any antimicrobial activity against the tested bacterial pathogens, with the only exception of Pst, whose growth was partially inhibited (by 50%) at a very high peptide concentration of 200 μg/mL (see FIG. 9, embodiment 900). Similarly, compared to its parent peptide, AMP2-DE was ineffective against bacterial pathogens (see FIG. 10, embodiment 1000). Similarly, no significant differences were observed between the two AMP2 peptides since the same behavior was observed in antifungal tests against *B. cinerea* (see FIG. 11, embodiment 1100). These results suggest that the antimicrobial activity is indeed carried out by the γ- core motif of this peptide regardless of the properties of flanking amino acids. Due to the antimicrobial activity observed for these peptides in the tested conditions, it is possible to consider them for their exposition on eCPMV surface.

[0167] Modification of eCPMV nanoparticles with AMPs through the genetic approach

[0168] eCPMV-AMP1 and eCPMV-AMP2

[0169] Due to highly cationic properties of antimicrobial peptides that are in contrast with the requirements for a successful exposition of eCPMV capsid, it has been hypothesized that an addition of acidic residues at the end of the peptides could enhance the expression of recombinant proteins as they could serve as "balancing" residues to reduce the pI. The production of eCPMV-AMP1 and eCPMV- AMP2DE particles *in planta* was assessed at 6 days post-infiltration (dpi) with *A. tumefaciens* strains carrying the different constructs and showed a significant difference between the particles (see FIG. 12, embodiment 1200). Indeed, while eCPMV-AMP2DE was clearly detected by western blot using the specific antibody raised against CPMV subunits, the signal corresponding to the eCPMV-AMP1 large coat protein (LCP) was only barely detectable after a long signal exposure. In both cases, the signal corresponding to the small coat protein (SCP) was not observed, suggesting that the inserted peptide may hamper the migration of the recombinant modified subunit into the gel. Since there is no difference in the sequence of the LCP, it is possible to assume that the differences in the SCP, due to peptide insertion, could affect both particle assembly and their solubility in the

plant extract. Indeed, the presence of cysteine residues in exposed AMPs could facilitate the formation of protein complexes leading to their precipitation. Accordingly, when the samples of eCPMV-AMP1 and eCPMV-AMP2DE were treated with a high concentration of a reducing agent (DTT 50 mM), the small coat protein was observed together with an expected increase in size (see FIG. 13. embodiment 1300).

[0170] Further trials of purification were unsuccessful irrespective of the method employed. FIG. 14, embodiment 1400, panel A shows two examples of purification trials for both eCPMV-AMP1 and eCPMV-AMP2DE. On the western blot on the right, a signal corresponding to eCPMV-AMP2DE on the last step of purification is present. However, the number of nanoparticles recovered was very low and the process itself was not reproducible (see FIG. 14, embodiment 1400, panel B). Indeed, eCPMV-AMP2DE particles were able to precipitate in presence of PEG (polyethylene glycol), suggesting that they are likely assembled into a viral capsid, but were not recovered from further steps of purification, or in very low amounts thus not enough to perform successive experiments. Likewise, the purification exploiting chromatographic techniques was worthless since nanoparticles were retained by the resin during the anion exchange chromatography and likely underwent some degradation process. Indeed, from western blot analyses, signals were detected at very low molecular weights (< 10kDa) in both column's wash samples (see FIG. 15, embodiment 1500). Then, eCPMV-AMP2DE was faintly detected in the fraction separated by size exclusion chromatography (SEC), while almost all eCPMV-AMP1 flowed into the elution with the counter-ion.

[0171] eCPMV-AMP3

[0172] eCPMV-AMP3 expression *in planta* was barely detectable, and these chimer particles showed the lowest expression level. However, the above-described rational modifications of AMP3 peptides, i.e., addition of acidic amino acid, cysteine-to-alanine substitutions, and complete cysteine, partially solved the problem of such low expression, since all the modified constructs showed a higher protein level in leaf extracts compared to the unmodified peptide (see FIG. 16, embodiment 1600). In particular, the hypothesis of loop rigidity due to the presence of disulfide bridges was supported by expression trials, as these modifications led to the highest expression of particles *in planta* regardless the length and the pI of the inserted sequence. Virus particles are not supposed to be completely degraded within plant leaves, and their accumulation should therefore be observed for the first 6-8 days. However, the expression level was very low, even when compared with eCPMV-AMP1. Moreover, the absence of a signal corresponding to the SCP in the eCPMV-AMP3-CDT sample led to the hypothesis that nanoparticles could be degraded within leaves. Indeed, if subunits

cannot properly assemble in a complete capsid, they may not take advantage of being organized in a viral structure, which confers high resistance to proteases or other mechanisms of protein degradation including the proteasome-mediated pathway. To assess this hypothesis, the proteasome pathway was inhibited by infiltrating the proteasome inhibitor MG132 24 h before sampling. Such treatment did not improve the level of protein level detected by western blot, suggesting that the proteasome pathway is very likely not involved in the degradation of eCPMV-AMP3 subunits (see FIG. 17, embodiment 1700). The role of proteases in this process could not be studied further, since the addition of protease inhibitor *in planta* would prevent the cleavage of the VP60 protein by the virus 24k protease, a process required for eCPMV subunits release. Thus, the very low expression level of all eCPMV-AMP3 particles should be ascribed to the AMP3 sequence itself, which hampers the proper assembly of the particles via a still unknown mechanism. This is further supported by an observation of SCP of both eCPMV-AMP1 and eCPMV-AMP2DE but not of eCPMV-AMP3-CDT upon extraction of protein.

[0173] Consistently, the purification of eCPMV-AMP3 particles was unsuccessful, likely due to both the very low expression level *in planta* and their inability to precipitate in the presence of PEG, which further strengthens the idea of unassembled particles.

[0174] Modification of eCPMV nanoparticles with AMPs through chemical conjugation to lysine residues

[0175] To address the issues of low nanoparticle yield when using a genetic fusion approach, another strategy was assessed to link the peptide to the surface eCPMV through a chemical conjugation approach. Chemical conjugation of eCPMV particles through exposed lysine residues was performed in collaboration with the Plant Virus Biotechnology group (P.I. Fernando Ponz Ascaso) at the Centro De Biotecnología y Genómica de Plantas (CBGP, Campus de Montegancedo, Pozuelo de Alarcón, Madrid). CPMV and eCPMV have been widely exploited for bioconjugation for many potential applications, ranging from material sciences to the medical field. Bioconjugation is a standard strategy to create new materials and relies on the activity of chemical groups located on the side chain of the target molecule to not interfere with their activity or properties. Commonly addressed amino acids for chemical conjugation include cysteines and lysines, both of which possess reactive chemical groups on their side chains, thiol and amine, respectively. CPMV has no externally accessible cysteine residues, while approximately 300 lysine residues can be targeted, 5 for each asymmetric unit. The strategy adopted here relies on a very specific reaction between a phosphine group and an azido group in the scheme of the Staudinger ligation. Indeed, since both groups are absent in living organisms, the reaction is very specific and can be performed *in vivo*. First, the

surface-exposed lysine residues may be addressed through a chemical linker carrying a phosphine group (NHS-phosphine). Then, the removal of the excess of the linker may be achieved by ultracentrifugation. The feasibility of this approach with eCPMV nanoparticles was first evaluated using the AlexaFluor488-azide fluorophore. In parallel, the conjugation was also performed using AlexaFluor555-NHS to assess which method was more suitable for conjugation with eCPMV (see FIGS. 18A-B, embodiments 1800a-b). In both cases, eCPMV was conjugated with a fluorescent molecule with similar yields. Indeed, an average conjugation loading of 27.6 and 25 molecules per CPMV was reached with AlexaFluor488-azide and AlexaFluor555-NHS, respectively. The efficiency of conjugation with the nanoparticles was assessed by evaluating the increase in size using SDS-PAGE, which was followed by gel staining or fluorophore excitation with UV-light. Subunit separation by SDS-PAGE was unable to reveal the expected slower band migration in comparison with unconjugated subunits, as showed by IstantBlue™ staining. In contrast, gel visualization under fluorophore excitation revealed a presence of conjugated proteins (see FIG. 19, embodiment 1900). Moreover, the absorbance of both Alexa molecules can be read using a spectrophotometer and used to estimate the number of conjugated molecules. Consequently, the Staudinger ligation system was considered suitable for the conjugation of the AMP3 peptide due to its higher reaction specificity. The AMP3 peptide has thus been synthesized with a modified N-term carrying the 5-azidopentanoic acid (Caslo ApS, Denmark). The eCPMV-AMP3 reaction was carried overnight at either room temperature or 4 °C, with constant shaking, using different molar excesses of the peptide with respect to the number of asymmetric units. The conjugation with AlexaFluor488 was used as a control for the overall process of conjugation. No significant migration shift was observed for either LCP or SCP after conjugation with AMP3 or AlexaFluor488. However, the band corresponding to SCP became broader and fainter, which could be due to an increase in the band number, thus suggesting a successful conjugation process (see FIG. 20, embodiment 2000). To confirm this observation, proteins were loaded into agarose gels and separated under native conditions. Indeed, the chemical conjugation could alter the surface charge of eCPMV due to both a presence of peptide and saturation of some Lys residues, as shown after conjugation with the phosphine linker (see FIG. 21, embodiment 2100, panel A). This approach did not reveal any migration shift of eCPMV conjugated with AMP3; instead, a progressively increasing tendency for nanoparticles to precipitate correlated with an increasing peptide molar excess (see FIG. 21, embodiment 2100, panel B). Together, these results strongly support a successful conjugation of the peptide to eCPMV, though a clear demonstration was not yet obtained. To further validate the suitability of peptide conjugation through the chemical approach, the vasoactive intestinal peptide (VIP, a peptide hormone having

multiple roles in the human brain and body) was used here as a positive control of conjugation reaction since a specific antibody is available for its detection. VIP was successfully conjugated to eCPMV following the same procedure (see FIG. 22A, embodiment 2200a). The chemically conjugated eCPMV-AMP3 nanoparticles were further tested on the growth of *P. syringae* pv. *actinidiae* and *A. tumefaciens* EHA105, but no antibacterial activity was observed. Therefore, the conjugation of AMP3 very likely led to the loss of antimicrobial activity in the peptide, likely due to the structural rigidity that prevents the peptide from accessing its target.

[0176] Evaluation of the antifungal activity of crude extracts expressing eCPMV-AMPs particles

[0177] The evaluation of the antifungal and antibacterial activity was not possible with purified nanoparticles harboring AMPs due to the technical issues described above, either using genetic or chemical approaches. However, assuming that nanoparticles were present in the extracts from agroinfiltrated plants, such extracts should display an antimicrobial activity. Moreover, since the antimicrobial activity is performed by a foreign peptide, the recombinant SCP, which carries the peptide, could display the activity, even in the absence of a nanoparticle assembly. Thus, the crude extracts from transiently transformed plants expressing eCPMV-AMPs were tested for their antimicrobial activity after a brief cleaning procedure to remove cell debris. Pant leaf tissues were homogenized in 0.1 M sodium phosphate buffer, filtered through 3 layers of miracloth, centrifuged to remove residual cell debris, filter-sterilized, and finally tested as "cleaned" extract against the fungus *Botrytis cinerea* in a liquid PDB medium. The presence of recombinant nanoparticles in the plant extracts were confirmed by western blot before fungus treatment (see FIG. 22B, embodiment 2200b). Filter-sterilized crude extracts were added in a 96-well plate 24 h after inoculation of *B. cinerea* conidia, and the growth of the mycelium was monitored 24, 48, and 72 h after the treatment. At 24 h post-treatment, no visible growth was detected, while at 48 h, a small white/grey mold started to form at the center of the well. Since the mycelium grows to form a mold and not as a planktonic culture, 16 measurements per well were taken to avoid bias due to uneven growth within the wells. The mycelium developed faster in the presence of plant extracts compared to the control that was grown only in a PDB medium. It is reasonable to assume that *B. cinerea*, as a saprophytic plant pathogen, may take advantage of plant-derived compounds present in the extracts, which may enhance its growth regardless of the presence of nanoparticles. Surprisingly, sodium phosphate itself seemed to enhance the growth of the fungal mycelium. Therefore, the growth in the presence of the control extract was used for proper comparison. The control extract was produced from leaves infiltrated with *A. tumefaciens* LBA4404 carrying only the vector containing the 24k proteinase

gene. Since there was a significant difference in terms of absolute absorbance values among the technical triplicates and among different biological replicas, the growth was further expressed as a percentage of mycelium increase between two time points. Altogether, these experiments showed that fungal growth was not influenced by any of the treatments (see FIG. 23, embodiment 2300). Accordingly, one-way ANOVA did not establish any significant difference among the samples. Therefore, it is not yet possible to fully determine any effect of recombinant eCPMV-AMP nanoparticles in crude leaf extracts on fungal growth.

[0178] To rule out the possibility that the fungus may grow better in the presence of plant extracts, eCPMV-AMP1, AMP2 and AMP3CDT nanoparticles, and eCPMV- ITP1 (used as additional control of modified eCPMV devoid of antimicrobial activity) have been further concentrated using centrifugal filters with 1000 kDa MW cut-off, in an attempt to remove plant-derived compounds. Figure 3.21 shows that only eCPMV-AMP2 and eCPMV-ITP1 have been successfully recovered in the upper fraction (> 1000 kDa), while eCPMV-AMP1 and eCPMV-AMP3CDT nanoparticles have been lost during the process, likely due to precipitation on the membrane (see FIG. 24, embodiment 2400). Their absence in the upper fraction may also indicate a lack of nanoparticle assembly, preventing them from being retained on the membrane. Indeed, the size of the unmodified eCPMV is already around 4 MDa, making it unlikely for properly assembled capsids to pass through those filters. This approach was unable to address the abovementioned issues but allowed for speculation over the assembly of eCPMV nanoparticles.

[0179] Discussion

[0180] The increasing emergence of antimicrobial resistance is threatening agriculture and the entire food production chain. Thus, the urgent need for alternatives to currently available agrochemicals, to address the fatal issue of antimicrobial resistance, led to the development of new strategies, in particular, by focusing on antimicrobial activities that can overcome the possibility of antimicrobial resistance occurrence. Antimicrobial peptides (AMPs) thus deserve attention since they represent a large class of antimicrobial compounds and an interesting source for novel therapies against infectious diseases. Particular modes of action pf AMPs are unlikely to lead to evolution of resistance in the microbial population. Indeed, the re-discovery of AMPs, particularly the of plant origin, is a leading strategy to achieve food security, since most AMPs show a broad-spectrum antimicrobial activity, which relies on strategies unlikely to be overcome in a short period. However, the broad-spectrum antimicrobial activity could be a double-edged sword, specifically in agriculture. Indeed, the inherent cationic feature of AMPs is considered responsible for the interaction with the negatively charged lipids of the cell membrane. However, such interaction may occur with specific

lipidic components. A specific interaction may exist between RsAFP2 and the glucosylceramide (GlcCer) sphingolipids, which are components of the fungal cell membrane of *Pichia pastoris* and *Candida albicans*. This is further suggested by deleting from these yeasts the GSC gene encoding a synthase involved in the production of GlcCer, which contributed to an increased resistance toward RsAFP2 peptide. Similarly, to develop a bioinformatic tool to predict the antimicrobial activity of AMPs, it has been highlighted that the abundance of negatively charged phospholipids can vary from 20% in *E. coli* to 100% in *Staphylococcus spp.* and *Streptococcus spp.* Thus, the antimicrobial activity is most likely strain-specific, or at least the composition and the abundance of different lipids in the cell membrane should be considered during the development of a new AMP candidate.

[0181] AMP2 was completely inactive against three different bacteria. This is in line with the fact it is classified as an antifungal peptide, although some plant defensins show also an antibacterial activity. Interestingly, as already reported, AMP2 performs its antifungal activity through a γ-core motif, and the addition of acidic amino acids at both ends of its sequence did not affect its activity. In contrast, AMP3 and AMP3-DE showed distinct antibacterial and antifungal activities, suggesting that the properties of the original peptide were compromised by the addition of foreign amino acids. Intriguingly, while a difference in the antibacterial activity of AMP3 was expected towards different bacteria, it was not expected between closely related pathovars (*actinidiae* and tomato). This further suggests the previous assumptions regarding the possibility of a strain-specific activity. It should be noted that in this case, it was a dose-dependent effect, and AMP3 was able to inhibit both *P. syringae* pathovars. AMP3 has indeed been selected for its strong antimicrobial activity besides other parameters, and the high efficacy against these pathosystems confirmed the potential of this antimicrobial peptide as a suitable candidate for exposition on eCPMV. The strain-specific activity is a fundamental feature for the development of sustainable pesticides. A high pesticide specificity is preferred than a broad activity which can negatively impact the ecosystem and cause adverse effects against, for example and without limitation, beneficial microbes. This approach with functionalizing eCMPV nanoparticles with selected AMPs was unsuccessful since the production of eCPMV-AMP nanoparticles was particularly cumbersome, and no purified nanoparticles have been obtained. The importance of the properties of the displayed peptide on the CPMV surface was extensively highlighted since the first attempts and it was proposed that the properties of amino acids, more than peptide length, were important for the proper exposition on CPMV surface. Later, a specific investigation into the peptide display system brought to the conclusion that the foreign sequence should have an isoelectric point lower than 9 and a sequence shorter than 30 amino acids. However, no such study has been carried out for eCPMV, likely because both nanoparticles maintain the same

structure and have a similar mechanism for capsid assembly. The C-terminus of the SCP may play a pivotal role, contributing in particular to the interaction between SCP and promoting the formation of pentons, which constitute the first element for capsid assembly. Intriguingly, from single-amino acid substitutions and complete replacement of the C-term with 3 or 5 arginine residues, it was observed that this region must be highly basic to allow proper capsid assembly. Therefore, the likely failure in capsid assembly of eCPMV-AMPs could be due to the interaction between foreign peptides of different coat proteins or within the same asymmetric unit. This technology may be exploited to present a peptide that leads to partial prevention of autoimmune diabetes in mice.

[0182] Putative role of cysteines in capsid assembly

[0183] Insertion of a thiol group on the exterior surface of eCPMV by the addition of a single cysteine within the βB-βC loop of the SCP led to drastic and irreversible virus aggregation even in presence of tris(2- carboxyethyl)phosphine hydrochloride (TCEP), a reducing agent. Accordingly, the removal of cysteines from AMP3 improved the expression of nanoparticles *in planta* regardless of the isoelectric point. Indeed, AMP3-CDT, which showed the highest expression level, displays the highest pI (11.84), quite far from the pI of 9 recommended for eCPMV functionalization. However, this is likely not the only reason for the prevented capsid formation. Indeed, the functionalization of eCPMV with AMP2DE, which carries 4 cysteines, could assemble into a complete capsid, according to nanoparticle recovery in the fraction of ultrafiltration corresponding to molecules with MW > 1000 kDa, thus in line with the assembled capsid size. Thus, in this case, the presence of Cys residues did not alter the capacity of subunits to form a capsid structure. In contrast, for the chemical conjugation of AMP3 to the surface of eCPMV, the presence of Cys residues induced an aggregation of nanoparticles. Another problem related to the presence of Cys residues is their possible role in leading to nanoparticle aggregation. Indeed, it is assumed that the unsuccessful chemical conjugation of the AMP3 peptide with eCPMV may be due to the fact that AMP3 carries 4 cysteines that may establish disulfide bridges either with neighboring nanoparticles and/or with free peptides. In accordance with this hypothesis, following the same procedure of chemical conjugation with the vasoactive intestinal peptide (VIP), which does not carry any cysteine, no aggregation occurred. However, the treatment of eCPMV-AMP3 nanoparticles with DTT to remove disulfide bridges and subsequently with iodoacetamide to prevent their re-formation did not improve the solubility of nanoparticles. Therefore, the chemical conjugation of AMP3 is most likely hampered by the presence of cysteines. Several aggregations of VNPs after chemical conjugation have been observed, and the data described herein therefore confirm that this approach may be suitable for most but no all compounds and chemistries. It requires further studies to fully understand the process

of eVLP functionalization through chemical conjugation. It's worth mentioning that cysteines are highly reactive amino acids which found extensive application in click chemistry strategies. Due to their high reactivity, cysteines may be difficult to control using chemical means. Indeed, the presence of thiol groups at the surface of eCPMV most likely affects particle assembly due to protein-protein interaction. This is not easily predictable by looking at the sequence inserted, as different behaviors were observed among the selected AMPs. Structural properties should be taken into consideration during nanoparticle design, and bioinformatics tools like protein modelling could help to predict the outcome of the modification. However, protein modelling may only work on single monomers of a capsid, and more powerful tools may be needed to predict their interactions during capsid assembly.

[0184] Purification of eCPMV-AMPs and their antifungal activity within leaf extracts

[0185] eCPMV nanoparticle purification takes advantage of the presence of an assembled capsid, which shows peculiar features with respect to plant proteins, e.g., net charge and large size. Moreover, a satisfactory level of expression is required to achieve nanoparticle purification since the process itself does not achieve a 100% recovery of nanoparticles. Despite the improvements in expression, particles were not purified by any method. In particular, PEG precipitation-based purification has been the only procedure followed and proved to be an efficient method with different peptides. However, the yield may still be related to the infectivity of VNPs and thus their abundance *in planta*, and these factors may affect the success of the purification. Nevertheless, since nanoparticles or eCPMV subunits could not be purified, their potential antifungal activity within plant extract was assessed. Unfortunately, no fungal growth reduction was observed. Besides the likely absence of particle formation, this result could be due to the mechanism of action of AMPs, which may be related both to the abundance and to the structure. At the same time, an evaluation of fungal growth inhibition in presence of a plant leaf extract proved to be not suitable for this purpose. *B. cinerea* grew better in the presence of plant extracts regardless of the presence of particles. Indeed, as a similar approach, vegetable extracts can be added to the growth medium to enhance its growth and favor its sporulation.

[0186] The unlikelihood in obtaining purified eCPMV nanoparticles with the selected AMPs leaves the question about the antimicrobial efficacy of eCPMV-AMPs. Altogether, these data suggest that the mechanism by which eCPMV particles can properly assemble is still puzzling. Moreover, to achieve antimicrobial activity, AMPs may need to reach a minimal concentration and be in close proximity to one another in order to allow for membrane permeabilization. The steric

hindrance of eCPMV carrying the AMPs together with the low expression level may be another explanation of the absence of antimicrobial activity within plant leaf extracts.

[0187]

[0188] EXAMPLE 3: eCPMV GENETIC ENGINEERING FOR TRIGGERING THE PLANT IMMUNE SYSTEM

[0189] Introduction

[0190] The Plant Immune System at a glance

[0191] The evolutionary dynamic concept of "Red Queen", as described above, explains the basis of plant-pathogen interactions: there is a continuous arms race between the host and the invaders for gaining a reproductive advantage. It is possible to assume that such interplay evolved since the appearance of plants on earth, approximately 500 million years ago, when microorganisms had already colonized almost any environment. Indeed, since there are also some pieces of evidence of bacteria infecting algae (for example, *Pseudomonas protegens* hampers the growth of *Chlamydomonas reinhardtii*), the establishment of symbiotic interactions started probably with the first emergence of plants. This interkingdom communication takes place by a continuous trade-off of molecules, which are perceived by receptors leading to a cooperative or antagonistic interaction. The first layer of plant stress response relies on receptor-like proteins (RLPs, also known as surface receptors) associated with the plasma membrane. The extracellular domain is devolved to the perception of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) or damage-associated molecular patterns (DAMPs) through leucine-rich repeat (LRR), lysine motif (LysM), lectin, and epidermal growth factor (EGF)-like domains. RLPs show also a transmembrane domain, and in some cases, an intracellular kinase domain, and are therefore termed RLKs (receptor-like kinases). On the other hand, microorganism pathogenicity can rely on the injection of molecules, which are termed virulence effectors, into plant cells. Such molecules can be, in turn, perceived by plants through intracellular nucleotide-binding site leucine-rich repeat receptors (NLRs), which constitute the second layer of pathogen perception, which ultimately trigger an immune response. Both types of receptors originated in caryophytes, suggesting that the evolution of the plant immune system through invader recognition started just before the colonization of terrestrial environments. A first proposal of the interplay between these two layers of plant defense is commonly known as the "zig-zag" model. According to this model, plants primarily detect pathogens thanks to RLKs, thus triggering an immune response, which hampers pathogen invasion through a process called PAMP-triggered immunity (PTI). From their side, pathogens secrete into the plant cells some effector molecules, restoring the pathogenicity in a phenomenon called effector-triggered susceptibility

(ETS). Resistant plants, in turn, evolve NLRs able to recognize specifically some effectors to mount up a strong immune response, termed effector-triggered immunity (ETI), characterized by a hypersensitive response (HR) leading to a programmed cell death able to stop biotrophic pathogen spread. In this frame, the NLR receptor responsible for specific effector recognition is termed R protein, where R stands for resistance, while the effector that is recognized is called avirulence factor (Avr). From an evolutionary point of view, plant-pathogen interactions are defined by this continuous arms race that is constantly evolving, therefore each avirulence factor is recognized by the product of an R gene, following a so-called "gene-for-gene" model and later the "guard model". However, these models of direct or indirect R-Avr interaction did not consider that different effectors could target the same R protein, and also that after R-Avr recognition, no immune response could be triggered. Another model, termed "decoy model", was thus proposed to solve these inconsistencies. The new assumptions were that R proteins are under continuous evolutionary pressure that may lead to an improved or reduced Avr recognition, leading to resistance reduction in the latter case. Therefore, it has been suggested that plant cells could display multiple decoy proteins, with the only function of binding effectors, by mimicking effector target features, and that the plant immune response is triggered only after decoy-R protein interaction. As an example, the RIN4 protein of *A. thaliana* plays a pivotal role in plant defense and is the target of at least 3 *P. syringae* effectors (AvrB, AvrRpm1 and AvrRpt2). Its role is associated with two R proteins (RPS2 and RPM1). The hypothesis is therefore that RIN4 acts as a decoy, since in knockout rin4 plants, AvrRpm1 and AvrRpt2 exploit virulence anyway. Moreover, a mutated AvrRpt2, unable to degrade RIN4, is not altered in its virulence function. Importantly, these two models assume that PTI, ETS and ETI are consecutive events that occur during a plant-pathogen interaction but do not consider the role played by DAMPs. Accordingly, the "ZigZag" model applies only to pathogens, which need their host to survive and exploit pathogenicity and, by contrast, does not apply to necrotrophs, insects, and symbionts. Moreover, this strict dichotomy between PTI and ETI is unlikely to occur during a real interaction, since it is not possible to have ETI without PTI, and both phenomena are likely to occur together. These observations led to the concept of the plant immune system as a "surveillance system", in which plants are constantly monitoring the presence of MAMPs or DAMPs and respond consistently, besides the strict difference between ETI and PTI. The benefit of this view is that plants can distinguish between invaders and beneficial microbes and that it considers necrotrophic pathogens, insects and viruses. The overlapping response between PTI and ETI, which share numerous responses, has been recently deeply investigated to suggest that plant-pathogen interaction includes a complex set of molecular events that are occurring simultaneously. Moreover,

a revision of the zig-zag model has been recently proposed in which ETI does not constitute a separate response but instead contributes positively to the PTI response, enhancing its effect and ultimately leading to resistance.

[0192] MAMP/PAMP/DAMP surface sensing and signal transduction

[0193] The plant cell membrane is the last physical barrier separating the plant cell from the external space and it is highly organized in nano and microdomains in which receptors are organized to increase their sensitivity and signal amplification. MAMPs and DAMPs are mostly small proteins or polysaccharides, that are sensed by different ectodomains of PRRs located at the plasma membrane. The most studied interaction among PAMPs is probably the one occurring between the peptide flg22, a minimal active motif located at the N-terminus of the bacterial flagellin, which is cleaved in the extracellular space by the action of the β- galactosidase (BGAL1) and then recognized by the co-receptors FLS2 (flagellin sensing 2) and BAK1 (BRI1-associated kinase). Another example of well-studied RLPs is the elongation factor Ef-Tu receptor (EFR), which detects the presence of the bacterial Ef-Tu through its N-terminal acetylated peptide elf18. Many other examples of PAMPs exist, such as the 25-amino acid peptide from *Botrytis cinerea* xylanase 11A that triggers an immune response in tomato and tobacco and an epitope of the transglutaminases of different *Phytophthora spp.* species, namely Pep13, which acts as an immunity-triggering peptide, although its receptor has not been already identified.

[0194] DAMPs represent another important category of general elicitors that are produced by the degradation of the plant cell wall or from protein precursors produced by the host. Rapid alkalization factors (RALFs) are a family of peptides, with 39 representatives in *A. thaliana*, generated from pre-proproteins perceived by the FERONIA RLKs, which act antagonistically respect to the FLS2-BAK1-mediated response. CAPE1 is a peptide isolated from tomato apoplastic fluid and corresponds to the C-terminal portion of tomato PR1. CAPE1 can repress the growth of Pseudomonas syringae pv. tomato DC3000, the feeding larvae *Sedopthera litura* or fungal pathogens in wheat, by activating defense genes. In *Arabidopsis thaliana*, another family of peptides, termed PAMP-induced (PIPs), enhances plant resistance against Pst DC3000. Interestingly, these peptides are released in the apoplast by a proteolytic cleavage of their precursors.

[0195] Since the mechanism of flg22 perception and signal transduction is the most well studied, it is described here as a general mechanism of elicitor signal transduction. Flg22 is a 22-amino acid peptide located in the N-terminal part of the bacterial flagellin. The flagellin-encoding FliC gene is present in the genome of many different bacterial species and the sequence of the minimal peptide flg22 is relatively conserved. Some key residues involved in flg22 recognition by

the FLS2 receptor (RLK with an LRR ectodomain), and the further formation of the PRR complex FLS2-BAK1, have been identified. Accordingly, the flg22 peptide from *Ralstonia solanacearum* (Rso) presents an I21A substitution that prevents its recognition by the FLS2-BAK1 complex of *A. thaliana*. However, the same peptide is perceived during the interaction between soybean and Rso, thus supporting the occurrence of mutations in the FLS2-BAK1 complex allowing the recognition of mutated flg22 peptides. The receptor FLS2 is widely spread among different plant species, implying that the recognition of the bacterial flagellum is a common mechanism to mount up a defense response against bacteria. Recently, an effort to build up the network of all known *A. thaliana* LRR ectodomains expressed in Drosophila Schneider S2 cells highlighted that FLS2- BAK1 complex interacts with FIR (FLS2-interacting receptor), promoting their interaction. Other PRRs have been proposed to interact with the FLS2-BAK1 complex, but their role in triggering immunity is still unknown. Following flg22 perception, receptor complex activation induces very rapid events, including membrane depolarization and extracellular alkalinization due to the efflux of Cl^-, NO_3^-, and a rapid increase of cytosolic Ca^{2+} concentration. The latter is one of the fastest responses upon pathogen perception and exhibits different amplitudes and durations giving rise to the so-called 'calcium signatures', which can be defined and monitored using luminescence-based assays. Due to the fast nature of this response, Ca^{2+} signaling is crucial in shaping the plant immune response. Indeed, the *A. thaliana* double mutant aca8 aca10, mutated in two Ca*2*+ ATPases, is impaired in Ca^{2+} signaling and unable to mount up a proper PAMP induced calcium burst as well as after flg22 treatment. Moreover, this mutant displayed a hypersusceptible phenotype after Pst DC3000 inoculation. Moreover, after FLS2-BAK1 complex formation, the activated intracellular kinase domains lead to BIK1 (*Botrytis*-induced kinase1) phosphorylation, which then phosphorylates RBOHD, leading to the burst of reactive oxygen species (ROS). As important players in stress signaling, it should be noted that ROS can directly modify proteins through post-translational modifications, resulting in an activation or inhibition of the target proteins. These target proteins may include different protein kinases, which can thus be considered as ROS sensors. Moreover, ROS production leads to an activation of an important signaling pathway driven by mitogen-activated protein kinases (MPKs), including MPK3 and MPK6. MAPK cascade activation leads in turn to hormone pathway regulation and differential gene expression, driven in particular by WRKY transcription factors upon phosphorylation by MAPKs. The transcriptional reprogramming leads to the activation of defense responses, including without limitation the production of pathogenesis-related (PR) proteins, antimicrobial activities, and strengthening of the above-mentioned signaling events. Last occurring events that are the result of the combined action of signal transduction and

represent the plant defense responses per se include the stomata closure and the deposition of callose, by which the plant aims to hamper pathogen colonization.

[0196] Plant hormone-mediated pathways, involving especially salicylic acid (SA) and jasmonic acid (JA), play a crucial role in mediating defense signal transduction during plant-pathogen interaction, contributing to the specificity of the triggered immunity and often depending on pathogen lifestyle. Indeed, both SA and JA are considered to play an important role in plant defense responses against biotrophic and necrotrophic pathogens, respectively. Interestingly, these hormones originate within the chloroplast. SA is produced for 90% by the isochorismate synthase gene (ISC1), which produces its precursor isochorismate (IC) in the chloroplast. Then IC is transported into the cytosol, where it is converted by PBS3 into an intermediate form and finally into salicylic acid, either spontaneously or through a reaction catalyzed by EPS1. The SA-dependent immune response is considered responsible for the immune signaling towards distant leaves, which results in a mechanism known as systemic acquired resistance (SAR). It is generally accepted that JA-dependent response antagonizes SA-mediated response. Indeed, some pathogens, like *P. syringae*, suppresses SA-mediated immunity through the activation of JA signaling due to the secretion of the effector HopZ1a. JA is produced through the conversion of lipids in the chloroplast; once transported in the cytosol in its active form JA-Ile, it is recognized by the receptor COI1 together with the co-receptor JAZ. JA-Ile perception leads to the degradation of the JAZ repressor proteins and, consequently, the activation of the MYC2 transcription factor, which ultimately activates the expression of JA- dependent genes and its repressors in a negative feedback loop. The mechanisms by which plants respond to pathogen invasion are thus the result of a complex, and still incompletely understood, cascade of events.

[0197] Materials and Methods

[0198] To avoid redundancy, the following methods are not reported here since they have been already described in EXAMPLE 2 above: Preparation of expression vectors, Colony PCR, Plasmid DNA isolation, Agrobacterium tumefaciens LBA4404 strain transformation, Production and purification of eCPMV and eCPMV-ITPs nanoparticles, eCPMV-AMPs nanoparticle purification by size exclusion chromatography, SDS-PAGE, Western blot, Silver staining.

[0199] Plant material

[0200] *Nicotiana benthamiana* plants have been grown in soil pots in controlled conditions for 4-5 weeks (22-24 °C, 120 µmol photons $m^{-2}s^{-1}$, 24 °C, 70% humidity, 10/14 h day/night).

[0201] *Arabidopsis thaliana* seeds sterilization and treatments for gene expression experiments

[0202] *A. thaliana* Columbia-0 (Col-0) plants were grown in soil under controlled conditions, humidity 70%, temperature 24 °C, 12 h of photoperiod. For *in vitro* experiments, *A. thaliana* seeds were surface sterilized with 90% ethanol for 1 minute then with 1.5% of sodium hypochlorite and 0.05% tween® 20 for 5 minutes, then rinsed three times with sterile Milli-Q water for 5 minutes, each with continuous agitation. Sterilized seeds were sown on liquid or solid half-strength MS agar supplemented with 1% of sucrose. Plates were transferred to a growth chamber with 16 h/8 h day-night photoperiods, at a constant temperature of 23 °C. For gene expression, *A. thaliana* plantlets were grown in a liquid half-strength MS medium supplemented with vitamins and 1% sucrose in 12-well transparent plates, sealed with breathable tape. 10-15 plantlets of 10-12 days old were challenged with 1000 nM, 100 nM, 10 nM or 1 nM of flg22 synthetic peptide (Biomatik Corporation, Canada), eCPMV-WT, or eCPMV-ITP1 (assuming that each asymmetric unit exposes a peptide) for 1 h, 6 h and 24 h, sodium phosphate buffer (NaP, 0,25 mM pH 7.0) was used as the negative control. Three individual biological replicates were used for each condition. After treatment, plants were flash-frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

[0203] *Arabidopsis thaliana* mesophyll protoplast isolation

[0204] For protoplast isolation, *A. thaliana* Aeqcyt/pMAQ2 in Col-0 background plants were grown in soil under controlled conditions for 5 weeks (70% humidity, 12 h day/night photoperiod, seeds were retrieved from professor M. Knight under MTA). Fully healthy expanded leaves from the younger portion of the "rosetta" were collected, surface-sterilized in sodium hypochlorite 0.5% for 5 minutes and rinsed three times with Milli-Q water for 5 minutes each. Mesophyll protoplasts were isolated. Briefly, leaves were immersed and cut in small pieces (squares of approximately 1 mm side) in the sterile enzyme solution (20 mM MES, pH 5.7, 1,5% w/v cellulase R10, Duchefa Biochemie, 0.4% Macerozyme R10, Duchefa Biochemie, 0.4 M mannitol, Sigma Aldrich, and 20 mM KCl). The solution was vacuum infiltrated into leaf pieces and the cell wall digestion was carried out for 3 h in the dark at room temperature. The protoplast solution was gently filtered through a sterile plastic sieve (70-µm pores) and diluted with an equal volume of W5 solution (2 mM MES, pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl). Protoplasts were kept in ice from this moment, washed once in this solution by centrifugation at 100×g for 2 minutes, and then resuspended in MMG solution (4 mM MES, pH 5.7, 0.4 M mannitol, and 15 mM $MgCl_2$). Protoplasts were checked for their integrity by light microscopy and diluted with MMG solution to 2 $\times 10^5$ protoplasts/mL for further use.

[0205] Aequorin-based measurements of intracellular Ca^{2+} variations

[0206] For apoaequorin assay, *A. thaliana* Aeqcyt/pMAQ2 in Col-0 background plants were grown *in vitro* in a liquid half-strength MS medium supplemented with 1% of sucrose for 8-10 days. Then, plantlets were individually transferred into each well of a white 96-well plate filled with 75 µL of sterile distilled water, avoiding any mechanical damage. Coelenterazine (PJK GmbH) was added at a 10 µM final concentration, and plants were incubated overnight in the dark. Eventually, 3 plants were transferred into the same well to enhance the photon emission. Plants were then treated with a 3-fold concentrated ITP1 solution to reach a final concentration of 1000 nM, 100 nM, 10 nM, and 1 nM, with eCPMV, eCPMV-ITP1 at 1000 nM. The release of photons was measured with TECAN infinite PRO200, at intervals of 6 seconds per well over a period of 20 minutes. For a long-incubation assay, a transparent lid was placed over the plate to prevent plants from dehydration, and luminescence was measured at intervals of 6 seconds for 16 h. Five individual plants were used for each condition, and the experiment was repeated three times. At the end of the measurement, 150 µL of the discharge (2M $CaCl_2$, 20% ethanol solution) was added to each well, and the emission of the luminescence was recorded for 5 minutes with a regular interval of 6 s per well. The discharge leads to a complete reconstitution of all apoaequorin protein produced by plants, allowing for a calibration of the detected signal against the maximum signal.

[0207] eCPMV nanoparticle characterization

[0208] Following purification, eCPMV particles were subjected to SDS-PAGE, dynamic light scattering (DLS), transmission electron microscopy (TEM) and agarose gel electrophoresis for particle characterization.

[0209] Transmission electron microscopy

[0210] TEM analysis of total plant protein extract was carried out to detect the presence of intact particles. Sampled leaves were pestled in sodium phosphate buffer (0.1 M, pH 7.0). TEM carbon-coated copper grids (200 mesh) were laid on a 10-µL drop of protein suspension and then washed with MilliQ water. UranyLESS EM stain (EMS) was used as a negative staining agent by deposition of a drop to each grid followed by a single wash with MilliQ water. Grids were analyzed at FEI TECNAI G2.

[0211] RNA extraction and cDNA synthesis

[0212] Plant tissues were ground in liquid nitrogen and stored at -80 °C until further manipulation. Total plant RNA was extracted alternatively using TRIzol (Invitrogen) or TRIfast (Euroclone) reagents according to manufacturer indications with minor modifications. RNA purity was assessed at NanoDrop One spectrofluorometer (ThermoFisher scientific), and samples with a low (< 1.8) 260/230 ratio (i.e., OD260/OD230) were subjected to LiCl treatment to remove

contaminants. Briefly, LiCl was added to RNA samples to a final concentration of 2.5 M, then samples were incubated at -20 °C for at least 30 minutes. RNA was pelleted by centrifugation at 12000×g for 20 minutes at 4 °C, and the supernatant was discarded. The pellet was washed with cold 70% ethanol and precipitated at 12000×g for 5 minutes at 4 °C. This step was repeated twice. The RNA pellet was air-dried and finally resuspended in Milli-Q water. The integrity of RNA was assessed by electrophoresis on 1% agarose gel. RNA was subjected to DNA removal by TurboDNase treatment (Ambion) following producers' indications. cDNA was synthesized from 2 µg of DNA depleted RNA using SuperScripIII (ThermoFisher) according to manufacturer instructions.

[0213] Real-time RT-qPCR and data analysis

[0214] Gene expression analysis of the selected defense-related genes was carried out by real-time RT-qPCR using diluted cDNA as the template and the GoTaq® qPCR Master Mix (Promega) as the master mix. The following PCR program was used for all PCR reactions: a cycle of 50 °C held for 2 minutes followed by 95 °C held for 10 minutes, 40 cycles at 95 °C for 15 seconds followed by 58 °C for 30 seconds, and the final stage in which dissociation curves were recorded. The PP2A gene (AT1G13320) was used as reference in all experimental conditions. Genes and their respective forward (F) and reverse (R) primer sequences are indicated in table 2.

Table 2. List of primers used for gene expression analysis.

Gene name	Locus	Primers
PR1	AT2G14610	F: ACGTGCAATGGAGTTTGTGG (SEQ ID NO: 1) R: ACACCTCAACTTTGGCACATC (SEQ ID NO: 2)
PR5	AT1G03230	F: TTCATCACAAGCGGCATTGC (SEQ ID NO: 3) R: GTCAATTCAAATCCTCCATCGC (SEQ ID NO: 4)
PDF1.2	AT5G44420	F: CTCTTTGCTGCTTTCGACGC (SEQ ID NO: 5) R: ATGTCCCACTTGGCTTCTCG (SEQ ID NO: 6)
ERF1	AT3G23240	F: ACGATCAAGCTGCTTTCTCG (SEQ ID NO: 7) R: TCCTCTTCAACGCCACAACC (SEQ ID NO: 8)
PP2A	AT1G13320	F: TAACGTGGCCAAAATGATGC (SEQ ID NO: 9) R: GTTCTCCACAACCGCTTGGT (SEQ ID NO: 10)

[0215] The amplification efficiency was calculated for each sample from raw data using the LinReg software. Then RT-qPCR data were analyzed by calculating the mean normalized expression and the standard error according to the Mean Normalized Expression (MNE) formula below:

$$MNE = \frac{(E_{reference})^{CT_{reference,mean}}}{(E_{target})^{CT_{target,mean}}}$$

$$SE_{MNE} = MNE \times ((lnE_{target}) \times SE_{CT_{target,mean}})^2 + ((lnE_{reference}) \times SE_{CT_{reference,mean}})^2)^{1/2}$$

[0216] Where: E stands for the reaction efficiency, CT for the threshold value, and SE for the standard error.

[0217] eCPMV nanoparticle penetration into *A. thaliana* leaves

[0218] *A. thaliana* plants were grown in a liquid half-strength MS medium supplemented with 1% sucrose for 14 days in a 12-well plate. eCPMV and eCPMV-ITP1 nanoparticles were added to reach a final concentration of 10 nM and 1 nM, with asymmetric units considered in the same manner as for the gene expression experiments. 10-12 plants were treated for each condition and 3 biological replicates were performed. Plants were sampled at 1, 6, and 24 h after particle treatment. To avoid nanoparticle contamination from the leaf surface, plants were washed in sterile distilled water before freezing in liquid nitrogen and weighted. Frozen tissues were homogenized by milling using glass beads, and a reducing extraction buffer was added to each sample with a ratio of 1:3 (w/v). The presence of eCPMV and eCPMV-ITP1 nanoparticles within protein leaf extracts were thus assessed by western blotting.

[0219] Assessing the activation of a hormone-mediated pathway by GUS assay reporter system

[0220] Transgenic *A. thaliana* Col-0 plants expressing the promoter of the AthJAZ2 gene were grown in a half-strength MS medium supplemented with 0.3% Phytagel and 1% sucrose for 10 days. The AthJAZ2 gene drives the expression of uidA gene encoding the β-glucuronidase (GUS). Then plants were transferred into a 12-well plate filled with a liquid half-strength MS medium (1% sucrose) avoiding mechanical stress. Four days after transfer, plants were challenged with 20 nM eCPMV, eCPMV-ITP1, and eCPMV-ITP2 nanoparticles for 24 h. The liquid medium was replaced with the GUS staining solution (2.5 mM NaP, pH 7.0, 0.1% Triton X-100, 0.5 mg/mL X- Gluc). Plants were vacuum infiltrated and incubated at 37 °C for 5 h. Finally, the staining solution was removed, and tissues were clarified by addition of 70% ethanol. Images were acquired using a Leica EZ4 light stereomicroscope, and Pixel saturation was evaluated with ImageJ. Images color mode was converted to HSB, then the saturation channel in the HSB stack was selected. The intensity of GUS staining was measured in the saturation channel.

[0221] *A. thaliana* flood and *syringae* inoculation of *Pseudomonas syringae* pv. tomato DC3000

[0222] *A. thaliana* Col-0 plants were grown on a half-strength MS medium supplemented with 0.3 % Phytagel and 1% sucrose for 14 days in 12-well plates. Each well, containing 10-12 plants,

was treated for 24 or 48 h with eCPMV and eCPMV- ITP1 nanoparticles or with flg22 synthetic peptide and NaP buffer (0.25 mM, pH 7.0) as the positive and the negative controls, respectively. Flg22 synthetic peptide, eCPMV and eCPMV-ITP1 nanoparticles were tested at 1 nM as the final concentration. The treatments were performed by adding the solution containing the particles to the wells avoiding plant submergence. After 24 h, the solutions were removed. Pst was flood inoculated. Alternatively, for Pst vacuum infiltration, the bacterial suspension was prepared as follows. Bacterial cells, from an overnight grown Pst culture, were collected by centrifugation at 4000×g for 5 minutes at room temperature. Cells were rinsed three times in sterile 10 mM $MgCl_2$ and diluted to 5×10^5 cfu/mL with the same solution. Then, *A. thaliana* plants were submerged and vacuum infiltrated with the bacterial suspension. After infiltration, the bacterial suspension was removed by pipetting. 2 or 3 days post-inoculation (dpi), plants were collected for bacterial count in KB agar plates supplemented with the appropriate antibiotic.

[0223] Results

[0224] Selection of ITP candidates

[0225] Plant immunity can be activated through different mechanisms following the perception of either microbe-derived or self damage-related molecules. The activation of plant immune response starts from the outside of the plant cell, in which the cell membrane has a pivotal role in perceiving external stimuli. In particular, plant cells recognize potential invaders by specific components of their structure, such as chitin for fungi and flagella for bacteria, or by the damage caused by pathogens. In both cases, plant cells have specific receptors located within the plasma membrane, able to detect the presence of such microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) or damage-associated molecular patterns (DAMPs). Only proteinaceous molecules have been considered and henceforth are called Immunity-Triggering Peptides or ITPs. Then, the signal perception can be mediated by the single receptor itself or in association with other co-receptors through extracellular domain interactions. In this second case, the MAMP or DAMP molecules can act as a molecular glue among different receptors, leading to signal perception. Accordingly, different peptides have been selected considering (i) their role in triggering a defense response, (ii) the mode of perception by the plant cell, and (iii) the presence of post-translational modifications. Indeed, some ITPs undergo post-translational modifications at their N- or C-terms, making them biologically active (e.g., elf18 is an epitope of the bacterial elongation factor Tu having an N-acetylated N terminus, which is essential for its activity). Since such PTM is very unlikely to occur on peptides fused to eCPMV particles, peptides requiring such modifications have been excluded from the selection.

[0226] Three peptides have been thus selected: ITP1 is a peptide of bacterial origin, while ITP2 and ITP3 are derived from plants and are thus considered as DAMPs (see Table 3). ITP1 is a well-known MAMP/PAMP, which is recognized by a two-receptor system on the cell surface, whose interaction leads to a well-known cascade of signaling events. ITP2 and ITP3 are part of plant defense mechanisms and are produced after the induction of the plant immune response. ITP2 and ITP3 were selected since their recognition relies on a single receptor on the plasma membrane, thus limiting the possibility of an incomplete recognition due to steric hindrance once exposed on the particle. However, even if a putative receptor for ITP2 has been proposed, further knowledge about the first signaling events occurring after its perception is still missing. Nevertheless, its role in plant defense is well recognized and different late responsive molecular events triggered by ITP2 have been already identified. The ITP sequences were not further modified to fit the requirements (length, pI, etc.) for the insertion in the βB-βC loop of the eCPMV.

Table 3. List of selected ITPs

Name	Lenght	pI	MW	Source	Role in plant defence
ITP1	22 AA	8.75	2216.58	Bacteria (PAMP)	Activation of SA pathway
ITP2	11 AA	9.18	1189.34	Plant (DAMP)	Activation of JA and SA pathways
ITP3	18 AA	9.70	2010.30	Plant (DAMP)	Activation of ET pathway

[0227] Expression and purification of eCPMV-ITPs nanoparticles

[0228] The expression of chimeric eCPMV nanoparticles in plant leaves has been evaluated at 6 days post-agroinfiltration by western blotting on total leaf protein extracts using an anti-CPMV polyclonal antibody. Briefly, total soluble proteins have been extracted from leaves homogenized in sodium phosphate buffer 0,1 M pH 7.0 with a ratio 1:3 (w/v). For each nanoparticle, both subunits showed a high level of expression in planta, though lower respect to the unmodified nanoparticles. This indicated that, though the presence of the peptides may decrease slightly nanoparticle expression efficiency, ITPs do not alter significantly the physio-chemical properties of the βB-βC loop, regardless of the length or the pI of each peptide (See FIG. 25, embodiment 2500). The absence of cysteine residues and charged amino acids likely excluded the possibility of establishing disulfide or saline bridges, both of which can contribute to the rigidity of the loop once the peptide is

inserted into the sequence. It is worth highlighting that the differences in size of the small coat proteins, observed by western blot, confirmed the presence of the peptides in the three recombinant protein-functionalized nanoparticles.

[0229] Nanoparticle purification was successfully achieved for eCPMV-ITP1 using both methods: PEG precipitation followed by centrifugal separation or by a combination of anion exchange chromatography and size exclusion chromatography, although the latter method may be preferred since it provided functionalized eVLPs with a higher degree of purity, besides being more reproducible (see FIG. 26, embodiment 2600). eCPMV-ITP1 was recovered with an estimated yield of 0.05 mg/g of fresh tissue, which is approximately 25% of the yield obtained with unmodified eCPMV.

[0230] eVLP purification was unsuccessful for eCPMV-ITP2 and eCPMV-ITP3 using the classical protocol (i.e., the first procedure) of PEG-mediated precipitation combined with centrifuge-based separation. In particular, nanoparticles precipitated together with other proteins just before the last step of purification (see FIG. 27, embodiment 2700). In contrast, using the second procedure, combining two different chromatography steps, eCPMV-ITP2 nanoparticles were successfully purified, even though with a lower reproducibility and yield compared with eCPMV-ITP1 (0.03 mg/g of fresh tissue, SEE FIG. 28, embodiment 2800). On the other hand, this second procedure did not improve eCPMV-ITP3 purification, since it couldn't be separated during gel filtration. Following the chromatography procedure, both CPMV-ITP2 and CPMV-ITP3 showed a high retention rate into the resin of the anion exchange column, since a significant amount of nanoparticles was found in the elution fraction (see FIG. 29, embodiment 2900). This was quite unexpected since nanoparticles are not supposed to interact with the resins due to their size, what makes less probable their retention by weak anionic interactions.

[0231] Characterization of eCPMV-ITPs

[0232] After purification, functionalized nanoparticles were characterized using different methods to assess their integrity, dimension, and structure. In particular, dynamic light scattering (DLS) proved to be an efficient technique to characterize the size of the nanoparticles and detect the presence of aggregates. eCPMV-ITP1 nanoparticles have been characterized several times and showed a high degree of stability, even when stored at room temperature for several weeks. When stored at 4 °C, the nanoparticles were stable for at least 1 year, although some precipitation occurred over time, as revealed by a broad peak in the size distribution starting from 20 nanometers and extending over 200 nm. Such aggregates have been easily removed after nanoparticle filtration through a 0.22-μm filter (see FIG. 30, embodiment 3000). In the same manner, eCPMV-ITP2

showed stability similar to eCPMV-ITP1, confirming the high stability of eVLPs functionalized with ITPs. All nanoparticles were present in a major single peak at around 30 nm, with a mean of 28.8 nanometers for unmodified and 28.2 nanometers for ITP1-functionalized nanoparticles, respectively. A low polydispersity index was detected, demonstrating that they were mainly monodisperse. Thus, the index of polydispersity of eVLPs may be interpreted to indicate a presence of aggregates instead of a presence of nanoparticles with different sizes. Moreover, unmodified eCPMV and eCPMV-ITP1 nanoparticles have been visualized by transmission electron microscopy, confirming that functionalized nanoparticles assembled into complete capsids, maintaining the same shape as the unmodified eCPMV (see FIG. 31, embodiment 3100). Both unmodified eCPMV and eCPMV-ITP1 displayed pale hexagonal structures and, occasionally, as dark-filled regions, since the absence of genetic material allowed the negative stain to accumulate inside the nanoparticle, thus only enabling a visualization of their profile. The analysis also confirmed the diameter of nanoparticles described above to be consistently within the range from 28 nanometers to 30 nm. Native agarose electrophoresis was also routinely performed to assess the integrity of the nanoparticles based on their rate of migration into the gel (see FIG. 32, embodiment 3200).

[0233] Assay of bioactivity of eCPMV-ITPs

[0234] The investigation of the role of functionalized eCPMV nanoparticles included an analysis of both early- and late-stage molecular events. The same analyses were conducted with unmodified eCPMV since, as a viral capsid it may be recognized by plant cells and induce defense responses by itself. The intracellular increase in Ca^{2+} concentration represents a very early plant defense molecular mechanism. Accordingly, the treatment of *A. thaliana* plantlets with the PAMP flg22 triggers a fast increase of intracellular Ca^{2+}, which reaches its peak after only 2-3 minutes, providing a specific Ca^{2+} signature. To monitor Ca^{2+} concentration variations, transgenic *A. thaliana* plants that constitutively express the apoaequorin protein in the cytoplasm have been used. After reconstitution, the aequorin binds to Ca^{2+}, leading to a conformational change that allows the oxidation of its substrate and further light emission. Thus, 9-day-old transgenic plantlets were challenged with eCPMV, eCPMV-ITP1 or eCPMV-ITP2 nanoparticles, and the synthetic flg22 peptide was used as a positive control due to its well-known capacity to induce Ca^{2+} concentration variations. eCPMV-ITP3 nanoparticles were not included in the assay since it was not possible to pursue their purification. Nanoparticles were applied in a range of concentration from 1 nM to 1 μM, while only lower doses of the peptide were applied to define a minimal detectable signal with this technique. According to the fact that the response intensity is proportional to peptide concentration, the reduction of flg22 concentration to 1 nM strongly reduced luminescence signal (see FIG. 33A,

embodiment 3300a). At this concentration, the Ca^{2+} spike was still present, although broader and delayed compared with higher concentrations. FIG. 33B, embodiment 3300b, shows that nanoparticles (functionalized or unmodified) did not induce an increase in Ca^{2+} concentration, regardless of the concentration used. It is speculated that that ITPs exposed to the surface of eCPMV capsid may be hampered in reaching its target receptor, preventing a rapid recognition of the PAMP within the time course of the experiment. Alternatively, the signal may be lower than the detection limit of the method. Therefore, three plantlets were transferred in the same well in an attempt to increase the signal. Unfortunately, this step did not result in a detected signal following treatment with the nanoparticles, thus suggesting that the nanoparticles may likely require more time than their free-peptide analogs to penetrate into plant tissues and reach the receptor located at the plasma membrane. Plantlet response was thus monitored overnight after challenging the plantlets with eCPMV-ITP2 to detect a possible delay in response induction due to the structure and/or properties nanoparticles, but results were unsuccessful (see FIG. 34, embodiment 3400). Since aequorin must be reconstituted just before its binding with Ca^{2+}, it is speculated that the method may not be suitable for analyzing Ca^{2+} variations over long periods. Another possible cause may be due to the presence of cell walls that decreases the efficiency of nanoparticles in binding to receptors. *A. thaliana* protoplasts were thus produced in an attempt to facilitate nanoparticle interaction with the receptor by removing plant cell wall (see FIG. 35, embodiment 3500). Such an approach did not provide insights regarding the elicitor activity of nanoparticles, since the removal of the cell wall by enzymatic digestion desensitized the cells, rendering them even irresponsive to treatments of free elicitor peptides. The absence of response with the positive control did not lead to conclusive insights on the effect of protoplast treatment with nanoparticles, suggesting that this technique is may not be suitable for the purposes described herein.

[0235] Nanoparticle penetration into plant leaves

[0236] The possibility for nanoparticles to enter the plant leaves was assayed using *A. thaliana* plantlets treated with eCPMV, dispensed directly into the wells at a final concentration of 1 nM. Leaves were sampled at 1 h, 6 h, and 24 h after nanoparticle administration and washed with Milli-Q water before protein extraction to avoid nanoparticle contamination from the surface of leaves. As shown in FIG. 36, embodiment 3600, nanoparticles were not detected by western blot with an anti-CPMV antibody. It is very likely that the low number of nanoparticles used in the experiment and recovered after protein extraction was not compatible with the detection limit of the western blot (see FIG. 36, embodiment 3600). An alternative could be the use of a fluorescent compound-labelled eCPMV to monitor the presence of the nanoparticles using confocal microscopy.

[0237] Activation of hormone-mediated pathway using a reporter system

[0238] Due to the issues encountered when assessing the induction of early-stage plant defense responses (i.e., Ca^{2+} variations) by the nanoparticles, their activity was further evaluated for late-stage event activation. For this purpose, plants of transgenic *A. thaliana* carrying the JAZ2 promoter was grown in a liquid medium for two weeks. The JAZ2 promoter drives the expression of the uidA gene, which encodes the β- glucuronidase. Plants were then challenged with 20 nM of eCPMV, eCPMV-ITP1 or eCPMV-ITP2 nanoparticles for 24 h and subjected to GUS staining (see FIG. 37, embodiment 3700). Plants treated with either eCPMV-ITP1 or eCPMV-ITP2 showed a stronger staining compared to plants treated with water or unmodified eCPMV, though a slight background was observed in the controls. Moreover, older leaves seemed more intensely stained compared to the younger ones, and newborn leaves appeared almost white, except for the treatment with MeJA, which was used as a positive control. Finally, no staining was observed with eCPMV-ITPs in the roots. On the contrary, roots turned blue following the treatment with MeJA, which can be taken up through the radical apparatus, indicating that the JAZ2:uidA was expressed in all plant tissues. The absence of root staining in response to the treatment with eCPMV-ITPs led to a proposed mechanism by which the nanoparticles are perceived by the plants only at the level of the leaves. To ensure the significance of staining differences, color intensity was quantified with ImageJ, by considering the whole picture, in terms of saturated pixels (see FIG. 38, embodiment 3800). Staining quantification confirmed the qualitative observations, showing that eCPMV-ITP1 and eCPMV-ITP2 induced a statistically significant activation of JAZ2 promoter compared to the controls ($p < 0.01$). Moreover, unmodified eCPMV and water treatments were not significantly different, indicating that unmodified nanoparticles did not activate the pathway mediated by jasmonate in the plant. Taken together, these data thus suggested that eCPMV nanoparticles functionalized with two different ITPs induce the JA-mediated pathway in plants.

[0239] Activation of defence related genes

[0240] To get a more complete overview of the elicitor activity of eCPMV nanoparticles functionalized with ITP1, at both early and late time points, defense-related gene expression was assessed in *Arabidopsis thaliana* Col-0 plants. Plants were challenged with unmodified eCPMV or eCPMV-ITP1, at concentrations ranging from 1 μM to 1 nM, while the free peptide and NaP buffer were used as positive and negative controls, respectively. Selected genes first included genes induced specifically by ITP1 (i.e., ITP-response specific genes, ISGs) at early and late time points, as well as well-known markers of resistance response induction. The free peptide induced the expected dose-dependent response, which was still active at the lowest dose. Thus, it was possible to

follow and validate the plant response to nanoparticle treatment. eCPMV-ITP1 nanoparticles induced a significant expression of ISG1, ISG2 and ISG3 genes, although such induction was higher at the lowest concentration (see FIG. 39, embodiment 3900). Since nanoparticles tend to aggregate at higher concentrations, as shown with DLS analyses, it is speculated that, at the highest concentration, the formation of nanoparticle aggregates may prevent their elicitor activity. Thus, in further experiments, nanoparticle concentration was reduced to 1 nM and 10 nM. The level of gene transcripts also increased following treatment with unmodified eCPMV nanoparticles, suggesting that the viral capsid itself may be responsible for defense activation, although with some differences compared with the functionalized eCPMV. In an attempt to improve the interaction between eCPMV-ITP1 with its receptor, nanoparticles were further vacuum-infiltrated in the whole plant, instead of contact treatment. Indeed, timing is often crucial in gene expression experiments, since some genes can be induced for a short period of time, as in the case of ISG1. Vacuum-infiltration proved to be an efficient method to evenly administrate nanoparticles in all plant tissues, but, at the same time, it appeared as a source of stress for the plant itself. Indeed, FIG. 40, embodiment 4000, shows a higher level of defense gene expression even in the controls. Although this did not affect the significance of the strong gene induction by the synthetic peptide, the effect of nanoparticles was not detectable with the vacuum- infiltration. As mentioned above, the effect of the lower nanoparticle concentration was again slightly higher compared to the higher nanoparticle concentration (see FIG. 40, embodiment 4000). In the same manner, eCPMV nanoparticles showed again the capacity to induce these early defense genes, to the same extent as eCPMV-ITP1 (see FIGS. 39-40). It was thus difficult to resolve the effect related to the capsid itself from the possible specific recognition of ITP1 exposed on its surface. Indeed, FIG. 41, embodiment 4100, shows the activation of the well-known defense marker PR1 at 24 h by all tested treatments, including eCPMV-WT, suggesting that eCPMV nanoparticles can induce an immune response in *A. thaliana*. Therefore, it is possible to assume that a defense response is effectively activated after treatment with nanoparticles even if to a lower extent respect to the free ITP1 peptide. Moreover, the simultaneous activation of PR5 further suggests that plants may perceive the viral capsid as a general elicitor of defense, leading to a salicylate-dependent pathway of induction. In contrast with JAZ2 results described above, the expression of PDF1.2, another marker of the jasmonate-mediated pathways, was not activated at 24 h post-treatment. Since, in the same manner, no expression of the ethylene pathway marker ERF1 was detected, it is speculated that the absence of ethylene in experimental conditions did not allow JA-mediated response enhancement, thus leading to a PDF1.2 expression that was too low to be detected. Although these results do not lead to a conclusion regarding a possible activation of JA-

mediated pathway, they however strengthen the hypothesis about a significant activation of the immunity-triggered salicylate pathway (see FIG. 41, embodiment 4100).

[0241] Expression of the early defense ISG1 gene was still observed at 24 h post-treatment, in particular with eCPMV-ITP1 nanoparticles, which showed an induction that was much higher compared with early time points and with other treatments (i.e., unmodified eCPMV and free ITP1) (see FIG. 42, embodiment 4200). The same pattern was also observed for the ISG2 and ISG3 genes, suggesting that: i) ITP1 exposed on the surface of eCPMV likely contributes positively to the induction of defense response, independently of eCPMV-related response, and ii) eCPMV-ITP1-induced response is delayed respect to the peptide alone. Indeed, these genes, especially ISG1, are transiently activated by the ITP1 elicitor and reach the maximum around 1 h post-treatment and vanish quickly thereafter. This situation is observed at 6 h post-treatment, in which almost none of these genes was still active compared to the control (see FIG. 43, embodiment 4300). We may thus assume that eCPMV-ITP1 nanoparticles induce a slower (and less intense) response compared to the free peptide, due to the time it takes for the nanoparticles to reach the ITP1 receptor, which would result in a cumulative response over time, which results from the higher persistence of eCPMV-ITP1 nanoparticles compared to the free peptide.

[0242] Evaluation of *Arabidopsis thaliana* protection against P. syringae pv. tomato DC3000

[0243] Given the ability of the nanoparticles to induce an immune response at a molecular level (i.e., defense gene induction) in *A. thaliana*, the ability of these nanoparticles in protecting plants from infection by *Pseudomonas syringae* pv. tomato DC3000 was evaluated using a flood-inoculation assay. This method simulates a natural mechanism of infection, in which bacteria are present on the leaf surface and need to penetrate through the plant leaf in order to colonize the apoplast. According to gene expression results, which suggested a slower and/or long-lasting induction of the plant response, plants were treated with eCPMV nanoparticles 24 h or 48 h before bacteria inoculation. Bacterial leaf population was then evaluated at 2 and 3 days post-inoculation. As shown in FIG. 44, embodiment 4400, unmodified eCPMV nanoparticles conferred full protection of the plants against Pst, regardless of the time of pre-treatment. In contrast, while both eCPMV-ITP1 and free ITP1 protected plants when applied 48 h before infection, they were not able to prevent bacterial colonization when applied only 24 h before bacteria inoculation (see FIG. 45, embodiment 4500). In the first set of experiments, it was noticed that plants appeared damaged (darkened and weakened) due to the presence of silwett L-77 added to bacteria inoculum. Considering that this may influence the outcome of the experiment, silwett L-77 has been thus excluded from further trials. To elucidate whether the observed total (eCPMV) or partial (eCPMV-

ITP1) protection is the outcome of the hampered bacterial penetration or, instead, of the inhibition of Pst growth in the apoplast, bacteria were further vacuum-infiltrated into plant leaves 48 h after nanoparticle (or free peptide) treatment instead of flood-inoculation. In these conditions, no difference was observed in terms of bacterial endophytic population at 2 days post-infiltration among different treatments (see FIG. 46, embodiment 4600). These results suggest that the penetration of bacteria into plant leaf is likely hindered following treatments, suggesting that the treatments may activate mechanisms leading, for instance, to stomatal closure, which is one of the outcomes of PTI.

[0244] Discussion

[0245] The perception of environmental clues by plant cells is fundamental for their survival, therefore plants evolved an intriguing and complex mechanism to perceive a huge variety of molecules. For instance, to cope with pathogens, plants developed different surface sensors to detect the presence of microorganisms directly through the recognition of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) or indirectly through the perception of damage-associated molecular pattern (DAMPs), and finally by sensing modifications occurring at the level of the cell wall caused by mechano- and osmoreceptors. These recognition events occur in the plant apoplast, which is considered the region outside the plant cell membrane crossing the plant cell wall and other intercellular spaces. Among elicitors perceived by plant cells, bioactive peptides, including without limitation MAMPs/PAMPs or DAMPs, represent an important class of molecules able to induce plant defense responses.

[0246] Three immunity-triggering peptides (ITP1 of bacterial origin, and ITP2/ITP3 of plant origin) were selected for their recognized role in plant immunity and inserted at the surface of eCPMV nanoparticles by genetically introducing their sequence within the βB-βC loop of the small coat protein. Although the three peptides did not fit all the suggested requirements for the exposition on eCPMV surface, i.e., a sequence shorter than 40 amino acids and a pI < 9 (e.g., ITP3 displays a pI of 9.7), all three peptides have been successfully expressed on the eCPMV surface, with similar results, suggesting that their properties did not interfere with capsid assembly. This was supported by their capacity to precipitate in the presence of PEG6000, similarly to the unmodified eCPMV. Nevertheless, only eCPMV-ITP1 nanoparticles, and to a lesser extent, eCPMV-ITP2, were efficiently and reproducibly purified, with an average yield of 0.05 mg/g of fresh tissue. Purified nanoparticles showed high stability over a long period of storage, and, despite a slight degree of aggregation, they were mainly monodisperse. This is a fascinating property of eCPMV nanoparticles, since most synthetic nanoparticles, as well as eVLPs from other viruses, may show a

significant degree of variability. Moreover, synthetic nanoparticle production in a narrow size range is difficult and expensive. Importantly, eCPMV-ITP1 nanoparticles display the same structure and size as the unmodified eCPMV, as confirmed by transmission electron microscopy (TEM). Nanoparticle characterization is fundamental to elucidating their properties over time and increasing the potential of their application in agriculture.

[0247] The first requirement for functionalized nanoparticles to induce plant defense responses is their capacity to enter within plant tissues to reach the plasma membrane where ITP1 receptor is located. However, neither unmodified eCPMV nor eCPMV-ITP1 were found by western blot in leaf extracts from plants treated with both types of nanoparticles for 1, 6, or 24 h. This is most likely due to the low concentration used for the treatment. Indeed, the use of different amounts of purified unmodified eCPMV showed that this technique, when using the anti-CPMV antibody instead, resulted in a detection of at least 40 ng of nanoparticles, which is far higher than the expected quantity of nanoparticles within plant leaves. The monitoring of eCPMV diffusion into plant leaves will thus require a more sensitive method, such as confocal microscopy, by exploiting eCPMV nanoparticle conjugated with a fluorophore.

[0248] Although it may not be possible to detect nanoparticles within plant leaves, several lines of evidence showed the ability of eCPMV-ITP1 to induce a significant plant defense response. Indeed, functionalized eCPMV nanoparticles (i.e., eCPMV-ITP1 and eCPMV-ITP2) were able to activate the promoter of the jasmonate-dependent gene JAZ2, as revealed using the reporter system pJAZ2:uidA in transgenic *A. thaliana plants*. Moreover, several early and late defense marker genes showed an increase in transcription level in response to eCPMV-ITP1, although it may be of a lower intensity and likely delayed compared to the free ITP1 peptide. According to the timing of induction for defense gene expression, showing a still significant expression of early defense genes after 24 h, it is speculated that eCPMV-ITP1may induce a delayed response or, more precisely, a slower response that becomes significant at later time points as a result of a cumulative effect of defense induction in several cells. Indeed, the steric hindrance due to the nanoparticle may reduce the efficiency of ITP1 to reach and/or bind its receptor, thus requiring more time to activate significant signals. Such delayed response may account for the absence of a rapid increase in Ca^{2+} concentration in the aequorin assay, which allows for detection of the rise in Ca^{2+} concentration within 2-3 minutes following elicitor perception but was never used for assessing defense induction after 24 h. Overall, the set of experiments showed that eCPMV functionalized with elicitor peptides can activate plant immunity, which involves in particular salicylic acid, as shown by the induction of two SA markers, namely PR1 and PR5. Some viruses are known to manipulate the SA pathway of plants, although the

mechanisms and the effects thereof are still under debate. Indeed, the activation of the SA pathway can be exploited by viruses to facilitate their infection, but it likely depends on specific virus-host interactions. In contrast, the activation of SA pathway is fundamental to enhancing plant defense against further invaders, especially biotrophic pathogens. Surprisingly enough, while eCPMV-IPT1 induces JAZ2 promoter, no expression of PDF1.2 (which is another jasmonate-mediated pathway marker) was detected in response to functionalized nanoparticles. Such inconsistency thus poses questions regarding the involvement of JA in response to eCPMV-ITP1. It could be thus assumed that JAZ2 promoter was induced by another signal that is different from JA. In this context, abscisic acid (ABA) may play an important role. Indeed, ABA can induce some JAZ genes like MYC2 and VSP1 while inhibiting PDF1.2 and ERF1 expression at the same time. Interestingly, a small JA-Ile accumulation is necessary for triggering ABA biosynthesis pathway, which further suggests the multiple interactions between these two hormones which share different molecular targets in their crosstalk.

[0249] While other experiments may still be needed to elucidate the precise events and key signaling molecules involved in eCPMV-ITP1-induced defense response, functionalized nanoparticles were able to partially protect *A. thaliana plants* against *P. syringae* pv. tomato DC3000, when used for plant treatment 48 h before flood-inoculation, while free ITP1 was already efficient in plant protection when used 24 h before bacterial infection. It is worth noting that this method of bacterial inoculation may be preferred for a more realistic plant infection. The higher efficiency after 48 h is in line with the possible delay of eCPMV-ITP1 to induce a full defense response, showing a maximum of early gene expression after 24 h instead of 1 to 6h. It will be worthwhile investigating the protection mediated by the nanoparticles to decipher whether this delayed response may also account for a longer-lasting resistance of the plants, by performing successive time courses with increasing time of pre-treatment. During the experiments to assess eCPMV-ITP1-induced defense responses, unmodified nanoparticles were used as a control to figure out the involvement of ITP1 exposed on the surface of the capsid. As expected, eCPMV-ITP1 induced different responses compared to the wild-type eVLP, particularly in term of pJAZ2:uidA activation and timing of gene expression, thus demonstrating the role of ITP1 in such responses.

[0250] eCPMV themselves were able to activate plant defense responses, including the induction of defense marker genes but more significantly a complete protection of plants from Pst DC3000 infection. Indeed, no bacteria were found in plant tissues following plant pre-treatment with eCPMV 24 or 48 h before flood-inoculation. This very drastic and reproducible protection suggests that Pst DC3000 was very likely hampered, or at least strongly delayed, when entering plant tissues.

Indeed, for the bacterial count, whole plants were collected 2 or 3 days after pathogen inoculation, thus the possibility may not be ruled out that endophytic bacterial population may be observed after 5-7 days post-inoculation. Moreover, the protective effect of eCPMV nanoparticles was lost when Pst DC3000 was vacuum infiltrated into plant tissues, further supporting the hypothesis that the protection mechanism may rely upon hampering bacteria invasion of leaf apoplast, for instance by triggering stomatal closure. Plant viruses are able to manipulate the plant hormone balance, especially of SA, and also to affect plant transpiration leading to drought resistance. For example, the infection with Plum pox virus expressing the RNA silencing inhibitor P25 of Potato virus X can induce stomatal closure in its host *N. benthamiana* but not in *A. thaliana*, which is not susceptible to the virus. In contrast, the Turnip Mosaic Virus induces a partial stomatal closure in *A. thaliana*. Consequently, it is speculated that the capsid of eCPMV could be somehow recognized by *A. thaliana*, leading to the inhibition of the colonization by Pst. However, this speculation needs to be further investigated since there is no evidence of proteinaceous elicitors of virus origin. Accordingly, using a similar approach, viral infection may induce resistance against Pst. In particular, the same flood-inoculation procedure may be used to inoculate Pst (specifically, the PPV-P25) strain into *A. thaliana* Col-0 plants after an appearance of viral symptoms. Three days post-inoculation, a reduction in the bacterial leaf population was observed when compared to mock-treated plants. This phenotype was absent when bacteria were syringe-infiltrated into the plant leaves or using *A. thaliana* mutants unable to perceive JA (coi1-1 line) or accumulate SA (NahG line). The same results were obtained with N. benthamiana, and it was proposed that this difference is likely due to a stomata closure induced by the plant virus. Accordingly, the same or a very similar mechanism may have occurred with the invention described herein that pertains to eVLPs instead of viruses. Finally, this mechanism may need further investigation since it represents a promising approach to achieve plant protection. Indeed, since Pst colonizes the apoplast of *A. thaliana*, by exploiting natural openings like stomata to enter the host, it is possible to speculate that eCPMV treatment could induce stomatal closure, through a mechanism dependent on ABA concentration in the guard cells. Stomata closure induced by ABA accumulation after a viral infection has been demonstrated for TuMV-*A. thaliana* pathosystem. However, the putative role of ABA in this interaction remains to be further elucidated, since eCPMV lacks any genetic material and should be considered merely as a proteinaceous nanoparticle. Thus, the interaction with the plant does not consider any genetic manipulation from the eCPMV, and potential mechanisms leading to the stomata closure are highly diverse.

[0251] The capacity of nanoparticles activating signaling pathways in plant cells may also require further elucidation. Indeed, a viral capsid may unlikely penetrate the plant cell wall, which is considered to have a pore size limit of 13 nanometers and a negative net charge, which may allow only small positively charged particles to cross the cell wall. However, the pore size of cell walls may vary among plant species. As a nonlimiting example, the exclusion limit for cotton is about 20 nm. Other factors may also affect the movement of nanoparticles into extracellular spaces, including without limitation the zeta-potential and plant-dependent modifications. Therefore, plant viruses are likely unable to penetrate within plant cells without external aids (e.g., feeding insects or physical damages) and therefore dependent on their host to complete their life cycle. In other words, they are strictly intracellular pathogens. Accordingly, while viral effectors are recognized to suppress or inhibit PTI through different mechanisms, to activate ETI, the possibility of plant virus patterns to induce PTI was excluded until recently. Nevertheless, the concept of virus-associated molecular patterns (VAMPs) was recently introduced, but to date, only dsRNAs have been identified as VAMP. Thus, the recognition of eCPMV and eCPMV-ITP1 may rely on other 'external' mechanisms, in which the extracellular space may be crucial since it is rich in proteins, and especially proteases, most of which are uncharacterized but have a role in plant defense activation. Indeed, recently, the upstream origin of flg22 peptide was unraveled in *N. benthamiana*, showing that it is released from bacterial flagellum through the action of plant secreted β-galactosidase 1 (BGAL1). In the same manner, during the development of CPMV for exposing peptides, the cleavage at the C-terminus of the inserted peptide in the βB-βC loop of the SCP was frequently observed without affecting the chimaera stability and infectivity. Moreover, peptide cleavage was also observed for sequences expressed on the LCP of CPMV, leading to a consideration of CPMV cleavage as a general phenomenon. Therefore, it should not be excluded, *a priori*, that a possible mechanism of capsid recognition exists. In this scenario, a portion of the loop may be released due to plant proteases, allowing the exposed peptide to reach the plasma membrane and to interact with its receptor. Such cleavage may be the reason why longer time is indeed required for CPMV- ITP1 to induce a marker of defense genes compared with the free peptide.

[0252]

[0253] The foregoing has been a detailed description of illustrative embodiments of the invention. Various modifications and additions can be made without departing from the spirit and scope of this invention. Features of each of the various embodiments described above may be combined with features of other described embodiments as appropriate in order to provide a multiplicity of feature combinations in associated new embodiments. Furthermore, while the

foregoing describes a number of separate embodiments, what has been described herein is merely illustrative of the application of the principles of the present invention. Additionally, although particular methods herein may be illustrated and/or described as being performed in a specific order, the ordering is highly variable within ordinary skill to achieve methods, systems, and software according to the present disclosure. Accordingly, this description is meant to be taken only by way of example, and not to otherwise limit the scope of this invention.

[0254] Exemplary embodiments have been disclosed above and illustrated in the accompanying drawings. It will be understood by those skilled in the art that various changes, omissions and additions may be made to that which is specifically disclosed herein without departing from the spirit and scope of the present invention.

What is claimed is:

1. A composition for inducing defense in a plant, the composition comprising:
a virus-like particle engineered to express at least a defense-inducing peptide; and
a buffer.

2. The composition of claim 1, wherein the at least a defense-inducing peptide includes one or more members selected from a group consisting of flg22, cape1, and systemin.

3. The composition of claim 1, wherein the plant includes one or more members selected from a group consisting of *Brassicaceae*, *Arabidopsis* including *Arabidopsis thaliana*, *Solanaceae*, *Solanum* including *Solanum lycopersicum*, *Rosaceae*, *Fragaria* including *Fragaria x ananassa*, and *Poaceae*.

4. A composition for inducing growth in a plant, the composition comprising:
a virus-like particle engineered to express at least a biostimulatory peptide; and
a buffer.

5. The composition of claim 4, wherein the plant includes one or more members selected from a group consisting of *Brassicaceae*, *Arabidopsis* including *Arabidopsis thaliana*, *Solanaceae*, *Solanum* including *Solanum lycopersicum*, *Rosaceae*, *Fragaria* including *Fragaria x ananassa*, and *Poaceae*.

6. A composition for treating a condition, the composition comprising:
a virus-like particle engineered to express at least a peptide at its surface;
a cargo encapsulated within the virus-like particle, wherein the cargo comprises at least an active substance; and
a buffer.

7. A method of manufacturing a composition for inducing defense or growth in a plant, the method comprising:
infecting a plant with a virus to produce a virus-like nanoparticle;
sampling symptomatic leaves from the plant;
homogenizing the virus-like nanoparticle;
incubating the virus-like nanoparticle;
centrifuging the virus-like nanoparticle; and
filtrating the virus-like nanoparticle.

8. A method of manufacturing a composition for inducing defense or growth in a plant, the method comprising:
agroinfiltrating a plant with a virus to produce a virus-like nanoparticle;

sampling symptomatic leaves of the plant;

homogenizing the virus-like nanoparticle;

centrifuging the virus-like nanoparticle; and

filtrating the virus-like nanoparticle.

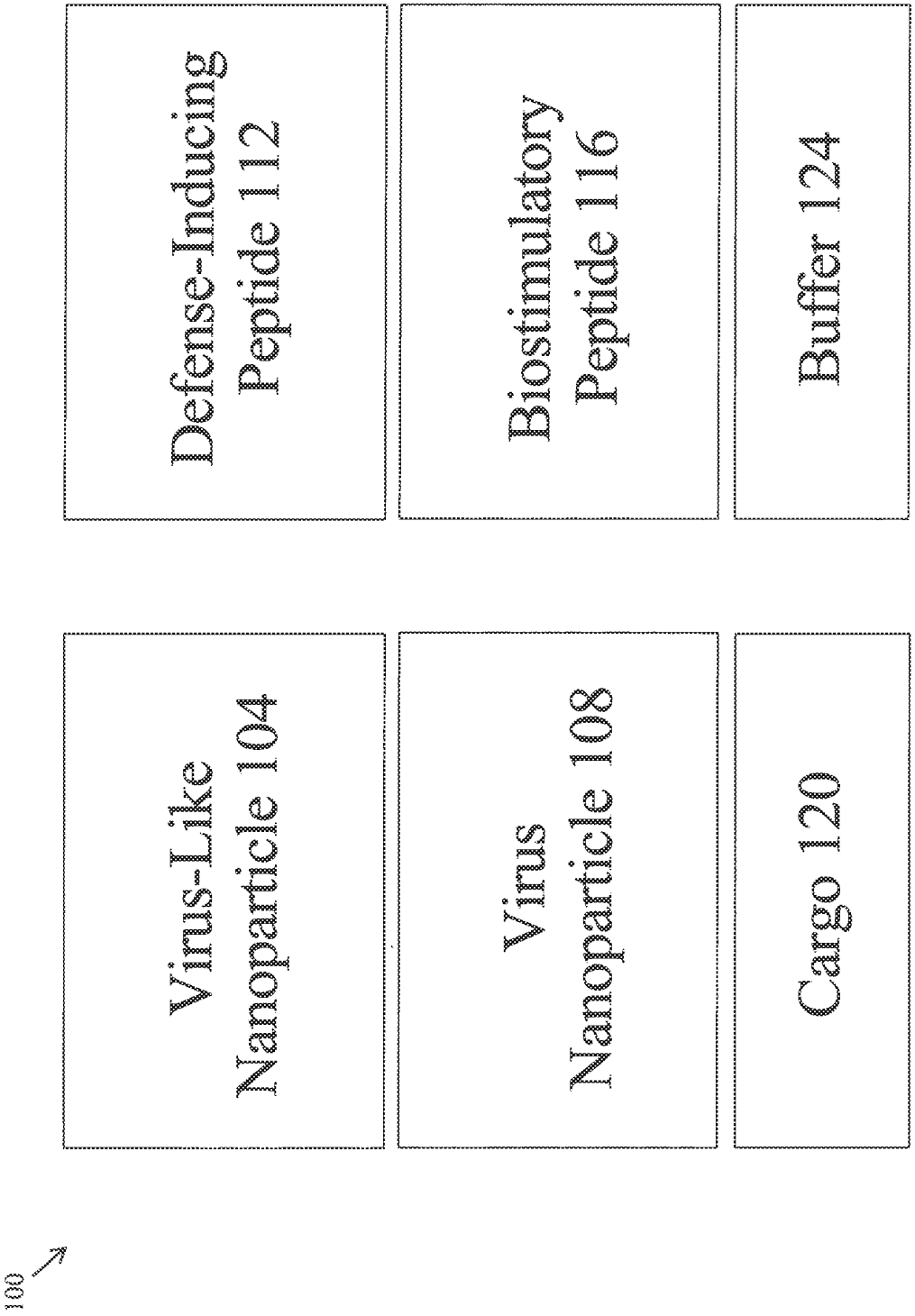

100
Virus-Like Nanoparticle 104
Virus Nanoparticle 108
Cargo 120
Defense-Inducing Peptide 112
Biostimulatory Peptide 116
Buffer 124

FIG. 1

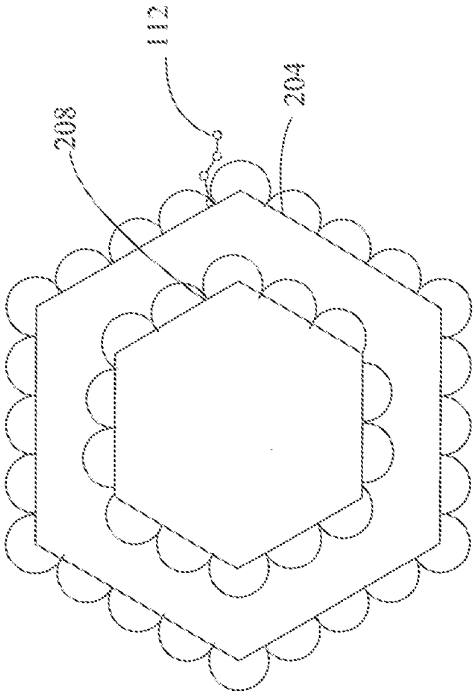
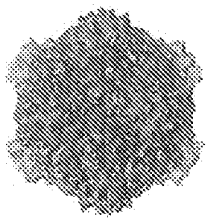


FIG. 2



COWPEA MOSAIC VIRUS
(CPMV)

FIG. 3

300

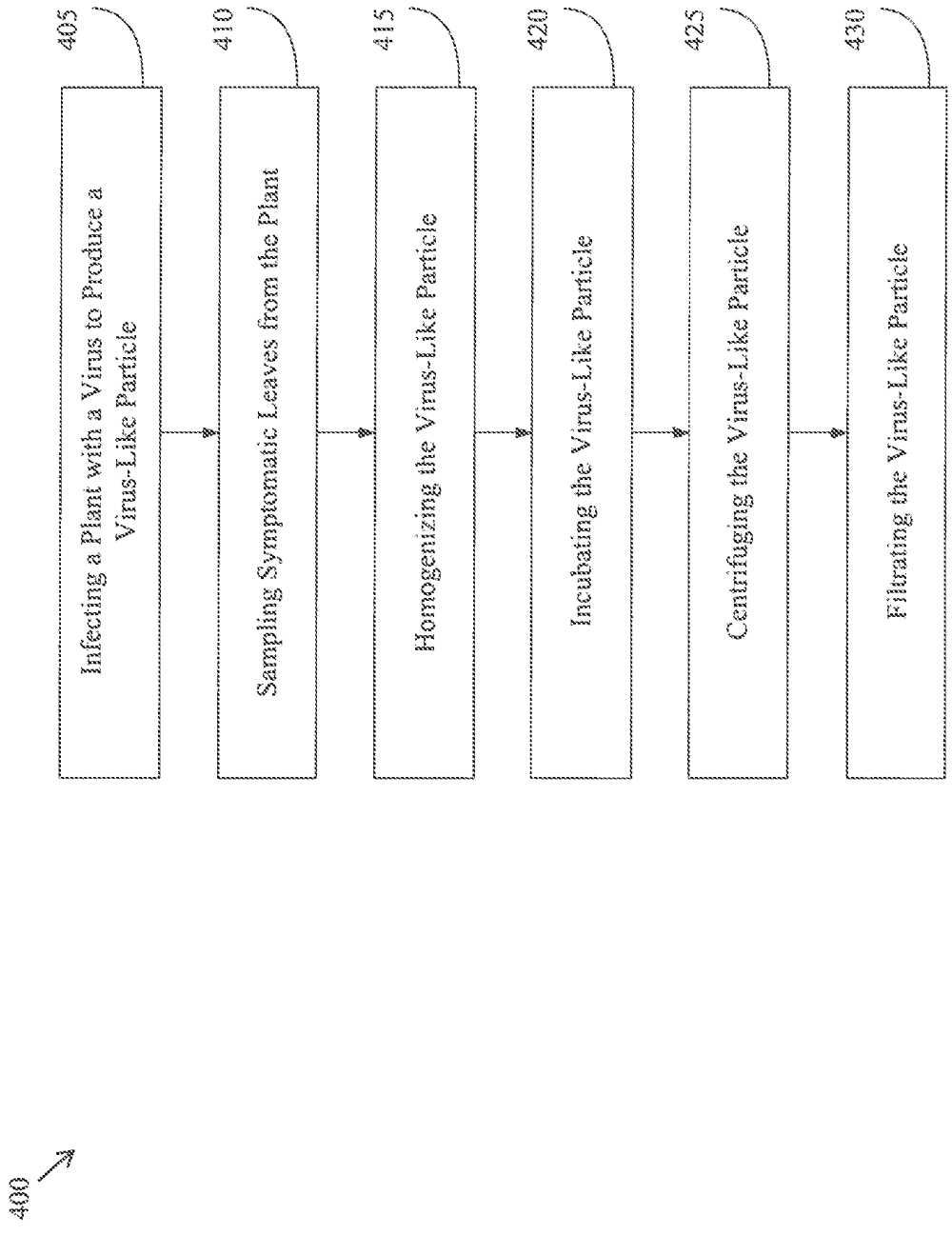

400
405
Infecting a Plant with a Virus to Produce a Virus-Like Particle
410
Sampling Symptomatic Leaves from the Plant
415
Homogenizing the Virus-Like Particle
420
Incubating the Virus-Like Particle
425
Centrifuging the Virus-Like Particle
430
Filtrating the Virus-Like Particle

FIG. 4

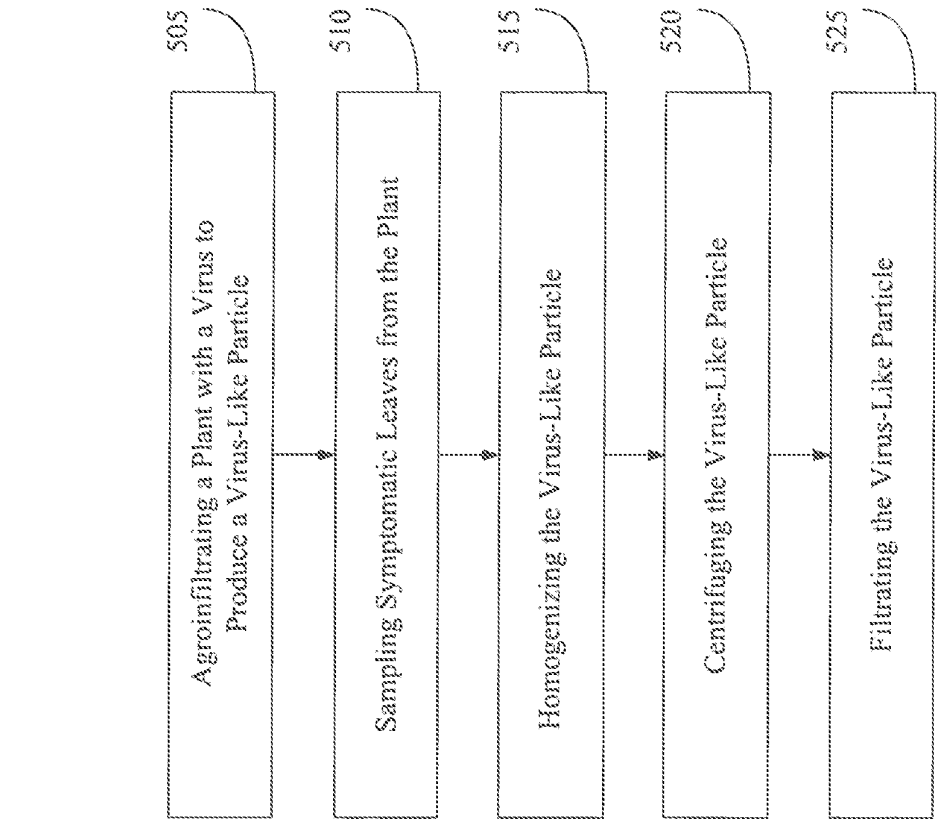

500
505
Agroinfiltrating a Plant with a Virus to Produce a Virus-Like Particle
510
Sampling Symptomatic Leaves from the Plant
515
Homogenizing the Virus-Like Particle
520
Centrifuging the Virus-Like Particle
525
Filtrating the Virus-Like Particle

FIG. 5

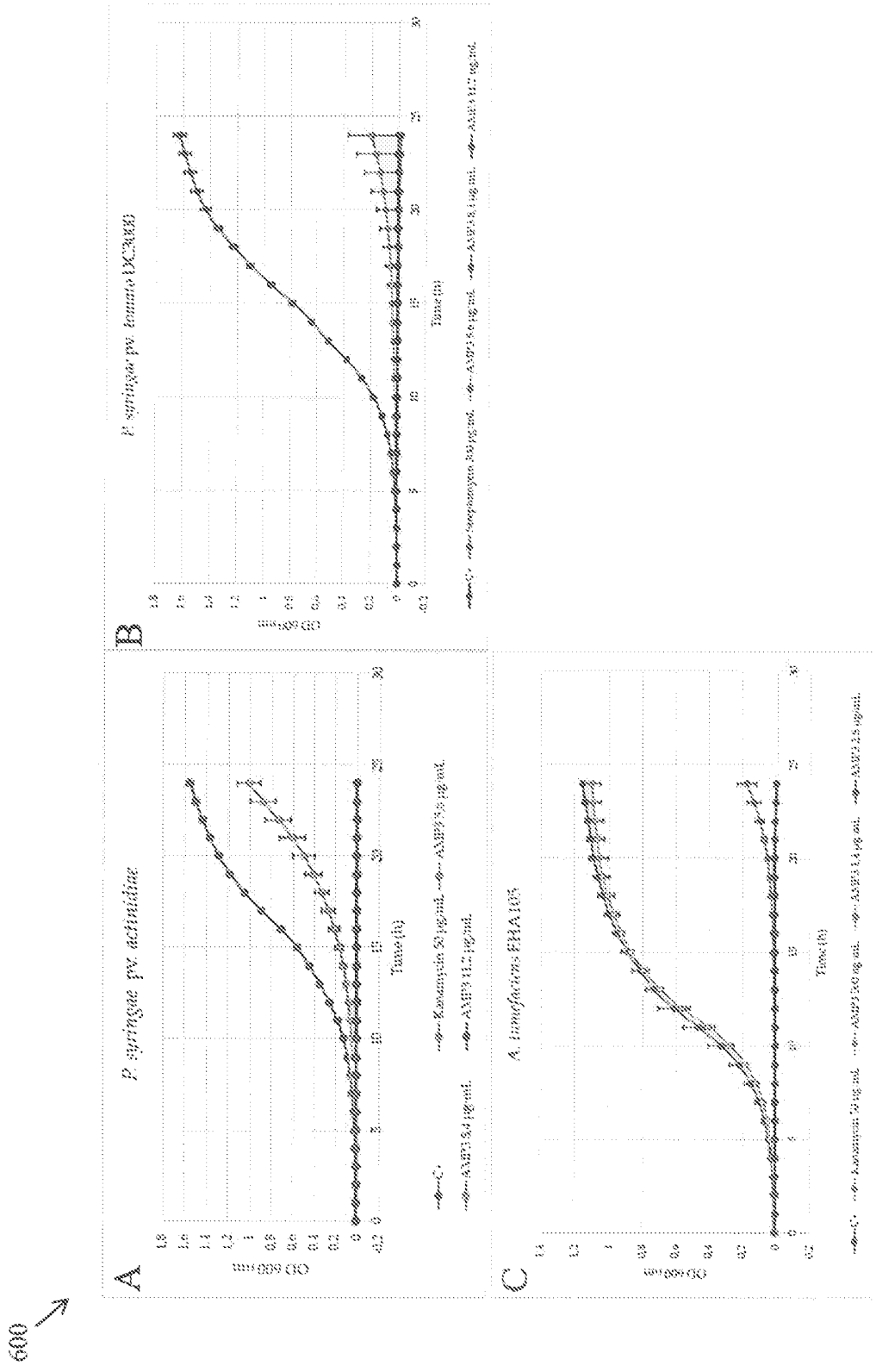

600
A
P. syringae pv. actinidiae
OD 600 nm
Time (h)
C-
Kanamycin 50 µg/mL
AMP3 50 µg/mL
AMP3 6.3 µg/mL
AMP3 12.5 µg/mL
B
P. syringae pv. tomato DC3000
OD 600 nm
Time (h)
C-
Streptomycin 100 µg/mL
AMP3 50 µg/mL
AMP3 25 µg/mL
AMP3 12.5 µg/mL
C
A. tumefaciens EHA105
OD 600 nm
Time (h)
C-
Kanamycin 50 µg/mL
AMP3 50 µg/mL
AMP3 25 µg/mL
AMP3 12.5 µg/mL

FIG. 6

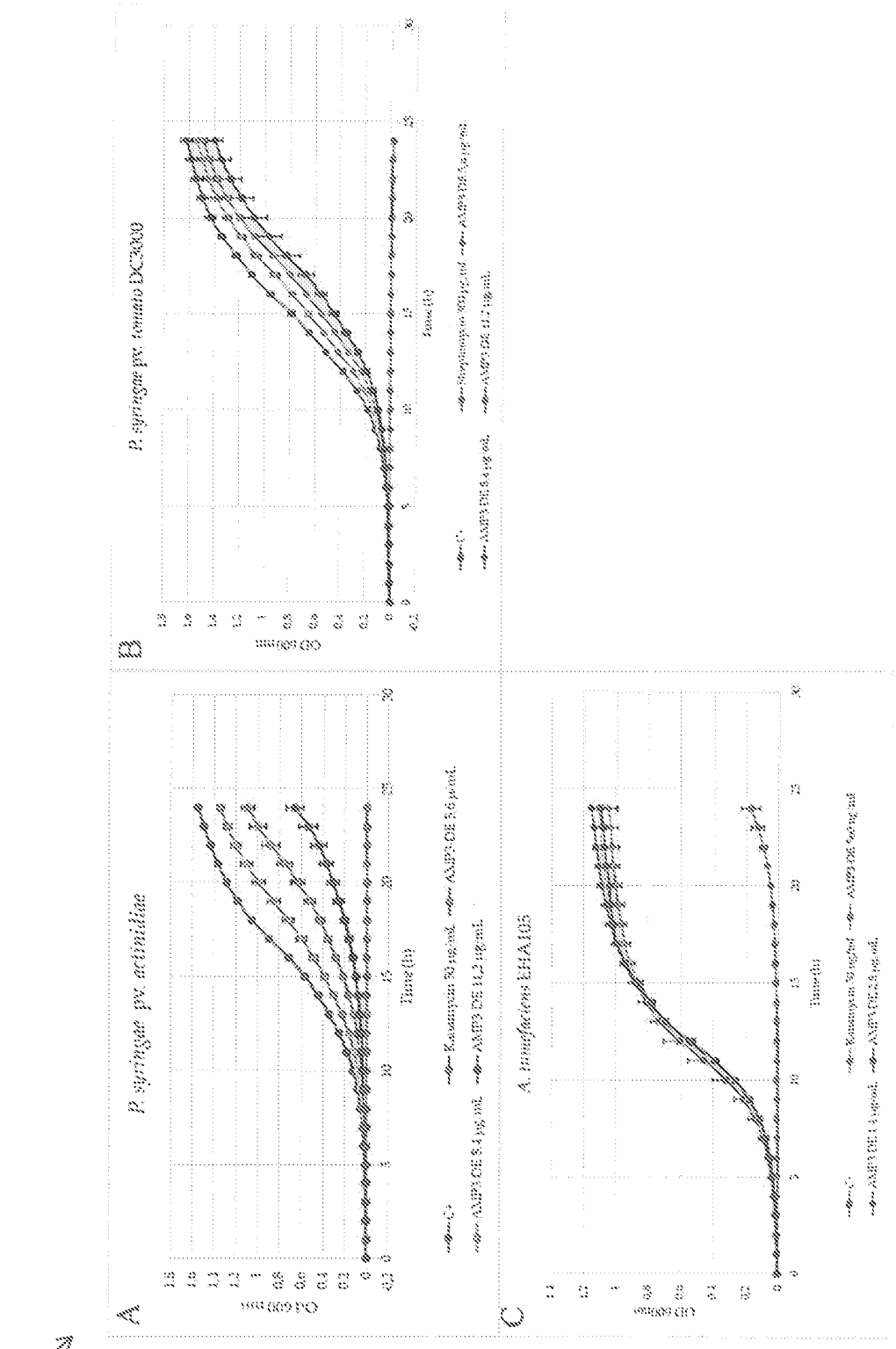

700
A
P. syringae pv. actinidiae
OD 600 nm
Time (h)
C-
Kanamycin 50 µg/mL
AMP3 OE 11.2 µg/mL
AMP3 OE 5.6 µg/mL
B
P. syringae pv. tomato DC3000
OD 600nm
Time (h)
C-
Kanamycin 50 µg/mL
AMP3 OE 11.2 µg/mL
AMP3 OE 5.6 µg/mL
C
A. tumefaciens LBA105
OD 600nm
Time (h)
C-
Kanamycin 50 µg/mL
AMP3 OE 11.2 µg/mL
AMP3 OE 5.6 µg/mL

FIG. 7

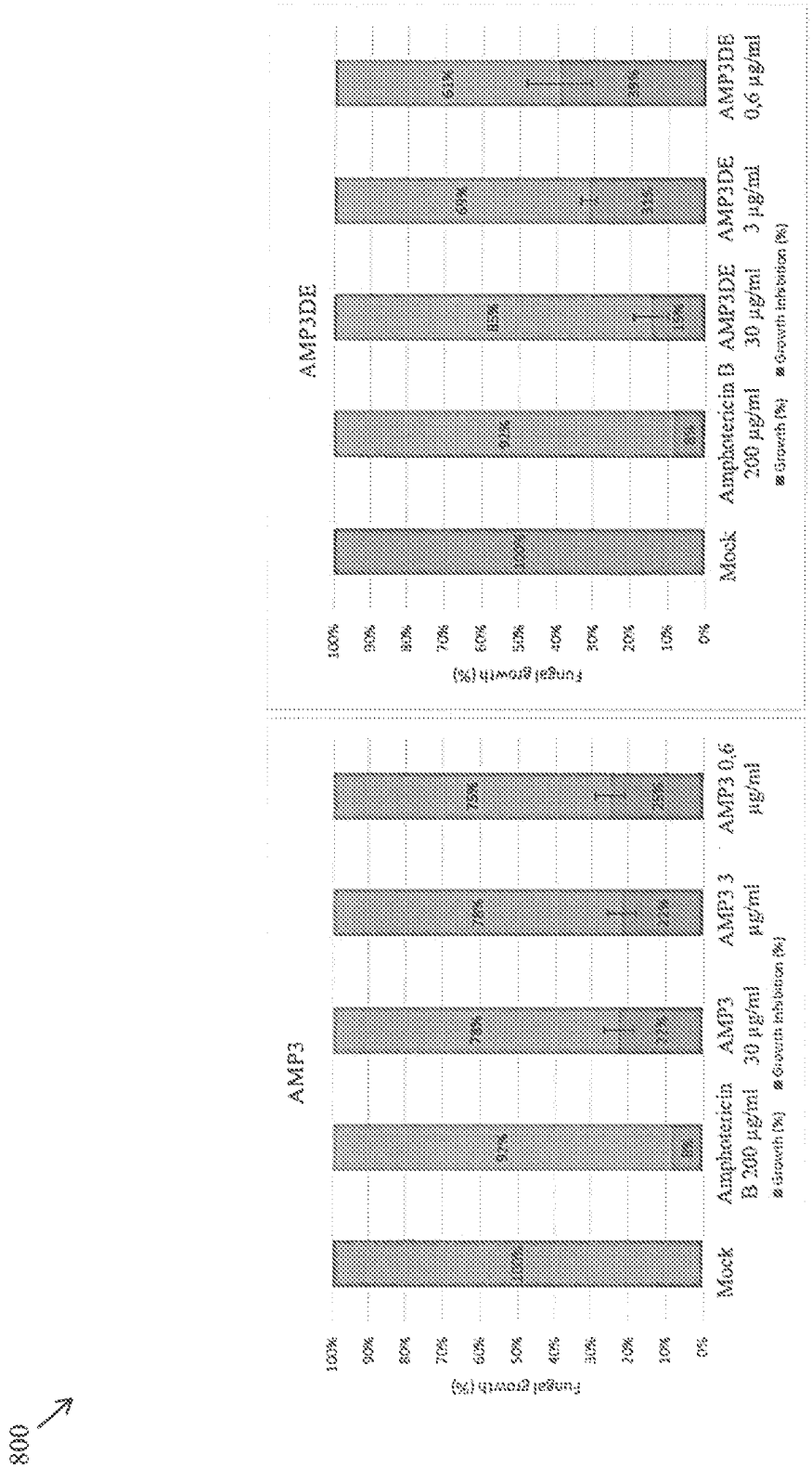

800
AMP3
Fungal growth (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
100%
51%
8%
76%
76%
75%
Mock
Amphotericin B 200 µg/ml
AMP3 30 µg/ml
AMP3 3 µg/ml
AMP3 0,6 µg/ml
Growth (%)
Growth inhibition (%)
AMP3DE
Fungal growth (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
100%
91%
8%
85%
69%
61%
Mock
Amphotericin B 200 µg/ml
AMP3DE 30 µg/ml
AMP3DE 3 µg/ml
AMP3DE 0,6 µg/ml
Growth (%)
Growth inhibition (%)

FIG. 8

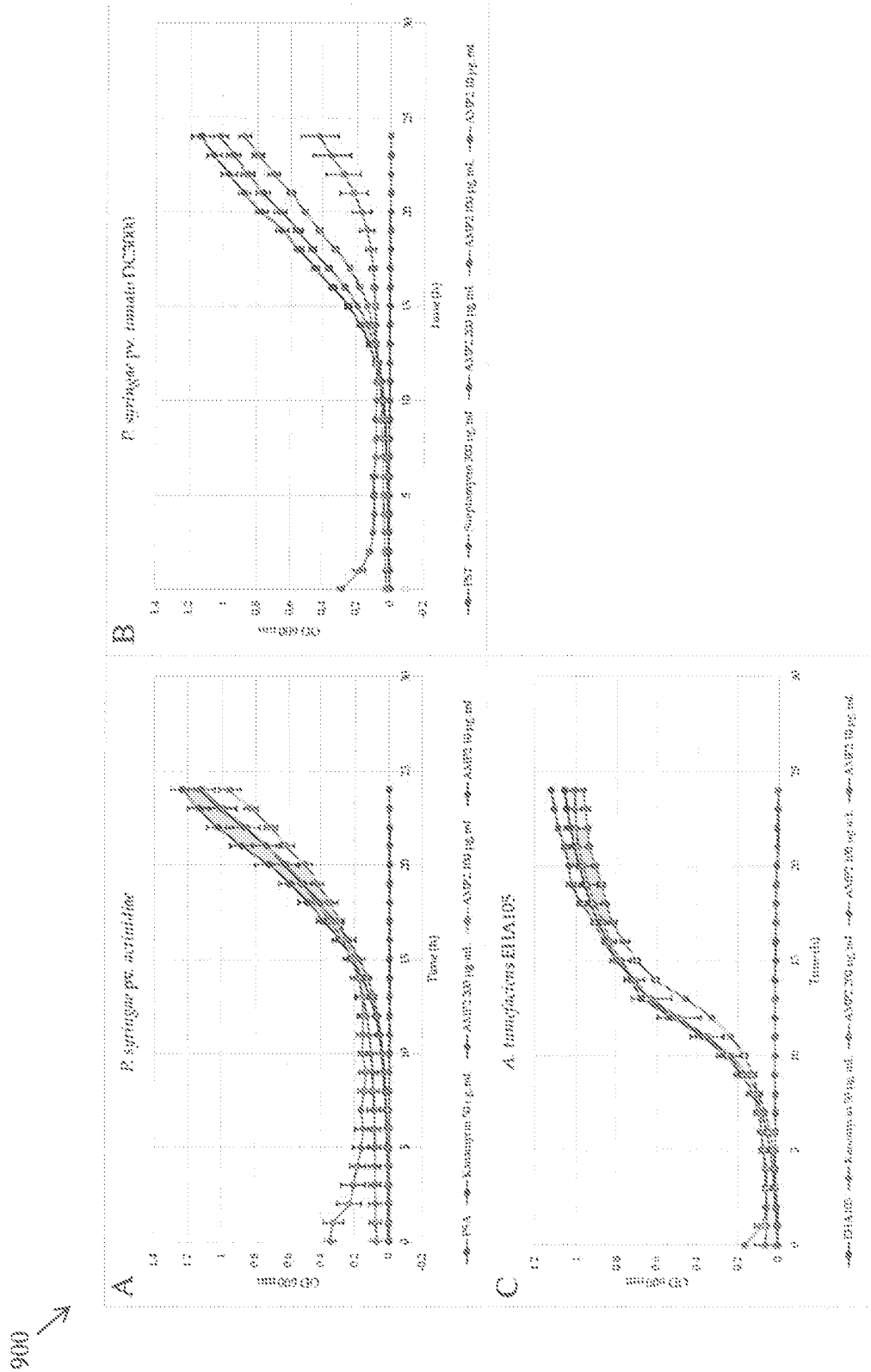

900
A
P. syringae pv. actinidiae
B
P. syringae pv. tomato DC3000
C
A. tumefaciens EHA105
OD 600 nm
Time (h)
EHA105
Kasugamycin 50 µg/mL
AMP1 300 µg/mL
AMP1 150 µg/mL
AMP1 75 µg/mL
Streptomycin 300 µg/mL
AMP1 300 µg/mL
AMP1 150 µg/mL
AMP1 75 µg/mL

FIG. 9

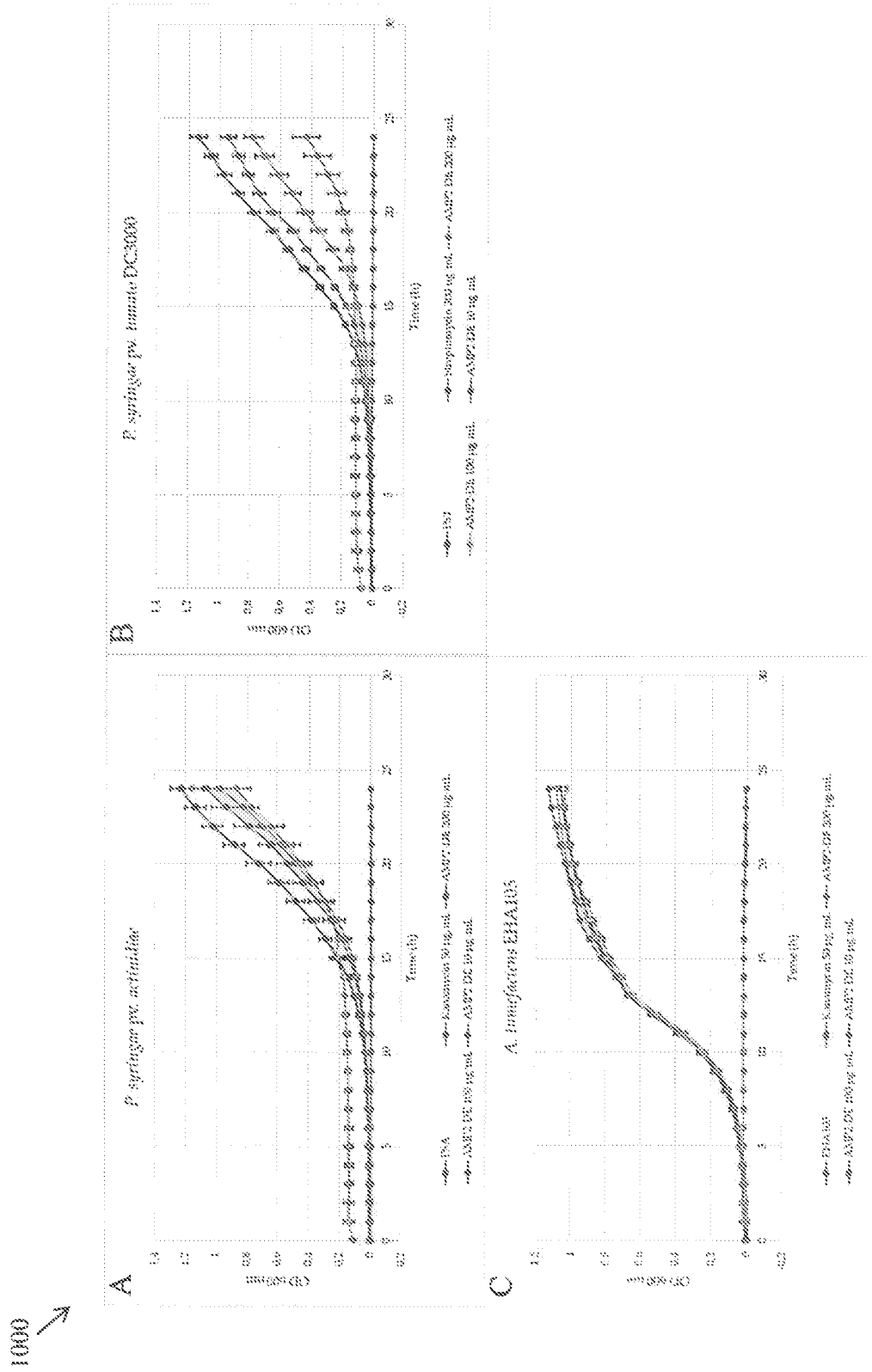

1000
A
P. syringae pv. actinidiae
B
P. syringae pv. tomato DC3000
C
A. tumefaciens EHA105
Time (h)
OD 600 nm
DMSO
Kasugamycin 50 µg/mL
AMP1 DE 100 µg/mL
AMP1 DE 300 µg/mL
Streptomycin 200 µg/mL
AMP1 DE 10 µg/mL

FIG. 10

1100

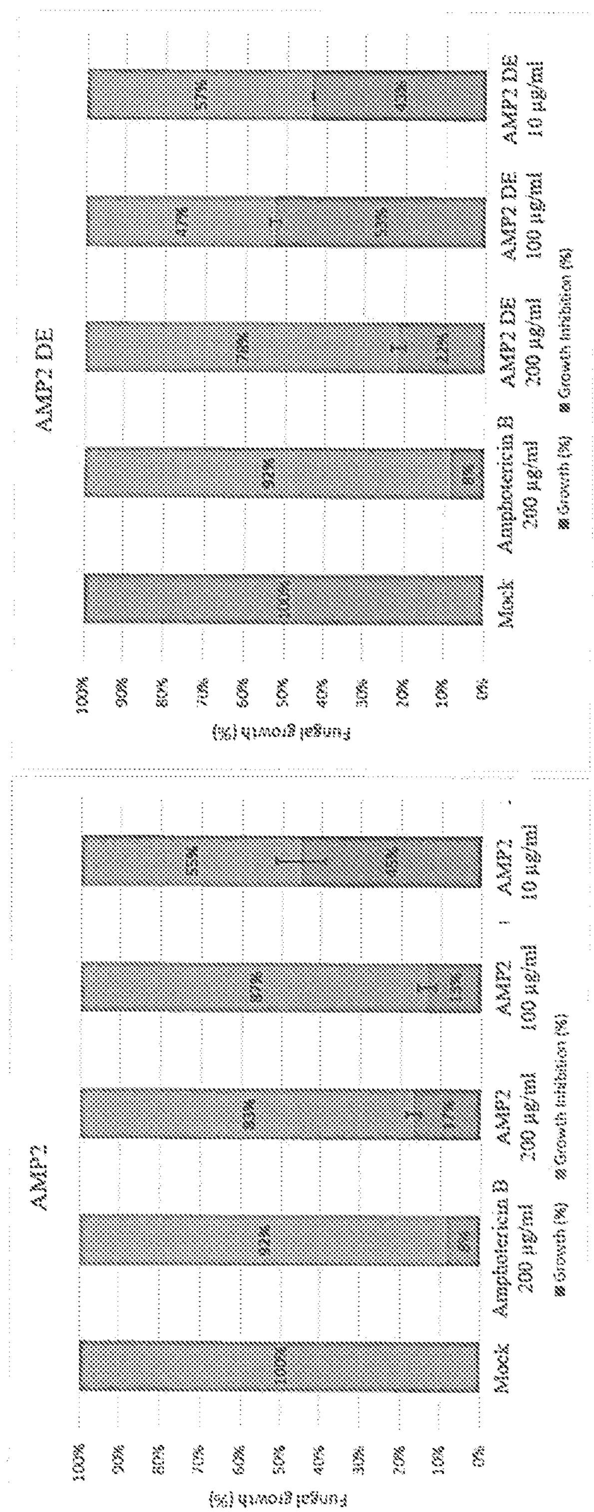

AMP2
Fungal growth (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Mock
Amphotericin B 200 µg/ml
AMP2 200 µg/ml
AMP2 100 µg/ml
AMP2 10 µg/ml
Growth (%)
Growth inhibition (%)
AMP2 DE
Fungal growth (%)
Mock
Amphotericin B 200 µg/ml
AMP2 DE 200 µg/ml
AMP2 DE 100 µg/ml
AMP2 DE 10 µg/ml
Growth (%)
Growth inhibition (%)

FIG. 11

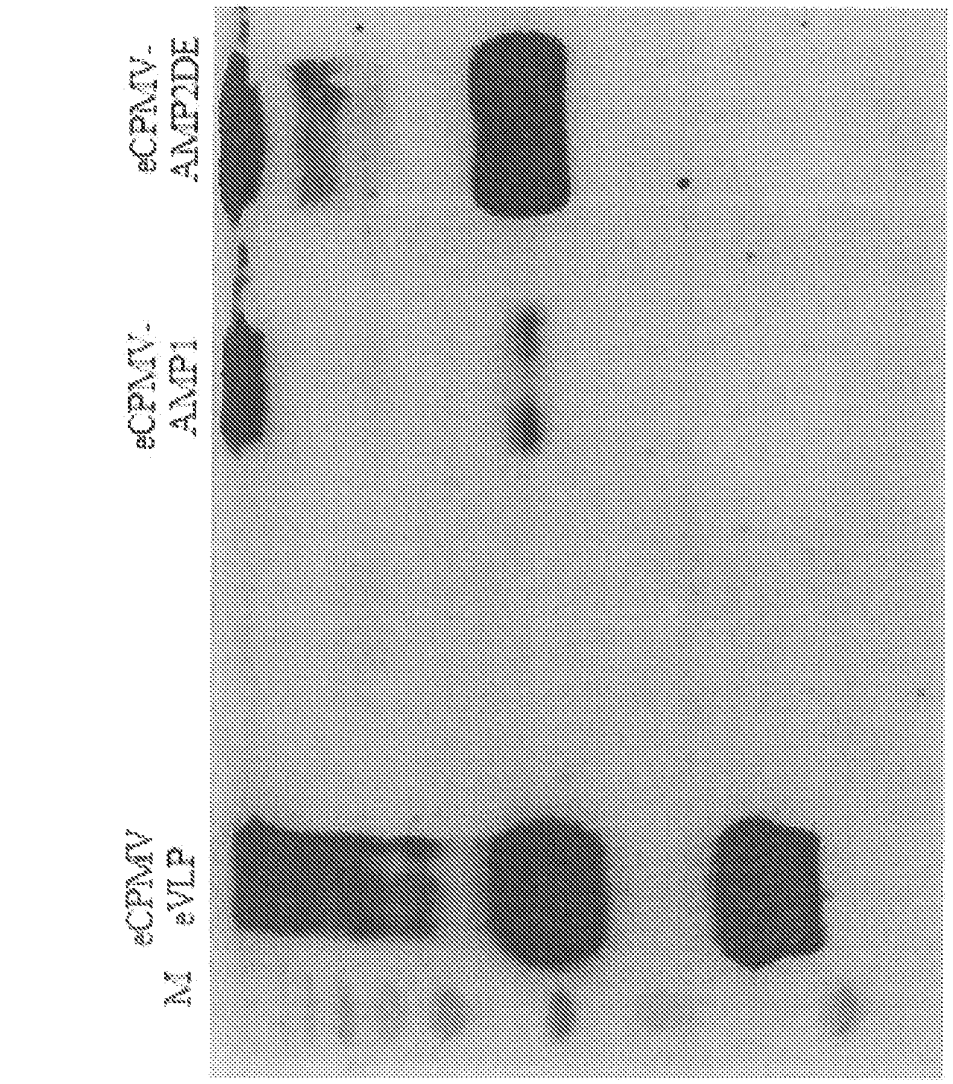


FIG. 12

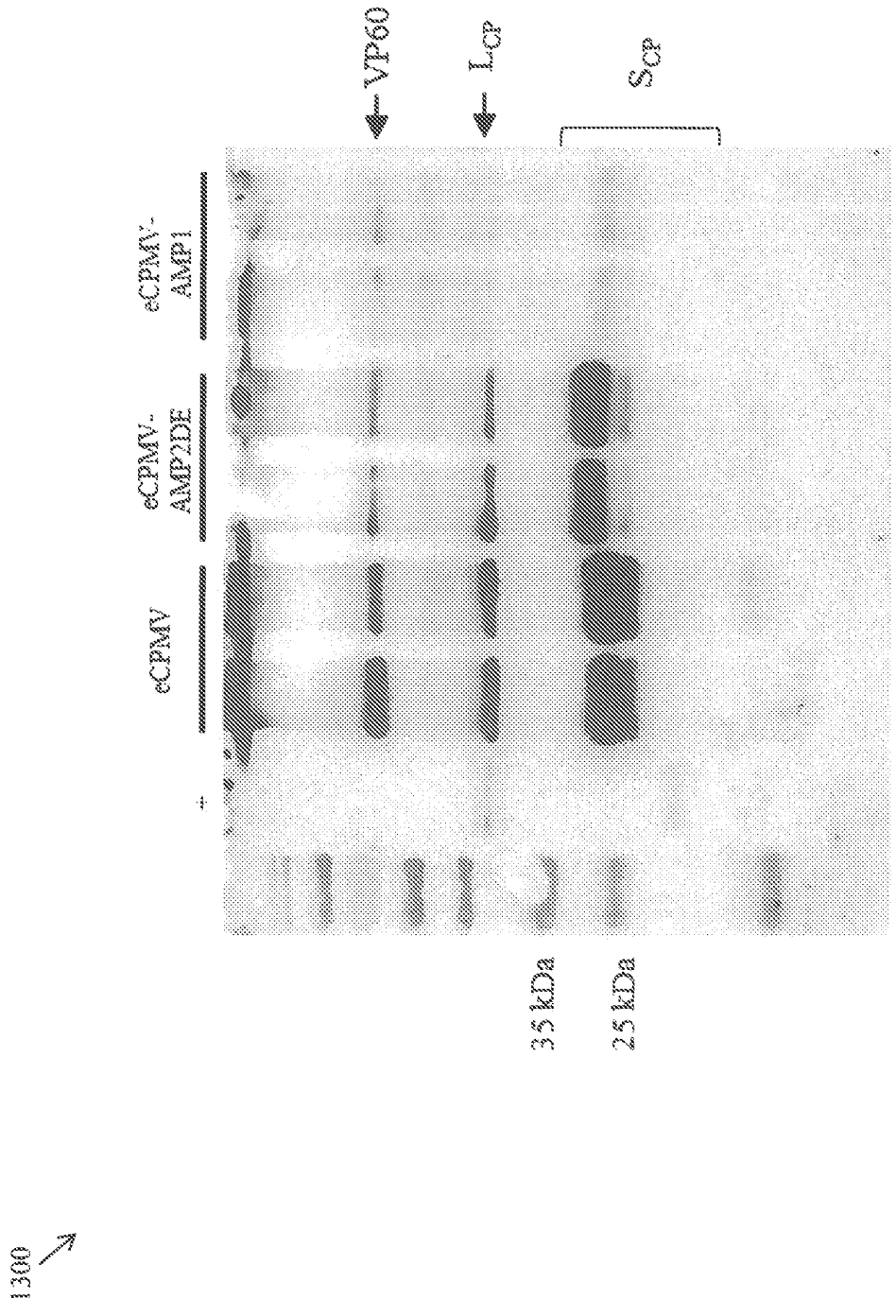


FIG. 13

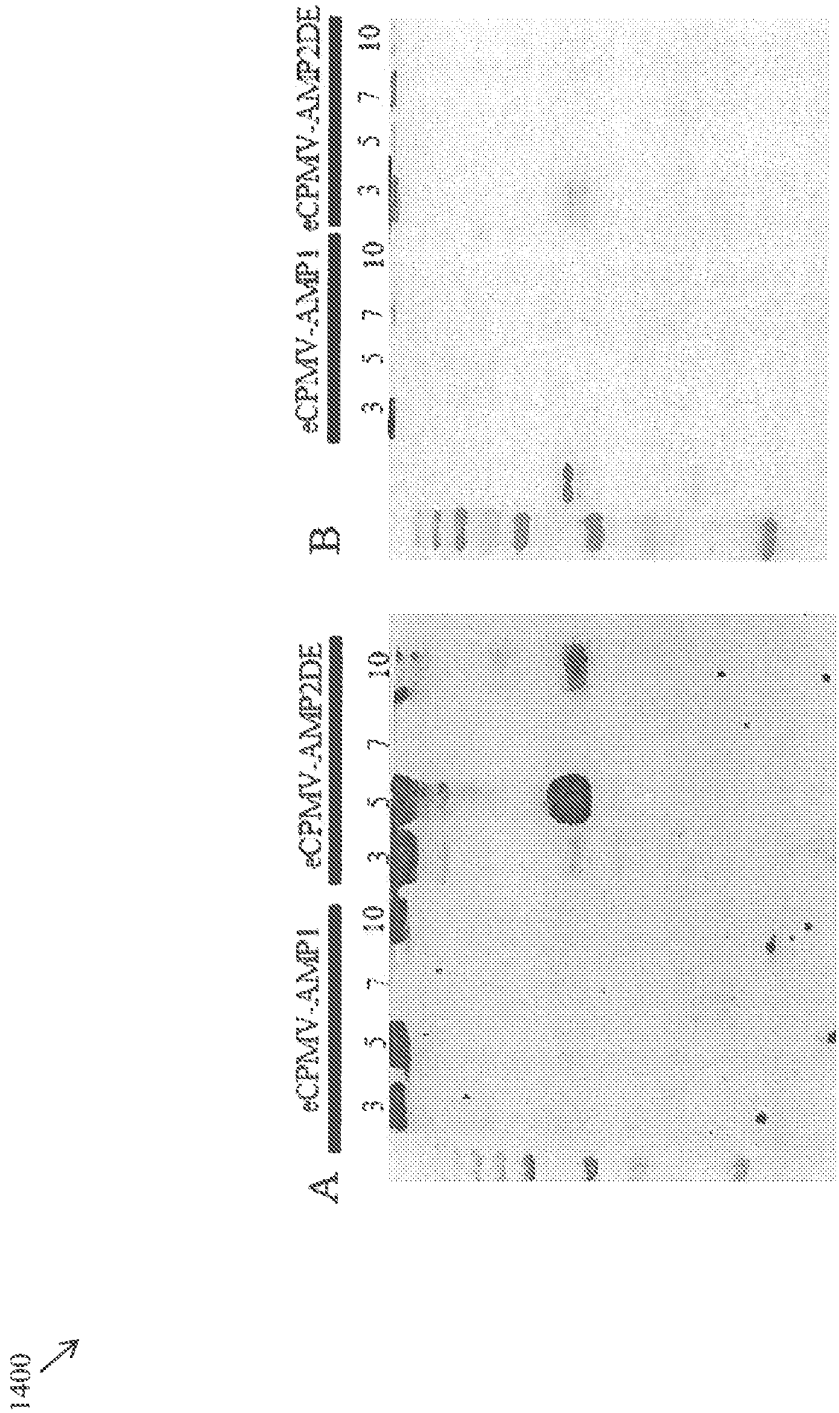

1400
A
eCPMV-AMP1
eCPMV-AMP2DE
3 5 7 10 3 5 7 10
B
eCPMV-AMP1
eCPMV-AMP2DE
3 5 7 10 3 5 7 10

FIG. 14

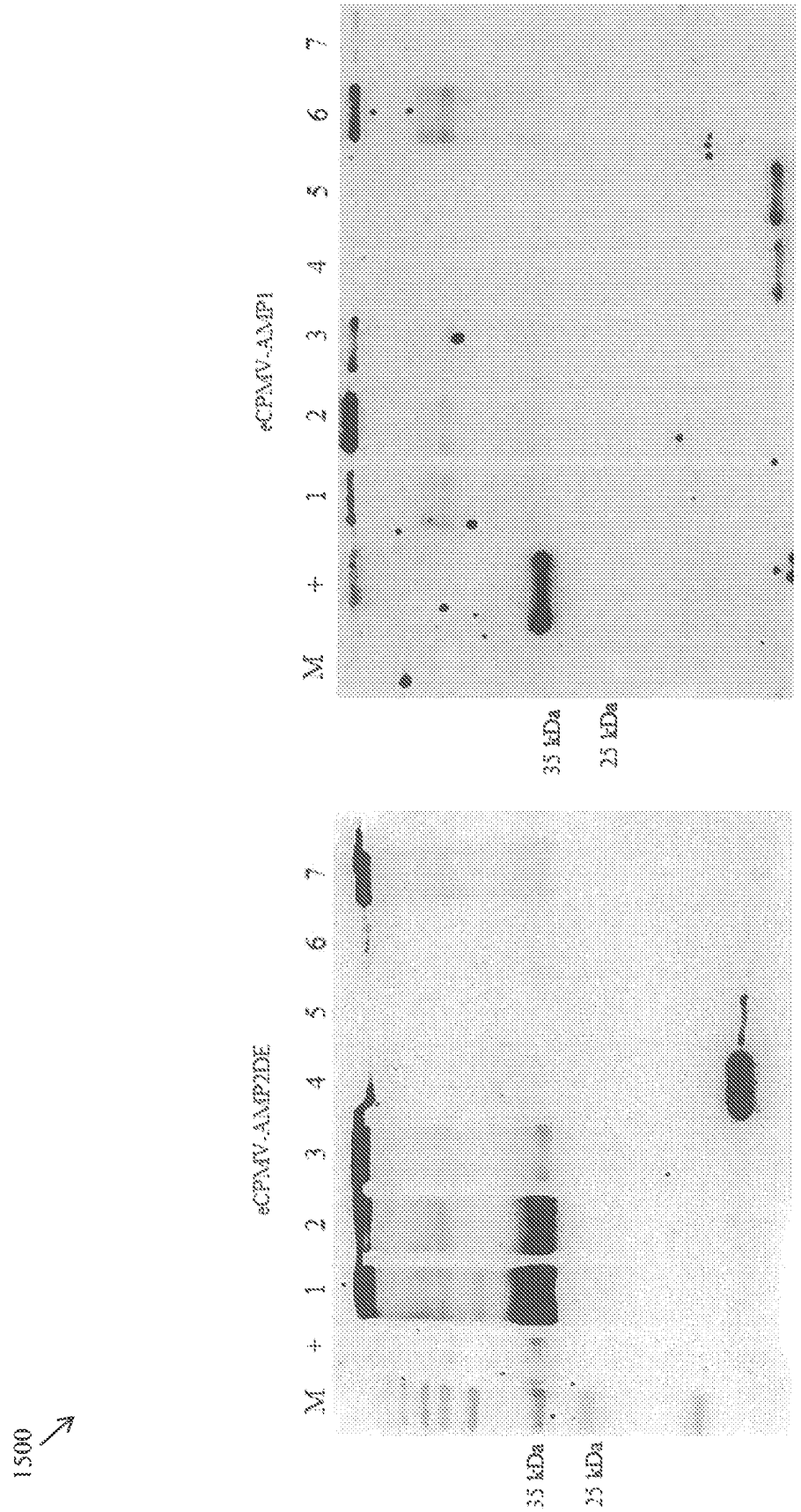

1500
eCPMV-AMP2DE
M + 1 2 3 4 5 6 7
35 kDa
25 kDa
eCPMV-AMP1
M + 1 2 3 4 5 6 7
35 kDa
25 kDa

FIG. 15

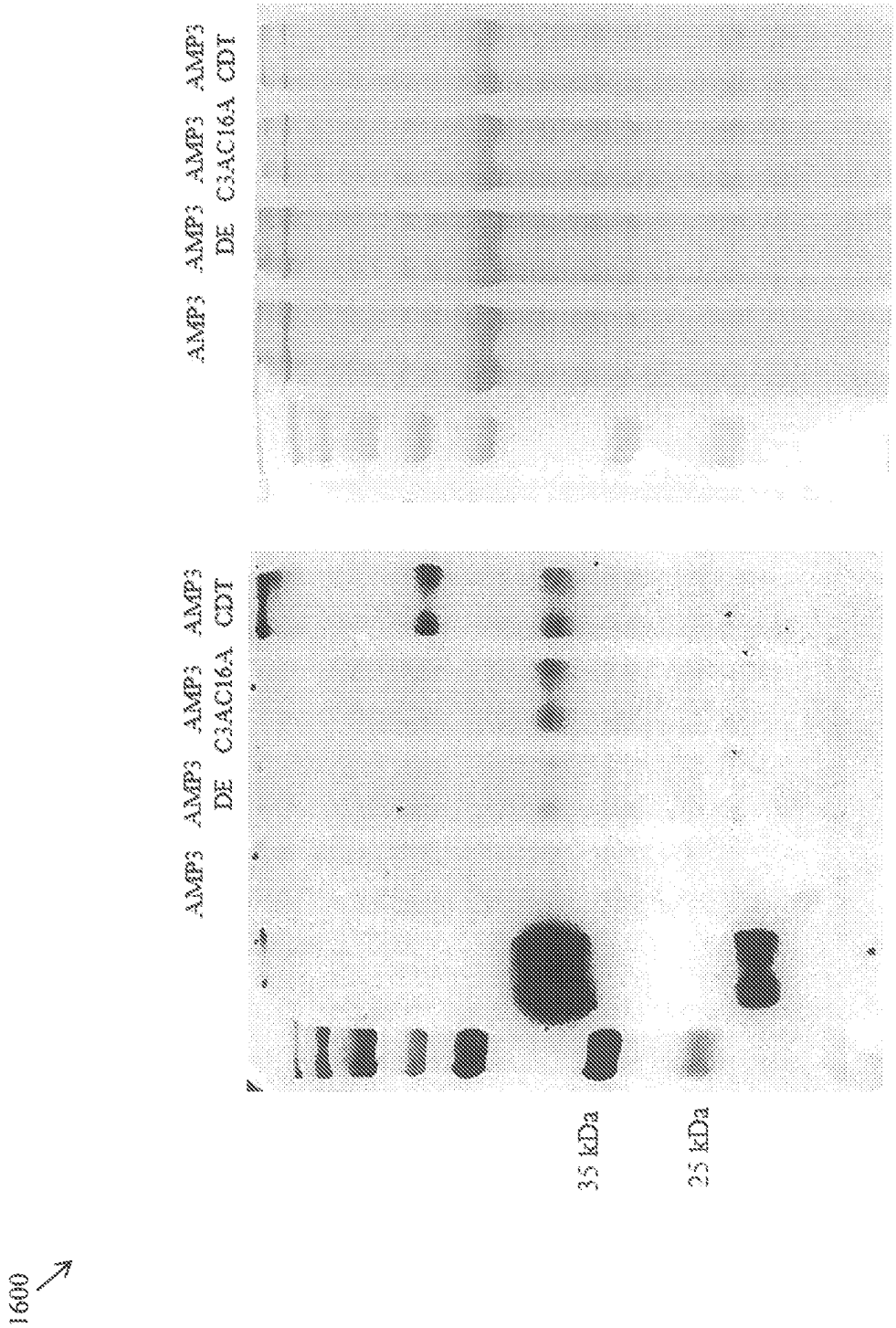

1600
AMP3
AMP3 DE
AMP3 C3AC16A
AMP3 CDT
35 kDa
25 kDa
AMP3
AMP3 DE
AMP3 C3AC16A
AMP3 CDT

FIG. 16

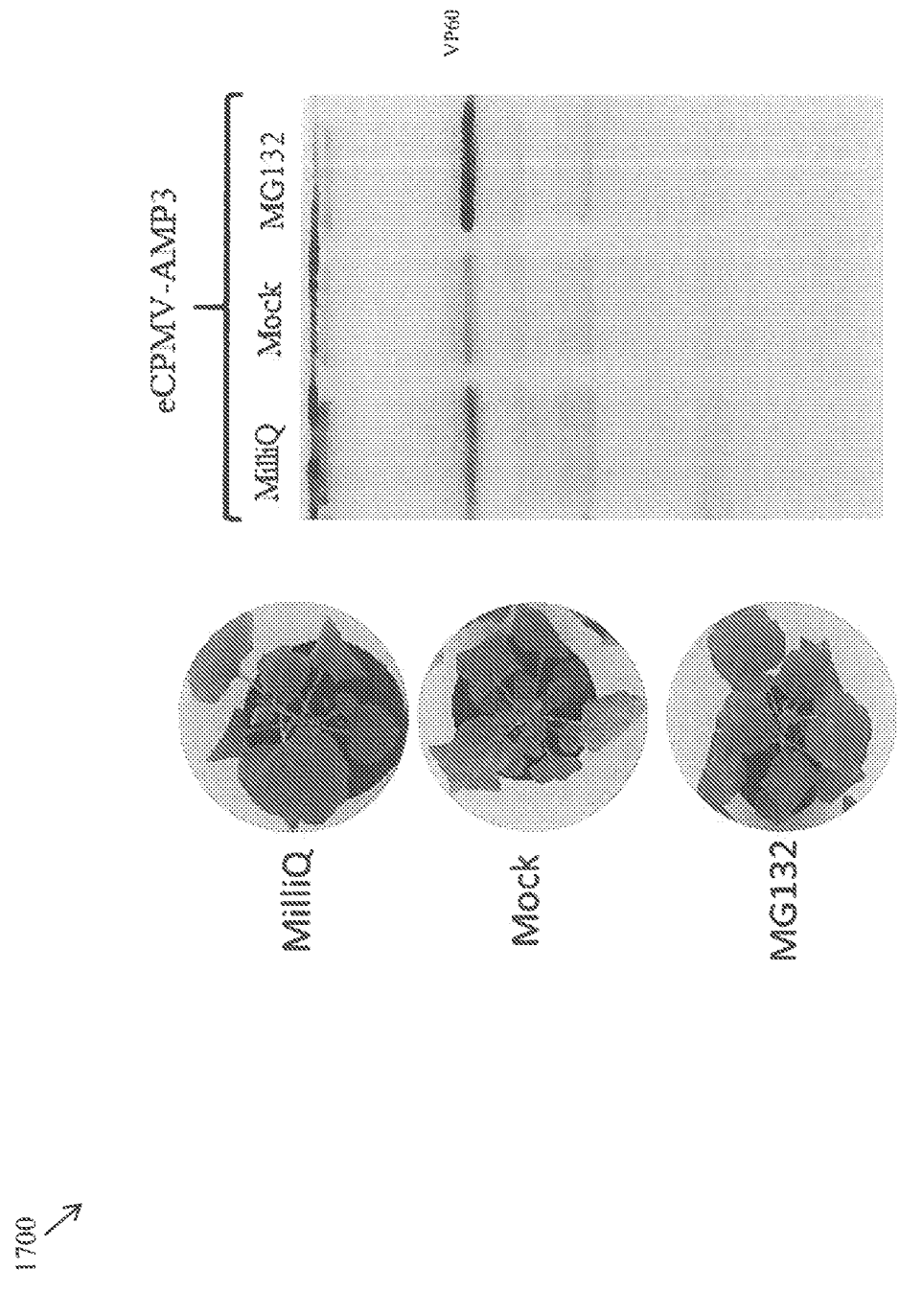


FIG. 17

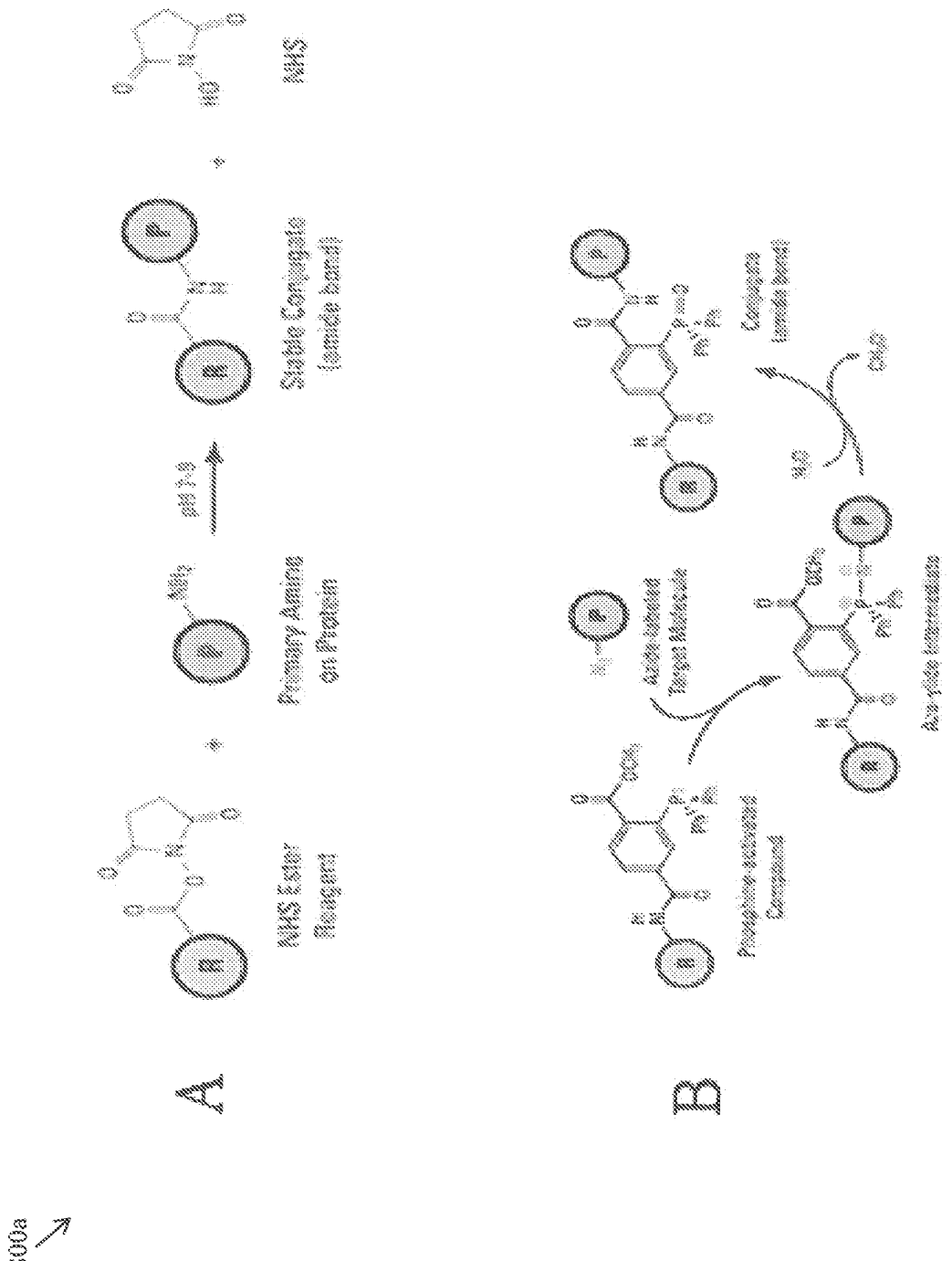

1800a
A
NHS Ester Reagent
Primary Amine on Protein
pH 7-9
Stable Conjugate (amide bond)
NHS
B
Propiolamide Compound
Azide-labeled Target Molecule
Cu(I)
Triazole Linkage
Bioconjugation

FIG. 18A

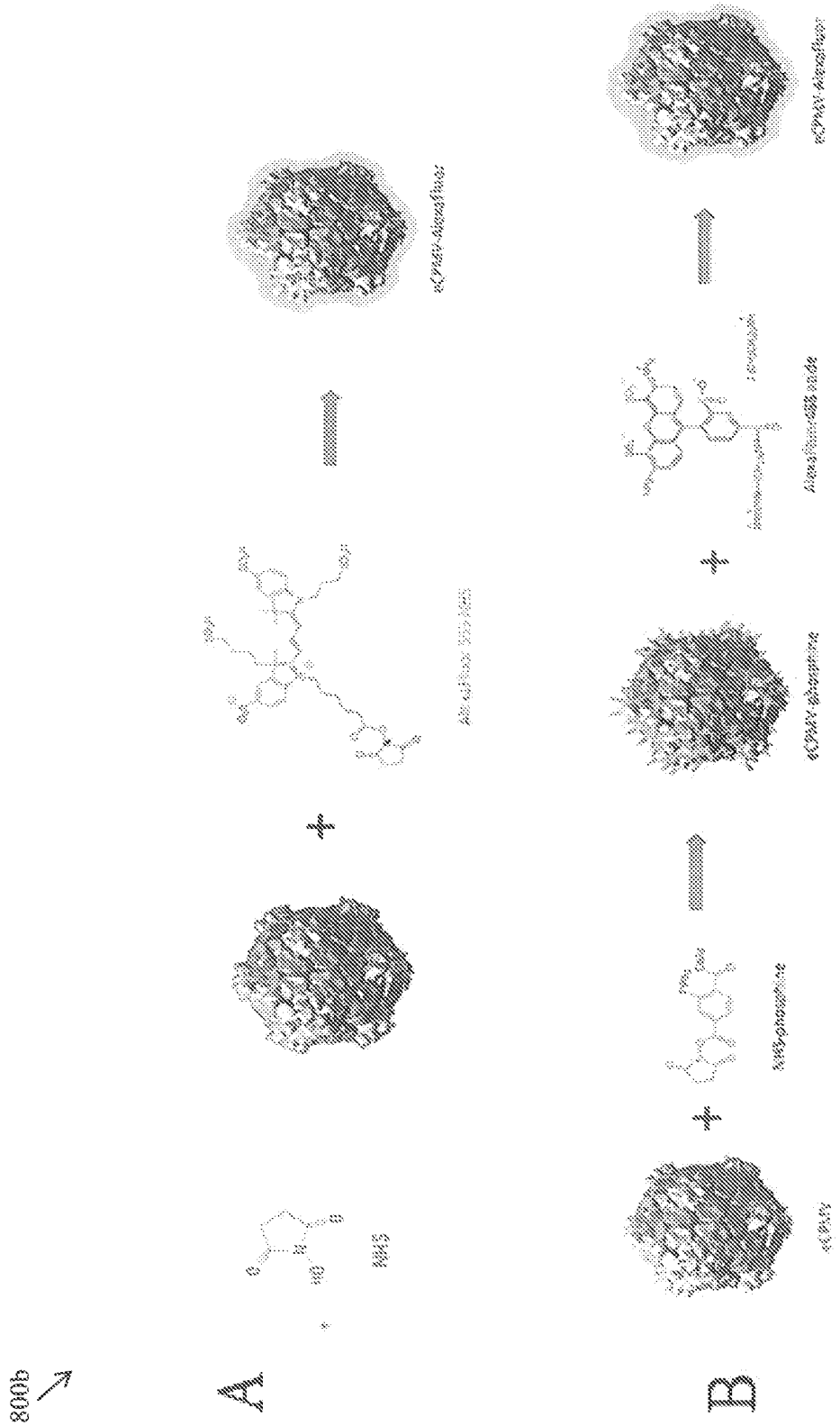


FIG. 18B

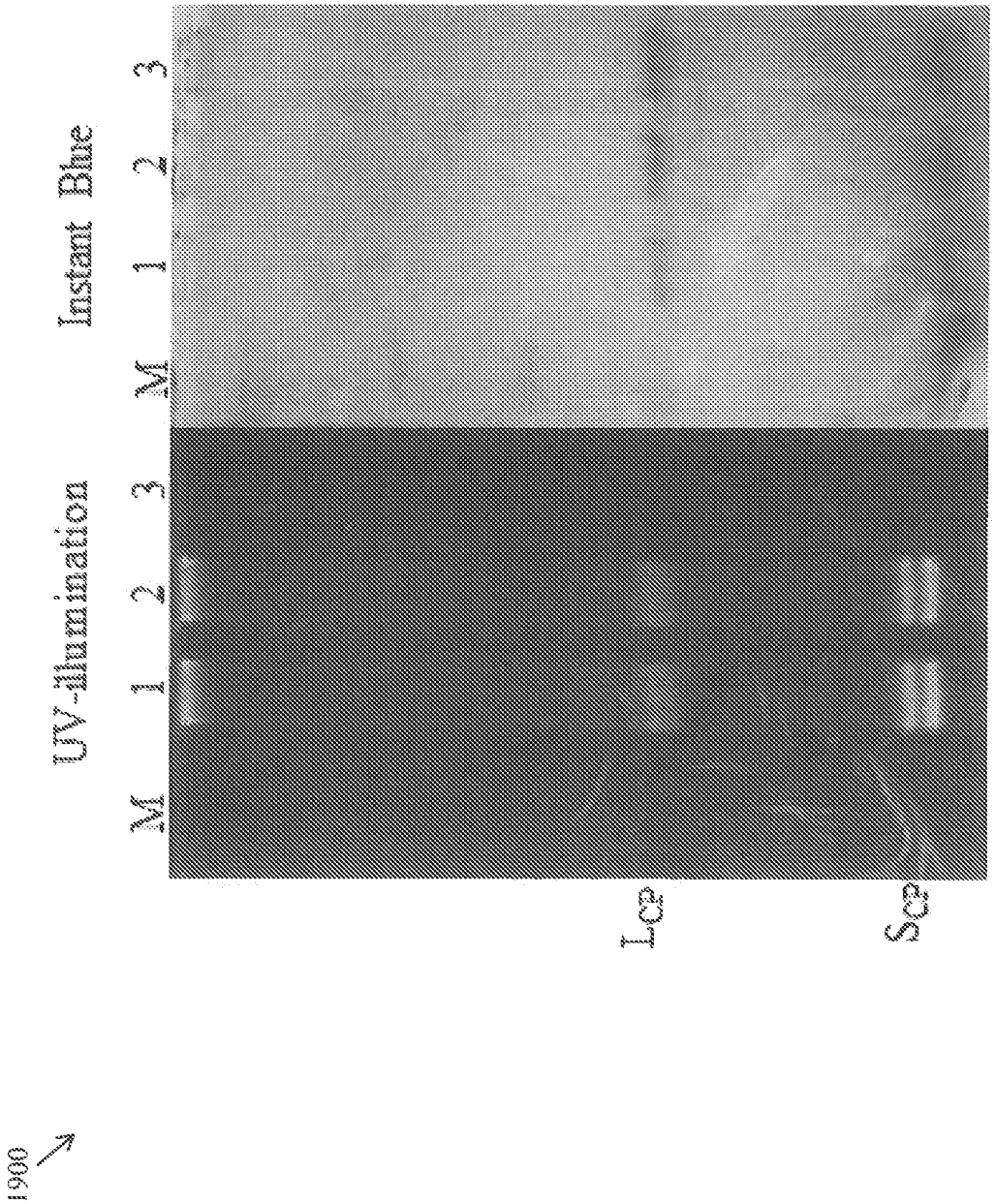


FIG. 19

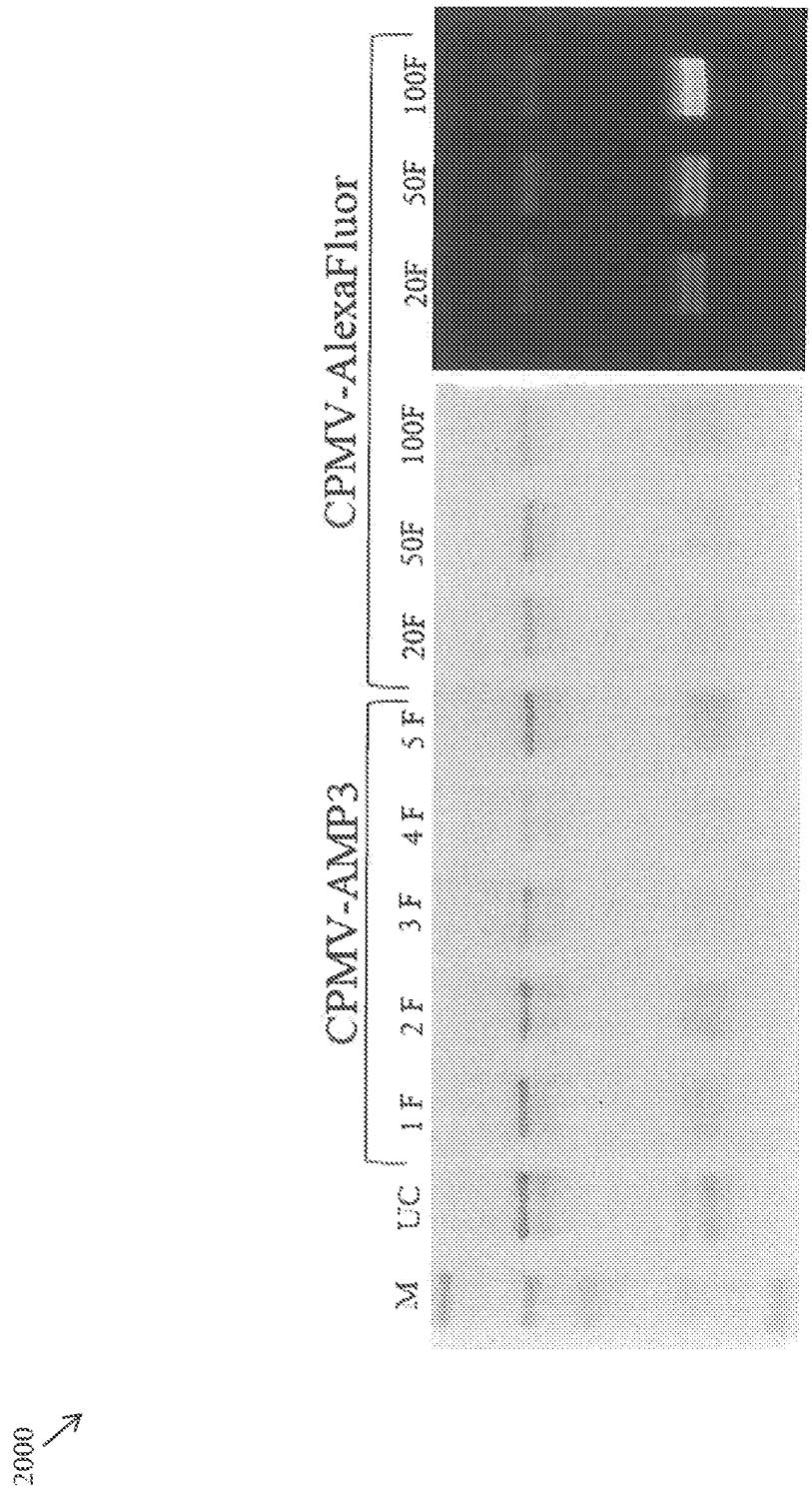

2000
M
UC
CPMV-AMP3
1 F
2 F
3 F
4 F
5 F
CPMV-AlexaFluor
20F
50F
100F
20F
50F
100F

FIG. 20

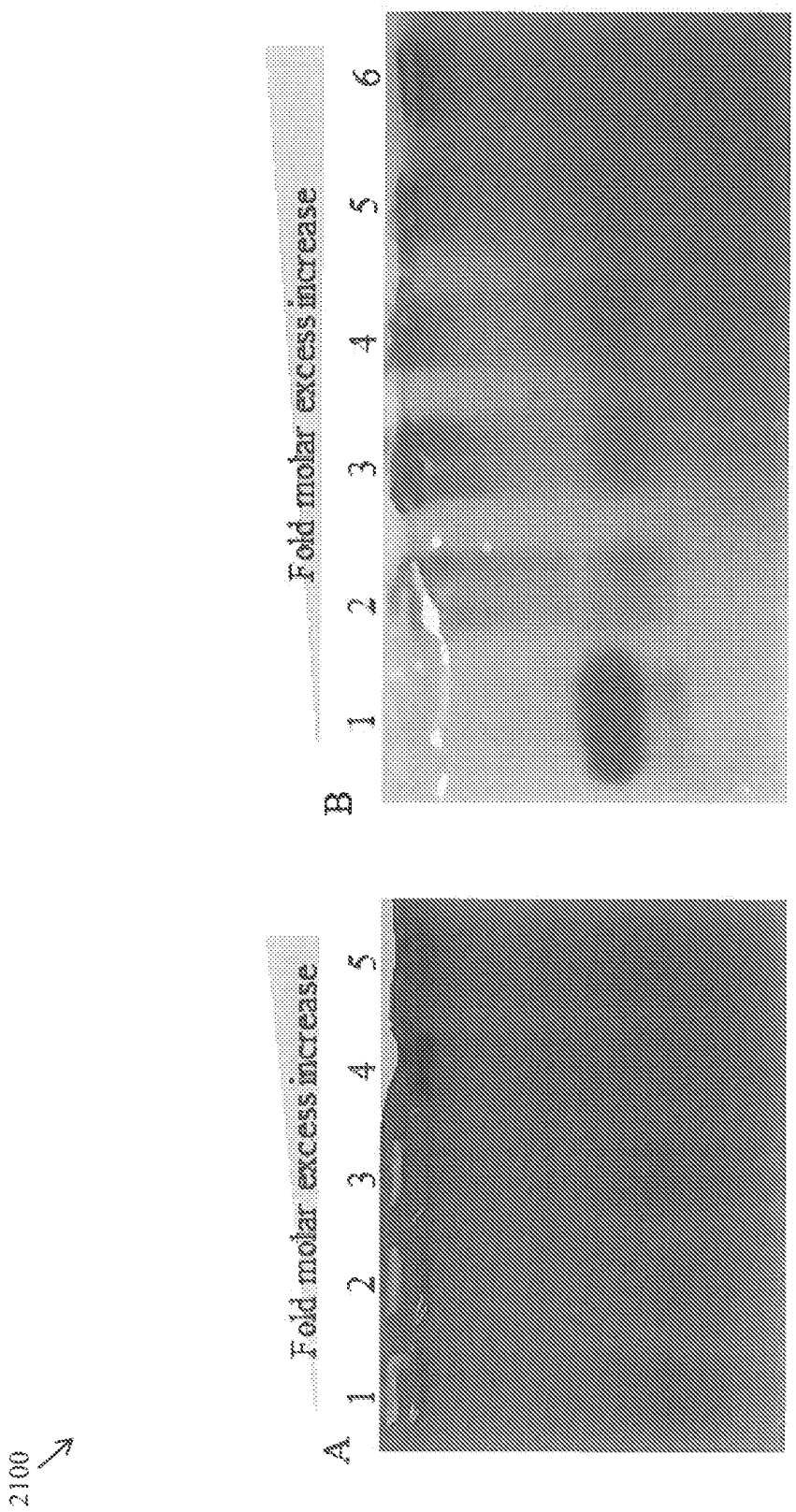

2100
A
Fold molar excess increase
1
2
3
4
5
B
Fold molar excess increase
1
2
3
4
5
6

FIG. 21

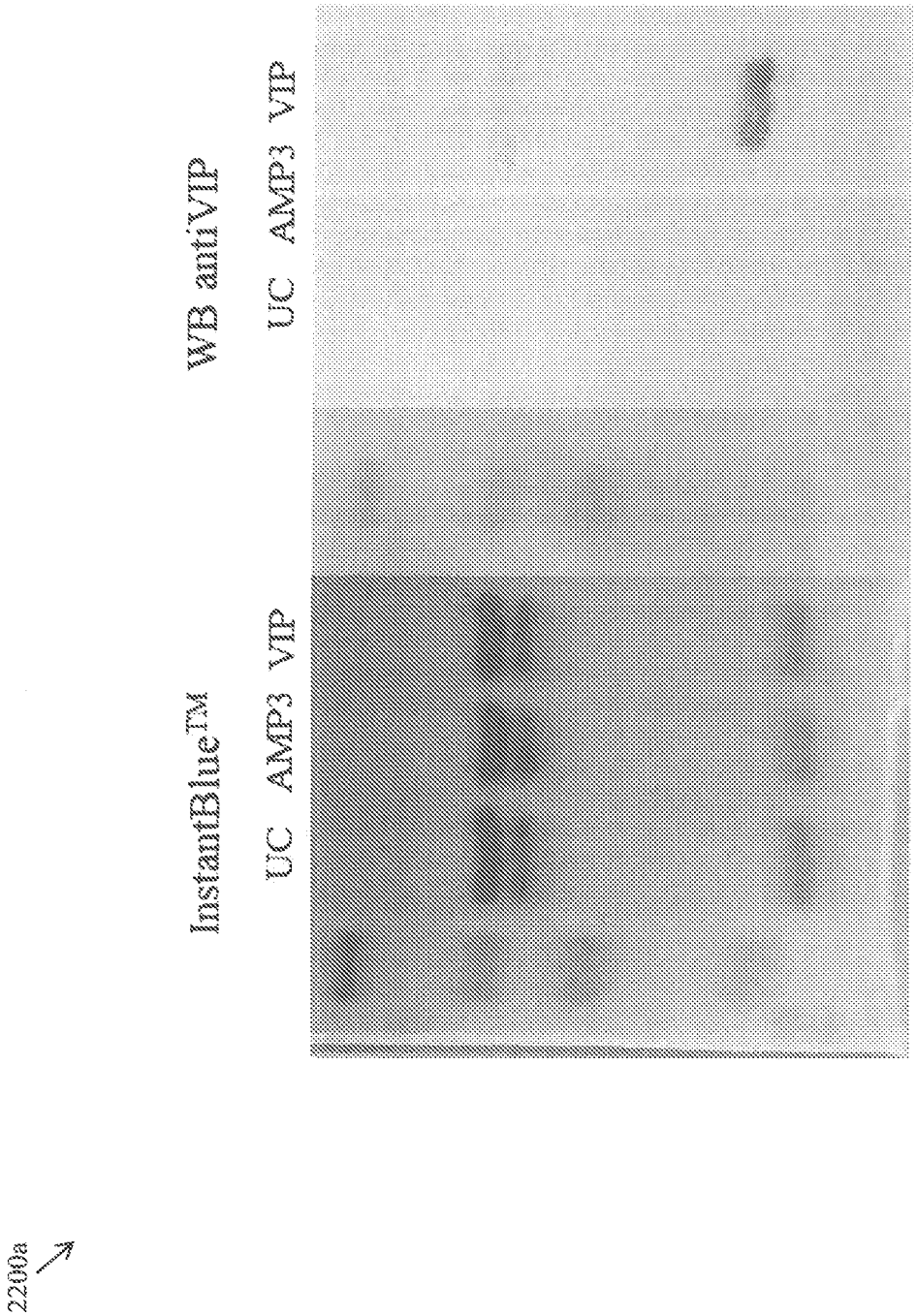


FIG. 22A

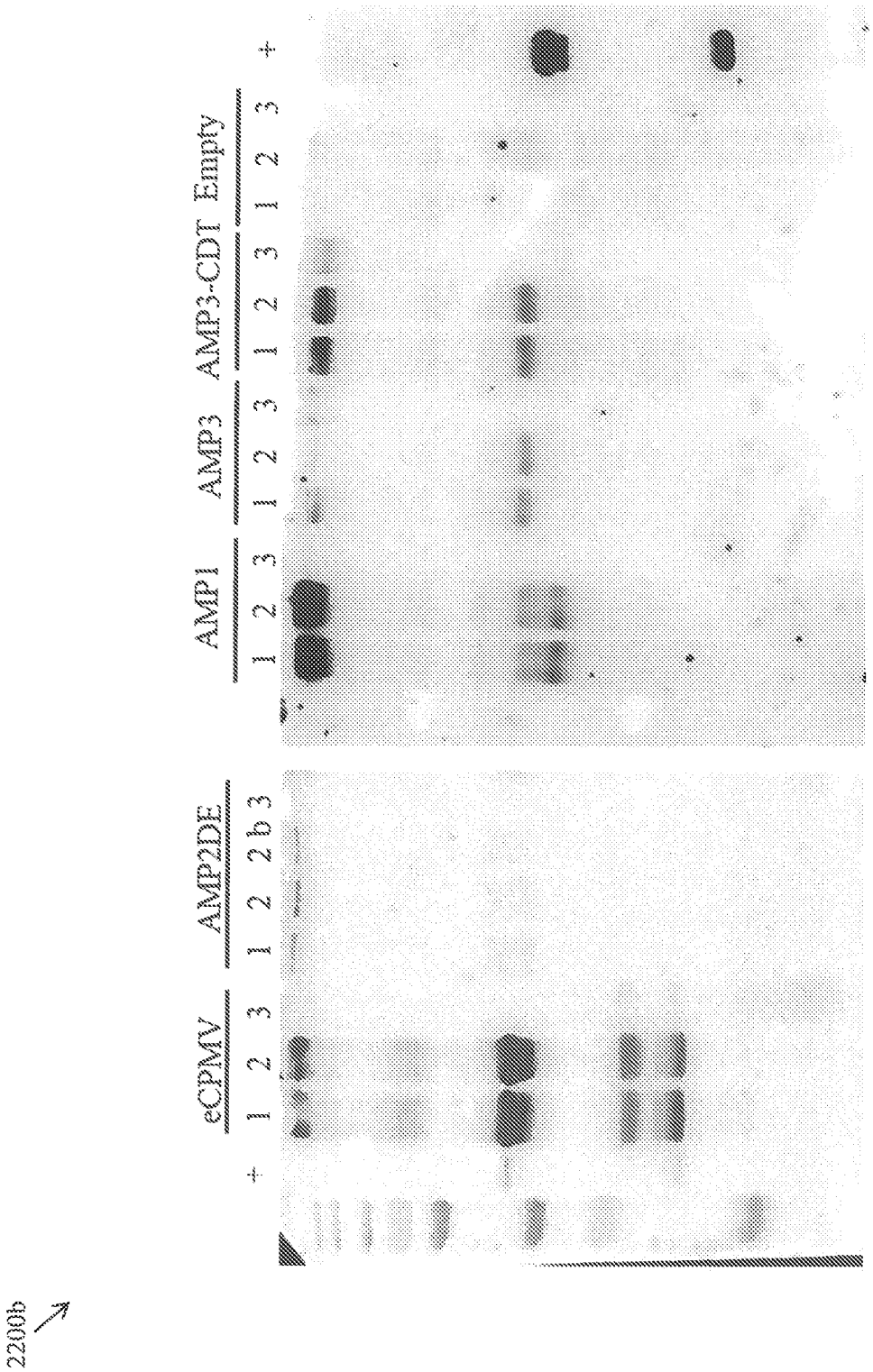

2200b
eCPMV
AMP2DE
+ 1 2 3 1 2 2b 3
AMP1
AMP3
AMP3-CDT
Empty
1 2 3 1 2 3 1 2 3 1 2 3 +

FIG. 22B

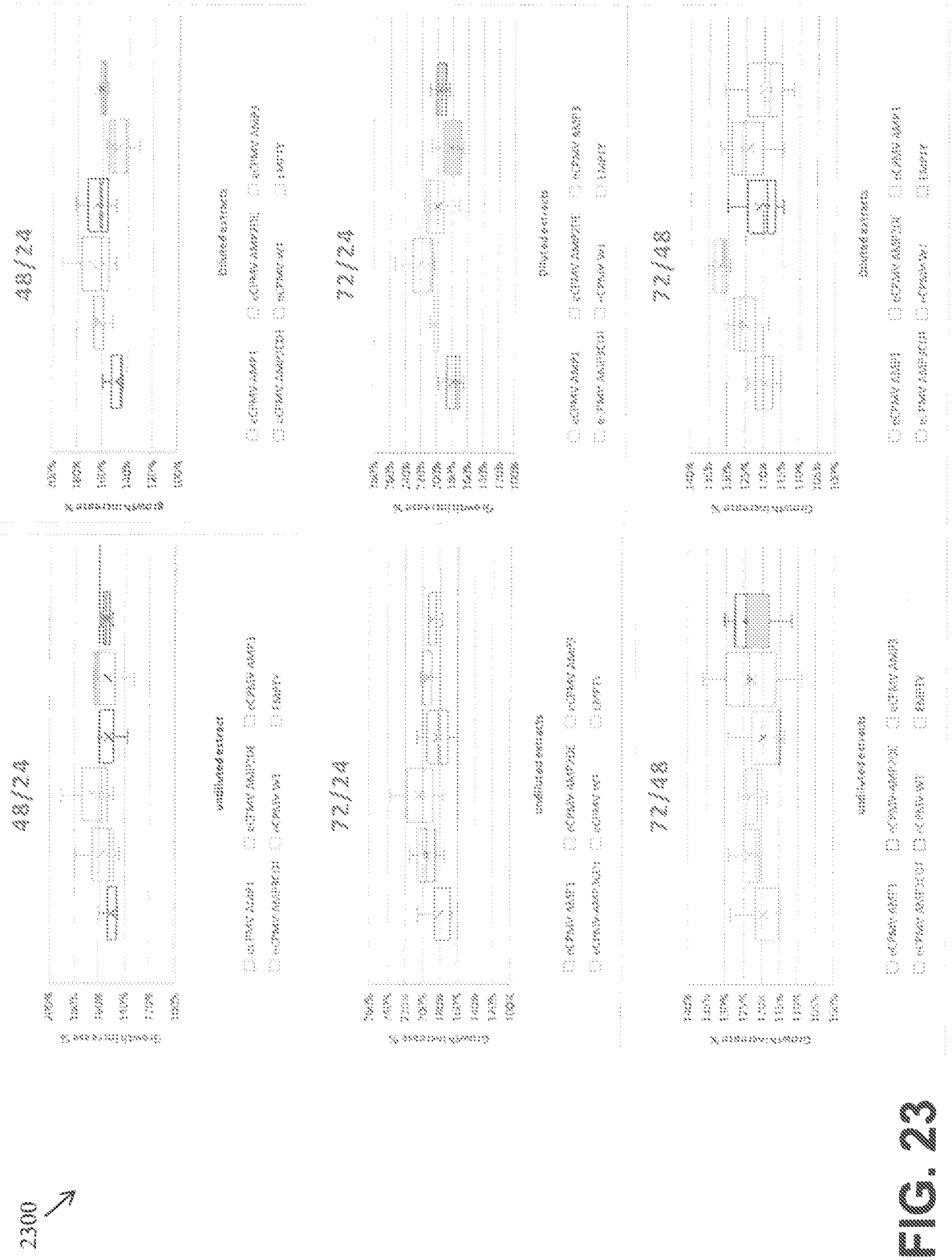

2300
48 / 24
72 / 24
72 / 48
Growth inhibition %
Growth increase %
Undiluted extracts
Diluted extracts
eCPMV AMP1
eCPMV AMP1(20)
eCPMV AMP2(20)
eCPMV AMP2
eCPMV WT
Empty

FIG. 23

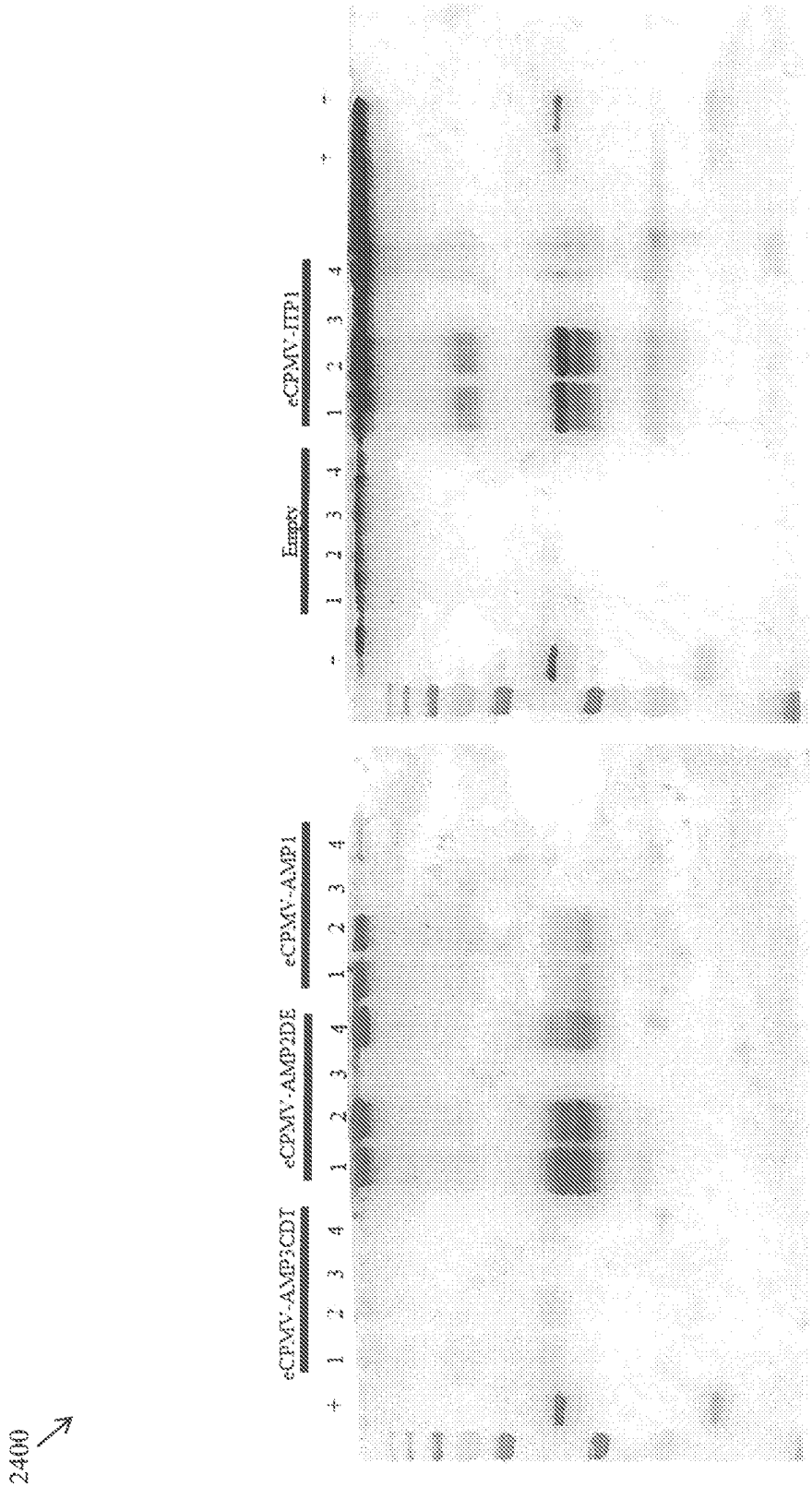

2400
eCPMV-AMP3CDT
eCPMV-AMP2DE
eCPMV-AMP1
Empty
eCPMV-ITP1
+ 1 2 3 4 1 2 3 4 1 2 3 4 - 1 2 3 4 1 2 3 4 + -

FIG. 24

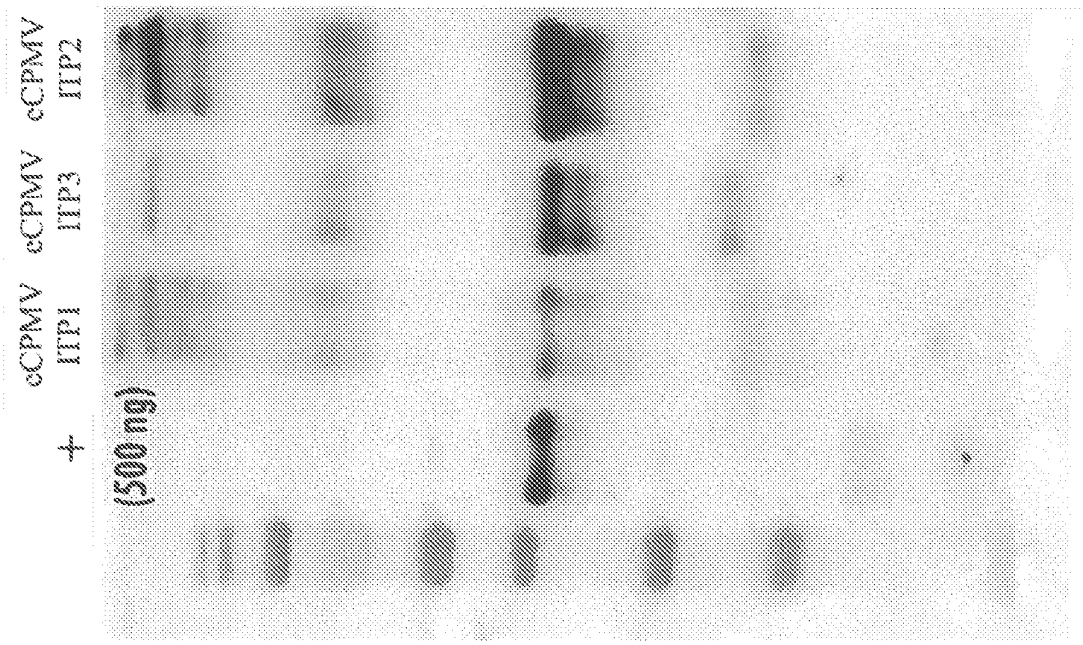


FIG. 25

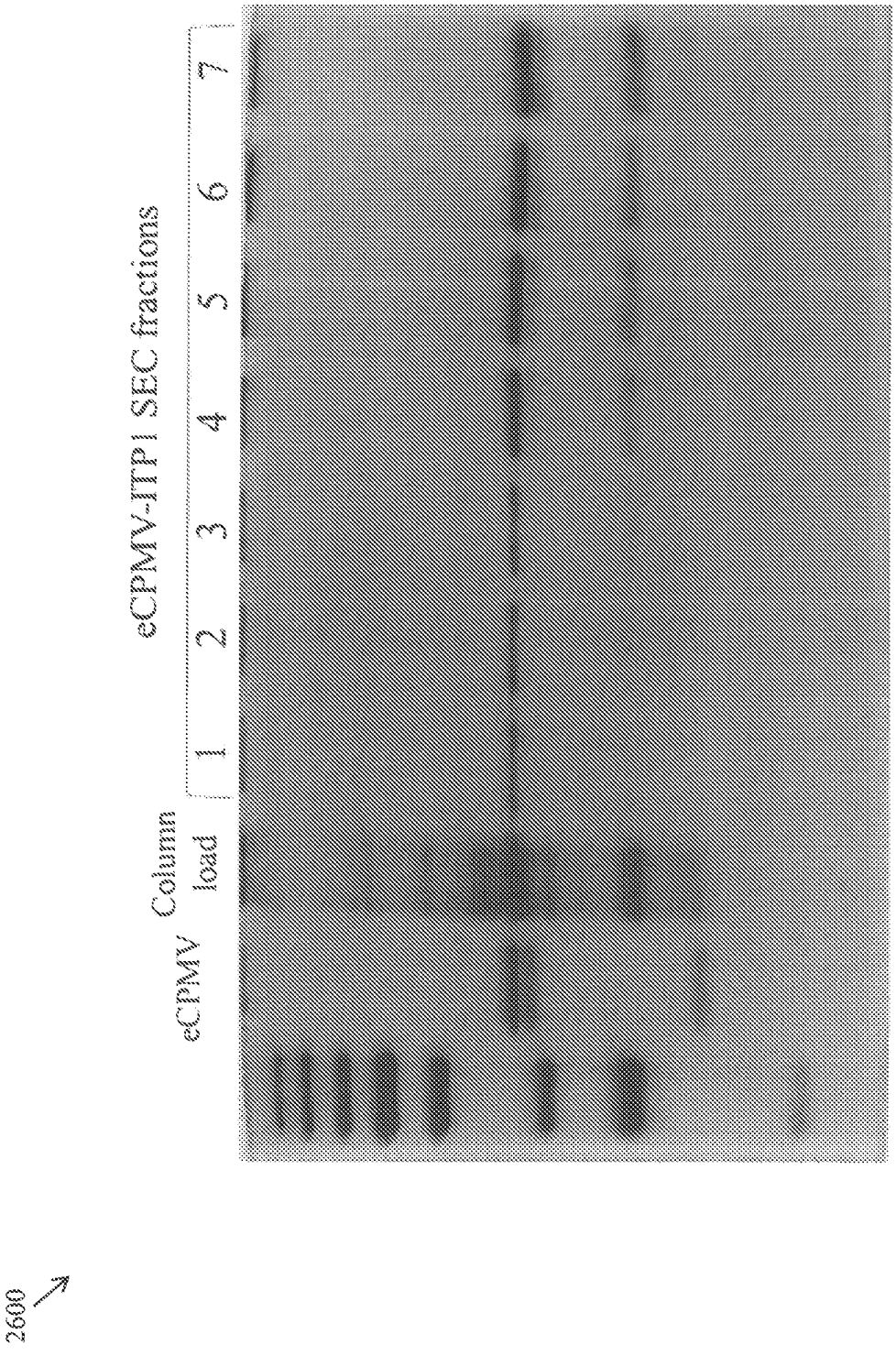


FIG. 26

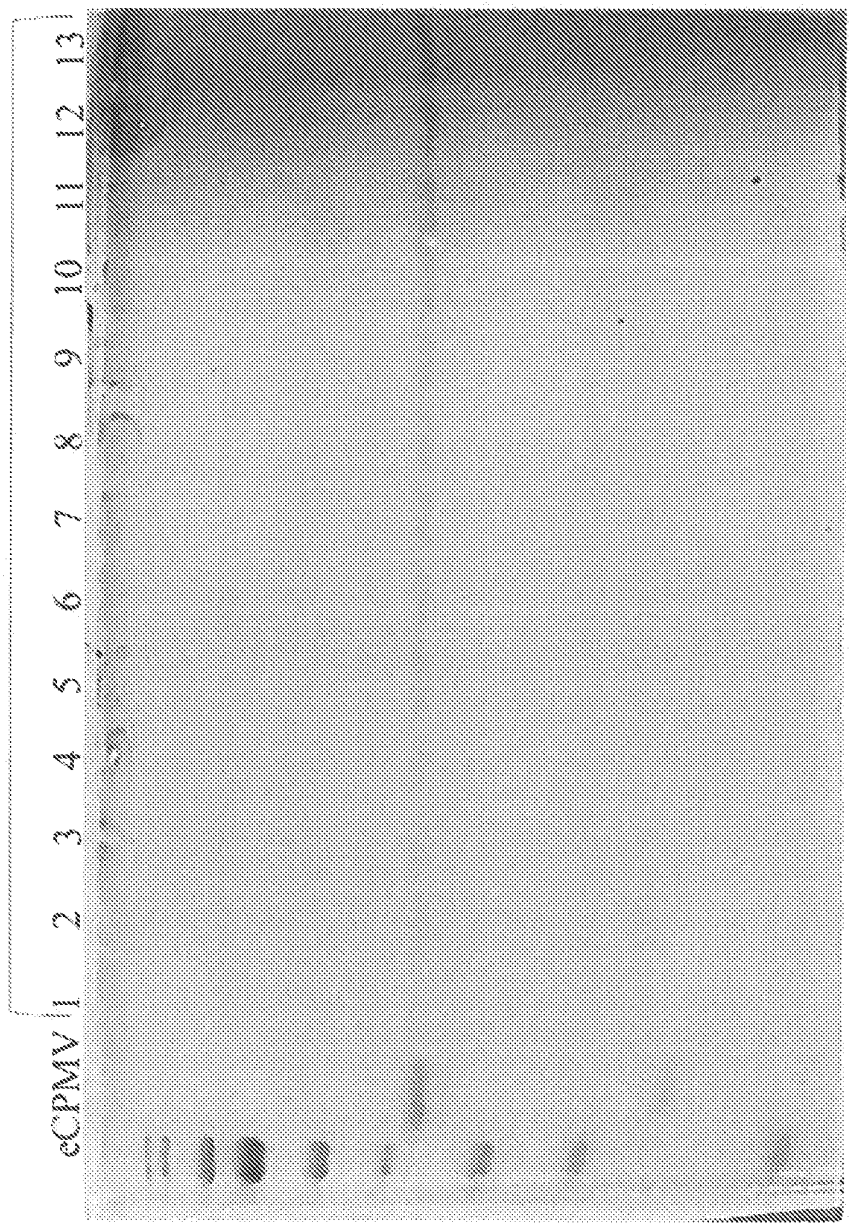


FIG. 27

2700

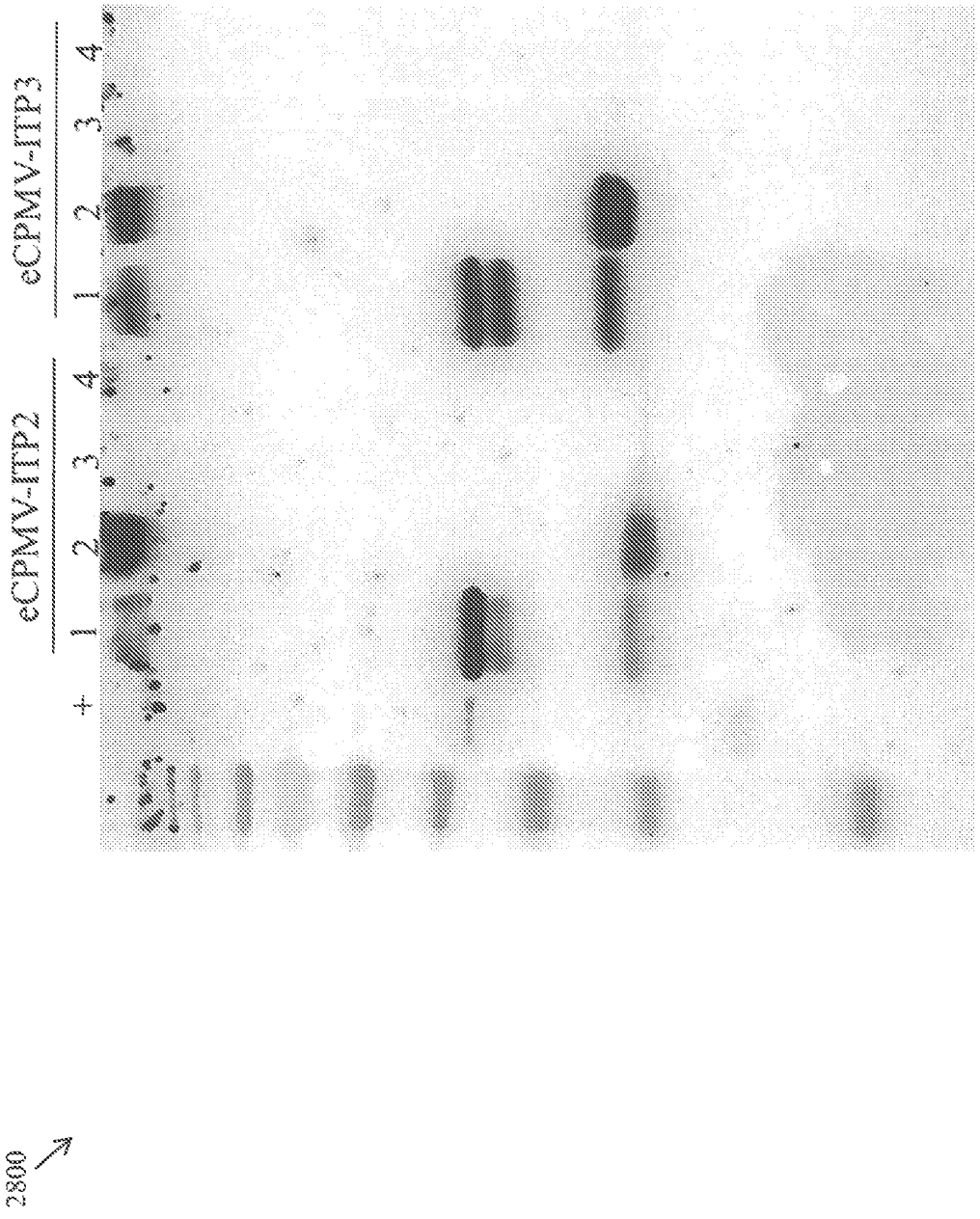


FIG. 28

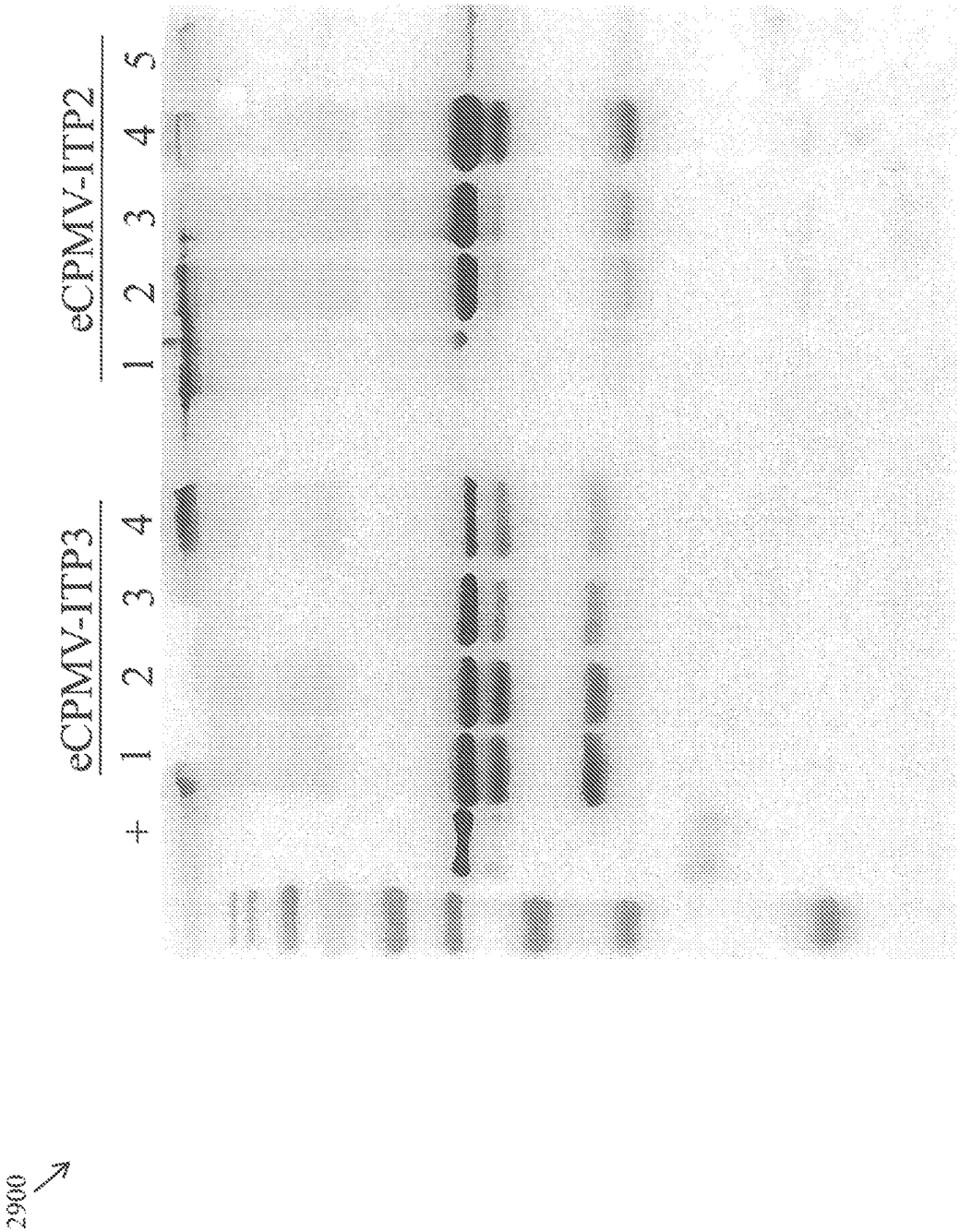


FIG. 29

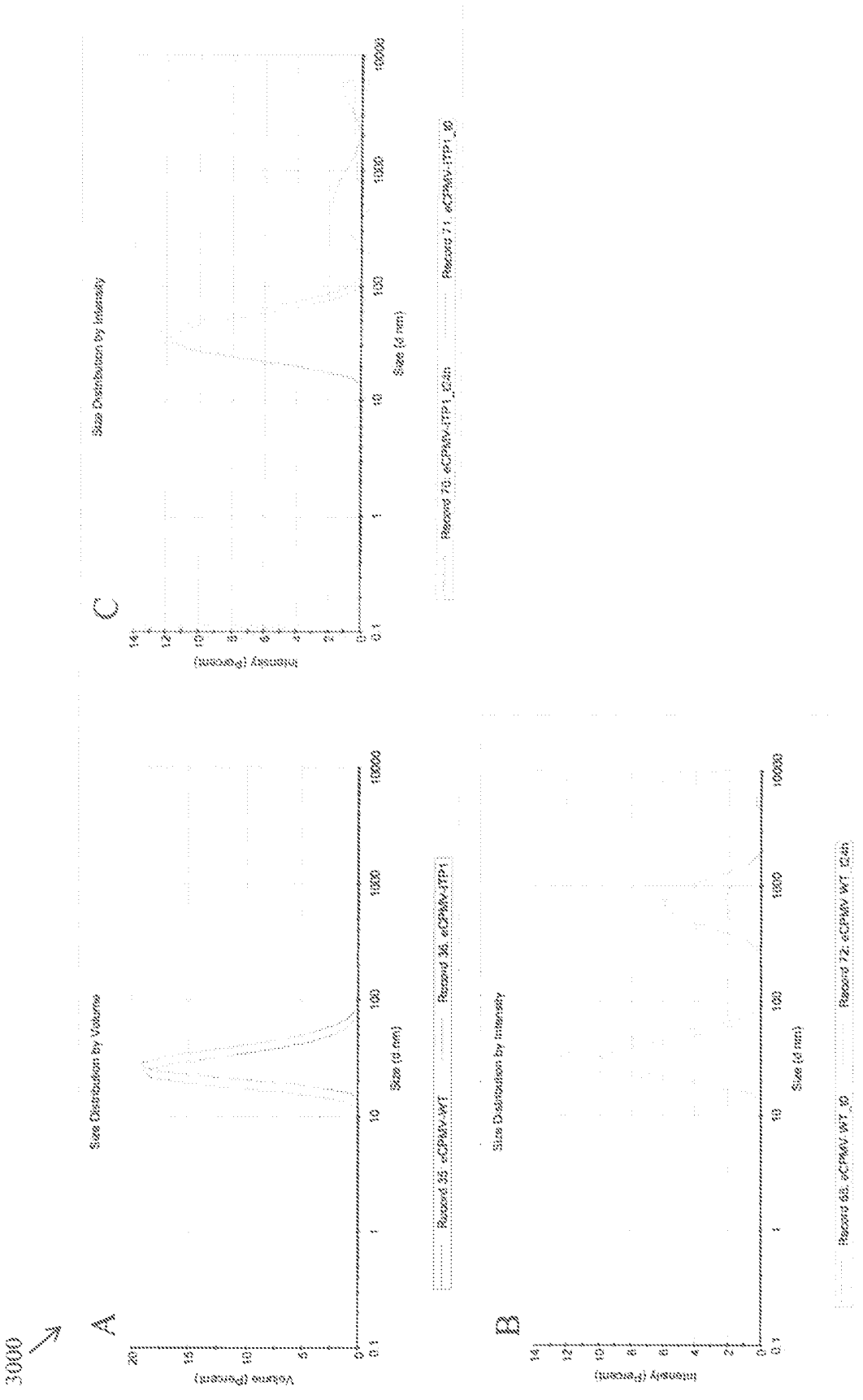

3000
A
Size Distribution by Volume
Volume (Percent)
Size (d.nm)
Record 35: eCPMV-WT
Record 36: eCPMV-ITP1
B
Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
Record 68: eCPMV-WT_10
Record 72: eCPMV-WT_12.4h
C
Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
Record 78: eCPMV-ITP1_12.4h
Record 71: eCPMV-ITP1_10

FIG. 30

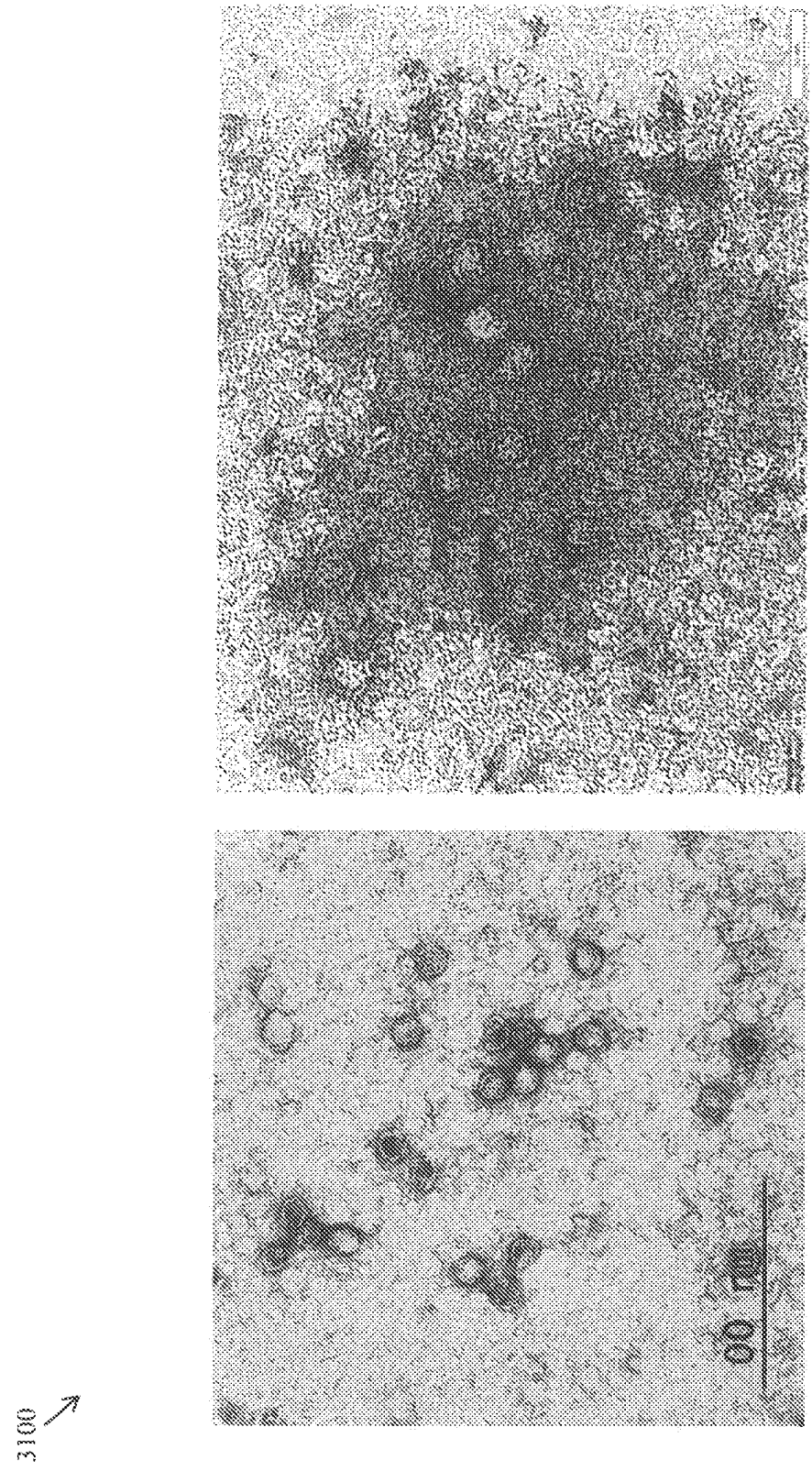


FIG. 31

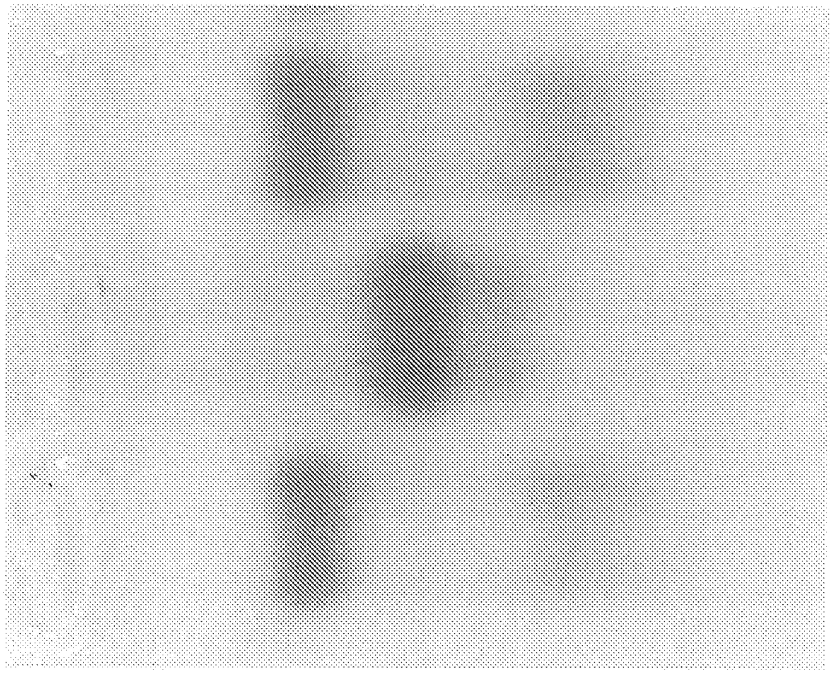


FIG. 32

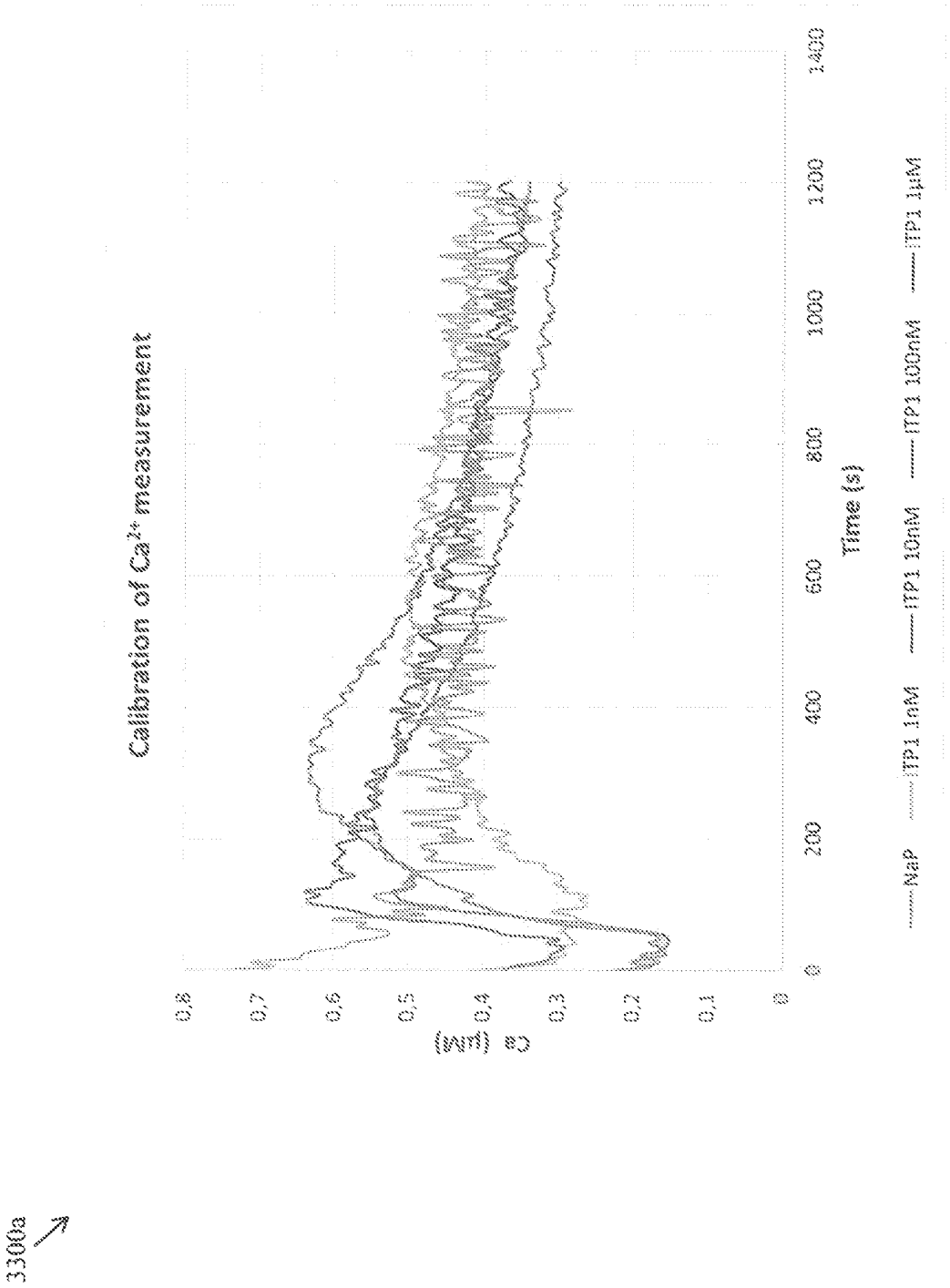

3300a
Calibration of Ca^{2+} measurement
Ca (µM)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
0
200
400
600
800
1000
1200
1400
Time (s)
NaP
ITP1 1nM
ITP1 10nM
ITP1 100nM
ITP1 1µM

FIG. 33A

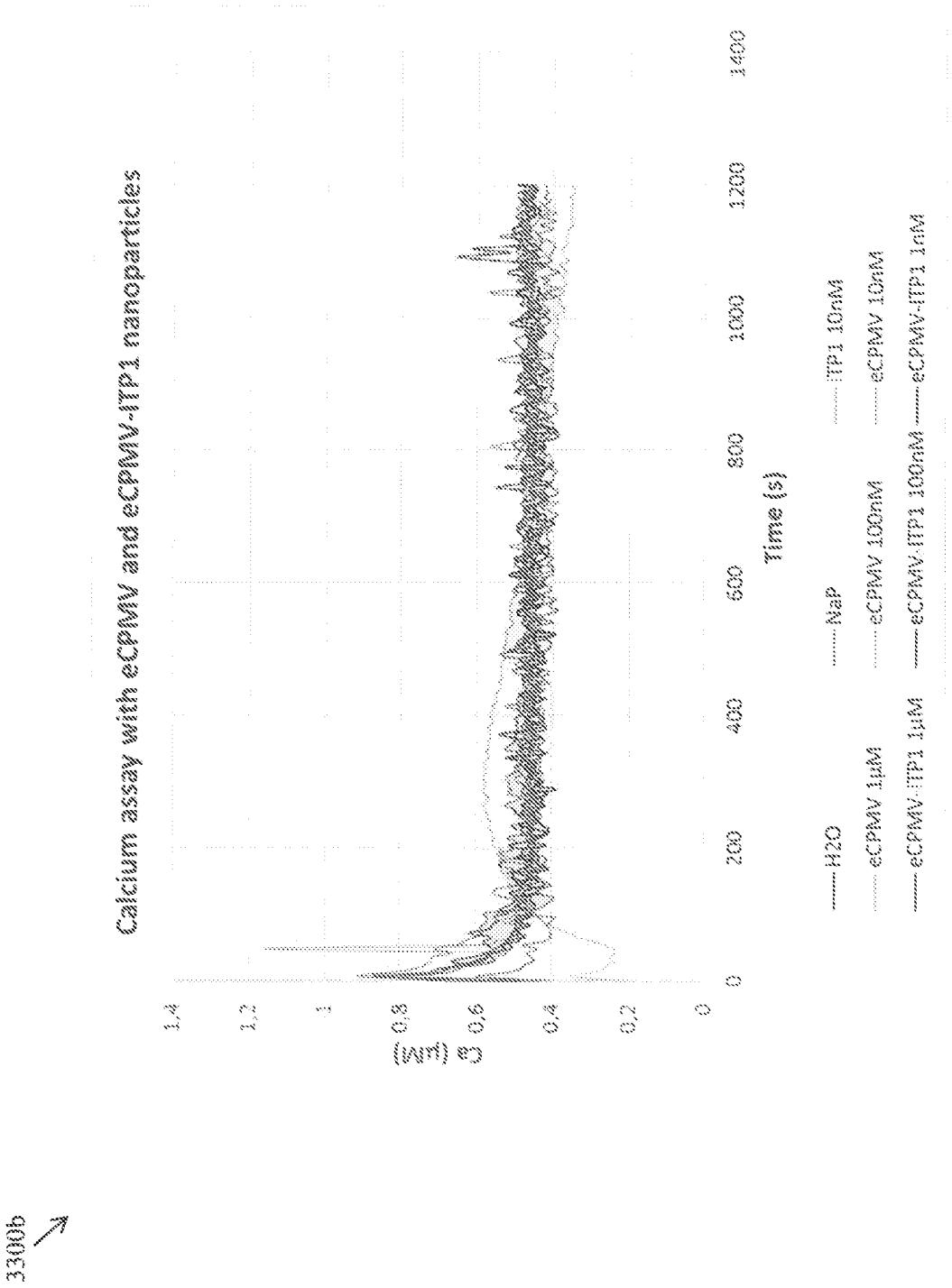

3300b
Calcium assay with eCPMV and eCPMV-ITP1 nanoparticles
Ca (μM)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
200
400
600
800
1000
1200
1400
Time (s)
H2O
NaP
ITP1 10nM
eCPMV 1μM
eCPMV 100nM
eCPMV 10nM
eCPMV-ITP1 1μM
eCPMV-ITP1 100nM
eCPMV-ITP1 1nM

FIG. 33B

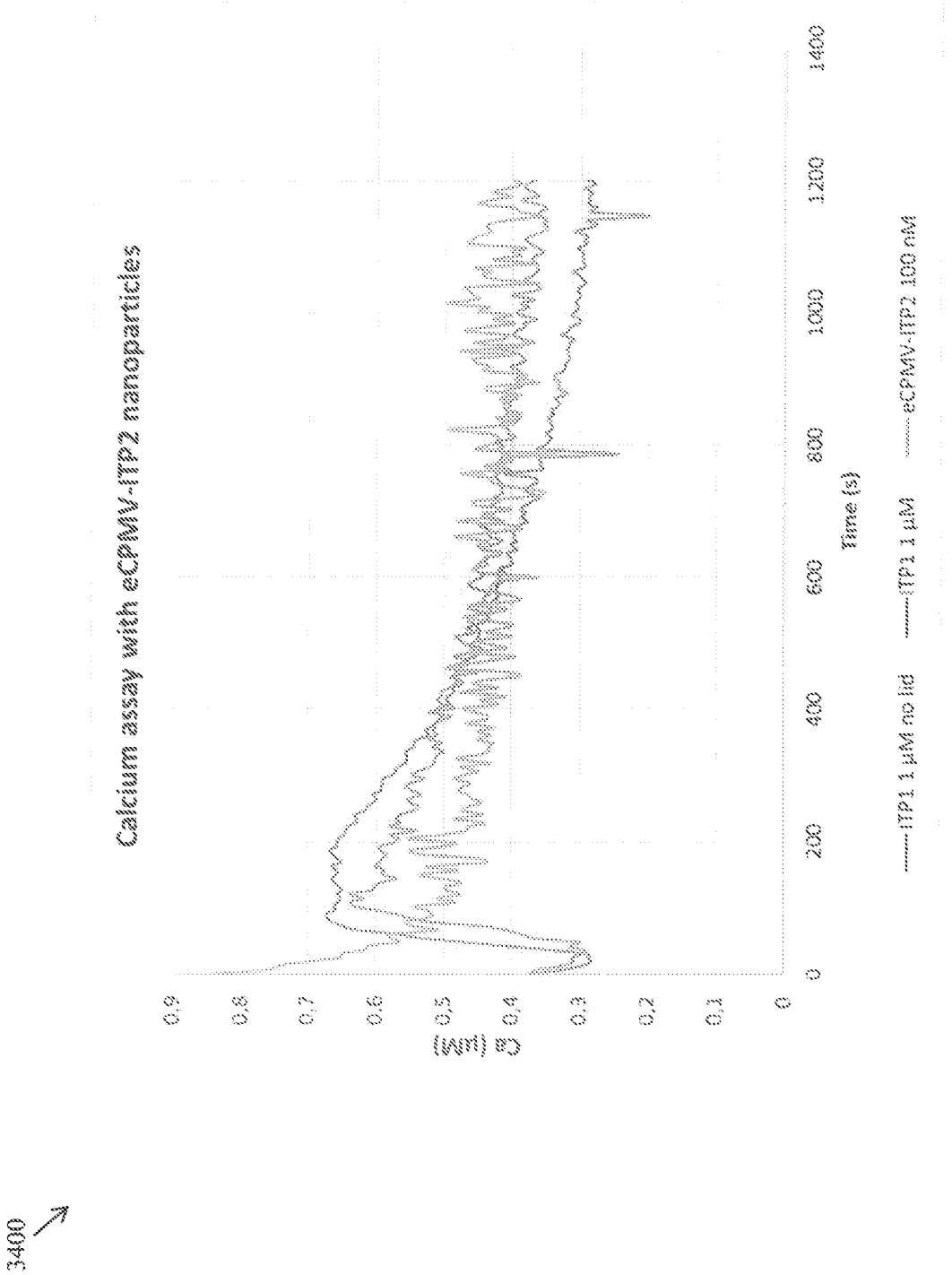

3400
Calcium assay with eCPMV-ITP2 nanoparticles
Ca (μM)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
200
400
600
800
1000
1200
1400
Time (s)
ITP1 1 μM no lid
ITP1 1 μM
eCPMV-ITP2 100 nM

FIG. 34

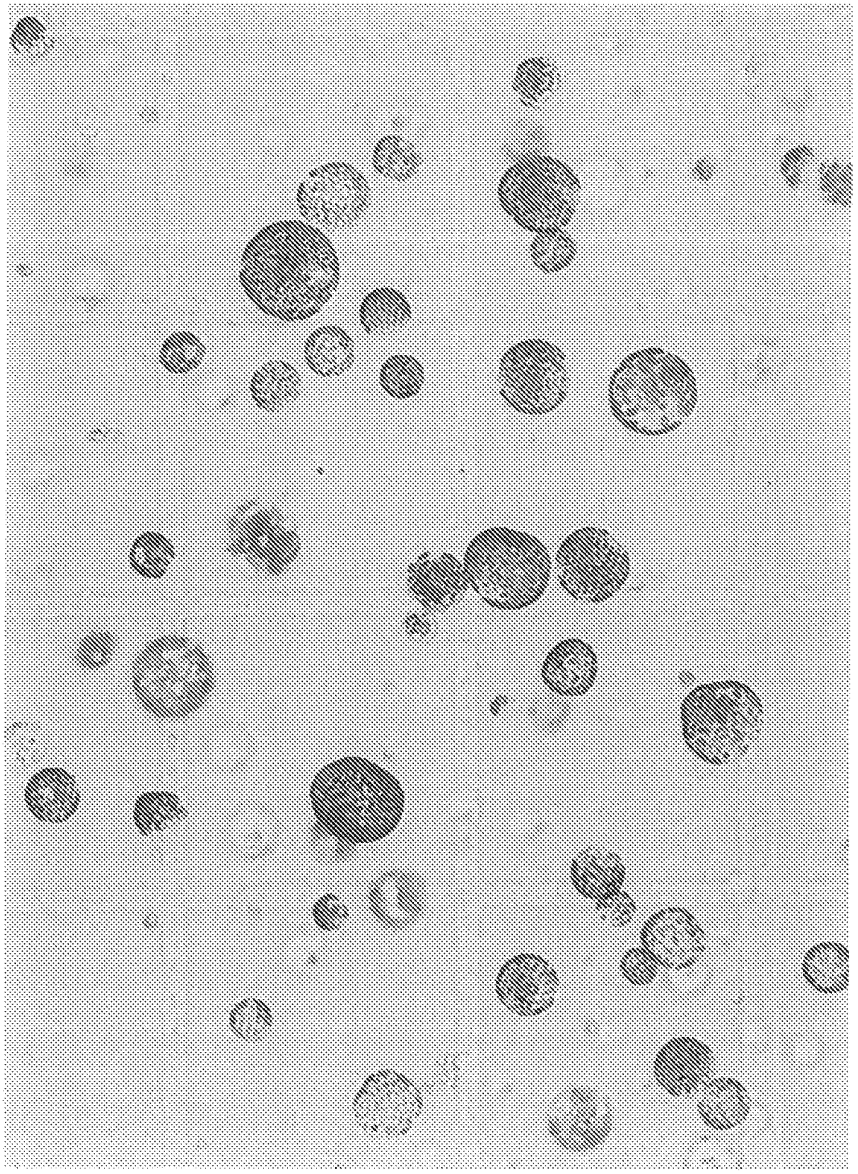


FIG. 35

3500

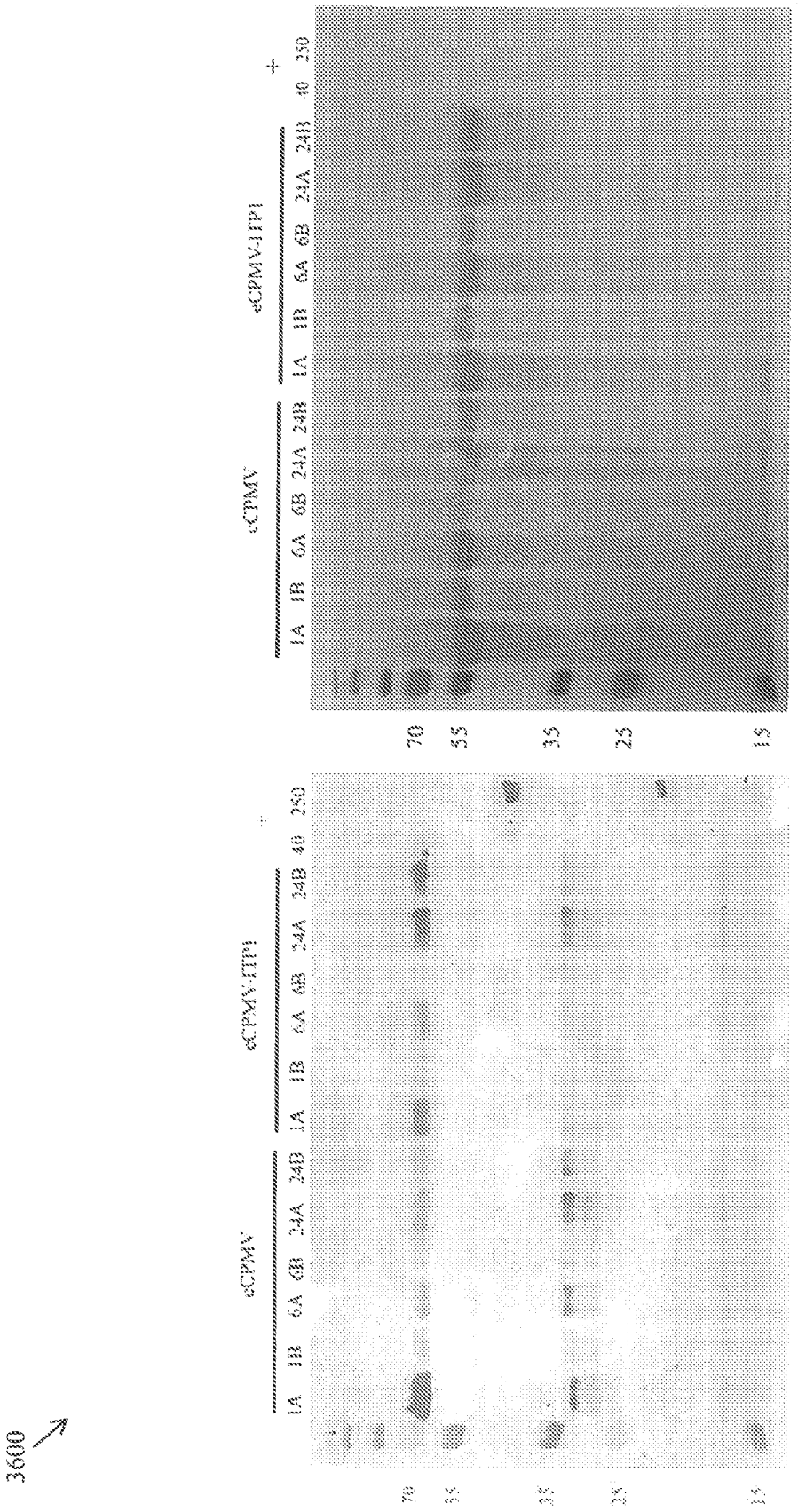


FIG. 36

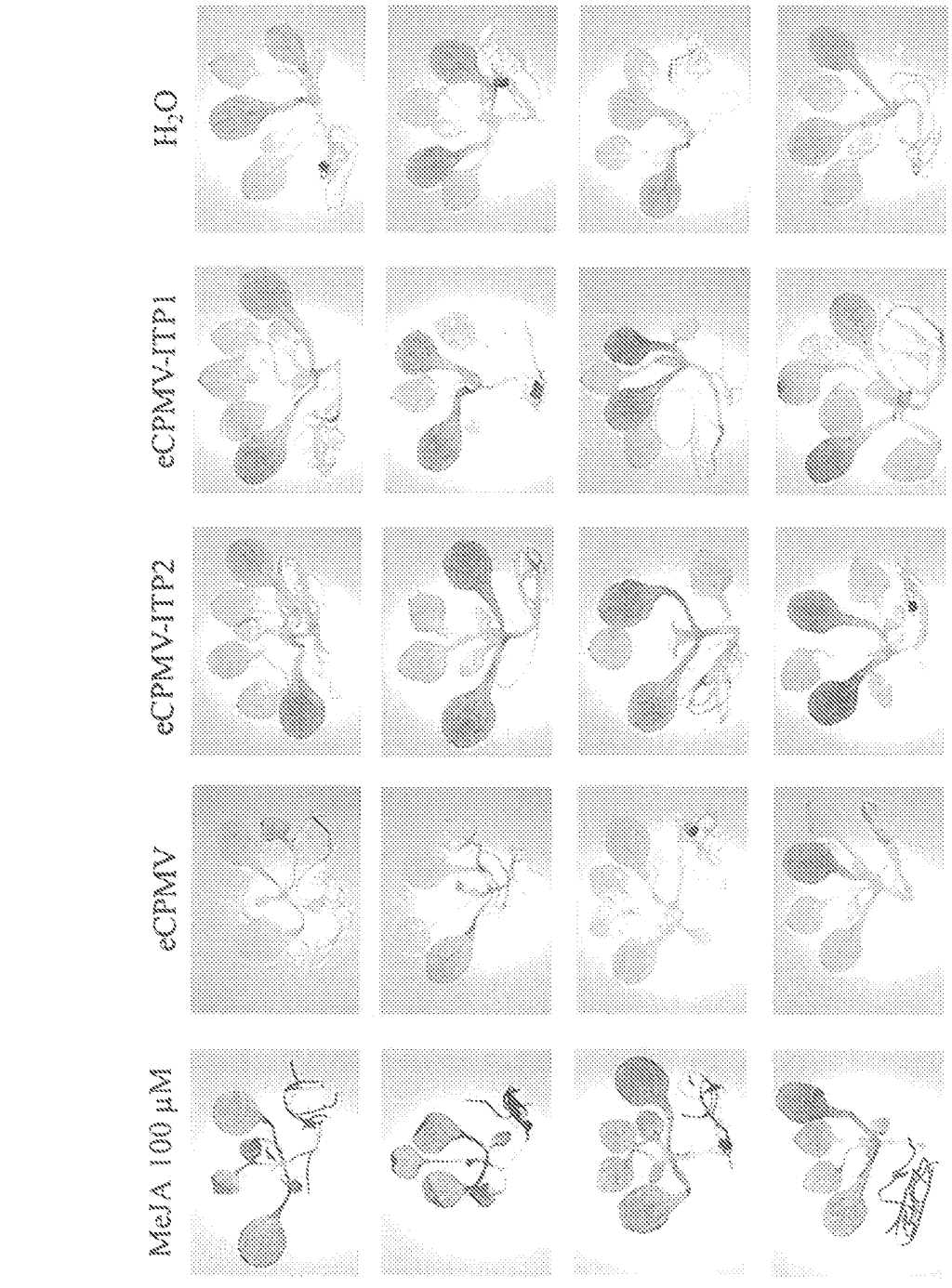


FIG. 37

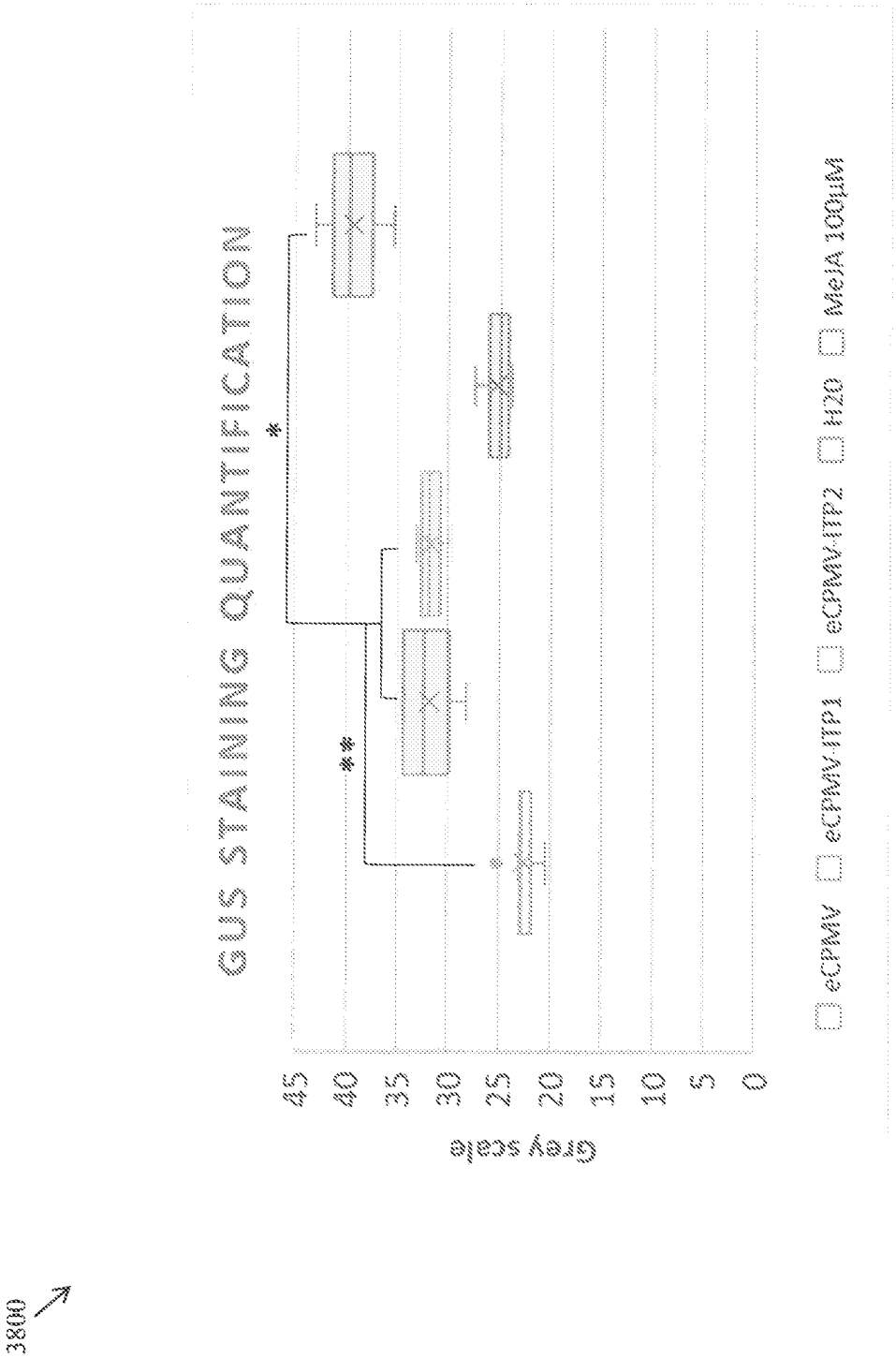

GUS STAINING QUANTIFICATION
Grey scale
45
40
35
30
25
20
15
10
5
0
*
**
eCPMV
eCPMV-ITP1
eCPMV-ITP2
H20
MeJA 100µM
3800

FIG. 38

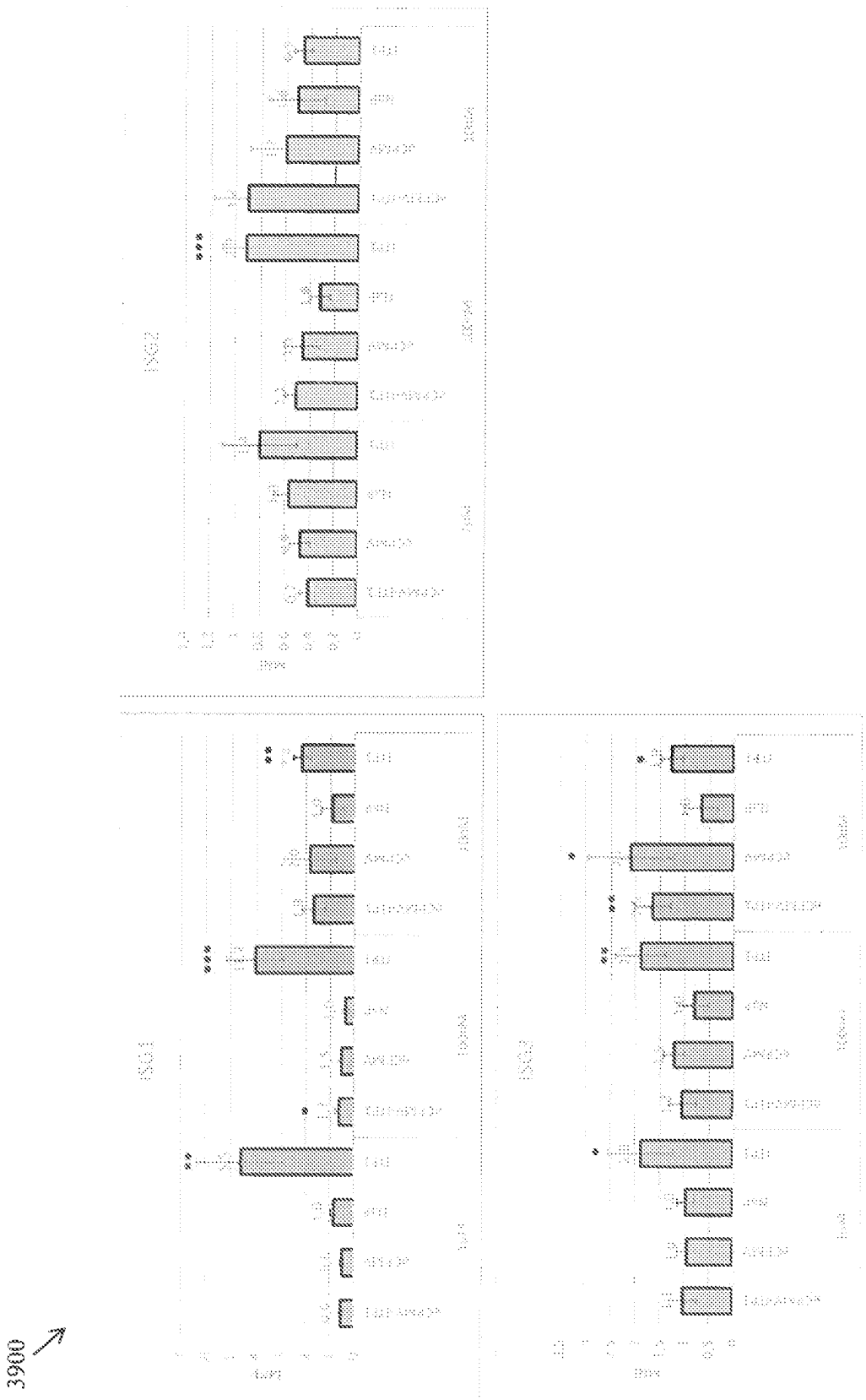
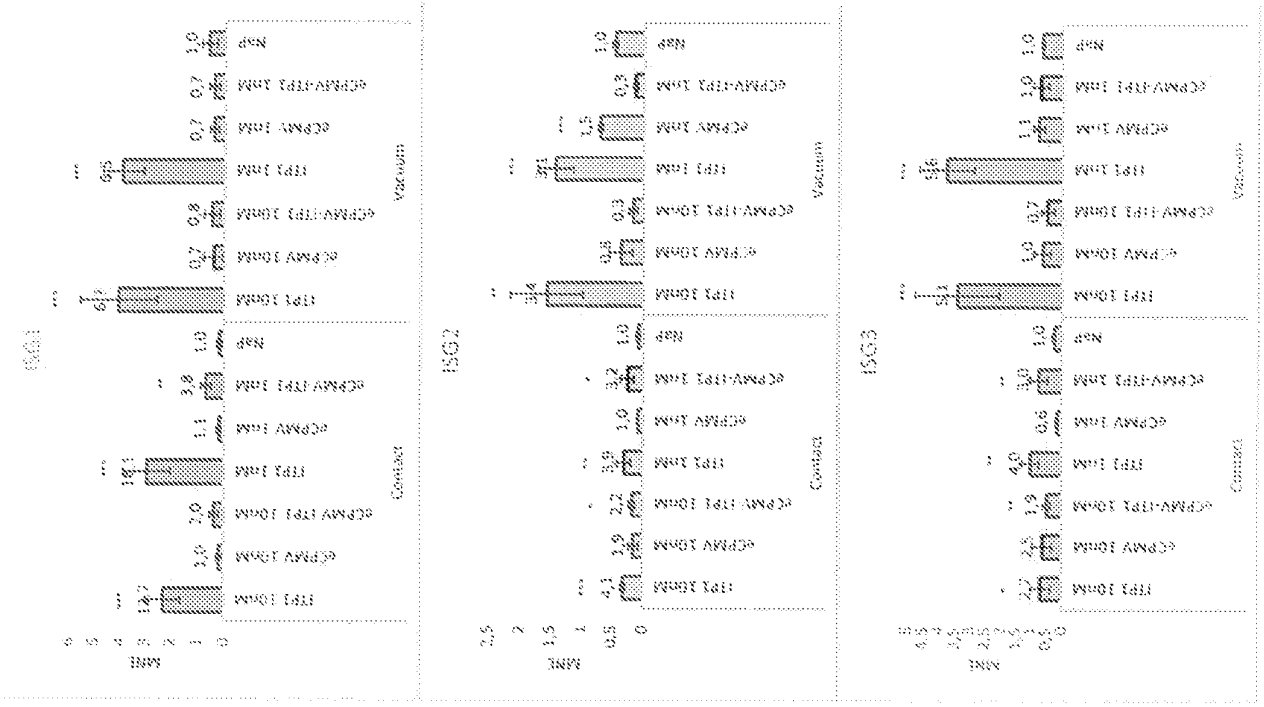


FIG. 39


ISG1
ISG2
ISG3
Contact
Vacuum
ITP1 10nM
eCPMV 10nM
eCPMV-ITP1 10nM
ITP1 1nM
eCPMV 1nM
eCPMV-ITP1 1nM
NaP
nM

4000

FIG. 40

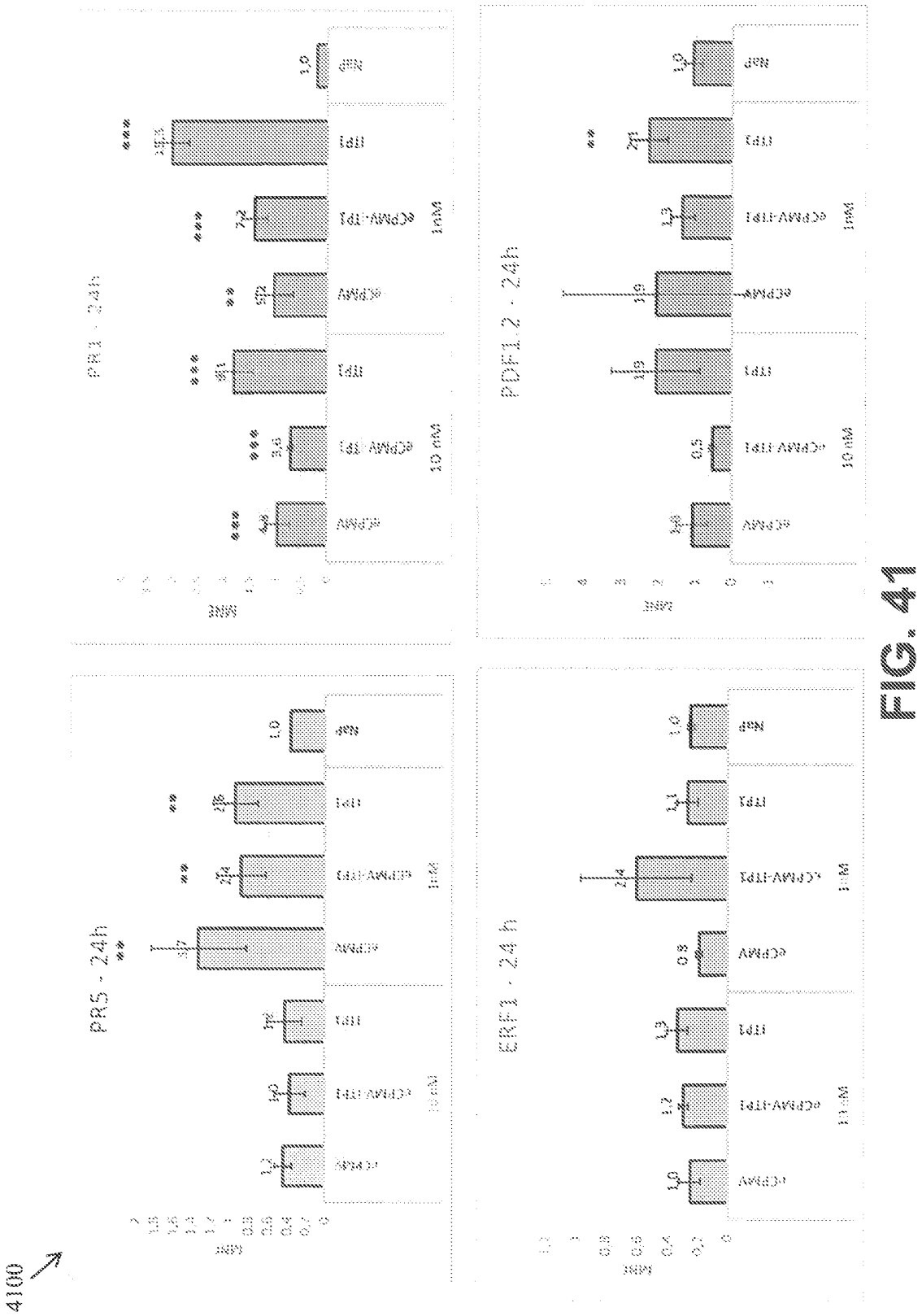

4100
PR1 - 24h
PDF1.2 - 24h
PR5 - 24h
ERF1 - 24h
MeJA
ITP1
cPMV-ITP1
cPMV
NaP
10 mM
1 mM

FIG. 41

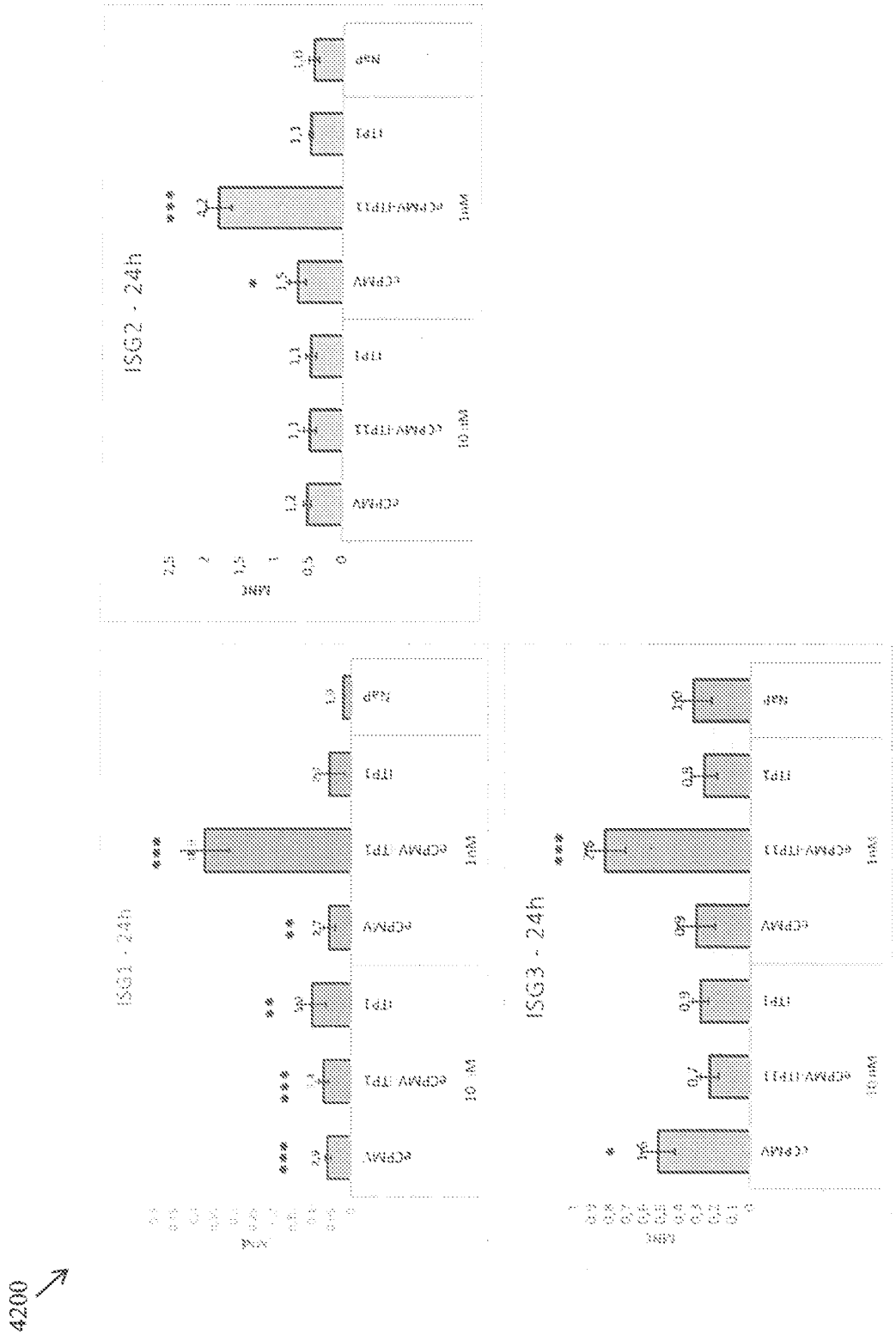

4200
ISG1 - 24h
ISG2 - 24h
ISG3 - 24h
eCPMV
eCPMV-ITP1.1
ITP1
Nap
10 nM
1 nM
MNE

FIG. 42

4300

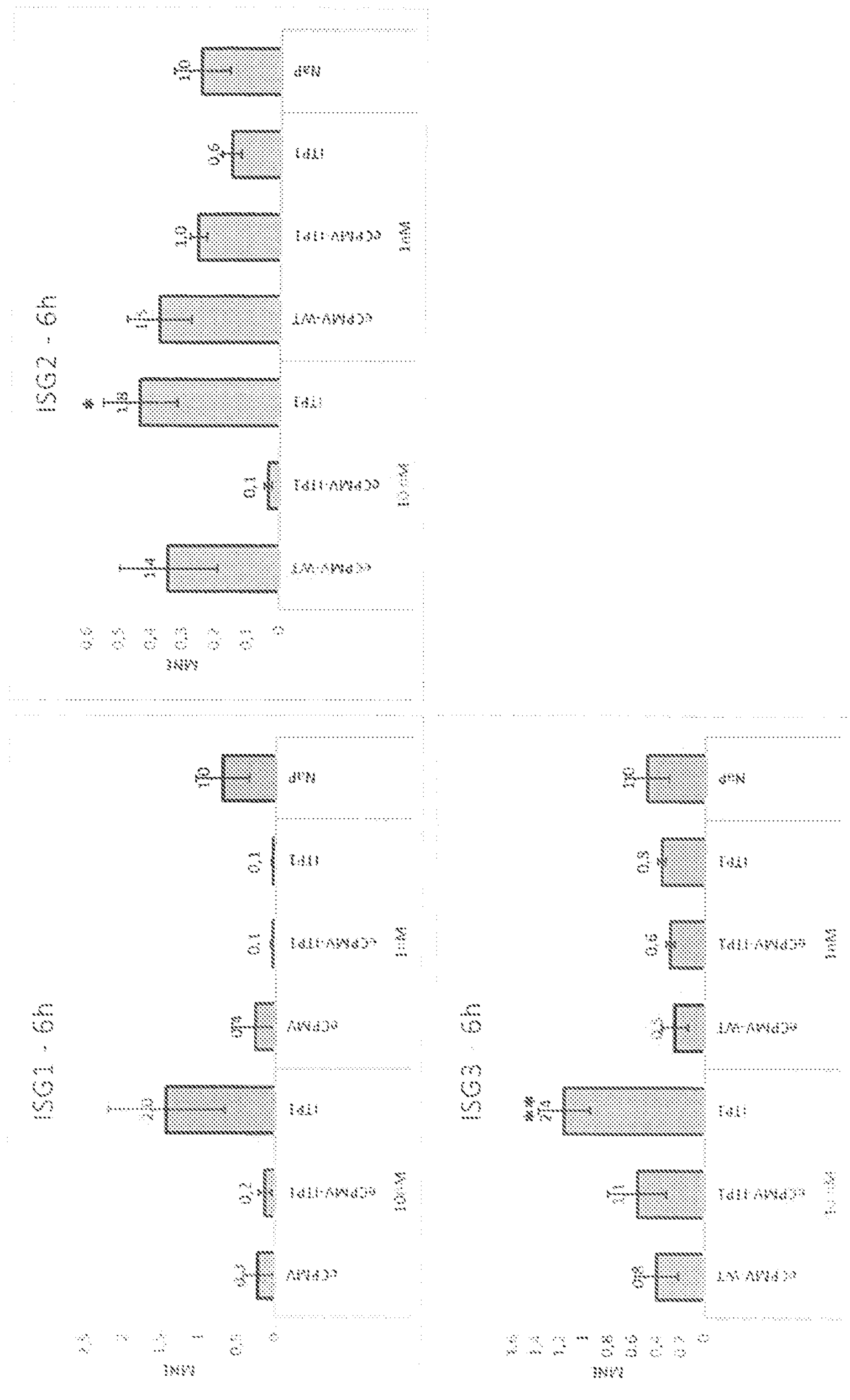


FIG. 43

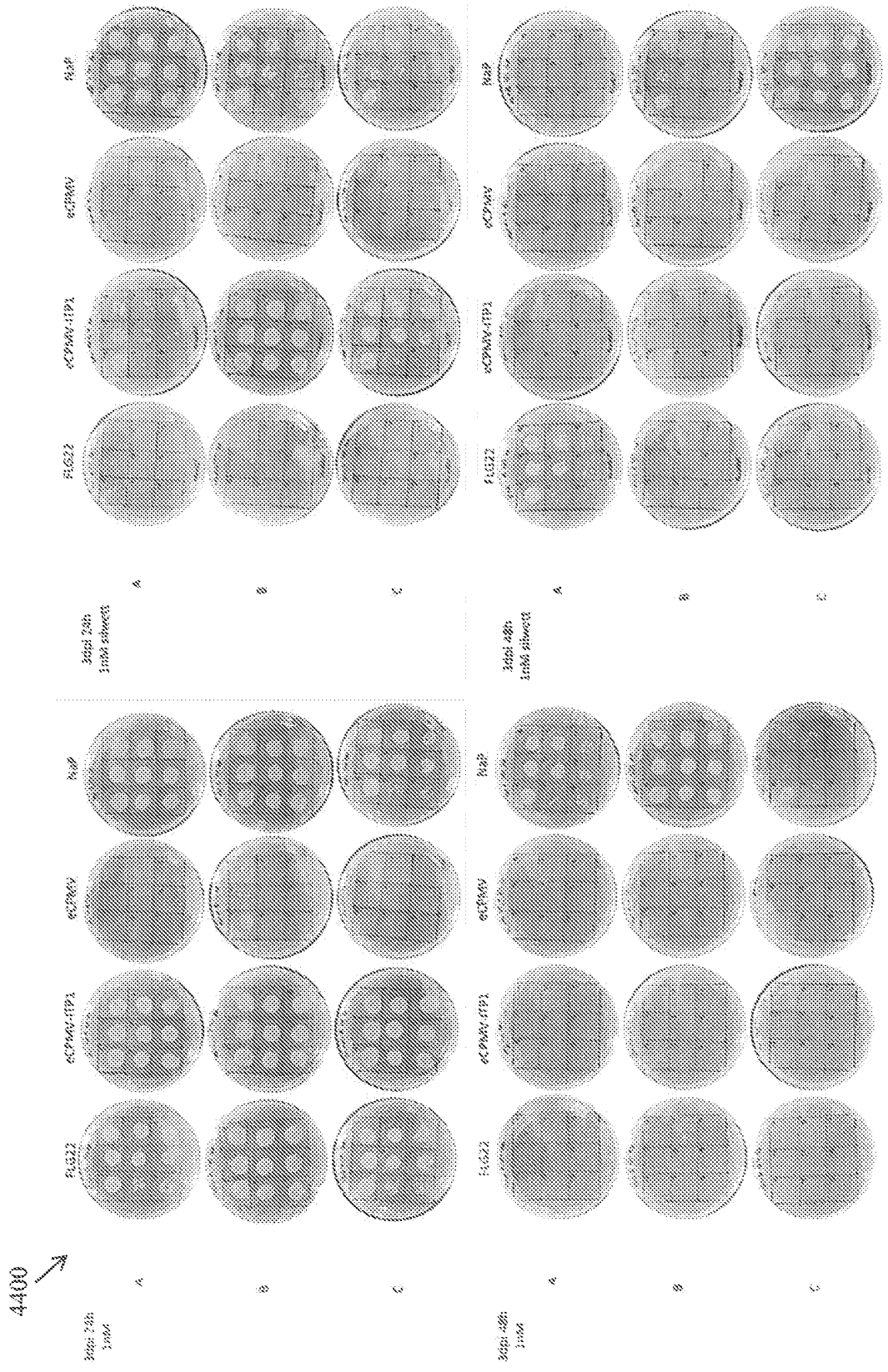

4400
3dpi 24h
1mM
3dpi 48h
1mM
A
B
C
FLG22
eCPMV-ITP1
eCPMV
NaP
3dpi 24h
1mM diweet
3dpi 48h
1mM diweet

FIG. 44

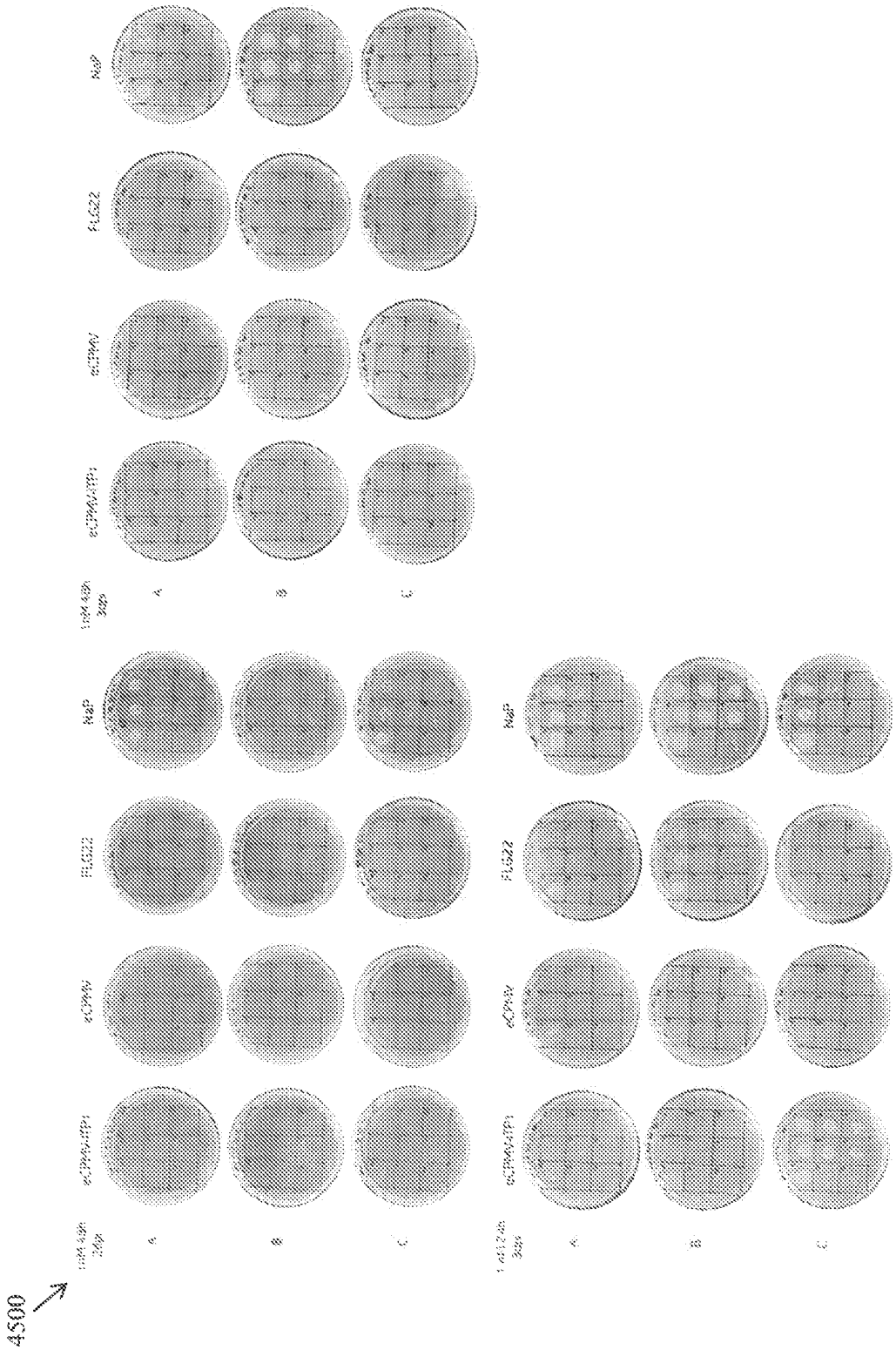


FIG. 45

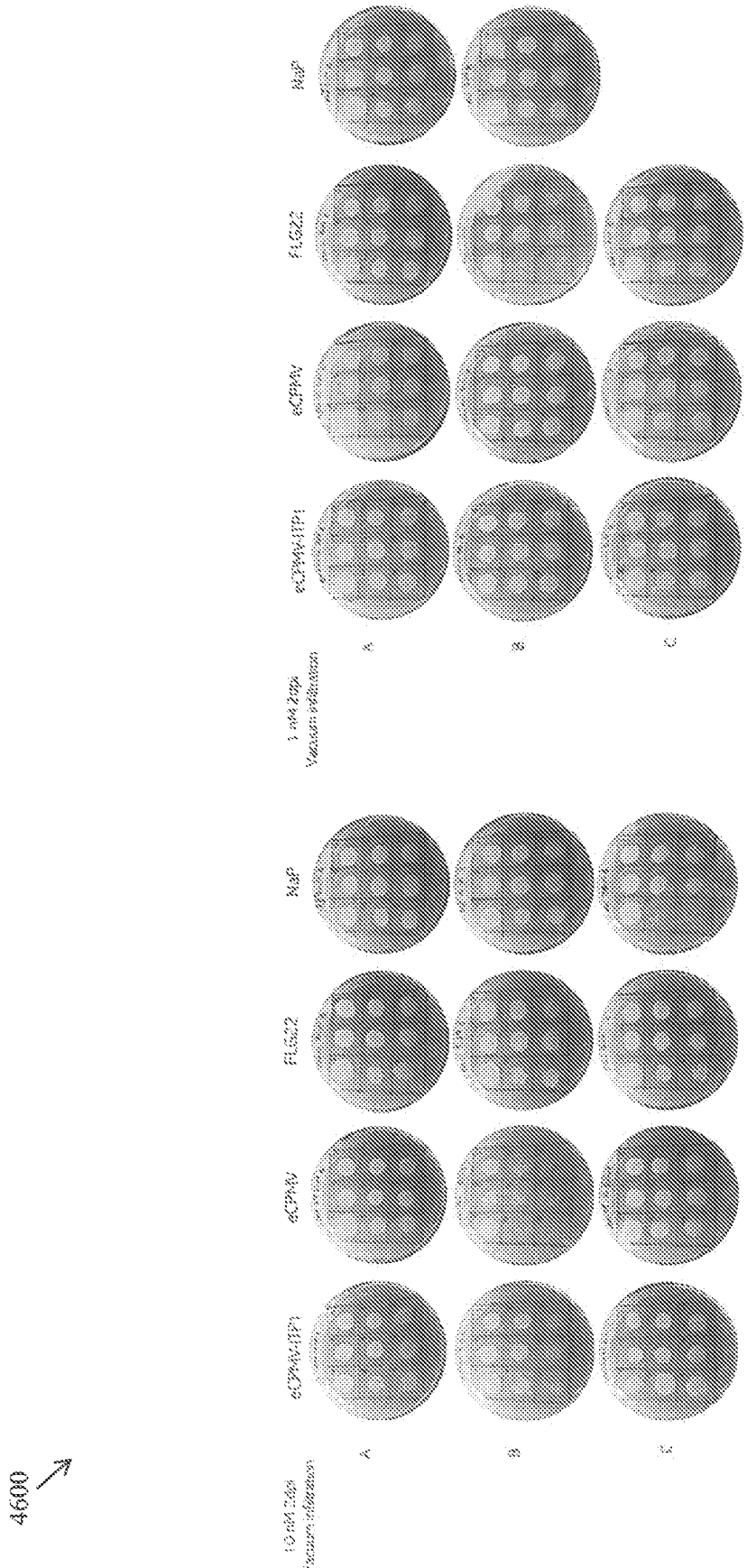
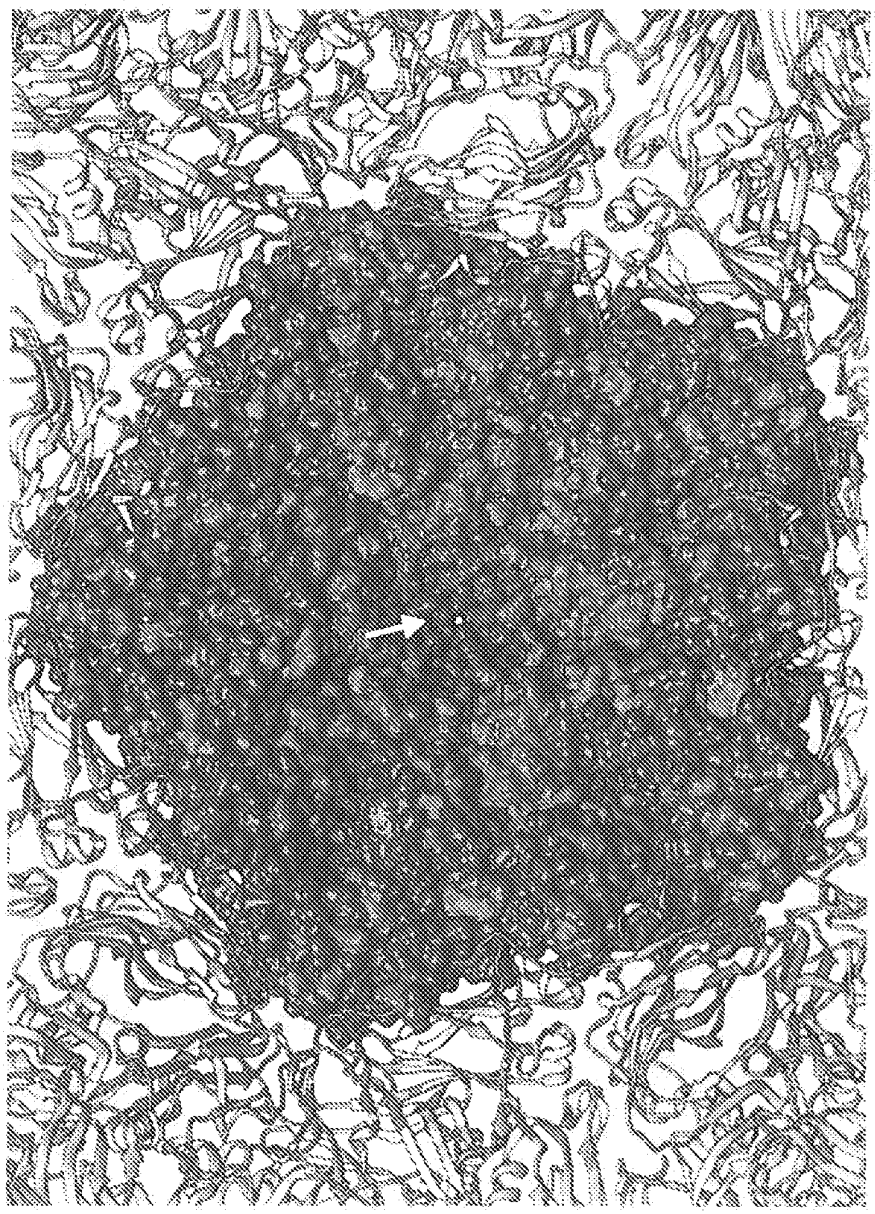
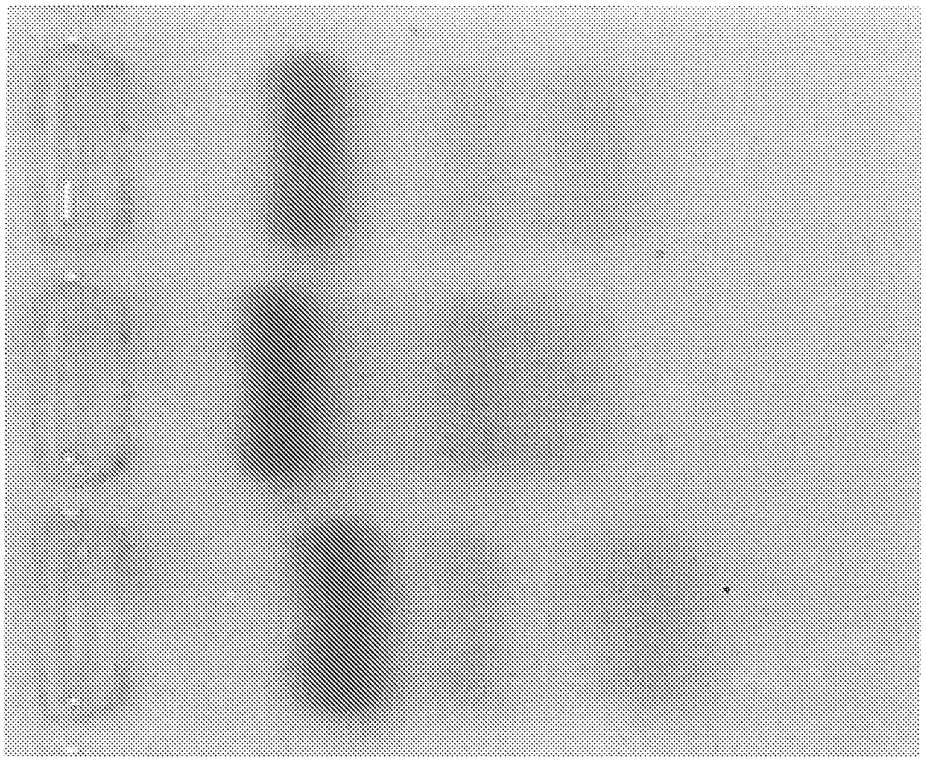

4600
10 mM Zeta
Vacuum filtration
eCPMV-ITP1
eCPMV
FLG22
NaP
A
B
C
1 mM Zeta
Vacuum filtration
eCPMV-ITP1
eCPMV
FLG22
NaP
A
B
C

FIG. 46



4700

FIG. 47



4800

FIG. 48

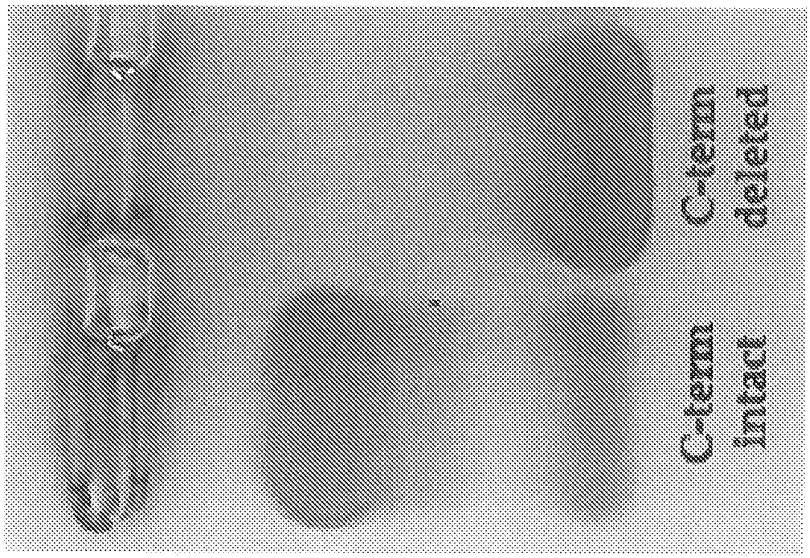


FIG. 49A

4900a

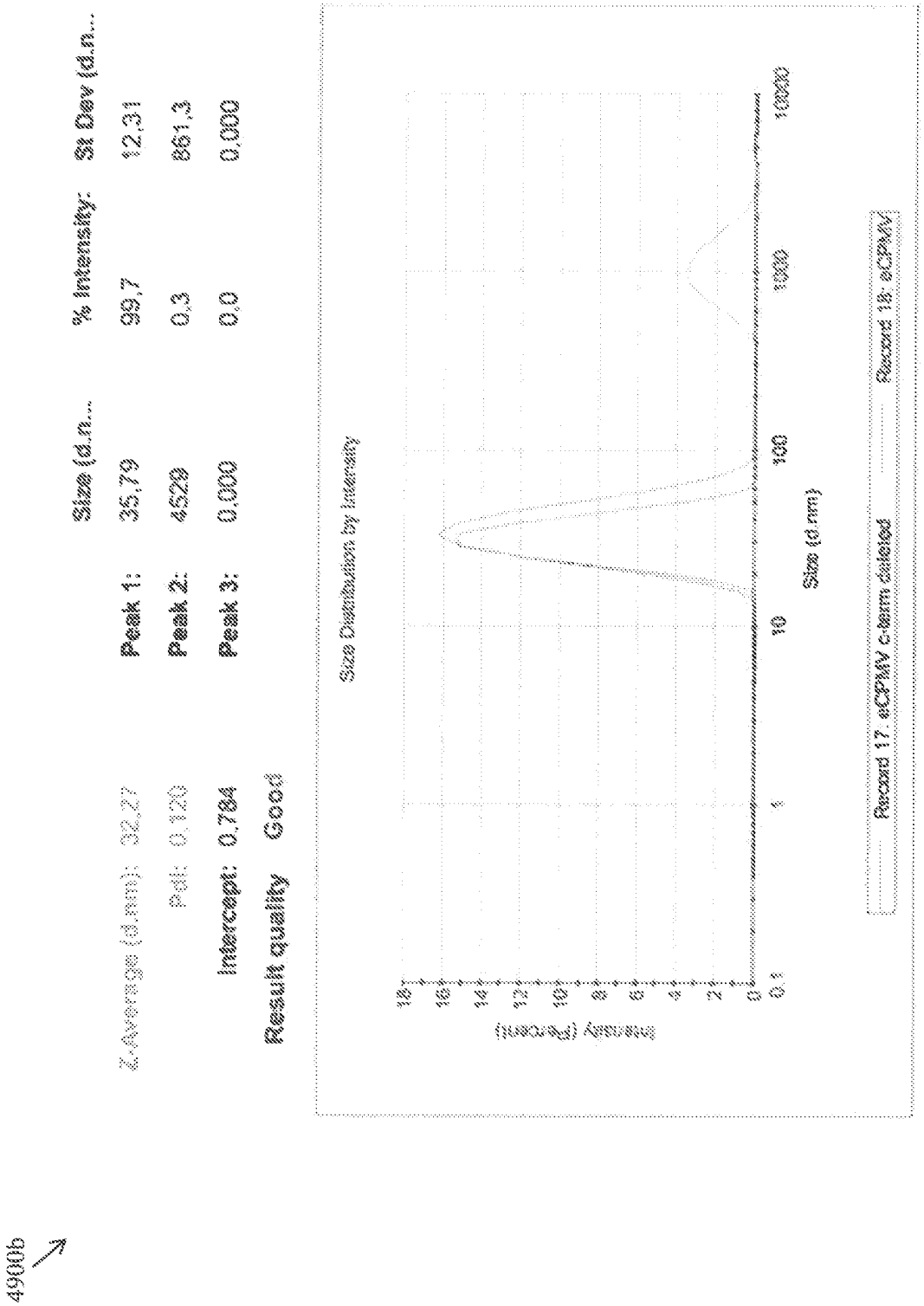

4900b
Size (d.n... % Intensity: St Dev (d.n...
Z-Average (d.nm): 32,27
Peak 1: 35,79 99,7 12,31
PdI: 0,120
Peak 2: 4529 0,3 861,3
Intercept: 0,784
Peak 3: 0,000 0,0 0,000
Result quality Good
Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
Record 17: eCPMV c-term deleted
Record 18: eCPMV

FIG. 49B

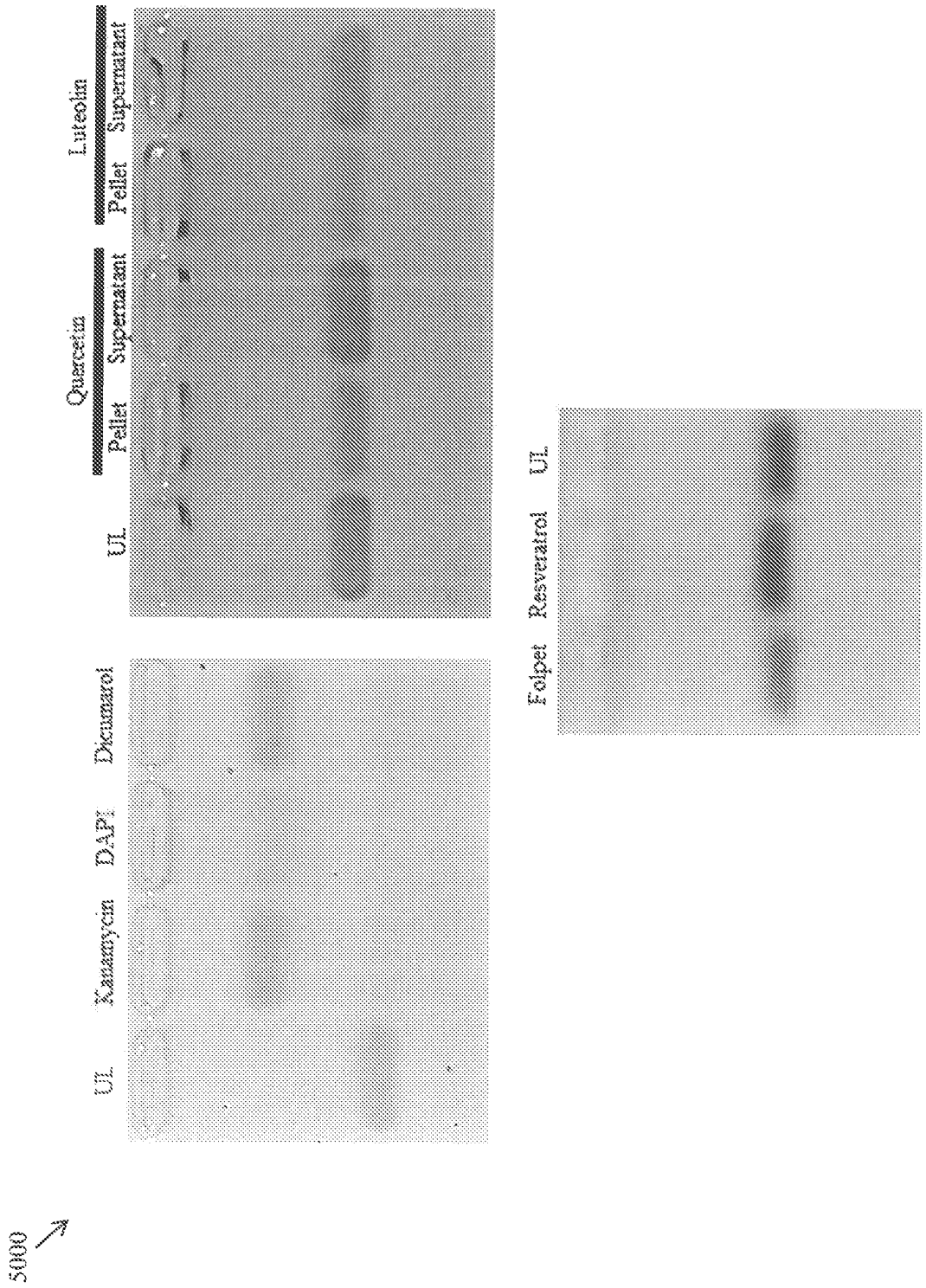

5000
UL
Kanamycin
DAPI
Dicumarol
UL
Quercetin
Pellet
Supernatant
Luteolin
Pellet
Supernatant
Folpet
Resveratrol
UL

FIG. 50

FIG. 51

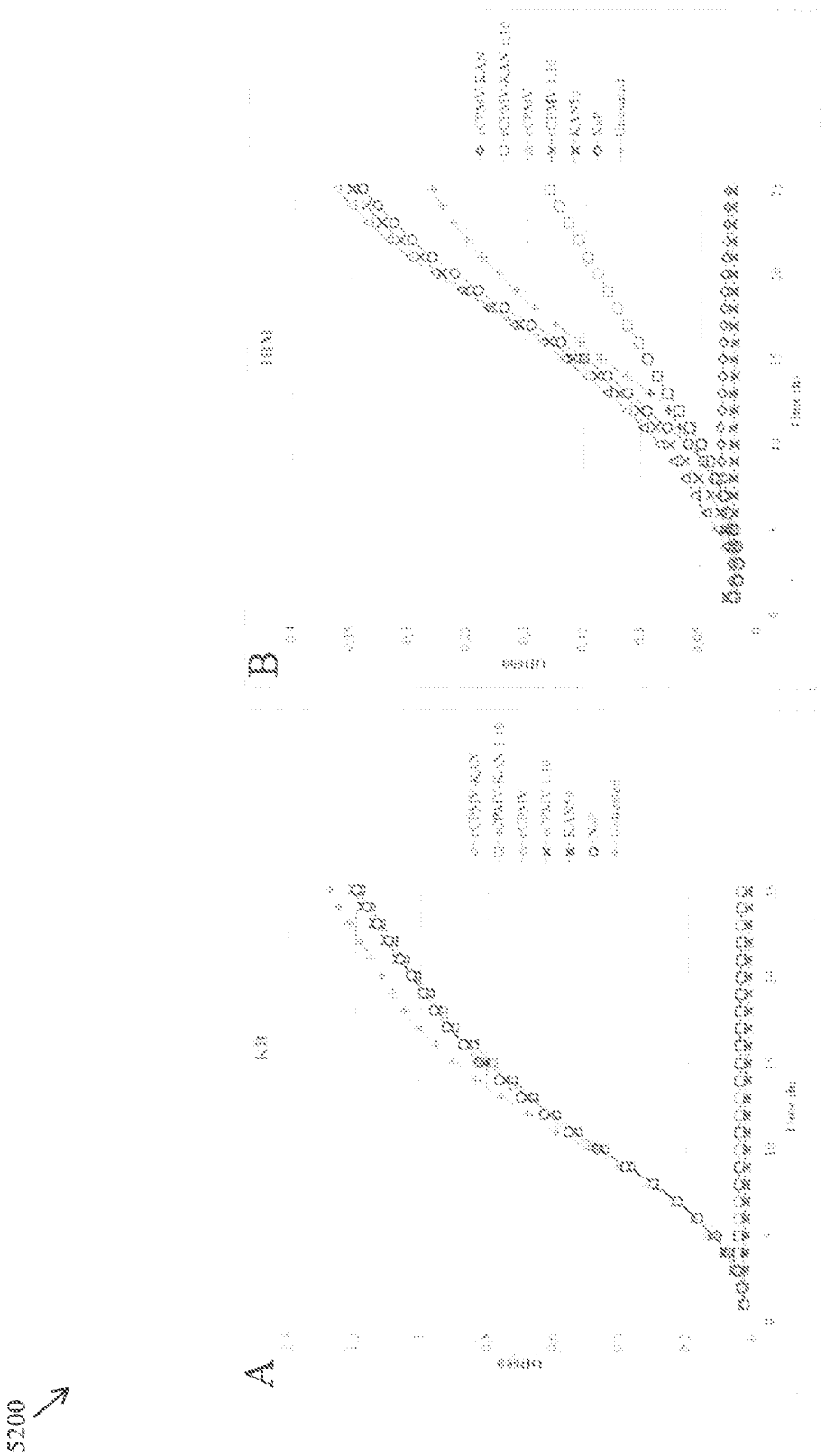


FIG. 52

INTERNATIONAL SEARCH REPORT

International application No
PCT/IT2024/000029

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01N25/28 A01N63/40 A01N65/00
ADD.

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)
A01N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Danzi David: "eCPMV Nanoparticles: the potential of a bio-inspired strategy for plant protection", CATALOGO DEI PRODOTTI DELLA RICERCA, 14 June 2021 (2021-06-14), pages 1-141, XP093272469, University of Verona Retrieved from the Internet: URL:https://iris.univr.it/handle/11562/1044775 [retrieved on 2025-04-25]	1-3,6-8
Y	abstract; tables 1, 3, 4 page 31, paragraph 1 page 35, paragraph 2 pages 79-81 figures 1.3.8, 1.3.15-16, 2.3.1, 2.3.17 page 115, paragraph 4 page 84, lines 1-9; figures 2.3.9, 2.3.13 pages 120, 121, lines 26-30, 1-3; figure	4,5

-/--

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 April 2025	13/05/2025

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Krüger, Julia

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Form PCT/ISA/210 (second sheet) (April 2005)

CORRECTED VERSION

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IT2024/000029

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. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.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:

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	3.3.6 -----	
X	BEATTY PERRIN H ET AL: "Cowpea mosaic virus nanoparticles for cancer imaging and therapy", ADVANCED DRUG DELIVERY REVIEWS, ELSEVIER, AMSTERDAM , NL, vol. 145, 17 April 2019 (2019-04-17), pages 130-144, XP085895278, ISSN: 0169-409X, DOI: 10.1016/J.ADDR.2019.04.005 [retrieved on 2019-04-17] abstract; figures 2-4 page 142, column 1, lines 12-15 -----	6
Y	XUE QI ET AL: "Plant and insect virus-like particles: emerging nanoparticles for agricultural pest management", PEST MANAGEMENT SCIENCE, vol. 79, no. 9, 3 June 2023 (2023-06-03), pages 2975-2991, XP093272829, Hoboken, USA ISSN: 1526-498X, DOI: 10.1002/ps.7514 abstract; figure 1 page 2985, column 1, lines 33-37, 45-49 -----	4,5

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