Influenza Virus-Like Particle Production in Plants

FIELD OF INVENTION

[0001] This invention relates to producing virus like particles in plants.

BACKGROUND OF THE INVENTION

[0002] Influenza is caused by an RNA virus of the orthomyxoviridae family. There are three types of these viruses and they cause three different types of influenza: type A, B and C. Influenza virus type A viruses infect mammals (humans, pigs, ferrets, horses) and birds. This is very important to mankind, as this is the type of virus that has caused worldwide pandemics. Influenza virus type B (also known simply as influenza B) infects only humans. It occasionally causes local outbreaks of flu. Influenza C viruses also infect only humans. They infect most people when they are young and rarely causes serious illness.

[0003] Vaccination provides protection against disease caused by a like agent by inducing a subject to mount a defense prior to infection. Conventionally, this has been accomplished through the use of live attenuated or whole inactivated forms of the infectious agents as immunogens. To avoid the danger of using the whole virus (such as killed or attenuated viruses) as a vaccine, recombinant viral proteins, for example subunits, have been pursued as vaccines. Both peptide and subunit vaccines are subject to a number of potential limitations. Subunit vaccines may exhibit poor immunogenicity, owing to incorrect folding or poor antigen presentation. A major problem is the difficulty of ensuring that the conformation of the engineered proteins mimics that of the antigens in their natural environment. Suitable adjuvants and, in the case of peptides, carrier proteins, must be used to boost the immune response. In addition these vaccines elicit primarily humoral responses, and thus may fail to evoke effective immunity. Subunit vaccines are often ineffective for diseases in which whole inactivated virus can be demonstrated to provide protection.

[0004] Virus-like particles (VLPs) are potential candidates for inclusion in immunogenic compositions. VLPs closely resemble mature virions, but they do not contain viral genomic material. Therefore, VLPs are nonreplicative in nature, which make them safe for administration as a vaccine. In addition, VLPs can be engineered

to express viral glycoproteins on the surface of the VLP, which is their most native physiological configuration. Moreover, since VLPs resemble intact virions and are multivalent particulate structures, VLPs may be more effective in inducing neutralizing antibodies to the glycoprotein than soluble envelope protein antigens.

[0005] VLPs have been produced in plants (WO2009/009876; WO 2009/076778; WO 2010/003225; WO 2010/003235; WO 2011/03522; WO 2010/148511; which are incorporated herein by reference), and in insect and mammalian systems (Noad, R. and Roy, P., 2003, *Trends Microbiol* 11: 438-44; Neumann et al., 2000, J. Virol., 74, 547-551). Latham and Galarza (2001, J. Virol., 75, 6154-6165) reported the formation of influenza VLPs in insect cells infected with recombinant baculovirus co-expressing hemagglutinin (HA), neuramindase (NA), M1, and M2 genes. This study demonstrated that influenza virion proteins self-assemble upon co-expression in eukaryotic cells and that the M1 matrix protein was required for VLP production. Gomez-Puertas et al., (1999, J. Gen. Virol, 80, 1635-1645) showed that overexpression of M2 completely blocked CAT RNA transmission to MDCK cultures.

[0006] The spike glycoprotein hemagglutinin (HA) of influenza viruses is of great importance for the uptake of virus particles by the host cell. It is responsible for their attachment to sialic acid-containing cellular receptors, and it is involved in virus penetration through fusion of the virus envelope with cellular membranes. Fusion activity and consequently virus infectivity depend on cleavage of the HA precursor molecule, HA0, into the disulfide-linked polypeptide chains, HA1 and HA2. Cleavage a subsequent pH-dependent conformational change result in the exposure and relocation of a highly conserved hydrophobic peptide at the amino terminus of the transmembrane polypeptide HA2, which mediates membrane fusion.

[0007] HA is synthesised as a precursor protein HA0, which undergoes proteolytic processing into two subunits (HA1 and HA2) linked together by a disulfide bridge. Two structural features are thought to be involved in HA cleavability: in HAs of restricted cleavability, the linker usually consists of a single arginine, whereas HAs cleavable in a broad range of different cell types have an insertion of a series of multiple basic residues in this position with the main enzyme recognition motif Arg-

X-Lys/Arg-Arg, whereby X is a nonbasic amino acid. HAs with a multiple basic cleavage site are cleaved on the exocytic transport route before they reach the budding site on the cell surface, in contrast to HAs with a monobasic linker that are activated on virus particles either in the extracellular space or, as shown for the WSN strain, at the stage of virus entry. A second determinant of HA cleavage appears to be a carbohydrate side chain that is present in close vicinity of the cleavage site and interferes with protease accessibility. Loss of this carbohydrate resulted in enhanced HA cleavability and viral pathogenicity.

[0008] Mammalian and apathogenic avian influenza virus strains cause anatomically localized infections as a result of the restricted range of cells secreting a protease that can cleave the HA0 precursor extracellularly (Chen J, et. al. 1998, Cell. Vol 95 :409-417). The proteases responsible for cleavage of HA0 in influenza infections of humans, are secreted by cells of the respiratory tract, or by coinfecting bacteria or mycoplasma, or they may be produced in inflammatory responses to infections. A major protease candidate is the tryptase Clara, which is produced by Clara cells of the bronchiolar epithelium, and has limited tissue distribution (upper respiratory tract). The protease is specific for the monobasic sequence Q/E-X-R found at the cleavage site of the H1, H2, H3, and H6. HA from H9 and B strains show a slightly different monobasic cleavage site with SSR and KER sequence respectively. No protease has been identified for the majority of influenza viruses that cause enteric and respiratory infection seen in aquatic birds. Most cell lines do not support multi-cycle replication unless exogenous protease (usually trypsin) is added.

[0009] In highly pathogenic avian strains, however, HA0 are cleaved by a family of more widespread intracellular proteases, resulting in systemic flu infections. This difference in pathogenicity correlates with structural differences at the HA0 cleavage site. Pathogenic strains have inserts of polybasic amino acids within, or next to, the monobasic site. Cleavage in this case occurs intracellularly and the proteases involved have been identified as furin, and other subtilisin-like enzymes, found in the Golgi and involved in the post-translational processing of hormone and growth factor precursors. The furin recognition sequence R-X-R/K-R is a frequent insertion amino acid at the HA0 cleavage sites of H5 and H7. The wide tissue distribution of the

enzyme, and the efficiency of intracellular cleavage, contribute to the wide-spread and virulent systemic infection caused by these viruses.

[0010] The HA cleavage site is a target for virus attenuation, since activation cleavage of the HA0 precursor into the HA1 and HA2 fragments by host proteases is a step in the replication cycle of all influenza A and B virus strains. Only the cleaved HA can undergo a conformational change in the acidic milieu of the endosome after receptor-mediated endocytosis to expose the hydrophobic N terminus of the HA2 fragment for mediating fusion between endosomal and virion membranes.

[0011] Horimoto T, et.al. (2006, Vaccine, Vol 24 : 3669-3676) describes the abolition of the polybasic cleavage site of H5 (RERRRKKR↓G) in H5. Selected mutants were submitted to immunogenicity study in mice, including a mutant with a deletion of the 4 first charged amino acids (RERR) and a modification to inactivate the polybasic cleavage site (RKKR with TETR). Abolition of the cleavage site did not affect the immunogenic properties of the mutant H5. Abolition the polybasic site (GERRRKKR↓G replaced by RETR) to produce mutant NIBSC 05/240 NIBSC influenza reference virus NIBG-23, has also been reported. Hoffman et. al. (2002, 2002, Vaccine, Vol 20 :3165-3170) replaced the polybasic cleavage site of a H5 HA with the monobasic site of H6 in order to boost the expression in eggs. The first 4 residues were deleted and replaced the four last amino acids of the polybasic site by IETR (replacement of RERRRKKR↓G with IETR↓G). This mutant H5 showed a high expression level, potential proteolysis and conformational change at low pH required for viral replication and production in the host cell, immunogenicity data were not reported. These studies show that modification of the cleavage site can be employed to diminishes the virulence of the viral particle (in cases where the true viruses is replicated), allowing the virus to replicate without killing the host egg. Without such mutations, viruses kill the egg before reaching high titers.

[0012] WO2013043067 by Sirko et al. describe a DNA vaccine for chicken which contains the cDNA encoding a modified H5 haemagglutinin (HA) protein wherein the proteolytic cleavage site between HA subunits is deleted. Sirko et al. state that this provides for greater safety of the vaccines and the expression of a "super antigen" in the form of a long, non-processed polypeptide. Sirko et al. further state that the

encoding region of the HA is modified in such a way that protein production in bird cells achieves maximal yield. The main modification is codon optimisation for chicken and deletion of the proteolysis sensitive region of HA.

[0013] WO 2013/044390 describes a method of producing a virus like particle (VLP) in a plant with modified hemagglutinin (HA) wherein the modified HA protein comprises a modified proteolytic loop. The modified HA is expressed in the presence of the regulatory region Cowpea mosaic virus (CPMV) HT and the geminivirus amplification element from Bean Yellow Dwarf Virus (BeYDV).

[0014] US 2008/0069821 by Yang et al. discloses polypeptides and polynucleotides variants of influenza HA for use in the production of influenza viruses as vaccines. Reassortant influenza viruses are obtained by introducing a subset of vectors corresponding to genomic segments of a master influenza virus, in combination with complementary segments derived from the variants of influenza HA. Typically, the master strains are selected on the basis of desirable properties relevant to vaccine administration. For example, for vaccine production, e.g., for production of a live attenuated vaccine, the master donor virus strain may be selected for an attenuated phenotype, cold adaptation and/or temperature sensitivity.

SUMMARY OF THE DISCLOSURE

[0015] This disclosure relates to producing virus like particles and modified HA proteins in plants.

[0016] It is an object of the disclosure to provide an improved production of virus like particles and HA proteins in plants.

[0016a]In one aspect, there is provided a nucleic acid comprising a regulatory region active in a plant and an expression enhancer active in a plant, the regulatory region and the expression enhancer operatively linked to a nucleotide sequence encoding a mutant influenza hemagglutinin (HA) comprising a fully deleted proteolytic loop, wherein the nucleic acid does not comprise a Bean Yellow Dwarf Virus long intergenic region (BeYDV LIR), and a BeYDV short intergenic region (BeYDV SIR).

[0016b] In one aspect, there is provided a method of producing influenza virus like particle (VLP) in a plant comprising,

a) introducing the nucleic acid as disclosed herein into the plant or portion of the plant; wherein the portion of the plant includes plant cells, tissue, plant leaves, stems, fruit, roots or a combination thereof;

b) incubating the plant or portion of the plant under conditions that permit the expression of the nucleic acids, thereby producing the VLP.

[0016c] In one aspect, there is provided a method of producing influenza virus like particles (VLPs) in a plant comprising:

a) providing a plant, or a portion of a plant, comprising the nucleic acid as disclosed herein, wherein the portion of the plant includes plant cells, tissue, plant leaves, stems, fruit, roots or a combination thereof and

b) incubating the plant or portion of the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLPs.

[0016d] In one aspect, there is provided a VLP produced by the method as disclosed herein, wherein the VLP comprises HA comprising the fully deleted proteolytic loop.

[0016e] In one aspect, there is provided a VLP comprising lipid derived from a plant and mutant HA, wherein the mutant HA comprise a fully deleted proteolytic loop and plant-specific N-glycans or modified N-glycans wherein the mutant HA is derived from an influenza type C, type A, subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, strain B Massachusetts/2/2012, B/Malaysia/2506/2004 or B/Florida/4/2006 HA.

[0016f] In one aspect, there is provided a method of producing a mutant HA protein comprising a fully deleted proteolytic loop in a plant or portion of a plant, wherein the portion of the plant includes plant cells, tissue, plant leaves, stems, fruit, roots or a combination thereof, the method comprising,

a) introducing the nucleic acid as disclosed herein into the plant or portion of the plant;

b) incubating the plant or portion of the plant under conditions that permit the expression of the HA protein, thereby producing the mutant HA protein,

c) harvesting the plant and purifying the mutant HA protein.

[0016g] In one aspect, there is provided an HA protein produced by the method as disclosed herein, wherein the HA comprises a fully deleted proteolytic loop and has hemagglutinin (HA) activity.

[0016h] In one aspect, there is provided an HA protein comprising a fully deleted proteolytic loop, wherein the HA is derived from an influenza type C, type A, subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, strain B Massachusetts/2/2012, B/Malaysia/2506/2004 or B/Florida/4/2006 HA.

[0016i] In one aspect, there is provided an HA encoded by the nucleic acid as disclosed herein.

[0016j] In one aspect, there is provided a plant comprising the nucleic acid as disclosed herein.

[0016k] In one aspect, there is provided a composition comprising an effective dose of the VLP as disclosed herein and a pharmaceutically acceptable carrier for use as a medicament for inducing an immune response.

[0016l] In one aspect, there is provided a vaccine comprising an effective dose of the VLP as disclosed herein for use in inducing an immune response.

[0016m] In one aspect, there is provided the use of the VLP as disclosed herein in the manufacture of a medicament for inducing immunity to an influenza virus infection in a subject.

[0016n] In one aspect, there is provided a method of increasing product yield of an HA from B or H3 influenza strain protein expressed in a plant or portion of a plant wherein the portion of the plant includes plant cells, tissue, plant leaves, stems, fruit, roots or a combination thereof, the method comprising,

a) introducing the nucleic acid as disclosed herein into the plant;

b) incubating the plant or portion of the plant under conditions that permit the expression of the HA protein, thereby producing a mutant HA protein,

c) harvesting the plant and purifying the mutant HA protein, wherein the mutant HA protein has increased product quality, when compared to a native HA.

[0016o] In one aspect, there is provided a nucleic acid comprising a regulatory region active in a plant and an expression enhancer active in a plant, the regulatory region and the expression enhancer operatively linked to a nucleotide sequence encoding a mutant influenza hemagglutinin (HA) from B or H3 influenza strain, the mutant HA comprising a partially or fully deleted proteolytic loop, wherein the nucleic acid does not comprise a Bean Yellow Dwarf Virus long intergenic region (BeYDV LIR), and a BeYDV short intergenic region (BeYDV SIR).

[0017] Described herein is a nucleic acid comprising an expression enhancer active in a plant and operatively linked to a nucleotide sequence encoding a modified influenza hemagglutinin (HA) comprising a modified proteolytic loop.

[0018] Furthermore described herein is a method (A) of producing a virus like particle (VLP) in a plant comprising,

a) introducing a nucleic acid comprising an expression enhancer active in a plant and operatively linked to a nucleotide sequence encoding a modified influenza

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hemagglutinin (HA) comprising a modified proteolytic loop into the plant, or portion of the plant;

b) incubating the plant or portion of the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLP.

[0019] In addition, described herein is a method (B) for producing influenza virus like particles (VLPs) in a plant comprising:

a) providing a plant, or a portion of a plant, comprising a nucleic acid comprising an expression enhancer active in a plant and operatively linked to a nucleotide sequence encoding a modified influenza hemagglutinin (HA) comprising a modified proteolytic loop, and

b) incubating the plant or portion of the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLPs

[0020] Furthermore described herein is a method (C) of producing a modified HA protein comprising a modified proteolytic loop comprising one or more protease cleavage sites exhibiting reduced or abolished cleavage in a plant comprising,

a) introducing a nucleic acid comprising an expression enhancer active in a plant and operatively linked to a nucleotide sequence encoding a modified influenza hemagglutinin (HA) comprising a modified proteolytic loop into the plant, or portion of the plant;

b) incubating the plant or portion of the plant under conditions that permit the expression of the HA protein, thereby producing the modified HA protein,

c) harvesting the plant and purifying the modified HA protein.

[0021] The methods (A), (B) or (C) as described above may further comprising the steps of

c) harvesting the plant, and

d) purifying the VLPs, wherein the VLPs range in size from 80-300 nm.

[0022] Furthermore, described herein is a method (D) of increasing the product yield of a HA protein in a plant, comprising ,

a) introducing a nucleic acid comprising an expression enhancer active in a plant and operatively linked to a nucleotide sequence encoding a modified influenza hemagglutinin (HA) comprising a modified proteolytic loop into the plant, or portion of the plant;

b) incubating the plant or portion of the plant under conditions that permit the expression of the HA protein, thereby producing the modified HA protein,

c) harvesting the plant and purifying the HA protein.

[0023] The expression enhancer may be CPMVX, CPMVX+ or CPMV-HT+. Furthermore, the nucleotide acid may not comprise a geminivirus amplification element. The nucleotide acid therefore may not comprise a Bean Yellow Dwarf Virus long intergenic region (BeYDV LIR), and a BeYDV short intergenic region (BeYDV SIR). The modified proteolytic loop may comprises one or more protease cleavage sites exhibiting reduced or abolished cleavage by a protease. The protease may be Clara-like or Furin-like. Furthermore the modified proteolytic loop may comprises a linker sequence and the linker sequence may have the amino acid sequence GG, TETQ or TETR. The modified HA may comprise a native or a non-native signal peptide. Furthermore the the nucleotide sequence encoding the modified HA may comprise a chimeric nucleotide sequence encoding, in series, a modified HA ectodomain comprising a modified proteolytic loop, an influenza transmembrane domain, and a cytoplasmic tail, wherein the modified HA ectodomain is from a first influenza strain and the transmembrane domain and the cytoplasmic tail are from a second influenza strain.

[0024] The modified proteolytic loop may comprise one or more protease cleavage sites exhibiting reduced or abolished cleavage by a protease. Furthermore, the nucleotide sequence encoding the HA is selected from the group consisting of B HA, C, H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16. Also described herein is a virus like particle (VLP) produced by the method (A). The

virus like particle (VLP) may comprise plant-specific N-glycans, or modified N-glycans.

[0025] The present disclosure also provides a composition comprising an effective dose of the VLP for inducing an immune response, and a pharmaceutically acceptable carrier.

[0026] Also described herein is a nucleic acid comprising a nucleotide sequence encoding an influenza hemagglutinin (HA), the nucleotide sequence operatively linked with a regulatory region that is active in a plant, wherein the HA comprises a modified proteolytic loop sequence. The nucleic acid may encode an HA comprising a modified proteolytic loop, where in the protein has hemagglutinin (HA) activity. A plant comprising the nucleic acid is also provided. Also included is a virus like particle (VLP) produced in a plant, the VLP comprising an influenza virus hemagglutinin (HA) encoded by the nucleic acid and one or more than one lipid derived from a plant.

[0027] This summary of the invention does not necessarily describe all features of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

[0029] **Figure 1** shows components used to prepare A-2X35S/CPMV-HT/H5 Indonesia/NOS (Construct number 489). **Figure 1A** shows primer IF-H5A-I-05.s1+3c (SEQ ID NO: 2). **Figure 1B** shows primer IF-H5dTm.r (SEQ ID NO: 3). **Figure 1C** shows a schematic representation of construct 1191. **Figure 1D** shows Construct 1191 (SEQ ID NO 4). **Figure 1E** shows expression cassette number 489 (SEQ ID NO 5). **Figure 1F** shows amino acid sequence of H5 from influenza A/Indonesia/5/2005 (H5N1) (SEQ ID NO: 6). **Figure 1G** shows a nucleotide sequence encoding H5 from influenza A/Indonesia/5/2005 (H5N1) (SEQ ID NO: 42).

[0030] **Figure 2** shows components used to prepare B-2X35S/CPMV HT/M2 New Caledonia/NOS (Construct number 1261). **Figure 2A** shows primer IF-S1-

M1+M2ANC.c (SEQ ID NO:7). **Figure 2B** shows primer IF-S1-4-M2ANC.r (SEQ ID NO: 8). **Figure 2C** shows the nucleotide sequence for the synthesized M2 gene (corresponding to nt 1-26 joined to 715-982 from Genbank accession number DQ508860) (SEQ ID NO: 9). **Figure 2D** shows the expression cassette number 1261 from 2X35S promoter to NOS terminator. M2 from influenza A/New Caledonia/20/1999 (H1N1) is underlined. (SEQ ID NO: 10). **Figure 2E** shows the amino acid sequence of M2 from influenza A/New Caledonia/20/1999 (H1N1) (SEQ ID NO: 11).

[0031] **Figure 3** shows components used to prepare C-2X35S/CPMV-HT/M2 Puerto Rico/NOS (Construct number 859). **Figure 3A** shows the nucleotide sequence of the synthesized M2 gene (corresponding to nt 26-51 joined to nt 740-1007 from Genebank accession number EF467824) (SEQ ID NO: 12). **Figure 3B** shows the expression cassette number 859 from 2X35S promoter to NOS terminator. M2 from Influenza A/Puerto Rico/8/1934 (H1N1) is underlined. (SEQ ID NO: 13). **Figure 3C** shows the amino acid sequence of M2 from influenza A/Puerto Rico/8/1934 (H1N1) (SEQ ID NO:14).

[0032] **Figure 4** shows components used to prepare G-2X35S/CPMV-HT/PDISP/HA B Brisbane/NOS into BeYDV+Replicase amplification system (Construct number 1008). **Figure 4A** shows a schematic representation of construct 1194. SacII and StuI restriction enzyme sites used for plasmid linearization are annotated on the representation. **Figure 4B** shows construct 1194 from left to right t-DNA borders (underlined). 2X35S/CPMV-HT/PDISP/NOS into BeYDV+Replicase amplification system with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette (SEQ ID NO: 31). **Figure 4C** shows expression cassette number 1008 from BeYDV left LIR to BeYDV right LIR. PDISP/HA from influenza B/Brisbane/60/2008 is underlined. (SEQ ID NO: 32).

[0033] **Figure 5** shows components used to prepare I-2X35S/CPMV-HT/PDISP/HA B Brisbane with deleted proteolytic loop/NOS into BeYDV+Replicase amplification system (Construct number 1059). **Figure 5A** shows primer 1039+1059.r (SEQ ID NO: 38). **Figure 5B** shows primer 1039+1059.c (SEQ ID NO: 39). **Figure 5C** shows expression cassette number 1059 from BeYDV left LIR to BeYDV right LIR.

PDISP/HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop is underlined (SEQ ID NO: 40). **Figure 5D** shows amino acid sequence of PDISP/HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop (SEQ ID NO: 41). **Figure 5E** shows nucleotide sequence of PDISP/HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop (SEQ ID NO: 43).

[0034] **Figure 6** shows components used to prepare B-2X35S/CPMV-HT/HA B Wisconsin/NOS into BeYDV(m)+Replicase amplification system (Construct number 1462). **Figure 6A** shows primer IF-HAB110.S1+3c (SEQ ID NO: 49). **Figure 6B** shows primer IF-HAB110.s1-4r (SEQ ID NO: 50). **Figure 6C** shows the nucleotide sequence of synthesized HA B Wisconsin (Genbank accession number JN993010) (SEQ ID NO: 51). **Figure 6D** shows a schematic representation of construct 193. **Figure 6E** shows construct 193 from left to right t-DNA borders (underlined). 2X35S/CPMV-HT/NOS into BeYDV(m)+Replicase amplification system with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette (SEQ ID NO: 52). **Figure 6F** shows the nucleotide sequence of expression cassette number 1462 from 2X35S promoter to NOS terminator. HA from influenza B/Wisconsin/1/2010 is underlined (SEQ ID NO: 53). **Figure 6G** shows the amino acid sequence of HA from influenza B/Wisconsin/1/2010 (SEQ ID NO: 54). **Figure 6H** shows a schematic representation of construct 1462.

[0035] **Figure 7** shows components used to prepare C-2X35S/CPMV-HT/HA B Wisconsin with deleted proteolytic loop/NOS into BeYDV(m)+Replicase amplification system (Construct number 1467). **Figure 7A** shows primer HAB110(PrL-).r (SEQ ID NO: 55). **Figure 7B** shows primer HAB110(PrL-).c (SEQ ID NO: 56). **Figure 7C** shows the nucleotide sequence of expression cassette number 1467 from 2X35S promoter to NOS terminator. HA from influenza B/Wisconsin/1/2010 with deleted proteolytic loop is underlined (SEQ ID NO: 57). **Figure 7D** shows the amino acid sequence of influenza B/Wisconsin/1/2010 with deleted proteolytic loop (SEQ ID NO: 58). **Figure 7E** shows a schematic representation of construct 1467.

[0036] **Figure 8** shows components used to prepare A-2X35S/CPMV-HT/PDISP/HA B Brisbane with deleted proteolytic loop/NOS (Construct number 1039). **Figure 8A**

shows the nucleotide sequence of expression cassette number 1039 from 2X35S promoter to NOS terminator. HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop is underlined (SEQ ID NO: 15). **Figure 8B** shows a schematic representation of construct 1039.

[0037] **Figure 9** shows the plasmid map of construct number 1008. Construct number 1008 directs the expression of wild-type HA from influenza strain B/Brisbane/60/2008. This construct comprises BeYDV-derived elements for DNA amplification.

[0038] **Figure 10** shows the plasmid map of construct number 1059. Construct number 1059 directs the expression of a mutant HA from influenza strain B/Brisbane/60/2008 with deleted proteolytic loop. This construct comprises BeYDV-derived elements for DNA amplification.

[0039] **Figure 11** shows the plasmid map of construct number 1261. Construct number 1261 directs the expression of wild-type M2 from influenza strain A/New Caledonia/20/99 (H1N1).

[0040] **Figure 12** shows the plasmid map of construct number 859. Construct number 859 directs the expression of wild-type M2 from influenza strain A/Puerto Rico/8/34 (H1N1).

[0041] **Figure 13A** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. "1008": expression of wild-type HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV); "1008+1261": co-expression of wild-type HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV) with M2 from A/New Caledonia/20/99; "1059": expression of mutant HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV); "1059+1261": co-expression of mutant HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV) with M2 from A/New Caledonia/20/99. Plants from three separate infiltrations were analyzed (A, B and C). Ratios indicate the proportion of *Agrobacterium* cultures used in co-expression experiments. **Figure 13B** shows a comparison of hemagglutination capacity of crude protein extracts from HA-producing plants.

[0042] **Figure 14** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. "1059": expression of mutant HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV); "1059+1261": co-expression of mutant HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV) with M2 from A/New Caledonia/20/99. "1059+859": co-expression of mutant HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV) with M2 from A/Puerto Rico/8/34. Plants from three separate infiltrations were analyzed (A, B and C). Ratios indicate the proportion of *Agrobacterium* cultures used in co-expression experiments.

[0043] **Figure 15** shows the amino acid sequence alignment of the region surrounding the linker of HAs from several strains of influenza: H1 New Cal (SEQ ID NO:22); H1 Brisbane (SEQ ID NO:23); H1 Sol Islands (SEQ ID NO:24); H2A Singapore (SEQ ID NO:25); H3A Brisbane (SEQ ID NO:26); H3A WCN (SEQ ID NO:27); H5 Anhui (SEQ ID NO:28); H5 Indo (SEQ ID NO:29); H5 Vietnam (SEQ ID NO:30); H6 Teal HK (SEQ ID NO:33); H7 Eq Prague (SEQ ID NO:34); H9A HK (SEQ ID NO:35); B Florida (SEQ ID NO:36); B Malaysia (SEQ ID NO:37). The cleavage site of the precursor HA0 is indicated by an arrow.

[0044] **Figure 16A** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. HA from B/Wisconsin/1/2010 is co-expressed with M2 from A/New Caledonia/20/99. Ten micrograms of protein extract were loaded per lane. "C+": positive control, semi-purified B/Wisconsin/1/2010 virus from the National Institute for Biological Standards and Control, United Kingdom; "1462": expression of wild-type HA from B/Wisconsin/1/2010 in the presence of amplification elements (BeYDV); "1467": expression of the mutant HA from B/Wisconsin/1/2010 in the presence of amplification elements (BeYDV); "1462+1261": co-expression of wild-type HA from B/Wisconsin/1/2010 in the presence of amplification elements (BeYDV) with M2; "1467+1261": co-expression of the mutant HA from B/Wisconsin/1/2010 in the presence of amplification elements (BeYDV) with M2. Ratios indicate the optical density for each *Agrobacterium* culture used in expression and co-expression experiments. **Figure 16B** shows a comparison of hemagglutination capacity of crude protein extracts from plants transformed with AGL1/1462, AGL1/1467, AGL1/1462+AGL1/1261 and AGL1/1467+AGL1/1261.

[0045] Figure 17A and 17B shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. "1008": expression of wild-type HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV); "1008+1261": co-expression of wild-type HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV) with M2 from A/New Caledonia/20/99;"1039":expression of mutant HA from B/Brisbane/60/2008 in the absence of amplification elements (BeYDV). "1039+1261": co-expression of mutant HA from B/Brisbane/60/2008 in the absence of amplification elements (BeYDV) with M2 from A/New Caledonia/20/99.HA from A/Brisbane/59/2007 (H1N1). "1059": expression of mutant HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV); "1059+1261": co-expression of mutant HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV) with M2 from A/New Caledonia/20/99.

Figure 18A shows the schematic representation of the cleavage of the HA0 by Clara-like and/or Furin like protease into HA1 and HA2. **Figure 18B** shows the sequence alignment of HAs from several strains of influenza: H1 New Cal (SEQ ID NO:22); H1 Brisbane (SEQ ID NO:23); H1 Sol Islands (SEQ ID NO:24); H2A Singapore (SEQ ID NO:25); H3A Brisbane (SEQ ID NO:26); H3A WCN (SEQ ID NO:27); H5 Anhui (SEQ ID NO:28); H5 Indo (SEQ ID NO:29); H5 Vietnam (SEQ ID NO:30); H6 Teal HK (SEQ ID NO:33); H7 Eq Prague (SEQ ID NO:34); H9A HK (SEQ ID NO:35); B Florida (SEQ ID NO:36); B Malaysia (SEQ ID NO:37). **Figure 18C** shows deletion of part of the cleavage site in H5 strains, A/Anhui/1/2005 (H5N1) SEQ ID NO: 69, A/Indonesia/5/2005 (H5N1) SEQ ID NO: 70, A/Vietnam/1194/2004 (H5N1) SEQ ID NO: 71, and type B strains, B/Florida/4/2006 SEQ ID NO: 72, and B/Malaysia/2506/2004 SEQ ID NO:73.

[0046] **Figure 19** shows the mutation of the cleavage site in H5/Indo. Native sequence (SEQ ID NO: 44); H5/Indo modified cleavage site comprising TETR (SEQ ID NO:45); H5/Indo modified cleavage site comprising TETQ (SEQ ID NO:46).

[0047] **Figure 20** shows the titers found in initial biomasses after enzyme-extraction of different modified H5/Indo HA's comprising mutations within the protelolytic loop. H5/Indo control (construct 489); H5/Indo proteolytic loop replaced with GG

linker (construct 928); H5/Indo proteolytic loop replaced with TETR linker (construct 676); H5/Indo proteolytic loop replaced with TETQ linker (construct 766).

[0048] **Figure 21** shows various approaches for modifying the proteolytic loop of type B HA. **Figure 21A** shows the amino acid sequence of native B/Brisbane/60/2008 (SEQ ID NO: 16). The underlined portion is the proteolytic loop and the HA2 domain. **Figure 21B** shows the amino acid sequence of B/Brisbane/60/2008 with the proteolytic loop modified (SEQ ID NO: 17), wherein 19 amino acid residues comprising the sequence AKLLKERGFFGAIAGFLEG have been replaced with a GG linker (italics). **Figure 21 C** shows the amino acid sequence of B/Brisbane/60/2008 (SEQ ID NO: 18), wherein 9 amino acid comprising the sequence PPAKLLKER has been replaced with a –GSSSGSSSG- linker (italics). **Figure 21 D** shows the amino acid sequence of native H3 A/Perth/16/2009 (SEQ ID NO: 19). **Figure 21 E** shows the amino acid sequence of H3 A/Perth/16/2009 (SEQ ID NO: 20) with 12 amino acid residues comprising the sequence RNVPEKQTRGIF replaced by a GS linker (italics). **Figure 21 F** shows the amino acid sequence of H3 A/Perth/16/2009 (SEQ ID NO: 21) with 9 amino acid residues comprising the sequence RNVPEKQTR replaced by a GSSGSSGSS- linker (italics).

[0049] **Figure 22** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. HA from H5/Indo. Upper panel listing of components loaded in each lane ; lower panel Western blot. C: Recombinant H5 Indonesia/5/05 S-STD-0002; primary antibody: Anti-HA A/Indonesia/05/2005 CBER # S-BIO-0003 1/50 000; blot. Lanes 1 and 2: H5/Indo control (construct 489); lanes 3 and 4 H5/Indo proteolytic loop replaced with GG linker (construct 928); lanes 5 and 6 H5/Indo proteolytic loop replaced with TETR linker (construct 676); lanes 7 and 8 H5/Indo proteolytic loop replaced with TETQ linker (construct 766); lane 9 MW marker; lane 10 H5 control.

[0050] **Figure 23** shows components used to prepare B-2X35S/CPMV-HT/H5 from A/Indonesia/5/2005 with TETR cleavage site mutation (Construct number 676). **Figure 23A** shows primer sequence MutCleavage-H5(Indo).r (SEQ ID NO:74). **Figure 23B** shows primer sequence MutCleavage-H5(Indo).c (SEQ ID NO:75). **Figure 23C** shows the nucleotide sequence (SEQ ID NO: 76) for expression cassette

676 from the 2X35S promoter to NOS terminator. H5 from influenza A/Indonesia/5/2005 (H5N1) TETR cleavage site mutant is underlined. **Figure 23D** shows the amino acid sequence (SEQ ID NO:77) of a TETR cleavage site mutant of H5 from influenza A/Indonesia/5/2005 (H5N1). **Figure 23E** shows a schematic representation of construct number 676.

[0051] **Figure 24** shows components used to prepare B-2X35S/CPMV-HT/H5 from A/Indonesia/5/2005 with TETQ cleavage site mutation (Construct number 766). **Figure 24A** shows primer sequence H5I505_TETQ.r (SEQ ID NO:78). **Figure 24B** shows primer sequence H5I505_TETQ.c (SEQ ID NO:79). **Figure 24C** shows the nucleotide sequence (SEQ ID NO: 80) for expression cassette 766 from the 2X35S promoter to NOS terminator. H5 from influenza A/Indonesia/5/2005 (H5N1) TETQ cleavage site mutant is underlined. **Figure 24D** shows the amino acid sequence (SEQ ID NO:81) of a TETQ cleavage site mutant of H5 from influenza A/Indonesia/5/2005 (H5N1). **Figure 24E** shows a schematic representation of construct number 766.

[0052] **Figure 25** shows components used to prepare B-2X35S/CPMV-HT/H5 from A/Indonesia/5/2005 with a deleted proteolytic loop (Construct number 928). **Figure 25A** shows primer sequence H5I505(PrL-).r (SEQ ID NO: 82). Figure **25B** shows primer sequence H5I505(PrL-).c (SEQ ID NO: 83). **Figure 25C** shows the nucleotide sequence (SEQ ID NO:84) for expression cassette 928 from the 2X35S promoter to NOS terminator. H5 from influenza A/Indonesia/5/2005 (H5N1) the deleted proteolytic loop is underlined. **Figure 25D** shows the amino acid sequence (SEQ ID NO:85) of a mutant of H5 from influenza A/Indonesia/5/2005 (H5N1) comprising a deleted proteolytic loop. **Figure 25E** shows a schematic representation of construct number 928.

[0053] **Figure 26A** shows a general schematic of an example of several enhancer sequences, CPMVX, and CPMVX+ (comprising CPMVX, and a stuffer fragment, which in this non-limiting example, comprises a multiple cloning site and plant kozak sequence), as described herein. CPMCX and CPMVX+ are each shown as operatively linked to plant regulatory region at their 5'ends, and at their 3' ends, in series, a nucleotide sequence of interest (including an ATG initiation site and STOP site), a 3'UTR, and a terminator sequence. An example of construct CPMVX as

described herein, is CPMV160. An example of construct CPMVX+ as described herein, is CPMV160+. **Figure 26B** shows the relative hemagglutination titres (HMG) in crude protein extracts of modified HA proteins produced in plants comprising CPMV-HT expression constructs, and CPMV160+ based expression constructs. Data for the expression of HA B Brisbane/60/08 with deleted proteolytic loop and with a PDI signal peptide (construct number 1039, 5'UTR: CMPV HT; and construct number 1937, 5'UTR: CMPV160+; see Example 5.7), B Brisbane/60/08+H1Tm, with deleted proteolytic loop, with transmembrane domain and cytoplasmic tail replaced by those of H1, B Massachusetts/2/2012 2012 with deleted proteolytic loop and with a PDI signal peptide (construct number 2072, 5'UTR: CMPV HT; and construct number 2050, 5'UTR: CMPV160+; see Example 5.14), B Massachusetts/2/2012+H1Tm with deleted proteolytic loop, with transmembrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009 and with a PDI signal peptide (construct number 2074, 5'UTR: CMPV HT; and construct number 2060, 5'UTR: CMPV160+; see Example 5.15), B Wisconsin/1/2010 with deleted proteolytic loop and with the native signal peptide (construct number 1445, 5'UTR: CMPV HT; and construct number 1975, 5'UTR: CMPV160+; see Example 5.16), and B Wisconsin/1/2010+H1Tm with deleted proteolytic loop, with transmembrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009 and with the native signal peptide (construct number 1454, 5'UTR: CMPV HT; and construct number 1893, 5'UTR: CMPV160+; see Example 5.18), are shown.

[0054] **FIGURE 27A** shows a general schematic of the enhancer sequence of the CPMV HT and CPMV HT+ fused to a nucleotide sequence of interest. Not all of the elements shown in this figure may be required within the enhancer sequence. Additional elements may be included at the 3'end of the nucleotide sequence of interest (not shown) including a sequence encoding a comovirus 3' untranslated region (UTR), a plastocyanin 3' UTR, or a combination of the comovirus 3' UTR and the plastocyanin 3' UTR. **FIGURE 27B** shows the relative hemagglutination titre (HMG) in crude protein extracts of proteins produced in plants comprising CPMV-HT expression constructs, and CPMV HT+ based expression constructs, operatively linked with a nucleotide sequence of interest. Data for the expression of

HA B Brisbane/60/08 with deleted proteolytic loop and with a PDI signal peptide (construct number 1039: CPMV HT; see Example 5.7 and construct number 1829: CPMV HT+; see example 5.12), B Brisbane/60/08 + H1TM with deleted proteolytic loop, with transmembrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009, and with a PDI signal peptide (construct number 1067: CPMV HT; see Example 5.14 and construct number 1875: CPMV HT+; see example 5.19), B Massachusetts/2/2012 with deleted proteolytic loop, and with a PDI signal peptide (construct number 2072: CMPV HT; see Example 5.15 and construct number 2052: CMPV HT+; see Example 5.20), B Massachusetts/2/2012+H1Tm with deleted proteolytic loop, with transmembrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009 and with a PDI signal peptide (construct number 2074: CMPV HT; see Example 5.16 and construct number 2062: CMPV HT+; see Example 5.21), B Wisconsin/1/2010 with deleted proteolytic loop and with the native signal peptide (construct number 1445: CMPV HT; see Example 5.17 and construct number 1839: CMPV HT+; see Example 5.22), and B Wisconsin/1/2010+H1Tm with deleted proteolytic loop, with transmembrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009 and with the native signal peptide (construct number 1454: CMPV HT; see Example 5.18 and construct number 1860: CMPV HT+; see Example 5.23), are shown.

[0055] **FIGURE 28A** shows a Western blot analysis of H3 Perth protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. Lane 1: (2019+1261) co-expression of native (wildtype) HA from H3 Perth-16-09 in the presence of expression enhancer (CPMV-HT+) with M2 from A/New Caledonia/20/99; Lane 2: (2139+1261) co-expression of native (wildtype) HA from H3 Perth-16-09 in the presence of expression enhancer (CPMV 160+) with M2 from A/New Caledonia/20/99; Lane 3 (2039+1261) co-expression of mutant (modified) HA from H3 Perth-16-09 in the presence of expression enhancer (CPMV HT+) with M2 from A/New Caledonia/20/99; Lane 4: (2159+1261) co-expression of mutant (modified) HA from H3 Perth-16-09 in the presence of expression enhancer (CPMV 160+) with M2 from A/New Caledonia/20/99.

[0056] **FIGURE 28B** shows a Western blot analysis of B Malaysia protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. Lane 2: (2013+1261) co-

expression of native (wildtype) HA from B Malaysia 2506-04 in the presence of expression enhancer (CPMV-160+) with M2 from A/New Caledonia/20/99; Lane 2: (2014+1261) co-expression of mutant (modified) HA from B Malaysia 2506-04 in the presence of expression enhancer (CPMV 160+) with M2 from A/New Caledonia/20/99.

[0057] **FIGURE 28C** shows a Western blot analysis of H9 Hong Kong protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. Lane 1: (1610+1261) co-expression of native (wildtype) HA from H9 Hong Kong -1037-99 in the presence of expression enhancer (CPMV-HT) with M2 from A/New Caledonia/20/99; Lane 2: (1630+1261) co-expression of native (wildtype) HA from H9 Hong Kong -1037-99 in the presence of expression enhancer (CPMV-HT+) and amplification element BEYDV with M2 from A/New Caledonia/20/99; Lane 3: (2244+1261) co-expression of native (wildtype) HA from H9 Hong Kong -1037-99 in the presence of expression enhancer (CPMV-HT+) with M2 from A/New Caledonia/20/99; Lane 4: (2226+1261): co-expression of native (wildtype) HA from H9 Hong Kong -1037-99 in the presence of expression enhancer (CPMV 160+) with M2 from A/New Caledonia/20/99. Lane 6: (2246+1261) co-expression of native (wildtype) HA from H9 Hong Kong -1037-99 in the presence of expression enhancer (CPMV-160+) and amplification element BeYDV with M2 from A/New Caledonia/20/99; Lane 7: (2225+1261) co-expression of mutant (modified) HA from H9 Hong Kong -1037-99 in the presence of expression enhancer (CPMV-HT+) with M2 from A/New Caledonia/20/99; Lane 8: (2245+1261) co-expression of mutant (modified) HA from H9 Hong Kong -1037-99 in the presence of expression enhancer (CPMV HT+) and amplification element BeYDV with M2 from A/New Caledonia/20/99. Lane 9: (2227+1261) co-expression of mutant (modified) HA from H9 Hong Kong -1037-99 in the presence of expression enhancer (CPMV 160+) with M2 from A/New Caledonia/20/99. Lane 10: (2247+1261) co-expression of mutant (modified) HA from H9 Hong Kong -1037-99 in the presence of expression enhancer (CPMV 160+) and amplification element BeYDV with M2 from A/New Caledonia/20/99.

[0058] **FIGURE 28D** shows a Western blot analysis of B Massachusetts protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. Lane 1: (2070+1261) co-expression of native (wildtype) HA from B Massachusetts -2-12 in the presence of

expression enhancer (CPMV-HT) with M2 from A/New Caledonia/20/99; Lane 2: (2080+1261) co-expression of native (wildtype) HA from B Massachusetts -2-12 in the presence of expression enhancer (CPMV-HT+) with M2 from A/New Caledonia/20/99; Lane 3: (2090+1261) co-expression of native (wildtype) HA from B Massachusetts -2-12 in the presence of expression enhancer (CPMV-160+) with M2 from A/New Caledonia/20/99; Lane 4: (2072+1261) co-expression of mutant (modified) HA from B Massachusetts -2-12 in the presence of expression enhancer (CPMV HT) with M2 from A/New Caledonia/20/99; Lane 5: (2052+1261) co-expression of mutant (modified) HA from B Massachusetts -2-12 in the presence of expression enhancer (CPMV HT+) with M2 from A/New Caledonia/20/99; Lane 6: (2050+1261) co-expression of mutant (modified) HA from B Massachusetts -2-12 in the presence of expression enhancer (CPMV 160+) with M2 from A/New Caledonia/20/99.

[0059] **FIGURE 28E** shows a Western blot analysis of H2 Sin protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. Lane 1: (2220+1261) co-expression of native (wildtype) HA from H2 Singapore -1-57 in the presence of expression enhancer (CPMV-HT+) with M2 from A/New Caledonia/20/99; Lane 2: (2222+1261) co-expression of native (wildtype) HA from H2 Singapore -1-57 in the presence of expression enhancer (CPMV 160+) with M2 from A/New Caledonia/20/99. Lane 3: (2221+1261) co-expression of mutant (modified) HA from H2 Singapore -1-57 in the presence of expression enhancer (CPMV-HT+) with M2 from A/New Caledonia/20/99; Lane 4: (2223+1261) co-expression of mutant (modified) HA from H2 Singapore -1-57 in the presence of expression enhancer (CPMV 160+) with M2 from A/New Caledonia/20/99.

[0060] FIGURE 28F shows a Western blot analysis of B/Forida protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. Lane 1: (1004+1261) co-expression of native (wildtype) HA from B/Florida in the presence of expression enhancer (CPMV-HT) with M2 from A/New Caledonia/20/99; Lane 2: (1003+1261) co-expression of native (wildtype) HA from B/Florida in the presence of expression enhancer (CPMV HT) and amplification element BeYDV with M2 from A/New Caledonia/20/99. Lane 3: (2102+1261) co-expression of mutant (modified) HA from B/Florida in the presence of expression enhancer (CPMV-HT+) with M2 from A/New

Caledonia/20/99; Lane 4: (2104+1261) co-expression of mutant (modified) HA from B/Florida in the presence of expression enhancer (CPMV HT+) and amplification element BeYDV with M2 from A/New Caledonia/20/99. Lane 5: (2106+1261) co-expression of mutant (modified) HA from B/Florida+H1 California TMCT in the presence of expression enhancer (CPMV HT+) with M2 from A/New Caledonia/20/99.Lane 6: (2108+1261) co-expression of mutant (modified) HA from B/Florida+H1 California TMCT in the presence of expression enhancer (CPMV HT+) and amplification element BeYDV with M2 from A/New Caledonia/20/99.

[0061] **Figure 29A** shows the relative HA titer of modified HA from various influenza strains that were expressed in the presence of enhancer element CPMV HT, CPMV HT+ or CPMV 160+. Activity is compared to the native HA protein expressed with the same enhancer element. H3 A Perth/16/09 (H3 Per1609), H3 Victoria/361/11 (H3 Vic26111), B Brisbane 60/2008 (HB Bris60008), B Malaysia 2506/04 (HB Mal 250604) and B Massachusetts 2/12 (Ma212) were co-expressed with influenza M2 protein.

[0062] **Figure 30A** shows primer IF-S2+S4-B Bris.c (SEQ ID NO: 86). **Figure 30B** shows primer IF-S1a4-B Bris.r (SEQ ID NO: 87). **Figure 30C** shows the nucleotide sequence of synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank accession number FJ766840) (SEQ ID NO: 88). **Figure 30D** shows the nucleotide sequence of expression cassette number 1029 from 2X35S promoter to NOS terminator. PDISP/HA from influenza B/Brisbane/60/2008 is underlined. (SEQ ID NO: 89). **Figure 30E** shows the amino acid sequence of PDISP/HA from influenza B/Brisbane/60/2008 (SEQ ID NO: 90). **Figure 30F** shows a schematic representation of construct 1029. SacII and StuI restriction enzyme sites used for plasmid linearization are annotated on the representation.

[0063] **FIGURE 31** shows sequence components used to prepare construct numbers 1039 and 1829 (2X35S/CPMV HT PDISP/HA B Brisbane (PrL-) NOS and 2X35S/CPMV HT+ PDISP/HA B Brisbane (PrL-) NOS, respectively; see Example 5.12). Construct number 1039 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between

the 5'UTR and the nucleotide sequence of interest (PDISP/HA B Brisbane (PrL-)). Construct number 1829 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment comprising an incomplete M protein ,a multiple cloning site, and a plant kozak sequence and is an example of a CPMV HT+ based construct. PDISP: protein disulfide isomerase signal peptide; NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop. **FIGURE 31A** shows the nucleotide sequence of PDISP/HA B Brisbane (PrL-) (SEQ ID NO: 91). **FIGURE 31B** shows the amino acid sequence of PDISP/HA B Brisbane (PrL-); SEQ ID NO: 92). **FIGURE 31C** shows a schematic representation of construct number 1829 (2X35S/CPMV HT+).

[0064] **FIGURE 32** shows sequence components used to prepare construct numbers 1039 and 1937 (2X35S/CPMV HT PDISP/HA B Brisbane (PrL-) NOS and 2X35S/CPMV160+ PDISP/HA B Brisbane (PrL-) NOS, respectively; see Example 5.7). Construct number 1039 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (PDISP/HA B Brisbane (PrL-)). Construct number 1937 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment (multiple cloning site), and a plant kozak sequence (this construct does not comprise a sequence encoding an incomplete M protein) and is an example of a CPMV160+ (CPMVX+, where X=160) based construct. PDISP: protein disulfide isomerase signal peptide; NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop. **FIGURE 32A** shows a schematic representation of construct number 1937 (2X35S/CPMV160+; a CPMVX+ based construct, where X=160).

[0065] **FIGURE 33** shows sequence components used to prepare construct numbers 1067 and 1977 (2X35S/CPMV HT PDISP/HA B Brisbane (Prl-)+H1 California TMCT NOS and 2X35S/CPMV160+ PDISP/HA B Brisbane (PrL-)+H1 California TMCT NOS, respectively; see Example 5.14). Construct number 1067 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (PDISP/HA B Brisbane (PrL-)+H1 California TMCT). Construct number 1977 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment

(multiple cloning site), and a plant kozak sequence (this construct does not comprise a sequence encoding an incomplete M protein) and is an example of a CPMV160+ (CPMVX+, where X=160) based construct. PDISP: protein disulfide isomerase signal peptide; NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop; TMCT: transmembrane domain cytoplasmic tail. **FIGURE 33A** shows the nucleotide sequence of PDISP/HA B Brisbane (PrL-)+H1 California TMCT (SEQ ID NO: 95). **FIGURE 33B** shows the amino acid sequence of PDISP/HA B Brisbane (PrL-)+H1 California TMCT (SEQ ID NO: 96). **FIGURE 33C** shows a schematic representation of construct number 1067 (2X35S/CPMV HT; reference construct). **FIGURE 33D** shows a schematic representation of construct number 1977 (2X35S/CPMV160+; a CPMVX+ based construct, where X=160).

[0066] **FIGURE 34** shows sequence components used to prepare construct numbers 2072 and 2050 (2X35S/CPMV HT PDISP/HA B Massachusetts (PrL-) NOS and 2X35S/CPMV160+ PDISP/HA B Massachusetts (PrL-) NOS, respectively; see Example 5.15). Construct number 2072 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (PDISP/HA B Massachusetts (PrL-)). Construct number 2050 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment (multiple cloning site), and a plant kozak sequence (this construct does not comprise a sequence encoding an incomplete M protein) and is an example of a CPMV160+ (CPMVX+, where X=160) based construct. PDISP: protein disulfide isomerase signal peptide; NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop. **FIGURE 34A** shows the nucleotide sequence of PDISP/HA B Massachusetts (PrL-) (SEQ ID NO: 97). **FIGURE 34B** shows the amino acid sequence of PDISP/HA B Massachusetts (PrL-) (SEQ ID NO: 98). **FIGURE 34C** shows a schematic representation of construct number 2072 (2X35S/CPMV HT; reference construct). **FIGURE 34D** shows a schematic representation of construct number 2050 (2X35S/CPMV160+; a CPMVX+ based construct, where X=160).

[0067] **FIGURE 35** shows sequence components used to prepare construct numbers 2074 and 2060 (2X35S/CPMV HT PDISP/HA B Massachusetts (PrL-)+H1 California

TMCT NOS and 2X35S/CPMV160+ PDISP/HA B Massachusetts (PrL-)+H1 California TMCT NOS, respectively; see Example 5.16). Construct number 2074 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (PDISP/HA B Massachusetts (PrL-)+H1 California TMCT). Construct number 2060 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment (multiple cloning site), and a plant kozak sequence (this construct does not comprise a sequence encoding an incomplete M protein) and is an example of a CPMV160+ (CPMVX+, where X=160) based construct. PDISP: protein disulfide isomerase signal peptide; NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop; TMCT: transmembrane domain cytoplasmic tail. **FIGURE 35A** shows the nucleotide sequence of PDISP/HA B Massachusetts (PrL-)+H1 California TMCT (SEQ ID NO: 99). **FIGURE 35B** shows the amino acid sequence of PDISP/HA B Massachusetts (PrL-)+H1 California TMCT (SEQ ID NO: 100). **FIGURE 35C** shows a schematic representation of construct number 2074 (2X35S/CPMV HT; reference construct). **FIGURE 35D** shows a schematic representation of construct number 2060 (2X35S/CPMV160+; a CPMVX+ based construct, where X=160).

[0068] **FIGURE 36** shows sequence components used to prepare construct numbers 1445, 1820 and 1975 (2X35S/CPMV HT HA B Wisconsin (PrL-) NOS, 2X35S/CPMV160+ HA B Wisconsin (PrL-) NOS and 2X35S/CPMV160 HA B Wisconsin (PrL-) NOS, respectively; see Example 15.17). Construct number 1445 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (HA B Wisconsin (PrL-)). Construct number 1820 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment (multiple cloning site), and a plant kozak sequence (this construct does not comprise a sequence encoding an incomplete M protein) and is an example of a CPMV160+ (CPMVX+, where X=160) based construct. Construct number 1975 includes a CPMV 5'UTR comprising 160 nucleotides, and does not include a stuffer fragment (multiple cloning site), or a plant

kozak sequence (this construct also does not comprise a sequence encoding an incomplete M protein) and is an example of a "CPMV160" (CPMVX) based construct. PrL-: deleted proteolytic loop; NOS: nopaline synthase terminator. **FIGURE 36A** shows the nucleotide sequence of HA B Wisconsin (PrL-) (SEQ ID NO: 101). **FIGURE 36B** shows the amino acid sequence of HA B Wisconsin (PrL-) (SEQ ID NO: 102). **FIGURE 36C** shows a schematic representation of construct number 1445 (2X35S/CPMV HT; reference construct). **FIGURE 36D** shows a schematic representation of construct number 1820 (2X35S/CPMV160+; a CPMVX+ based construct). **FIGURE 36E** shows a schematic representation of construct number 1975 (2X35S/CPMV160; a CPMVX based construct, where X=160).

[0069] **FIGURE 37** shows sequence components used to prepare construct numbers 1454 and 1893 (2X35S/CPMV HT HA B Wisconsin (PrL-)+H1 California TMCT NOS and 2X35S/CPMV160+ HA B Wisconsin (PrL-)+H1 California TMCT NOS, respectively; see Example 5.18). Construct number 1454 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (HA B Wisconsin (PrL-)+H1 California TMCT). Construct number 1893 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment (multiple cloning site), and a plant kozak sequence (this construct does not comprise a sequence encoding an incomplete M protein) and is an example of a CPMV160+ (CPMVX+, where X=160) based construct. NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop; TMCT: transmembrane domain cytoplasmic tail. **FIGURE 37A** shows the nucleotide sequence of HA B Wisconsin (PrL-)+H1 California TMCT (SEQ ID NO: 103). **FIGURE 37B** shows the amino acid sequence of PDISP/HA B Wisconsin (PrL-)+H1 California TMCT (SEQ ID NO: 104). **FIGURE 37C** shows a schematic representation of construct number 1454 (2X35S/CPMV HT; reference construct). **FIGURE 37D** shows a schematic representation of construct number 1893 (2X35S/CPMV160+; a CPMVX+ based construct, where X=160).

[0070] **FIGURE 38** shows sequence components used to prepare construct numbers 1067 and 1875 (2X35S/CPMV HT PDISP/HA B Brisbane (Prl-)+H1 California TMCT NOS and 2X35S/CPMV HT+ PDISP/HA B Brisbane (PrL-)+H1 California

TMCT NOS, respectively; see Example 5.19). Construct number 1067 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (PDISP/HA B Brisbane (PrL-)+H1 California TMCT). Construct number 1875 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment comprising an incomplete M protein, a multiple cloning site, and a plant kozak sequence and is an example of a CPMV HT+ based construct. PDISP: protein disulfide isomerase signal peptide; NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop; TMCT: transmembrane domain cytoplasmic tail. **FIGURE 38A** shows the nucleotide sequence of PDISP/HA B Brisbane (PrL-)+H1 California TMCT (SEQ ID NO: 105). **FIGURE 38B** shows the amino acid sequence of PDISP/HA B Brisbane (PrL-)+H1 California TMCT (SEQ ID NO: 106). **FIGURE 38C** shows a schematic representation of construct number 1875 (2X35S/CPMV160+).

[0071] **FIGURE 39** shows sequence components used to prepare construct numbers 2072 and 2052 (2X35S/CPMV HT PDISP/HA B Massachusetts (PrL-) NOS and 2X35S/CPMV HT+ PDISP/HA B Massachusetts (PrL-) NOS, respectively; see Example 5.20). Construct number 2072 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (PDISP/HA B Massachusetts (PrL-)). Construct number 2052 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment comprising an incomplete M protein, a multiple cloning site, and a plant kozak sequence and is an example of a CPMV HT+ based construct. PDISP: protein disulfide isomerase signal peptide; NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop. **FIGURE 39A** shows the nucleotide sequence of PDISP/HA B Massachusetts (PrL-) (SEQ ID NO: 107). **FIGURE 39B** shows the amino acid sequence of PDISP/HA B Massachusetts (PrL-) (SEQ ID NO: 108). **FIGURE 39C** shows a schematic representation of construct number 2052 (2X35S/CPMV HT+).

[0072] **FIGURE 40** shows sequence components used to prepare construct numbers 2074 and 2062 (2X35S/CPMV HT PDISP/HA B Massachusetts (PrL-)+H1 California TMCT NOS and 2X35S/CPMV HT+ PDISP/HA B Massachusetts (PrL-)+H1 California TMCT NOS, respectively; see Example 5.21). Construct number 2074 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (PDISP/HA B Massachusetts (PrL-)+H1 California TMCT). Construct number 2062 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment comprising an incomplete M protein, a multiple cloning site, and a plant kozak sequence and is an example of a CPMV HT+ based construct. PDISP: protein disulfide isomerase signal peptide; NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop; TMCT: transmembrane domain cytoplasmic tail. **FIGURE 40A** shows the nucleotide sequence of PDISP/HA B Massachusetts (PrL-)+H1 California TMCT (SEQ ID NO: 109). **FIGURE 40B** shows the amino acid sequence of PDISP/HA B Massachusetts (PrL-)+H1 California TMCT (SEQ ID NO: 110). **FIGURE 40C** shows a schematic representation of construct number 2062 (2X35S/CPMV HT+).

[0073] **FIGURE 41** shows sequence components used to prepare construct numbers 1445 and 1839 (2X35S/CPMV HT HA B Wisconsin (PrL-) NOS, and 2X35S/CPMV HT+ HA B Wisconsin (PrL-) NOS, respectively; see Example 5.22). Construct number 1445 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (HA B Wisconsin (PrL-)). Construct number 1839 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment comprising an incomplete M protein, a multiple cloning site, and a plant kozak sequence and is an example of a CPMV HT+ based construct. PrL-: deleted proteolytic loop; NOS: nopaline synthase terminator. **FIGURE 41A** shows the nucleotide sequence of HA B Wisconsin (PrL-) (SEQ ID NO: 111). **FIGURE 41B** shows the amino acid sequence of HA B Wisconsin (PrL-) (SEQ ID NO: 112).

FIGURE 41C shows a schematic representation of construct number 1839 (2X35S/CPMV HT+).

[0074] **FIGURE 42** shows sequence components used to prepare construct numbers 1454 and 1860 (2X35S/CPMV HT HA B Wisconsin (PrL-)+H1 California TMCT NOS and 2X35S/CPMV HT+ HA B Wisconsin (PrL-)+H1 California TMCT NOS, respectively; see Example 5.23). Construct number 1454 incorporates a prior art CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (HA B Wisconsin (PrL-)+H1 California TMCT). Construct number 1860 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment comrpsing n incomplete M protein, a multiple cloning site, and a plant kozak sequence and is an example of a CPMV HT+ based construct. NOS: nopaline synthase terminator; PrL-: deleted proteolytic loop; TMCT: transmembrane domain cytoplasmic tail. **FIGURE 42A** shows the nucleotide sequence of HA B Wisconsin (PrL-)+H1 California TMCT (SEQ ID NO: 113). **FIGURE 42B** shows the amino acid sequence of PDISP/HA B Wisconsin (PrL-)+H1 California TMCT (SEQ ID NO: 114). **FIGURE 42C** shows a schematic representation of construct number 1893 (2X35S/CPMV HT+).

[0075] **FIGURE 43** shows sequence components used to prepare construct numbers 489 (2X35S/CPMV HT H5 Indonesia NOS see Example 5.24). Construct number 489 incorporates a CPMV-HT sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a sequence encoding an incomplete M protein) and does not comprise a heterologous kozak sequence between the 5'UTR and the nucleotide sequence of interest (PDISP/H1 California). **FIGURE 43A** shows the nucleotide sequence of native H5 Indonesia (SEQ ID NO: 115). **FIGURE 43B** shows the amino acid sequence of native H5 Indonesia (SEQ ID NO: 116). **FIGURE 43C** shows a schematic representation of construct number 489 (2X35S/CPMV HT; reference construct).

[0076] **FIGURE 44** shows the sequence components used to prepare construct number 1800 (A-2X35S CPMV160+ PDISP H3Victoria NOS; see example 5.25).

Construct number 1800 includes a CPMV 5'UTR comprising 160 nucleotides, a stuffer fragment (multiple cloning site), and a plant kozak sequence (this construct does not comprise a sequence encoding an incomplete M protein) and is an example of a CPMV160+ (CPMVX+, where X=160) based construct. PDISP: protein disulfide isomerase signal peptide. NOS: nopaline synthase terminator. **FIGURE 44A** shows primer sequence IF**(SacII)-PDI.s1+4c (SEQ ID NO:117). **FIGURE 44B** shows primer sequence IF-H3V36111.s1-4r (SEQ ID NO: 118). **FIGURE 44C** shows the sequence of PDISP/H3 Victoria (SEQ ID NO:119). **FIGURE 44D** shows a schematic representation of construct 2171 (SacII and StuI restriction enzyme sites used for plasmid linearization are indicated). **FIGURE 44E** shows construct 2171 from left to right t-DNA borders (underlined), 2X35S/CPMV160+/NOS with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette, an H1 California transmembrane cytoplasmic tail, and the CPMV3'UTR (SEQ ID NO: 120). **FIGURE 44F** shows expression cassette number 1800 from 2X35S promoter to NOS terminator. PDISP/H3 Victoria nucleotide sequence is underlined; 5'UTR is shown in bold; plant kozak sequence double underline; a stuffer fragment (multiple cloning site) of 16 base pairs is positioned between the 5'UTR and plant kozak sequence (SEQ ID NO:121). **FIGURE 44G** shows the amino acid sequence of PDISP/H3 Victoria (SEQ ID NO:122). **FIGURE 44H** shows a schematic representation of construct number 1800 (a CPMVX+ based construct, where X=160).

[0077] **FIGURE 45** shows the sequence components used to prepare construct number 1819 (2X35S CPMV-HT+ PDISP H3Victoria NOS). Construct number 1819 incorporates a CPMV-HT+ sequence (CMPV 5'UTR with mutated start codon at position 161 fused to a stuffer fragment encoding an incomplete M protein, a multiple cloning site, and comprises a plant kozak sequence between the multiple cloning site and the nucleotide sequence of interest (PDISP/H3 Victoria)). PDISP: protein disulfide isomerase signal peptide. NOS: nopaline synthase terminator. **FIGURE 45A** shows primer sequence IF(SacII)-Kozac_PDI.c (SEQ ID NO: 123). **FIGURE 45B** shows primer sequence IF-H3V36111.s1-4r (SEQ ID NO:124). **FIGURE 45C** shows a schematic representation of construct 2181. **FIGURE 45D** shows the sequence for construct 2181 (from left to right t-DNA borders, underlined; 2X35S/CPMV-HT+/NOS with Plastocyanine-P19-Plastocyanine silencing inhibitor

expression cassette; SEQ ID NO:126). **FIGURE 45E** shows expression cassette number 1819 from 2X35S promoter to NOS terminator. The PDISP/H3 Victoria nucleotide sequence is underlined (SEQ ID NO:127). **FIGURE 45F** shows a schematic representation of construct 1819.

[0078] **Figure 46A** shows the relative hemagglutination activity of native H7 Hangzhou HA and modified H7 Hangzhou HA, with the proteolytic loop deleted when co-expressed with M2. Native and modified H7 Hangzhou HA (constructs #2142 and 2152, see Examples 5.33 and 5.34) were expressed in the presence of M2 (construct #1261 see Example 5.1) and purified from plants. **Figure 46B** shows the protein yield of native H7 Hangzhou HA (construct #2142) and modified H7 Hangzhou HA, with the proteolytic loop deleted (construct #2152). **Figure 46C** shows an SDS-PAGE analysis, with lane 2 showing purified modified H7 Hangzhou HA with a removed proteolytic loop (construct #2152) and lane 3 showing the purified native H7 Hangzhou HA (construct #2142). For each lane, 2µg of total protein were loaded on the gel. The purity of the proteins profiles are similar for both constructs.

[0079] **Figure 47A** shows the Trypsin resistance between native HA protein and modified HA with the proteolytic loop replaced with a GG liker (prl-), modified HA with the proteolytic loop replaced with a TETQ liker (TETQ) and modified HA with the proteolytic loop replaced with a TETR liker (TETR). Native (# 489), PRL- (# 928), TETQ (#766) and TETR (#676) H5 Indonesia HA VLP constructs were purified. For each lot, two samples of HA VLPs were resuspended in buffer (100 mM Na/KP_{O4}, 150 mM NaCl, 0.01% TWEEN 80) at pH 7.4 at a target concentration of 150µg/mL. Trypsin was added in a 1:100 protein ratio to one resuspended sample. Samples were incubated for 30, 60 and 120 minutes at room temperature. 30µL of the non-digested extract (control) and 30µL of the digested extracts were loaded on SDS-PAGE gel, which was stained with Coomassie blue. **Figure 47B** shows immunogenicity (HI titer) of native H5 VLP and its mutant counterparts (prl-, TETQ and TETR) in mice after two doses. Bars represent relative (%) HI titers comparison of each H5 mutants VLP with the native H5 VLP.

[0080] **FIGURE 48** shows sequence components used to prepare construct numbers 2220 (2X35S/CPMV HT+/ PDISP/H2 Singapore/ NOS see Example 5.27). **FIGURE 48A** shows the nucleotide sequence of primer IF**-H2S157.s1-6r (SEQ ID NO: 127). **FIGURE 48B** shows the nucleotide sequence of PDISP/H2 Singapore (SEQ ID NO: 128) **FIGURE 48C** shows the nucleotide sequence of expression cassette number 2220 from 2X35S promoter to NOS terminator. PDISP/H2 Singapore nucleotide sequence is underlined. **FIGURE 48D** shows the Amino acid sequence of PDISP/H2 Singapore. **FIGURE 48E** shows a schematic representation of construct number 2220.

[0081] **FIGURE 49** shows sequence components used to prepare construct numbers 2221 (2X35S/CPMV HT+/ PDISP/H2 Singapore with deleted proteolytic loop/ NOS see Example 5.28). **FIGURE 49A** shows the nucleotide sequence of primer H2S157(Prl-).r (SEQ ID NO: 131). **FIGURE 49B** shows the nucleotide sequence of primer H2S157(Prl-).c (SEQ ID NO: 132) **FIGURE 49C** shows the nucleotide sequence of expression cassette number 2221 from 2X35S promoter to NOS terminator. PDISP/H2 Singapore nucleotide sequence is underlined (SEQ ID NO: 133). **FIGURE 49D** shows the Amino acid sequence of PDISP/H2 Singapore with deleted proteolytic loop (SEQ ID NO:134) . **FIGURE 49E** shows a schematic representation of construct number 2221.

[0082] **FIGURE 50** shows sequence components used to prepare construct numbers 2222 (2X35S/CPMV 160+/ PDISP/H2 Singapore) and 2223 (2X35S/CPMV 160+/ PDISP/H2 Singapore with deleted proteolytic loop/ NOS) see Example 5.29). **FIGURE 50A** shows the nucleotide sequence of expression cassette number 2222 from 2X35S promoter to NOS terminator. PDISP/H2 Singapore nucleotide sequence is underlined (SEQ ID NO:135). **FIGURE 50B** shows the nucleotide sequence of expression cassette number 2223 from 2X35S promoter to NOS terminator. PDISP/H2 Singapore with deleted proteolytic loop nucleotide sequence is underlined (SEQ ID NO: 136). **FIGURE 50C** a schematic representation of construct number 2222 **FIGURE 50D** a schematic representation of construct number 2223.

[0083] **FIGURE 51** shows sequence components used to prepare construct numbers 2219 (2X35S/CPMV HT+ PDISP/H3 Perth) and 2139 (2X35S/CPMV 160+/

PDISP/H3 Perth) see Example 5.30). **FIGURE 51A** shows the nucleotide sequence of PDISP/H3 Perth (SEQ ID NO: 137). **FIGURE 51B** shows the nucleotide sequence of primer IF**-H3P1609.S1-6r (SEQ ID NO: 138). **FIGURE 51C**shows the Amino acid sequence of PDISP/H3 Perth (SEQ ID NO: 139). **Figure 51D** shows a schematic representation of construct number 2219. **FIGURE 51E** shows a schematic representation of construct number 2139.

[0084] **FIGURE 52** shows sequence components used to prepare construct numbers 2039 (2X35S/CPMV HT+ PDISP/H3 Perth with deleted proteolytic loop) and 2159 (2X35S/CPMV 160+ PDISP/H3 Perth with deleted proteolytic loop) see Example 5.31). **FIGURE 52A** shows the nucleotide sequence of PDISP/H3 Perth with deleted proteolytic loop (SEQ ID NO: 140). **FIGURE 52B** shows the nucleotide sequence of primer H3P1609(Prl-)#2.r (SEQ ID NO: 141). **FIGURE 52C** shows the nucleotide sequence of primer H3P1609(Prl-)#2.c (SEQ ID NO: 142). **FIGURE 52D**shows the amino acid sequence of PDISP/H3 Perth with deleted proteolytic loop (SEQ ID NO: 143). **Figure 52E** shows a schematic representation of construct number 2039. **FIGURE 52F** shows a schematic representation of construct number 2159.

[0085] **FIGURE 53** shows sequence components used to prepare construct numbers 2230 (2X35S/CPMV HT+ PDISP/H3 Victoria with deleted proteolytic loop) and 2250 (2X35S/CPMV 160+ PDISP/H3 Victoria with deleted proteolytic loop) see Example 5.32). **FIGURE 53A** shows the nucleotide sequence of nucleotide sequence of PDISP/H3 Victoria with deleted proteolytic loop (SEQ ID NO: 144). **FIGURE 53B** shows the nucleotide sequence of primer H3V36111(Prl-).r (SEQ ID NO: 145). **FIGURE 53C** shows the nucleotide sequence of primer H3V36111(Prl-).c (SEQ ID NO: 146). **FIGURE 53D**shows the amino acid sequence of PDISP/H3 Victoria with deleted proteolytic loop (SEQ ID NO: 147). **FIGURE 53E** shows a schematic representation of construct number 2230. **FIGURE 53F** shows a schematic representation of construct number 2250.

[0086] **FIGURE 54** shows sequence components used to prepare construct number 2142 (2X35S/CPMV HT+/PDISP/H7 Hangzhou/NOS) see Example 5.33). **FIGURE 54A** shows the nucleotide sequence of PDISP/H7 Hangzhou (SEQ ID NO: 148). **FIGURE 54B** shows the nucleotide sequence of primer IF*-H7H113.s1-6r (SEQ ID

NO: 149). **FIGURE 54C** shows the amino acid sequence of PDISP/H7 Hangzhou (SEQ ID NO: 150). **FIGURE 53D** shows a schematic representation of construct number 2142.

[0087] **FIGURE 55** shows sequence components used to prepare construct number 2152 (2X35S/CPMV HT+/PDISP/H7 Hangzhou with deleted proteolytic loop/NOS) see Example 5.34). **FIGURE 55A** shows the nucleotide sequence of PDISP/H7 Hangzhou with deleted proteolytic loop (SEQ ID NO: 151). **FIGURE 55B** shows the nucleotide sequence of primer H7H113(PrL-).r (SEQ ID NO: 152). **FIGURE 55C** shows the nucleotide sequence of primer H7H113(PrL-).c (SEQ ID NO: 153). **FIGURE 55D** shows the amino acid sequence of PDISP/H7 Hangzhou with deleted proteolytic loop (SEQ ID NO: 154). **FIGURE 53E** shows a schematic representation of construct number 2152.

[0088] **FIGURE 56** shows sequence components used to prepare construct numbers 2224 (2X35S/CPMV HT+ PDISP/H9 Hong Kong) and 2226 (2X35S/CPMV 160+ PDISP/H9 Hong Kong) see Example 5.35). **FIGURE 56A** shows the nucleotide sequence of PDISP/H9 Hong Kong (SEQ ID NO: 155). **FIGURE 56B** shows the nucleotide sequence of primer IF**-H9HK107399.S1-6r (SEQ ID NO: 156). **FIGURE 56C** shows the amino acid sequence of PDISP/H9 Hong Kong (SEQ ID NO: 157). **FIGURE 56D** shows a schematic representation of construct number 2224. **FIGURE 56E** shows a schematic representation of construct number 2226.

[0089] **FIGURE 57** shows sequence components used to prepare construct numbers 2225 (2X35S/CPMV HT+ PDISP/H9 Hong Kong with deleted proteolytic loop) and 2227 (2X35S/CPMV 160+ PDISP/H9 Hong Kong with deleted proteolytic loop) see Example 5.36. **FIGURE 57A** shows the nucleotide sequence of PDISP/H9 Hong Kong with deleted proteolytic loop (SEQ ID NO: 158). **FIGURE 57B** shows the nucleotide sequence of primer H9HK107399(Prl-).r (SEQ ID NO: 159). **FIGURE 57C** shows the nucleotide sequence of primer H9HK107399(Prl-).c (SEQ ID NO: 160). **FIGURE 57D** shows the amino acid sequence of PDISP/H9 Hong Kong with deleted proteolytic loop (SEQ ID NO: 161). **FIGURE 57E** shows a schematic representation of construct number 2225. **FIGURE 57F** shows a schematic representation of construct number 2227.

[0090] **FIGURE 58** shows sequence components used to prepare construct number 2013 (2X35S/CPMV 160+/PDISP/HA B Malaysia/NOS) see Example 5.37. **FIGURE 58A** shows the nucleotide sequence of PDISP/HA B Malaysia (SEQ ID NO: 162). **FIGURE 58B** shows the nucleotide sequence of primer IF**-HBM250604.S1-6r (SEQ ID NO: 163). **FIGURE 58C** shows the amino acid sequence of PDISP/HA B Malaysia (SEQ ID NO: 164). **FIGURE 58D** shows a schematic representation of construct number 2013.

[0091] **FIGURE 59** shows sequence components used to prepare construct number 2014 (2X35S/CPMV 160+/PDISP/HA B Malaysia with deleted proteolytic loop/NOS) see Example 5.38. **FIGURE 59A** shows the nucleotide sequence of PDISP/HA B Malaysia with deleted proteolytic loop (SEQ ID NO: 165). **FIGURE 59B** shows the nucleotide sequence of primer HBM250604(PrL-).r (SEQ ID NO: 166). **FIGURE 59C** shows the nucleotide sequence of primer HBM250604(PrL-).c (SEQ ID NO: 167). **FIGURE 59D** shows the amino acid sequence of PDISP/HA B Malaysia with deleted proteolytic loop (SEQ ID NO: 168). **FIGURE 59E** shows a schematic representation of construct number 2014.

[0092] **FIGURE 60** shows sequence components used to prepare construct numbers 2070 (2X35S/CPMV HT PDISP/HA B Massachusetts), 2080 (2X35S/CPMV HT+ PDISP/HA B Massachusetts) and 2090 (2X35S/CPMV 160+ PDISP/HA B Massachusetts) see Example 5.39. **FIGURE 60A** shows the nucleotide sequence of PDISP/HA B Massachusetts (SEQ ID NO: 169). **FIGURE 60B** shows amino acid sequence of PDISP/HA B Massachusetts (SEQ ID NO: 170). **FIGURE 60C** shows a schematic representation of construct number 2070. **FIGURE 60D** shows a schematic representation of construct number 2080. **FIGURE 60E** shows a schematic representation of construct number 2090.

[0093] **FIGURE 61** shows sequence components used to prepare construct numbers 2102 (2X35S/CPMV HT PDISP/HA B Florida with proteolytic loop deleted) and 2104 (2X35S/CPMV HT+ /BeYDV/PDISP/HA B Florida with proteolytic loop deleted) see Example 5.40. **FIGURE 61A** shows the nucleotide sequence of primer HBF406(PrL-).r (SEQ ID NO: 190). **FIGURE 61B** shows the nucleotide sequence of primer HBF406(PrL-).c (SEQ ID NO: 191). **FIGURE 61C** shows the nucleotide

sequence of primer IF*-HBF406.s1-6r (SEQ ID NO: 192). **FIGURE 61D** shows the nucleotide sequence of PDISP/HA B Florida with deleted proteolytic loop. **FIGURE 61E** shows the amino acid sequence of PDISP/HA B Florida with deleted proteolytic loop. **FIGURE 61F** shows the nucleotide sequence of expression cassette number 2102. **FIGURE 61G** shows the schematic representation of construct number 2102. **FIGURE 61H** shows the nucleotide sequence of expression cassette number 2104. **FIGURE 61I** shows the schematic representation of construct number 2104.

[0094] **FIGURE 62** shows sequence components used to prepare construct numbers 2106 (2X35S/CPMV HT+/ PDISP/B Florida +H1 California TMCT with proteolytic loop deleted / NOS) and 2108 (2X35S/CPMV HT+/ BeYDV/PDISP/B Florida +H1 California TMCT with proteolytic loop deleted/ NOS) see Example 5.41. **FIGURE 62A** shows the nucleotide sequence of primer IF-H1cTMCT.S1-4r (SEQ ID NO: 197). **FIGURE 62B** shows the nucleotide sequence of PDISP/HA B Florida+H1Cal TMCT with deleted proteolytic loop (SEQ ID NO: 198). **FIGURE 62C** shows the amino acid sequence of PDISP/HA B Florida+H1Cal TMCT with deleted proteolytic loop. **FIGURE 62D** shows the nucleotide sequence of expression cassette number 2106. **FIGURE 62E** shows the schematic representation of construct number 2106. **FIGURE 62F** shows the nucleotide sequence of expression cassette number 2108. **FIGURE 62G** shows the schematic representation of construct number 2108.

DETAILED DESCRIPTION

[0095] The following description is of a preferred embodiment.

[0096] The present invention relates to virus-like particles (VLPs) and methods of producing and increasing VLP yield, accumulation and production in plants.

[0097] The present invention provides, in part, a method of producing a virus like particle (VLP) in a plant, or portion of the plant. The method involves introducing a nucleic acid into the plant or portion of the plant. The nucleic acid comprises comprising a regulatory region active in the plant and operatively linked to a nucleotide sequence encoding an influenza hemagglutinin (HA). The HA comprises a modified proteolytic loop or cleavage site. The plant or portion of the plant is incubated under conditions that permit the expression of the nucleic acid, thereby

producing the VLP. If desired, the plant or portion of the plant may be harvested and the VLP purified.

[0098] The present invention also provides a VLP produced by this method. The VLP may comprise one or more than one lipid derived from a plant.

[0099] The VLP may be used to prepare a composition comprising an effective dose of the VLP for inducing an immune response, and a pharmaceutically acceptable carrier.

[00100] Also provided herein is a modified hemagglutinin, wherein the proteolytic loop or cleavage site has been modified.

[00101] The present invention also provides plant matter comprising the VLP produced by expressing the nucleic acids described above. The plant matter may be used in inducing immunity to an influenza virus infection in a subject. The plant matter may also be admixed as a food supplement.

[00102] The VLP of the present invention may also be produced by providing a plant or portion of the plant comprising a nucleic acid as defined above, and incubating the plant or portion of the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLP. The VLP may comprise one or more than one lipid derived from a plant. The VLP may be used to prepare a composition comprising an effective dose of the VLP for inducing an immune response, and a pharmaceutically acceptable carrier. The present invention also provides plant matter comprising the VLP produced by expressing the first and second nucleic acids. The plant matter may be used in inducing immunity to an influenza virus infection in a subject. The plant matter may also be admixed as a food supplement.

[00103] The VLP of the present invention comprises one or more modified influenza hemagglutinin (HA). The modified HA may be derived from any HA, for example an H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 or type B HA as described in WO 2009/009876; WO 2009/076778; WO 2010/003225; WO 2010/003235; WO 2011/03522; which are incorporated herein by reference).

[00104] The current invention includes VLPs comprising HA sequences of influenza strains, where the HA sequences comprise modified polybasic cleavage sites including for example, the modifications as described herein.

HA Protein

[00105] The term "hemagglutinin" or "HA" as used herein refers to a glycoprotein found on the outside of influenza viral particles. HA is a homotrimeric membrane type I glycoprotein, generally comprising a signal peptide, an HA1 domain, and an HA2 domain comprising a membrane-spanning anchor site at the C-terminus and a small cytoplasmic tail. Nucleotide sequences encoding HA are well known and are available--see, for example, the BioDefence Public Health base (Influenza Virus; see URL: biohealthbase.org) or National Center for Biotechnology Information (see URL: ncbi.nlm.nih.gov), both of which are incorporated herein by reference. HA may include any HA, for example an H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 or type B HA as described in WO 2009/009876; WO 2009/076778; WO 2010/003225; WO 2010/003235; WO 2011/03522; which are incorporated herein by reference). Furthermore, the HA may be based on the sequence of a hemagglutinin that is isolated from one or more emerging or newly-identified influenza viruses. The present invention also includes VLPs that comprise modified HAs obtained from one or more than one influenza subtype.

[00106] The HA monomer may be subdivided in three functional domains - a stem domain, or stem domain cluster (SDC), a globular head domain, or head domain cluster (HDC) and a transmembrane domain cluster (TDC). The SDC and HDC may be collectively referred to as the 'ectodomain'. A publication by Ha et al. 2002 (EMBO J. 21 :865-875; which is incorporated herein by reference) illustrates the relative orientation of the various subdomains of the SDC and HDC in several influenza subtypes, based on Xray crystallographic structures.

[00107] HA protein is synthesized as a precursor protein (HA0) of about 75 kDa, which assembles at the surface into an elongated trimeric protein. The precursor protein is cleaved at a conserved activation cleavage site into 2 polypeptide chains, HA1 and HA2 (comprising the transmembrane region), linked by a disulfide bond.

Figure 15 provides non-limiting examples of amino acid sequences of the linker region for several HAs.

[00108] The term "homotrimer" or "homotrimeric" indicates that an oligomer is formed by three HA protein molecules. Without wishing to be bound by theory, HA protein is synthesized as monomeric precursor protein (HA0) of about 75 kDa, which assembles at the surface into an elongated trimeric protein. Before trimerization occurs, the precursor protein is cleaved at a conserved activation cleavage site (also referred to as fusion peptide) into 2 polypeptide chains, HA1 and HA2 (comprising the transmembrane region), linked by a disulfide bond. The HA1 segment may be 328 amino acids in length, and the HA2 segment may be 221 amino acids in length. Although this cleavage may be important for virus infectivity, it may not be essential for the trimerization of the protein or for immunogenicity. Insertion of HA within the endoplasmic reticulum (ER) membrane of the host cell, signal peptide cleavage and protein glycosylation are co-translational events. Correct refolding of HA requires glycosylation of the protein and formation of 5-6 intra-chain disulfide bonds. The HA trimer assembles within the cis- and trans-Golgi complex, the transmembrane domain playing a role in the trimerization process. The crystal structures of bromelain-treated HA proteins, which lack the transmembrane domain, have shown a highly conserved structure amongst influenza strains. It has also been established that HA undergoes major conformational changes during the infection process, which requires the precursor HA0 to be cleaved into the 2 polypeptide chains HA1 and HA2. The HA protein may be processed (i.e., comprise HA1 and HA2 domains), or may be unprocessed (i.e. comprise the HA0 domain). The unprocessed precursor protein of HA is synthesized as a precursor protein (HA0) of about 75 kDa, which assembles at the surface into an elongated trimeric protein. The precursor protein is cleaved at a conserved cleavage site (also known as a proteolytic loop) into 2 polypeptide chains, HA1 and HA2 (comprising the transmembrane region), linked by a disulfide bond.

[00109] The HA protein as described herein may further be a modified HA (also referred to as “mutant HA”) protein, for example a modified precursor protein (HA0), in which the proteolytic loop or cleavage site is modified.

Modified HA/cleavage site

[00110] Following cleavage of HA0, HA becomes sensitive to pH, undergoing irreversible conformational change at the pH of endosome (<pH 6.0). The conformation of the precursor HA0 is stable at low pH, but the cleaved HA1-HA2 form, is metastable (Bullough PA et. al., 1994, Nature. Vol 371 :37-43). The pH threshold that induce conformational change in different HA's is approximately pH 5.8-5.9 for the B strains, whereas it is more acidic, pH 5.1 to 5.8, for type A HA's (Beyer WEP et al, 1986, Archives Virol, vol 90: 173). Following cleavage, the amino terminal of HA2 is a nonpolar sequence of 23 amino acids that then become a transmembrane domain spraining cross the host cell membrane (called the fusion peptide; Figure 15). The cleavage site of an HA is located on a protruding loop at the surface of the HA, and this site is accessible by proteases.

[00111] In order to optimize the production of vaccine in eggs and maintain an active but attenuated virus, modification of the polybasic cleavage site of H5 (RERRRKKR↓G) has been studied (Horimoto T, et. al, 2006, Vaccine, Vol 24 : 3669-3676). Mutants of interest contained a deletion of the 4 first charged amino acids (RERR) and a replacement of amino acids RKKR with TETR that inactivate the polybasic cleavage site but maintained the possibility to process HA0 to HA1-HA2 through the Arginin residue of the TETR motif (see Figure 19). A similar strategy to produce attenuated virus is employed by NIBSC to abolish the polybasic site allowing producing at high yields the A/Turkey/Turkey/1/2005 H5N1 strain without killing the eggs. The polybasic site sequence (GERRRKKR↓G) is replaced by RETR in their mutant (NIBSC 05/240 NIBSC influenza reference virus NIBG-23). The polybasic cleavage site of a H5 HA has also been replaced by the monobasic site of H6 for expression in eggs. In this example, the first 4 residues and the four last amino acids of the polybasic site are replaced by IETR (replacement of RERRRKKR↓G with IETR↓G; Hoffman E, et. al., 2002, Vaccine, Vol 20 :3165-3170). In each of the examples provided above, the modification was performed to attenuate the virus while maintaining production of the HA within eggs. That is, the cleavage of HA0 precursor was not totally inactivated in order to allow the HA0 to be processed to HA1-HA2 and undergo pH conformational change, thereby permitting virus replication in the host cell.

[00112] As used herein, the term "modified hemagglutinin" or "modified HA", "mutated hemagglutinin" or "mutated HA" refers to an HA in which the HA has a modification or mutation, for example a substitution, insertion, deletion, or a combination thereof, that results in an altered amino acid sequence in the proteolytic loop or cleavage site of the HA protein.

[00113] The crystal structure of HA0 from A/Hong Kong/68 has been determined (Chen, J., 1998. Cell 95:409-417; incorporated herein by reference). Residues that are exposed to solvent are generally thought of being part of the cleavage site which forms an extended, highly exposed surface loop. A consensus sequence may be determined in this chosen region for example, but not limited to:

A/H3/HA0 Consensus: NVPEKQTR/GIFGAIAGFIE (SEQ ID NO: 66)

A/H1/HA0 Consensus: NIPSIQSR/GLFGAIAGFIE (SEQ ID NO: 67)

Avian H5 Consensus QRESRRKKR/GLFGAIAGFIEG (SEQ ID NO: 1)

B/HA0 Consensus: PAKLLKER/GFFGAIAGFLE (SEQ ID NO: 68)

Where the cleavage between HA1 and HA2 is indicated by "/" (see Bianchi et al., 2005, Journal of Virology, 79:7380-7388; incorporated herein by reference), and also Figures 15 and 18A.

[00114] The HA protein may be an influenza type B hemagglutinin or Influenza type A hemagglutinin protein with a modification in the proteolytic loop region, for example a deletion, insertion, substitution or a combination thereof of the proteolytic loop (cleavage site). Without wishing to be bound by theory, modification of the proteolytic loop may ensures that the HA molecule is maintained as an HA0 precursor. Thereby producing a more homogenous and consistent VLP comprising HA0 proteins.

[00115] By "proteolytic loop" or "cleavage site" is meant the consensus sequence of the proteolytic site that is involved in precursor HA0 cleavage. "Consensus" or "consensus sequence" as used herein means a sequence (either amino acid or nucleotide sequence) that comprises the sequence variability of related

sequences based on analysis of alignment of multiple sequences, for example, subtypes of a particular influenza HA0 sequence. Consensus sequence of the influenza HA0 cleavage site may include influenza A consensus hemagglutinin amino acid sequences, including for example consensus H1, consensus H3, consensus H5, or influenza B consensus hemagglutinin amino acid sequences, for example but not limited to B Florida and B Malaysia. Non limiting examples of sequences of the proteoloytic loop region are shown in Figure 15 and 18B (and see Bianchi et al., 2005, Journal of Virology, 79:7380-7388; incorporated herein by reference).

[00116] Residues in the proteolytic loop or cleavage site might be either mutated, for example but not limited to point mutation, substitution, insertion, or deletion. The term "amino acid mutation" or “amino acid modification” as used herein is meant to encompass amino acid substitutions, deletions, insertions, and modifications. Any combination of substitution, deletion, insertion, and modification can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., reduced or abolished cleavage of the proteolytic loop or cleavage site by a protease.

[00117] By “modified proteolytic loop” it is meant that the proteolytic loop may include one or more point mutations, be partially deleted, fully deleted, partially replaced with a linker sequence, fully replaced by a linker sequence, comprise a partial or complete replacement of amino acids within the cleavage site with one or more non-protein amino acids, or a combination thereof. Similarly, by “modified cleavage site”, it is meant that the cleavage site within the proteolytic loop may include one or more point mutations, be partially deleted, fully deleted, partially replaced with a linker sequence, fully replaced by a linker sequence, comprising a partial or complete replacement of amino acids within the cleavage site with one or more non-protein amino acids, or a combination thereof. Modifications to the proteolytic loop , cleavage site, or both, may also involve the deletion, replacement, or substitution of one or more amino acids that are located outside of, or adjacent to, the proteolytic loop or cleavage site sequence. By “linker” it is meant an amino acid sequence comprising one or more amino acids that may be introduced within a proteolytic loop or a cleavage site, or that may replace some or all of the amino acids with the proteolytic loop or cleavage site. A linker may be designed to ensure that

any amino acids deletions within the proteolytic loop or cleavage site do not disrupt the expression or subsequent activity of the modified HA.

[00118] By stabilizing the HA protein by modifying or deleting the proteolytic loop increased product or protein yields may be achieved, when expressing the modified HA in a plant, when compared to a native HA expressed in a plant under the same conditions. Furthermore, by modifying or deleting the proteolytic loop the variability of expression of the expressed modified HA is reduced and the consistency of the produced modified HA is increased, when compared to a native HA expressed in a plant under the same conditions.

[00119] Therefore, the present invention also includes a method of increasing the product yield of a HA protein in a plant. Without wishing to be bound by theory, it is believed that by modifying or deleting the proteolytic loop in an HA protein, improved stability against proteolytic degradation in the plant, stabilization during passage of the HA in the golgi apparatus secretion process, and during the purification process is provided.

[00120] Furthermore, the present invention also includes a method of increasing the product quality of an HA protein expressed in a plant. By product quality, it is meant for example an increased product yield of an HA expressed in a plant, stability of the product for example increased stability of the HA expressed in a plant, consistency of the product for example the production of a homogenous product for example HA0 or a combination thereof.

[00121] By an increase in product or protein yield, it is meant an increase in relative protein yield by about 20% to about 100%, or any amount therebetween as determined using standard techniques in the art, for example, from about 40% to about 70% or any value therebetween for example about 20, 22, 24, 25, 26, 28, 30, 32, 34, 35, 36, 38, 40, 42, 44, 45, 46, 48, 50, 52, 54, 55, 56, 58, 60, 65, 70, 75, 80, 85, 90, 95, or 100%, or any amount therebetween, when compared to the product or protein yield of the same HA protein that does not have its proteolytic loop removed.

[00122] As shown in Figures 13A and 14, HA from B/Brisbane/60/2008 is poorly expressed in agroinfiltrated *Nicotiana benthamiana* leaves (see lane 1008).

However, expression of HA type B that has been modified to delete the proteolytic loop (see lane 1059, Figure 13A, Figure 14) resulted in increased expression. Furthermore, co-expression of HA-type B with M2 from A/New Caledonia/20/99, results in an increase in HA expression (see lanes "1008+1261"; and 1059+1261"). Co-expression of HA type B comprising a deletion in the proteolytic loop, with a M2 from A/Puerto Rico/8/34 also resulted in increased expression (1059+859; Figure 14).

[00123] As further shown in Figure 46B, protein yield of HA protein from H7 A/Hangzhou/1/13 expressed in agroinfiltrated *Nicotiana benthamiana* is increased in HA that has been modified to delete or modify the proteolytic loop. Co-expression of native (wildtype) HA H7 A/Hangzhou/1/13 with M2 from A/New Caledonia/20/99 lead to a 100% relative protein yield, whereas co-expression of HA H7 A/Hangzhou/1/13 comprising a deletion in the proteolytic loop, with M2 from A/New Caledonia/20/99, lead to a 182% relative protein yield (Figure 46B). The increase of relative protein yield of HA comprising a deletion in the proteolytic loop is, however, not dependent on M2. As for example shown in Figure 29A, H7 A/Hangzhou/1/13 comprising a deletion in the proteolytic loop showed increased expression (as measure by the relative HA titer) when compared to native H7 A/Hangzhou/1/13.

[00124] Several strategies were evaluated in order to inactivate the cleavage of HA0 for both A and B strains. The consensus sequence that is recognized by proteases is enclosed on a extended loop, exposed to the solvent, and closed to the membrane distal part to the protein. In the B strain, this loop contains 2 sequence motifs recognized by proteases and the first N-terminal amino acids of the HA2 domain. A point mutation approach (for examples see Table 2, below) to inactivate the cleavage of HA0 precursor resulted in HA0 production, without an increase accumulation of B strain VLP. Deletion of the sequence motifs comprising the 2 protease cleavage motifs (7 amino acids) abolished the accumulation of the B HA. Remove the entire 18-amino acid loop from the HA protein of the B strain and inserting a linker to conserve structural features (beta strands) of the protein structure was effective (see below; Figures 13A, 14, 16A, 17B). Removal or replacement of the proteolytic loop in HA protein of A strains was also effective (see Figures 20, 22).

[00125] Amino acid sequence deletions and insertions include amino acid deletions and insertions of amino acids. A non-limiting example of a deletion in influenza B is the deletion of 17 amino acids (AKLLKERGFFGAIAGFLE) from position 340 to 357 of mature HA protein for example as shown in Figure 18C for influenza B Florida and B Malaysia. This deletion may be replaced by an appropriate linker to link the polypeptide chains for proper expression, for example but not limited to, using the sequence "GG", as shown in Figure 21B (SEQ ID NO:17; modified B/Brisbane/60/2008; replacing AKLLKERGFFGAIAGFLEG with GG; e.g. Construct 1059, Figures 5D, 10; Construct 1039, Figure 8B, or Construct 1467; Figure 7D, 7E). An alternate replacement make comprise replacing "PPAKLLKER" with "GSSSGSSSG", as shown in Figure 21C (SEQ ID NO: 18). Furthermore, the sequence "RESRRKKR" may be replaced with "TETR" or "TETQ", as shown in Figure 19 for influenza H5/Indonesia.

[00126] Alternate amino acid mutations for HA from the A strain include amino acid substitutions, insertions and deletions, for example but not limited to a deletion in the proteolytic loop region of H5 Anhui of the amino acid sequence "RERRRKRGLFGAIAGFIE", a deletion of the amino acid sequence of the proteolytic loop region of H5 Indo comprising "RESRRKKRGLFGAIAGFIE" or a deletion of the amino acid sequence of the proteolytic loop region of H5 Vietnam "RERRRKKRGLFGAIAGFIE". For H3, the sequence "RNVPEKQTRGIF" may be deleted and replaced by an appropriate linker sequence, for example but not limited to "GS" as shown in Figure 21E (SEQ ID NO:20). Alternatively, the sequence "RNVPEKQTR" in H3 may be replaced by "GSSGSSGSS" as shown in Figure 21F (SEQ ID NO: 21; modified H3 A/Perth/16/2009).

[00127] Furthermore, modifying or altering the proteolytic loop or cleavage site of a HA to reduce or abolish cleavage of the proteolytic loop or cleavage site by a protease, may also comprise non-conservative amino acid substitutions, i.e. replacing one amino acid with another amino acid having different structural and/or chemical properties. Non-protein amino acids may also be used for substitution. For example, amino acid substitutions may include replacing a hydrophobic by a hydrophilic amino acid. Amino acid substitutions may include replacement by non-naturally occurring

amino acids or by naturally occurring amino acid derivatives of the protein amino acids.

[00128] Amino acid mutations for HA from the B strain and/or A strains may include amino acid deletions. For example in order to reduce or abolish cleavage of the proteolytic loop or cleavage site by a protease, one or more amino acid are deletion or removal within the proteolytic loop or cleavage site sequence. Non-limiting examples of deletions include removal of amino acids 323 to 341 of native HA H5 protein, for example H5 Anhui (RERRRKRGLFGAIAGFIE), H5 Indo (RESRRKKRGLFGAIAGFIE), or H5 Vietnam (RERRRKKRGLFGAIAGFIE), as shown in Figure 18C. For H3, the sequence "RNVPEKQTRGIF" may be replaced by "GS" (Figure 21E; SEQ ID NO:20), or the H3 sequence "RNVPEKQTR" may be replaced by "GSSGSSGSS" (Figure 21F; SEQ ID NO: 21). For B strains, the sequence "AKLLKERGFFGAIAGFLE" may be deleted and/or replaced by the sequence "GG", as shown in Figure 21B (SEQ ID NO:17), the sequence "AKLLKERGFFGAIAGFLEG" may be replaced with "GG"), or the sequence "PPAKLLKER" replaced with "GSSSGSSSG"(Figure 21C; SEQ ID NO: 18).

[00129] Amino acid mutations can be generated using genetic or chemical methods well known in the art. Genetic methods may include site-directed mutagenesis, PCR, gene synthesis and the like. It is contemplated that methods of altering the side chain group of an amino acid by methods other than genetic engineering, such as chemical modification, may also be useful.

[00130] Therefore the hemagglutinin (HA) sequences of the invention may comprise modified proteolytic loop sequences or cleavage sites, thereby having reduced or abolished cleavage of the proteolytic loop or cleavage site by a protease. The hemagglutinin polypeptide sequences may comprise modified proteolytic loop or modified cleavage site sequences as, for example, set forth in Figures 5D, 7D, 8A, 18C, 19, 21B, 21C, 21E, 21F, 24D, 25D, and 26D. The cleavage sites of any hemagglutinin polypeptide sequence of any influenza strain can be determined or predicted using any number of methods known in the art, including sequence alignment (see for example Figure 15).

[00131] Analysis of sequence from H1, H3 and B HAs reveals that H1 possess one monobasic proteolytic site (Clara type monobasic: Q/EXR) that directly precedes the fusion peptide, whereas H3 and B HAs have 2 proteolytic sites, one that is recognized by Clara-like proteases (as found in H1), and another site recognized by trypsine and chymotrypsine-like proteases (P-E/A-K). The consensus sequence for cleavage of these HA is presented in Table 1.

Table 1 : Consensus sequence of the proteolytic site for precursor HA_0 cleavage. The sequences recognized by Clara tryptases or trypsine/chimotrypsine are italicized, and bolded respectively. Several HA strains comprise polybasic Furin type cleavage sites (RKKR; plain text, underlined).

H1	NIPSI*QSR*↓GLF	SEQ ID NO: 47
H3	NV**PEK***QTR*↓GIF	SEQ ID NO: 48
H5	TGLRNSPQRESRRKKR↓GLF	SEQ ID NO: 60
B	**PAK**LL*KER*↓GFF	SEQ ID NO: 59

[00132] In order to avoid a potential proteolytic cleavage of HA0 precursor of the HA, only one proteolytic site may need to be modified from the sequence of H1, whereas, in the case of H3 and B, two monobasic sites may need to be modified.

[00133] For example, a first cleavage site of HA_0 of B/Florida and B/Brisbane may for example be eliminated by replacing the Lys 341 (mature protein numbering) with an Ile (see Table 2). The second monobasic site may be abolished by replacing three amino acids prior to the fusion peptide, KER (344-346), with NIQ. Sequences of several modified proteolytic loops of HA are provided in Table 2.

Table 2: Illustration of examples of mutations to destroy the cleavage of the precursor HA_0. The monobasic site are italicized (Clara-like recognition) and in bold (no underlining; trypsine/chymotrypsine-like). The mutation are shown as bolded and underlined. The arrow represents the site for cleavage for conversion of HA_0 into HA1-HA2.

Strain	Natural sequence	Abolition of precursor cleavage site
H5/Indo	TGLRNSPQ**RESRRKKR**↓GLF SEQ ID NO:60	TGLRNSPQ***TETR***GLF SEQ ID NO:61 TGLRNSPQ***TETQ***GLF SEQ ID NO:62
H1/Brisbane	NIPSI*QSR*↓GLF SEQ ID NO:47	NIPSIQS***Q***GLF SEQ ID NO:63
H3/Brisbane	NV**PEK***QTR*↓GIF SEQ ID NO:48	NVPE***I***QT***Q***GIF SEQ ID NO:64
B/Florida, B/Brisbane	**PAK**LL*KER*↓GFF SEQ ID NO:59	PA***I***LL***NIQ***GFF SEQ ID NO:65

[00134] In further examples, the sequences comprising the proteolytic loop in HA0 may be replaced or deleted. For example, an H3 variant containing a deletion of the sequence RNVPEKQT at the C-terminus of HA1 in addition of deletion of the N-terminus amino acids GIFGIA of HA2 is provided in Figure 21 E. The shortened HA1-HA2 may be linked together by a GS linker.

[00135] In another example, the loop contain the proteolytic cleavage site in, for example H3, may have been replaced by a flexible linker, and the HA2 part may be left intact. A (GSS)3 linker may be designed in order to accommodate the shortened HA1 to HA2. (see Figure 21F).

[00136] In another example, HA from influenza B may contain a deletion of sequence ALKLLKER at the C-terminus of HA1 in addition of deletion of the N-terminus amino acids GFFGAIAGFLEG of HA2. The shortened HA1-HA2 may be linked together by a GG linker (see for example Figure 21B; Construct 1008). The expression of this construct is shown in Figures 13A and B.

[00137] In another example, HA from influenza B the loop containing the proteolytic site may have been replaced by a flexible linker, and the HA2 part was left

intact. A longer GSSS linker may be designed in order to accommodate the shortened HA1 to HA2. (see for example Figure 21 C).

[00138] As shown in Figures 13A and 14, HA from B/Brisbane/60/2008 is poorly expressed in agroinfiltrated *Nicotiana benthamiana* leaves (see lane 1008). However, expression of HA type B that has been modified to delete the proteolytic loop (see lane 1059, Figure 13A, Figure 14) resulted in increased expression. Furthermore, co-expression of HA-type B with M2 from A/New Caledonia/20/99, results in an increase in HA expression (see lanes "1008+1261"; and 1059+1261"). Co-expression of HA type B comprising a deletion in the proteolytic loop, with a M2 from A/Puerto Rico/8/34 also resulted in increased expression (1059+859; Figure 14).

[00139] In a similar manner, deletion of the proteolytic loop in H5/Indo, and replacement with either a "GG" (Construct 928; see Figure 46D), "TETR" (Construct 676; also see Figures 19, 24D) or "TETQ" (Construct 766; also see Figures 19, 25D) sequence resulted in expression levels that matched or increased over the level of expression observed with native H5/Indo (Construct 489; see Figures 20 and 23).

[00140] As show in Figure 13B, by deleting the proteolytic loop of HA0 (sequence shown in Figure 21B), the resultant HA0 protein exhibits an increased activity as shown by a greater hemagglutination capacity, when compared to a HA protein that does not have its proteolytic loop removed.

[00141] By an increase in activity, it is meant an increase in hemagglutination capacity by about 2% to about 100%, or any amount therebetween as determined using standard techniques in the art, for example, from about 10% to about 50% or any value therebetween for example about 2, 5, 8, 10, 12, 15, 18, 20, 22, 24, 25, 26, 28, 30, 32, 34, 35, 36, 38, 40, 42, 44, 45, 46, 48, 50, 52, 54, 55, 56, 58, 60, 65, 70, 75, 80, 85, 90, 95, or 100%, or any amount therebetween, when compared to the activity of the same HA protein that does not have its proteolytic loop removed.

[00142] The present invention also includes nucleotide sequences encoding modified HA from for example modified H1,H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 or type B HA, or any nucleotide sequences that hybridize to H1,H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15,

H16 or type B HA under stringent conditions, or a nucleotide sequence that hybridizes under stringent hybridisation conditions to a compliment of H1,H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 or type B HA, wherein the nucleotide sequence encodes a hemagglutinin protein that when expressed forms a VLP, and that the VLP induces the production of an antibody. For example, expression of the nucleotide sequence within a plant cell forms a VLP, and the VLP may be used to produce an antibody that is capable of binding HA, including mature HA from B or H3. The VLP, when administered to a subject, induces an immune response. Preferably, the VLP induces the production of an antibody and the VLP, when administered to a subject, induces an immune response.

[00143] For example, expression of the nucleotide sequence within a plant cell forms a VLP, and the VLP may be used to produce an antibody that is capable of binding a virus protein such for example HA, including but not limited to HA0, HA0 protein with its proteolytic loop deleted or modified, HA1 or HA2 of one or more influenza types or subtypes, such for example but not limited to subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, type B HA . The VLP, when administered to a subject, induces an immune response.

[00144] Hybridization under stringent hybridization conditions is known in the art (see for example Current Protocols in Molecular Biology, Ausubel et al., eds. 1995 and supplements; Maniatis *et al.*, in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982; Sambrook and Russell, in Molecular Cloning: A Laboratory Manual, 3rd edition 2001; each of which is incorporated herein by reference). An example of one such stringent hybridization conditions may be about 16-20 hours hybridization in 4 X SSC at 65ºC, followed by washing in 0.1 X SSC at 65ºC for an hour, or 2 washes in 0.1 X SSC at 65°C each for 20 or 30 minutes. Alternatively, an exemplary stringent hybridization condition could be overnight (16-20 hours) in 50% formamide, 4 X SSC at 42ºC, followed by washing in 0.1 X SSC at 65ºC for an hour, or 2 washes in 0.1 X SSC at 65°C each for 20 or 30 minutes, or overnight (16-20 hours), or hybridization in Church aqueous phosphate buffer (7% SDS; 0.5M $NaPO_4$ buffer pH 7.2; 10 mM EDTA) at 65ºC, with 2 washes either at 50ºC in 0.1 X SSC, 0.1% SDS for 20 or 30 minutes each, or 2 washes at 65ºC in 2 X SSC, 0.1% SDS for 20 or 30 minutes each.

[00145] Additionally, the present invention includes nucleotide sequences that are characterized as having about 70, 75, 80, 85, 87, 90, 91, 92, 93 94, 95, 96, 97, 98, 99, 100% or any amount therebetween, sequence identity, or sequence similarity, with the nucleotide sequence encoding H1,H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 or type B HA, wherein the nucleotide sequence encodes a hemagglutinin protein (modified HA) with a modified proteolytic loop sequences or cleavage sites which has reduced or abolished cleavage of the proteolytic loop or cleavage site by a protease. When nucleotide sequence encoding the modified HA is expressed it forms a VLP, and the VLP induces the production of an antibody. For example, expression of the nucleotide sequence within a plant cell forms a VLP, and the VLP may be used to produce an antibody that is capable of binding HA, including unprocessed HA (HA0) or unprocessed wherein the proteolytic loop has been deleted. The VLP, when administered to a subject, induces an immune response.

[00146] Additionally, the present invention includes nucleotide sequences that are characterized as having about 70, 75, 80, 85, 87, 90, 91, 92, 93 94, 95, 96, 97, 98, 99, 100% or any amount therebetween, sequence identity, or sequence similarity, with the nucleotide sequence of SEQ ID NO: 43, 91, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 137, 140, 144, 151, 158, 165, wherein the nucleotide sequence encodes a modified HA protein that when expressed forms a VLP, and that the VLP induces the production of an antibody that is capable of binding HA, including unprocessed HA (HA0) or unprocessed wherein the proteolytic loop has been deleted or modified. The VLP, when administered to a subject, induces an immune response.

[00147] Furthermore, the present invention includes amino acid sequences that are characterized as having about 70, 75, 80, 85, 87, 90, 91, 92, 93 94, 95, 96, 97, 98, 99, 100% or any amount therebetween, sequence identity, or sequence similarity, with the amino acid sequences of SEQ ID NO: 17, 18, 20, 21, 41, 58, 77, 81, 85, 92, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 134, 143, 147, 154, 161,168, 194 and 199. wherein the amino acid sequence encodes a modified HA protein that when expressed forms a VLP, and that the VLP induces the production of an antibody that is capable of binding HA, including unprocessed HA (HA0) or unprocessed wherein the proteolytic loop has been deleted or modified. The VLP, when administered to a subject, induces an immune response.

[00148] Sequence identity or sequence similarity may be determined using a nucleotide sequence comparison program, such as that provided within DNASIS (for example, using, but not limited to, the following parameters: GAP penalty 5, #of top diagonals 5, fixed GAP penalty 10, k-tuple 2, floating gap 10, and window size 5). However, other methods of alignment of sequences for comparison are well-known in the art for example the algorithms of Smith & Waterman (1981, Adv. Appl. Math. 2:482), Needleman & Wunsch (J. Mol. Biol. 48:443, 1970), Pearson & Lipman (1988, Proc. Nat'l. Acad. Sci. USA 85:2444), and by computerized implementations of these algorithms (e.g. GAP, BESTFIT, FASTA, and BLAST)., or by manual alignment and visual inspection. An example of sequence alignment of HAs from different strains of influenza can be found in Figure 24.

For example, but is not limited to, nucleotide sequences encoding:

- a type B HA with a modified proteolytic loop as defined by SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 58, SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114 and SEQ ID NO: 168, or nucleotide sequences encoding type B HAs comprising modified proteolytic loop regions as defined in SEQ ID NO: 65, SEQ ID NO: 72, SEQ ID NO:73, SEQ ID NO:95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113 and SE ID NO: 165.

- an H1 with a modified proteolytic loop include sequences comprising a modified cleavage site as defined by SEQ ID NO: 63.

- an H2 with a modified proteolytic loop include sequences comprising a modified cleavage site as defined by SEQ ID NO: 134.

- an H3 with a modified proteolytic loop include sequences defined by SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO 143, SEQ ID NO: 147 or comprising a modified cleavage site as defined by SEQ ID NO: 64.

an H5 with a deleted proteolytic loop include sequences comprising a modified cleavage site as defined by SEQ ID NO: 61, SEQ ID NO:62, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71.

- an H7 with a modified proteolytic loop include sequences comprising a modified cleavage site as defined by SEQ ID NO: 154 or nucleotide sequences encoding type H7 HAs comprising modified proteolytic loop regions as defined in SEQ ID NO: 151.

- an H9 with a modified proteolytic loop include sequences comprising a modified cleavage site as defined by SEQ ID NO: 161 or nucleotide sequences encoding type H9 HAs comprising modified proteolytic loop regions as defined in SEQ ID NO: 158.

[00149] The present invention pertains to the use of an HA protein comprising the transmembrane domain and includes HA1 and HA2 domains, for example the HA protein may be HA0, or processed HA comprising HA1 and HA2. The HA protein may be used in the production or formation of VLPs using a plant, or plant cell, expression system.

Amplification Elements and Enhancer Elements/ Regulatory Elements

[00150] In another example the modified HA protein may be expressed in an expression system that comprises amplification elements and/or regulatory elements or regions (also referred to herein as enhancer elements). For example an amplification element from a geminivirus such as for example, an amplification element from the bean yellow dwarf virus (BeYDV) may be used to express the modified HA. BeYDV belongs to the Mastreviruses genus adapted to dicotyledonous plants. BeYDV is monopartite having a single-strand circular DNA genome and can replicate to very high copy numbers by a rolling circle mechanism. BeYDV-derived DNA replicon vector systems have been used for rapid high-yield protein production in plants.

[00151] As used herein, the phrase “amplification elements” refers to a nucleic acid segment comprising at least a portion of one or more long intergenic regions (LIR) of a geminivirus genome. As used herein, “long intergenic region” refers to a

region of a long intergenic region that contains a rep binding site capable of mediating excision and replication by a geminivirus Rep protein. In some aspects, the nucleic acid segment comprising one or more LIRs, may further comprises a short intergenic region (SIR) of a geminivirus genome. As used herein, "short intergenic region" refers to the complementary strand (the short IR (SIR) of a Mastreviruses). Any suitable geminivirus-derived amplification element may be used herein. See, for example, WO2000/20557; WO2010/025285; Zhang X. et al. (2005, *Biotechnology and Bioengineering*, Vol. 93, 271-279), Huang Z. et al. (2009, *Biotechnology and Bioengineering,* Vol. 103, 706-714), Huang Z. et al.(2009, *Biotechnology and Bioengineering,* Vol. 106, 9-17); which are herein incorporated by reference). If more than one LIR is used in the construct, for example two LIRs, then the promoter, CMPV-HT regions and the nucleic acid sequence of interest and the terminator are bracketed by each of the two LIRs.

[00152] As described herein, co-delivery of bean yellow dwarf virus (BeYDV)-derived vector and a Rep/RepA-supplying vector, by agroinfiltration of *Nicotiana benthamiana* leaves results in efficient replicon amplification and robust protein production. Western blot analysis of protein extracts from plants transformed with gene constructs driving the expression of modified influenza B HA (from B/Brisbane/60/2008) with or without the proteolytic loop removed (see Figure 17A for constructs) and in the presence or absence of the amplification element BeYDV (construct no. 1059 and 1039) showed that in the absence of BeYDV no accumulation of influenza B HA could be detected (Figure 17B), when the regulatory element was CPMV-HT.

[00153] As shown in Figures 17B, expression of HA from B/Brisbane/60/2008 with the proteolytic loop removed in the absence of BeYDV does not lead to detectable expression by Western Blot analysis (see lane 1039 in Figure 17B). However, expression of HA type B with the proteolytic loop removed in the presence of amplification element BeYDV, results in increased expression (see lane 1059). Similarly, in the absence of BeYDV, co-expression of mutant HA-type B comprising a deletion in the proteolytic loop, with M2 from A/New Caledonia/20/99, does not result in detectable HA expression (see lanes "1039+1261" in Figure 17B). Co-expression of mutant HA type B comprising a deletion in the proteolytic loop in

the presence of BeYDV, with a M2 from A/New Caledonia/20/99 on the other hand resulted in increased expression (see lane "1059+1261"; Figure 17B).

[00154] However, the presence of BeyDV is not required when an enhancer element is present in the expression system and when the enhance element is not CPMV-HT. As for example shown in Figures 29A, expression of various B HA strains under the control of an enhancer element, such for example CPMV 160, CPMV160+ or CPMV HT+, leads to the production of HA proteins that show increased hemagglutination titre (HMG) in the absence of BeYDV.

[00155] Therefore, the mutant (modified) HA protein may be expressed in the absence of an amplification element, such as a geminivirus-based amplification element for example BeYDV, but in the presence of an enhancer element, such for example CPMV 160, CPMV160+ or CPMV HT+.

[00156] The mutant (modified HA) may be expressed in the presence of an enhancer element, such for example CPMV 160, CPMV160+ or CPMV HT+, but in the absence or presence of an amplification element, such for example BeYDV. As shown in Figures 28B, 28C and 28F mutant (modified) HA may be expressed in the presence of an enhancer element, with or without the presence of an amplification element. Therefore the present invention is also directed to the expression of a mutant (modified) HA in the presence of an enhancer element and optionally an amplification element.

[00157] HA constructs comprising an enhancer element (either CMPV HT+ or CMPV 160+) and a proteolytic loop replaced with a GG linker (deleted proteolytic loop) exhibit increased expression when compared to wild type or HA constructs comprising CPMV HT (Figure 28A, H3 Per; Figure 28B, B Malaysia; Figure 28C, H9 HK; Figure 29D, B Mass; Figure 28E, H2 Sin).

[00158] Figure 29A present summary data for hemagglutination titre of modified HA proteins produced in plants comprising CPMV HT, CPMV HT+ , CPMV 160 or CPMV160+, based enhancer elements operatively linked with a nucleotide sequence encoding either modified HA with a deleted proteolytic loop (GG linker) or a native HA. In most cases, the expression (determined as

hemagglutination titer) were higher for the CPMV HT+, CPMV 160 or CPMV160+ based construct demonstrates significant expression levels.

[00159] Enhancer elements may be used to achieve high level of transient expression of mutant (modified) HA proteins with modified proteolytic loops. Enhancer elements may be based on RNA plant viruses, including comoviruses, such as *Cowpea mosaic virus* (CPMV; see, for example, WO2007/135480; WO2009/087391; US 2010/0287670, Sainsbury F. et al., 2008, *Plant Physiology;* 148: 121-1218; Sainsbury F. et al., 2008, *Plant Biotechnology Journal;* 6: 82-92; Sainsbury F. et al., 2009, *Plant Biotechnology Journal;* 7: 682-693; Sainsbury F. et al. 2009, *Methods in Molecular Biology, Recombinant Proteins From Plants*, vol. 483: 25-39).

***CPMV 160 (*CPMVX) *and CPMV 160+* (CPMVX+)**

[00160] In one embodiment the Enhancer Elements are "CPMVX" (also referred as "CPMV 160") and/ or "CPMVX+" (also referred to as "CPMV 160+") as described in US 61/925,852, which is incorporated herein by reference.

[00161] Expression enhancer "CPMVX" comprises a comovirus cowpea mosaic virus (CPMV) 5' untranslated region (UTR). The 5'UTR from nucleotides 1-160 of the CPMV RNA -2 sequence (SEQ ID NO: 93), starts at the transcription start site to the first in frame initiation start codon (at position 161), which serve as the initiation site for the production of the longer of two carboxy coterminal proteins encoded by a wild-type comovirus genome segment. Furthermore a 'third' initiation site at (or corresponding to) position 115 in the CPMV RNA-2 genomic sequence may also be mutated, deleted or otherwise altered. It has been shown that removal of AUG 115 in addition to the removal of AUG 161 enhances expression when combined with an incomplete M protein (Sainsbury and Lomonossoff, 2008, *Plant Physiology;* 148: 1212-1218; WO 2009/087391; which are incorporated herein by reference).

[00162] CPMVX comprises X nucleotides of SEQ ID NO:93, where X=160, 155, 150, or 114 of SEQ ID NO:93, or a sequence that comprises between 80% to 100% sequence similarity with CPMVX, where X=160, 155, 150, or 114 of SEQ ID

NO:93. This expression enhancer is generally referred to as CPMVX (see Figure 26A).

[00163] The expression enhancer CPMVX, where X=160, consists of nucleotides 1-160 of SEQ ID NO: 93:

```
1   tattaaaatc ttaataggtt ttgataaaag cgaacgtggg gaaacccgaa ccaaaccttc
61  ttctaaactc tctctcatct ctcttaaagc aaacttctct cttgtctttc ttgcgtgagc
121 gatcttcaac gttgtcagat cgtgcttcgg caccagtaca (SEQ ID NO:93)
```

[00164] The CPMVX enhancer sequence may further be fused to a stuffer sequence, wherein the CMPVX comprises X nucleotides of SEQ ID NO:1, where X=160, 155, 150, or 114 of SEQ ID NO:1, or a sequence that comprises between 80 to 100 % sequence similarity with CPMVX, where X=160, 155, 150, or 114 of SEQ ID NO:93, and the stuffer sequence comprises from 1-100 nucleotides fused to the 3' end of the CMPVX sequence. For example, the stuffer sequence may comprise from about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides, or any number of nucleotides therebetween.

[00165] If the CMPVX sequence comprises a stuffer fragment, then this expression enhancer may be referred to as CPMVX+ (see Figure 26A), where X=160, 155, 150, 114 of SEQ ID NO:1, it may also be referred to as CMPVX comprising a stuffer sequence, or it may be referred to as CPMV160+; CPMV155+; CPMV150+; CPMV114+, when X-160, 155, 150, or 114, respectively. Constructs comprising CPMVX that do not comprise a stuffer sequence may be termed CPMVX+, where X=160, 155, 150, 114 of SEQ ID NO:93, and where the stuffer sequence is of 0 nucleotides in length.

[00166] The stuffer sequence may be modified by truncation, deletion, or replacement of the native CMPV5'UTR sequence that is located 3'to nucleotide 160. The modified stuffer sequence may be removed, replaced, truncated or shortened when compared to the initial or unmodified (i.e. native) stuffer sequence associated with the 5'UTR (as described in Sainsbury F., and Lomonossoff G.P., 2008, Plant Physiol. 148: pp. 1212-1218). The stuffer sequence may comprise a one or more restriction sites (polylinker, multiple cloning site, one or more cloning sites), one or

more plant kozak sequences, one or more linker sequences, one or more recombination sites, or a combination thereof. For example, which is not to be considered limiting, a stuffer sequence may comprise in series, a multiple cloning site of a desired length fused to a plant kozak sequence. The stuffer sequence does not comprise a nucleotide sequence from the native 5'UTR sequence that is positioned 3' to nucleotide 160 of the native CPMV 5'UTR, for example nucleotides 161 to 512 as shown in Figure 1 of Sainsbury F., and Lomonossoff G.P. (2008, Plant Physiol. 148: pp. 1212-1218; which is incorporated herein by reference), or nucleotides 161-509 of SEQ ID NO:4. That is, the incomplete M protein present in the prior art CPMV HT sequence (Figure 1; of Sainsbury F., and Lomonossoff G.P., 2008) is removed from the 5'UTR in the present invention.

[00167] Plant Kozak consensus sequences are known in the art (see for example Rangan et al. Mol. Biotechnol., 2008, July 39(3), pp. 207-213). Both naturally occurring and synthetic Kozak sequences may be used in the expression enhancer or may be fused to the nucleotide sequence of interest as described herein.

[00168] The plant kozak sequence may be any known plant kozak sequences (see for example L. Rangan et. al. Mol. Biotechnol.2008), including, but not limited to the following plant consensus sequences:

caA(A/C)a (SEQ ID NO:174; plant kingdom)

aaA(A/C)a (SEQ ID NO:175; dicots)

aa(A/G)(A/C)a (SEQ ID NO:176; arabidopsis)

The plant kozak sequence may also be selected from the group of:

AGAAA (SEQ ID NO: 177)
AGACA (SEQ ID NO: 178)
AGGAA (SEQ ID NO: 179)
AAAAA (SEQ ID NO: 180)
AAACA (SEQ ID NO: 181)
AAGCA (SEQ ID NO: 182)
AAGAA (SEQ ID NO: 183)
AAAGAA (SEQ ID NO: 184)
AAAGAA (SEQ ID NO: 185)

(A/-)A(A/G)(A/G)(A/C)A. (SEQ ID NO: 186; Consensus sequence)

[00169] The expression enhancer CPMVX, or CPMVX+, may be operatively linked at the 5'end of the enhancer sequence with a regulatory region that is active in a plant, and operatively linked to a nucleotide sequence of interest at the 3'end of the expression enhancer (Figure 26A), in order to drive expression of the nucleotide sequence of interest within a plant host.

CPMV HT+ ,CPMV HT+[WT115], CPMV HT+ [511]

[00170] In another embodiment the Enhancer Elements is "CPMV HT+" as described in US 61/971,274, which is incorporated herein by reference. Expression enhancer "CPMV HT+" (see Figure 27A) comprises a comovirus 5' untranslated region (UTR) and a modified, lengthened, or truncated stuffer sequence.

[00171] A plant expression system comprising a first nucleic acid sequence comprising a regulatory region, operatively linked with one or more than one expression enhancer as described herein (e.g. CPMV HT+, CPMV HT+[WT115], CPMV HT+ [511]), and a nucleotide sequence encoding a modified HA is also provided. Furthermore, a nucleic acid comprising a promoter (regulatory region) sequence, an expression enhancer (e.g. CPMV HT+ or CPMV HT+[WT115]) comprising a comovirus 5'UTR and a stuffer sequence with a plant kozak sequence fused to one or more nucleic acid sequences encoding a modified HA are described. The nucleic acid may further comprise a sequence comprising a comovirus 3' untranslated region (UTR), for example, a plastocyanin 3' UTR, or other 3'UTR active in a plant, and a terminator sequence, for example a NOS terminator, operatively linked to the 3'end of the nucleotide sequence encoding a modified HA (referred to as nucleotide of interest in Figure 27A), so that the nucleotide sequence encoding the modified HA is inserted upstream from the comovirus 3' untranslated region (UTR), plastocyanin 3' UTR, or other 3'UTR sequence.

[00172] SEQ ID NO:173 comprises a "CPMV HT" expression enhancer as known in the prior art (e.g. Figure 1 of Sainsbury and Lomonossoff 2008, Plant Physiol. 148: pp. 1212-1218; which is incorporated herein by reference). CPMV HT includes the 5'UTR sequence from nucleotides 1-160 of SEQ ID NO:173 with modified nucleotides at position 115 (cgt), and an incomplete M protein with a

modified nucleotide at position 162 (acg), and lacks a plant kozak sequence (5'UTR: nucleotides 1-160; incomplete M protein underlined, nucleotides 161 – 509). SEQ ID NO:173 also includes a multiple cloning site (italics, nucleotides 510-528) which is not present in the prior art CPMV HT sequence:

1 tattaaaatc ttaataggtt ttgataaaag cgaacgtggg gaaacccgaa ccaaaccttc

61 ttctaaactc tctctcatct ctcttaaagc aaacttctct cttgtctttc ttgcgtgagc

121 gatcttcaac gttgtcagat cgtgcttcgg caccagtaca ***acg***ttttctt tcactgaagc

181 gaaatcaaag atctctttgt ggacacgtag tgcggcgcca ttaaataacg tgtacttgtc

241 ctattcttgt cggtgtggtc ttgggaaaag aaagcttgct ggaggctgct gttcagcccc

301 atacattact tgttacgatt ctgctgactt tcggcgggtg caatatctct acttctgctt

361 gacgaggtat tgttgcctgt acttctttct tcttcttctt gctgattggt tctataagaa

421 atctagtatt ttctttgaaa cagagttttc ccgtggtttt cgaacttgga gaaagattgt

481 taagcttctg tatattctgc ccaaatttgt *cgggccc* SEQ ID NO: 173

[00173] CPMV HT+ with a plant kozak consensus sequence is provided in SEQ ID NO:187 (nucleotide 1-160, 5'UTR, including modified ATG at positions 115 (GTG) lower case bold and italics; stuffer fragment comprising: an incomplete M protein underlined, nucleotides 161 – 509, with modified nucleotide at 162 (ACG); a multiple cloning site, italics, nucleotides 510-528; and a consensus plant kozak sequence, caps and bold, nucleotides 529-534).

1 tattaaaatc ttaataggtt ttgataaaag cgaacgtggg gaaacccgaa ccaaaccttc

61 ttctaaactc tctctcatct ctcttaaagc aaacttctct cttgtctttc ttgc***gtg***agc

121 gatcttcaac gttgtcagat cgtgcttcgg caccagtaca ***acg***ttttctt tcactgaagc

181 gaaatcaaag atctctttgt ggacacgtag tgcggcgcca ttaaataacg tgtacttgtc

241 ctattcttgt cggtgtggtc ttgggaaaag aaagcttgct ggaggctgct gttcagcccc

301 atacattact tgttacgatt ctgctgactt tcggcgggtg caatatctct acttctgctt

361 gacgaggtat tgttgcctgt acttctttct tcttcttctt gctgattggt tctataagaa

421 atctagtatt ttctttgaaa cagagttttc ccgtggtttt cgaacttgga gaaagattgt

481 taagcttctg tatattctgc ccaaatttgt *tcgggcccaa taccgcgg*(***A/-)A(A/G)***

(A/G) (A/C)A (SEQ ID NO:187)

[00174] SEQ ID NO:188 ("CPMV HT+ 511") comprises a segment of the native sequence of the CPMV RNA 2 genome from nucleotides 1-154. The 5'UTR sequence from nucleotides 1-511 of SEQ ID NO:188 comprises modified "atg" sequences at positions 115 ("g" in place of "a"; italics bold) and 162 ("c" in place of "t"; italics bold), and an incomplete M protein (underlined) from nucleotides 161 – 511. CPMV HT+ 511 comprises a native M protein kozak consensus sequence (nucleotides 508-511; bold):

1 tattaaaatc ttaataggtt ttgataaaag cgaacgtggg gaaacccgaa ccaaaccttc

61 ttctaaactc tctctcatct ctcttaaagc aaacttctct cttgtctttc ttgc***gtg***agc

121 gatcttcaac gttgtcagat cgtgcttcgg caccagtaca ***acg***ttttctt tcactgaagc

181 gaaatcaaag atctctttgt ggacacgtag tgcggcgcca ttaaataacg tgtacttgtc

241 ctattcttgt cggtgtggtc ttgggaaaag aaagcttgct ggaggctgct gttcagcccc

301 atacattact tgttacgatt ctgctgactt tcggcgggtg caatatctct acttctgctt

361 gacgaggtat tgttgcctgt acttctttct tcttcttctt gctgattggt tctataagaa

421 atctagtatt ttctttgaaa cagagttttc ccgtggtttt cgaacttgga gaaagattgt

481 taagcttctg tatattctgc ccaaatt**tga** **a**... SEQ ID NO: 188

[00175] Another non-limiting example of a CPMV HT+ enhancer sequence is provided by the sequence of SEQ ID NO:189 (CPMV HT+[WT115]). Expression cassettes or vectors comprising CPMV HT+ and including a plant regulatory region in operative association with the expression enhancer sequence of SEQ ID NO: 189, and the transcriptional start site (ATG) at the 3' end fused to a nucleotide sequence encoding modified HA are also part o the present invention.

[00176] SEQ ID NO: 189 (CPMV HT+[WT115]) nucleotide 1-160, 5'UTR, with an ATG at position 115-117, lower case bold; stuffer fragment comprising: an incomplete M protein underlined, nucleotides 161 - 509; with a modified ATG at position 161-153 lower case bold, and underlined, a multiple cloning site, italics, nucleotides 510-528; and a plant kozak sequence, caps and bold, nucleotides 529-534).

1 tattaaaatc ttaataggtt ttgataaaag cgaacgtggg gaaacccgaa ccaaaccttc

61 ttctaaactc tctctcatct ctcttaaagc aaacttctct cttgtctttc ttgc***atg***agc

121 gatcttcaac gttgtcagat cgtgcttcgg caccagtaca ***acg***ttttctt tcactgaagc

181 gaaatcaaag atctctttgt ggacacgtag tgcggcgcca ttaaataacg tgtacttgtc

241 ctattcttgt cggtgtggtc ttgggaaaag aaagcttgct ggaggctgct gttcagcccc

301 atacattact tgttacgatt ctgctgactt tcggcgggtg caatatctct acttctgctt

361 gacgaggtat tgttgcctgt acttctttct tcttcttctt gctgattggt tctataagaa

421 atctagtatt ttctttgaaa cagagttttc ccgtggtttt cgaacttgga gaaagattgt

481 taagcttctg tatattctgc ccaaatttgt *tcgggcccaa taccgcgg****AG AAAA***

(SEQ ID NO:189)

[00177] The plant kozak sequence of SEQ ID NO:189 may be any plant kozak sequence, including but not limited, to one of the sequences of SEQ ID NO's: 174-186.

"Chimeric protein"

[00178] The modified HA might further be a chimeric protein. By "chimeric virus protein" or "chimeric virus polypeptide", also referred to as "chimeric protein" or "chimeric polypeptide", or "chimeric HA" it is meant a protein or polypeptide that comprises amino acid sequences from two or more than two sources, for example but not limited to, two or more influenza types or subtypes, or influenza's of a different origin, that are fused as a single polypeptide. The chimeric protein or polypeptide may include a signal peptide that is the same as, or heterologous with, the remainder of the polypeptide or protein. The chimeric protein or chimeric polypeptide may be produced as a transcript from a chimeric nucleotide sequence, and following synthesis, and as required, may associate to form a multimeric protein. Therefore, a chimeric protein or a chimeric polpypeptide also includes a protein or polypeptide comprising subunits that are associated via disulphide bridges (i.e. a multimeric protein). For example, a chimeric polypeptide comprising amino acid sequences from two or more than two sources may be processed into subunits, and the subunits associated via disulphide bridges to produce a chimeric protein or chimeric

polypeptide. A chimeric HA protein may also comprises an antigenic protein or a fragment thereof of a first influenza virus, and a transmembrane domain complex (TDC) from an second virus influenza HA, including a transmembrane domain and cytosolic tail domains (TM/CT). The polypeptide may be a modified HA, and each of the two or more than two amino acid sequences that make up the polypeptide may be obtained from different HA's to produce a chimeric HA, chimeric influenza HA, chimeric modified HA or chimeric modified influenza HA. A chimeric HA may also include an amino acid sequence comprising heterologous signal peptide (a chimeric HA preprotein) that is cleaved after or during protein synthesis. Preferably, the chimeric polypeptide, or chimeric influenza HA is not naturally occurring. A nucleic acid encoding a chimeric polypeptide may be described as a "chimeric nucleic acid", or a "chimeric nucleotide sequence". For example a chimeric nucleic acid may comprise a nucleotide sequence encoding the modified HA comprises a chimeric nucleotide sequence encoding, in series, a modified HA ectodomain comprising a modified proteolytic loop, an influenza transmembrane domain, and a cytoplasmic tail, wherein the modified HA ectodomain is from a first influenza strain and the transmembrane domain and the cytoplasmic tail are from a second influenza strain. Examples of chimeric nucleotide acids, wherein the modified HA ectodomain is from a first influenza strain and the transmembrane domain and the cytoplasmic tail are from a second influenza strain are given in Examples 5.14, 5.16, 5.18, 5.19, 5. 21 and 5.23. A virus-like particle comprised of chimeric HA may be described as a “chimeric VLP”.

[00179] As described above, the chimeric protein, chimeric polypeptide, or chimeric HA may include a signal peptide that is the same as, or heterologous with, the remainder of the polypeptide or protein. The term "signal peptide" is well known in the art and refers generally to a short (about 5-30 amino acids) sequence of amino acids, found generally at the N-terminus of a polypeptide that may direct translocation of the newly-translated polypeptide to a particular organelle, or aid in positioning of specific domains of the polypeptide chain relative to others. As a non-limiting example, the signal peptide may target the translocation of the protein into the endoplasmic reticulum and/or aid in positioning of the N-terminus proximal domain relative to a membrane-anchor domain of the nascent polypeptide to aid in cleavage

and folding of the mature protein, for example a modified HA or chimeric modified HA.

[00180] The HA may also be a chimeric HA or chimeric modified HA, wherein a native transmembrane domain of the HA or modified HA is replaced with a heterologous transmembrane domain. The transmembrane domain of HA proteins is highly conserved (see for example Figure 1C of WO 2010/148511; which is incorporated herein by reference). The heterologous transmembrane domain may be obtained from any HA transmembrane domain, for example but not limited to the transmembrane domain from H1 California, B/Florida/4/2006 (GenBank Accession No. ACA33493.1), B/Malaysia/2506/2004 (GenBank Accession No. ABU99194.1), H1/Bri (GenBank Accession No. ADE28750.1), H1 A/Solomon Islands/3/2006 (GenBank Accession No. ABU99109.1), H1/NC (GenBank Accession No. AAP34324.1), H2 A/Singapore/1/1957 (GenBank Accession No. AAA64366.1), H3 A/Brisbane/10/2007 (GenBank Accession No. ACI26318.1), H3 A/Wisconsin/67/2005 (GenBank Accession No. ABO37599.1), H5 A/Anhui/1/2005 (GenBank Accession No. ABD28180.1), H5 A/Vietnam/1194/2004 (GenBank Accession No. ACR48874.1), H5-Indo (GenBank Accession No. ABW06108.1),. The transmembrane domain may also be defined by the following consensus amino acid sequence:

iLXiYystvAiSslXlXXmlagXsXwmcs (SEQ ID NO:94)

[00181] Examples of constructs comprising a chimeric HA with a heterologous trans-membrane domain include: construct number 1875 (CPMV-HT+ B Brisbane/60/08 with deleted proteolytic loop + H1TM, with trans-membrane domain and cytoplasmic tail replaced by H1 A/California/07/2009; see example 5.19), construct number 1977 (CPMV-160+ B Brisbane/60/08 with deleted proteolytic loop + H1TM, with trans-membrane domain and cytoplasmic tail replaced by H1 A/California/07/2009; see example 5.14), construct number 1067 (CPMV-HT B Brisbane/60/08 with deleted proteolytic loop + H1TM, with trans-membrane domain and cytoplasmic tail replaced by H1 A/California/07/2009; see example 5.14), construct number 2074 (CPMV HT B Massachusetts/2/2012+H1Tm, with trans-

membrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009; see Example 5.16), construct number 2060 (CPMV HT160+ Massachusetts/2/2012+H1Tm, with trans-membrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009; see Example 5.16), construct number 2062 (CPMV 160+ B Massachusetts/2/2012+H1Tm, with trans-membrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009; see Example 5.21), construct number 1860 (CPMV HT+ B Wisconsin/1/2010+H1Tm with trans-membrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009; see Example 5.23), construct number 1454 (CPMV HT B Wisconsin/1/2010+H1Tm with trans-membrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009, see Example 5.18) and construct number 1893 (CPMV 160+ B Wisconsin/1/2010+H1Tm with trans-membrane domain and cytoplasmic tail replaced by those of H1 A/California/07/2009) see Example 5.18. . Activity of these chimeric modified HA's is shown in Figures 26B and 27B.

Signal Peptide

[00182] A signal peptide (SP) may be native to the modified HA or chimeric modified HA, or a signal peptide may be heterologous with respect to the primary sequence of the modified HA being expressed. The modified HA may comprise a signal peptide from a first influenza type, subtype or strain with the balance of the HA from one or more than one different influenza type, subtype or strain. For example the native signal peptide of HA subtypes H1, H2, H3, H5, H6, H7, H9 or influenza type B may be used to express the modified HA in a plant system. In some embodiments of the invention, the SP may be of an influenza type B, H1, H3 or H5; or of the subtype H1/Bri, H1/NC, H5/Indo, H3/Bri or B/Flo.

[00183] Furthermore, the modified HA or chimeric modified HA may comprise a native, or a non-native signal peptide; the non-native signal peptide may be of plant origin or obtained from an animal or bacterial polypeptide. The native signal peptide may correspond to that of the HA or modified HA being expressed, additionally, the signal peptide may be from a structural protein or hemagglutinin of a virus other than influenza. Non-limiting examples of a signal peptide that may be used is that of alfalfa protein disulfide isomerase (PDI SP; nucleotides 32-103 of Accession No.

Z11499 also see WO 2009/076778; WO 2010/148511, or WO 2010/003235), or the patatin signal peptide (PatA SP; located nucleotides 1738 - 1806 of GenBank Accession number A08215). The nucleotide sequence of PatA SP for this accession number is:

ATGGCAACTACTAAAACTTTTTTAATTTTATTTTTTATGATATTAGCAACTACTAGTTCAACA TGTGCT (SEQ ID NO: 171)

the amino acid sequence of patatin A signal peptide is :

MATTKTFLILFFMILATTSSTCA (SEQ ID NO: 172)

[00184] . The present invention therefore provides for a modified HA or chimeric modified HA comprising a native, or a non-native signal peptide, and nucleic acids encoding such chimeric modified HA proteins.

Co-expression with channel protein

[00185] The mutant (modified) HA may be produced in a plant by co-expressing a first nucleic acid encoding the modified HA with a second nucleic acid encoding a channel protein, for example but not limited to a proton channel protein. The first and second nucleic acids may be introduced to the plant in the same step, or they may be introduced to the plant sequentially. The first and second nucleic acids may be introduced in the plant in a transient manner, or in a stably manner. Furthermore, a plant that expresses a first nucleic acid encoding the modified HA may transformed with a channel protein, for example but not limited to a proton channel protein, (second nucleic acid) so that both the first and the second nucleic acids are co-expressed in the plant. Alternatively, a plant that expresses a channel protein, for example but not limited to a proton channel protein, (second nucleic acid) may transformed with a first nucleic acid encoding the modified HA so that both the first and the second nucleic acids are co-expressed in the plant. Additionally, a first plant expressing the first nucleic acid encoding modified HA, may be crossed with a second plant expressing the second nucleic acid encoding the channel proteinfor example but not limited to a proton channel protein, to produce a progeny plant that co-expresses the first and second nucleic acids encoding the modified HA and the channel protein, for example but not limited to a proton channel protein, respectively.

[00186] Without wishing to be bound by theory, the pH of a cellular compartment comprising modified HA, including the Golgi apparatus, may be important for the folding, stability and /or proteolysis of HA. Proton channel proteins, such as for example influenza M2 and BM2 protein may regulate the pH in cellular compartments. For example, M2 regulates the potentiation of membrane fusion by buffering intracellular compartments both in late and early stages of influenza viral replication.

[00187] By co-expressing a channel protein, for example but not limited to a proton channel protein, along with a modified HA, the pH within the Golgi apparatus may increase, and result in an increase in stability, reduction of degradation, or a combination thereof, and increase expression levels and yield of modified HA and/or VLPs.

[00188] By co-expressing a modified HA along with a channel protein, for example but not limited to a proton channel protein, in a plant, increased yield of HA and/or VLPs are observed, when compared to a plant that expressed the modified without co-expression of the channel protein, for example but not limited to a proton channel protein (see Figures 13A and 14). As shown for example in Figure 13A, the co-expression of M2 with the modified influenza B HA increased HA accumulation level (Figure 13A, 1059 vs 1059+1261) .

[00189] Furthermore, the efficacy of M2 from influenza A/Puerto Rico/8/1934 to increase accumulation of the modified influenza B HA and H3 was compared to that of M2 from influenza A/New Caledonia/20/1999. For the modified influenza B HA, the comparison was undertaken by western blot analysis of protein extracts from plants transformed with constructs 1059, 1059+1261 and 1059+859. The results obtained demonstrated that the co-expression of M2 from influenza A/Puerto Rico/8/1934 (encoded by construct no. 859) was as efficient as the co-expression of M2 from influenza A/New Caledonia/20/1999 (encoded by construct no. 1261) for increasing accumulation of the modified influenza B HA (Figure 14).

[00190] As used herein, the terms “M2,” “M2 protein,” “M2 sequence” and “M2 domain” refer to all or a portion of an M2 protein sequence isolated from, based upon or present in any naturally occurring or artificially produced influenza virus

strain or isolate. Thus, the term M2 and the like include naturally occurring M2 sequence variants produced by mutation during the virus life-cycle or produced in response to a selective pressure (e.g., drug therapy, expansion of host cell tropism or infectivity, etc.), as well as recombinantly or synthetically produced M2 sequences. Non-limiting example of sequences that may be used with the present invention include M2 from A/Puerto Rico/8/1934 and M2 from A/New Caledonia/20/1999.

Immune Response

[00191] An "immune response" generally refers to a response of the adaptive immune system. The adaptive immune system generally comprises a humoral response, and a cell-mediated response. The humoral response is the aspect of immunity that is mediated by secreted antibodies, produced in the cells of the B lymphocyte lineage (B cell). Secreted antibodies bind to antigens on the surfaces of invading microbes (such as viruses or bacteria), which flags them for destruction. Humoral immunity is used generally to refer to antibody production and the processes that accompany it, as well as the effector functions of antibodies, including Th2 cell activation and cytokine production, memory cell generation, opsonin promotion of phagocytosis, pathogen elimination and the like. The terms "modulate" or "modulation" or the like refer to an increase or decrease in a particular response or parameter, as determined by any of several assays generally known or used, some of which are exemplified herein.

[00192] A cell-mediated response is an immune response that does not involve antibodies but rather involves the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. Cell-mediated immunity is used generally to refer to some Th cell activation, Tc cell activation and T-cell mediated responses. Cell mediated immunity is of particular importance in responding to viral infections.

[00193] For example, the induction of antigen specific CD8 positive T lymphocytes may be measured using an ELISPOT assay; stimulation of CD4 positive T-lymphocytes may be measured using a proliferation assay. Anti-influenza antibody titres may be quantified using an ELISA assay; isotypes of antigen-specific or cross reactive antibodies may also be measured using anti-isotype antibodies (e.g. anti -IgG,

IgA, IgE or IgM). Methods and techniques for performing such assays are well-known in the art.

[00194] Cross-reactivity HAI titres may also be used to demonstrate the efficacy of an immune response to other strains of virus related to the vaccine subtype. For example, serum from a subject immunized with a vaccine composition of a first strain (e.g. VLPs of A/Indonesia 5/05) may be used in an HAI assay with a second strain of whole virus or virus particles (e.g. A/Vietnam/1194/2004), and the HAI titer determined.

[00195] Cytokine presence or levels may also be quantified. For example a T-helper cell response (Th1/Th2) will be characterized by the measurement of IFN-γ and IL-4 secreting cells using by ELISA (e.g. BD Biosciences OptEIA kits). Peripheral blood mononuclear cells (PBMC) or splenocytes obtained from a subject may be cultured, and the supernatant analyzed. T lymphocytes may also be quantified by fluorescence-activated cell sorting (FACS), using marker specific fluorescent labels and methods as are known in the art.

[00196] A microneutralization assay may also be conducted to characterize an immune response in a subject, see for example the methods of Rowe et al., 1973. Virus neutralization titers may be obtained several ways, including: 1) enumeration of lysis plaques (plaque assay) following crystal violet fixation/coloration of cells; 2) microscopic observation of cell lysis in culture; 3) ELISA and spectrophotometric detection of NP virus protein (correlate with virus infection of host cells).

[00197] The term “virus like particle” (VLP), or "virus-like particles" or "VLPs" refers to structures that self-assemble and comprise virus proteins for example an influenza HA protein or modified HA protein such for example an HA0 protein, wherein the proteolytic loop has been modified. VLPs are generally morphologically and antigenically similar to virions produced in an infection, but lack genetic information sufficient to replicate and thus are non-infectious. In some examples, VLPs may comprise a single protein species, or more than one protein species. For VLPs comprising more than one protein species, the protein species may be from the same species of virus, or may comprise a protein from a different species, genus, subfamily or family of virus (as designated by the ICTV

nomenclature). In other examples, one or more of the protein species comprising a VLP may be modified from the naturally occurring sequence, such for example a modified HA as described herein. VLPs may be produced in suitable host cells including plant and insect host cells. Following extraction from the host cell and upon isolation and further purification under suitable conditions, VLPs may be purified as intact structures.

[00198] Furthermore, VLPs may be produced that comprise a combination of HA subtypes. For example, VLPs may comprise one or more than one HA or one or more than one modified HA from the subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, subtype B HA or a combination thereof. Selection of the combination of HAs or modified HAs may be determined by the intended use of the vaccine prepared from the VLP. For example a vaccine for use in inoculating birds may comprise any combination of HA subtypes or modified HA subtypes, while VLPs useful for inoculating humans may comprise subtypes one or more than one of subtypes or modified subtype of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, subtype B HA . However, other HA subtype or modified HA subtype combinations may be prepared depending upon the use of the VLP. In order to produce VLPs comprising combinations of HA subtypes or modified subtype HAs, the desired HA subtype or modified HA subtype may be co-expressed within the same cell, for example a plant cell.

[00199] The VLPs produced from influenza derived proteins, in accordance with the present invention do not comprise M1 protein. The M1 protein is known to bind RNA (Wakefield and Brownlee, 1989) which is a contaminant of the VLP preparation. The presence of RNA is undesired when obtaining regulatory approval for the VLP product, therefore a VLP preparation lacking RNA may be advantageous.

[00200] The VLPs produced as described herein do not typically comprise neuramindase (NA). However, NA may be co-expressed with HA should VLPs comprising HA and NA be desired.

[00201] The invention also includes, but is not limited to, virus derived VLPs that obtain a lipid envelope from the plasma membrane of the cell in which the VLP

proteins are expressed. For example, if the VLP is expressed in a plant-based system, the VLP may obtain a lipid envelope from the plasma membrane of the cell.

[00202] Generally, the term "lipid" refers to a fat-soluble (lipophilic), naturally-occurring molecules. The term is also used more specifically to refer to fatty-acids and their derivatives (including tri-, di-, and monoglycerides and phospholipids), as well as other fat-soluble sterol-containing metabolites or sterols. Phospholipids are a major component of all biological membranes, along with glycolipids, sterols and proteins. Examples of phospholipids include phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and the like. Examples of sterols include zoosterols (e.g., cholesterol) and phytosterols. Over 200 phytosterols have been identified in various plant species, the most common being campesterol, stigmasterol, ergosterol, brassicasterol, delta-7-stigmasterol, delta-7-avenasterol, daunosterol, sitosterol, 24-methylcholesterol, cholesterol or beta-sitosterol. As one of skill in the art would understand, the lipid composition of the plasma membrane of a cell may vary with the culture or growth conditions of the cell or organism from which the cell is obtained.

[00203] Cell membranes generally comprise lipid bilayers, as well as proteins for various functions. Localized concentrations of particular lipids may be found in the lipid bilayer, referred to as 'lipid rafts'. Without wishing to be bound by theory, lipid rafts may have significant roles in endo and exocytosis, entry or egress of viruses or other infectious agents, inter-cell signal transduction, interaction with other structural components of the cell or organism, such as intracellular and extracellular matrices.

[00204] In plants, influenza VLPs bud from the plasma membrane therefore the lipid composition of the VLPs reflects their origin. The VLPs produced according to the present invention comprise HA of one or more than one type or subtype of influenza, complexed with plant derived lipids. Plant lipids can stimulate specific immune cells and enhance the immune response induced. Plant membranes are made of lipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and also contain glycosphingolipids, saponins, and phytosterols. Additionally, lipid rafts are also found in plant plasma membranes - these microdomains are enriched in

sphingolipids and sterols. In plants, a variety of phytosterols are known to occur, including stigmasterol, sitosterol, 24-methylcholesterol and cholesterol (Mongrand et al., 2004).

[00205] PC and PE, as well as glycosphingolipids can bind to CD1 molecules expressed by mammalian immune cells such as antigen-presenting cells (APCs) like dendritic cells and macrophages and other cells including B and T lymphocytes in the thymus and liver (Tsuji M,. 2006). CD1 molecules are structurally similar to major histocompatibility complex (MHC) molecules of class I and their role is to present glycolipid antigens to NKT cells (Natural Killer T cells). Upon activation, NKT cells activate innate immune cells such as NK cells and dendritic cells and also activate adaptive immune cells like the antibody-producing B cells and T-cells.

[00206] A variety of phytosterols may be found in a plasma membrane – the specific complement may vary depending on the species, growth conditions, nutrient resources or pathogen state, to name a few factors. Generally, beta-sitosterol is the most abundant phytosterol.

[00207] The phytosterols present in an influenza VLP complexed with a lipid bilayer, such as an plasma-membrane derived envelope may provide for an advantageous vaccine composition. Without wishing to be bound by theory, plant-made VLPs complexed with a lipid bilayer, such as a plasma-membrane derived envelope, may induce a stronger immune reaction than VLPs made in other expression systems, and may be similar to the immune reaction induced by live or attenuated whole virus vaccines.

[00208] The VLP as described herein may be complexed with a plant-derived lipid bilayer. In some embodiments the plant-derived lipid bilayer may comprise the envelope of the VLP. The plant derived lipids may comprise lipid components of the plasma membrane of the plant where the VLP is produced, including, but not limited to, phosphatidylcholine (PC), phosphatidylethanolamine (PE), glycosphingolipids, phytosterols or a combination thereof. A plant-derived lipid may alternately be referred to as a ‘plant lipid’. Examples of phytosterols are known in the art, and include, for example, stigmasterol, sitosterol, 24-methylcholesterol and cholesterol – see, for example, Mongrand et al., 2004.

[00209] VLPs may be assessed for structure and size by, for example, hemagglutination assay, electron microscopy, or by size exclusion chromatography.

[00210] For size exclusion chromatography, total soluble proteins may be extracted from plant tissue by homogenizing (Polytron) sample of frozen-crushed plant material in extraction buffer, and insoluble material removed by centrifugation. Precipitation with PEG may be used. The soluble protein is quantified, and the extract passed through a size exclusion matrix, for example but not limited to Sephacryl™. Following chromatography, fractions may be further analyzed by immunoblot to determine the protein complement of the fraction.

[00211] Without wishing to be bound by theory, the capacity of HA to bind to RBC from different animals is driven by the affinity of HA for sialic acids α2,3 or α2,3 and the presence of these sialic acids on the surface of RBC. Equine and avian HA from influenza viruses agglutinate erythrocytes from all several species, including turkeys, chickens, ducks, guinea pigs, humans, sheep, horses and cows; whereas human HAs will bind to erythrocytes of turkey, chickens, ducks, guina pigs, humans and sheep (see also Ito T. et al, 1997, Virology, vol 227, p493-499; and Medeiros R et al, 2001, Virology, vol 289 p.74-85).

[00212] Correct folding of the expressed virus protein may be important for stability of the protein, formation of multimers, formation of VLPs, function of the virus protein and recognition of the virus protein by an antibody, among other characteristics. Folding and accumulation of a protein may be influenced by one or more factors, including, but not limited to, the sequence of the protein, the relative abundance of the protein, the degree of intracellular crowding, the pH in a cell compartment, the availability of cofactors that may bind or be transiently associated with the folded, partially folded or unfolded protein, the presence of one or more chaperone proteins, or the like.

[00213] Heat shock proteins (Hsp) or stress proteins are examples of chaperone proteins, which may participate in various cellular processes including protein synthesis, intracellular trafficking, prevention of misfolding, prevention of protein aggregation, assembly and disassembly of protein complexes, protein folding, and protein disaggregation. Examples of such chaperone proteins include, but are not

limited to, Hsp60, Hsp65, Hsp 70, Hsp90, Hsp100, Hsp20-30, Hsp10, Hsp100-200, Hsp100, Hsp90, Lon, TF55, FKBPs, cyclophilins, ClpP, GrpE, ubiquitin, calnexin, and protein disulfide isomerases (see, for example, Macario, A.J.L., *Cold Spring Harbor Laboratory Res.* 25:59-70. 1995; Parsell, D.A. & Lindquist, S. *Ann. Rev. Genet.* 27:437-496 (1993); U.S. Patent No. 5,232,833). As described herein, chaperone proteins, for example but not limited to Hsp40 and Hsp70 may be used to ensure folding of a virus protein.

[00214] Examples of Hsp70 include Hsp72 and Hsc73 from mammalian cells, DnaK from bacteria, particularly mycobacteria such as *Mycobacterium* leprae, *Mycobacterium tuberculosis,* and *Mycobacterium bovis* (such as Bacille-Calmette Guerin: referred to herein as Hsp7l). DnaK from *Escherichia coli,* yeast and other prokaryotes, and BiP and Grp78 from eukaryotes, such as *A. thaliana* (Lin et al. 2001 (Cell Stress and Chaperones 6:201-208). A particular example of an Hsp70 is *A. thaliana* Hsp70 (encoded by Genbank ref: AY120747.1). Hsp70 is capable of specifically binding ATP as well as unfolded polypeptides and peptides, thereby participating in protein folding and unfolding as well as in the assembly and disassembly of protein complexes.

[00215] Examples of Hsp40 include DnaJ from prokaryotes such as *E. coli* and mycobacteria and HSJ1, HDJl and Hsp40 from eukaryotes, such as alfalfa (Frugis et al., 1999. Plant Molecular Biology 40:397-408). A particular example of an Hsp40 is *M. sativa* MsJ1 (Genbank ref: AJ000995.1). Hsp40 plays a role as a molecular chaperone in protein folding, thermotolerance and DNA replication, among other cellular activities.

[00216] Among Hsps, Hsp70 and its co-chaperone, Hsp40, are involved in the stabilization of translating and newly synthesized polypeptides before the synthesis is complete. Without wishing to be bound by theory, Hsp40 binds to the hydrophobic patches of unfolded (nascent or newly transferred) polypeptides, thus facilitating the interaction of Hsp70-ATP complex with the polypeptide. ATP hydrolysis leads to the formation of a stable complex between the polypeptide, Hsp70 and ADP, and release of Hsp40. The association of Hsp70-ADP complex with the hydrophobic patches of the polypeptide prevents their interaction with other hydrophobic patches, preventing

the incorrect folding and the formation of aggregates with other proteins (reviewed in Hartl, FU. 1996. Nature 381:571-579).

[00217] Native chaperone proteins may be able to facilitate correct folding of low levels of recombinant protein, but as the expression levels increase, the abundance of native chaperones may become a limiting factor. High levels of expression of virus protein in the agroinfiltrated leaves may lead to the accumulation of virus protein in the cytosol, and co-expression of one or more than one chaperone proteins such as Hsp70, Hsp40 or both Hsp70 and Hsp40 may reduce the level of misfolded or aggregated proteins, and increase the number of proteins exhibiting tertiary and quaternary structural characteristics that allow for formation of virus-like particles.

[00218] Therefore, the present invention also provides for a method of producing virus protein VLPs in a plant, wherein a first nucleic acid encoding a virus protein is co-expressed with a second nucleic acid encoding a channel protein, for example but not limited to a proton channel protein, and a third nucleic acid encoding a chaperone. The first, second and third nucleic acids may be introduced to the plant in the same step, or may be introduced to the plant sequentially.

[00219] The VLP produced within a plant may induce an virus protein comprising plant-specific N-glycans. Therefore, this invention also provides for a VLP comprising virus protein having plant specific N-glycans.

[00220] Furthermore, modification of N-glycan in plants is known (see for example WO 2008/151440; WO 2010/006452; or U.S. 60/944,344; which are incorporated herein by reference) and virus protein having modified N-glycans may be produced. Virus protein comprising a modified glycosylation pattern, for example with reduced fucosylated, xylosylated, or both, fucosylated and xylosylated, N-glycans may be obtained, or virus protein having a modified glycosylation pattern may be obtained, wherein the protein lacks fucosylation, xylosylation, or both, and comprises increased galatosylation. Furthermore, modulation of post-translational modifications, for example, the addition of terminal galactose may result in a reduction of fucosylation and xylosylation of the expressed virus protein when compared to a wild-type plant expressing virus protein.

[00221] For example, which is not to be considered limiting, the synthesis of virus protein having a modified glycosylation pattern may be achieved by co-expressing the protein of interest along with a nucleotide sequence encoding beta-1.4galactosyltransferase (GalT), for example, but not limited to mammalian GalT, or human GalT however GalT from another sources may also be used. The catalytic domain of GalT may also be fused to a CTS domain (i.e. the cytoplasmic tail, transmembrane domain, stem region) of N-acetylglucosaminyl transferase (GNT1), to produce a GNT1-GalT hybrid enzyme, and the hybrid enzyme may be co-expressed with virus protein. The virus protein may also be co-expressed along with a nucleotide sequence encoding N-acetylglucosaminyltrasnferase III (GnT-III), for example but not limited to mammalian GnT-III or human GnT-III, GnT-III from other sources may also be used. Additionally, a GNT1-GnT-III hybrid enzyme, comprising the CTS of GNT1 fused to GnT-III may also be used.

[00222] Therefore the present invention also includes VLP's comprising one or more virus protein having modified N-glycans.

[00223] Non-limiting example of sequences that may be used with the present invention to produce modified HA's also include those described in WO 2009/009876; WO 2009/076778; WO 2010/003225; WO 2010/148511; WO 2010/003235; WO 2010/006452 (which are herein incorporated by reference), for example, but not limited to:

H1 protein encoded by the nucleic acid molecule for example from A/Brisbane/59/2007 (H1N1), A/New Caledonia/20/99 (H1N1) , A/Solomon Islands 3/2006 (H1N1), /PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), strain;

H2 protein encoded by the nucleic acid molecule may be from the A/Singapore/1/57 (H2N2) strain;

H3 protein encoded by the nucleic acid molecule may be from the A/Brisbane 10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2) strain, A/Victoria/361/2011 (H3N2) or A/Perth/16/2009 (H3N2);

H5 protein encoded by the nucleic acid molecule may be from the A/Anhui/1/2005 (H5N1), A/Indonesia/5/2005 (H5N1), A/Vietnam/1194/2004 (H5N1)

H6 protein encoded by the nucleic acid molecule may be from the A/Teal/HongKong/W312/97 (H6N1) strain;

H7 protein encoded by the nucleic acid molecule may also be from the A/ Hangzhou/1/13 (H7N9), A/Equine/Prague/56 (H7N7) strain;

H9 protein encoded by the nucleic acid molecule may be from the A/HongKong/1073/99 (H9N2) strain;

HA protein from B subtype encoded by the nucleic acid may be from the B/Florida/4/2006, B/Massachusetts/2/12, B/Malaysia/2506/2004, B/Wisconsin/1/2010, or B/Brisbane/60/2008 strain.

[00224] Table 3: Examples of constructs that have been prepared as described herein:

Constr. #	Expression Enhancer	Ampl. Element	Description	Example
1261	CPMV-HT		A/New Caledonia/20/1999 (H1N1)	Example 5.1
859	CPMV-HT		A/Puerto Rico/8/1934 (H1N1)	Example 5.2
1008	CPMV-HT	BeYDV	B/Brisbane/60/2008	Example 5.3
1059	CPMV-HT	BeYDV	B/Brisbane/60/2008 with deleted proteolytic loop	Example 5.4
1462	CPMV-HT	BeYDV	B/Wisconsin/1/2010	Example 5.5
1467	CPMV-HT	BeYDV	B/Wisconsin/1/2010 with deleted proteolytic loop	Example 5.6
1039	CPMV-HT		B/Brisbane/60/2008 with deleted proteolytic loop	Example 5.7
1029	CPMV-HT		B/Brisbane/60/2008	Example 5.11
1829	CPMV-HT+		B/Brisbane/60/2008 with deleted proteolytic loop	Example 5.12
1937	CPMV-160+		B/Brisbane/60/2008 with deleted proteolytic loop	Example 5.13
1067	CPMV-HT		B/Brisbane/60/2008 with deleted proteolytic loop +H1 California TMCT	Example 5.14
1977	CPMV-160+		B/Brisbane/60/2008 with deleted proteolytic loop +H1 California TMCT	Example 5.14
1875	CPMV-HT+		B/Brisbane/60/2008 with deleted proteolytic loop +H1 California TMCT	Example 5.19
676	CPMV-HT		H5 A/Indonesia/5/2005 with TETR cleavage site	Example 5.8
766	CPMV-HT		H5 A/Indonesia/5/2005 with TETQ cleavage site	Example 5.9
928	CPMV-HT		H5 A/Indonesia/5/2005 with deleted proteolytic loop	Example 5.10
489	CPMV-HT		H5 A/Indonesia/5/2005 (native)	Example 5.24
2220	CPMV-		H2 A/Singapore/1/57 (native)	Example 5.27

	HT+			
2221	CPMV-HT+		H2 A/Singapore/1/57 with deleted proteolytic loop	Example 5.28
2222	CPMV-160+		H2 A/Singapore/1/57 (native)	Example5.29
2223	CPMV-160+		H2 A/Singapore/1/57 with deleted proteolytic loop	Example5.29
2019	CPMV-HT+		H3 A/Perth/16/09 (native)	Example 5.30
2039	CPMV-HT+		H3 A/Perth/16/09 with deleted proteolytic loop	Example 5.31
2139	CPMV-160+		H3 A/Perth/16/09 (native)	Example 5.30
2159	CPMV-160+		H3 A/Perth/16/09 with deleted proteolytic loop	Example 5.31
1819	CPMV-HT+		H3 A/Victoria /361/11 (native)	Example 5.26
2230	CPMV-HT+		H3 A/Victoria /361/11 with deleted proteolytic loop	Example 5.32
1800	CPMV-160+		H3 A/Victoria /361/11 (native)	Example 5.25
2250	CPMV-160+		H3 A/Victoria /361/11 with deleted proteolytic loop	Example 5.32
2142	CPMV-HT+		H7 A/Hangzhou/1/13 (native)	Example 5.33
2152	CPMV-HT+		H7 A/Hangzhou/1/13 with deleted proteolytic loop	Example 5.34
2224	CPMV-HT+		H9 A/Hong Kong/1073/99 (native)	Example 5.35
2225	CPMV-HT+		H9 A/Hong Kong/1073/99 with deleted proteolytic loop	Example 5.36
2226	CPMV-160+		H9 A/Hong Kong/1073/99 (native)	Example 5.35
2227	CPMV-160+		H9 A/Hong Kong/1073/99 with deleted proteolytic loop	Example 5.36
2013	CPMV-160+		B/Malaysia /2506/04 (native)	Example 5.37
2014	CPMV-160+		B/Malaysia /2506/04 with deleted proteolytic loop	Example 5.38
2070	CPMV-HT		B/Massachusetts/2/12 (native)	Example 5.39
2072	CPMV-HT		B/Massachusetts/2/12 with deleted proteolytic loop	Example 5.15
2080	CPMV-HT+		B/Massachusetts/2/12 (native)	Example 5.39
2052	CPMV-HT+		B/Massachusetts/2/12 with deleted proteolytic loop	Example 5.20
2090	CPMV-160+		B/Massachusetts/2/12 (native)	Example 5.39
2050	CPMV-160+		B/Massachusetts/2/12 with deleted proteolytic loop	Example 5.15
2074	CPMV HT		HA B/Massachusetts/2/12 (PrL-)+H1 California TMCT	Example 5.16
2060	CPMV-		HA B/Massachusetts/2/12 (PrL-)+H1	Example 5.16

	160+		California TMCT	
2062	CPMV-HT+		HA B/Massachusetts/2/12 (PrL-)+H1 California TMCT	Example 5.21
1445	CPMV HT		B/Wisconsin/1/2010 with deleted proteolytic loop (PrL-)	Example 5.17
1839	CPMV-HT+		B/Wisconsin/1/2010 with deleted proteolytic loop (PrL-)	Example 5.22
1820	CPMV160+		B/Wisconsin/1/2010 with deleted proteolytic loop (PrL-)	Example 5.17
1975	CPMV160		B/Wisconsin/1/2010 with deleted proteolytic loop (PrL-)	Example 5.17
1454	CPMV-HT		HA B Wisconsin with deleted proteolytic loop (PrL-)+H1 California TMCT	Example 5.18
1893	CPMV-160+		HA B Wisconsin with deleted proteolytic loop (PrL-)+H1 California TMCT	Example 5.18
1860	CPMV-HT+		HA B Wisconsin with deleted proteolytic loop (PrL-)+H1 California TMCT	Example 5.23
2102	CPMV-HT+		B Florida with deleted proteolytic loop (PrL-)	Example 5.40
2104	CPMV-HT+	BeYDV	B Florida with deleted proteolytic loop (PrL-)	Example 5.40
2016	CPMV-HT+		B Florida +H1 California TMCT with deleted proteolytic loop (PrL-)	Example 5.41
2108	CPMV-HT+	BeYDV	B Florida +H1 California TMCT with deleted proteolytic loop (PrL-)	Example 5.41

Table: 4: Description of sequences

SEQ ID NO:	**Description**	**Figure**	**SEQ ID NO:**	**Description**	**Figure**
1	Avian H5 proteolytic loop consensus sequence		102	Amino acid sequence of HA B Wisconsin (PrL-).	36B
2	IF-H5A-I-05.si+3c	1A	103	Nucleotide sequence of HA B Wisconsin (PrL-)+H1 California TMCT	37A
3	IF-H5dTm.r	1B	104	Amino acid sequence of HA B Wisconsin (PrL-)+H1 California TMC.	37B
4	Construct 1191	1D	105	Nucleotide sequence of PDISP/HA B Brisbane (PrL-)+H1 California TMCT.	38A
5	Cassette 489	1E	106	Amino acid sequence of PDISP/HA B Brisbane (PrL-	38B

SEQ ID NO:	Description	Figure	SEQ ID NO:	Description	Figure
				)+H1 California TMCT.	
6	Amino acid sequence H5 A/Indonesia/5/2005 (H5N1)	1F	107	Nucleotide sequence of PDISP/HA B Massachussetts (PrL-).	39A
7	IF-S1-M1+M2ANC.c	2A	108	Amino acid sequence of PDISP/HA B Massachussetts (PrL-).	39B
8	IF-S1-4-M2ANC.r	2B	109	Nucleotide sequence of PDISP/HA B Massachussetts (PrL-)+H1 California TMCT.	40A
9	Synthetic M2 (nt 1-26 joined to 715-982 from DQ508860)	2C	110	Amino acid sequence of PDISP/HA B Massachussetts (PrL-)+H1 California TMCT.	40B
10	Cassette 1261	2D	111	Nucleotide sequence of HA B Wisconsin (PrL-).	41A
11	M2 from influenza A/New Caledonia/20/1999 (H1N1)	2E	112	Amino acid sequence of HA B Wisconsin (PrL-).	41B
12	Synthetic M2 (nt 26-51 joined to nt 740-1007 from EF467824)	3A	113	Nucleotide sequence of HA B Wisconsin (PrL-)+H1 California TMCT	42A
13	Cassette 859	3B	114	Amino acid sequence of HA B Wisconsin (PrL-)+H1 California TMC.	42B
14	Amino acid sequence M2 influenza A/Puerto Rico/8/1934 (H1N1)	3C	115	Nucleotide sequence of native H5 Indonesia.	43A
15	Cassette 1039	8A	116	Amino acid sequence of native H5 Indonesia	43B
16	Amino acid sequence	21A	117	IF**(SacII)-PDI.s1+4c	44A

SEQ ID NO:	Description	Figure	SEQ ID NO:	Description	Figure
	B/Brisbane/60/2008				
17	Amino acid sequence delta-proteolytic loop of type B HA (with linker GG)	21B	118	IF-H3V36111.s1-4r	44B
18	Amino acid sequence replacing cleavage site of B HA with linker	21C	119	Nucleotide sequence of PDISP/H3 Victoria.	44C
19	Amino acid sequence H3 A/Perth/16/2009	21D	120	Construct 2171	44E
20	Amino acid sequence delta-proteolytic loop H3 (with linker GS)	21E	121	Construct 1800	44F
21	Amino acid sequence Replacing cleavage site of H3 with linker	21F	122	Amino acid sequence of PDISP/H3 Victoria	44G
22	H1 New Cal linker region	15	123	IF(SacII)-Kozac_PDI.c	45A
23	H1 Brisbane linker region	15	124	IF-H3V36111.s1-4r	45B
24	H1 Sol Islands linker region	15	125	Construct 2181	45D
25	H2A Singapore linker region	15	126	Construct 1819	45E
26	H3A Brisbane linker region	15	127	IF**-H2S157.s1-6r	48A
27	H3A WCN linker region	15	128	Nucleotide sequence of PDISP/H2 Singapore.	48B
28	H5 Anhui linker region	15	129	Expression cassette number 2220	48C
29	H5 Indo linker	15	130	Amino acid sequence of	48D

SEQ ID NO:	Description	Figure	SEQ ID NO:	Description	Figure
	region			PDISP/H2 Singapore	
30	H5 Vietnam linker region	15	131	H2S157(Prl-).r	49A
31	Construct 1194	4B	132	H2S157(Prl-).c	49B
32	Cassette 1008	4C	133	Expression cassette number 2221	49C
33	H6 Teal HK linker region	15	134	Amino acid sequence of PDISP/H2 Singapore with deleted proteolytic loop	49D
34	H7 Eq Prague linker region	15	135	Expression cassette number 2222	50A
35	H9A HK linker region	15	136	Expression cassette number 2223	50B
36	B Florida linker region	15	137	Nucleotide sequence of PDISP/H3 Perth	51A
37	B Malaysia	15	138	IF**-H3P1609.S1-6r	51B
38	1039+1059.r	5A	139	Amino acid sequence of PDISP/H3 Perth	51C
39	1039+1059.c	5B	140	Nucleotide sequence of PDISP/H3 Perth with deleted proteolytic loop	52A
40	Cassette 1059	5C	141	H3P1609(Prl-)#2.r	52B
41	Amino acid sequence PDISP/HA influenza B/Brisbane/60/2008 (deleted proteolytic loop)	5D	142	H3P1609(Prl-)#2.c	52C
42	Nucleotide sequence H5 A/Indonesia/5/2005 (H5N1)	1G	143	Amino acid sequence of PDISP/H3 Perth with deleted proteolytic loop	52D
43	nucleotide sequence PDISP/HA influenza	5E	144	Nucleotide sequence of PDISP/H3 Victoria with	53A

SEQ ID NO:	Description	Figure	SEQ ID NO:	Description	Figure
	B/Brisbane/60/2008 (deleted proteolytic loop)			deleted proteolytic loop	
44	H5/Indo cleavage site natural sequence	19	145	H3V36111(Prl-).r	53B
45	H5/Indo modified cleavage site (TETR)	19	146	H3V36111(Prl-).c	53C
46	H5/Indo modified cleavage site (TETQ)	19	147	Amino acid sequence of PDISP/H3 Victoria with deleted proteolytic loop	53D
47	H1 cleavage site	Table 1	148	Nucleotide sequence of PDISP/H7 Hangzhou	54A
48	H3 cleavage site	Table 1	149	IF*-H7H113.s1-6r	54B
49	IF-HAB110.S1+3c	6A	150	Amino acid sequence of PDISP/H7 Hangzhou	54C
50	IF-HAB110.s1-4r	6B	151	Nucleotide sequence of PDISP/H7 Hangzhou with deleted proteolytic loop	55A
51	Synthetic HA B Wisconsin	6C	152	H7H113(PrL-).r	55B
52	Construct 193	6E	153	H7H113(PrL-).c	55C
53	Cassette 1462	6F	154	Amino acid sequence of PDISP/H7 Hangzhou with deleted proteolytic loop	55D
54	Amino acid sequence HA influenza B/Wisconsin/1/2010	6G	155	Nucleotide sequence of PDISP/H9 Hong Kong	56A
55	HAB110(PrL-).r	7A	156	IF**-H9HK107399.S1-6r	56B
56	HAB110(PrL-).c	7B	157	Amino acid sequence of PDISP/H9 Hong Kong	56C
57	Cassette 1467	7C	158	Nucleotide sequence of PDISP/H9 Hong Kong with	57A

SEQ ID NO:	Description	Figure	SEQ ID NO:	Description	Figure
				deleted proteolytic loop	
58	Amino acid sequence HA influenza B/Wisconsin/1/2010 (deleted PL)	7D	159	H9HK107399(Prl-).r	57B
59	B cleavage site	Table 1	160	H9HK107399(Prl-).c	57C
60	H5/Indo natural cleavage site	Table 2	161	Amino acid sequence of PDISP/H9 Hong Kong with deleted proteolytic loop	57D
61	H5/Indo modified cleavage site	Table 2	162	Nucleotide sequence of PDISP/HA B Malaysia	58A
62	H5/Indo modified cleavage site	Table 2	163	IF**-HBM250604.S1-6r	58B
63	H1/Brisbane modified cleavage site	Table 2	164	Amino acid sequence of PDISP/HA B Malaysia	58C
64	H3/Brisbane modified cleavage site	Table 2	165	Nucleotide sequence of PDISP/HA B Malaysia with deleted proteolytic loop	59A
65	B/Florida, B/Brisbane modified cleavage site	Table 2	166	HBM250604(PrL-).r	59B
66	A/H3/HA0 Consensus		167	HBM250604(PrL-).c	59C
67	A/H1/HA0 Consensus		168	Amino acid sequence of PDISP/HA B Malaysia with deleted proteolytic loop	59D
68	B/HA0 Consensus		169	Nucleotide sequence of PDISP/HA B Massachusetts	60A
69	H5 Anhui proteolytic loop deletion	18C	170	Amino acid sequence of PDISP/HA B Massachusetts	60B
70	H5 Indo proteolytic loop deletion	18C	171	nucleotide sequence of PatA SP	

SEQ ID NO:	Description	Figure	SEQ ID NO:	Description	Figure
71	H5 Vietnam proteolytic loop deletion	18C	172	amino acid sequence of patatin A signal peptide	
72	B Florida proteolytic loop deletion	18C	173	CPMV HT sequence	
73	B Malaysia proteolytic loop deletion	18C	174	Plant consensus kozak sequence - plant kingdom	
74	MutCleavage-H5(Indo).r	23A	175	Plant consensus kozak sequence- dicots	
75	MutCleavage-H5(Indo).c	23B	176	Plant consensus kozak sequence -arabidopsis	
76	Cassette 676	23C	177	Plant consensus kozak sequence	
77	Amino acid sequence influenza A/Indonesia/5/2005 (H5N1) TETR cleavage site mutant.	23D	178	Plant consensus kozak sequence	
78	H5I505_TETQ.r	24A	179	Plant consensus kozak sequence	
79	H5I505_TETQ.c	24B	180	Plant consensus kozak sequence	
80	Cassette 766	25C	181	Plant consensus kozak sequence	
81	Amino acid sequence influenza A/Indonesia/5/2005 (H5N1) TETQ cleavage site mutant.	25D	182	Plant consensus kozak sequence	
82	H5I505(PrL-).r	26A	183	Plant consensus kozak sequence	
83	H5I505(PrL-).c	26B	184	Plant consensus kozak sequence	

SEQ ID NO:	Description	Figure	SEQ ID NO:	Description	Figure
84	Cassette 928	26C	185	Plant consensus kozak sequence	
85	Amino acid sequence influenza A/Indonesia/5/2005 (H5N1) with deleted proteolytic loop.	26D	186	Kozak consensus sequence	
86	IF-S2+S4-B Bris.c	30A	187	Nucleotide sequence of CPMV HT+	
87	IF-S1a4-B Bris.r	30B	188	Nucleotide sequence of CPMV HT+ 511	
88	Synthesized HA B Brisbane gene	30C	189	Nucleotide sequence ofCPMV HT+[WT115]	
89	Construct 1029	30D	190	HBF406(PrL-).r	61A
90	Amino acid sequence of PDISP/HA from influenza B/Brisbane/60/2008	30E	191	HBF406(PrL-).c	61B
91	Nucleotide sequence of PDISP/HA B Brisbane (PrL-).	31A	192	IF*-HBF406.s1-6r	61C
92	Amino acid sequence of PDISP/HA B Brisbane (PrL-)	31B	193	Nucleotide sequence of PDISP/HA B Florida with deleted proteolytic loop	61D
93	Nucleotide sequence of CPMVX/CPMVX+		194	Amino acid sequence of PDISP/HA B Florida with deleted proteolytic loop	61E
94	consensus amino acid sequence of transmembrane domain		195	Expression cassette number 2102	61F
95	Nucleotide sequence of PDISP/HA B Brisbane (PrL-)+H1	33A	196	Expression cassette number 2104	61H

SEQ ID NO:	Description	Figure	SEQ ID NO:	Description	Figure
	California TMCT.				
96	Amino acid sequence of PDISP/HA B Brisbane (PrL-)+H1 California TMCT.	33B	197	IF-H1cTMCT.S1-4r	62A
97	Nucleotide sequence of PDISP/HA B Massachussetts (PrL-)	34A	198	Nucleotide sequence of PDISP/HA B Florida+H1Cal TMCT with deleted proteolytic loop	62B
98	Amino acid sequence of PDISP/HA B Massachussetts (PrL-)	34B	199	Amino acid sequence of PDISP/HA B Florida+H1Cal TMCT with deleted proteolytic loop	62C
99	Nucleotide sequence of PDISP/HA B Massachussetts (PrL-)+H1 California TMCT.	35A	200	Expression cassette number 2106	62D
100	Amino acid sequence of PDISP/HA B Massachussetts (PrL-)+H1 California TMCT.	35B	201	Expression cassette number 2108	62F
101	Nucleotide sequence of HA B Wisconsin (PrL-).	36A			

Examples

Example 1

Agrobacterium transfection

[00225] *Agrobacterium* strain AGL1 was transfected by electroporation with the DNA constructs using the methods described by D'Aoust et al 2008 (Plant Biotechnology Journal 6:930-940). Transfected *Agrobacterium* were grown in YEB medium supplemented with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), 20 μM acetosyringone, 50 μg/ml kanamycin and 25 μg/ml of carbenicillin pH5.6 to an OD_{600} between 0.6 and 1.6. *Agrobacterium* suspensions were centrifuged before use and resuspended in infiltration medium (10 mM $MgCl_2$ and 10 mM MES pH 5.6).

Preparation of plant biomass, inoculum and agroinfiltration

[00226] The terms "biomass" and "plant matter" as used herein are meant to reflect any material derived from a plant. Biomass or plant matter may comprise an entire plant, tissue, cells, or any fraction thereof. Further, biomass or plant matter may comprise intracellular plant components, extracellular plant components, liquid or solid extracts of plants, or a combination thereof. Further, biomass or plant matter may comprise plants, plant cells, tissue, a liquid extract, or a combination thereof, from plant leaves, stems, fruit, roots or a combination thereof. A portion of a plant may comprise plant matter or biomass.

[00227] *Nicotiana benthamiana* plants were grown from seeds in flats filled with a commercial peat moss substrate. The plants were allowed to grow in the greenhouse under a 16/8 photoperiod and a temperature regime of 25ºC day/20ºC night. Three weeks after seeding, individual plantlets were picked out, transplanted in pots and left to grow in the greenhouse for three additional weeks under the same environmental conditions.

[00228] *Agrobacteria* transfected with each construct were grown in a YEB medium supplemented with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), 20 μM acetosyringone, 50 μg/ml kanamycin and 25 μg/ml of carbenicillin pH5.6 until they reached an OD_{600} between 0.6 and 1.6. *Agrobacterium* suspensions were centrifuged before use and resuspended in infiltration medium (10 mM $MgCl_2$ and 10 mM MES pH 5.6) and stored overnight at 4°C. On the day of infiltration, culture batches were diluted in 2.5 culture volumes and allowed to warm before use. Whole plants of *N. benthamiana* were placed upside down in the bacterial suspension in an

air-tight stainless steel tank under a vacuum of 20-40 Torr for 2-min. Plants were returned to the greenhouse for a 2-6 day incubation period until harvest.

Leaf harvest and total protein extraction

[00229] Following incubation, the aerial part of plants was harvested, frozen at -80ºC and crushed into pieces. Total soluble proteins were extracted by homogenizing (Polytron) each sample of frozen-crushed plant material in 3 volumes of cold 50 mM Tris pH 8.0, 0.5 M NaCl, 0.1% Triton X-100 and 1 mM phenylmethanesulfonyl fluoride. After homogenization, the slurries were centrifuged at 10,000 g for 10 min at 4ºC and these clarified crude extracts (supernatant) kept for analyses.

Protein analysis and immunoblotting

[00230] The total protein content of clarified crude extracts was determined by the Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the reference standard. Proteins were separated by SDS-PAGE and electrotransferred onto polyvinylene difluoride (PVDF) membranes (Roche Diagnostics Corporation, Indianapolis, IN) for immunodetection. Prior to immunoblotting, the membranes were blocked with 5% skim milk and 0.1% Tween-20 in Tris-buffered saline (TBS-T) for 16-18h at 4ºC.

[00231] Immunoblotting was performed with a first incubation with a primary antibody (Table 4 presents the antibodies and conditions used for the detection of each HA), in 2 µg/ml in 2% skim milk in TBS-Tween 20 0.1%. Secondary antibodies used for chemiluminescence detection were as indicated in Table 4, diluted as indicated in 2% skim milk in TBS-Tween 20 0.1%. Immunoreactive complexes were detected by chemiluminescence using luminol as the substrate (Roche Diagnostics Corporation). Horseradish peroxidase–enzyme conjugation of human IgG antibody was carried out by using the EZ-Link Plus® Activated Peroxidase conjugation kit (Pierce, Rockford, IL).

Table 4: Electrophoresis conditions, antibodies, and dilutions for immunoblotting of expressed proteins.

HA subtype	Influenza strain	Electro-phoresis condition	Primary antibody	Dilution	Secondary antibody	Dilution
B	B/Brisbane/60/2008	Non-reducing	TGA, AS397	1:20000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
B	B/Wisconsin/1/2010	Non-reducing	NIBSC 07/356	1 :2000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
B	B/Malaysia/2506/2004	Non-reducing	NIBSC 07/184	1 :2000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H3	A/Perth/16/2009 (H3N2)	Non-reducing	TGA, AS400	1:20000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H3	A/Victoria/361/2011	Non-reducing	TGA, AS400	1 :2000 0	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H1	A/California/07/2009 (H1N1)	Reducing	Sino, 11055-MMO1	1 µg/ml	Goat anti-mouse (JIR 115-035-146)	1:7 500
H5	A/Indonesia/05/2005 (H5N1)	Reducing	CBER, S-7858	1:4000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000

JIR: Jackson ImmunoResearch, West Grove, PA, USA;
CBER: Center for Biologics Evaluation and Research, Rockville, MD, USA.
Sino: Sino Biological inc., Beijing, China.
TGA: Therapeutic Goods Administration, Australia.
NIBSC: National Institute for Biological Standards and Control, United Kingdom

Hemagglutination assay

[00232] Hemagglutination assay was based on a method described by Nayak and Reichl (2004). Briefly, serial double dilutions of the test samples (100 µL) were made in V-bottomed 96-well microtiter plates containing 100 µL PBS, leaving 100 µL of diluted sample per well. One hundred microliters of a 0.25% turkey red blood cells suspension (Bio Link Inc., Syracuse, NY) were added to each well, and plates were incubated for 2h at room temperature. The reciprocal of the highest dilution showing complete hemagglutination was recorded as HA activity. In parallel, a recombinant HA standard (A/Vietnam/1203/2004 H5N1) (Protein Science Corporation, Meriden, CT) was diluted in PBS and run as a control on each plate.

VLP extraction by cell wall digestion

[00233] Leaf tissue was collected from the *Nicotiana benthamiana* plants and cut into ~1 cm^2 pieces. The leaf pieces were soaked in 500 mM mannitol for 30 minutes at room temperature (RT). The mannitol solution was then removed and changed with the enzyme mix (mixture of cellulases from *Trichoderma viride* (Onozuka R-10; 3% v/v) and a mixture of pectinases from *Rhizopus* sp. (MACEROZYME™; 0.75% v/v; both from Yakult Pharmaceuticals) in protoplasting solution (500 mM mannitol, 10mM $CaCl_2$ and 5 mM MES/KOH (pH 5.6)). The ratio used was 20 g of leaf pieces per 100 mL solution. This preparation was spread evenly into a shallow vessel (~11x18 cm) and incubated for 16 hours on a rotary shaker at 40 rpm and 26°C.

[00234] Alternately, VLP extraction may be performed as follows: plants were agroinfiltrated with AGL1/#489, 928, 676 and 766. Leaf tissue was collected from the *N. benthamiana* plants at day 7 post-infiltration and cut into ~1 cm^2 pieces. Pectinase 162L (Biocatalysts), Multifect CX CG and Multifect CX B (Genencor) were added to a 200 mM Mannitol, 75 mM Citrate, 0.04% sodium bisulfite pH 6.0 buffer; digestion buffer. The biomasses were digested in duplicate overnight at room temperature in an orbital shaker. .

[00235] Following enzyme-assisted extraction, leaf debris was removed by filtration (nylon filter of 250 or 400 μm mesh). The coarse filtered extract was centrifuged at 5000 xg for 5 minutes. Supernatant was submitted to detection of HA expression (hemagglutination activity (see Figure 20) and Western blotting (see Figure 22).

Example 2:

Effect of modified proteolytic loop on accumulation of HA

[00236] As shown in Figure 13A, expression of native B/Brisbane (Construct No:1008) was lower than the expression of B/Brisbane comprising a modified proteolytic loop (Construct No: 1059). Increased hemagglutination activity was also observed with B/Brisbane comprising a modified proteolytic loop (Construct No:

1059) when compared to the native B/Brisbane HA (Construct No: 1008; Figure 13B).

[00237] Similar results were observed in the accumulation level of B/ Wisconsin comprising a modified proteolytic loop (Construct No: 1467), which is greater than that observed for the native B/Wisconsin HA (Construct No: 1462; Figure 16A). Increased hemagglutination activity was also observed with B/Wisconsin comprising a modified proteolytic loop (Construct No: 1467) when compared to the native B/Wisconsin HA (Construct No: 1462; Figure 16B) indicating a greater accumulation for the mutant protein.

[00238] Expression of H5/Indo comprising a modified proteolytic loop was also observed with modifications including a proteolytic loop comprising a GG linker (Construct No: 928; SEQ ID NO:85), a TETR linker (Construct No: 676; SEQ ID NO:77), or a TETQ linker (Construct No: 766; SEQ ID NO: 8; Figure 22).

Effect of influenza M2 co-expression on the accumulation level of HA

[00239] The co-expression of M2 was evaluated for its impact on the accumulation level of a modified influenza B HA. Construct no. 1059 encodes an influenza B HA in which the proteolytic loop is replaced by a 2 amino acid linker (GG in place of aa 341-359). The results from western blot analysis presented in figure 13A show that the removal of the proteolytic loop resulted in increased influenza B HA accumulation level (compare 1008 with 1059) and that the co-expression of M2 with the modified influenza B HA also increased HA accumulation level (Figure 13A, 1059 vs 1059+1261). An analysis of hemagglutination activity on crude protein extracts from plants transformed with influenza B HA with or without modification and with or without co-expression of M2 confirmed the positive effect of M2 co-expression on the accumulation level of the native influenza B HA (Figure 13B, 1008 vs 1008+1261) and the modified influenza B HA (Figure 13B, 1059 vs 1059+1261).

[00240] Co-expression of M2 with type A HA comprising a modified proteolytic loop also resulted in HA expression. For example, co-expression of

modified H3, with the proteolytic loop replaced with a GS linker or a $(GSS)_3$ linker (see Figure 21E, 21F), along with M2 may also result in HA accumulation in a plant.

[00241] The efficacy of M2 from influenza A/Puerto Rico/8/1934 to increase accumulation of the modified influenza B HA and H3 was compared to that of M2 from influenza A/New Caledonia/20/1999. For the modified influenza B HA, the comparison was undertaken by western blot analysis of protein extracts from plants transformed with constructs 1059, 1059+1261 and 1059+859. The results obtained demonstrated that the co-expression of M2 from influenza A/Puerto Rico/8/1934 (encoded by construct no. 859) was as efficient as the co-expression of M2 from influenza A/New Caledonia/20/1999 (encoded by construct no. 1261) for increasing accumulation of the modified influenza B HA (Figure 14).

Effect of influenza M2 co-expression on the accumulation level of different strains of B HA

[00242] Western blot analysis of protein extracts from plants transformed with gene constructs driving the expression of influenza B HA (from B/Wisconsin/1/2010) (constructs no. 1462) in the presence or absence of M2-expression construct (construct no. 1261) showed that M2 co-expression results in increased accumulation of influenza B HA (Figure 16A).

[00243] The co-expression of M2 was also evaluated for its impact on the accumulation level of a modified influenza B HA. Construct no. 1467 encodes an influenza B HA in which the proteolytic loop is replaced by a 2 amino acid linker (GG in place of aa 341-359). The results from western blot analysis presented in figures 16A show that the removal of the proteolytic loop resulted in increased influenza B HA accumulation level (compare 1462 with 1467) and that the co-expression of M2 with the modified influenza B HA also increased HA accumulation level (Figure 16A, 1467 vs 1467+1261). An analysis of hemagglutination activity on crude protein extracts from plants transformed with influenza B HA with or without modification and with or without co-expression of M2 confirmed the positive effect of M2 co-expression on the accumulation level of the native influenza B HA (Figure

16B, 1462 vs 1462+1261) and the modified influenza B HA (Figure 16B, 1467 vs 1467+1261).

Effect of amplification element BeYDV and modified proteolytic loop on accumulation of HA

[00244] Western blot analysis of protein extracts from plants transformed with gene constructs driving the expression of modified influenza B HA (from B/Brisbane/60/2008) with or without the proteolytic loop removed (see Figure 17A for constructs) and in the presence or absence of the amplification element BeYDV (construct no. 1059 and 1039) showed that in the absence of BeYDV no accumulation of influenza B HA could be detected (Figure 17B), when the regulatory element was CPMV-HT.

Effect of modified proteolytic loop on relative HA titer and hemagglutination

[00245] With reference to Figure 29A, there is shown a comparison of the activity of modified HA proteins produced in plants comprising CPMV HT, CPMV HT+ , CPMV 160 or CPMV160+, based enhancer elements operatively linked with a nucleotide sequence encoding either modified HA with the proteolytic loop deleted (replaced with a GG linker) or a native HA. In most cases, the expression (determined as hemagglutination titrer or activity) were higher for the CPMV HT+, CPMV 160 or CPMV160+ based construct demonstrates significant expression levels.

Table 5a: Relative HA titer (wt HA=1) (see Figure 29A)

	Fct = 1					
	wt			PrL-		
Description (n=)	GM	MD	n=	GM	MD	n=
H2 Sin157 (HT+) (n = 2)	1.0	0.4	2	1.2	0.1	2
H2 Sin157 (160+) (n = 2)	1.0	0.3	2	1.9	0.9	2
H3 Per1609 (HT+) (n = 4)	1.0	0.0	4	4.9	4.2	4
H3 Per1609 (160+) (n = 4)	1.0	0.3	4	6.1	3.6	4
H3 Vic36111 (HT+) (n = 6)	1.0	0.3	6	0.9	0.3	6
H3 Vic36111 (160+) (n = 6)	1.0	0.3	6	1.2	0.4	6
H5 Indo505 (HT) (n = 3)	1.0	0.1	3	1.2	0.3	3
H7 Han113 (HT+) (n = 5)	1.0	0.2	5	1.0	0.1	5
H9 HK107399 (HT+) (n = 2)	1.0	0.4	2	1.2	0.2	2
H9 HK107399 (160+) (n = 2)	1.0	0.2	2	0.7	0.1	2

HB Bri6008 (HT) (n = 1)	1.0	0.6	1	0.6	0.1	1
HB Mal250604 (160+) (n = 2)	1.0	0.3	2	8.4	2.4	2
HB Mas212 (HT) (n = 3)	1.0	0.1	3	1.4	1.3	3
HB Mas212 (HT+) (n = 3)	1.0	0.6	3	6.7	3.5	3
HB Mas212 (160+) (n = 2)	1.0	0.8	2	11.5	4.2	2

Example 3

Increased H7 Hangzhou HA VLP yields when the proteolytic loop is removed (PrL-) compared to the native construct.

[00246] *N. benthamiana* plants were infiltrated with AGL1/#2142+1261 and #2152+1261 and the leaves were harvested after a seven-day incubation period. Leaf tissue was collected and cut into ~1 cm^2 pieces. Pectinase 162L and Pectinase 444L (Biocatalysts), Multifect CX CG and Multifect CX B (Genencor) were added in a 200 mM Mannitol, 125 mM Citrate, 0.04% sodium bisulfite pH 6.0 buffer. The biomass was digested overnight at room temperature in an orbital shaker.

[00247] Following digestion, the apoplastic fraction was filtered through a 400 µm nylon filter to remove coarse undigested vegetal tissue (<5% of starting biomass). The filtered extract was then centrifuged at room temperature for 15 min at 5000xg to remove protoplasts and intracellular contaminants (proteins, DNA, membranes, vesicles, pigments, etc). Next, the supernatant was depth-filtered (for clarification) using a 1.2µm glass fiber filter (Sartopore GF plus/Sartorius Stedim), and a 0.45/0.2µm filter (Sartopore 2/Sartorius Stedim), before being subjected to chromatography.

[00248] The clarified apoplastic fraction was loaded over a cation exchange column (Poros HS Applied Biosystems) equilibrated with an equilibration/elution buffer (50 mM NaPO4, 100 mM NaCl, 0.005% Tween 80 pH 6.0). Once the UV was back to zero, the extract was step-eluted with the equilibration/elution buffer containing increasing concentrations of NaCl (500 mM). The purified VLPs were concentrated by TFF, diafiltered against formulation buffer (100 mM PO4, 150 mM NaCl, 0.01% Tween 80 at pH 7.4) and passed through a 0.22µm filter.

[00249] Hemagglutination assay for H7 was performed based on a method described by Nayak and Reichl (2004). Briefly, successive double dilutions of the test

samples (100 µL) were made in V-bottomed 96-well microtiter plates containing 100 µL PBS, leaving 100 µL of diluted sample per well. One hundred microliters of a 0.25% turkey red blood cells suspension (Bio Link Inc., Syracuse, NY) were added to each well, and plates were incubated for 2h at room temperature. The reciprocal of the highest dilution showing complete hemagglutination was recorded as hemagglutination activity.

[00250] Total protein content of clarified crude extracts was determined using bovine serum albumin as the reference standard. Relative yields were obtained by comparing the PrL- construct to the native construct used as control. Separation by SDS-PAGE, with denaturing sample loading buffer (0.1M Tris pH 6.8, 0.05% bromophenol blue, 12.5% glycerol, 4% SDS and 5% beta-mercaptoethanol), was performed under reducing conditions and Coomassie Brillant Blue R-250 was used for protein staining.

[00251] Figure 46A shows that the hemagglutination activity in plant extracts is greater for the H7 Hangzhou construct where the proteolytic loop is removed (#2152 + #1261, see Example 5.34) compared to the native construct (# 2142 + # 1261, see Example 5.33).

[00252] Figure 46B shows that the relative total protein yield in purified VLP is greater for the H7 Hangzhou construct where the proteolytic loop is removed (#2152 + #1261) compared to the native construct (# 2142 + # 1261). This example demonstrate a good correlation between the improvement in the VLP accumulated in plants vs the final yields when performing the complete process.

[00253] Figure 46C shows a SDS-PAGE analysis, with lane 2 showing the purified H7 Hangzhou construct with a removed proteolytic loop and lane 3 showing the purified native H7 Hangzhou construct. For each lane, 2µg of total protein were loaded on the gel. The purity of the proteins profiles is similar for both constructs and greater than 90%.

Example 4.1.

Trypsin resistance of mutants H5 Indonesia VLP where the proteolytic loop is modified or removed is greater than native H5 Indonesia.

[00254] *N. benthamiana* plants were agroinfiltrated with AGL1/#489, #928, #766 and # 676 as described in Example 1 (above). Leaves were collected from the plants 7 days post-infiltration, cut into ~1 cm2 pieces. Pectinase 162L (Biocatalysts), Multifect CX CG and Multifect CX B (Genencor) were added in a 200 mM Mannitol, 75 mM Citrate, 0.04% sodium bisulfite pH 6.0 buffer. The biomass was digested overnight at room temperature in an orbital shaker. The digested extracts were coarse-filtered, centrifuged, clarified and purified as described in Example 3 (H7 Hangzhou).

[00255] For each of the native (# 489), PRL- (# 928), TETQ (# 766) and TETR (# 676), H5 Indonesia HA VLP extracts, two samples of HA VLPs were resuspended in buffer (100 mM Na/KPO_4, 150 mM NaCl, 0.01% TWEEN 80) at pH 7.4. Trypsin was added in a 1:100 protein ratio. Samples were grabbed after 30, 60 and 120 minutes of incubation at room temperature, then boiled in sample loading buffer to stop the reaction. The non-digested extracts (control) and the trypsin-digested extracts analysed by SDS-PAGE gel as described in Example 3.

[00256] Figure 47A shows an SDS-PAGE analysis of trypsin-digested samples, with lanes 2 through 5 showing the native H5 Indonesia VLP (#489), with lanes 6 through 9 showing PrL- H5 Indonesia VLP (#928), with lanes 10 through 13 showing TETQ H5 Indonesia VLP (#766) and with lanes 14 through 17 showing the TETR H5 Indonesia VLP (#676) at different time points in the digestion (0, 30, 60, and 120 minutes). The native H5 Indonesia VPL, with a band corresponding to the HA0 monomer being detectable at approximately 75 kDa in the non-digested extract in lane 2, was rapidly processed into HA1 and HA2 bands through addition of trypsine, detectable at approximately 50 and 25kDa respectively during the trypsin digestion in lanes 3 through 5. Both the PrL- and the TETQ H5 Indonesia VLPs, stabilized by the removal or modification of the proteolytic site, showed trypsin resistance as the HA0 band did not cleave into HA1 and HA2 bands. The TETR H5 Indonesia VLPs were partially stabilized by the modification of the proteolytic site and HA0 monomers were cleaved into HA1 and HA2 slower than in the native H5 Indonesia VLPS.

[00257] These data demonstrate the successful protection of the HA0 protein at its proteolytic site within HA1-HA2, by either deleting the proteolytic loop (prl-) or replacing the proteolytic loop with a linker sequence (TETQ) approach.

[00258] **Example 4.2. Immunogenicity of native H5 Indonesia VLPs is similar to its mutant counterparts (PrL-, TETQ and TETR) in mice.**

[00259] The native, PrL-, TETR and TETQ H5 Indonesia VLPs extracts were purified as described in Example 4.1 (above).

[00260] Figure 47B shows immunogenicity (HI titer) of native H5 VLP and its mutant counterparts (prl-, TETQ and TETR) in mice after two doses. BALB/c mice (n=8/group) were injected twice intramuscularly, 21 days apart, with 10ug dose of plant based H5 VLP vaccines (native, prl-, TETQ or TETR) based on its HA content. HI titers analysis was done from sera of each animal, 42 dpv (21 days after de 2nd dose) and H5 VLP A/Indonesia/5/2005 (H5N1) was used as antigen. Bars represent relative (%) HI titers comparison of each H5 mutants VLP with the H5 VLP native (calculated with the log2 of the HI titer GMT and 95% CI). Statistical differences between groups for each dose were compared by using a one-way ANOVA followed by a Tukey's post-hoc analysis on Log2- transformed data (assuming a normal distribution of them). * $p < 0.05$ was considered significant. No difference between groups for each dose was observed.

Example 5.1: B-2X35S/CPMV-HT/M2 New Caledonia/NOS (Construct number 1261)

[00261] A sequence encoding M2 from influenza A/New Caledonia/20/1999 (H1N1) was cloned into 2X35S/CPMV-HT/NOS expression system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the complete M2 coding sequence was amplified using primers IF-S1-M1+M2ANC.c (Figure 2A, SEQ ID NO: 7) and IF-S1-4-M2ANC.r (Figure 2B, SEQ ID NO: 8) using synthesized M2 gene (corresponding to nt 1-26 joined to nt 715-982 from GenBank accession number DQ508860; Figure 2C, SEQ ID NO: 9) as template. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1191 (Figure 1C) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in a CPMV HT-based expression cassette. It also incorporates a gene

construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 1D (SEQ ID NO: 4). The resulting construct was given number 1261 (Figure 2D, SEQ ID NO: 10). The amino acid sequence of M2 from influenza A/New Caledonia/20/1999 (H1N1) is presented in Figure 2E (SEQ ID NO: 11). A representation of plasmid 1261 is presented in Figure 11.

Example 5.2: C-2X35S/CPMV-HT/M2 Puerto Rico/NOS (Construct number 859)

[00262] A sequence encoding M2 from influenza A/Puerto Rico/8/1934 (H1N1) was cloned into 2X35S/CPMV-HT/NOS expression system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing the complete M2 coding sequence was amplified using primers IF-S1-M1+M2ANC.c (Figure 2A, SEQ ID NO: 7) and IF-S1-4-M2ANC.r (Figure 2B, SEQ ID NO: 8), using synthesized M2 gene (corresponding to nt 26-51 joined to nt 740-1007 from Genbank accession number EF467824) (Figure 3A, SEQ ID NO: 12) as template. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1191 (Figure 1C) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The vector is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 1D (SEQ ID NO: 4). The resulting construct was given number 859 (Figure 3B, SEQ ID NO: 13). The amino acid sequence of M2 from influenza A/Puerto Rico/8/1934 (H1N1) is presented in Figure 3C (SEQ ID NO: 14). A representation of plasmid 859 is presented in Figure 17.

Example 5.3: G-2X35S/CPMV-HT/PDISP/HA B Brisbane/NOS into BeYDV+Replicase amplification system (Construct number 1008)

[00263] The preparation of construct 1008 is described in US 61/541,780. Briefly, a sequence encoding HA from influenza B/Brisbane/60/2008 was cloned into 2X35S/CPMV-HT/PDISP/NOS comprising the BeYDV+replicase amplification system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using a PCR-based method using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank accession number FJ766840). The PCR product was cloned in-frame with alfalfa PDI signal peptide in 2X35S/CPMV-HT/NOS expression cassette into the BeYDV amplification system. Construct 1194 (see Figures 4A, 4B) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for an assembly reaction to produce construct number 1008 (Figure 4C, Figure 9; SEQ ID NO: 32).

[00264] Construct number 1194 (Figure 4A) is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in frame with an alfalfa PDI signal peptide in a CPMV-HT-based expression cassette into the BeYDV amplification system. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid.

Example 5.4: I-2X35S/CPMV-HT/PDISP-HA B Brisbane with deleted proteolytic loop into BeYDV+Replicase amplification system (Construct number 1059)

[00265] The preparation of construct 1059 is described in US 61/541,780. Briefly, a sequence encoding HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop was cloned into 2X35S/CPMV-HT/PDISP/NOS comprising the BeYDV+replicase amplification system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the a PCR-based ligation method (Darveau et al., 1995, Methods in Neuroscience 26: 77-85). In a first round of PCR, a fragment containing HA B Brisbane coding sequence from nt 46 to nt 1065 was amplified using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genebank accession number FJ766840) as template. A second fragment, containing HA B Brisbane coding sequence from nt 1123 to nt 1758, was amplified using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank

accession number FJ766840) as template. The PCR products from both amplifications were then mixed and used as template for a second round of amplification. The resulting fragment (encoding HA B/Brisbane/60/2008 Δa.a. 356-374 with a GG linker between fragments; see Figure 21B) was cloned in-frame with alfalfa PDI signal peptide in 2X35S/CPMV-HT/NOS expression cassette comprising the BeYDV amplification system to produce construct 1194 (Figures 4A, 4B) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for an assembly reaction. The resulting construct was given number 1059 (Figure 5C; SEQ ID NO: 40).

[00266] The amino acid sequence of PDISP-HA B/Brisbane/60/2008 with deleted proteolytic loop is presented in Figure 5D (SEQ ID NO: 41).

Example 5.5: B-2X35S/CPMV-HT/HA B Wisconsin/NOS into BeYDV(m)+Replicase amplification system (Construct number 1462)

[00267] The preparation of construct 1462 is described in US 61/541,780. Briefly, a sequence encoding HA from influenza B/Wisconsin/1/2010 was cloned into 2X35S/CPMV-HT/NOS comprising the BeYDV(m)+replicase amplification system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using a PCR-based method. A fragment containing the complete HA B Wisconsin coding sequence was amplified using synthesized HA B Wisconsin gene (Genbank accession number JN993010) as template. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression cassette into the BeYDV(m) amplification system. Construct 193 (Figure 6D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for an assembly reaction.

[00268] Construct number 193 (Figure 6D, 6E) is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in a CPMV-HT-based expression cassette into the BeYDV(m) amplification system. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 6E (SEQ ID NO: 52). The resulting construct was given number 1462 (Figure 6F, SEQ ID NO: 53). The amino acid sequence of PDISP/HA from Influenza B/Wisconsin/1/2010 is

presented in Figure 6G (SEQ ID NO: 54). A representation of plasmid 1462 is presented in Figure 6H.

Example 5.6: C-2X35S/CPMV-HT/HA B Wisconsin with deleted proteolytic loop into BeYDV(m)+Replicase amplification system (Construct number 1467)

[00269] The preparation of construct 1467 is described in US 61/541,780. Briefly, a sequence encoding HA from influenza B/Wisconsin/1/2010 with deleted proteolytic loop was cloned into 2X35S/CPMV-HT/NOS comprising the BeYDV(m)+replicase amplification system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using a PCR-based ligation method (Darveau et al. 1995, Methods in Neuroscience 26: 77-85). In a first round of PCR, a fragment containing HA B Wisconsin coding sequence from nt 1 to nt 1062 was amplified using primers IF-HAB110.S1+3c (Figure 6A, SEQ ID NO: 49) and HAB110(PrL-).r (Figure 7A, SEQ ID NO: 55) , using synthesized HA B Wisconsin gene (Genbank accession number JN993010) (Figure 6C, SEQ ID NO: 51) as template. A second fragment, containing HA B Wisconsin coding sequence from nt 1120 to nt 1755, was amplified using primers HAB110(PrL-).c (Figure 7B, SEQ ID NO: 56) and and IF-HAB110.s1-4r (Figure 6B, SEQ ID NO: 50), using synthesized HA B Wisconsin gene (Genbank accession number JN993010) (Figure 6C, SEQ ID NO: 51) as template. The PCR products from both amplifications were then mixed and used as template for a second round of amplification using IF-HAB110.S1+3c (Figure 6A, SEQ ID NO: 49) and IF-HAB110.s1-4r (Figure 6B, SEQ ID NO: 50) as primers. The resulting fragment (encoding HA B/Wisconsin/1/2010 Δa.a. 340-358 with a GG linker between fragments) was cloned in 2X35S/CPMV-HT/NOS expression cassette comprising the BeYDV(m) amplification system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 193 (Figure 6D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction.

[00270] Construct number 193 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in a CPMV-HT-based expression cassette into the BeYDV(m) amplification system. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin

gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 6E (SEQ ID NO: 52). The resulting construct was given number 1467 (Figure 7C, SEQ ID NO: 57). The amino acid sequence of HA from Influenza B/Wisconsin/1/2010 with deleted proteolytic loop is presented in Figure 7D (SEQ ID NO: 58). A representation of plasmid 1467 is presented in Figure 7E.

Example 5.7: A-2X35S/CPMV-HT/PDISP-HA B Brisbane with deleted proteolytic loop (Construct number 1039)

[00271] The preparation of construct 1192 is described in US 61/541,780. Briefly, a sequence encoding HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop was cloned into 2X35S/CPMV-HT/PDISP/NOS in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based ligation method (Darveau et al., 1995, Methods in Neuroscience 26: 77-85). In a first round of PCR, a fragment containing HA B Brisbane coding sequence from nt 46 to nt 1065 was amplified using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genebank accession number FJ766840) as template. A second fragment, containing HA B Brisbane coding sequence from nt 1123 to nt 1758, was amplified using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank accession number FJ766840) as template. The PCR products from both amplifications were then mixed and used as template for a second round of amplification. The resulting fragment (encoding HA B/Brisbane/60/2008 Δa.a. 356-374 with a GG linker between fragments) was cloned in-frame with alfalfa PDI signal peptide in 2X35S/CPMV-HT/NOS expression cassette. Construct 1192 was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction.

[00272] Construct number 1192 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in frame with an alfalfa PDI signal peptide in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid The resulting construct was given number 1039 (Figure 8B). The amino acid sequence of

PDISP-HA B/Brisbane/60/2008 with deleted proteolytic loop is presented in Figure 5D (SEQ ID NO: 41). A representation of plasmid 1039 is presented in Figure 8A (SEQ ID NO: 15).

Example 5.8: A-2X35S/CPMV-HT/H5 from A/Indonesia/5/2005 with TETR cleavage site mutation (Construct number 676)

[00273] A sequence encoding H5 from A/Indonesia/5/2005 with TETR cleavage site mutation was cloned into 2X35S/CPMV-HT/ NOS in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based ligation method (Darveau et al., 1995, Methods in Neuroscience 26: 77-85). In a first round of PCR, a fragment containing H5 from A/Indonesia/5/2005 coding sequence from nt 1 to nt 1015 was amplified using primers IF-H5A-I-05.s1+3c (Figure 1A, SEQ ID NO: 2) and MutCleavage-H5(Indo).r (Figure 23A, SEQ ID NO: 74), using synthesized H5 from A/Indonesia/5/2005 (Figure 1G, SEQ ID NO: 42) as template. A second fragment, containing H5 from A/Indonesia/5/2005 coding sequence from nt 1038 to nt 1707, was amplified using primers MutCleavage-H5(Indo).c (Figure 23B, SEQ ID NO : 75) and IF-H5dTm.r (Figure 1B, SEQ ID NO: 3), using synthesized H5 from A/Indonesia/5/2005 (Figure 1G, SEQ ID NO: 42) as template. The PCR products from both amplifications were then mixed and used as template for a second round of amplification using IF-H5A-I-05.s1+3c (Figure 1A, SEQ ID NO: 2) and IF-H5dTm.r (Figure 1B, SEQ ID NO: 3) as primers. The resulting fragment (encoding H5 from A/Indonesia/5/2005 Δa.a. 339-346 with a TETR linker between fragments) was cloned in 2X35S/CPMV-HT/NOS expression cassette using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1191 (Figure 1D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in frame in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 1D (SEQ ID NO: 4). The resulting construct was given number 676 (Figure 23C, SEQ ID NO: 76). The amino acid sequence of H5 from A/Indonesia/5/2005 TETR cleavage

site mutant is presented in Figure 23D (SEQ ID NO: 77). A schematic representation of plasmid 676 is presented in Figure 23E.

Example 5.9: B-2X35S/CPMV-HT/H5 from A/Indonesia/5/2005 with TETQ cleavage site mutation (Construct number 766)

[00274] A sequence encoding H5 from A/Indonesia/5/2005 with TETQ cleavage site mutation was cloned into 2X35S/CPMV-HT/ NOS in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based ligation method (Darveau et al., 1995, Methods in Neuroscience 26: 77-85). In a first round of PCR, a fragment containing H5 from A/Indonesia/5/2005 coding sequence from nt 1 to nt 1015 was amplified using primers IF-H5A-I-05.s1+3c (Figure 1A, SEQ ID NO: 2) and H5I505_TETQ.r (Figure 24A, SEQ ID NO: 78), using synthesized H5 from A/Indonesia/5/2005 (Figure 1G, SEQ ID NO: 42) as template. A second fragment, containing H5 from A/Indonesia/5/2005 coding sequence from nt 1038 to nt 1707, was amplified using primers H5I505_TETQ.c (Figure 24B, SEQ ID NO : 79) and IF-H5dTm.r (Figure 1B, SEQ ID NO: 3), using synthesized H5 from A/Indonesia/5/2005 (Figure 1G, SEQ ID NO: 42) as template. The PCR products from both amplifications were then mixed and used as template for a second round of amplification using IF-H5A-I-05.s1+3c (Figure 1A, SEQ ID NO: 2) and IF-H5dTm.r (Figure 1B, SEQ ID NO: 3) as primers. The resulting fragment (encoding H5 from A/Indonesia/5/2005 Δa.a. 339-346 with a TETQ linker between fragments) was cloned in 2X35S/CPMV-HT/NOS expression cassette using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1191 (Figure 1D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in frame in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 1D (SEQ ID NO: 4). The resulting construct was given number 766 (Figure 24C, SEQ ID NO: 80). The amino acid sequence of H5 from A/Indonesia/5/2005 TETQ cleavage site mutant is

presented in Figure 24D (SEQ ID NO: 81). A schematic representation of plasmid 766 is presented in Figure 24E.

Example 5.10: C-2X35S/CPMV-HT/H5 from A/Indonesia/5/2005 with deleted proteolytic loop (Construct number 928)

[00275] A sequence encoding H5 from A/Indonesia/5/2005 with deleted proteolytic loop was cloned into 2X35S/CPMV-HT/ NOS in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based ligation method presented by Darveau et al. (Methods in Neuroscience 26: 77-85 (1995)). In a first round of PCR, a fragment containing H5 from A/Indonesia/5/2005 coding sequence from nt 1 to nt 1011 was amplified using primers IF-H5A-I-05.s1+3c (Figure 1A, SEQ ID NO: 2) and H5I505(PrL-).r (Figure 25A, SEQ ID NO: 82), using synthesized H5 from A/Indonesia/5/2005 (Figure 1G, SEQ ID NO: 42) as template. A second fragment, containing H5 from A/Indonesia/5/2005 coding sequence from nt 1075 to nt 1707, was amplified using primers H5I505(PrL-).c (Figure 25B, SEQ ID NO : 83) and IF-H5dTm.r (Figure 1B, SEQ ID NO: 3), using synthesized H5 from A/Indonesia/5/2005 (Figure 1G, SEQ ID NO: 42) as template. The PCR products from both amplifications were then mixed and used as template for a second round of amplification using IF-H5A-I-05.s1+3c (Figure 1A, SEQ ID NO: 2) and IF-H5dTm.r (Figure 1B, SEQ ID NO: 3) as primers. The resulting fragment (encoding H5 from A/Indonesia/5/2005 Δa.a. 338-358 with a GG linker between fragments) was cloned in 2X35S/CPMV-HT/NOS expression cassette using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1191 (Figure 1D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1191 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in frame in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 1D (SEQ ID NO: 4). The resulting construct was given number 928 (Figure 25C, SEQ ID NO: 84). The amino acid sequence of H5 from A/Indonesia/5/2005 with deleted proteolytic loop is presented in Figure 25D (SEQ ID NO: 85). A representation of plasmid 928 is presented in Figure 25E.

Example 5.11 - F-2X35S/CPMV-HT/PDISP/HA B Brisbane/NOS (Construct number 1029)

[00276] A sequence encoding HA from influenza B/Brisbane/60/2008 was cloned into 2X35S/CPMV-HT/PDISP/NOS expression system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing HA B Brisbane coding sequence without his wild type signal peptide was amplified using primers IF-S2+S4-B Bris.c (Figure 30A, SEQ ID NO: 86) and IF-S1a4-B Bris.r (Figure 30B, SEQ ID NO: 87), using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank accession number FJ766840) (Figure 30C, SEQ ID NO: 88) as template. The PCR product was cloned in-frame with alfalfa PDI signal peptide in 2X35S/CPMV-HT/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1192 was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1192 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in frame with an alfalfa PDI signal peptide in a CPMV-HT-based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders. The resulting construct was given number 1029 (Figure 30D, SEQ ID NO: 89). The amino acid sequence of PDISP/HA from influenza B/Brisbane/60/2008 is presented in Figure 30E (SEQ ID NO: 90). A representation of plasmid 1029 is presented in Figure 30F.

Example 5.12 - 2X35S/CPMV HT (construct no 1039) and HT+ (construct no 1829) for PDISP/HA B Brisbane (PrL-)

[00277] A coding sequence corresponding to HA from Influenza B/Brisbane/60/2008 with deleted proteolytic loop (PrL-) in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/HA B Brisbane (PrL-; Figure 31A, SEQ ID NO: 91) was cloned into original HT and modified HT+ using the same PCR-based method described in Examples 5.7 and 5.11, but with modified PCR primers specifically designed for PDISP/HA B Brisbane (PrL-). The amino acid sequence of mature HA B Brisbane (PrL-) fused with PDISP

is presented in Figure 31B (SEQ ID NO: 92). Representations of plasmid 1039 and 1829 are presented in Figure 8B and 31D.

Example 5.13 - 2X35S/CPMV HT (construct no 1039) and 2X35S/CPMV160+ (construct no 1937) for PDISP/HA B Brisbane (PrL-)

[00278] A coding sequence corresponding to HA from Influenza B/Brisbane/60/2008 with deleted proteolytic loop (PrL-) (see US provisional application No.61/806,227 Filed March 28, 2013, which is incorporated herein by reference, for additional information re: deleted proteolytic loop regions in HA sequences) in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/HA B Brisbane (PrL-)) (Figure 32A, SEQ ID NO: 93) was cloned into original CPMV-HT and CPMV160+ using the same PCR-based method as described in Example 5.7 and in Example 5.11, but with modified PCR primers specifically designed for PDISP/HA B Brisbane (PrL-). The amino acid sequence of mature HA B Brisbane (PrL-) fused with PDISP is presented in Figure 31B (SEQ ID NO: 92). Representations of plasmid 1039 and 1937 are presented in Figure 8B and Figure 32C.

Example 15.14 - 2X35S/CPMV HT (construct no 1067) and 2X35S/CPMV160+ (construct no 1977) for PDISP/HA B Brisbane (PrL-)+H1 California TMCT

[00279] A chimer hemagglutinin coding sequence corresponding to the ectodomain of HA from Influenza B/Brisbane/60/08 with deleted proteolytic loop (PrL-) (see US provisional application No.61/806,227 Filed March 28, 2013, which is incorporated herein by reference, for additional information re: deleted proteolytic loop regions in HA sequences) fused to the transmembrane domain and cytoplasmic tail (TMCT) of H1 from influenza A/California/7/2009 and with the signal peptide of alfalfa protein disulfide isomerase (PDISP/HA B Brisbane (PrL-)+H1 California TMCT) (Figure 33A, SEQ ID NO: 95) was cloned into original CPMV-HT and CPMV160+ using the same PCR-based method as described in Examples 5.7 and 5.11, but with modified PCR primers specifically designed for PDISP/HA B Brisbane (PrL-)+H1 California TMCT. The amino acid sequence of mature HA B Brisbane (PrL-)+H1 California TMCT fused with PDISP is presented in Figure 33B (SEQ ID NO: 96). Representations of plasmid 1067 and 1977 are presented in Figure 33C and Figure 33D.

Example 5.15 - 2X35S/CPMV HT (construct no 2072) and 2X35S/CPMV160+ (construct no 2050) for PDISP/HA B Massachussetts (PrL-)

[00280] A coding sequence corresponding to HA from Influenza B/Massachussetts/2/2012 with deleted proteolytic loop (PrL-) (see US provisional application No.61/806,227 Filed March 28, 2013 for additional information re: deleted proteolytic loop regions in HA sequences, which is incorporated herein by reference) in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/HA B Massachussetts (PrL-)) (Figure 34A, SEQ ID NO: 97) was cloned into original CPMV-HT and CPMV160+ using the same PCR-based method as described in Examples 5.7 and 5.11, but with modified PCR primers specifically designed for PDISP/HA B Massachussetts (PrL-). The amino acid sequence of mature HA B Massachussetts (PrL-) fused with PDISP is presented in Figure 34B (SEQ ID NO: 98). Representations of plasmid 2072 and 2050 are presented in Figure 34C and Figure 34D.

Example 5.16 - 2X35S/CPMV HT (construct no 2074) and 2X35S/CPMV160+ (construct no 2060) for PDISP/HA B Massachussetts (PrL-)+H1 California TMCT

[00281] A chimer hemagglutinin coding sequence corresponding to the ectodomain of HA from Influenza B/Massachussetts/2/2012 with deleted proteolytic loop (PrL-) (see US provisional application No.61/806,227 Filed March 28, 2013 for additional information re: deleted proteolytic loop regions in HA sequences, which is incorporated herein by reference) fused to the transmembrane domain and cytoplasmic tail (TMCT) of H1 from influenza A/California/7/2009 and with the signal peptide of alfalfa protein disulfide isomerase (PDISP/HA B Massachussetts (PrL-)+H1 California TMCT) (Figure 35A, SEQ ID NO: 99) was cloned into original CPMV-HT and CPMV160+ using the same PCR-based method as described in Examples 5.7 and 5.11, but with modified PCR primers specifically designed for PDISP/HA B Massachussetts (PrL-)+H1 California TMCT. The amino acid sequence of mature HA B Massachussetts (PrL-)+H1 California TMCT fused with PDISP is presented in Figure 35B (SEQ ID NO: 100). Representations of plasmid 2074 and 2060 are presented in Figure 35C and 35D.

Example 5.17 - 2X35S/CPMV HT (construct no 1445), 2X35S/CPMVHT+ (construct no 1820) and CPMV160+ (construct no 1975) for HA B Wisconsin (PrL-)

[00282] A coding sequence corresponding to HA from Influenza B/Wisconsin/1/2010 with deleted proteolytic loop (PrL-) (see US provisional application No.61/806,227 Filed March 28, 2013 for additional information re: deleted proteolytic loop regions in HA sequences, which is incorporated herein by reference) with his native signal peptide (HA B Wisconsin (PrL-)) (Figure 36AA, SEQ ID NO: 101) was cloned into original CPMV-HT, CPMVHT+, and CPMV160 using the same PCR-based method as described in Examples 5.7 and 5.11, but with modified PCR primers specifically designed for HA B Wisconsin (PrL-). The amino acid sequence of HA B Wisconsin (PrL-) with his native signal peptide is presented in Figure 36B (SEQ ID NO: 102). Representations of plasmid 1445, 1820 and 1975 are presented in Figures 36C, 36D and 36E, respectively.

Example 5.18 - 2X35S/CPMV HT (construct no 1454) and 2X35S/CPMV160+ (construct no 1893) for HA B Wisconsin (PrL-)+H1 California TMCT

[00283] A chimer hemagglutinin coding sequence corresponding to the ectodomain of HA from Influenza B/ Wisconsin /2/2012 with deleted proteolytic loop (PrL-) (see US provisional application No.61/806,227 Filed March 28, 2013 for additional information re: deleted proteolytic loop regions in HA sequences, which is incorporated herein by reference) fused to the transmembrane domain and cytoplasmic tail (TMCT) of H1 from influenza A/California/7/2009 with the native signal peptide of HA B Wisconsin (HA B Wisconsin (PrL-)+H1 California TMCT) (Figure 37A, SEQ ID NO: 103) was cloned into original CPMV-HT and CPMV160+ using the same PCR-based method as described in Examples 5.7 and 5.11, but with modified PCR primers specifically designed for HA B Wisconsin (PrL-)+H1 California TMCT. The amino acid sequence of HA B Wisconsin (PrL-)+H1 California TMCT is presented in Figure 37 (SEQ ID NO: 104). Representations of plasmid 1454 and 1893 are presented in Figure 37C and 37D.

Example 5.19: 2X35S/CPMV HT (construct no 1067) and HT+ (construct no 1875) for PDISP/HA B Brisbane (PrL-)+H1 California TMCT

[00284] A chimer hemagglutinin coding sequence corresponding to the ectodomain of HA from Influenza B/Brisbane/60/08 with deleted proteolytic loop

(PrL-) fused to the transmembrane domain and cytoplasmic tail (TMCT) of H1 from influenza A/California/7/2009 and with the signal peptide of alfalfa protein disulfide isomerase (PDISP/HA B Brisbane (PrL-)+H1 California TMCT) (Figure 38A, SEQ ID NO: 105) was cloned into original HT and modified HT+ using the same PCR-based method as described in Example 5.26, but with modified PCR primers specifically designed for PDISP/HA B Brisbane (PrL-)+H1 California TMCT. The amino acid sequence of mature HA B Brisbane (PrL-)+H1 California TMCT fused with PDISP is presented in Figure 38B (SEQ ID NO: 106). Representations of plasmid 1067 and 1875 are presented in Figure 33C and 39C.

Example 5.20: 2X35S/CPMV HT (construct no 2072) and HT+ (construct no 2052) for PDISP/HA B Massachussetts (PrL-)

[00285] A coding sequence corresponding to HA from Influenza B/Massachussetts/2/2012 with deleted proteolytic loop (PrL-) in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/HA B Massachussetts (PrL-)) (Figure 39A, SEQ ID NO: 107) was cloned into original HT and modified HT+ using the same PCR-based method as as described in Example 5.26, but with modified PCR primers specifically designed for PDISP/HA B Massachussetts (PrL-). The amino acid sequence of mature HA B Massachussetts (PrL-) fused with PDISP is presented in Figure 39B (SEQ ID NO: 108). Representations of plasmid 2072 and 2052 are presented in Figure 34C and Figure 39C.

Example 5.21: 2X35S/CPMV HT (construct no 2074) and HT+ (construct no 2062) for PDISP/HA B Massachussetts (PrL-)+H1 California TMCT

[00286] A chimer hemagglutinin coding sequence corresponding to the ectodomain of HA from Influenza B/Massachussetts/2/2012 with deleted proteolytic loop (PrL-) fused to the transmembrane domain and cytoplasmic tail (TMCT) of H1 from influenza A/California/7/2009 and with the signal peptide of alfalfa protein disulfide isomerase (PDISP/HA B Massachussetts (PrL-)+H1 California TMCT) (Figure 40A, SEQ ID NO: 109) was cloned into original HT and modified HT+ using the same PCR-based method as as described in Example 5.26, but with modified PCR primers specifically designed for PDISP/HA B Massachussetts (PrL-)+H1 California TMCT. The amino acid sequence of mature HA B Massachussetts (PrL-)+H1

California TMCT fused with PDISP is presented in Figure 40B (SEQ ID NO: 110). Representations of plasmid 2074 and 2062 are presented in Figure 35C and Figure 40C.

Example 5.22: 2X35S/CPMV HT (construct no 1445) and HT+ (construct no 1839) for HA B Wisconsin (PrL-)

[00287] A coding sequence corresponding to HA from Influenza B/Wisconsin/1/2010 with deleted proteolytic loop (PrL-) with his native signal peptide (HA B Wisconsin (PrL-)) (Figure 41A, SEQ ID NO: 111) was cloned into original HT and modified HT+ using the same PCR-based method as described in Example 5.26, but with modified PCR primers specifically designed for HA B Wisconsin (PrL-). The amino acid sequence of HA B Wisconsin (PrL-) with his native signal peptide is presented in Figure 41B (SEQ ID NO: 112). Representations of plasmid 1445 and 1839 are presented in Figure 36C and 41C.

Example 5.23: 2X35S/CPMV HT (construct no 1454) and HT+ (construct no 1860) for HA B Wisconsin (PrL-)+H1 California TMCT

[00288] A chimer hemagglutinin coding sequence corresponding to the ectodomain of HA from Influenza B/ Wisconsin /2/2012 with deleted proteolytic loop (PrL-) fused to the transmembrane domain and cytoplasmic tail (TMCT) of H1 from influenza A/California/7/2009 with the native signal peptide of HA B Wisconsin (HA B Wisconsin (PrL-)+H1 California TMCT) (Figure 42A, SEQ ID NO: 113) was cloned into original HT and modified HT+ using the same PCR-based method as described in Example 5.26 but with modified PCR primers specifically designed for HA B Wisconsin (PrL-)+H1 California TMCT. The amino acid sequence of HA B Wisconsin (PrL-)+H1 California TMCT is presented in Figure 42B (SEQ ID NO: 114). Representations of plasmid 1454 and 1860 are presented in Figure 37C and 42C.

Example 5.24 - 2X35S/CPMV HT (construct no 489), 2X35S/CPMV160+ (construct no 1880) and 2X35S/CPMV160 (construct no 1885) for H5 Indonesia

[00289] A coding sequence corresponding to native H5 from Influenza A/Indonesia/5/2005 (Figure 43A, SEQ ID NO: 115) was cloned into original CPMV-HT, CPMV160+ and CPMV160 using the same PCR-based method as described in Example 5.25 but with modified PCR primers specifically designed for H5 Indonesia.

The amino acid sequence of native H5 from Influenza A/Indonesia/5/2005 is presented in Figure 43B (SEQ ID NO: 116). Representation of plasmid 489 is presented in Figure 43C.

Example 5.25 - 2X35S/CPMV160+/PDISP/H3 Victoria/ NOS (Construct number 1800)

[00290] A sequence encoding H3 from Influenza A/Victoria/361/2011 in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H3 Victoria) was cloned into 2X35S/CPMV160+/NOS expression system (CPMV160+) using the following PCR-based method. A fragment containing the PDISP/H3 Victoria coding sequence was amplified using primers IF**(SacII)-PDI.s1+4c (Figure 44A, SEQ ID NO: 117) and IF-H3V36111.s1-4r (Figure 44B, SEQ ID NO: 118), using PDISP/H3 Victoria sequence (Figure 44C, SEQ ID NO: 119) as template. The PCR product was cloned in 2X35S/CPMV160+/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 2171 (Figure 44D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 2171 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in a CPMV160+ based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 44E (SEQ ID NO: 120). The resulting construct was given number 1800 (Figure 44F, SEQ ID NO: 121). The amino acid sequence of mature H3 from Influenza A/Victoria/361/2011 fused with PDISP is presented in Figure 44G (SEQ ID NO: 122). A representation of plasmid 1800 is presented in Figure 44H.

Example 5.26: 2X35S/CPMV-HT+/ PDISP/H3 Victoria/ NOS (Construct number 1819)

[00291] A sequence encoding H3 from Influenza A/Victoria/361/2011 in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H3 Victoria) was cloned into 2X35S-CPMV-HT+/NOS expression using the following PCR-based method. A fragment containing the PDISP/H3 Victoria coding sequence was amplified using primers IF(SacII)-Kozac_PDI.c (Figure

45A, SEQ ID NO: 123) and IF-H3V36111.s1-4r (Figure 45B, SEQ ID NO: 124), using PDISP/H3 Victoria sequence (Figure 44C, SEQ ID NO: 119) as template. The PCR product was cloned in 2X35S/CPMV-HT+/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 2181 (Figure 45D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 2181 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in a CPMV-HT+ based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 45E (SEQ ID NO: 125). The resulting construct was given number 1819 (Figure 45E, SEQ ID NO: 126). The amino acid sequence of mature H3 from Influenza A/Victoria/361/2011 fused with PDISP is presented in Figure 44G (SEQ ID NO: 122). A representation of plasmid 1819 is presented in Figure 45F.

Example 5.27 2X35S/CPMV HT+/ PDISP/H2 Singapore/ NOS (Construct number 2220)

[00292] A sequence encoding H2 from Influenza A/Singapore/1/1957 in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H2 Singapore) was cloned into 2X35S/CPMV HT+/NOS expression system using the following PCR-based method. A fragment containing the PDISP/H2 Singapore coding sequence was amplified using primers IF(SacII)-Kozac_PDI.c (described for construct 1819 in Example 5.26) and IF**-H2S157.s1-6r (Figure 48A, SEQ ID NO: 127), using PDISP/H2 Singapore sequence (Figure 48B, SEQ ID NO : 128) as template. The PCR product was cloned in 2X35S/CPMV HT+/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 2181 (described for construct 1819 in Example 5.26) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 2181 is an acceptor plasmid intended for "In Fusion" cloning of genes of interest in a CPMV HT+ based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and

terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 45D. The resulting construct was given number 2220 (Figure 48C, SEQ ID NO: 129). The amino acid sequence of mature H2 from Influenza A/Singapore/1/1957 fused with PDISP is presented in Figure 48D (SEQ ID NO: 130). A representation of plasmid 2220 is presented in Figure 48E.

Example 5.28 2X35S/CPMV HT+/ PDISP/H2 Singapore with deleted proteolytic loop/ NOS (Construct number 2221)

[00293] A sequence encoding H2 from Influenza A/Singapore/1/1957 with deleted proteolytic loop in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H2 Singapore with deleted proteolytic loop) was cloned into 2X35S/CPMV HT+/NOS expression system using the following PCR-based ligation method presented by Darveau et al. (Methods in Neuroscience 26: 77-85 (1995)). In a first round of PCR, a fragment containing H2 from Influenza A/Singapore/1/1957 coding sequence from nt 1 to nt 1032 was amplified with primers IF(SacII)-Kozac_PDI.c (described for construct 1819 in Example 5.26) and H2S157(Prl-).r (Figure 49A, SEQ ID NO: 131), using PDISP/H2 Singapore sequence (Figure 48B, SEQ ID NO: 128) as template. A second fragment, containing H2 from Influenza A/Singapore/1/1957 coding sequence from nt 1084 to nt 1716, was amplified with primers H2S157(Prl-).c (Figure 49B, SEQ ID NO : 132) and IF**-H2S157.s1-6r (Figure 48A, SEQ ID NO: 127) using PDISP/H2 Singapore sequence (Figure 48B, SEQ ID NO :128) as template. The PCR products from both amplifications were then mixed and used as template for a second round of amplification using IF(SacII)-Kozac_PDI.c (described for construct 1819 in Example 5.26) and IF**-H2S157.s1-6r (Figure 48A, SEQ ID NO: 127) as primers. The PCR product (comprising PDISP/H2 Singapore coding sequence with aa 321 to 337 replaced by a GG linker) was cloned in 2X35S/CPMV HT+/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 2181 (described for construct 1819 in Example 5.26) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 2181 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in a CPMV HT+ based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of

silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in Figure 45D (described for construct 1819, in Example 5.26). The resulting construct was given number 2221 (Figure 49C, SEQ ID NO: 133). The amino acid sequence of mature H2 from Influenza A/Singapore/1/1957 with deleted proteolytic loop fused with PDISP is presented in Figure 49D (SEQ ID NO: 134). A representation of plasmid 2221 is presented in Figure 49E.

Example 5.29 PDISP/H2 Singapore (Construct number 2222) and PDISP/H2 Singapore with deleted proteolytic loop (Construct number 2223) in 2X35S/CPMV 160+/NOS expression system

[00294] Sequences encoding H2 from Influenza A/Singapore/1/1957 with or without proteolytic loop in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H2 Singapore and PDISP/H2 Singapore with deleted proteolytic loop) were cloned into 2X35S/CPMV 160+/NOS expression system using the same PCR-based method as construct 2220 and 2221, respectively, but using modified forward primer IF**(SacII)-PDI.s1+4c (described for construct 1800 in Example 5.25) for amplification and a different acceptor plasmid. Resulting PCR products were cloned in 2X35S/CPMV 160+/NOS expression system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct number 2171 (described for construct 1800 in Example 5.25) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 2171 is an acceptor plasmid intended for “In Fusion” cloning of genes of interest in a CPMV 160+ based expression cassette. It also incorporates a gene construct for the co-expression of the TBSV P19 suppressor of silencing under the alfalfa Plastocyanin gene promoter and terminator. The backbone is a pCAMBIA binary plasmid and the sequence from left to right t-DNA borders is presented in (described for construct 1800 in Example 5.25). The resulting constructs were given number 2222 for PDISP/H2 Singapore (Figure 50A, SEQ ID NO: 135) and 2223 for PDISP/H2 Singapore with deleted proteolytic loop (Figure 50B, SEQ ID NO: 136). Representations of plasmid 2222 and 2223 are presented in figure 50C and 50D respectively.

Example 5.30 2X35S/CPMV HT+ (construct no 2019) and 160+ (construct no 2139) for PDISP/H3 Perth

[00295] A coding sequence corresponding to H3 from Influenza A/Perth/16/2009 in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H3 Perth) (Figure 51A, SEQ ID NO: 137) was cloned into modified CPMV HT+ and 160+ using the same In Fusion-based approach as construct 2220 and 2222, respectively, but with modified PCR primer specifically designed for PDISP/H3 Perth (Figure 51B, SEQ ID NO: 138). The amino acid sequence of mature H3 from Influenza A/Perth/16/2009 fused with PDISP is presented in Figure 51C (SEQ ID NO: 139). Representations of plasmid 2019 and 2139 are presented in Figure 51D and Figure 51E.

Example 5.31 2X35S/CPMV HT+ (construct no 2039) and 160+ (construct no 2159) for PDISP/H3 Perth with deleted proteolytic loop

[00296] A coding sequence corresponding to H3 from Influenza A/Perth/16/2009 with deleted proteolytic loop in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H3 Perth with deleted proteolytic loop) (Figure 52, SEQ ID NO: 140) was cloned into modified CPMV HT+ and 160+ using the same In Fusion-based approach as construct 2221 and 2223, respectively, but with modified PCR primers specifically designed for PDISP/H3 Perth with deleted proteolytic loop (Figure 51B (SEQ ID NO: 138), 52B (SEQ ID NO: 141) and 53C (SEQ ID NO: 142). The amino acid sequence of mature H3 from Influenza A/Perth/16/2009 with deleted proteolytic loop fused with PDISP is presented in Figure 52D (SEQ ID NO: 143). Representations of plasmid 2039 and 2159 are presented in Figure 52E and Figure 52F.

Example 5.32 2X35S/CPMV HT+ (construct no 2230) and 160+ (construct no 2250) for PDISP/H3 Victoria with deleted proteolytic loop

[00297] A coding sequence corresponding to H3 from Influenza A/Victoria/361/2011 with deleted proteolytic loop in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H3 Victoria with deleted proteolytic loop) (Figure 53, SEQ ID NO: 144) was cloned into modified

CPMV HT+ and 160+ using the same In Fusion-based approach as construct 2221 and 2223 (see Examples 5.28 and 5.29), respectively, but with modified PCR primer specifically designed for PDISP/H3 Victoria with deleted proteolytic loop (Figures 53B (SEQ ID NO: 145) and 53C (SEQ ID NO: 146). The amino acid sequence of mature H3 from Influenza A/Victoria/361/2011 with deleted proteolytic loop fused with PDISP is presented in Figure 53D (SEQ ID NO: 147). Representations of plasmid 2230 and 2250 are presented in Figure 53E and Figure 53F.

Example 5.33 2X35S/CPMV HT+/PDISP/H7 Hangzhou/NOS (Construct no 2142)

[00298] A coding sequence corresponding to H7 from Influenza A/Hangzhou/1/2013 in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H7 Hangzhou) (Figure 54A, SEQ ID NO: 148) was cloned into modified CPMV HT+ using the same In Fusion-based approach as construct 2220 but with modified PCR primer specifically designed for PDISP/H7 Hangzhou (Figure 54B, SEQ ID NO: 149). The amino acid sequence of mature H7 from Influenza A/Hangzhou/1/2013 fused with PDISP is presented in Figure 54C (SEQ ID NO: 150). Representation of plasmid 2142 is presented in Figure 54E.

Example 5.34 2X35S/CPMV HT+/PDISP/H7 Hangzhou with deleted proteolytic loop/NOS (Construct no 2152)

[00299] A coding sequence corresponding to H7 from Influenza A/Hangzhou/1/2013 with deleted proteolytic loop in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/ H7 Hangzhou with deleted proteolytic loop) (Figure 55A, SEQ ID NO: 151) was cloned into modified CPMV HT+ using the same In Fusion-based approach as construct 2221 but with modified PCR primers specifically designed for PDISP/ H7 Hangzhou with deleted proteolytic loop (Figure 54B (SEQ ID NO: 149), Figure 55B (SEQ ID NO: 152) and Figure 55C (SEQ ID NO: 153)). The amino acid sequence of mature H7 from Influenza A/Hangzhou/1/2013 with deleted proteolytic loop fused with PDISP is presented in Figure 55D (SEQ ID NO: 154). Representation of plasmid 2152 is presented in Figure 55E.

Example 5.35 2X35S/CPMV HT+ (construct no 2224) and 160+ (construct no 2226) for PDISP/H9 Hong Kong

[00300] A coding sequence corresponding to H9 from Influenza A/Hong Kong/1073/1999 in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H9 Hong Kong) (Figure 56A, SEQ ID NO: 155) was cloned into modified CPMV HT+ and 160+ using the same In Fusion-based approach as construct 2220 and 2222, respectively, but with modified PCR primer specifically designed for PDISP/H9 Hong Kong (Figure 56B, SEQ ID NO: 156). The amino acid sequence of mature H9 from Influenza A/Hong Kong/1073/1999 fused with PDISP is presented in Figure 56C (SEQ ID NO: 157). Representations of plasmid 2224 and 2226 are presented in Figures 56D and Figure 56E.

Example 5.36 2X35S/CPMV HT+ (construct no 2225) and 160+ (construct no 2227) for PDISP/H9 Hong Kong with deleted proteolytic loop

[00301] A coding sequence corresponding to H9 from Influenza A/Hong Kong/1073/1999 with deleted proteolytic loop in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/H9 Hong Kong with deleted proteolytic loop) (Figure 57A, SEQ ID NO: 158) was cloned into modified CPMV HT+ and 160+ using the same In Fusion-based approach as construct 2221 and 2223, respectively, but with modified PCR primers specifically designed for PDISP/H9 Hong Kong with deleted proteolytic loop (Figure 56B (SEQ ID NO: 156), Figure 57B (SEQ ID NO: 159) and Figure 57C (SEQ ID NO: 160)). The amino acid sequence of mature H9 from Influenza A/Hong Kong/1073/1999 with deleted proteolytic loop fused with PDISP is presented in Figure 57D (SEQ ID NO: 161). Representations of plasmid 2225 and 2227 are presented in Figure 57E and Figure 57F.

Example 5.37 2X35S/CPMV 160+/PDISP/HA B Malaysia/NOS (Construct no 2013)

[00302] A coding sequence corresponding to HA from Influenza B/Malaysia/2506/2004 in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/HA B Malaysia) (Figure 58A, SEQ ID

NO: 162) was cloned into modified CPMV 160+ using the same In Fusion-based approach as construct 2222 but with modified PCR primer specifically designed for PDISP/HA B Malaysia (Figure 58B, SEQ ID NO: 163). The amino acid sequence of mature HA from Influenza B/Malaysia/2506/2004 fused with PDISP is presented in Figure 58C (SEQ ID NO: 164). Representation of plasmid 2013 is presented in Figure 58D.

Example 5.38 2X35S/CPMV 160+/PDISP/HA B Malaysia with deleted proteolytic loop/NOS (Construct no 2014)

[00303] A coding sequence corresponding to HA from Influenza B/Malaysia/2506/2004 with deleted proteolytic loop in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/HA B Malaysia with deleted proteolytic loop) (Figure 59A, SEQ ID NO: 165) was cloned into modified CPMV 160+ using the same In Fusion-based approach as construct 2223 but with modified PCR primers specifically designed for PDISP/HA B Malaysia with deleted proteolytic loop (Figure 58B (SEQ ID NO: 163), Figure 59B (SEQ ID NO: 166), Figure 59C (SEQ ID NO: 167). The amino acid sequence of mature HA from Influenza B/Malaysia/2506/2004 with deleted proteolytic loop fused with PDISP is presented in Figure 59D (SEQ ID NO: 168). Representation of plasmid 2014 is presented in Figure 59E.

Example 5.39 2X35S/CPMV HT (construct no 2070), HT+ (construct no 2080) and 160+ (-Mprot) (construct no 2090) for PDISP/HA B Massachusetts

[00304] A coding sequence corresponding to HA from Influenza B/Massachusetts/2/2012 in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/ HA B Massachusetts) (Figure 60A, SEQ ID NO: 169) was cloned into original HT, modified HT+ and 160+ using the same In Fusion-based approach as construct 2072, 2220 and 2222, respectively, but with modified PCR primers specifically designed for PDISP/ HA B Massachusetts. The amino acid sequence of mature HA from Influenza B/Massachusetts/2/2012 fused with PDISP is presented in Figure 60B (SEQ ID NO: 170). Representations of plasmid 2070, 2080 and 2090 are presented in Figure 60C, Figure 60D and Figure 60E.

Example 5.40 2X35S/CPMV HT+ (construct no 2102), HT+ with BeYDV (construct no 2104) for PDISP/HA B Florida with deleted proteolytic loop

[00305] A coding sequence corresponding to HA from Influenza B/Florida with the proteolytic loop deleted and in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/ HA B Florida) (Figure 61D, SEQ ID NO: 193) was cloned into modified HT+ the same In Fusion-based approach as described above, but with modified PCR primers specifically designed for PDISP/ HA B/Florida (see Figures Fig 61A (SEQ ID No:190), Fig 61B (SEQ ID NO:191) and Fig 61C (SEQ ID NO: 192). The nucleotide sequence of the resulting expression cassette 2102 is given in Fig 61F (SEQ ID NO: 195). Similarly, a coding sequence corresponding to HA from Influenza B/Florida with the proteolytic loop deleted and in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase was cloned into modified HT+ together with amplification element BeYDV. The nucleotide sequence of the resulting expression cassette 2104 is given in Fig 61F (SEQ ID NO: 196). The amino acid sequence of mature HA from Influenza B/Florida with deleted proteolytic loop fused with PDISP is presented in Figure 61E (SEQ ID NO: 194). Representations of plasmid 2102 and 2104 are presented in Figure 61G and 61I.

Example 5.41 2X35S/CPMV HT+ (construct no 2106), HT+ with BeYDV (construct no 2108) for PDISP/HA B Florida +H1 California TMCT with deleted proteolytic loop

[00306] A coding sequence corresponding to HA from Influenza B/Florida +H1 California TMCT with the proteolytic loop deleted and in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase (PDISP/ HA B Florida +H1 California TMCT) (Figure 62B, SEQ ID NO: 198) was cloned into modified HT+ the same In Fusion-based approach as described above, but with modified PCR primers specifically designed for PDISP/ HA B/Florida+H1 California TMCT (see Figures Fig 61A (SEQ ID No:197). The nucleotide sequence of the resulting expression cassette 2106 is given in Fig 62D (SEQ ID NO: 200). Similarly, a coding sequence corresponding to HA from Influenza B/Florida +H1 California TMCT with the proteolytic loop deleted and in which the native signal peptide has been replaced by that of alfalfa protein disulfide isomerase was cloned into modified HT+ together with amplification element BeYDV. The nucleotide sequence of the

resulting expression cassette 2108 is given in Fig 62F (SEQ ID NO: 201). The amino acid sequence of mature HA from Influenza B/Florida+H1 California TMCT with deleted proteolytic loop fused with PDISP is presented in Figure 62C (SEQ ID NO: 199). Representations of plasmid 2106 and 2108 are presented in Figure 62E and 62G.

[00307] All citations are hereby incorporated by reference.

[00308] The present invention has been described with regard to one or more embodiments. However, it will be apparent to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as defined in the claims.

CLAIMS

1. A nucleic acid comprising a regulatory region active in a plant and an expression enhancer active in a plant, the regulatory region and the expression enhancer operatively linked to a nucleotide sequence encoding a mutant influenza hemagglutinin (HA) comprising a fully deleted proteolytic loop, wherein the nucleic acid does not comprise a Bean Yellow Dwarf Virus long intergenic region (BeYDV LIR), and a BeYDV short intergenic region (BeYDV SIR).

2. The nucleic acid of claim 1, wherein the expression enhancer is selected from the group consisting of CPMVX, CPMVX+ , CPMV-HT+, CPMV HT+[WT115] and CPMV HT+ [511] wherein:

CPMVX comprises X nucleotides of SEQ ID NO:93, where X= nucleotides 1-160, 1-155, 1-150, or 1-114 of SEQ ID NO:93, or a sequence that comprises between 80% to 100% sequence similarity with CPMVX, where X= nucleotides 1- 160, 1-155, 1-150, or 1-114 of SEQ ID NO:93,

CPMVX+ comprises X nucleotides of SEQ ID NO:1, where X= nucleotides 1-160, 1-155, 1-150, or 1-114 of SEQ ID NO:1, or a sequence that comprises between 80 to 100 % sequence similarity with CPMVX, where X= nucleotides 1-160, 1-155, 1-150, or 1-114 of SEQ ID NO:93, and a stuffer sequence comprises from 1-100 nucleotides fused to the 3' end of the CMPVX sequence,

CPMV-HT+ comprises a comovirus 5' untranslated region (UTR) and a modified, lengthened, or truncated stuffer sequence,

CPMV HT+[WT115] comprises the sequence of SEQ ID NO:189, and

CPMV HT+ [511] comprises the sequence of SEQ ID NO:188.

3. The nucleic acid of claim 1, wherein the expression enhancer is not CPMV HT.

4. The nucleic acid of claim 1, wherein the mutant HA is maintained as a HA0 precursor.

5. The nucleic acid of claim 1, wherein the proteolytic loop is fully replaced with a linker sequence.

6. The nucleic acid of claim 5, wherein the linker sequence has the amino acid sequence GG, TETQ or TETR.

7. The nucleic acid of claim 1, wherein the HA encoded by the nucleotide sequence is selected from the group consisting of HA from influenza types B, C, A, and subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16.

8. The nucleic acid of claim 1, wherein the HA encoded by the nucleotide sequence is selected from the group consisting of HA from influenza types C, A, subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, or strains B Massachusetts/2/2012, B/Malaysia/2506/2004 and B/Florida/4/2006.

9. The nucleic acid of claim 1, wherein the nucleotide sequence encoding the influenza hemagglutinin (HA) protein has at least 70% sequence identity to a sequence selected from the group consisting of SEQ ID NO:. 17, 18, 20, 21, 41, 58, 77, 81, 85, 92, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 134, 143, 147, 154, 161, 168, 194 and 199.

10. The nucleic acid of claim 1, wherein the HA is an HA0.

11. The nucleic acid of claim 1, wherein the mutant HA comprises a native or a non-native signal peptide.

12. The nucleic acid of claim 1, wherein the nucleotide sequence encoding the mutant HA comprises a chimeric nucleotide sequence encoding, in series, a mutant HA ectodomain comprising the fully deleted proteolytic loop, an influenza

transmembrane domain, and a cytoplasmic tail, wherein the mutant HA ectodomain is from a first influenza strain and the transmembrane domain and the cytoplasmic tail is from a second influenza strain.

13. A method of producing influenza virus like particle (VLP) in a plant comprising,
a) introducing the nucleic acid of claim 1 into the plant or portion of the plant; wherein the portion of the plant includes plant cells, tissue, plant leaves, stems, fruit, roots or a combination thereof;
b) incubating the plant or portion of the plant under conditions that permit the expression of the nucleic acids, thereby producing the VLP.

14. The method of claim 13, wherein in step a) a second nucleic acid is introduced into the plant or portion of the plant, the second nucleic acid comprising a second regulatory region active in the plant and operatively linked to a nucleotide sequence encoding a proton channel protein wherein the proton channel protein is selected from influenza M2 or BM2.

15. A method of producing influenza virus like particles (VLPs) in a plant comprising:
a) providing a plant, or a portion of a plant, comprising the nucleic acid of claim 1, wherein the portion of the plant includes plant cells, tissue, plant leaves, stems, fruit, roots or a combination thereof and
b) incubating the plant or portion of the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLPs.

16. The method of claim 15, further comprising the steps of
c) harvesting the plant, and
d) purifying the VLPs, wherein the VLPs range in size from 80-300 nm.

17. The method of claim 13, wherein the HA encoded by the nucleotide sequence is selected from the group consisting of HA from influenza types C, A, subtypes H1, H2,

H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 or strains B Massachusetts/2/2012, B/Malaysia/2506/2004 or B/Florida/4/2006.

18. A VLP produced by the method of claim 17, wherein the VLP comprises HA comprising the fully deleted proteolytic loop.

19. The VLP of claim 18 further comprising one or more than one lipid derived from a plant.

20. The VLP of claim 19, wherein the mutant HA protein comprises plant-specific N-glycans or modified N-glycans.

21. A VLP comprising lipid derived from a plant and mutant HA, wherein the mutant HA comprise a fully deleted proteolytic loop and plant-specific N-glycans or modified N-glycans
wherein the mutant HA is derived from an influenza type C, type A, subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, strain B Massachusetts/2/2012, B/Malaysia/2506/2004 or B/Florida/4/2006 HA.

22. A method of producing a mutant HA protein comprising a fully deleted proteolytic loop in a plant or portion of a plant, wherein the portion of the plant includes plant cells, tissue, plant leaves, stems, fruit, roots or a combination thereof, the method comprising,
a) introducing the nucleic acid of claim 1 into the plant or portion of the plant;
b) incubating the plant or portion of the plant under conditions that permit the expression of the HA protein, thereby producing the mutant HA protein,
c) harvesting the plant and purifying the mutant HA protein.

23. The method of claim 21, wherein the HA encoded by the nucleotide sequence is selected from the group consisting of HA from influenza types C, A, subtypes H1, H2,

H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 or B Massachusetts/2/2012, B/Malaysia/2506/2004 or B/Florida/4/2006.

24. An HA protein produced by the method of claim 23, wherein the HA comprises a fully deleted proteolytic loop and has hemagglutinin (HA) activity.

25. An HA protein comprising a fully deleted proteolytic loop, wherein the HA is derived from an influenza type C, type A, subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, strain B Massachusetts/2/2012, B/Malaysia/2506/2004 or B/Florida/4/2006 HA.

26. The HA of claim 25, wherein the HA further comprises plant-specific N-glycans or modified N-glycans

27. An HA encoded by the nucleic acid of claim 8.

28. The method of claim 13, wherein in the step of introducing (step a), the nucleic acid is introduced in the plant in a transient manner, or the nucleic acid is introduced in the plant so that it is stable.

29. plant comprising the nucleic acid of claim 1.

30. A composition comprising an effective dose of the VLP of claim 18 or 21 and a pharmaceutically acceptable carrier for use as a medicament for inducing an immune response.

31. A vaccine comprising an effective dose of the VLP of claim 18 or 21 for use in inducing an immune response.

32. Use of the VLP of claim 18 or 21 in the manufacture of a medicament for inducing immunity to an influenza virus infection in a subject.

33. The use of claim 32, wherein the VLP is to be administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

34. A method of increasing product yield of an HA from B or H3 influenza strain protein expressed in a plant or portion of a plant wherein the portion of the plant includes plant cells, tissue, plant leaves, stems, fruit, roots or a combination thereof, the method comprising,

a) introducing the nucleic acid of claim 1 into the plant;

b) incubating the plant or portion of the plant under conditions that permit the expression of the HA protein, thereby producing a mutant HA protein,

c) harvesting the plant and purifying the mutant HA protein, wherein the mutant HA protein has increased product quality, when compared to a native HA.

35. A nucleic acid comprising a regulatory region active in a plant and an expression enhancer active in a plant, the regulatory region and the expression enhancer operatively linked to a nucleotide sequence encoding a mutant influenza hemagglutinin (HA) from B or H3 influenza strain, the mutant HA comprising a partially or fully deleted proteolytic loop, wherein the nucleic acid does not comprise a Bean Yellow Dwarf Virus long intergenic region (BeYDV LIR), and a BeYDV short intergenic region (BeYDV SIR).