Increasing Virus-Like Particle Yield in Plants

FIELD OF INVENTION

[0001] The present invention relates to producing viral proteins in plants. More specifically, the present invention relates to producing and increasing virus-like particles production 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. However, Gomez-Puertas et al., (1999, J. Gen. Virol, 80, 1635-1645) also showed that overexpression of M2 completely blocked CAT RNA transmission to MDCK cultures.

[0006] M2 functions as an ion channel protein and it has been shown that, when this protein is overexpressed, the intracellular transport of co-expressed HA is inhibited and the accumulation of heamaglutinin (HA) at the plasma membrane is reduced by 75 - 80% (Sakaguchi et al., 1996; Henkel & Weisz, 1998). Furthermore, by overexpressing M2, the accumulation of virus membrane proteins at the plasma membrane is reduced and hence there is a drastic reduction in the number of functional VLPs produced.

[0007] The M2 protein is abundantly expressed at the cell surface of influenza A infected cells (Lamb et al. (1985) Cell, 40, 627 to 633). The protein is also found in the membrane of the virus particle itself, but in much smaller quantities, 14 to 68 molecules of M2 per virion (Zebedee and Lamb (1988) J. Virol. 62, 2762 to 72). The M2 protein is posttranslationally modified by the addition of a palmitic acid on cysteine at position 50 (Sugrue et al. (1990) Virology 179, 51 to 56).

[0008] The M2 protein is a homotetramer composed of two disulfide-linked dimers, which are held together by noncovalent interactions (Sugrue and Hay (1991) Virology 180, 617 to 624). By site-directed mutagenesis, Holsinger and Lamb, (1991) Virology 183, 32 to 43, demonstrated that the cysteine residues at positions 17 and 19 are involved in disulfide bridge formation. Only the cysteine at position 17 is present in all viruses analyzed. In the virus strains where cysteine 19 is also present, it is not known whether a second disulfide bridge is formed in the same dimer (already linked by Cys 17-Cys 17) or with the other dimer.

[0009] Smith et al. (US Patent Application 2010/0143393) and Song et al. (Plos ONE 2011 6(1):e14538) describe vaccines and VLPs that comprise influenza M2 protein. The VLPs comprise at least a viral core protein such as M1. This core protein drives budding and release of the particles from the insect host cells.

[0010] Szecsi et al. (Virology Journal, 2006, 3:70) assembled VLPs on replication-defective core particles derived from murine leukaemia virus (MLV). The engineered influenza VLP are derived by transiently co-expressing cells surface (HA, NA, M2) and internal viral components (Gag, GFP marker genome) and harbour at their surface HA, HA and either NA or M2, or all three proteins derived from the H7N1 or H5N1 virus. According to Szecsi et al. the expression of M2 during Flu-VLP production did not influence the incorporation of HA or NA onto viral particles (page 2, right column, second paragraph in Szecsi et al.).

SUMMARY OF THE INVENTION

[0011] The present invention relates to producing viral proteins in plants. More specifically, the present invention relates to producing and increasing virus-like particles production in plants.

[0012] It is an object of the invention to provide an improved method to increase virus like particle production in plants.

[0013] According to the present invention there is provided a method (A) of producing a virus like particle (VLP) in a plant comprising,

a) introducing a first nucleic acid comprising a first regulatory region active in the plant and operatively linked to a nucleotide sequence encoding a structural virus protein into the plant, or portion of the plant,

b) introducing a second nucleic acid comprising a second regulatory region active in the plant and operatively linked to a nucleotide sequence encoding a channel protein

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

The first regulatory region active in the plant, and the second regulatory region active in the plant may be the same or different.

[0014] The channel protein of the method (A) described above may be a proton channel protein. The proton channel protein may be selected from M2 or BM2. Furthermore, the proton channel protein may comprise the proton channel signature sequence HXXXW. The M2 protein may be an M2 protein obtained from influenza A/Puerto Rico/8/1934 (SEQ ID NO:14) or from influenza A/New Caledonia/20/1999 (SEQ ID NO:11).

[0015] The present invention also provides the method (A) as described above , wherein the structural virus protein comprises a trimerization domain. Furthermore, the nucleotide sequence encoding the structural virus protein comprises a chimeric nucleotide sequence encoding, in series, an antigenic viral protein or fragment thereof, an influenza transmembrane domain, and a cytoplasmic tail. The structural virus protein may comprise an influenza HA protein. Furthermore one or more proteolytic loop of the influenza HA protein may be deleted .

[0016] The present invention provides the method (A) as described above wherein, the nucleotide sequence encoding the structural virus protein may be selected from the group consisting of B HA, C, HA, H2, H3, H4, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16. For example, the nucleotide sequence encoding the structural virus protein may be Type B HA or H3. The nucleotide sequence encoding the structural virus protein may be for example HA from influenza B/Brisbane/60/2008, B/Malaysia/2506/2004 or B/Wisconsin/1/2010, or H3 from

influenza A/Perth/16/2009 or A/Victoria/361/2011. Furthermore, the nucleotide sequence encoding a structural virus protein has at least 70% sequence identity to SEQ ID NO: 23, 28, 43, 46, 51, 57 or 61. The sequence of the structural virus protein may also be comprise the sequence of SEQ ID NO:25, 30, 41, 48, 54, 58 or 64.

[0017] The present invention also includes the method (A) as described above, wherein the first nucleic acid sequence comprises the first regulatory region operatively linked with a one or more than one comovirus enhancer, the nucleotide sequence encoding the structural virus protein, and one or more than one geminivirus amplification element, and a third nucleic acid encoding a geminivirus replicase is introduced into the plant or portion of the plant. The one or more than one comovirus enhancer may be a comovirus UTR, for example, a Cowpea Mosaic Virus hyperanslatable (CPMV-HT) UTR such as the CPMV-HT 5' and/or 3'UTR. The one or more than one geminivirus amplification element may be selected from a Bean Yellow Dwarf Virus long intergenic region (BeYDV LIR), and a BeYDV short intergenic region (BeYDV SIR). Furthermore, the nucleotide sequence encoding the structural virus protein may be Type B HA or H3, for example, the nucleotide sequence encoding a structural virus protein may have at least 70% sequence identity to SEQ ID NO: 23, 28, 43, 46, 51, 57 or 61. The sequence of the structural virus protein may also be comprise the sequence of SEQ ID NO:25, 30, 41, 48, 54, 58 or 64.

[0018] The method as described above (Method A) may also involving introducing another nucleic acid sequence encoding a suppressor of silencing, for example HcPro or p19.

[0019] The present invention also includes the method (A) as described above, wherein in the step of introducing (step a), the nucleic acid is transiently expressed in the plant. Alternatively, in the step of introducing (step a), the nucleic acid is stably expressed in the plant.

[0020] The method (A) as described above may further comprising a step of:

d) harvesting the plant and purifying the VLPs.

[0021] The present invention also includes the method (A) as described above, wherein the VLP does not contain a viral matrix or a core protein.

[0022] The present invention provides a VLP produced by the method (A) as described above. The VLP may further comprising one or more than one lipid derived from a plant. The VLP may also be characterized by not containing the channel protein. Furthermore, the structural virus protein of the VLP may be an HA0 protein. The one or more virus protein comprises of the VLP may comprise plant-specific N-glycans, or modified N-glycans. The present invention also provides a polyclonal antibody prepared using the VLP.

[0023] The present invention includes a composition comprising an effective dose of the VLP as just described for inducing an immune response, and a pharmaceutically acceptable carrier.

[0024] The present invention also includes a method of inducing immunity to an influenza virus infection in a subject, comprising administering the VLP as just described to the subject. The VLP may be administered to a subject orally, intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously.

[0025] The present invention also provides plant matter comprising a VLP produced by the method (A) 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.

[0026] The present invention also provides a method (B) of producing a virus like particle (VLP) comprising,

a) providing a plant or portion of the plant comprising a first nucleic acid comprising a first regulatory region active in the plant and operatively linked to a nucleotide sequence encoding a structural virus protein into the plant, or portion of the plant, and a second nucleic acid comprising a second regulatory region active in the plant and operatively linked to a nucleotide sequence encoding a channel protein

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

The first regulatory region active in the plant, and the second regulatory region active in the plant may be the same or different.

[0027] The channel protein of the method (B) described above may be a proton channel protein. The proton channel protein may be selected from M2 or BM2. Furthermore, the proton channel protein may comprise the proton channel signature sequence HXXXW.

[0028] The present invention also provides the method (B) as described above , wherein the structural virus protein comprises a trimerization domain. Furthermore, the nucleotide sequence encoding the structural virus protein comprises a chimeric nucleotide sequence encoding, in series, an antigenic viral protein or fragment thereof, an influenza transmembrane domain, and a cytoplasmic tail. The structural virus protein may comprise an influenza HA protein. Furthermore one or more proteolytic loop of the influenza HA protein may be deleted .

[0029] The present invention provides the method (B) as described above wherein, the nucleotide sequence encoding the structural virus protein may be selected from the group consisting of B HA, C, HA, H2, H3, H4, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16. For example, the nucleotide sequence encoding the structural virus protein may be Type B HA or H3. The nucleotide sequence encoding the structural virus protein may be for example HA from influenza B/Brisbane/60/2008, B/Malaysia/2506/2004 or B/Wisconsin/1/2010, or H3 from influenza A/Perth/16/2009 or A/Victoria/361/2011.

[0030] The Present invention also includes the method (B) as described above, wherein the first nucleic acid sequence comprises the first regulatory region operatively linked with a one or more than one comovirus enhancer, the nucleotide sequence encoding the structural virus protein, and one or more than one geminivirus amplification element, and a third nucleic acid encoding a geminivirus replicase is introduced into the plant or portion of the plant. The one or more than one comovirus enhancer may be a comovirus UTR, for example, a Cowpea Mosaic Virus

hyperanslatable (CPMV-HT) UTR such as the CPMV-HT 5' and/or 3'UTR. Additionally, the one or more than one geminivirus amplification element may be selected from a Bean Yellow Dwarf Virus long intergenic region (BeYDV LIR), and a BeYDV short intergenic region (BeYDV SIR).

[0031] The method as described above (Method B) may also involving introducing another nucleic acid sequence encoding a suppressor of silencing, for example HcPro or p19.

[0032] The present invention also includes the method (B) as described above, wherein in the step of introducing (step a), the nucleic acid is transiently expressed in the plant. Alternatively, in the step of introducing (step a), the nucleic acid is stably expressed in the plant.

[0033] The method (B) as described above may further comprising a step of:

d) harvesting the plant and purifying the VLPs.

[0034] The present invention also includes the method (B) as described above, wherein the VLP does not contain a viral matrix or a core protein.

[0035] The present invention provides a VLP produced by the method (B) as described above. The VLP may further comprising one or more than one lipid derived from a plant. The VLP may also be characterized by not containing the channel protein. Furthermore, the structural virus protein of the VLP may be an HA0 protein. The, one or more virus protein comprises of the VLP may comprise plant-specific N-glycans, or modified N-glycans. The present invention also provides a polyclonal antibody prepared using the VLP.

[0036] The present invention includes a composition comprising an effective dose of the VLP made by the method (B) as just described, for inducing an immune response, and a pharmaceutically acceptable carrier.

[0037] The present invention also includes a method of inducing immunity to an influenza virus infection in a subject, comprising administering the VLP as just described, to the subject. The VLP may be administered to a subject orally,

intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously.

[0038] The present invention also provides plant matter comprising a VLP produced by the method (B) 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.

[0039] The present invention provides a polypeptide comprising the amino acid sequence of SEQ ID NO:41 (PDISP/HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop), and a nucleic acid sequence encoding the polypeptide of SEQ ID NO:41. The nucleic acid sequence may comprises the nucleotide sequence of SEQ ID NO:43. The present invention provides a VLP comprising the polypeptide comprising the amino acid sequence of SEQ ID NO:41. The VLP may further comprising one or more than one lipid derived from a plant. The VLP may also be characterized by not containing the channel protein. The VLP may comprise plant-specific N-glycans, or modified N-glycans. The present invention provides a composition comprising an effective dose of the VLP comprising the amino acid sequence of SEQ ID NO:41, for inducing an immune response, and a pharmaceutically acceptable carrier. The present invention also includes a method of inducing immunity to an influenza virus infection in a subject, comprising administering the VLP comprising the amino acid sequence of SEQ ID NO:41, to the subject. The VLP may be administered to a subject orally, intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously. The present invention also provides plant matter comprising a VLP comprising the amino acid sequence of SEQ ID NO:41. 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.

[0040] By co-expressing a structural virus protein along with a channel protein, for example but not limited to a proton channel protein, increased yield of the structural virus protein and VLPs are observed. HA's are known to under go pH-dependant conformation change. Without wishing to bound by theory, the pH within the Golgi apparatus of the HA producing cells during maturation and migration may influence

HA folding, effects stability and increase degradation, or a combination thereof, of the HA. By co-expressing a channel protein, for example but not limited to a proton channel protein, along with an 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 HA yield.

[0041] This summary of the invention does not necessarily describe all features of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] 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:

[0043] **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).

[0044] **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).

[0045] **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).

[0046] **Figure 4A** shows primer IF-H1A-C-09.s2+4c (SEQ ID NO: 15). **Figure 4B** shows primer IF-H1A-C-09.s1-4r (SEQ ID NO: 16). **Figure 4C** shows the nucleotide sequence of the synthesized H1 gene (Genbank accession number FJ966974) (SEQ ID NO: 17). **Figure 4D** shows a schematic representation of construct 1192. SacII and StuI restriction enzyme sites used for plasmid linearization are annotated on the representation. **Figure 4E** shows construct 1192 from left to right t-DNA borders (underlined). 2X35S/CPMV-HT/PDISP/NOS with Plastocyanine-P19-Plastocyanine silencing inhibitor expression cassette (SEQ ID NO: 18). **Figure 4F** shows expression cassette number 484 from 2X35S promoter to NOS terminator. PDISP/H1 from influenza A/California/7/2009 (H1N1) is underlined. (SEQ ID NO: 19). **Figure 4G** shows amino acid sequence of PDISP-H1 from influenza A/California/7/2009 (H1N1) (SEQ ID NO: 20).

[0047] **Figure 5A** shows primer IF-S2+S4-H3 Per.c (SEQ ID NO: 21). **Figure 5B** shows primer IF-S1a4-H3 Per.r (SEQ ID NO: 22). **Figure 5C** shows the nucleotide sequence of the synthesized H3 gene (corresponding to nt 26-1726 from Genbank accession number GQ293081) (SEQ ID NO: 23). **Figure 5D** shows the expression cassette number 1019 from 2X35S promoter to NOS terminator. PDISP/H3 from influenza A/Perth/16/2009 (H3N2) is underlined. (SEQ ID NO: 24). **Figure 5E** shows the amino acid sequence of PDISP/H3 from influenza A/Perth/16/2009 (H3N2) (SEQ ID NO: 25).

[0048] **Figure 6A** shows primer IF-S2+S4-B Bris.c (SEQ ID NO: 26). **Figure 6B** shows primer IF-S1a4-B Bris.r (SEQ ID NO: 27). **Figure 6C** shows the nucleotide sequence of synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank accession number FJ766840) (SEQ ID NO: 28). **Figure 6D** 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: 29). **Figure 6E** shows the amino acid sequence of PDISP/HA from influenza B/Brisbane/60/2008 (SEQ ID NO: 30). **Figure 6F** shows a schematic representation of construct 1194. SacII and StuI restriction enzyme sites used for plasmid

linearization are annotated on the representation. **Figure 6G** 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 6H** 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).

[0049] **Figure 7A** shows primer dTmH5I-B Bris.r (SEQ ID NO: 33). **Figure 7B** shows primer B Bris-dTmH5I.c (SEQ ID NO: 34). **Figure 7C** shows primer IF-S1aS4-dTmH5I.r (SEQ ID NO: 35). **Figure 7D** shows expression cassette number 1009 from BeYDV left LIR to BeYDV right LIR. PDISP/HA B Brisbane/H5Indo TMCT is underlined. (SEQ ID NO:36). **Figure 7E** shows amino acid sequence of PDISP/HA B Brisbane/H5Indo TMCT (SEQ ID NO: 37).

[0050] **Figure 8A** shows primer 1039+1059.r (SEQ ID NO: 38). **Figure 8B** shows primer 1039+1059.c (SEQ ID NO: 39). **Figure 8C** 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 8D** shows amino acid sequence of PDISP/HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop (SEQ ID NO: 41). **Figure 8E** shows nucleotide sequence of PDISP/HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop (SEQ ID NO: 43).

[0051] **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.

[0052] **Figure 10** shows the plasmid map of construct number 1009. Construct number 1009 directs the expression of a chimeric HA from influenza strain B/Brisbane/60/2008 in which the transmembrane domain and cytosolic tail are replaced with those of H5 from influenza A/Indonesia/05/2005. This construct comprises BeYDV-derived elements for DNA amplification.

[0053] **Figure 11** shows the plasmid map of construct number 1029. Construct number 1029 directs the expression of wild-type HA from influenza strain B/Brisbane/60/2008.

[0054] **Figure 12** 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.

[0055] **Figure 13** shows the plasmid map of construct number 1019. Construct number 1019 directs the expression of wild-type H3 from influenza strain A/Perth/16/2009 (H3N2).

[0056] **Figure 14** shows the plasmid map of construct number 484. Construct number 484 directs the expression of wild-type H1 from influenza strain A/California/07/2009 (H1N1).

[0057] **Figure 15** shows the plasmid map of construct number 489. Construct number 489 directs the expression of wild-type H5 from influenza strain A/Indonesia/05/2005 (H5N1).

[0058] **Figure 16** 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).

[0059] **Figure 17** 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).

[0060] **Figure 18** shows Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. HA from B/Brisbane/60/2008 is co-expressed with M2 from A/New Caledonia/20/99. "C+": positive control, semi-purified B/Brisbane/60/2008 virus from the Therapeutic Goods Administration, Australia; "C-": negative control, mock-infiltrated plants; "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; "1009+1261": co-expression of chimeric HA from B/Brisbane/60/2008 in the presence of amplification elements (BeYDV) with M2; "1029": expression of wild-type HA from B/Brisbane/60/2008 in the absence of amplification elements (BeYDV); "1029+1261": co-expression of wild-type HA from B/Brisbane/60/2008 in the absence of amplification elements(BeYDV) with M2 from A/New Caledonia/20/99. Ratios indicate the proportion of *Agrobacterium* cultures used in co-expression experiments.

[0061] **Figure 19** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. Lane "C+": Positive control, semi-purified A/Wisconsin/15/2009 (H3N2) virus from the Therapeutic Goods Administration, Australia; "C-": negative control, mock-infiltrated plants; "`1019": expression of wild-type HA from A/Perth/16/2009 (H3N2); "1019+1261": co-expression of wild-type HA from A/Perth/16/2009 (H3N2) with M2 from A/New Caledonia/20/99. The ratio indicates the proportion of *Agrobacterium* cultures used in co-expression experiments.

[0062] **Figure 20** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. Lane "C+": Positive control, semi-purified A/California/7/2009 (H1N1) NYMC X-179A from NIBSC virus (NIBSC code 09/146); "C-": negative control, mock-infiltrated plants; "484": expression of wild-type HA from A/California/7/2009 (H1N1); "484+1261": co-expression of wild-type HA from A/California/7/2009 (H1N1) with M2 from A/New Caledonia/20/99. The ratio indicates the proportion of *Agrobacterium* cultures used in co-expression experiments.

[0063] **Figure 21** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. Lane "C+": Positive control, purified recombinant H5 from A/Indonesia/05/2005, Immune Technology Corporation (product no. IT-003-052p) ; "C-": negative control, mock-infiltrated plants; "489": expression of wild-type HA from A/Indonesia/5/05 (H5N1); "489+1261": co-expression of wild-type HA from A/Indonesia/5/05 (H5N1) with M2 from A/New Caledonia/20/99.

[0064] **Figure 22A** 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 22B** shows a comparison of hemagglutination capacity of crude protein extracts from HA-producing plants.

[0065] **Figure 23** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. **Figure 23A**: "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. **Figure 23B**: "1019": expression of wild-type HA from A/Perth/16/2009 (H3N2); "1019+1261": co-expression of wild-type HA from A/Perth/16/2009 (H3N2) with M2 from A/New Caledonia/20/99; "1019+859": co-expression of wild-type HA from A/Perth/16/2009 (H3N2) with M2 from A/Puerto Rico/8/34. Ratios indicate the proportion of *Agrobacterium* cultures used in co-expression experiments.

[0066] **Figure 24** shows the sequence alignment of HAs from several strains of influenza. The cleavage site of the precursor HA0 is indicated by an arrow.

[0067] **Figure 25A** shows primer IF-H3V36111.S2+4c (SEQ ID NO: 44). **Figure 25B** shows primer IF-H3V36111.s1-4r (SEQ ID NO: 45). **Figure 25C** shows the nucleotide sequence of synthesized H3 gene (corresponding to nt 25 to 1725 from

GISAID isolate number EPI_ISL_101506 HA sequence) (SEQ ID NO: 46). **Figure 25D** shows the nucleotide sequence of expression cassette number 1391 from 2X35S promoter to NOS terminator. PDISP/H3 from influenza A/Victoria/361/2011 (H3N2) is underlined. (SEQ ID NO: 47). **Figure 25E** shows the amino acid sequence of PDISP-H3 from influenza A/ Victoria/361/2011 (H3N2) (SEQ ID NO: 48). **Figure 25F** shows a schematic representation of construct 1391.

[0068] **Figure 26A** shows primer IF-HAB110.S1+3c (SEQ ID NO: 49). **Figure 26B** shows primer IF-HAB110.s1-4r (SEQ ID NO: 50). **Figure 26C** shows the nucleotide sequence of synthesized HA B Wisconsin (Genbank accession number JN993010) (SEQ ID NO: 51). **Figure 26D** shows a schematic representation of construct 193. **Figure 26E** 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 26F** 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 26G** shows the amino acid sequence of HA from influenza B/Wisconsin/1/2010 (SEQ ID NO: 54). **Figure 26H** shows a schematic representation of construct 1462.

[0069] **Figure 27A** shows primer HAB110(PrL-).r (SEQ ID NO: 55). **Figure 27B** shows primer HAB110(PrL-).c (SEQ ID NO: 56). **Figure 27C** 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 27D** shows the amino acid sequence of influenza B/Wisconsin/1/2010 with deleted proteolytic loop (SEQ ID NO: 58). **Figure 27E** shows a schematic representation of construct 1467.

[0070] **Figure 28A** shows primer IF-HB-M-04.s2+4c (SEQ ID NO: 59). **Figure 28B** shows primer IF-HB-M-04.s1-4r (SEQ ID NO: 60). **Figure 28C** shows the nucleotide sequence of synthesized HA B Malaysia (corresponding to nt 31-1743 from Genbank accession number EU124275) with T759C and C888G mutations being underlined. (SEQ ID NO: 61). **Figure 28D** shows a schematic representation of construct 194, with SacII and StuI restriction enzyme sites used for plasmid

linearization being annotated on the representation. **Figure 28E** shows construct 194 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: 62). **Figure 28F** shows the nucleotide sequence of expression cassette number 1631 from 2X35S promoter to NOS terminator. PDISP-HA from influenza B/ Malaysia/2506/2004 is underlined. (SEQ ID NO: 63). **Figure 28G** shows the amino acid sequence of PDISP-HA from influenza B/Malaysia/2506/2004 (SEQ ID NO: 64). **Figure 28H** shows a schematic representation of construct 1631.

[0071] **Figure 29** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. HA from B/Malaysia/2506/2004 is co-expressed with M2 from A/New Caledonia/20/99. Twenty micrograms of protein extract were loaded per lane. "C+": positive control, semi-purified B/Malaysia/2506/2004 virus from the National Institute for Biological Standards and Control, United Kingdom; "1631": expression of wild-type HA from B/Malaysia/2506/2004 in the presence of amplification elements (BeYDV); "1631+1261": co-expression of wild-type HA from B/Malaysia/2506/2004 in the presence of amplification elements (BeYDV) with M2. Ratios indicate the proportion of *Agrobacterium* cultures used in co-expression experiments.

[0072] **Figure 30A** 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 30B** 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.

[0073] **Figure 31** shows a Western blot analysis of HA protein expression in agroinfiltrated *Nicotiana benthamiana* leaves. HA from H3/Victoria/361/2011 is co-expressed with M2 from A/New Caledonia/20/99. Twenty micrograms of protein extract were loaded per lane. "C+": positive control, semi-purified H3/Wisconsin/15/2009 virus from the Therapeutic Goods Administration, Australia; "1391": expression of wild-type HA from H3/Victoria/361/2011; "1391+1261": co-expression of wild-type HA from H3/Victoria/361/2011 with M2. Ratios indicate the optical density for each *Agrobacterium* culture used in expression and co-expression experiments.

DETAILED DESCRIPTION

[0074] The following description is of a preferred embodiment.

[0075] The present invention relates to virus-like particles (VLPs) and methods of producing and increasing VLP yield and production in plants.

[0076] 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 first nucleic acid and a second nucleic acid into the plant. The first nucleic acid comprises a first regulatory region active in the plant or portion of the plant, and operatively linked to a nucleotide sequence encoding a structural virus protein. The second nucleic acid comprising a second regulatory region active in the plant and operatively linked to a nucleotide sequence encoding a channel protein, for example but not limited to a proton channel protein. The first regulatory region and the second regulatory region may be the same or different. The plant or portion of the plant is incubated under conditions that permit the expression of the nucleic acids, thereby producing the VLP. If desired, the plant or portion of the plant may be harvested and the VLP purified. Preferably, the VLP does not contain M1, a viral matrix or a core protein. 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.

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.

[0077] The present invention also provides plant matter comprising the VLP produced by expressing the first and second 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.

[0078] The VLP of the present invention may also be produced by providing a plant or portion of the plant comprising a first nucleic acid and second nucleic acid as defined above, and incubating the plant or portion of the plant under conditions that permit the expression of the first and second nucleic acids, 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.

[0079] The VLPs of the present invention comprise one or more virus proteins. For example, which is not to be considered limiting, the one or more virus protein may be a structural viral protein such as influenza hemagglutinin (HA) or a channel protein, for example but not limited to a proton channel protein, such as for example M2. The HA may be any HA, for example an H2, H3, H4, 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).

[0080] As described in more detail below, VLPs may be produced in a plant by co-expressing a first nucleic acid encoding a virus protein 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 a viral protein 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 a viral protein 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 a viral protein, 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 viral protein and the channel protein, for example but not limited to a proton channel protein, respectively.

[0081] The present invention also provides a method of increasing expression and yield of a virus protein in plant by co-expressing a first nucleic acid encoding a virus protein 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 a viral protein 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 a viral protein 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 a viral protein, may be crossed with a second plant expressing the second nucleic acid encoding the channel protein, for 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 viral protein and the channel protein for example but not limited to a proton channel protein, respectively.

Channel Protein

[0082] By "channel protein" it is meant a protein that is capable of forming a channel across a phospholipid membrane that allows for the crossing of ions and/or small molecules through the membrane. Channel proteins may be selective for size and/or charge of the ions and/or small molecules. Non limiting examples of channel proteins are non-specific channel protein that alter permeability of membranes to low molecular weight compounds and ion channel protein, such as for example chloride channel, potassium channel, sodium channel, calcium channel and proton channel.

[0083] By "proton channel protein" it is meant a protein that is capable of forming a proton selective channel across a phospholipid bilayer. The proton channel protein may be a single pass membrane protein with a transmembrane (TM) domain flanked by hydrophobic domains. The TM domain of the proton channel may comprise the sequence HXXXW (SEQ ID NO. 1).

[0084] 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). Studies on the pH threshold that induce conformational changes in different HAs, show that this threshold is approx pH 5.8-5.9 for the B strains, whereas it is more acidic (pH 5.1 to 5.3) for type A HAs (Beyer WEP et al, 1986, Archives Virol, vol 90: 173). During extraction of the plant biomass (between pH 5-6), a conformational change of HA1-HA2 may also take place with type B HA.

[0085] Without wishing to be bound by theory, the pH of a cellular compartment comprising HA, including the Golgi apparatus, may therefore 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. Early in infection of new cells after endocytic uptake of viral particles, activation of M2 proton channel activity leads to acidification of the interior of the virion during the uncoating process. Late in infection during virus production, M2 acts to raise the pH during transit through the trans-Golgi network and prevents the

low pH-induced inactivation of co-transported proteins, such as HA in the case of influenza. By co-expressing a structural virus protein along with a channel protein, for example but not limited to a proton channel protein, increased yield of the structural virus protein and VLPs are observed. HA's are known to under go pH-dependent confirmation change. Without wishing to bound by theory, the pH within the Golgi apparatus of the HA producing cells during maturation and migration may influence HA folding, effects stability and increase degradation, or a combination thereof, of the HA. By co-expressing a channel protein, for example but not limited to a proton channel protein, along with an 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 HA and/or VLPs.

[0086] By co-expressing a structural virus protein along with a channel protein, for example but not limited to a proton channel protein, in a plant, increased yield of the structural virus protein and/or VLPs are observed, when compared to a plant that expressed the structural virus protein without co-expression of the channel protein, for example but not limited to a proton channel protein.

[0087] Furthermore, by co-expressing a structural virus protein such as HA with a channel protein, for example but not limited to a proton channel protein, in a plant, the HA protein may exhibits an increased activity as shown by a greater hemagglutination capacity, when compared to a HA protein that is not co-expressed with a channel protein, for example but not limited to a proton channel protein. 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%, when compared to the activity of the same HA protein produced in the absence of a channel protein, for example but not limited to a proton channel protein.

[0088] 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. Examples of channel proteins that may be used include, but are not limited to proton channel proteins for example those listed in Table 1. 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. An exemplary M2 protein consists of the amino acid sequence as shown in SEQ ID NO: 11 or 14.

[0089] As used herein, the terms "BM2," "BM2 protein," "BM2 sequence" and "BM2 domain" refer to all or a portion of a BM2 protein sequence isolated from, based upon or present in any naturally occurring or artificially produced influenza virus strain or isolate. Thus, the term BM2 and the like include naturally occurring BM2 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 BM2 sequences. Examples of channel proteins that may be used include, but are not limited to proton channel proteins those listed in Table 2.

[0090] Additional exemplary proton channel protein sequences consist of the sequences deposited under the GenBank accession numbers shown in Table 1 and Table 2.

Table 1: Accession numbers for amino acids sequences M2 proton channel proteins

GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number
ABA42438.1	ABB54697.1	ABI36079.1	ADM95491.1	ADM29632.1
ABA42436.1	AAA43253.1	ABI36077.1	ADM95489.1	ADM29566.1
ABA42434.1	BAB19809.1	ABI36075.1	ADM95487.1	ADM29555.1
AAD51268.1	ABD59884.1	ABI36073.1	ADM95485.1	ADM29544.1
AAD51264.1	ABD59882.1	ABI36071.1	ADM95483.1	ADM29533.1
AAC60735.1	ABD59880.1	ABI36069.1	ADM95481.1	ADM29445.1
BAI77393.1	BAD89348.1	ABI36067.1	ADM95479.1	ADM29434.1
BAI77450.1	BAD89338.1	ABI36065.1	ADM95477.1	ADM29423.1
CAP58009.1	BAD89328.1	ABI36063.1	ADM95475.1	ADM29412.1
CAP58007.1	BAE47133.1	ABI36061.1	ADM95473.1	ADM29401.1
CAP58005.1	ABD59890.1	ABI36059.1	ADM95471.1	ADM29379.1
BAH84754.1	ABD59888.1	ABI36037.1	BAF37390.1	ADM29368.1
BAH86619.1	ABD59886.1	ABI36027.1	ADG59188.1	ADM29357.1
BAH86616.1	ABD59900.1	ABI36016.1	ADG59186.1	ADM29313.1

GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number
BAH84985.1	ABD59898.1	ABI36005.1	ADG59184.1	ADM29302.1
YP_308853.1	ABD59896.1	AAY87447.1	ADG59182.1	ADM29291.1
AAD49092.1	ABD59894.1	AAY87431.1	ADG59180.1	ADM29280.1
AAD49090.1	ABD59892.1	AAV32647.1	ADG59178.1	ADM29269.1
AAD49088.1	AAC79578.1	AAV32639.1	ADG59176.1	ADM29258.1
ABQ12378.1	ABB51968.1	AAU00829.1	ADG59174.1	ADM29698.1
AAO33518.1	ABY75105.1	AAU00827.1	ADG59172.1	ADM29687.1
AAO33516.1	ABY75039.1	AAA91324.1	ADG59170.1	ADM29676.1
AAO33514.1	ABY75037.1	ABG75620.1	ADG59168.1	ADM29665.1
AAO33512.1	AAL60446.1	ACR09361.1	ADG59166.1	ADM29654.1
AAO33510.1	ABB00351.1	ACR09359.1	ADG59164.1	ADM29621.1
AAO33508.1	AAA43312.1	ACR09355.1	ADG59162.1	ADM29610.1
AAO33506.1	ABB00339.1	ACR09353.1	ADG59160.1	ADM29599.1
AAO33504.1	ABW38094.1	ACQ99604.1	ADG59158.1	ADM29522.1
AAO33502.1	AAM09299.1	ACQ99602.1	ADG59156.1	ADM29511.1
ABS52607.1	AAM09297.1	ACQ99600.1	ADG59154.1	ADM29500.1
ABS52597.1	ABS00915.1	ACQ99592.1	ADG59152.1	ADM29489.1
ABS52587.1	ABS00914.1	ACQ99590.1	ADG59150.1	ADM29478.1
ABM21873.1	ABS00913.1	ACQ99588.1	ADG59148.1	ADM29467.1
ABM21871.1	ABS00912.1	ACQ99586.1	ADG59146.1	ADM29456.1
ABM21869.1	ABS00911.1	ACP41965.1	ADG59144.1	ADM29390.1
ABM21867.1	ABS00910.1	ACP41955.1	ADG59142.1	ADM29346.1
ABM21865.1	ABS00909.1	ACP41951.1	ADG59140.1	ADM29335.1
ABM21863.1	ABS00908.1	ACP41946.1	ADG59138.1	ADM29324.1
ABM21861.1	ABS00907.1	ACR49258.1	ADG59136.1	ADM29247.1
AAO33500.1	ABS00906.1	ACR49256.1	ADG59134.1	AEE73588.1
AAD49094.1	ABS00905.1	ACR49254.1	ADG59132.1	AEB89880.1
AAD49086.1	ABS00904.1	ACR49252.1	ADG59130.1	AEB89869.1
AAD49084.1	ABS00903.1	ACR49250.1	ADG59128.1	AEB89858.1
AAD49082.1	ABS00902.1	ACR49248.1	ADG59126.1	AEA74023.1
AAD49080.1	ABB51974.1	ACR49246.1	ADG59124.1	AEA74013.1
AAD49078.1	ABB51972.1	ACR49244.1	ADG59122.1	ADF42731.1
AAD49076.1	ABB51970.1	ACR38840.1	ADG59120.1	ADF42721.1
AAD49074.1	AAD00150.1	ACR38838.1	ADG59118.1	ADF28007.1
AAD49072.1	AAD00148.1	ACR38836.1	ADG59116.1	ADF27997.1
AAD49070.1	AAD00146.1	ACR38834.1	ADG59114.1	ADF27987.1
AAD49068.1	AAD00144.1	ACR38832.1	ADG59112.1	ADF27977.1
ACA25333.1	AAD00142.1	ACR18965.1	ADG59110.1	ADF27967.1
ACA25323.1	AAD00140.1	ACR18963.1	ADG59108.1	ADF27957.1
ACA25313.1	AAD00138.1	ACR18958.1	ADG59106.1	ADF27947.1
CAJ12148.1	AAD00136.1	ACR18957.1	ADG59104.1	ADF27937.1
CAJ12154.1	AAD00134.1	ACR18953.1	ADG59102.1	ADF27927.1
CAJ12152.1	AAD00132.1	ACR18949.1	ADG59100.1	ADF27917.1
CAJ12150.1	AAD00130.1	ACR18946.1	ADG59098.1	ADF27907.1
ACP41109.1	AAC80168.1	ACR18945.1	ADG59096.1	ADF27897.1
ADG59536.1	AAC80166.1	ACR18943.1	ADG59094.1	ADF27887.1
AAK14988.1	AAC80164.1	ACR08560.1	ABO21713.1	ACS87931.1
AAK14984.1	AAC80162.1	ACR08556.1	ADG59717.1	ACU44926.1
ACR67209.1	AAC80160.1	NP_040979.2	ADG59706.1	ACU44922.1
ACP41929.2	AAC80158.1	ABZ91697.1	AAF74335.1	ACU44920.1
ACR18961.1	AAC80156.1	ABZ91685.1	AAF74333.1	ACU44918.1
ACR18955.1	ABY75159.1	ACB54711.1	ADF56637.1	ACU44916.1
ACR18941.1	ABY75157.1	ABM90504.1	ADF56636.1	ACU44914.1
ACR08564.1	ABY75155.1	ABM90493.1	ADF56635.1	ACU44912.1

GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number
ACR08562.1	ABY75153.1	ABM90482.1	ADF29921.1	ACU44910.1
ACR08558.1	ABY75151.1	ABM90471.1	ADE75385.1	ACU44908.1
ACQ99594.1	ABY75149.1	ABM90460.1	ADE75374.1	ACU44906.1
ACQ83308.1	ABY75147.1	ABM90449.1	ADE75365.1	ACU44904.1
ACQ76400.1	ABY75145.1	ABM90438.1	ADE75354.1	ACU44902.1
ACQ76382.1	ABY75143.1	ABI49411.1	ADE75344.1	ACU44900.1
ACQ76375.1	ABY75141.1	ABI49400.1	ADE75327.1	ACU44898.1
ACQ76369.1	ABY75139.1	ABO31433.1	ADE75298.1	ACU44896.1
ACQ76361.1	ABY75137.1	ABM90548.1	ADE75287.1	ACU44894.1
ACQ76355.1	ABY75135.1	ABM90537.1	ADE75276.1	ACU44892.1
ACQ76346.1	ABY75133.1	ABM90526.1	ADE75265.1	ACU44890.1
ACQ76332.1	ABY75131.1	ABM90515.1	ADE75254.1	ACU44888.1
ACQ76325.1	ABY75129.1	ABL31784.1	ADE75244.1	ACU44886.1
ACQ76313.1	ABY75127.1	ABL31770.1	ADE75235.1	ACU44883.1
ACQ76303.1	ABY75125.1	ABL31759.1	ADE75228.1	ACU44881.1
ACQ76293.1	ABY75123.1	ABL31748.1	ADE75218.1	ACU44879.1
ACQ63288.1	ABY75121.1	ABI49419.1	ADE75207.1	ACU44877.1
ACQ63259.1	ABY75119.1	ABL07034.1	ADE75196.1	ACU44875.1
ACQ63250.1	ABY75117.1	ABL07023.1	ADE75187.1	ACU44873.1
ACQ63217.1	ABY75115.1	ABL07012.1	ADE75178.1	ACU44871.1
ACQ63211.1	ABY75113.1	ACC55276.2	ADE75170.1	ACU44869.1
ACQ55364.1	ABY75111.1	ABV53559.1	ADE75152.1	ACU44867.1
ACQ55353.1	ABY75109.1	AEB71385.1	ADE75143.1	ACU44865.1
ACP44171.1	ABY75107.1	AEB66897.1	ADE75134.1	ACU44863.1
ACP44160.1	ABY75103.1	AEB40208.1	ADE75124.1	ACU44861.1
ACP44153.1	ABY75101.1	ADX36111.1	ADE75115.1	ACU44859.1
ACP44149.1	ABY75099.1	ADX21100.1	ADE75095.1	ACU44857.1
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AAY87421.1	ABY75095.1	ADX21080.1	ADE75075.1	ACU44853.1
AAY87413.1	ABY75093.1	ADW93762.1	ADE75057.1	ACU44851.1
ACQ63284.1	ABY75091.1	ADW82270.1	ADE75046.1	ACU44849.1
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ACQ63266.1	ABY75087.1	ADW82250.1	ACL11961.1	ACU44845.1
ACQ63225.1	ABY75085.1	ADW82240.1	ABY40439.1	ACU44843.1
ACP44185.1	ABY75083.1	ADW82230.1	ABY40432.1	ACU44841.1
ACP44178.1	ABY75081.1	ADW82220.1	AAD25212.1	ACU44839.1
ACA28776.1	ABY75079.1	ADW82210.1	AAD25206.1	ACU44837.1
ACA28772.1	ABY75077.1	ADW82200.1	AAD25172.1	ACU44835.1
ACA28768.1	ABY75075.1	ADW82190.1	BAF36962.1	ACU44833.1
ACR49240.1	ABY75073.1	ADW82179.1	ABI94583.1	ACU44831.1
ACQ84453.1	ABY75071.1	ADW82168.1	ACT21522.1	ACU44829.1
ACU00946.2	ABY75069.1	ADW82157.1	ABY81638.1	ACU44827.1
ACR46665.1	ABY75067.1	ADW82148.1	ACF40971.1	ACU44825.1
ACZ81655.1	ABY75065.1	ADW82137.1	ACD88518.1	ACU44823.1
ACZ81651.1	ABY75063.1	ADW82126.1	ACD88507.1	ACU44821.1
ACR46675.1	ABY75061.1	ADV19021.1	ABW97453.1	ACU44819.1
ACU00956.1	ABY75059.1	ADL41167.1	ACZ81646.1	ACU44817.1
ACU00936.1	ABY75057.1	AAF74337.1	YP_308670.1	ACU44815.1
ACT21587.1	ABY75055.1	ACS92616.1	AAA56808.1	ACU44813.1
ACT21581.1	ABY75053.1	ACC94117.1	AAA56806.1	ACU44811.1
ACT21576.1	ABY75051.1	ACC94089.1	ABS00311.1	ACU44809.1
ACR19302.2	ABY75049.1	ACC94087.1	ABS00320.1	ACU44807.1
ACR19300.2	ABY75047.1	ACC94085.1	ACR08491.1	ACU44805.1
ACR19298.2	ABY75045.1	ACC94071.1	ACR01010.1	ACU44803.1

GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number
ACR19296.2	ABY75043.1	ACC94067.1	ACR01006.1	ACU44801.1
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ABJ90284.2	ABY75035.1	ACC94059.1	ABG78553.1	ACU44797.1
ABJ90273.2	ABY75033.1	ACC94057.1	ABG78550.1	ACU44795.1
ABJ90230.1	ABY75031.1	ACC94051.1	ACD37773.1	ACU44793.1
ADD21567.1	ABY75029.1	ACC94041.1	ACD37763.1	ACU44791.1
ACU44924.1	ABY75027.1	ACC94033.1	ACA64013.1	ACU44789.1
ACU44779.1	ABY75025.1	ABW97496.1	ABX10519.1	ACU44787.1
ACU44773.1	ABY75023.1	ACA28780.1	ABW95953.1	ACU44785.1
ADE48138.1	ABY75021.1	ACA28778.1	ABW95942.1	ACU44783.1
ACG80349.1	ABY75019.1	ACA28774.1	ABJ90263.1	ACU44781.1
ADN34731.1	ABY75017.1	ACA28770.1	ABJ90251.1	ACU44777.1
ADN34711.1	ABY75015.1	ACA28766.1	ABJ90241.1	ACU44775.1
ADG59534.1	ABY75013.1	ACZ81636.1	BAF38386.1	ACU44771.1
ADG59532.1	ABY75011.1	ACU27045.1	BAF37824.1	ACU44769.1
ADG59530.1	ABY75009.1	ACR54040.1	BAF33431.1	ACU44767.1
ACX43975.1	ABY75007.1	ACH68522.1	BAF33417.1	ACU44765.1
ACX43973.1	ABY75005.1	ACF04730.1	BAF33412.1	ACU44763.1
ABG91471.1	ABY75003.1	ACF04728.1	BAF33401.1	ACU44761.1
ABG91467.1	ABY75001.1	ACF04726.1	ACN22341.1	ACU44759.1
ABF21313.1	ABY74999.1	ACF04724.1	ACV49525.1	ACU44757.1
ABF21301.1	ABY74997.1	ACF04722.1	ACV49503.1	ACU44755.1
ABF21299.1	ABY74995.1	ACC69091.1	ACU79906.1	AEA92622.1
ABF21297.1	ABY74993.1	ABV53579.1	ACU79895.1	AEA35548.1
ABQ57382.1	ABY74991.1	ABV53569.1	ACU79884.1	ADM29588.1
ACR09357.1	ABY74989.1	ABV53549.1	ACU79873.1	ADM29577.1
ACQ99606.1	ABY74987.1	ABV53539.1	ACI25792.1	BAK08628.1
ACQ99598.1	ABY74985.1	ABV53529.1	ACI25781.1	BAK08626.1
ACQ99596.1	ABY74983.1	ABV53519.1	ACI25770.1	ADZ75331.1
ACU43624.2	ABV45404.1	ABV53509.1	ACI25759.1	ADZ75320.1
ACR67240.1	AAC63486.1	ABV53499.1	ACI25748.1	ADP07242.1
ACR67238.1	AAC63484.1	ABV53489.1	ACF54468.1	ACZ54004.1
ACR67235.1	AAC63482.1	ABV53479.1	ACF54457.1	ACX93288.1
ACR67234.1	AAC63480.1	ABV53470.1	ACF54446.1	ACX93222.1
ACR67232.1	ABB00355.1	ADP37370.1	ACF54435.1	ACD65198.1
ACR67230.1	ABB00353.1	ADG21464.1	ACF54424.1	ACD65196.1
ACR67228.1	ABB00349.1	ADG21457.1	ACF54413.1	ACD65194.1
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ACR67224.1	ABB00345.1	ACF17943.1	ACF41825.1	ACD65189.1
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ACR67218.1	ABB00337.1	ADM95565.1	ACF41792.1	ABD79034.1
ACR67216.1	ABB00335.1	ADM95563.1	ACF41781.1	ABJ16853.1
ACR67214.1	ABB00333.1	ADM95561.1	ACF41770.1	ABJ16842.1
ACR67212.1	CAA30889.1	ADM95559.1	ACF41759.1	ADI99547.1
ACR67208.1	CAA30887.1	ADM95557.1	ACF41748.1	ADI99536.1
ACR67206.1	CAA30885.1	ADM95555.1	ACF41737.1	ADI34045.1
ACR54054.1	CAA30893.1	ADM95553.1	ACF22399.1	ADD21471.1
ACR09363.1	CAA30891.1	ADM95551.1	ACF22388.1	ADD21461.1
ACP41961.1	AAA43091.1	ADM95549.1	ACF22377.1	ADD21451.1
ACP41938.1	AAA43577.1	ADM95547.1	ACF22366.1	ACZ48112.1
ABI36484.1	BAB19808.1	ADM95545.1	ACF22355.1	ACF25678.1
ABI36475.1	CAA30883.1	ADM95543.1	ACF22344.1	ACF25666.1
ABI36464.1	AAD51929.1	ADM95541.1	ACF22333.1	ACF25466.1

GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number
ABI36456.1	BAA99398.1	ADM95539.1	ACF22322.1	ACF25065.1
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ABI36434.1	AAF99671.1	ADM95535.1	ACF22300.1	ACF24971.1
ABI36425.1	ABH04389.1	ADM95533.1	ACF22278.1	ACV74288.1
ABI36412.1	ABB90274.1	ADM95531.1	ACF22256.1	ACV74286.1
ABI36401.1	AAD51270.1	ADM95529.1	ACF22245.1	ABV56243.1
ABI36390.1	AAM70004.1	ADM95527.1	ACF22234.1	ACI25712.1
ABI36379.1	AAB19772.1	ADM95525.1	ACF22223.1	ACI25710.1
ABI36368.1	AAD51266.1	ADM95523.1	ACF22212.1	ADA81213.1
ABI36357.1	ABD59885.1	ADM95521.1	ACF22201.1	ACZ56084.1
ABI36346.1	AAM70001.1	ADM95519.1	ACF22190.1	ACZ45024.1
ABI36335.1	AAM69992.1	ADM95517.1	ACF22172.1	ACV91683.1
ABI36324.1	AAM69982.1	ADM95515.1	ADL41185.1	ACV91679.1
ABI36313.1	AAM69972.1	ADM95513.1	ADM07115.1	ACV91675.1
ABI36297.1	AAM69961.1	ADM95511.1	ADM07104.1	ACV72405.1
ABI36277.1	AAZ38741.1	ADM95509.1	ADM07093.1	ACV72403.1
ABI36202.1	AAZ38739.1	ADM95507.1	ADM07082.1	ACV72401.1
ABI36191.1	AAZ38737.1	ADM95505.1	ADM07071.1	ACV72399.1
ABI36181.1	AAZ38735.1	ADM95503.1	ADM07060.1	ACV72397.1
ABI36170.1	AAZ38733.1	ADM95501.1	ADE62289.1	ACV72395.1
ABI36159.1	AAZ38731.1	ADM95499.1	AEG65177.1	ACV72393.1
ABI36148.1	AAZ38729.1	ADM95497.1	AEC46386.1	ACV72391.1
ABI36083.1	ABA42442.1	ADM95495.1	ADR78653.1	ACV72389.1
ABI36081.1	ABA42440.1	ADM95493.1	ADM29643.1	ACV72349.1

Table 2: Accession numbers for amino acids sequences BM2 proton channel proteins

GenBank accession number	GenBank accession number	GenBank accession number	GenBank accession number
AAU01002.1	ACR15701.1	ACA96576.1	ABN50549.1
BAC54010.1	ACR15690.1	ACA96565.1	ABN50538.1
BAC53999.1	ACR15679.1	ACA96554.1	ABN50527.1
P0C0X4.1	ACR15668.1	ACA65099.1	ABN50516.1
P03493.2	ACR15657.1	ACA65088.1	ABN50505.1
P08383.2	ACR15646.1	ACA65077.1	ABN50494.1
P13882.2	ACR15635.1	ACA65066.1	ABN50483.1
P13881.2	ACR15624.1	ACA65055.1	ABN50472.1
Q80DN6.1	ACO94663.1	ACA65044.1	ABN50450.1
ABF21319.1	ACO06025.1	ACA65033.1	ABN50439.1
ABN50461.1	ACO06014.1	ACA65022.1	ABN50428.1
YP_419283.1	ACO06003.1	ACA65011.1	ABN50417.1
ACN32784.1	ACO05992.1	ACA65000.1	ABN50406.1
ACN32773.1	ACO05981.1	ACA64989.1	ABN50395.1
ACN32719.1	ACO05970.1	ACA64978.1	ABN50384.1
ACN32613.1	ACO05959.1	ACA64967.1	ABL77389.1
ACN32602.1	ACO05937.1	ACA64956.1	ABL77378.1
ACN32591.1	ACO05926.1	ACA64945.1	ABL77367.1
ACN32580.1	ACF54369.1	ACA64934.1	ABL77356.1
ACN32569.1	ACF54358.1	ACA64923.1	ABL77345.1
ACN32558.1	ACF54347.1	ACA64912.1	ABL77334.1
ABL77103.1	ACF54336.1	ACA64901.1	ABL77323.1
ABN50725.1	ACF54325.1	ABR16019.1	ABL77312.1
ABX71689.1	ACF54314.1	ABR16008.1	ABL77301.1

ABF21321.1	ACF54303.1	ABR15997.1	ABL77290.1
AAD29209.1	ACF54292.1	ABR15986.1	ABL77279.1
AAD29207.1	ACF54281.1	ABR15975.1	ABL77268.1
AAD29205.1	ACF54270.1	ABO72379.1	ABL77257.1
AAD29203.1	ACF54259.1	ABN50637.1	ABL77246.1
AAD29201.1	ACF54248.1	ABN59447.1	ABL77235.1
AAD29199.1	ACF54226.1	ABN58663.1	ABL77224.1
AAD29197.1	ACF54215.1	ABN51197.1	ABL77213.1
AAD29195.1	ACF54204.1	ABN51186.1	ABL77202.1
AAD29193.1	ACF54182.1	ABN50747.1	ABL77191.1
AAD29191.1	ACF54160.1	ABN50736.1	ABL77180.1
AAD29189.1	ACF54149.1	ABN50714.1	ABL77169.1
AAD29185.1	ACF54138.1	ABN50703.1	ABL77158.1
AAD29183.1	ACF41660.1	ABN50692.1	ABL77147.1
AAD29181.1	ACD56579.1	ABN50681.1	ABL77136.1
AAD29179.1	ACD56568.1	ABN50670.1	ABL77125.1
AAD29177.1	ACB06477.1	ABN50659.1	ABL77114.1
AAD29175.1	ACA96664.1	ABN50648.1	ABL77092.1
AAD29173.1	ACA96653.1	ABN50626.1	ABL77081.1
AAT69452.1	ACA96642.1	ABN50615.1	ABL77070.1
AAT69441.1	ACA96631.1	ABN50604.1	ABL77059.1
AAT69430.1	ACA96620.1	ABN50593.1	ABL77048.1
ACR39338.1	ACA96609.1	ABN50582.1	ABL77037.1
ACR15734.1	ACA96598.1	ABN50571.1	ABL77026.1
ACR15723.1	ACA96587.1	ABN50560.1	ABL77015.1
ACR15712.1	ACA96576.1	ABN50549.1	ABL77004.1

Structural Virus protein

[0091] The structural virus protein (also referred to as structural viral protein) may be a viral antigenic protein or fragment thereof, for example but not limited to a virus glycoprotein or virus envelop protein. The structural virus protein may be a chimeric virus protein. The viral protein may exist as a monomer, a dimer, a trimer, or a combination thereof. A trimer is a macromolecular complex formed by three, usually non-covalently bound proteins. Without wishing to be bound by theory, the trimerization domain of a protein may be important for the formation such trimers. Therefore the structural viral protein or fragment thereof may comprise a trimerization domain. A non-limiting example of a structural virus protein is influenza hemagglutinin (HA), or a fragment of HA. Non-limiting examples of HA, or fragments of HA that may be used according to the present invention include those described in WO2009/009876, WO 2009/076778; WO 2010/003225, WO 2010/003235, WO 2011/03522, WO 2010/006452, WO 2010/148511, WO 2011/035422 (which are incorporated herein by reference).

[0092] Furthermore the structural virus protein may be the unprocessed precursor protein of HA. 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.

Proteolytic Loop (cleavage site) modification

[0093] The structural virus protein may be an influenza B hemagglutinin or Influenza A hemagglutinin protein with a deletion or modification of the proteolytic loop (cleavage site) within the hemagglutinin protein. Deletion or modification of the proteolytic loop ensures that the HA molecule is mostly maintained as HA0 precursor.

[0094] HA is synthesised as a precursor protein HA0, which undergoes proteolytic processing into two subunits (HA1 and HA2) linked together by a disulfide bridge. 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 (see Fig. 24). No protease has been identified for the majority of influenza viruses that cause enteric and respiratory infection seen in aquatic birds. In the laboratory, most cell lines do not support multi-cycle replication unless exogenous protease (usually trypsin) is added.

[0095] Highly pathogenic avian strains, however, are cleaved by a family of more widespread intracellular proteases, resulting in systemic 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 (see Figure 24). 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.

[0096] 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, 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.

[0097] During the folding of HA and secretion thorough the Golgi, the hemagglutinin precursor cleavage site, which is located on a loop at the surface of HA, is well accessible for proteolysis by proteases. Without wishing to be bound by theory, if proteolysis of precursor HA0 occurs at the mono or the polybasic site during folding of the HA in the ER, a conformational change of the protein may take place in the Golgi apparatus during secretion, because the pH environment inside the Golgi of the plant and in the apoplast is slightly acidic. A low-pH conformation HA may be produced, decreasing both the level of expression and intrinsic stability of the particle. Thus, mostly uncleaved HA0 precursor protein would be budding from plasma membrane.

[0098] 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, or influenza B consensus hemagglutinin amino acid sequences. Non limiting examples of consensus sequences are shown in Figure 24.

[0099] In the amino acid sequence of the HA the proteolytic loop is located, before the fusion peptide that consist of the 20 first amino acids of the HA2 part. 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. From this specific peptide sequence, the consensus sequence may be determined in this chosen region (Bianchi et al., 2005, Journal of Virology, 79:7380-7388; incorporated herein by reference).

[00100] In order to abolish the proteolytic loop, the structure of a B HA was examined. Deletion of only the proteolytic cleavage site of the HA would have left the C-terminal of HA1 and N-terminal of HA2 left apart and a long linker would have needed to be designed. However deleting part of the fusion peptide along with the proteotic cleave site allowed to remove the complete proteolytic loop and join the remaining HA1 and HA2 sequence by a minimal peptide linker of 2 amino acids. In summary, the B variant contains 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 were linked together by a GG linker.

[00101] As show in Figure 22B, by deleting the proteolytic loop of HA0, 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. 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%, when compared to the activity of the same HA protein that does not have its proteolytic loop removed.

[00102] By “chimeric virus protein” or “chimeric virus polypeptide”, also referred to as “chimeric protein” or “chimeric polypeptide”, 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 the chimeric protein or chimeric polypeptide cleaved following synthesis, and as required, associated 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 virus 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 hemagglutinin (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, or chimeric 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". A virus-like particle comprised of chimeric HA may be described as a “chimeric VLP”.

[00103] 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 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 which is not to be considered limiting, a mature HA protein.

[00104] Non limiting examples of chimeric virus proteins or chimieric virus nucleic acids that may be used according to the present invention are described in, WO 2009/076778, WO 2010/003235, or WO 2010/148511 (which are incorporated herein by reference).

Signal peptide

[00105] A signal peptide (SP) may be native to the antigenic protein or virus protein, or a signal peptide may be heterologous with respect to the primary sequence of the antigenic protein or virus protein being expressed. A antigenic protein or virus protein 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 chimeric virus protein 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.

[00106] A signal peptide may also be non-native, for example, from a antigenic protein, viral protein or hemagglutinin of a virus other than virus protein, or from a

plant, animal or bacterial polypeptide. A non limiting example of a signal peptide that may be used is that of alfalfa protein disulfide isomerase ("PDISP"; nucleotides 32-103 of Accession No. Z11499; also see WO 2009/076778; WO 2010/148511, or WO 2010/003235, which are incorporated herein by reference). The present invention therefore provides for a chimeric virus protein comprising a native, or a non-native signal peptide, and nucleic acids encoding such chimeric virus proteins.

[00107] The present invention therefore also provides for a method of producing chimeric VLP in a plant, wherein a first nucleic acid encoding a chimeric virus protein is co-expressed 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 may be introduced to the plant sequentially.

HA

[00108] With reference to influenza virus, 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.

[00109] 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 in animal cells, 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. 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 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 HA protein may be used in the production or formation of VLPs using a plant, or plant cell, expression system.

[00110] The HA of the present invention may be obtained from any subtype. For example, the HA may be of subtype H2, H3, H4, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, or influenza type B HA. The recombinant HA of the present invention may also comprise an amino acid sequence based on the sequence any hemagglutinin known in the art– 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). 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.

[00111] Non-limiting examples of HA, or fragments of HA that may be used according to the present invention include those described in WO2009/009876, WO 2009/076778; WO 2010/003225, WO 2010/003235, WO 2010/006452, WO 2011/035422 or WO 2010/148511 (which are incorporated herein by reference).

[00112] As shown in Figure 18, HA from B/Brisbane/60/2008 is poorly expressed in agroinfiltrated *Nicotiana benthamiana* leaves (see lanes "1008" or "1029"). However, co-expression of HA-type B with M2 from A/New Caledonia/20/99, results in a significant increase in HA expression (see lanes "1008+1261"; "1009+1261" and 1029+1261"). The increase in HA expression was

observed in with both native type B HA or a chimeric HA type B. HA expression was observed in the presence or absence of amplification elements (BeYDV), and across various dilutions of *Agrobacterium*. A similar increase in H3 expression was observed when H3 from A/Perth/16/2009 was co-expressed with M2 from A/New Caledonia/20/99 (Figure 19; compare lane "1019" H3 alone, with "1019+1261" H3 co-expressed with M2).

VLP

[00113] The term "virus like particle" (VLP), or "virus-like particles" or "VLPs" refers to structures that self-assemble and comprise virus proteins for example a structural viral protein such as influenza HA protein or a channel protein, for example but not limited to a proton channel protein, such as M2 or a combination of those proteins. 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. 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.

[00114] Furthermore, VLPs may be produced that comprise a combination of HA subtypes. For example, VLPs may comprise one or more than one HA from the subtype H2, H3, H4, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, subtype B HA or a combination thereof. Selection of the combination of 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, while VLPs useful for inoculating humans may comprise subtypes one or more than one of subtypes H2, H3, H4, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, subtype B HA . However, other HA subtype combinations may be prepared

depending upon the use of the VLP. In order to produce VLPs comprising combinations of HA subtypes, the desired HA subtype may be co-expressed within the same cell, for example a plant cell.

[00115] As described in more detail below, the expression of a type B HA or H3 from B/Brisbane/60/2008 in agroinfiltrated *Nicotiana benthamiana* leaves is increased when co-expressed M2 from A/New Caledonia/20/99 (see Figures 18 and 19). A similar increase is not observed when H1 or H5 is co-expressed with M2, as high levels of H1 or H5 expression are observed in the presence or absence of M2 (Figures 20 and 21 respectively). HA are known to be processed in a pH-dependent manner (see Reed M. L. et. al. Journal of Virology, February 2010, p. 1527-1535, Vol. 84, No. 3), and to under go pH-dependent confirmation change (Skehel J.J. et. al. 1982, PNAS79: 968-972). H1 and H5 exhibit a conformational change at a pH lower than that observed with either H3 and type B HA. Without wishing to bound by theory, the pH within the Golgi Apparatus during maturation and migration may not have an effect on H1 or H5 folding, however, a low pH within the Golgi Apparatus may effect H3 and type B HA folding. By co-expressing a channel protein, for example but not limited to a proton channel protein, long with H3 or Type B HA, the pH within the Golgi Apparatus may increase and result in HA folding leading to increased HA yield. Furthermore, H1 and H5 may be more stable at lower pH, than H3 and Type B HA. Therefore, by co-expressing a channel protein, for example but not limited to a proton channel protein, along with H3 or Type B HA, less HA is degraded within the Golgi apparatus.

[00116] 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.

[00117] 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.

[00118] 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.

[00119] 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.

[00120] 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.

[00121] 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).

[00122] 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.

[00123] 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.

[00124] 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.

[00125] Therefore, in some embodiments, the invention provides for a VLP 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.

[00126] VLPs may be assessed for structure and size by, for example, hemagglutination assay, electron microscopy, or by size exclusion chromatography.

[00127] 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.

[00128] 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).

Folding (Chaperon)

[00129] 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.

[00130] 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.

[00131] 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.

[00132] 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.

[00133] 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).

[00134] 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.

[00135] 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.

N-Glycans

[00136] 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.

[00137] 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.

[00138] 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.

[00139] Therefore the present invention also includes VLP's comprising one or more virus protein having modified N-glycans.

Sequences

[00140] Non-limiting example of sequences that may be used with the present invention include:

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);

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/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/Malaysia/2506/2004, B/Wisconsin/1/2010, or B/Brisbane/60/2008 strain.

[00141] Non-limiting example of sequences that may be used with the present invention 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). Examples of sequences of amino acid molecules encoding such HA proteins from H2, H3, H4, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 and type B HA, which are known in the art. For example H3 or B subtypes include SEQ ID Nos: 25 or 30. The sequence encoding the structural virus protein may be for example HA from influenza B/Brisbane/60/2008, B/Malaysia/2506/2004 or B/Wisconsin/1/2010, or H3 from influenza A/Perth/16/2009 or A/Victoria/361/2011. Other examples include sequences of nucleic acid molecules that encode HA proteins wherein the proteolytic loop of the HA protein has been deleted such as for example, but not limited to the sequence defined by SEQ ID NO: 41.

[00142] The present invention also includes, but is not limited to, nucleotide sequences encoding HA from for example H2, H3, H4, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 or type B HA. For example SEQ ID NO: 28, 43, 23, encoding an HA from B, B with deleted proteolytic loop or H3. respectively, a nucleotide sequence that hybridizes under stringent hybridisation conditions to SEQ ID NO: 28, 43, 23, or a nucleotide sequence that hybridizes under stringent hybridisation conditions to a compliment of SEQ ID NO: 28, 43, 23, 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. The nucleotide sequence may also be co-expressed with a second nucleotide sequence encoding a channel protein, for example but not limited to, nucleotide sequences SEQ ID NO: 9, 12, a nucleotide sequence that hybridizes under stringent hybridisation conditions to SEQ ID NO: 9, 12, or a nucleotide sequence that hybridizes under stringent hybridisation conditions to a compliment of SEQ ID NO: 9, 12, wherein the second nucleotide sequence encodes a proton channel protein forms a VLP. 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 H2, H3, H4, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, subtype 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 HA from B (SEQ ID NO: 28), B with deleted or modified proteolytic loop (SEQ ID NO: 43), H3 (SEQ ID NO:23), or an HA encoded by any one or more of SEQ ID NO:23, 28, 43, 46, 51, 57, or 61, 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 unprocessed and/or mature HA from B or H3, or unprocessed and/or mature HA wherein the proteolytic loop has been deleted. The VLP, when administered to a subject, induces an immune response.

[00146] The present invention also 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 M2 (SEQ ID NO: 9, 12), wherein the nucleotide sequence encodes a channel protein, for example but not limited to a proton channel protein, that when co-expressed with a structural virus protein forms a VLP. Preferably, the VLP induces the production of an antibody and the VLP, when administered to a subject, induces an immune response.

[00147] 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.

[00148] 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.

[00149] 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.

[00150] 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.

[00151] 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.

[00152] 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.

[00153] 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).

Constructs

[00154] The present invention is further directed to a gene construct comprising a nucleic acid encoding a channel protein, for example but not limited to a proton channel protein or a structural virus protein, as described above, operatively linked to a regulatory element that is operative in a plant. Examples of regulatory elements operative in a plant cell and that may be used in accordance with the present invention include but are not limited to a plastocyanin regulatory region (US 7,125,978; which is incorporated herein by reference), or a regulatory region of Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO; US 4,962,028; which is incorporated herein by reference), chlorophyll a/b binding protein (CAB; Leutwiler et al; 1986; which is incorporated herein by reference), ST-LS1 (associated with the oxygen-evolving complex of photosystem II and described by Stockhaus et al.1987, 1989; which is incorporated herein by reference).

Regulatory Elements

[00155] The use of the terms "regulatory region", "regulatory element" or "promoter" in the present application is meant to reflect a portion of nucleic acid typically, but not always, upstream of the protein coding region of a gene, which may be comprised of either DNA or RNA, or both DNA and RNA. When a regulatory region is active, and in operative association, or operatively linked, with a gene of interest, this may result in expression of the gene of interest. A regulatory element may be capable of mediating organ specificity, or controlling developmental or temporal gene activation. A "regulatory region" may includes promoter elements, core promoter elements exhibiting a basal promoter activity, elements that are inducible in response to an external stimulus, elements that mediate promoter activity such as negative regulatory elements or transcriptional enhancers. "Regulatory region", as used herein, may also includes elements that are active following transcription, for example, regulatory elements that modulate gene expression such as translational and transcriptional enhancers, translational and transcriptional repressors, upstream activating sequences, and mRNA instability determinants. Several of these latter elements may be located proximal to the coding region.

[00156] In the context of this disclosure, the term "regulatory element" or "regulatory region" typically refers to a sequence of DNA, usually, but not always, upstream (5') to the coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at a particular site. However, it is to be understood that other nucleotide sequences, located within introns, or 3' of the sequence may also contribute to the regulation of expression of a coding region of interest. An example of a regulatory element that provides for the recognition for RNA polymerase or other transcriptional factors to ensure initiation at a particular site is a promoter element. Most, but not all, eukaryotic promoter elements contain a TATA box, a conserved nucleic acid sequence comprised of adenosine and thymidine nucleotide base pairs usually situated approximately 25 base pairs upstream of a transcriptional start site. A promoter element comprises a basal promoter element, responsible for the initiation of transcription, as well as other regulatory elements (as listed above) that modify gene expression.

[00157] There are several types of regulatory regions, including those that are developmentally regulated, inducible or constitutive. A regulatory region that is developmentally regulated, or controls the differential expression of a gene under its control, is activated within certain organs or tissues of an organ at specific times during the development of that organ or tissue. However, some regulatory regions that are developmentally regulated may preferentially be active within certain organs or tissues at specific developmental stages, they may also be active in a developmentally regulated manner, or at a basal level in other organs or tissues within the plant as well. Examples of tissue-specific regulatory regions, for example see-specific a regulatory region, include the napin promoter, and the cruciferin promoter (Rask et al., 1998, J. Plant Physiol. 152: 595-599; Bilodeau et aI., 1994, Plant Cell 14: 125-130). An example of a leaf-specific promoter includes the p1astocyanin promoter (see US 7,125,978, which is incorporated herein by reference).

[00158] An inducible regulatory region is one that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor that binds specifically to an inducible regulatory region to activate transcription may be present in an inactive form, which is then directly or indirectly converted to the active form by the inducer. However, the protein factor may also be absent. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible regulatory region may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods. Inducible regulatory elements may be derived from either plant or non-plant genes (e.g. Gatz, C. and Lenk, LR.P., 1998, Trends Plant Sci. 3, 352-358; which is incorporated by reference). Examples, of potential inducible promoters include, but not limited to, tetracycline-inducible promoter (Gatz, C.,1997, Ann. Rev. Plant Physiol. Plant Mol. BioI. 48,89-108; which is incorporated by reference), steroid inducible promoter (Aoyama. T. and Chua, N.H.,1997, Plant 1. 2, 397-404; which is incorporated by reference) and ethanol-inducible promoter (Salter,

M.G., et aI, 1998, Plant 10urnal 16, 127-132; Caddick, M.X., et al,1998, Nature Biotech. 16, 177-180, which are incorporated by reference) cytokinin inducible IB6 and CKI 1 genes (Brandstatter, I. and K.ieber, 1.1.,1998, Plant Cell 10, 1009-1019; Kakimoto, T., 1996, Science 274,982-985; which are incorporated by reference) and the auxin inducible element, DR5 (Ulmasov, T., et aI., 1997, Plant Cell 9, 1963-1971; which is incorporated by reference).

[00159] A constitutive regulatory region directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive regulatory elements include promoters associated with the CaMV 35S transcript (Odell et aI., 1985, Nature, 313: 810-812), the rice actin 1 (Zhang et aI, 1991, Plant Cell, 3: 1155-1165), actin 2 (An *et al.,* 1996, *Plant J.,* 10: 107-121), or tms 2 (U.S. 5,428,147, which is incorporated herein by reference), and triosephosphate isomerase 1 (Xu et. aI., 1994, Plant Physiol. 106: 459-467) genes, the maize ubiquitin 1 gene (Cornejo et ai, 1993, Plant Mol. BioI. 29: 637-646), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et aI, 1995, Plant Mol. BioI. 29: 637-646), and the tobacco translational initiation factor 4A gene (Mandel et aI, 1995, Plant Mol. BioI. 29: 995-1004).

[00160] The term "constitutive" as used herein does not necessarily indicate that a gene under control of the constitutive regulatory region is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types even though variation in abundance is often observed. Constitutive regulatory elements may be coupled with other sequences to further enhance the transcription and/or translation of the nucleotide sequence to which they are operatively linked. For example, the CPMV-HT system is derived from the untranslated regions of the Cowpea mosaic virus (CPMV) and demonstrates enhanced translation of the associated coding sequence. By "native" it is meant that the nucleic acid or amino acid sequence is naturally occurring, or "wild type". By "operatively linked" it is meant that the particular sequences, for example a regulatory element and a coding region of interest, interact either directly or indirectly to carry out an intended function, such as mediation or modulation of gene expression. The interaction of operatively linked sequences may, for example, be mediated by proteins that interact with the operatively linked sequences.

[00161] The one or more virus protein such as a structural virus protein or channel protein, for example but not limited to a proton channel protein may be expressed in an expression system comprising a viral based, DNA or RNA, expression system, for example but not limited to, a comovirus-based expression cassette and geminivirus-based amplification element.

[00162] The expression system as described herein may comprise an expression cassette based on a bipartite virus, or a virus with a bipartite genome. For example, the bipartite viruses may be of the Comoviridae family. Genera of the Comoviridae family include Comovirus, Nepovirus, Fabavirus, Cheravirus and Sadwavirus. Comoviruses include Cowpea mosaic virus (CPMV), Cowpea severe mosaic virus (CPSMV), Squash mosaic virus (SqMV), Red clover mottle virus (RCMV), Bean pod mottle virus (BPMV), Turnip ringspot virus (TuRSV), Broad bean true mosaic virus (BBtMV), Broad bean stain virus (BBSV), Radish mosaic virus (RaMV). Examples of comoviruse RNA-2 sequences comprising enhancer elements that may be useful for various aspects of the invention include, but are not limited to: CPMV RNA-2 (GenBank Accession No. NC_003550), RCMV RNA-2 (GenBank Accession No. NC_003738), BPMV RNA-2 (GenBank Accession No. NC_003495), CPSMV RNA-2 (GenBank Accession No.NC_003544), SqMV RNA-2 (GenBank Accession No.NC_003800), TuRSV RNA-2 (GenBank Accession No. NC_013219.1). BBtMV RNA-2 (GenBank Accession No. GU810904), BBSV RNA2 (GenBank Accession No. FJ028650), RaMV (GenBank Accession No. NC_003800)

[00163] Segments of the bipartite comoviral RNA genome are referred to as RNA-1 and RNA-2. RNA-1 encodes the proteins involved in replication while RNA-2 encodes the proteins necessary for cell-to-cell movement and the two capsid proteins. Any suitable comovirus-based cassette may be used including CPMV, CPSMV, SqMV, RCMV, or BPMV, for example, the expression cassette may be based on CPMV.

[00164] "Expression cassette" refers to a nucleotide sequence comprising a nucleic acid of interest under the control of, and operably (or operatively) linked to, an appropriate promoter or other regulatory elements for transcription of the nucleic acid of interest in a host cell.

[00165] It has been shown that transformation of *Nicotiana benthamiana* with full-length, replication-competent cDNA copies of both genomic RNAs of CPMV can result in a productive infection (Liu et al., 2004, *Virology* 323, 37-48, herein incorporated by reference). Examples of CPMV-based expression cassettes are described in WO2007/135480; WO2009/087391; and Sainsbury F. et al., (2008, *Plant Physiology;* 148: 1212-1218; Sainsbury F. et al., (2008, *Plant Biotechnology Journal;* 6: 82-92; Sainsbury F. et al., 2009, *Plant Biotechnology Journal;* 7: 682-693; which documents are herein incorporated by reference). As an example, which is not to be considered limiting, the untranslated regions (UTRs) obtained from the genomic RNA 2 of the cowpea mosaic virus (CPMV) in which the two first translation initiation codons found in the 5'leader sequence have been deleted, may be used as described in WO 2009/087391. When combined to the CaMV 35S promoter and the nopaline synthase (NOS) terminator, the modified CPMV UTRs enhanced translation of the flanking coding region. The CPMV-based expression system was named CPMV-HT (hyperanslatable). Expression cassettes, expression constructs and expression systems of the invention may therefore also comprise an CPMV-based expression system such as for example an CPMV-HT expression system.

[00166] As described herein, an expression enhancer sequence, which sequence is derived from (or shares homology with) the RNA-2 genome segment of a bipartite RNA virus, such as a comovirus, in which a target initiation site has been mutated, may be used for expressing a nucleic acid sequence of interest. The present invention further provides processes for increasing the expression, or translational enhancing activity, of a sequence derived from an RNA-2 genome segment of a bipartite virus, which processes comprise mutating a target initiation site therein.

[00167] "Enhancer" sequences (or enhancer elements), include sequences derived from (or sharing homology with) the RNA-2 genome segment of a bipartite RNA virus, such as a comovirus, in which a target initiation site has been mutated. Such sequences can enhance downstream expression of a heterologous ORF to which they are attached. Without limitation, it is believed that such sequences when present in transcribed RNA, can enhance translation of a heterologous ORF to which they are attached.

[00168] The expression systems may also comprise amplification elements from a geminivirus for example, an amplification element from the bean yellow dwarf virus (BeYDV). 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.

[00169] As used herein, the phrase "amplification elements" refers to a nucleic acid segment comprising at least a portion of one ore 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.

[00170] 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.

[00171] A comovirus-based expression cassette and a geminivirus-derived amplification element may be comprised in respective, first and second vectors, or the component parts may be included in one vector. If two vectors are used, the first and second vectors may be introduced into a plant cell simultaneously or separately.

[00172] A viral replicase may also be included in the expression system as described herein to increase expression of the nucleic acid of interest.. An non-limiting example of a replicase is a BeYDV replicase (pREP110) encoding BeYDV Rep and RepA (C2/C1; Huang et al., 2009, *Biotechnol. Bioeng*. 103, 706-714; which is incorporated herein by reference).

[00173] Post-transcriptional gene silencing (PTGS) may be involved in limiting expression of transgenes in plants, and co-expression of a suppressor of silencing from the p19 of Tomato bushy stunt virus (TBSV p19) or potato virus Y (HcPro) may be used to counteract the specific degradation of transgene mRNAs (Brigneti et al., 1998).

[00174] Alternate suppressors of silencing are well known in the art and may be used as described herein (Chiba et al., 2006, Virology 346:7-14; which is incorporated herein by reference), for example but not limited to, TEV -p1/HC-Pro (Tobacco etch virus-p1/HC-Pro), BYV -p21, capsid protein of Tomato crinkle virus (TCV -CP), 2b of Cucumber mosaic virus; CMV-2b), p25 of Potato virus X (PVX-p25), p11 of Potato virus M (PVM-p11), p11 of Potato virus S (PVS-p11), p16 of Blueberry scorch virus, (BScV –p16), p23 of Citrus tristexa virus (CTV-p23), p24 of Grapevine leafroll-associated virus-2, (GLRaV-2 p24), p10 of Grapevine virus A, (GVA-p10), p14 of Grapevine virus B (GVB-p14), p10 of Heracleum latent virus (HLV-p10), or p16 of Garlic common latent virus (GCLV-p16). Therefore, a suppressor of silencing, for example, but not limited to, HcPro, TEV -p1/HC-Pro, BYV-p21, TBSV p19, TCV-CP, CMV-2b, PVX-p25, PVM-p11, PVS-p11, BScV-p16, CTV-p23, GLRaV-2 p24, GBV-p14, HLV-p10, GCLV-p16 or GVA-p10, may be co-expressed along with the nucleic acid sequence encoding the protein of interest to further ensure high levels of protein production within a plant.

[00175] By “co-expressed” it is meant that two, or more than two, nucleotide sequences are expressed at about the same time within the plant, and within the same tissue of the plant. However, the nucleotide sequences need not be expressed at exactly the same time. Rather, the two or more nucleotide sequences are expressed in a manner such that the encoded products have a chance to interact. For example, the protein that modifies glycosylation of the protein of interest may be expressed either

before or during the period when the protein of interest is expressed so that modification of the glycosylation of the protein of interest takes place. The two or more than two nucleotide sequences can be co-expressed using a transient expression system, where the two or more sequences are introduced within the plant at about the same time under conditions that both sequences are expressed. Alternatively, a platform plant comprising one of the nucleotide sequences, for example the sequence encoding the protein that modifies the glycosylation profile of the protein of interest, may be transformed, either transiently or in a stable manner, with an additional sequence encoding the protein of interest. In this case, the sequence encoding the protein that modifies the glycosylation profile of the protein of interest may be expressed within a desired tissue, during a desired stage of development, or its expression may be induced using an inducible promoter, and the additional sequence encoding the protein of interest may be expressed under similar conditions and in the same tissue, to ensure that the nucleotide sequences are co-expressed.

[00176] The one or more virus protein may be produced as a transcript from a nucleotide sequence, and the protein cleaved following synthesis, and as required, associated to form a multimeric protein. Therefore, the one or more virus protein also includes a protein or polypeptide comprising subunits that are associated via disulphide bridges (i.e. a multimeric protein). For example, a protein 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 protein.

[00177] The one or more nucleic acid sequences or genetic constructs of the present invention may be expressed in any suitable plant host that is transformed by the nucleotide sequence, or constructs, or vectors of the present invention. Examples of suitable hosts include, but are not limited to, agricultural crops including alfalfa, canola, *Brassica* spp., maize, *Nicotiana* spp., potato, ginseng, pea, oat, rice, soybean, wheat, barley, sunflower, cotton and the like.

[00178] The one or more genetic constructs of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The

polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon. Non-limiting examples of suitable 3' regions are the 3' transcribed nontranslated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene, plant genes such as the soybean storage protein genes, the small subunit of the ribulose-I, 5-bisphosphate carboxylase gene (ssRUBISCO; US 4,962,028; which is incorporated herein by reference), the promoter used in regulating plastocyanin expression, described in US 7,125,978 (which is incorporated herein by reference).

[00179] One or more of the genetic constructs of the present invention may also include further enhancers, either translation or transcription enhancers, as may be required. Enhancers may be located 5' or 3' to the sequence being transcribed. Enhancer regions are well known to persons skilled in the art, and may include an ATG initiation codon, adjacent sequences or the like. The initiation codon, if present, may be in phase with the reading frame ("in frame") of the coding sequence to provide for correct translation of the transcribed sequence.

[001] By “transformation” it is meant the interspecific transfer of genetic information (nucleotide sequence) that is manifested genotypically, phenotypically or both. The interspecific transfer of genetic information from a construct to a host may be transient and the transfer of genetic information is not inheritable or the transfer may be heritable and the transfer of genetic information considered stable.

[00180] The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); Geierson and Corey, *Plant Molecular Biology*, 2d Ed. (1988); and Miki and Iyer, Fundamentals of Gene Transfer in Plants. In *Plant Metabolism*, 2d Ed. DT. Dennis, DH Turpin, DD Lefebrve, DB Layzell (eds), Addison Wesly, Langmans Ltd. London, pp. 561-579 (1997). Other methods include

direct DNA uptake, the use of liposomes, electroporation, for example using protoplasts, micro-injection, microprojectiles or whiskers, and vacuum infiltration. See, for example, Bilang, et al. (Gene 100: 247-250 (1991), Scheid et al. (Mol. Gen. Genet. 228: 104-112, 1991), Guerche et al. (Plant Science 52: 111-116, 1987), Neuhause et al. (Theor. Appl Genet. 75: 30-36, 1987), Klein et al., Nature 327: 70-73 (1987); Howell et al. (Science 208: 1265, 1980), Horsch et al. (Science 227: 1229-1231, 1985), DeBlock et al., Plant Physiology 91: 694-701, 1989), Methods for Plant Molecular Biology (Weissbach and Weissbach, eds., Academic Press Inc., 1988), Methods in Plant Molecular Biology (Schuler and Zielinski, eds., Academic Press Inc., 1989), Liu and Lomonossoff (J Virol Meth, 105:343-348, 2002,), U.S. Pat. Nos. 4,945,050; 5,036,006; and 5,100,792, U.S. patent application Ser. Nos. 08/438,666, filed May 10, 1995, and 07/951,715, filed Sep. 25, 1992, (all of which are hereby incorporated by reference).

[00181] As described below, transient expression methods may be used to express the constructs of the present invention (see Liu and Lomonossoff, 2002, Journal of Virological Methods, 105:343-348; which is incorporated herein by reference). Alternatively, a vacuum-based transient expression method, as described by Kapila et al., 1997, which is incorporated herein by reference) may be used. These methods may include, for example, but are not limited to, a method of Agro-inoculation or Agro-infiltration, syringe infiltration, however, other transient methods may also be used as noted above. With Agro-inoculation, Agro-infiltration, or synringe infiltration, a mixture of *Agrobacteria* comprising the desired nucleic acid enter the intercellular spaces of a tissue, for example the leaves, aerial portion of the plant (including stem, leaves and flower), other portion of the plant (stem, root, flower), or the whole plant. After crossing the epidermis the *Agrobacteria* infect and transfer t-DNA copies into the cells. The t-DNA is episomally transcribed and the mRNA translated, leading to the production of the protein of interest in infected cells, however, the passage of t-DNA inside the nucleus is transient.

[00182] To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes that provide for resistance to chemicals such as an antibiotic for example, gentamycin, hygromycin, kanamycin, or herbicides such as

phosphinothrycin, glyphosate, chlorosulfuron, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as GUS (beta-glucuronidase), or luminescence, such as luciferase or GFP, may be used.

[002] By the term "plant matter", it is meant any material derived from a plant. Plant matter may comprise an entire plant, tissue, cells, or any fraction thereof. Further, plant matter may comprise intracellular plant components, extracellular plant components, liquid or solid extracts of plants, or a combination thereof. Further, 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. Plant matter may comprise a plant or portion thereof which has not been subjected to any processing steps. However, it is also contemplated that the plant material may be subjected to minimal processing steps as defined below, or more rigorous processing, including partial or substantial protein purification using techniques commonly known within the art including, but not limited to chromatography, electrophoresis and the like.

[003] By the term "minimal processing" it is meant plant matter, for example, a plant or portion thereof comprising a protein of interest which is partially purified to yield a plant extract, homogenate, fraction of plant homogenate or the like (i.e. minimally processed). Partial purification may comprise, but is not limited to disrupting plant cellular structures thereby creating a composition comprising soluble plant components, and insoluble plant components which may be separated for example, but not limited to, by centrifugation, filtration or a combination thereof. In this regard, proteins secreted within the extracellular space of leaf or other tissues could be readily obtained using vacuum or centrifugal extraction, or tissues could be extracted under pressure by passage through rollers or grinding or the like to squeeze or liberate the protein free from within the extracellular space. Minimal processing could also involve preparation of crude extracts of soluble proteins, since these preparations would have negligible contamination from secondary plant products. Further, minimal processing may involve aqueous extraction of soluble protein from leaves, followed by precipitation with any suitable salt. Other methods may include large scale maceration and juice extraction in order to permit the direct use of the extract.

[004] The plant matter, in the form of plant material or tissue may be orally delivered to a subject. The plant matter may be administered as part of a dietary supplement, along with other foods, or encapsulated. The plant matter or tissue may also be concentrated to improve or increase palatability, or provided along with other materials, ingredients, or pharmaceutical excipients, as required.

[005] It is contemplated that a plant comprising the protein of interest, or expressing the VLP comprising the protein of interest may be administered to a subject or target organism, in a variety of ways depending upon the need and the situation. For example, the protein of interest obtained from the plant may be extracted prior to its use in either a crude, partially purified, or purified form. If the protein is to be purified, then it may be produced in either edible or non-edible plants. Furthermore, if the protein is orally administered, the plant tissue may be harvested and directly feed to the subject, or the harvested tissue may be dried prior to feeding, or an animal may be permitted to graze on the plant with no prior harvest taking place. It is also considered within the scope of this invention for the harvested plant tissues to be provided as a food supplement within animal feed. If the plant tissue is being feed to a subject or an animal with little or not further processing it is preferred that the plant tissue being administered is edible.

[00183] The VLP's produced according to the present invention may be purified, partially purified from a plant, portion of a plant or plant matter, or may be administered as an oral vaccine, using methods as know to one of skill in the art. Purification may include production of an apoplast fraction as described in WO 2011/035422 (which is incorporated herein by reference). For preparative size exclusion chromatography, a preparation comprising VLPs may be obtained and insoluble material removed by centrifugation. Precipitation with PEG may also be used. The recovered protein may be quantified using conventional methods (for example, Bradford Assay, BCA), and the extract passed through a size exclusion column, using for example SEPHACRYL™, SEPHADEX™, or similar medium, and the fractions collected. Blue Dextran 2000 or a suitable protein, may be used as a calibration standard. The extract may also be passed through a cation exchange column and active fractions collected. Following chromatography, fractions may be

further analyzed by protein electrophoresis, immunoblot, or both, to confirm the presence of VLPs and the protein complement of the fraction.

[00184] Also considered part of this invention are transgenic plants, plant cells, seeds or any fraction thereof containing the nucleotide sequences of the present invention. Methods of regenerating whole plants from plant cells are also known in the art. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques. Transgenic plants can also be generated without using tissue cultures.

[00185] As shown in Figure 18, HA from B/Brisbane/60/2008 is poorly expressed in agroinfiltrated *Nicotiana benthamiana* leaves (see lanes "1008" or "1029"). However, co-expression of HA-type B with M2 from A/New Caledonia/20/99, results in a significant increase in HA expression (see lanes "1008+1261"; "1009+1261" and 1029+1261"). The increase in HA expression was observed in with both native type B HA or a chimeric HA type B. HA expression was observed in the presence or absence of amplication elements (BeYDV), and across various dilutions of *Agrobacterium*. A similar increase in H3 expression was observed when H3 from A/Perth/16/2009 was co-expressed with M2 from A/New Caledonia/20/99 (Figure 19; compare lane "1019" H3 alone, with "1019+1261" H3 co-expressed with M2).

[00186] The present invention includes nucleotide sequences as set forth in Table 3:

Table 3. List of Sequence Identification numbers.

SEQ ID NO:	Description	Figure

SEQ ID NO:	Description	Figure
1	proton channel signature sequence HXXXW	
2	primer IF-H5A-I-05.s1+3c	Fig. 1A
3	primer IF-H5dTm.r	Fig. 1B
4	Construct 1191	Fig. 1D
5	Expression cassette number 489	Fig. 1E
6	Amino acid sequence of H5 from influenza A/Indonesia/5/2005 (H5N1)	Fig. 1F
7	primer IF-S1-M1+M2ANC.c	Fig. 2A
8	primer IF-S1-4-M2ANC.r	Fig. 2B
9	nucleotide sequence of synthesized M2 gene (DQ508860)	Fig. 2C
10	Expression cassette number 1261	Fig. 2D
11	Amino acid sequence of M2 from influenza A/New Caledonia/20/1999 (H1N1)	Fig. 2E
12	nucleotide sequence of synthesized M2 gene	Fig. 3A
13	Expression cassette number 859	Fig. 3B
14	Amino acid sequence of M2 from influenza A/Puerto Rico/8/1934 (H1N1)	Fig. 3C
15	primer IF-H1A-C-09.s2+4c	Fig. 4A
16	primer IF-H1A-C-09.s1-4r	Fig. 4B
17	nucleotide sequence of synthesized H1 gene	Fig. 4C
18	Construct 1192	Fig. 4E
19	Expression cassette number 484	Fig. 4F
20	Amino acid sequence of PDISP-H1 from influenza A/California/7/2009 (H1N1)	Fig. 4G
21	primer IF-S2+S4-H3 Per.c	Fig. 5A
22	primer IF-S1a4-H3 Per.r	Fig. 5B
23	nucleotide sequence of synthesized H3 gene	Fig. 5C
24	Expression cassette number 1019	Fig. 5D
25	Amino acid sequence of PDISP/H3 from influenza A/Perth/16/2009 (H3N2)	Fig. 5E
26	primer IF-S2+S4-B Bris.c	Fig. 6A
27	primer IF-S1a4-B Bris.r	Fig. 6B
28	nucleotide sequence of synthesized HA B Brisbane gene	Fig. 6C

SEQ ID NO:	Description	Figure
29	Expression cassette number 1029	Fig. 6D
30	Amino acid sequence of PDISP/HA from influenza B/Brisbane/60/2008	Fig. 6E
31	Construct 1194	Fig. 6G
32	Expression cassette number 1008	Fig. 6H
33	primer dTmH5I-B Bris.r	Fig. 7A
34	primer B Bris-dTmH5I.c	Fig. 7B
35	primer IF-S1aS4-dTmH5I.r	Fig. 7C
36	Expression cassette number 1009	Fig. 7D
37	Amino acid sequence of PDISP/HA B Brisbane/H5Indo TMCT	Fig. 7E
38	primer 1039+1059.r	Fig. 8A
39	primer 1039+1059.c	Fig. 8B
40	Expression cassette number 1059 from BeYDV left LIR to BeYDV right LIR. PDISP/HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop	Fig. 8C
41	Amino acid sequence of PDISP/HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop	Fig. 8D
42	nucleotide sequence encoding H5 from influenza A/Indonesia/5/2005 (H5N1)	Fig. 1G
43	nucleotide sequence of PDISP/HA from influenza B/Brisbane/60/2008 with deleted proteolytic loop	Fig 8E
44	primer IF-H3V36111.S2+4c	Fig. 25A
45	primer IF-H3V36111.s1-4r	Fig. 25B
46	nucleotide sequence of synthesized H3 gene	Fig. 25C
47	expression cassette number 1391	Fig. 25D
48	Amino acid sequence of PDISP-H3 from influenza A/ Victoria/361/2011	Fig. 25E
49	primer IF-HAB110.S1+3c	Fig. 26A
50	primer IF-HAB110.s1-4r	Fig. 26B
51	nucleotide sequence of synthesized HA B/Wisconin (JN993010)	Fig. 26C
52	Construct 193	Fig. 26E
53	Expression cassette number 1462	Fig. 26F

SEQ ID NO:	Description	Figure
54	Amino acid sequence of HA from influenza B/Wisconsin/1/2010	Fig. 26G
55	primer HAB110(PrL-).r	Fig. 27A
56	primer HAB110(PrL-).c	Fig. 27B
57	Expression cassette number 1467	Fig. 27C
58	Amino acid sequence of HA from influenza B/Wisconsin/1/2010 with deleted proteolytic loop	Fig. 27D
59	primer IF-HB-M-04.s2+4c	Fig. 28A
60	primer IF-HB-M-04.s1-4r	Fig. 28B
61	nucleotide sequence of synthesized HA B Malaysia	Fig. 28C
62	Construct 194	Fig. 28E
63	Expression cassette number 1631	Fig. 28F
64	Amino acid sequence of PDISP-HA from influenza B/Malaysia/2506/2004	Fig. 28G

[00187] The present invention will be further illustrated in the following examples.

[00188] **Examples**

Material and Methods:: **Assembly of expression cassettes with influenza protein**

A-2X35S/CPMV-HT/H5 Indonesia/NOS (Construct number 489)

[00189] A sequence encoding H5 from influenza A/Indonesia/5/2005 (H5N1) 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 H5 coding sequence was amplified using primers IF-H5A-I-05.s1+3c (Figure 1A, SEQ ID NO: 2) and IF-H5dTm.r (Figure 1B, SEQ ID NO: 3) using construct number 972 (see Figure 94, SEQ ID NO: 134 of WO 2010/003225, which is incorporated herein by reference, for the sequence of construct number 972) 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 1D, SEQ ID NO: 4) 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 489 (Figure 1E, SEQ ID NO: 5). The amino acid sequence of H5 from influenza A/Indonesia/5/2005 (H5N1) is presented in Figure 1F (SEQ ID NO: 6). A representation of plasmid 489 is presented in Figure 15.

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

[00190] 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 16.

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

[00191] 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.

D-2X35S/CPMV-HT/PDISP/H1 California/NOS (Construct number 484)

[00192] A sequence encoding H1 from influenza A/California/7/2009 (H1N1) 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 the H1 coding sequence without his wild type signal peptide was amplified using primers IF-H1A-C-09.s2+4c (Figure 4A, SEQ ID NO: 15) and IF-H1A-C-09.s1-4r (Figure 4B, SEQ ID NO: 16), using synthesized H1 gene (Genbank accession number FJ966974) (Figure 4C, SEQ ID NO: 17) 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 (Figure 4D) 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 is presented in Figure 4E (SEQ ID NO: 18). The resulting construct was given number 484 (Figure 4F, SEQ ID NO: 19). The amino acid sequence of PDISP/H1from influenza A/California/7/2009 (H1N1) is presented in Figure 4G (SEQ ID NO: 20). A representation of plasmid 484 is presented in Figure 14.

E-2X35S/CPMV-HT/PDISP/H3 Perth/NOS (Construct number 1019)

[00193] A sequence encoding H3 from influenza A/Perth/16/2009 (H3N2) 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 the H3 coding sequence without his wild type signal peptide was amplified using primers IF-S2+S4-H3 Per.c (Figure 5A, SEQ ID NO: 21) and IF-S1a4-H3 Per.r (Figure 5B, SEQ ID NO: 22), using synthesized H3 gene (corresponding to nt 26-1726 from Genbank accession number GQ293081) (Figure 5C, SEQ ID NO: 23) 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 (Figure 4D) 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 is presented in Figure 4E (SEQ ID NO: 18). The resulting construct was given number 1019 (Figure 5D, SEQ ID NO: 24). The amino acid sequence of PDISP/H3 from influenza

A/Perth/16/2009 (H3N2) is presented in Figure 5E (SEQ ID NO: 25). A representation of plasmid 1019 is presented in Figure 13.

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

[00194] 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 6A, SEQ ID NO: 26) and IF-S1a4-B Bris.r (Figure 6B, SEQ ID NO: 27), using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank accession number FJ766840) (Figure 6C, SEQ ID NO: 28) 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 (Figure 4D) 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 is presented in Figure 4E (SEQ ID NO: 18). The resulting construct was given number 1029 (Figure 6D, SEQ ID NO: 29). The amino acid sequence of PDISP/HA from influenza B/Brisbane/60/2008 is presented in Figure 6E (SEQ ID NO: 30). A representation of plasmid 1029 is presented in Figure 11.

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

[00195] 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 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 6A, SEQ ID NO: 26) and IF-S1a4-B Bris.r (Figure 6B, SEQ ID NO: 27), using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank accession number FJ766840) (Figure 6C, SEQ ID NO: 28) as template. The PCR product was cloned in-frame with alfalfa PDI signal peptide in 2X35S/CPMV-HT/NOS expression cassette into the BeYDV amplification system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1194 (see Figures 6F and 6G) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1194 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 and the sequence from left to right t-DNA borders is presented in Figure 6G (SEQ ID NO: 31). The resulting construct was given number 1008 (Figure 6H SEQ ID NO: 32). The amino acid sequence of Influenza PDISP/HA from B/Brisbane/60/08 is presented in Figure 6E (SEQ ID NO: 30). A representation of plasmid 1008 is presented in Figure 9.

H-2X35S/CPMV-HT/PDISP/HA B Brisbane/H5 Indonesia transmembrane domain and cytoplasmic tail (H5Indo TMCT)/NOS into BeYDV+Replicase amplification system (Construct number 1009)

[00196] A sequence encoding HA from influenza B/Brisbane/60/2008 ectodomain fused to the transmembrane and cytosolic domains of H5 from A/Indonesia/5/2005 (H5N1) 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 as follows using the 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 HA B Brisbane ectodomain coding sequence without the native signal peptide, transmembrane and cytoplasmic domains was amplified using primers IF-S2+S4-B Bris.c (Figure 6A, SEQ ID NO: 26) and dTmH5I-B Bris.r (Figure 7A, SEQ ID NO: 33), using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank accession number FJ766840)

(Figure 6C, SEQ ID NO: 28) as template. A second fragment containing the transmembrane and cytoplasmic domains of H5 Indonesia was amplified using primers B Bris-dTmH5I.c (Figure 7B, SEQ ID NO: 34) and IF-S1aS4-dTmH5I.r (Figure 7C, SEQ ID NO: 35), using construct number 489 (see Figure 1E, SEQ ID NO: 5) as template. The PCR products from both amplifications were then mixed and used as template for a second round of amplification using IF-S2+S4-B Bris.c (Figure 6A, SEQ ID NO: 26) and IF-H5dTm.r (Figure 7C, SEQ ID NO: 34) as primers. The resulting fragment was cloned in-frame with alfalfa PDI signal peptide in 2X35S/CPMV-HT/NOS expression cassette into the BeYDV amplification system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1194 (Figures 6F and 6G) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1194 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 and the sequence from left to right t-DNA borders is presented in Figure 6G (SEQ ID NO: 31). The resulting construct was given number 1009 (Figure 7D, SEQ ID NO: 36). The amino acid sequence of PDISP/HA B Brisbane/H5indo TMCT is presented in Figure 7E (SEQ ID NO: 37). A representation of plasmid 1009 is presented in Figure 10.

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

[00197] 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 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 HA B Brisbane coding sequence from nt 46 to nt 1065 was amplified using primers IF-S2+S4-B Bris.c (Figure 6A, SEQ ID NO: 26) and 1039+1059.r (Figure 8A, SEQ ID NO: 38), using synthesized HA B Brisbane

gene (corresponding to nt 34-1791 from Genebank accession number FJ766840) (Figure 6C, SEQ ID NO: 28) as template. A second fragment, containing HA B Brisbane coding sequence from nt 1123 to nt 1758, was amplified using primers 1039+1059.c (Figure 8B, SEQ ID NO: 39) and IF-S1a4-B Bris.r (Figure 6B, SEQ ID NO: 27), using synthesized HA B Brisbane gene (corresponding to nt 34-1791 from Genbank accession number FJ766840) (Figure 6C, SEQ ID NO: 28) as template. The PCR products from both amplifications were then mixed and used as template for a second round of amplification using IF-S2+S4-B Bris.c (Figure 6A, SEQ ID NO: 26) and IF-H5dTm.r IF-S1a4-B Bris.r (Figure 6B, SEQ ID NO: 27) as primers. 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 comprising the BeYDV amplification system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 1194 (Figure 6F and 6G) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 1194 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 and the sequence from left to right t-DNA borders is presented in Figure 6G (SEQ ID NO: 31). The resulting construct was given number 1059 (Figure 8C, SEQ ID NO: 40). The amino acid sequence of PDISP-HA B/Brisbane/60/2008 with deleted proteolytic loop is presented in Figure 8D (SEQ ID NO: 41). A representation of plasmid 1059 is presented in Figure 12.

A-2X35S/CPMV-HT/PDISP/H3 Victoria/NOS (Construct number 1391)

[00198] A sequence encoding H3 from influenza A/Victoria/361/2011 (H3N2) 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 the H3 coding sequence without his wild type signal peptide was amplified using primers IF-H3V36111.S2+4c (Figure 25A, SEQ ID NO: 44) and IF-H3V36111.s1-4r (Figure 25B, SEQ ID NO: 45), using synthesized

H3 gene (corresponding to nt 25 to 1725 from GISAID EPI_ISL_101506 isolate HA sequence) (Figure 25C, SEQ ID NO: 46) 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 (Figure 4D) 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 is presented in Figure 4E (SEQ ID NO: 18). The resulting construct was given number 1391 (Figure 25D, SEQ ID NO: 47). The amino acid sequence of PDISP/H3 from Influenza A/Victoria/361/2011 (H3N2) is presented in Figure 25E (SEQ ID NO: 48). A representation of plasmid 1391 is presented in Figure 25F.

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

[00199] 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 the following PCR-based method. A fragment containing the complete HA B Wisconsin coding sequence was amplified using primers IF-HAB110.S1+3c (Figure 26A, SEQ ID NO: 49) and IF-HAB110.s1-4r (Figure 26B, SEQ ID NO: 50), using synthesized HA B Wisconsin gene (Genbank accession number JN993010) (Figure 26C, SEQ ID NO: 51) as template. The PCR product was cloned in 2X35S/CPMV-HT/NOS expression cassette into the BeYDV(m) amplification system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 193 (Figure 26D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. 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 26E (SEQ ID NO: 52). The resulting construct was given number 1462 (Figure 26F, SEQ ID NO: 53). The amino acid sequence of PDISP/HA from Influenza B/Wisconsin/1/2010 is presented in Figure 26G (SEQ ID NO: 54). A representation of plasmid 1462 is presented in Figure 26H.

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

[00200] 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 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 HA B Wisconsin coding sequence from nt 1 to nt 1062 was amplified using primers IF-HAB110.S1+3c (Figure 26A, SEQ ID NO: 49) and HAB110(PrL-).r (Figure 27A, SEQ ID NO: 55) , using synthesized HA B Wisconsin gene (Genbank accession number JN993010) (Figure 26C, 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 27B, SEQ ID NO: 56) and and IF-HAB110.s1-4r (Figure 26B, SEQ ID NO: 50), using synthesized HA B Wisconsin gene (Genbank accession number JN993010) (Figure 26C, 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 26A, SEQ ID NO: 49) and IF-HAB110.s1-4r (Figure 26B, 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 26D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. 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 26E (SEQ ID NO: 52). The resulting construct was given number 1467 (Figure 27C, SEQ ID NO: 57). The amino acid sequence of HA from Influenza B/Wisconsin/1/2010 with deleted proteolytic loop is presented in Figure 27D (SEQ ID NO: 58). A representation of plasmid 1467 is presented in Figure 27E.

D-2X35S/CPMV-HT/PDISP/HA B Malaysia/NOS into BeYDV(m)+Replicase amplification system (Construct number 1631)

[00201] A sequence encoding HA from influenza B/Malaysia/2506/2004 was cloned into 2X35S/CPMV-HT/PDISP/NOS comprising the BeYDV(m)+replicase amplification system in a plasmid containing Plasto_pro/P19/Plasto_ter expression cassette using the following PCR-based method. A fragment containing HA B Malaysia coding sequence without his wild type signal peptide was amplified using primers IF-HB-M-04.s2+4c (Figure 28A, SEQ ID NO: 59) and IF-HB-M-04.s1-4r (Figure 28B, SEQ ID NO: 60), using synthesized HA B Malaysia gene (corresponding to nt 31-1743 from Genbank accession number EU124275. Silent mutations T759C and C888G were inserted in synthesized sequence in order to modify DraIII and BamHI restriction enzyme recognition sites) (Figure 28C, SEQ ID NO: 61) as template. The PCR product was cloned in-frame with alfalfa PDI signal peptide in 2X35S/CPMV-HT/NOS expression cassette into the BeYDV(m) amplification system using In-Fusion cloning system (Clontech, Mountain View, CA). Construct 194 (Figure 28D) was digested with SacII and StuI restriction enzyme and the linearized plasmid was used for the In-Fusion assembly reaction. Construct number 194 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(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 28E (SEQ ID NO: 62). The resulting construct was given number 1631 (Figure

28F, SEQ ID NO: 63). The amino acid sequence of PDISP/HA from Influenza B/Malaysia/2506/2004 is presented in Figure 28G (SEQ ID NO: 64). A representation of plasmid 1631 is presented in Figure 28H.

Agrobacterium transfection

[00202] *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

[00203] 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.

[00204] *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 25°C day/20°C 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.

[00205] *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

[00206] Following incubation, the aerial part of plants was harvested, frozen at -80°C 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, 0.15 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 4°C and these clarified crude extracts (supernatant) kept for analyses.

Protein analysis and immunoblotting

[00207] 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 4°C.

[00208] 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

[00209] 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.

Example 1: Effect of influenza M2 co-expression on the accumulation level of B HA and H3

[00210] The effect of influenza M2 co-expression on the accumulation level of HA from different influenza strains was analyzed by co-transferring constructs driving expression of HA with a construct for the expression of M2 from influenza A/New Caledonia/20/1999 (H1N1) in the agroinfiltration-based transient transformation system.

[00211] Western blot analysis of protein extracts from plants transformed with gene constructs driving the expression of influenza B HA (from B/Brisbane/60/2008) (constructs no. 1008, 1009 and 1029) 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 18). Similarly, the co-expression of M2 with H3 from influenza A/Perth/16/2009 (construct no. 1019+1261) resulted in increased accumulation of H3 in transformed plants when compared to plants transformed with H3-expression construct only (construct no. 1019) as shown in Figure 19.

[00212] Western blot analysis of protein extracts from plants co-expressing M2 with H1 from influenza A/California/07/2009 showed that the co-expression of M2 with H1 resulted in a slight decrease in H1 accumulation level (Figure 20, 484 vs 484+1261). The co-expression of M2 with H5 from influenza A/Indonesia/05/2005 also resulted in a reduced H5 accumulation when compared to H5 expressed alone (Figure 21, 489 vs 489+1261).

[00213] The co-expression of M2 was further 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 22A 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 further increased HA accumulation level (Figure 22A, 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 22B, 1008 vs 1008+1261) and the modified influenza B HA (Figure 22B, 1059 vs 1059+1261).

[00214] 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. For H3, a similar comparison was performed on protein extracts from plants transformed with 1019, 1019+1261 and 1019+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 both the modified influenza B HA (Figure 23A) and H3 (Figure 23B).

Example 2: Effect of influenza M2 co-expression on the accumulation level of different strains of B HA and H3

[00215] Western blot analysis of protein extracts from plants transformed with gene constructs driving the expression of influenza B HA (from B/Malaysia/2506/2004) (constructs no. 1631) 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 29).

[00216] 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 30).

[00217] The co-expression of M2 was further 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 30A 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 further increased HA accumulation level (Figure 30A, 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 30B, 1462 vs 1462+1261) and the modified influenza B HA (Figure 26B, 1467 vs 1467+1261).

[00218] Western blot analysis of protein extracts from plants transformed with gene constructs driving the expression of influenza H3 (from H3/Victoria/361/2011) (constructs no. 1391) in the presence or absence of M2-expression construct (construct no. 1261) showed that M2 co-expression results in increased accumulation of influenza H3 (Figure 31).

All citations are hereby incorporated by reference.

[00219] 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 method of producing a virus like particle (VLP) in a plant comprising,

a) introducing a first nucleic acid comprising a first regulatory region active in the plant and operatively linked to a nucleotide sequence encoding an influenza hemagglutinin (HA) protein selected from the group consisting of B and H3 into the plant, or portion of the plant;

b) introducing a 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 M2 or BM2; and

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

2. The method of claim 1, wherein the proton channel protein comprises the proton channel signature sequence HXXXW.

3. The method of claim 1, wherein one or more proteolytic loop of the influenza HA protein has been deleted.

4. The method of claim 1, wherein the influenza B HA is from influenza B/Brisbane/60/2008, B/Malaysia/2506/2004 or B/Wisconsin/1/2010.

5. The method of claim 4, wherein one or more proteolytic loop of the influenza B HA protein has been deleted.

6. The method of claim 1, wherein the influenza H3 is from influenza A/Perth/16/2009 or from influenza A/Victoria/361/2011.

7. The method 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: 28, SEQ ID NO:51 and SEQ ID NO:61.

8. The method of claim 1, wherein the sequence of the influenza hemagglutinin (HA) protein is as shown as in at least one of SEQ ID NO:30, SEQ ID NO:54 and SEQ ID NO:64.

9. The method 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: 43 and the underlined portion of SEQ ID NO:57.

10. The method of claim 1, wherein the sequence of the influenza hemagglutinin (HA) protein is as shown as in at least one of SEQ ID NO:41 and SEQ ID NO:58.

11. The method 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: 23 and SEQ ID NO:46.

12. The method of claim 1, wherein the sequence of the influenza hemagglutinin (HA) protein is as shown as in at least one of SEQ ID NO:25 and SEQ ID NO:48.

13. The method of claim 1, wherein the first nucleic acid sequence comprises the first regulatory region operatively linked with one or more than one comovirus enhancer, the nucleotide sequence encoding the influenza hemagglutinin (HA) protein, and one or more than one geminivirus amplification element, and a third nucleic acid encoding a geminivirus replicase is introduced into the plant or portion of the plant.

14. The method of claim 13, wherein the one or more than one comovirus enhancer is a comovirus UTR.

15. The method of claim 14, wherein the comovirus UTR is a Cowpea Mosaic Virus (CPMV) UTR.

16. The method of claim 13, wherein the one or more than one geminivirus amplification element is selected from a Bean Yellow Dwarf Virus long intergenic region (BeYDV LIR), and a BeYDV short intergenic region (BeYDV SIR).

17. The method of claim 13, 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: 28, SEQ ID NO: 51 and SEQ ID NO: 61.

18. The method of claim 13, wherein the sequence of the influenza hemagglutinin (HA) protein is as shown as in at least one of SEQ ID NO: 30, SEQ ID NO: 54 and SEQ ID NO: 64.

19. The method of claim 13, 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: 43 and the underlined portion of SEQ ID NO: 57.

20. The method of claim 13, wherein the sequence of the influenza hemagglutinin (HA) protein is as shown as in at least one of SEQ ID NO: 41 and SEQ ID NO: 58.

21. The method of claim 13, 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: 23 and SEQ ID NO: 46.

22. The method of claim 13, wherein the sequence of the influenza hemagglutinin (HA) protein is as shown as in at least one of SEQ ID NO: 25 and SEQ ID NO: 48.

23. The method of claim 1, wherein in the step of introducing (step a), the nucleic acid is transiently expressed in the plant.

24. The method of claim 1, wherein, in the step of introducing (step a), the nucleic acid is stably expressed in the plant.

25. The method of claim 1 further comprising a step of

d) harvesting the plant and purifying the VLPs.

26. The method of claim 1, wherein the VLP does not contain a viral matrix, a core protein or a channel protein.

27. A method of producing a virus like particle (VLP) comprising,

a) providing a plant or portion of the plant comprising a first nucleic acid comprising a first regulatory region active in the plant and operatively linked to a nucleotide sequence encoding an influenza hemagglutinin (HA) protein selected from the group consisting of B and H3, and a 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 M2 or BM2; and

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

28. The method of claim 27, wherein the proton channel protein comprises the proton channel signature sequence HXXXW.

29. The method of claim 27, wherein one or more proteolytic loop of the influenza HA protein has been deleted.

30. A VLP produced by the method of claim 3.

31. A VLP produced by the method of claim 29.

32. The VLP of claim 30 or 31, further comprising one or more than one lipid derived from a plant.

33. The VLP of claim 30 or 31, wherein one or more virus protein comprises plant-specific N-glycans, or modified N-glycans.

34. A composition comprising an effective dose of the VLP of claim 30 or 31 for inducing an immune response, and a pharmaceutically acceptable carrier.

35. Use of a composition comprising an effective dose of the VLP of claim 30 or 31 in the manufacture of a medicament for inducing immunity to an influenza virus infection in a subject, wherein the VLP of claim 30 or 31 is to be administered.

36. The use of claim 35, wherein the VLP is to be administered to a subject orally, intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously.

37. A plant matter comprising a VLP produced by the method of claim 1 or 28.

38. The plant matter of claim 37 for use in inducing immunity to an influenza virus infection in a subject.

39. A food supplement comprising the plant matter of claim 38.

40. The method of claim 1, further comprising introducing a third nucleic acid sequence, the third nucleic acid sequence encoding a suppressor of silencing.

41. The method of claim 13, further comprising introducing a fourth nucleic acid sequence, the fourth nucleic acid sequence encoding a suppressor of silencing.

42. The method of claim 40 or 41 wherein the suppressor of silencing is selected from the group HcPro and p19.

43. The method of claim 1 or 27, wherein the VLP does not contain the proton channel protein.

44. The method of claim 1 or 27, wherein the influenza hemagglutinin (HA) protein is an HA0 protein.

45. The method of claim 1, wherein the M2 protein is selected from the group comprising influenza A/Puerto Rico/8/1934 and influenza A/New Caledonia/20/1999.