

CHIMERIC INFLUENZA VIRUS-LIKE PARTICLES COMPRISING HEMAGGLUTININ

FIELD OF INVENTION

[0001] This application claims priority from US Provisional Application No. 61/220,161 filed
5 June 24, 2009.

[0002] The present invention relates to virus-like particles. More specifically, the present invention is directed to virus-like particles comprising chimeric influenza hemagglutinin, and methods of producing chimeric influenza virus-like particles.

BACKGROUND OF THE INVENTION

10 [0003] Influenza is the leading cause of death in humans due to a respiratory virus, and during “flu season”, it is estimated that 10-20% of the population worldwide may be infected, leading to 250-500,000 deaths annually.

[0004] The current method of combating influenza in humans is by annual vaccination. The vaccine is usually a combination of several strains that are predicted to be the dominant strains
15 for the coming flu-season, however the number of vaccine doses produced annually is not sufficient to vaccinate the world’s population. For example, Canada and the United-States obtain enough vaccine doses to immunize about one third of their population, and in Europe, only about 17% can be vaccinated given current production – in the face of a worldwide flu pandemic, this production would be insufficient. Even if the necessary annual production
20 could somehow be met in a given year, the dominant strains change from year to year, thus stockpiling at low-need times in the year is not practical. Economical, large scale production of an effective influenza vaccine is of significant interest to government and private industry alike.

[0005] Influenza haemagglutinin (HA) surface glycoprotein is both a receptor-binding and
25 membrane fusion protein. It is a trimer of identical subunits, each containing two disulphide-linked polypeptides, HA1 and HA2, that are derived by proteolytic cleavage of a precursor, HA0, that has a signal peptide sequence at its N-terminus and a membrane anchor sequence at

its C-terminus. Cleavage to form HA1 and HA2 generates the N-terminus of the smaller polypeptide, HA2, which has the membrane anchor sequence at its C-terminus. Cleavage is required for membrane fusion activity but not for immunogenicity. The HA2 N-terminal sequence is called the 'fusion peptide' because cleavage at similar hydrophobic sequences is also required for the activity of other virus fusion proteins, and because 20-residue synthetic peptide analogues of the sequence fuse membranes *in vitro*.

[0006] Generally, the surface of the globular 'head' comprises several flexible loops with well-characterized and variable antigenic regions designated as sites A, B, C, D and E (reviewed in Wiley et al., 1987. *Annu. Rev Biochem* 56:365-394). Insertion or replacement of short peptide sequences at some sites (e.g. B and E) for immunity studies have been described (Garcia-Sastré et al. 1995. *Biologicals* 23:171-178). Epidermal growth factor (EGF), single chain antibody (scFV) and the Fc domain of an IgG, ranging in size from 53 to 246 amino acids, have been inserted at the N-terminal end of a H7 and chimeras has been successfully expressed (Hatzioannou et al., 1999. *Human Gene Therapy* 10:1533-1544). More recently, 90 and 140 amino acid domains of *Bacillus anthracis* protective antigen have been fused to the amino terminus of a H3 (Li et al., 2005. *J. Virol* 79:10003-1002). Copeland (Copeland et al., 2005. *J. Virol* 79:6459-6471) describes the expression of the gp120 Env HIV surface glycoprotein on a H3 stalk, where the gp120 domain replaced the whole globular head of HA.

[0007] Several recombinant products have been developed as recombinant influenza vaccine candidates. These approaches have focused on the expression, production, and purification of influenza type A HA and NA proteins, including expression of these proteins using baculovirus infected insect cells (Crawford et al, 1999 *Vaccine* 17:2265-74; Johansson, 1999 *Vaccine* 17:2073-80), viral vectors, and DNA vaccine constructs (Olsen et al., 1997 *Vaccine* 15:1149-56).

[0008] Production of a non-infectious influenza virus strain for vaccine purposes is one way to avoid inadvertent infection. Alternatively, virus-like particles (VLPs) as substitutes for the cultured virus have been investigated. VLPs mimic the structure of the viral capsid, but lack a genome, and thus cannot replicate or provide a means for a secondary infection. Current

influenza VLP production technologies rely on the co-expression of multiple viral proteins, and this dependence represents a drawback of these technologies since in case of a pandemic and of yearly epidemics, response time is crucial for vaccination. A simpler VLP production system, for example, one that relies on the expression of only one or a few viral proteins without requiring expression of non-structural viral proteins is desirable to accelerate the development of vaccines.

[0009] Enveloped viruses may obtain their lipid envelope when ‘budding’ out of the infected cell and obtain the membrane from the plasma membrane, or from that of an internal organelle. In mammalian or baculovirus cell systems, for example, influenza buds from the plasma membrane (Quan et al 2007 J. Virol 81:3514-3524). Only a few enveloped viruses are known to infect plants (for example, members of the Tospoviruses and Rhabdoviruses). Of the known plant enveloped viruses, they are characterized by budding from internal membranes of the host cell, and not from the plasma membrane. Although a small number of recombinant VLPs have been produced in plant hosts, none were derived from the plasma membrane, raising the question whether plasma membrane-derived VLPs, including influenza VLPs can be produced in plants.

[0010] Formation of VLPs, in any system, places considerable demands on the structure of the proteins – altering short stretches of sequence that correspond to selected surface loops of a globular structure may not have much of an effect on expression of the polypeptide itself, however structural studies are lacking to demonstrate the effect of such alterations on the formation of VLPs. The cooperation of the various regions and structures of HA (e.g. the membrane anchor sequences, the stalk or stem regions of the trimer that separate the globular head from the membranes) has evolved with the virus and may not be amendable to similar alterations without loss of HA trimer integrity and VLP formation.

[0011] The production of influenza HA VLPs has been previously described by the inventors in WO 2009/009876.

SUMMARY OF THE INVENTION

[0012] The present invention relates to virus-like particles. More specifically, the present invention is directed to virus-like particles comprising chimeric influenza hemagglutinin, and methods of producing chimeric influenza hemagglutinin virus-like particles.

5 [0013] It is an object of the invention to provide an improved chimeric influenza virus-like particle (VLP).

[0014] The present invention provides a polypeptide comprising a chimeric influenza HA comprising a stem domain cluster (SDC), a head domain cluster (HDC) and a transmembrane domain cluster (TDC) wherein: the SDC comprises an F'1, F'2 and F subdomain; the HDC
10 comprises an RB, E1 and E2 subdomain; the TDC comprises a TmD and Ctail subdomain; and wherein at least one subdomain is of a first influenza HA and the other subdomains are of one or more second influenza HA. The first and second influenza HA may independently be selected from the group comprising H1, H3, H5 and B. Furthermore, the polypeptide may comprise a signal peptide.

15 [0015] The present invention also provides a nucleic acid encoding the polypeptide comprising a chimeric influenza HA comprising a stem domain cluster (SDC), a head domain cluster (HDC) and a transmembrane domain cluster (TDC) wherein: the SDC comprises an F'1, F'2 and F subdomain; the HDC comprises an RB, E1 and E2 subdomain; the TDC comprises a TmD and Ctail subdomain; and wherein at least one subdomain is of a first
20 influenza HA, and the other subdomains are of one or more second influenza HA. The nucleic acid may also encode the polypeptide that comprises a signal peptide in addition to the SDC, HDC and TDC as defined.

[0016] A method of producing chimeric influenza virus like particles (VLPs) in a plant is also provided, the method comprising:

25 a) introducing a nucleic acid encoding a chimeric influenza HA comprising a signal peptide, a stem domain cluster (SDC), a head domain cluster (HDC) and a transmembrane domain cluster (TDC) wherein: the SDC comprises an F'1, F'2 and F subdomain; the HDC

comprises an RB, E1 and E2 subdomain; the TDC comprises a TmD and Ctail subdomain; and wherein at least one subdomain is of a first influenza HA, and the other subdomains are of one or more second influenza HA into the plant, or portion thereof, and

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

[0017] The present invention includes the method described above wherein in the step of introducing (step a), the nucleic acid is introduced in the plant in a transient manner.

Alternatively, in the step of introducing (step a), the nucleic acid is introduced in the plant and is stably integrated. The method may further comprise a step of: c) harvesting the host and purifying the VLPs.

[0018] The present invention provides a plant, or portion thereof, comprising a chimeric influenza HA, or a nucleotide sequence encoding the chimeric influenza HA, the chimeric influenza HA comprising a stem domain cluster (SDC), a head domain cluster (HDC) and a transmembrane domain cluster (TDC) wherein: the SDC comprises an F'1, F'2 and F subdomain; the HDC comprises an RB, E1 and E2 subdomain; the TDC comprises a TmD and Ctail subdomain; and wherein at least one subdomain is of a first influenza HA and the other subdomains are of one or more second influenza HA.

[0019] The plant, or portion thereof, may further comprise a nucleic acid comprising a nucleotide sequence encoding one or more than one chaperone protein operatively linked to a regulatory region active in a plant. The one or more than one chaperon proteins may be selected from the group comprising Hsp40 and Hsp70.

[0020] The present invention pertains to a virus like particle (VLP) comprising a chimeric influenza HA, the chimeric influenza HA comprising a stem domain cluster (SDC), a head domain cluster (HDC) and a transmembrane domain cluster (TDC) wherein: the SDC comprises an F'1, F'2 and F subdomain; the HDC comprises an RB, E1 and E2 subdomain; the TDC comprises a TmD and Ctail subdomain; and wherein at least one subdomain is of a

first influenza HA and the other subdomains are of one or more second influenza HA. The VLP may further comprise plant-specific N-glycans, or modified N-glycans.

[0021] A composition comprising an effective dose of the VLP as just described and a pharmaceutically acceptable carrier is also provided.

- 5 [0022] In an alternate aspect of the present invention there is provided a method of inducing immunity to an influenza virus infection in a subject, comprising administering the VLP to the subject. The VLP may administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

- 10 [0023] Regulatory regions that may be operatively linked to a sequence encoding a chimeric HA protein include those that are operative in a plant cell, an insect cell or a yeast cell. Such regulatory regions may include a plastocyanin regulatory region, a regulatory region of Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), chlorophyll a/b binding protein (CAB) or ST-LS1. Other regulatory regions include a 5' UTR, 3' UTR or terminator sequences. The plastocyanin regulatory region may be an alfalfa plastocyanin regulatory
15 region; the 5' UTR, 3'UTR or terminator sequences may also be alfalfa sequences.

- [0024] The present invention provides a chimeric influenza HA polypeptide comprised of a first influenza and a second influenza, the first influenza and the second influenza may be independently selected from the group comprising B, H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16; with the proviso that the first influenza and the
20 second influenza are not the same influenza type, subtype, or of the same origin.

- [0025] In accordance with some aspects of the invention, the chimeric influenza HA polypeptide comprises a signal peptide sequence, the signal peptide sequence may be selected from the group comprising a native signal peptide sequence, an alfalfa PDI signal peptide sequence, an influenza H5 signal peptide sequence and an influenza H1 signal peptide
25 sequence

[0026] The present invention provides a method for producing a VLP containing chimeric influenza hemagglutinin (HA) within a host capable of producing a VLP, including a plant, insect, or yeast comprising, introducing a nucleic acid encoding a chimeric influenza HA comprising a stem domain cluster (SDC), a head domain cluster (HDC) and a transmembrane domain cluster (TDC) wherein: the SDC comprises an F'1, F'2 and F subdomain; the HDC comprises an RB, E1 and E2 subdomain; the TDC comprises a TmD and Ctail subdomain; and wherein at least one subdomain is of a first influenza HA and the other subdomains are of one or more second influenza HA, into the host, and incubating the host under conditions that permit the expression of the nucleic acid, thereby producing the VLPs.

[0027] The production of VLPs in plants presents several advantages over the production of these particles in insect cell culture. 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 that are unique to plants and some bacteria and protozoa. Sphingolipids are unusual in that they are not esters of glycerol like PC or PE but rather consist of a long chain amino alcohol that forms an amide linkage to a fatty acid chain containing more than 18 carbons. 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. Furthermore, in addition to the potential adjuvant effect of the presence of plant lipids, the ability of plant N-glycans to facilitate the capture of glycoprotein antigens by antigen presenting cells, may be advantageous of the production of chimeric VLPs in plants. Without wishing to be bound by theory, it is anticipated that plant-made chimeric VLPs induce a stronger immune reaction than chimeric VLPs made in other manufacturing systems and that the immune reaction induced by these plant-made chimeric VLPs is stronger when compared to the immune reaction induced by live or attenuated whole virus vaccines.

[0028] Contrary to vaccines made of whole viruses, chimeric VLPs provide the advantage as they are non-infectious, thus restrictive biological containment is not as significant an issue as it would be working with a whole, infectious virus, and is not required for production. Plant-

made chimeric VLPs provide a further advantage again by allowing the expression system to be grown in a greenhouse or field, thus being significantly more economical and suitable for scale-up.

[0029] Additionally, plants do not comprise the enzymes involved in synthesizing and adding sialic acid residues to proteins. VLPs may be produced in the absence of neuraminidase (NA), and there is no need to co-express NA, or to treat the producing cells or extract with sialidase (neuraminidase), to ensure VLP production in plants

[0030] This summary of the invention does not necessarily describe all features of the invention of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] 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:

[0032] Figure 1A shows a schematic diagram of HA subdomains. SP: signal peptide, F'1, F'2, and F: fusion subdomains; RB: receptor-binding subdomain, E1 and E2: esterase subdomains, TMD/CT: transmembrane and cytoplasmic tail subdomains. Figure 1B shows schematic representations of plastocyanin-based expression cassettes (construct numbers: 774, 540, 660, 690, 691, 696) for the expression of hemagglutinin H1 A/Brisbane/59/2007 (H1/Bri), hemagglutinin H1 A/New Caledonia/20/99 (H1/NC) and hemagglutinin H5 A/Indonesia/5/05 (H5/Indo) in native and chimeric forms. Plasto pro: alfalfa plastocyanin promoter, Plasto ter: alfalfa plastocyanin terminator, SP: signal peptide, RB: receptor-binding subdomain, E1-RB-E2: esterase and receptor-binding subdomains, TMD/CT: transmembrane and cytoplasmic tail subdomains, PDI: alfalfa protein disulfide isomerase. Figure 1C shows the amino acid sequence alignment superimposed with a structural alignment for several influenza HAs (B/Florida/4/2006 (BFlorida), SEQ ID NO:94 (GenBank Accession No. ACA33493.1; B/Malaysia/2506/2004 (B-Malaysia), SEQ ID NO:95 (GenBank Accession No. ABU99194.1; H1/Bri (A-Brisbane), SEQ ID NO:96 (GenBank Accession No. ADE28750.1; H1 A/Solomon Islands/3/2006 (A-Sol.Is), SEQ ID NO:97 (GenBank

Accession No. ABU99109.1); H1/NC (A-NewCal), SEQ ID NO:98 (GenBank Accession No. AAP34324.1); H2 A/Singapore/1/1957 (A-Singapore), SEQ ID NO:99 (GenBank Accession No. AAA64366.1); H3 A/Brisbane/10/2007 (A-Brisbane), SEQ ID NO:100 (GenBank Accession No. ACI26318.1); H3 A/Wisconsin/67/2005(A-WCN), SEQ ID NO:101 (GenBank Accession No. ABO37599.1); H5 A/Anhui/1/2005 (A-Anhui), SEQ ID NO:102 (GenBank Accession No. ABD28180.1); H5 A/Vietnam/1194/2004(A-Vietnam), SEQ ID NO:103 (GenBank Accession No. ACR48874.1); H5-Indo, SEQ ID NO:104 (GenBank Accession No. ABW06108.1. The boundaries between the F'1, Esterase 1, Receptor binding, Esterase 2, F'2, Peptide fusion, TMD/CT subdomains, and di-sulfide bridges are indicated.

[0033] Figure 2 shows the amino acid sequence of the indicated subdomains of chimeric HA expressed with, upper panel, constructs 690, 734 (SEQ ID NO: 11), 696 (SEQ ID NO: 112), and lower panel, 691 (SEQ ID NO: 113. Amino acids 1-92 of SEQ ID NO: 111 is the F'1+E1 domain of H5/Indo; amino acids 93-263 is an RB head domain of H1/Brisbane and amino acids 264-552 is an E2+F'2 domain of H5/Indo. Amino acids 1-92 of SEQ ID NO: 112 is the F'1+E1 domain of H5/NC; amino acids 93-301 is an RB head domain of H5/Indo and amino acids 302-586 is an E2+F'2 domain of H1/NC. Amino acids 1-42 of SEQ ID NO: 113 is the F'1 domain of H5/Indo; amino acids 43-273 is an E1- RB-E2 head domain of H1/Brisbane and amino acids 274-552 is an F'2 domain of H5/Indo.

[0034] Figure 3 shows the amino acid sequence of the coding region of constructs 690 and 734 (SEQ. ID. NO. 80) comprising an RB subdomain of H1/Bri, an H5/Indo signal peptide, and a stem domain complex (SDC) comprising an H5/Indo F'1, E1, E2, F'2 and F subdomains.

[0035] Figure 4 shows the amino acid sequence of the coding region of construct 691 (SEQ. ID. NO. 81) comprising H1/Bri head domain complex (HDC) comprising E1, RB, E2, an H5/Indo signal peptide, and H5/Indo stem domain complex (SDC) comprising H5/Indo F'1, F'2 and F subdomains.

[0036] Figure 5 shows the amino acid sequence of the coding region of construct 696 (SEQ. ID. NO. 82) comprising an RB subdomain of H5/Indo, a PDI signal peptide, and H1/NC stem domain complex comprising F'1, E1, E2 and F'2.

[0037] Figure 6 shows an immunoblot analysis of expression of H1/Bri in native form, construct 774 (comprising H1/Bri), construct 692 (comprising the head domain complex (HDC) of H1/Bri), and construct 690 (comprising the RB sub domain of H1/Bri fused with H5/Indo stem domain complex (SDC) in plants. For each construct, total protein extracts from 3 separate plants were analyzed. Twenty micrograms of protein were loaded for each plant analyzed. The Western blot was revealed with anti-HA monoclonal antibodies (anti H1-Brisbane; FII 10-I50). Construct 774 expresses H1/Bri with the native signal peptide of H1/Bri; constructs 690, 691 express the HA with the native signal peptide of H5/Indo.

[0038] Figure 7 shows an immunoblot analysis of expression of H5/Indo in native form, construct 660 (comprising H5/Indo, or construct 696 (comprising H1/Indo RB subdomain fused with H1/NC SDC, E1 and E2 subdomains). For each construct, total protein extracts from 3 separate plants were analyzed. Twenty micrograms of proteins were loaded for each plant analyzed. The Western blot was revealed with anti-H5 Indonesia polyclonal antibodies (ITC IT-003-005V). Construct 660 expresses H5/Indo with its native signal peptide; construct 696 expresses the chimeric HA with a PDI signal peptide.

[0039] Figure 8 shows a schematic representation of 35SCPMV/HT-based expression cassettes for the expression of H1/Bri in native (construct 732) and chimeric (constructs 733 and 734) forms. Construct 733, comprising PDI signal peptide and HDC, SDC and transmembrane domain complex (TDC) of H1/Bri, and construct 734 comprising an H5/Indo signal peptide, F'1, E1, E2, F'2, F, and an RB from H1/Bri. 35S pro: CaMV 35S promoter, NOS ter: nopaline synthase terminator, SP: signal peptide, RB: receptor-binding subdomain, E1-RB-E2: esterase and receptor-binding subdomains, TMD/CT: transmembrane and cytoplasmic tail subdomains, PDI: alfalfa protein disulfide isomerase; CPMV-HT: 5' and 3' elements of the hyper translatable Cowpea Mosaic Virus expression system.

[0040] Figure 9 shows immunoblot analysis of expression of H1/Bri in native form, construct 732 (comprising H1/Bri under the control of the 35SCPMV/HT-based expression cassette), construct 733 (comprising a PDI signal peptide fused with H1/Bri; under the control of the 35SCPMV/HT-based expression cassette), or construct 734 comprising an H1/Bri RB subdomain fused with an H5/Indo SDC, E1 and E2 subdomains; under the control of the 35SCPMV/HT-based expression cassette). For each construct, total protein extracts from 3 separate plants were analyzed. Five micrograms of protein were loaded for each plant analyzed. The Western blot was revealed with anti-HA monoclonal antibodies (FII 10-I50).

[0041] Figure 10 shows schematic representation of 35SCPMV/HT-based expression cassettes for the expression of H3 A/Brisbane/10/2007 HA (H3/Bri) and B/Florida/4/2006 HA (B/Flo) hemagglutinins. Construct 736 comprises H3/Bri fused to a PDI signal peptide. Construct 737 comprises the H3/Bri fused to a PDI signal peptide and an H5/Indo TMD/CT. Construct 739 comprises B/Flo fused to a PDI signal peptide. Construct 745 comprises the B/Flo fused to a PDI signal peptide and an H5/Indo TMD/CT. 35S pro: CaMV 35S promoter, NOS ter: nopaline synthase terminator, SP: signal peptide, RB: receptor-binding subdomain, E1-RB-E2: esterase and receptor-binding subdomains, TMD/CT: transmembrane and cytoplasmic tail subdomains, PDI: alfalfa protein disulfide isomerase; CPMV-HT: 5' and 3' elements of the hyper translatable Cowpea Mosaic Virus expression system.

[0042] Figure 11 shows the fusion border in constructs number 745 and 737. Origin of HA sequence is indicated by bullet-ended arrows. Amino acids of the transmembrane domain are QILSIYSTVA, and are preceded by amino acids that are part of the ectodomain.

[0043] Figure 12 shows amino acid sequence of the chimeric H5/H3 hemagglutinin (SEQ. ID. NO. 83; construct 737) comprising a PDI signal peptide, an ectodomain of H3 A/Brisbane/10/2007 and a TMD/CT of H5 A/Indonesia/5/2005.

[0044] Figure 13 shows the amino acid sequence of the chimeric H5/B hemagglutinin (SEQ. ID. NO. 84) comprising an ectodomain of B/Florida/4/2006 and a TMD/CT of H5 A/Indonesia/5/2005 encoded by the open reading frame in construct number 745.

[0045] Figure 14 shows immunoblot analysis of expression of B/Flo in native form, construct 739 (comprising PDI-B/Flo), or with construct 745 (comprising B/Flo HDC and SDC fused with an H5/Indo TDC). For each construct, total protein extracts from 3 separate plants were analyzed. Twenty micrograms of protein were loaded for each plant analyzed. The Western blot was revealed with anti-HA B/Florida polyclonal antibodies (NIBSC 07/356).

[0046] Figure 15 shows immunoblot analysis of expression of H3/Bri in native form, construct 736 (comprising PDI sp - H3/Bri), or with construct 737 (H3/Bri HDC and SCD fused with an H5/Indo TDC). For each construct, total protein extracts from 3 separate plants were analyzed. Twenty micrograms of protein were loaded for each plant analyzed. The Western blot was revealed with anti-H3 Brisbane polyclonal antibodies (NIBSC 08/124).

[0047] Figure 16 shows size exclusion chromatography of leaf protein extracts from plants infiltrated with construct number 745. Relative protein content of elution fractions is presented for each fraction. Immunodetection (Western blot) of hemagglutinin using anti-HA B/Florida polyclonal antibodies (NIBSC 07/356) in fractions 7 to 15 is presented under the graph. The elution peak of Blue Dextran 2000 is indicated by the arrow (fraction 8).

[0048] Figure 17 shows the nucleic acid sequence (SEQ ID NO: 52) of the synthesized fragment comprising the complete H5 (A/Indonesia/5/05 (H5N1)) coding region (including the signal peptide and the stop codon) flanked, in 5' by a HindIII site and, in 3', by a SacI site.

[0049] Figure 18 shows the nucleic acid sequence (SEQ ID NO: 53) of construct 660, an HA expression cassette comprising an alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H5 form A/Indonesia/5/05 (H5N1), alfalfa plastocyanin 3' UTR and terminator sequences.

[0050] Figure 19 shows the nucleic acid sequence (SEQ ID NO: 54) of the wild-type H1 (A/New Caledonia/20/99 (H1N1) (GenBank acc. no. AY289929) coding sequence without a TmD and Ctail.

[0051] Figure 20 shows the nucleic acid sequence (SEQ ID NO: 55) of a synthesized fragment comprising H1 (A/New Caledonia/20/99 (H1N1) coding sequence lacking TmD and Ctail. In the 5' region, the last nucleotides originate from PDI SP and include a BglII restriction site and in 3', a dual SacI/StuI site is found immediately downstream of the stop codon.

5 [0052] Figure 21 shows the nucleic acid sequence (SEQ ID NO: 56) of the synthesized fragment comprising the C-ter H1 (A/New Caledonia/20/99 (H1N1) coding sequence including the TmD and Ctail from the KpnI site to the stop codon (flanked in 3' by a dual SacI/StuI site).

10 [0053] Figure 22 shows the nucleotide sequence for *Medicago sativa* mRNA for protein disulfide isomerase. GenBank Accession No. Z11499 (SEQ ID NO: 57). Nucleotides 32-103 encode the PDI signal peptide.

[0054] Figure 23 shows the nucleotide sequence for PromPlasto-PDISP-Plasto 3'UTR plasmid. Figure 23A shows the nucleotide sequence for PromPlasto-PDISP (SEQ ID NO:58). Figure 23B shows the nucleotide sequence from Plasto 3'UTR (SEQ ID NO:85). Protein
15 disulfide isomerase (PDI) signal peptide is underlined. BglII (AGATCT) and SacI (GAGCTC) restriction sites used for cloning are shown in bold.

[0055] Figure 24 shows the nucleic acid sequence (SEQ ID NO: 59; construct 540) of the an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, coding
20 sequence of the signal peptide from PDI and of H1 from A/New Caledonia/20/99 (H1N1), alfalfa plastocyanin 3' UTR and terminator sequences. H1 from A/New Caledonia/20/1999 coding sequence is underlined.

[0056] Figure 25 shows the nucleic acid sequence (SEQ ID NO: 60) of the synthesized
25 fragment comprising the complete H1 (A/Brisbane/59/07 (H1N1)) coding region (including the signal peptide and the stop codon) flanked, in 5' by alfalfa plastocyanin gene sequences corresponding to the first 84 nucleotides upstream of the initial ATG, starting with a DraIII site and, in 3', by a SacI site.

[0057] Figure 26 shows the nucleic acid sequence (SEQ ID NO: 61; construct 774) of the an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H1 form A/Brisbane/59/07 (H1N1), alfalfa plastocyanin 3' UTR and terminator sequences.

- 5 [0058] Figure 27 shows the nucleic acid sequence of expression cassette number 828 (SEQ ID NO: 62), from PacI (upstream of the promoter) to AscI (immediately downstream of the NOS terminator). CPMV HT 3' UTR sequencer underlined with mutated ATG in bold. ApaI restriction site underlined and italic.

- 10 [0059] Figure 28 shows the nucleic acid sequence (SEQ ID NO: 63; construct 690) of a chimeric H5/H1 expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, chimeric hemagglutinin coding sequence, alfalfa plastocyanin 3' UTR and terminator sequences. Chimeric HA coding sequence is underlined.

- 15 [0060] Figure 29 shows the nucleic acid sequence (SEQ ID NO: 64; construct 691) of a chimeric H5/H1 expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, chimeric hemagglutinin coding sequence, alfalfa plastocyanin 3' UTR and terminator sequences. Chimeric HA coding sequence is underlined.

- 20 [0061] Figure 30 shows the nucleic acid sequence (SEQ ID NO: 65; construct 696) of a chimeric H1/H5 expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, chimeric hemagglutinin coding sequence, alfalfa plastocyanin 3' UTR and terminator sequences. Chimeric HA coding sequence is underlined.

[0062] Figure 31 shows the nucleic acid sequence (SEQ ID NO: 66; construct 732) of the an HA expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, hemagglutinin coding sequence of H1 form A/Brisbane/59/07 (H1N1), CPMV-HT 3' UTR and NOS terminator sequences. Coding sequence of H1/Bri is underlined.

- 25 [0063] Figure 32 shows the nucleic acid sequence (SEQ ID NO: 67) of the coding sequence, from ATG to stop, of intermediate construct number 787.

[0064] Figure 33 shows the nucleic acid sequence (SEQ ID NO: 68; construct no. 733) of SpPDI H1/Bri expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, coding sequence of the signal peptide from PDI, hemagglutinin coding sequence of H1 form A/Brisbane/59/07 (H1N1), CPMV-HT 3' UTR and NOS terminator sequences. Coding sequence of SpPDI H1/Bri is underlined.

[0065] Figure 34 shows the nucleic acid sequence (SEQ ID NO: 69; construct 734) of a chimeric H5/H1 expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, chimeric hemagglutinin coding sequence, CPMV-HT 3' UTR and NOS terminator sequences. Coding sequence of chimeric HA is underlined.

[0066] Figure 35 shows the nucleic acid sequence (SEQ ID NO: 70) of the synthesized fragment comprising the complete H3 (A/Brisbane/10/07 (H3N2)) coding region (including the signal peptide and the stop codon) flanked, in 5' by alfalfa plastocyanin gene sequences corresponding to the first 84 nucleotides upstream of the initial ATG, starting with a DraIII site and, in 3', by a SacI site.

[0067] Figure 36 shows the nucleic acid sequence (SEQ ID NO: 71; construct 736) of the an HA expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, coding sequence of the signal peptide from PDI, hemagglutinin coding sequence of H3 form A/Brisbane/10/07 (H2N3), CPMV-HT 3' UTR and NOS terminator sequences. Coding sequence of Sp PDI H3/Bris is underlined.

[0068] Figure 37 shows the nucleic acid sequence (SEQ ID NO: 72; construct no. 737) of a chimeric H5/H3 expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, chimeric hemagglutinin coding sequence, CPMV-HT 3' UTR and NOS terminator sequences. Coding sequence of chimeric HA is underlined.

[0069] Figure 38 shows the nucleic acid sequence (SEQ ID NO: 73) of the synthesized fragment comprising the complete HA (B/Florida/4/06) coding region (including the signal peptide and the stop codon) flanked, in 5' by alfalfa plastocyanin gene sequences

corresponding to the first 84 nucleotides upstream of the initial ATG, starting with a DraIII site and, in 3', by a SacI site.

[0070] Figure 39 shows the nucleic acid sequence (SEQ ID NO: 74; construct 739) of the an HA expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, coding
5 sequence of the signal peptide from PDI, hemagglutinin coding sequence of HA form B/Florida/4/06, CPMV-HT 3' UTR and NOS terminator sequences. Coding sequence of Sp PDI B/Flo is underlined.

[0071] Figure 40 shows the nucleic acid sequence (SEQ ID NO: 75; construct 745) of a chimeric H5/B expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR,
10 chimeric hemagglutinin coding sequence, CPMV-HT 3' UTR and NOS terminator sequences. Coding sequence of chimeric HA is underlined.

[0072] Figure 41 shows the nucleic acid sequence encoding Msj1 (SEQ ID NO: 76).

[0073] Figure 42 shows the nucleic acid sequence (SEQ ID NO: 77) of a portion of construct number R850, from HindIII (in the multiple cloning site, upstream of the promoter) to EcoRI
15 (immediately downstream of the NOS terminator). HSP40 coding sequence is underlined.

[0074] Figure 43 shows the nucleic acid sequence (SEQ ID NO: 78) of a portion of construct number R860, from HindIII (in the multiple cloning site, upstream of the promoter) to EcoRI (immediately downstream of the NOS terminator). The HSP70 coding sequence is underlined.

[0075] Figure 44 shows the nucleic acid sequence (SEQ ID NO: 79) of a portion of construct
20 number R870, from HindIII (in the multiple cloning site, 5 upstream of the promoter) to EcoRI (immediately downstream of the NOS terminator). The HSP40 coding sequence is in underlined italic and the HSP70 coding sequence is underlined. A) nucleotides 1-4946; B) nucleotides 4947-9493.

[0076] Figure 45 shows a schematic representation of construct number R472.

[0077] Figure 46 shows the disulfide bridge pattern of influenza type A. Bridge numbering: 1) Cys4HA1-Cys137HA2, 2) Cys60HA1-Cys72HA1, 3) Cys94HA1-Cys143HA1, 4) Cys292HA1-Cys318HA1 5) Cys144HA2-Cys148HA2 and 6) Cys52HA1-Cys277HA1. The disulfide bridges that differ between A and B (Figure 47) subtypes are indicated with arrows.

5 The numbering from mature H3 protein was used.

[0078] Figure 47 shows the disulfide bridge pattern of influenza type B HA. Bridge numbering: 1) Cys4HA1-Cys137HA2, 2) Cys60HA1-Cys72HA1, 3) Cys94HA1-Cys143HA1, 4) Cys292HA1-Cys318HA1 5) Cys144HA2-Cys148HA2, 6) Cys52HA1-Cys277HA1, 7) Cys54HA1-Cys57HA1 and 8) Cys178HA1-Cys272HA1. The disulfide bridges that differ between A (Figure 46) and B subtypes are indicated with arrows. The numbering from mature H3 protein was used.

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[0079] Figure 48 shows a schematic diagram of domain swap fusion junctions. Figure 48A shows the fusion of RB subdomain from H1/Bri, H3/Bri, and B/Flo with H5/Indo SDC's, and the RB subdomain of H5/Indo with H1/NC stem domain. Figure 48B shows the fusion of E1-RB-E2 subdomains (HDC) from H1/Bri, H3/Bri or B/Flo with H5/Indo SDC, and of H5/Indo HDC withn H1/NC SDC.

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[0080] Figure 49A shows the nucleotide sequence SEQ ID NO: 86) of H1 A/California/04/09. Alfalfa protein disulfide isomerase signal peptide coding sequence is underlined and mature H1 coding sequence is highlighted in bold. Figure 49B shows the amino acid sequence (SEQ ID NO: 87) of the H1 A/California/04/09. Alfalfa protein disulfide isomerase signal peptide is underlined.

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[0081] Figure 50 shows an immunoblot analysis of expression of H5/B chimeric hemagglutinin (construct number 747; comprising B/Flo HDC and SDC fused with an H5/Indo TDC) after infiltration of AGL1/747 undiluted, co-infiltrated with AGL1/443 (empty vector) and co-infiltrated with AGL1/R870 (HSP40/HSP70). For each construct, total protein extracts from 3 separate plants were analyzed. Twenty micrograms of proteins were loaded for

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each plant analyzed. The Western blot was revealed with anti-B Florida polyclonal antibodies (NIBSC).

[0082] Figure 51A shows the nucleotide sequence for the 2X35S promoter sequence (SEQ ID NO:88). Figure 51B shows the nucleotide sequence for construct 747 (SEQ ID NO:93) from
5 PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator). Coding sequence of chimeric HA is underlined. 2X35S promoter sequence is indicated in italics.

DETAILED DESCRIPTION

[0083] The present invention relates to virus-like particles. More specifically, the present
10 invention is directed to virus-like particles comprising chimeric influenza hemagglutinin, and methods of producing chimeric influenza virus-like particles.

[0084] The following description is of a preferred embodiment.

[0085] The present invention provides a nucleic acid comprising a nucleotide sequence encoding a chimeric influenza hemagglutinin (HA) operatively linked to a regulatory region
15 active in a plant.

[0086] Furthermore, the present invention provides a method of producing virus like particles (VLPs) in a plant. The method involves introducing a nucleic acid encoding a chimeric influenza HA operatively linked to a regulatory region active in the plant, into the plant, or portion of the plant, and incubating the plant or a portion of the plant under conditions that
20 permit the expression of the nucleic acid, thereby producing the VLPs.

[0087] The present invention further provides for a VLP comprising a chimeric influenza HA. The VLP may be produced by the method as provided by the present invention.

[0088] By "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
25 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 polypeptide 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 (see Figures 46 and 47). 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".

[0089] The chimeric influenza HA according to various embodiments of the present invention may comprise a stem domain complex (SDC) a head domain complex (HDC) and a transmembrane domain complex (TDC), where one or more than one subdomain of either the SDC, HDC or TDC is of a first influenza HA type, subtype or from one origin, and one or more than one subdomain of either the SDC, HDC or TDC is from a second influenza HA type, subtype, or from a second or different origin. As described herein, the "SDC" comprises an F'1, F'2 and F subdomain, the "HDC" comprises an RB, E1 and E2 subdomain, the "TDC" comprises a TmD and Ctail subdomain (TMD/CT; see Figures 1A, 46 and 47).

[0090] The term "virus like particle" (VLP), or "virus-like particles" or "VLPs" refers to structures that self-assemble and comprise structural proteins such as influenza HA protein, or chimeric influenza HA protein. VLPs and chimeric 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. VLPs and chimeric VLPs may be produced

in suitable host cells including plant host cells. Following extraction from the host cell and upon isolation and further purification under suitable conditions, VLPs and chimeric VLPs may be purified as intact structures.

[0091] Chimeric VLPs, or VLPs, produced from influenza derived proteins, in accordance
5 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 VLP preparation. The presence of RNA is undesired when obtaining regulatory approval for the chimeric VLP product, therefore a chimeric VLP preparation lacking RNA may be advantageous.

[0092] The chimeric VLPs of the present invention may be produced in a host cell that is
10 characterized by lacking the ability to sialylate proteins, for example a plant cell, an insect cell, fungi, and other organisms including sponge, coelenterata, annelida, arthropoda, mollusca, nemathelminthea, trochelmintes, plathelminthes, chaetognatha, tentaculate, chlamydia, spirochetes, gram-positive bacteria, cyanobacteria, archaebacteria, or the like. See, for example Gupta et al., 1999. Nucleic Acids Research 27:370-372; Toukach et al., 2007.
15 Nucleic Acids Research 35:D280-D286; Nakahara et al., 2008. Nucleic Acids Research 36:D368-D371. The chimeric VLPs produced as described herein do not typically comprise neuraminidase (NA). However, NA may be co-expressed with HA should VLPs comprising HA and NA be desired.

[0093] The invention also provides VLPs comprising chimeric HA that obtain a lipid
20 envelope from the plasma membrane of the cell in which the chimeric HA are expressed. For example, if the chimeric HA is expressed in a plant-based system, the resulting VLP may obtain a lipid envelope from the plasma membrane of the plant cell.

[0094] Generally, the term "lipid" refers to a fat-soluble (lipophilic), naturally-occurring
25 molecules. A chimeric VLP produced in a plant according to some aspects of the invention may be complexed with plant-derived lipids. The plant-derived lipids may be in the form of a lipid bilayer, and may further comprise an envelope surrounding the VLP. The plant derived lipids may comprise lipid components of the plasma membrane of the plant where the VLP is

produced, including phospholipids, tri-, di- and monoglycerides, as well as fat-soluble sterol or metabolites comprising sterols. Examples include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol, phosphatidylserine, glycosphingolipids, phytosterols or a combination thereof. A plant-derived lipid may alternately be referred to as a 'plant lipid'. Examples of phytosterols include campesterol, stigmasterol, ergosterol, brassicasterol, delta-7-stigmasterol, delta-7-avenasterol, daunosterol, sitosterol, 24-methylcholesterol, cholesterol or beta-sitosterol – see, for example, Mongrand et al., 2004. 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, or species, from which the cell is obtained. Generally, beta-sitosterol is the most abundant phytosterol.

[0095] 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'. These lipid raft microdomains may be enriched in sphingolipids and sterols. 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.

[0096] The invention includes VLPs comprising chimeric HA, of which the subdomains may be obtained from any type, subtype of influenza virus which may infect humans, including, for example, B, H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16 types or subtypes. In some embodiments, the influenza virus may be of an H1, H3, H5 or B types or subtypes. Non limiting examples of H1, H3, H5 or B types or subtypes include the A/New Caledonia/20/99 subtype (H1N1) ("H1/NC"; SEQ ID NO:56), the H1 A/California 04/09 subtype (H1N1) ("H1/Cal"; SEQ ID NO:86), the A/Indonesia/5/05 sub-type (H5N1) ("H5/Indo"), A/Brisbane/59/2007 ("H1/Bri"), and B/Florida/4/2006 ("B/Flo") and H3 A/Brisbane/10/2007 ("H3/Bri"). Furthermore, the chimeric HA may comprise one or more subdomains of a hemagglutinin that is isolated from one or more emerging or newly-identified influenza viruses.

[0097] The present invention also pertains to influenza viruses which infect other mammals or host animals, for example humans, primates, horses, pigs, birds, avian water fowl, migratory birds, quail, duck, geese, poultry, chicken, camel, canine, dogs, feline, cats, tiger, leopard, civet, mink, stone marten, ferrets, house pets, livestock, mice, rats, seal, whale and the like.

5 Some influenza viruses may infect more than one host animal.

[0098] With reference to influenza virus, the term “hemagglutinin” or “HA” as used herein refers to a structural glycoprotein of influenza viral particles. The structure of influenza hemagglutinin is well-studied and demonstrates a high degree of conservation in secondary, tertiary and quaternary structure. This structural conservation is observed even though the amino acid sequence may vary (see, for example, Skehel and Wiley, 2000 *Ann Rev Biochem* 69:531-69; Vaccaro et al 2005; which is incorporated herein by reference). Nucleotide sequences encoding HA are well known, and are available for example, from the BioDefense and Public Health Database (for example at URL: biohealthbase.org/GSearch/home.do?decorator=Influenza) or the databases maintained by the National Center for Biotechnology Information (NCBI; for example at URL: ncbi.nlm.nih.gov/sites/entrez?db=nuccore&cmd=search&term=influenza) both of which are incorporated herein by reference.

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[0099] The HA monomer may be subdivided in three functional domains - a stem domain, or stem domain cluster (SDC), a globular head domain, or head domain cluster (HDC) and a transmembrane domain cluster (TDC). The SDC comprises four subdomains, a fusion peptide F, F'1 and F'2 (this subdomain may be generally referred to as a ‘backbone’). The TDC comprises two subdomains, the transmembrane (TmD) and a C terminal tail (CT). The HDC comprises three subdomains, vestigial esterase domains E1' and E2, and a receptor binding domain RB. The SDC and HDC may be collectively referred to as the ‘ectodomain’. A publication by Ha et al. 2002 (*EMBO J.* 21:865-875; which is incorporated herein by reference) illustrates the relative orientation of the various subdomains of the SDC and HDC in several influenza subtypes, based on Xray crystallographic structures. A schematic diagram of the subdomains relative to N and C termini of the HA1 and HA2 polypeptides is shown in Figure 1A. An annotated structural alignment of various influenza subtypes is provided in Figure 1C.

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[00100] Amino acid variation is tolerated in hemagglutinins of influenza viruses. This variation provides for new strains that are continually being identified. Infectivity between the new strains may vary. However, formation of hemagglutinin trimers, which subsequently form VLPs is maintained. The present invention, therefore, provides for a hemagglutinin amino acid sequence comprising chimeric HA, or a nucleic acid encoding a chimeric hemagglutinin amino acid sequence, that forms VLPs in a plant, and includes known sequences and variant HA sequences that may develop. The present invention also pertains to the use of a chimeric HA polypeptide comprising a TDC, SDC and HDC. For example the chimeric HA protein may be HA0, or a cleaved chimeric HA comprising subdomains of HA1 and HA2 from two or more influenza types. The chimeric HA protein may be used in the production or formation of VLPs using a plant, or plant cell, expression system.

[00101] HA0 may be expressed and folded to form a trimer, which may subsequently assemble into VLPs. Cleavage of HA0 yields HA1 and HA2 polypeptides linked by a disulfide bridge (see Figures 1C, 46 and 47 for illustration of disulfide bridge patterns). For an infectious virus particle, cleavage of precursor HA0 is required to trigger the conformational change of HA2 that release the fusion peptide (at the N terminus of the HA2 polypeptide) and make it available for fusion of the cell and viral membranes. However, VLPs are not infectious, and cleavage of the HA into HA1 and HA2 is not required, for example, for vaccine production. Uncleaved HA0 precursor also assembles in trimers and bud from plasma membrane to form VLP nanoparticles.

[00102] The HA0 polypeptide comprises several domains. The RB subdomain of the HDC comprises several loops in antigenic regions designated as site A-E. Antibodies that may neutralize infectious influenza virus are frequently targeted to one or more of these sites. The vestigial esterase subdomains (E1 and E2) may have a role in fusion, and may bind Ca⁺⁺. The F, F'1 and F'2 domains interact and cooperate to form a stem, raising the head of the HA trimer above the membrane. A TmD and CT may be involved in anchoring of the folded HA to a membrane. The TmD may have a role in affinity of HA for lipid rafts, while the CT may have a role in secretion of HA, while some of the cysteine residues found in the CT subdomain may be palmitoylated. A signal peptide (SP) may also be found at the N terminus

of the HA0 polypeptide. Figure 2, and Tables 4 and 5 provide examples of the amino acid sequences of SP, F'1, F'2, E1, RB, E2 and F domains of some influenza virus subtypes.

[00103] Processing of an N-terminal signal peptide (SP) sequence during expression and/or secretion of influenza hemagglutinins may have a role in the folding of the HA. The term "signal peptide" refers generally to a short (about 5-30 amino acids) sequence of amino acids, found generally at the N-terminus of a hemagglutinin 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. The signal peptide of hemagglutinins target the translocation of the protein into the endoplasmic reticulum and have been proposed to aid in positioning of the N-terminus proximal domain relative to a membrane-anchor domain of the nascent hemagglutinin polypeptide to aid in cleavage and folding of the mature hemagglutinin.

[00104] Insertion of HA within the endoplasmic reticulum (ER) membrane of the host cell, signal peptide cleavage and protein glycosylation are co-translational events. Correct folding of HA requires glycosylation of the protein and formation of at least 6 intra-chain disulfide bonds (see Figures 46 and 47). In Figure 46, the HA from subtype A is shown to have 6 conserved disulfide bridges per monomer. By comparison, the monomer of B HA (Figure 47) has seven disulfide bridges, and five of these disulfide bridges have a counterpart in A (reviewed in Skehel and Wiley, 2000. *Ann Rev Biochem.* 69:531-569; examples of structures illustrating intra- and intermolecular disulfide bridges and other conserved amino acids and their relative positions are described in, for example, Gamblin et al 2004, *Science* 303:1838-1842; both of which are incorporated herein by reference). As one of skill in the art would realize, it is important to ensure a similar arrangement of disulfide bridges is obtained when preparing chimeric HAs.

[00105] A signal peptide may be native to the hemagglutinin, or a signal peptide may be heterologous with respect to the primary sequence of hemagglutinin being expressed. A chimeric HA may comprise a signal peptide from a first influenza type, subtype or strain with the balance of the HA from one or more than one different influenza type, subtype or strain.

For example the native SP of HA subtypes B H1, H2, H3, H5, H6, H7, H9 or influenza type B may be used to express the HA in a plant system. In some embodiments of the invention, the SP may be of an influenza type B, H1, H3 or H5; or of the subtype H1/Bri, H1/NC, H5/Indo, H3/Bri or B/Flo.

- 5 [00106] A SP may also be non-native, for example, from a structural protein or hemagglutinin of a virus other than influenza, 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 (PDI SP; nucleotides 32-103 of Accession No. Z11499; SEQ ID NO: 34; Figure 17) having the amino acid sequence:

10 MAKNV AIFGLLFSLLLLVP SQIF AEE (nucleotides 32-103; SEQ ID NO: 34)

The present invention therefore provides for a chimeric influenza hemagglutinin comprising a native, or a non-native signal peptide, and nucleic acids encoding such chimeric hemagglutinins.

- [00107] Correct folding of the hemagglutinins may be important for stability of the protein, formation of multimers, formation of VLPs and function of the HA (ability to hemagglutinate), among other characteristics of influenza hemagglutinins. Folding 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 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.
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- [00108] 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, FKBP, cyclophilins, ClpP, GrpE, ubiquitin, calnexin, and protein disulfide isomerases (see, for example, Macario,
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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 chimeric HA.

5 [00109] 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 Hsp71). 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
10 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.

[00110] Examples of Hsp40 include DnaJ from prokaryotes such as *E. coli* and
15 mycobacteria and HSPJ1, HDJ1 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 (AJ000995.1 or SEQ ID NO: 76). Hsp40 plays a role as a molecular chaperone in protein folding, thermotolerance and DNA replication, among other cellular activities. Figure 41 shows the nucleic acid sequence encoding Msj1 (SEQ ID NO: 76).

20 [00111] 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
25 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).

[00112] 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 hemagglutinin in the agroinfiltrated leaves may lead to the accumulation of hemagglutinin polypeptides 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 hemagglutinin polypeptides, and increase the number of polypeptides exhibiting tertiary and quaternary structural characteristics that allow for hemagglutination and/or formation of virus-like particles. SEQ ID NO: 77 is a nucleic acid sequence of a portion of construct number R850, from HindIII (in the multiple cloning site, upstream of the promoter) to EcoRI (immediately downstream of the NOS terminator), encoding HSP40 (underlined). SEQ ID NO: 78 is a nucleic acid sequence of a portion of construct number R860, from HindIII (in the multiple cloning site, upstream of the promoter) to EcoRI (immediately downstream of the NOS terminator), encoding HSP70 (underlined). SEQ ID NO: 79 is a nucleic acid sequence of a portion of construct number R870, from HindIII (in the multiple cloning site, 5 upstream of the promoter) to EcoRI (immediately downstream of the NOS terminator) encoding HSP40 (underlined italic) and HSP70 (underlined).

[00113] Therefore, the present invention also provides for a method of producing chimeric influenza VLPs in a plant, wherein a first nucleic acid encoding a chimeric influenza HA is co-expressed with a second nucleic acid encoding a chaperone. The first and second nucleic acids may be introduced to the plant in the same step, or may be introduced to the plant sequentially.

[00114] VLPs may be assessed for structure and size by, for example, hemagglutination assay, electron microscopy, or by size exclusion chromatography.

[00115] 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 also be of benefit. The soluble protein is quantified, and the extract passed through a

SephacrylTM column. Blue Dextran 2000 may be used as a calibration standard. Following chromatography, fractions may be further analyzed by immunoblot to determine the protein complement of the fraction.

[00116] The present invention also provides for a plant comprising a nucleic acid
5 encoding one, or more than one chimeric influenza hemagglutinin and a nucleic acid encoding one or more than one chaperones.

[00117] The present invention includes nucleotide sequences:

[00118] SEQ ID NO: 63 (construct 690; a chimeric H5/H1 expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, chimeric hemagglutinin coding
10 sequence, alfalfa plastocyanin 3' UTR and terminator sequences) and the underlined portion of SEQ ID NO:63 encoding SP, F'1, E1 of H5/Indo-RB of H1/Bri-E2, F'2, F, TMD/CT of H5/Indo;

[00119] SEQ ID NO: 64 (construct 691; a chimeric H5/H1 expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, chimeric hemagglutinin coding
15 sequence, alfalfa plastocyanin 3' UTR and terminator sequences), and the underlined portion of SEQ ID NO:64, encoding SP, F'1, of H5/Indo-E1, RB.E2 of H1/Bri-F'2, F, TMD/CT of H5/Indo;

[00120] SEQ ID NO: 65 (construct 696;a chimeric H1/H5 expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, chimeric hemagglutinin coding
20 sequence, alfalfa plastocyanin 3' UTR and terminator sequences)and the underlined portion of SEQ ID NO:65 encoding PDI SP-F'1, E1 of H1/NC-RB of H5/Indo-E2, F'2, F, TMD/CT of H1/NC;

[00121] SEQ ID NO: 68 (construct 733; the SpPDI H1/Bri expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, coding sequence of the signal
25 peptide from PDI, hemagglutinin coding sequence of H1 form A/Brisbane/59/07 (H1N1),

CPMV-HT 3' UTR and NOS terminator sequences), and the underlined portion of SEQ ID NO:68, encoding PDI SP-F'1, E1, RB,E2, F'2, F, TMD/CT of H1/BRI;

[00122] SEQ ID NO: 69 (construct 734; a chimeric H5/H1 expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, chimeric hemagglutinin coding sequence, CPMV-HT 3' UTR and NOS terminator sequences). The coding sequence of chimeric HA is underlined, encoding the same chimieric HA as SEQ ID NO:63;

[00123] SEQ ID NO: 71 (construct 736; an HA expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, coding sequence of the signal peptide from PDI, hemagglutinin coding sequence of H3 form A/Brisbane/10/07 (H2N3), CPMV-HT 3' UTR and NOS terminator sequences), and the underlined portion of SEQ ID NO: 71 encoding PDI SP-F'1, E1, RB,E2, F'2, F, TMD/CT of H3/Bri;

[00124] SEQ ID NO: 72 (construct 737; a chimeric H5/H3 expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, chimeric hemagglutinin coding sequence, CPMV-HT 3' UTR and NOS terminator sequences), and the underlined portion of SEQ ID NO:72 encoding PDI SP-F'1, E1, RB,E2, F'2, F, TMD/CT of H5/Indo;

[00125] SEQ ID NO: 74 (construct 739; an HA expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, coding sequence of the signal peptide from PDI, hemagglutinin coding sequence of HA form B/Florida/4/06, CPMV-HT 3' UTR and NOS terminator sequences), and the underlined portion of SEQ ID NO:74 encoding PDI SP-F'1, E1, RB,E2, F'2, F, TMD/CT of B/Flo;

[00126] SEQ ID NO: 75 (construct 734; a chimeric H5/B expression cassette comprising the CaMV 35S promoter, CPMV-HT 5' UTR, chimeric hemagglutinin coding sequence, CPMV-HT 3' UTR and NOS terminator sequences), and the underlined portion of SEQ ID NO:75 encoding PDI SP-F'1, E1, RB,E2, F'2, F of B/Flo-TND/CT of H5/Indo.

[00127] The present invention also includes a nucleotide sequence that hybridizes under stringent hybridisation conditions to the underlined portions of any one of SEQ ID

NOs:63-65, 68, 69, and 71-75. The present invention also includes a nucleotide sequence that hybridizes under stringent hybridisation conditions to a complement of the underlined portions of any one of SEQ ID NOs:63-65, 68, 69, and 71-75. These nucleotide sequences that hybridize to the underlined portions of SEQ ID NOs:63-65, 68, 69, and 71-75, or a complement of the underlined portions of SEQ ID NOs:63-65, 68, 69, and 71-75, encode a chimeric hemagglutinin protein that, when expressed forms a chimeric VLP, and the chimeric VLP induces production of an antibody when administered to a subject. For example, expression of the nucleotide sequence within a plant cell forms a chimeric VLP, and the chimeric VLP may be used to produce an antibody that is capable of binding HA, including mature HA, HA0, HA1, or HA2 of one or more influenza types or subtypes. The chimeric VLP, when administered to a subject, induces an immune response.

[00128] 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 65°C, followed by washing in 0.1 X SSC at 65°C 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 42°C, followed by washing in 0.1 X SSC at 65°C 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 NaPO₄ buffer pH 7.2; 10 mM EDTA) at 65°C, with 2 washes either at 50°C in 0.1 X SSC, 0.1% SDS for 20 or 30 minutes each, or 2 washes at 65°C in 2 X SSC, 0.1% SDS for 20 or 30 minutes each.

[00129] 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 chimeric HA according to the underlined portions of any one of SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 75, wherein the nucleotide sequence encodes a hemagglutinin protein that when expressed forms a chimeric VLP, and that the chimeric VLP induces the production of an antibody. For example, expression of the nucleotide sequence within a plant cell forms a chimeric VLP, and the chimeric VLP may be used to produce an antibody that is capable of binding HA, including mature HA, HA0, HA1, or HA2. The VLP, when administered to a subject, induces an immune response.

[00130] 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.

[00131] 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.

[00132] 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.

5 [00133] A hemagglutination inhibition (HI, or HAI) assay may also be used to demonstrate the efficacy of antibodies induced by a vaccine, or vaccine composition comprising chimeric HA or chimeric VLP can inhibit the agglutination of red blood cells (RBC) by recombinant HA. Hemagglutination inhibitory antibody titers of serum samples may be evaluated by microtiter HAI (Aymard et al 1973). Erythrocytes from any of several species
10 may be used – e.g. horse, turkey, chicken or the like. This assay gives indirect information on assembly of the HA trimer on the surface of VLP, confirming the proper presentation of antigenic sites on HAs.

[00134] 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
15 from a subject immunized with a vaccine composition comprising a chimeric hemagglutinin comprising an HDC of a first influenza type or subtype may be used in an HAI assay with a second strain of whole virus or virus particles, and the HAI titer determined.

[00135] 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 bound with $\alpha 2,3$ or $\alpha 2,6$
20 linkages 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, guinea pigs, humans and sheep (Ito T. et al, 1997, Virology, 227:493-499; Medeiros R et al, 2001. Virology 289:74-85).

25 [00136] 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.

[00137] 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)

[00138] Sequence identity or sequence similarity may be determined using a 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 (Altschul et al., 1990. J. Mol Biol 215:403-410), or by manual alignment and visual inspection. Nucleic acid or amino acid sequences may be compared or aligned and consensus sequences may be determined using any of several software packages known in the art, for example MULTALIN (Corpet F., 1988, Nucl. Acids Res., 16 (22), 10881-10890), BLAST, CLUSTAL or the like; alternately sequences may be aligned manually and similarities and differences between the sequences determined.

[00139] A fragment or portion of a protein, fusion protein or polypeptide includes a peptide or polypeptide comprising a subset of the amino acid complement of a particular protein or polypeptide, provided that the fragment can form a chimeric VLP when expressed. The fragment may, for example, comprise an antigenic region, a stress-response-inducing region, or a region comprising a functional domain of the protein or polypeptide. The

fragment may also comprise a region or domain common to proteins of the same general family, or the fragment may include sufficient amino acid sequence to specifically identify the full-length protein from which it is derived.

[00140] For example, a fragment or portion may comprise from about 60% to about 100%, of the length of the full length of the protein, or any amount therebetween, provided that the fragment can form a chimeric VLP when expressed. For example, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 95% to about 100%, of the length of the full length of the protein, or any amount therebetween. Alternately, a fragment or portion may be from about 150 to about 500 amino acids, or any amount therebetween, depending upon the chimeric HA, and provided that the fragment can form a chimeric VLP when expressed. For example, a fragment may be from 150 to about 500 amino acids, or any amount therebetween, from about 200 to about 500 amino acids, or any amount therebetween, from about 250 to about 500 amino acids, or any amount therebetween, from about 300 to about 500 or any amount therebetween, from about 350 to about 500 amino acids, or any amount therebetween, from about 400 to about 500 or any amount therebetween, from about 450 to about 500 or any amount therebetween, depending upon the chimeric HA, and provided that the fragment can form a chimeric VLP when expressed. For example, about 5, 10, 20, 30, 40 or 50 amino acids, or any amount therebetween may be removed from the C terminus, the N terminus or both the N and C terminus of a chimeric HA protein, provided that the fragment can form a chimeric VLP when expressed.

[00141] Numbering of amino acids in any given sequence are relative to the particular sequence, however one of skill can readily determine the 'equivalency' of a particular amino acid in a sequence based on structure and/or sequence. For example, if 6 N terminal amino acids were removed, this would change the specific numerical identity of the amino acid (e.g. relative to the full length of the protein), but would not alter the relative position of the amino acid in the structure.

[00142] The present invention describes, but is not limited to, expression of a nucleic acid encoding a chimeric HA in a plant portion of a plant, or a plant cell, and the production of chimeric influenza VLPs from the plant, suitable for vaccine production. Examples of such nucleic acids include, for example, but are not limited to, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 75.

[00143] The present invention further provides expression of a nucleic acid encoding a chimeric HA, for example but not limited to SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 75 in a plant, a portion of a plant, or a plant cell, and production of influenza vaccine candidates or reagents comprised of recombinant influenza structural proteins that self-assemble into functional and immunogenic homotypic macromolecular protein structures, including subviral influenza particles and chimeric influenza VLP, in transformed plant cells.

[00144] Therefore, the invention provides for chimeric VLPs, and a method for producing chimeric VLPs in a plant expression system, from the expression of a single chimeric envelope protein.

[00145] The nucleic acid encoding the chimeric HA of influenza subtypes, for example SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 75 may be synthesized by reverse transcription and polymerase chain reaction (PCR) using HA RNA. As an example, the RNA may be isolated from H1/NC, H1/Bri, H3/Bri, B/Flo or H5/Indo, or from cells infected with these or other influenza virus types or subtypes. For reverse transcription and PCR, oligonucleotide primers specific for the HA RNA may be used. Additionally, a nucleic acid encoding a chimeric HA may be chemically synthesized using methods as would be known to one of skill in the art.

[00146] The present invention is further directed to a gene construct comprising a nucleic acid encoding a chimeric HA, 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
5 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).

[00147] The gene construct of the present invention may also comprise a constitutive
10 promoter that directs the expression of a gene that is operatively linked to the promoter throughout the various parts of a plant and continuously throughout plant development. A non-limiting example of a constitutive promoter is that associated with the CaMV 35S transcript (e.g. Odell et al., 1985, *Nature*, **313**: 810-812, which is incorporated by reference).

[00148] An example of a sequence comprising a plastocyanin regulatory region is the
15 sequence 5' to the underlined sequence encoding a PDI signal peptide of SEQ ID NO: 58. A regulatory element or regulatory region may enhance translation of a nucleotide sequence to which it is operatively linked, where the nucleotide sequence may encode a protein or polypeptide. Another example of a regulatory region, is that derived from the untranslated regions of the Cowpea Mosaic Virus (CPMV), which may be used to preferentially translate
20 the nucleotide sequence to which it is operatively linked. This CPMV regulatory region is exploited in a hyper-translatable CPMV system (CPMV-HT; see, for example, Sainsbury et al, 2008, *Plant Physiology* 148: 1212-1218; Sainsbury et al., 2008 *Plant Biotechnology Journal* 6:82-92; both of which are incorporated herein by reference).

[00149] Therefore, an aspect of the invention provides for a nucleic acid comprising a
25 regulatory region operatively linked to a sequence encoding a chimeric influenza HA. The regulatory region may be a plastocyanin regulatory element, and the chimeric influenza HA may comprise subdomains from H5/Indo, H1/Bri, H3/Bri, H1/NC, B/Flo influenza types, subtypes or strains. Nucleic acid sequences comprising a plastocyanin regulatory element and

a chimeric influenza HA are exemplified herein by SEQ ID NOs: 63 and 64. Nucleic acid sequences comprising a 35S regulatory element and a chimeric influenza HA are exemplified herein by SEQ ID NOs: 68, 69 and 71-75.

[00150] In another aspect, the invention provides for a nucleic acid comprising a
5 CPMV regulatory region and a chimeric influenza HA, comprising subdomains from H5/Indo, H1/Bri, H3/Bri, H1/NC, B/Flo influenza types, subtypes or strains. Nucleic acid sequences comprising a CPMP regulatory element and a chimeric HA are exemplified herein by SEQ ID NOs: 66-69 and 71-75.

[00151] Plant-produced chimeric influenza VLPs bud from the plasma membrane and
10 the lipid composition of the chimeric VLPs reflects that of the plant cell or plant tissue type from which they are produced. The VLPs produced according to the present invention comprise chimeric HA of two or more than two types or subtypes of influenza, complexed with plant derived lipids. Plant lipids can stimulate specific immune cells and enhance the immune response induced.

[00152] Plant lipids such as PC (phosphatidyl choline) and PE (phosphatidyl
15 ethanolamine), 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 (reviewed in Tsuji M., 2006 Cell Mol Life Sci 63:1889-98). CD1 molecules are structurally similar to
20 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.

[00153] The phytosterols present in an influenza VLP complexed with a lipid bilayer,
25 such as an plasma-membrane derived envelope may provide for an advantageous vaccine composition. Without wishing to be bound by theory, plant-made VLPs, including those comprising chimeric HA, 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.

[00154] Therefore, in some embodiments, the invention provides for a VLP comprising
5 a chimeric HA, complexed with a plant-derived lipid bilayer. In some embodiments the plant-derived lipid bilayer may comprise the envelope of the VLP.

[00155] The VLP produced within a plant may include a chimeric HA comprising plant-specific N-glycans. Therefore, this invention also provides for a VLP comprising a chimeric HA having plant specific N-glycans.

10 [00156] Furthermore, modification of N-glycan in plants is known (see for example WO 2008/151440; which is incorporated herein by reference) and chimeric HA having modified N-glycans may be produced. A chimeric HA comprising a modified glycosylation pattern, for example with reduced fucosylated, xylosylated, or both, fucosylated and xylosylated, N-glycans may be obtained, or chimeric HA having a modified glycosylation
15 pattern may be obtained, wherein the protein lacks fucosylation, xylosylation, or both, and comprises increased galatossylation. 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 chimeric HA when compared to a wild-type plant expressing chimeric HA.

20 [00157] For example, which is not to be considered limiting, the synthesis of chimeric HA 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
25 (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 HA. The HA may also be co-expressed along with a nucleotide sequence

encoding N-acetylglucosaminyltransferase 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.

5 [00158] Therefore the present invention also includes VLP's comprising chimeric HA having modified N-glycans.

[00159] Without wishing to be bound by theory, the presence of plant N-glycans on a chimeric HA may stimulate the immune response by promoting the binding of HA by antigen presenting cells. Stimulation of the immune response using plant N glycan has been proposed
10 by Saint-Jore-Dupas et al. (Trends Biotechnol 25: 317-23, 2007). Furthermore, the conformation of the VLP may be advantageous for the presentation of the antigen, and enhance the adjuvant effect of VLP when complexed with a plant derived lipid layer.

[00160] By "regulatory region", "regulatory element" or "promoter" it is meant a portion of nucleic acid typically, but not always, upstream of the protein coding region of a
15 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" includes promoter elements, core promoter elements
20 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, 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
25 repressors, upstream activating sequences, and mRNA instability determinants. Several of these latter elements may be located proximal to the coding region.

[00161] 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.

[00162] 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 al., 1994, Plant Cell 14: 125-130). An example of a leaf-specific promoter includes the plastocyanin promoter (see, for example SEQ ID NO: 58); US 7,125,978, which is incorporated herein by reference).

[00163] 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, I.R.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. Biol. 48, 89-108; which is incorporated by reference), steroid inducible promoter (Aoyama, T. and Chua, N.H., 1997, Plant J. 2, 397-404; which is incorporated by reference) and ethanol-inducible promoter (Salter, M.G., et al, 1998, Plant Journal 16, 127-132; Caddick, M.X., et al, 1998, Nature Biotech. 16, 177-180, which are incorporated by reference) cytokinin inducible IB6 and CKI1 genes (Brandstatter, I. and Kieber, J.J., 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 al., 1997, Plant Cell 9, 1963-1971; which is incorporated by reference).

[00164] 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 al., 1985, Nature, 313: 810-812), the rice actin 1 (Zhang et al, 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. al., 1994, Plant Physiol. 106: 459-467) genes, the maize ubiquitin 1 gene (Cornejo et al, 1993, Plant Mol. Biol. 29: 637-646), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et al, 1995, Plant Mol. Biol. 29: 637-646), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995

Plant Mol. Biol. 29: 995-1004). 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
5 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.

[00165] By "native" it is meant that the nucleic acid or amino acid sequence is naturally
10 occurring, or "wild type".

[00166] 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
15 interact with the operatively linked sequences.

[00167] The one or more than one nucleotide sequence 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.,
20 alfalfa, potato, ginseng, pea, oat, rice, soybean, wheat, barley, sunflower, cotton and the like.

[00168] The one or more chimeric 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
25 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.

[00169] Non-limiting examples of suitable 3' regions are the 3' transcribed non-translated 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-1, 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).

10 [00170] One or more of the chimeric 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
15 reading frame ("in frame") of the coding sequence to provide for correct translation of the transcribed sequence.

[00171] 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
20 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.

[00172] Also considered part of this invention are transgenic plants, plant cells or seeds
25 containing the chimeric gene construct 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.

[00173] Also considered part of this invention are transgenic plants, trees, yeast, bacteria, fungi, insect and animal cells containing the chimeric gene construct comprising a nucleic acid encoding recombinant, chimeric HA or HA0 for VLP production, in accordance with the present invention.

[00174] The regulatory elements of the present invention may also be combined with coding region of interest for expression within a range of host organisms that are amenable to transformation, or transient expression. Such organisms include, but are not limited to plants, both monocots and dicots, for example but not limited to corn, cereal plants, wheat, barley, oat, *Nicotiana* spp, *Brassica* spp, soybean, bean, pea, alfalfa, potato, tomato, ginseng, and *Arabidopsis*.

[00175] Methods for stable transformation, and regeneration of these organisms are established in the art and known to one of skill in the art. The method of obtaining transformed and regenerated plants is not critical to the present invention.

[00176] 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 chimeric construct to a host may be heritable and the transfer of genetic information considered stable, or the transfer may be transient and the transfer of genetic information is not inheritable.

[00177] 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. A portion of a plant may comprise plant matter.

5 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.

[00178] By the term "minimal processing" it is meant plant matter, for example, a plant
10 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,
15 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
20 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.

[00179] The plant matter, in the form of plant material or tissue may be orally delivered
25 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.

[00180] Examples of a subject or target organism that the VLPs of the present invention may be administered to include, but are not limited to, humans, primates, birds, water fowl, migratory birds, quail, duck, geese, poultry, chicken, swine, sheep, equine, horse, camel, canine, dogs, feline, cats, tiger, leopard, civet, mink, stone marten, ferrets, house pets,
5 livestock, rabbits, mice, rats, guinea pigs or other rodents, seal, whale and the like. Such target organisms are exemplary, and are not to be considered limiting to the applications and uses of the present invention.

[00181] It is contemplated that a plant comprising the chimeric HA according to some embodiments of the invention, or expressing the VLP comprising the chimeric HA according
10 to some embodiments of the invention, may be administered to a subject or target organism, in a variety of ways depending upon the need and the situation. For example, the chimeric HA obtained from the plant may be extracted prior to its use in either a crude, partially purified, or purified form. If the chimeric HA is to be at least partially purified, then it may be produced in either edible or non-edible plants. Furthermore, if the chimeric HA is orally administered, the
15 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 an animal with little or not further processing it is preferred that the plant tissue
20 being administered is edible.

[00182] 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 potato virus Y (HcPro) may be used to counteract the specific degradation of transgene
mRNAs (Brigneti et al., 1998). Alternate suppressors of silencing are well known in the art
25 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, p19 of Tomato bushy stunt virus (TBSV p19), 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 tristeza 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.

[00183] Furthermore, VLPs produced as described herein do not comprise neuraminidase (NA). However, NA may be co-expressed with HA should VLPs comprising HA and NA be desired.

[00184] Therefore, the present invention further includes a suitable vector comprising the chimeric HA sequence suitable for use with either stable or transient expression systems.

The genetic information may be also provided within one or more than one construct. For example, a nucleotide sequence encoding a protein of interest may be introduced in one construct, and a second nucleotide sequence encoding a protein that modifies glycosylation of the protein of interest may be introduced using a separate construct. These nucleotide sequences may then be co-expressed within a plant. However, a construct comprising a nucleotide sequence encoding both the protein of interest and the protein that modifies glycosylation profile of the protein of interest may also be used. In this case the nucleotide sequence would comprise a first sequence comprising a first nucleic acid sequence encoding the protein of interest operatively linked to a promoter or regulatory region, and a second sequence comprising a second nucleic acid sequence encoding the protein that modifies the glycosylation profile of the protein of interest, the second sequence operatively linked to a promoter or regulatory region.

[00185] 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.

[00186] 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, infiltration, and the like. 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); Geierman 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 Lefebvre, DB Layzell (eds), Addison-Wesley, 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), Neuhauser 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), , Liu and Lomonossoff (J. Virol Meth, 105:343-348, 2002,), U.S. Pat. Nos. 4,945,050; 5,036,006; 5,100,792; 6,403,865; 5,625,136, (all of which are hereby incorporated by reference).

[00187] 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 Plant Science 122:101-108 (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, however, other transient methods may also be used as noted above. With either Agro-inoculation or Agro-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 *Agrobacterium* 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.

[00188] The VLPs comprising chimeric HA provided by the present invention may be used in conjunction with an existing influenza vaccine, to supplement the vaccine, render it more efficacious, or to reduce the administration dosages necessary. As would be known to a person of skill in the art, the vaccine may be directed against one or more than one influenza virus. Examples of suitable vaccines include, but are not limited to, those commercially available from Sanofi-Pasteur, ID Biomedical, Merial, Sinovac, Chiron, Roche, MedImmune, GlaxoSmithKline, Novartis, Sanofi-Aventis, Serono, Shire Pharmaceuticals and the like.

[00189] If desired, the VLPs of the present invention may be admixed with a suitable adjuvant as would be known to one of skill in the art. Furthermore, the VLP may be used in a vaccine composition comprising an effective dose of the VLP for the treatment of a target organism, as defined above. Furthermore, the VLP produced according to the present

invention may be combined with VLPs obtained using different influenza proteins, for example, neuraminidase (NA).

[00190] Therefore, the present invention provides a method for inducing immunity to influenza virus infection in an animal or target organism comprising administering an effective dose of a vaccine comprising one or more than one VLP. The vaccine may be administered orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

[00191] Compositions according to various embodiments of the invention may comprise VLPs of two or more influenza strains or subtypes. "Two or more" refers to two, three, four, five, six, seven, eight, nine, 10 or more strains or subtypes. The strains or subtypes represented may be of a single subtype (e.g. all H1N1, or all H5N1), or may be a combination of subtypes. Exemplary subtype and strains include H5/Indo, H1/Bri, H1/NC, H3/Bri, B/ Flo. The choice of combination of strains and subtypes may depend on the geographical area of the subjects likely to be exposed to influenza, proximity of animal species to a human population to be immunized (e.g. species of waterfowl, agricultural animals such as swine, etc) and the strains they carry, are exposed to or are likely to be exposed to, predictions of antigenic drift within subtypes or strains, or combinations of these factors. Examples of combinations used in past years are available in the databases maintained by the World Health Organization (WHO) (see URL: who.int/csr/disease/influenza/vaccine_recommendations1/en).

[00192] The two or more VLPs may be expressed individually, and the purified or semi-purified VLPs subsequently combined. Alternately, the VLPs may be co-expressed in the same host, for example a plant, portion of plant, or plant cell. The VLPs may be combined or produced in a desired ratio, for example about equivalent ratios, or may be combined in such a manner that one subtype or strain comprises the majority of the VLPs in the composition.

[00193] Therefore, the invention provides for compositions comprising VLPs of two or more strains or subtypes.

[00194] Also provided is an article of manufacture, comprising packaging material and a composition comprising a VLP comprising a chimeric HA. The composition includes a physiologically or pharmaceutically acceptable excipient, and the packaging material may include a label which indicates the active ingredients of the composition (e.g. the VLP).

[00195] A kit comprising a composition comprising a nucleic acid encoding a chimeric HA as provided herein, along with instructions for use of the nucleic acid for production of chimeric HA, or VLPs comprising the chimeric HA is also provided. The kit may be useful for production of VLPs comprising the chimeric HA, and the instructions may include, for example, information on expressing the nucleic acid in a plant or a plant cell, instructions for harvesting and obtaining the VLPs from the plant or plant tissue.

[00196] In another embodiment, a kit for the preparation of a medicament, comprising a VLP comprising a chimeric HA, along with instructions for its use is provided. The instructions may comprise a series of steps for the preparation of the medicament, the medicament being useful for inducing a therapeutic or prophylactic immune response in a subject to whom it is administered. The kit may further comprise instructions addressing dose concentrations, dose intervals, preferred administration methods or the like.

[00197] The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

[00198] The sequences described herein are summarized below.

SEQ ID NO	Description	SEQ ID NO:	Description
1	primer XmaI-pPlas.c	55	nucleic acid seq of synthetic BglII - PDI SP-H1/NC-SacI/StuI (lacking TMD/CT)
2	primer SacI-ATG-pPlas.r	56	nucleic acid seq of KpnI - H1/NC TmD/CT-SacI/StuI comprising the C-ter H1 (A/New Caledonia/20/99

			(H1N1) coding sequence including the TmD and Ctail from the KpnI site to the stop codon (flanked in 3' by a dual SacI/StuI site)
3	primer SacI-PlasTer.c	57	nucleic acid seq of protein disulphide isomerase Nucleotides 32-103 encode the PDI signal peptide
4	primer EcoRI-PlasTer.r	58	nucleic acid seq of plasto promoter - PDI SP-BglI
5	primer Plasto-443c	59	nucleic acid seq of construct 540 comprising alfalfa plastocyanin promoter and 5' UTR, coding sequence of the signal peptide from PDI and of H1 form A/New Caledonia/20/99 (H1N1), alfalfa plastocyanin 3' UTR and terminator sequences. H1 from A/New Caledonia/20/1999 coding sequence is underlined.
6	primer SpHA(Ind)-Plasto.r	60	nucleic acid seq of DraI - Plasto promoter-H1/Bri-SacI comprising the complete H1 (A/Brisbane/59/07 (H1N1)) coding region (including the signal peptide and the stop codon) flanked, in 5' by alfalfa plastocyanin gene sequences corresponding to the first 84 nucleotides upstream of the initial ATG,
7	primer Plasto-SpHA	61	nucleic acid seq of construct 774 comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H1 form A/Brisbane/59/07 (H1N1), alfalfa plastocyanin 3' UTR and terminator sequences
8	primer HA(Ind)-Sac.r	62	nucleic acid seq of construct 828, from PacI (upstream of the promoter) to AscI (immediately downstream of the NOS terminator).CPMV HT 3' UTR sequencer underlined with mutated ATG in bold.
9	primer pBinPlus.2613c	63	nucleic acid seq of construct 690
10	primer Mut-ATG115.r	64	nucleic acid seq of construct 691
11	primer Mut-ATG161.c	65	nucleic acid seq of construct 696
12	primer LC-C5-1.110r	66	nucleic acid seq of construct 732 comprising the CaMV 35S promoter, CPMV-HT 5' UTR, hemagglutinin coding sequence of H1 form A/Brisbane/59/07 (H1N1), CPMV-HT 3' UTR and NOS terminator sequences. Coding sequence of H1/Bri is underlined.

13	primer E1 H1B-E1 H5L.r	67	nucleic acid seq of intermediate construct 787
14	primer E1 H5N-E1 H1B.c	68	nucleic acid seq of construct 733
15	primer E2 H5I-RB H1B.r	69	nucleic acid seq of construct 734
16	primer RB H1B-E2 H5L.c	70	nucleic acid seq of DraIII-plasto promoter - H3/Bri-SacI comprising the complete H3 (A/Brisbane/10/07 (H3N2)) coding region (including the signal peptide and the stop codon) flanked, in 5' by alfalfa plastocyanin gene sequences corresponding to the first 84 nucleotides upstream of the initial ATG
17	primer E1 H1B-F'1 H5L.r	71	nucleic acid seq of construct 736
18	primer F'1 H5N-E1 H1B.c	72	nucleic acid seq of construct 737
19	primer F'2 H5I-E2 H1B.r	73	nucleic acid seq of DraIII-plasto promoter - B/Flo-SacI
20	primer E2 H1B-F'2 H5L.c	74	nucleic acid seq of construct 739
21	primer E1 H5I-E1 H1NC.r	75	nucleic acid seq of construct 745
22	primer E1 H1NC-E1 H5L.c	76	nucleic acid seq of MspI
23	primer E2 H1NC-RB H5L.r	77	nucleic acid seq of construct 850
24	primer RB H5I-E2 H1NC.c	78	nucleic acid seq of construct 860
25	primer HA-SacL.r	79	nucleic acid seq of construct 870
26	primer ApaI-H1B.c	80	Amino acid sequence comprising an RB subdomain of H1/Bri, an H5/Indo signal peptide, and a stem domain complex (SDC) comprising an H5/Indo F'1, E1, E2, F'2 and F subdomains
27	primer StuI-H1B.r	81	amino acid sequence comprising H1/Bri head domain complex (HDC) comprising E1, RB, E2, an H5/Indo signal peptide, and H5/Indo stem domain complex (SDC) comprising H5/Indo F'1, F'2 and F subdomains
28	primer SpPDI-H1B.c	82	amino acid sequence of comprising an RB subdomain of H5/Indo, a PDI signal peptide, and H1/NC stem domain complex comprising F'1, E1, E2 and F'2
29	primer SacI-H1B.r	83	amino acid sequence of comprising a PDI signal peptide, an ectodomain of H3 A/Brisbane/10/2007 and a TMD/CT of H5 A/Indonesia/5/2005
30	primer ApaI-SpPDI.c	84	amino acid sequence comprising an ectodomain of B/Florida/4/2006 and a TMD/CT of H5 A/Indonesia/5/2005 encoded by the open reading frame in construct number 745
31	primer ApaI-H5 (A-Indo).1c	85	nucleic acid seq of SacI - plastocyanin 3'UTR
32	primer H5 (A-Indo)-	86	nucleotide seq of PDI SP-H1

	StuI.1707r		A/California/04/09 Alfalfa protein disulfide isomerase signal peptide coding sequence is underlined and mature H1 coding sequence is highlighted in bold.
33	primer H3B-SpPDI.r	87	amino acid sequence of PDI SP-H1 A/California/04/09 Alfalfa protein disulfide isomerase signal peptide is underlined
34	primer SpPDI-H3B.c	88	2X35S promoter.
35	primer StuI-H3B.r	89	primer PacI-MCS-2X35S.c
36	primer TmD H5I-H3B.r	90	primer CPMV 5'UTR- 2X35S.r
37	primer H3B-TmD H5I.c	91	primer 2X35S -CPMV 5'UTR.c
38	primer HBF-SpPDI.r	92	primer Apal -M prot.r
39	primer SpPDI-HBF.c	93	nucleic acid sequence of construct 747. Coding sequence of chimeric HA is underlined. 2X35S promoter sequence is indicated in italics
40	primer StuI-HBF.r	94	amino acid sequence of B-Florida
41	primer TmD H5I-B Flo.r	95	amino acid sequence of B-Malaysia
42	primer B Flo-TmD H5I.c	96	amino acid sequence of H1-Brisbane
43	primer Hsp40Luz.1c	97	amino acid sequence of H1-Sol.Isl.
44	primer Hsp40Luz-SacI.1272r	98	amino acid sequence of H1-New Cal.
45	primer Hsp40Luz-Plasto.r	99	amino acid sequence of H2-Singapore
46	primer Hsp70Ara.1c	100	amino acid sequence of H3-Brisbane
47	primer Hsp70Ara-SacI.1956r	101	amino acid sequence of H3A-WCN
48	primer Hsp70Ara-Plasto.r	102	amino acid sequence of H5A-Anhui
49	primer supP19-plasto.r	103	amino acid sequence of H5A-Vietnam
50	primer supP19-1c	104	amino acid sequence of H5-Indo
51	primer SupP19-SacI.r		
52	nucleic acid sequence of complete H5 (A/Indonesia/5/05 (H5N1)) coding region (including the signal peptide and the stop codon) flanked, in 5' by a HindIII site and, in 3', by a SacI site		
53	nucleic acid seq of construct 660 - an HA expression cassette comprising an alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H5 form A/Indonesia/5/05 (H5N1), alfalfa plastocyanin 3' UTR and terminator sequences		
54	nucleic acid seq of native H1/NC (lacking TMD/CT)		

Methods and Materials

1. Assembly of HA expression cassettes

A- pCAMBIAPlasto

5 [00199] All manipulations were done using the general molecular biology protocols of Sambrook and Russell (2001; which is incorporated herein by reference). Table 1 presents oligonucleotide primers used for expression cassettes assembly. The first cloning step consisted in assembling a receptor plasmid containing upstream and downstream regulatory elements of the alfalfa plastocyanin gene. The plastocyanin promoter and 5'UTR sequences
10 were amplified from alfalfa genomic DNA using oligonucleotide primers XmaI-pPlas.c (SEQ ID NO:1) and SacI-ATG-pPlas.r (SEQ ID NO:2). The resulting amplification product was digested with XmaI and SacI and ligated into pCAMBIA2300 (Cambia, Canberra, Australia), previously digested with the same enzymes, to create pCAMBIApromoPlasto. Similarly, the 3'UTR sequences and terminator of the plastocyanin gene was amplified from alfalfa genomic
15 DNA using the following primers: SacI-PlasTer.c (SEQ ID NO:3) and EcoRI-PlasTer.r (SEQ ID NO:4), and the product was digested with SacI and EcoRI before being inserted into the same sites of pCAMBIApromoPlasto to create pCAMBIAPlasto.

B- Plasto-Native SP-H5 A/Indonesia/5/05 (construct number 660)

[00200] A fragment encoding hemagglutinin from influenza strain A/Indonesia/5/05
20 (H5N1; Acc. No. LANL ISDN125873) was synthesized by Epoch Biolabs (Sugar Land, TX, USA). The fragment produced, containing the complete H5 coding region including the native signal peptide flanked by a HindIII site immediately upstream of the initial ATG, and a SacI site immediately downstream of the stop (TAA) codon, is presented in (SEQ ID NO:52; Figure 17). The H5 coding region was cloned into a plastocyanin-based expression cassette by
25 the PCR-based ligation method presented in Darveau et al. (1995). Briefly, a first PCR amplification was obtained using primers Plasto-443c (SEQ ID NO:5;) and SpHA(Ind)-

Plasto.r (SEQ ID NO:6) and pCAMBIApromoPlasto as template. In parallel, a second amplification was performed with primers Plasto-SpHA (SEQ ID NO:7) and HA(Ind)-Sac.r (SEQ ID NO:8) with H5 coding fragment (SEQ ID NO:52; Figure 17) as template. The amplification obtained from both reactions were mixed together and the mixture served as
 5 template for a third reaction (assembling reaction) using Plasto-443c (SEQ ID NO:5) and HA(Ind)-Sac.r (SEQ ID NO:8) as primers. The resulting fragment was digested with BamHI (in the plastocyanin promoter) and SacI (at the 3' end of the fragment) and cloned into pCAMBIAPlasto previously digested with the same enzymes. The resulting plasmid, named 660, is presented in Figure 18 (SEQ ID NO:53).

10 **C- Plasto-PDI SP-H1 A/New Caledonia/20/99 (construct number 540)**

[00201] The open reading frame from the H1 gene of influenza strain A/New Caledonia/20/99 (H1N1) was synthesized in two fragments (Plant Biotechnology Institute, National Research Council, Saskatoon, Canada). A first fragment synthesized corresponds to the wild-type H1 coding sequence (GenBank acc. No. AY289929; SEQ ID NO: 54; Figure 19)
 15 lacking the signal peptide coding sequence at the 5' end and the transmembrane domain coding sequence at the 3' end. The 5' end of the fragment is composed of the last nucleotides encoding PDISP (including a BglII restriction site) and a dual SacI/StuI site was added immediately downstream of the stop codon at the 3' terminal end of the fragment, to yield SEQ ID NO: 55 (Figure 20). A second fragment encoding the C-terminal end of the H1
 20 protein (comprising a transmembrane domain and cytoplasmic tail) from the KpnI site to the stop codon, and flanked in 3' by SacI and StuI restriction sites was also synthesized (SEQ ID NO. 56; Figure 21).

[00202] The first H1 fragment was digested with BglII and SacI and cloned into the same sites of a binary vector (pCAMBIAPlasto) containing the plastocyanin promoter and 5' UTR fused to the signal peptide of alfalfa protein disulfide isomerase (PDI) gene (nucleotides
 25 32-103; Accession No. Z11499; SEQ ID NO: 57; Figure 22) resulting in a PDI-H1 chimeric gene downstream of the plastocyanin regulatory elements. The sequence of the plastocyanin-based cassette, containing the promoter and PDI signal peptide up to the BglII restriction site

and the plastocyanin terminator downstream of a SacI site, is presented in SEQ ID NO. 58 (Figure 23). The addition of the C-terminal end of the H1 coding region (encoding the transmembrane domain and the cytoplasmic tail) was obtained by inserting the synthesized fragment (SEQ ID NO. 56; Figure 21) previously digested with KpnI and SacI, into the H1 expression plasmid. The resulting construct, named 540, is presented in SEQ ID NO. 59 (Figure 24).

D- Plasto-Native SP-H1 A/Brisbane/59/07 (construct number 774)

[00203] Expression cassette number 774, driving the expression of H1 from A/Brisbane/59/07, was assembled as follows. A synthetic fragment was synthesized comprising the complete hemagglutinin coding sequence (from ATG to stop) flanked in 3' by alfalfa plastocyanin gene sequences corresponding to the first 84 nucleotides upstream of the plastocyanin ATG starting with a DraIII restriction site. The synthetic fragments also comprised a SacI site immediately downstream of the stop codon.

[00204] The synthetic fragment was synthesized by Top Gene Technologies (Montreal, QC, Canada). The fragment synthesized is presented in SEQ ID NO. 60 (Figure 25). For the assembly of the complete expression cassette, the synthetic fragment was digested with DraIII and SacI and cloned into pCAMBIAPlasto previously digested with the same enzymes to give construct 774 (SEQ ID NO. 61; Figure 26).

E- CPMV HT-LC C51 (construct number 828)

[00205] CPMV-*HT* expression cassettes use the 35S promoter to control the expression of an mRNA comprising a coding sequence of interest flanked, in 5', by nucleotides 1–512 from the Cowpea mosaic virus (CPMV) RNA2 with mutated ATG at positions 115 and 161, and in 3', by nucleotides 3330–3481 from the CPMV RNA2 (corresponding to the 3' UTR) followed by the NOS terminator. Plasmid pBD-C5-1LC, (Sainsbury et al. 2008; Plant Biotechnology Journal 6: 82-92 and PCT Publication WO 2007/135480), was used for the assembly of CPMV-*HT*-based hemagglutinin expression cassettes. The mutation of ATGs at position 115 and 161 of the CPMV RNA2 was done using a PCR-based ligation method

presented in Darveau et al. (Methods in Neuroscience 26: 77-85 (1995)). Two separate PCRs were performed using pBD-C5-1LC as template. The primers for the first amplification were pBinPlus.2613c (SEQ ID NO: 9) and Mut-ATG115.r (SEQ ID NO: 10). The primers for the second amplification were Mut-ATG161.c (SEQ ID NO: 11) and LC-C5-1.110r (SEQ ID NO: 12). The two fragments obtained were mixed and used as template for a third amplification using pBinPlus.2613c (SEQ ID NO: 9) and LC-C5-1.110r (SEQ ID NO: 12) as primers. The resulting fragment was digested with PacI and ApaI and cloned into pBD-C5-1LC digested with the same enzymes. The construct generated, named 828, is presented in Figure 27 (SEQ ID NO: 62).

F- H1 A/Brisbane/59/07 receptor-binding (RB) domain in H5 A/Indonesia/5/05 backbone (construct number 690)

[00206] A chimeric HA was made by replacing the RB domain in the H5 A/Indonesia/5/05 with that of H1 A/Brisbane/59/07 using the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, a segment of the plastocyanin promoter fused to the natural signal peptide, the F'1 and E1 domains of the H5 A/Indonesia/5/05 was amplified using primers Plasto-443c (SEQ ID NO: 5) and E1 H1B-E1 H5L.r (SEQ ID NO:13) with construct number 660 (SEQ ID NO:53, Figure 18) as template. A second fragment, comprising the H1 A/Brisbane/59/07 RB domain coding sequence, was amplified with primers E1 H5N-E1 H1B.c (SEQ ID NO:14) and E2 H5L-RB H1B.r (SEQ ID NO:15) using construct number 774 (SEQ ID NO:61; Figure 26) as template. A third fragment comprising E2, F'2, F, transmembrane and cytoplasmic domains from H5 A/Indonesia/5/05 was amplified using primers RB H1B-E2 H5L.c (SEQ ID NO:16) and HA(Ind)-SacI.r (SEQ ID NO:8) with construct number 660 (SEQ ID NO: 53; Figure 18) as template. Amplification products were then mixed and used as template for a second round of amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO:5) and HA(Ind)-SacI.r (SEQ ID NO:8). The resulting fragment was digested with BamHI (in the plastocyanin promoter) and SacI (after the stop codon) and cloned into construct number 660 (SEQ ID NO: 53; Figure 18), previously digested with the same restriction enzymes to give construct number 690 (SEQ ID NO: 63). The construct is presented in Figure 28.

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G- H1 A/Brisbane/59/07 esterase and receptor-binding domains (E1-RB-E2) in H5 A/Indonesia/5/05 backbone (construct number 691)

[00207] A chimeric HA was assembled by replacing the E1-RB-E2 domains in H5 A/Indonesia/5/05 with those of H1 A/Brisbane/59/07 using the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, a segment of the plastocyanin promoter fused to the natural signal peptide and the F'1 domain of H5 A/Indonesia/5/05 was amplified using primers Plasto-443c (SEQ ID NO:5) and E1 H1B-F'1 H5I.r (SEQ ID NO: 17) with construct number 660 (SEQ ID NO: 53; Figure 18) as template. In parallel, two other fragments were amplified. The second fragment, containing the H1 A/Brisbane/59/07 E1-RB-E2 domains coding sequence, was amplified with primers F'1 H5N-E1 H1B.c (SEQ ID NO: 18) and F'2 H5I-E2 H1B.r (SEQ ID NO: 19) using construct number 774 (SEQ ID NO:61; Figure 26) as template. For the third fragment, F'2, F, transmembrane and cytoplasmic domains from H5 A/Indonesia/5/05 were amplified using primers E2 H1B-F'2 H5I.c (SEQ ID NO: 20) and HA(Ind)-SacI.r (SEQ ID NO: 8) with construct number 660 (SEQ ID NO: 53; Figure 18) as template. Amplification products were then mixed and used as template for a second round of amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO:5) and HA(Ind)-SacI.r (SEQ ID NO: 8). The resulting fragment was digested with BamHI (in the plastocyanin promoter) and SacI (after the stop codon) and cloned into construct number 660 (SEQ ID NO: 53; Figure 18), previously digested with the same restriction enzymes to give construct number 691 (SEQ ID NO: 64). The construct is presented in Figure 29.

H- H5 A/Indonesia/5/05 receptor-binding (RB) domain in H1 A/New Caledonia/20/99 backbone (construct number 696)

[00208] A chimeric HA was made by replacing the RB domain in the H1 A/New Caledonia/20/99 with that of H5 A/Indonesia/5/05 using the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, a segment of the plastocyanin promoter fused to the signal peptide of alfalfa protein disulfide isomerase (PDISP; Accession No. Z11499; nucleotides 32-103 of SEQ ID NO: 57;

Figure 22), the F'1 and E1 domains of H1 A/New Caledonia/20/99 were amplified using primers Plasto-443c (SEQ ID NO: 5) and E1 H5I-E1 H1NC.r (SEQ ID NO: 21) with construct number 540 (SEQ ID NO: 59; Figure 24) as template. A second fragment, comprising the H5 A/Indonesia/5/05 RB domain coding sequence, was amplified with primers E1 H1NC-E1 H5I.c (SEQ ID NO: 22) and E2 H1NC-RB H5I.r (SEQ ID NO: 23) using construct number 660 (SEQ ID NO: 53; Figure 18) as template. A third fragment comprising E2, F'2, F, transmembrane and cytoplasmic domains from H1 A/New Caledonia/20/99 was amplified using primers RB H5I-E2 H1NC.c (SEQ ID NO: 24) and HA-SacI.r (SEQ ID NO: 25) with construct number 540 (SEQ ID NO: 59; Figure 24) as template. Amplification products were then mixed and used as template for a second round of amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO: 5) and HA-SacI.r (SEQ ID NO: 25). The resulting fragment was digested with BglII and SacI and cloned into construct number 540 (SEQ ID NO: 59; Figure 24) previously digested with the same restriction enzymes to give construct number 696 (SEQ ID NO: 65). The construct is presented in Figure 30.

I- Assembly of H1 A/Brisbane/59/2007 in CPMV-*HT* expression cassette (construct number 732).

[00209] The coding sequence of HA from H1 A/Brisbane/59/2007 was cloned into CPMV-*HT* as follows. Restriction sites ApaI (immediately upstream of ATG) and StuI (immediately downstream of the stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-H1B.c (SEQ ID NO: 26) and StuI-H1B.r (SEQ ID NO: 27) using construct number 774 (SEQ ID NO: 61; Figure 26) as template. The resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 62; Figure 27) digested with the same enzymes. Resulting cassette was named construct number 732 (SEQ ID NO: 66; Figure 31).

J- Assembly of SpPDI-H1 A/Brisbane/59/2007 in CPMV-HT expression cassette (construct number 733).

[00210] A sequence encoding the signal peptide of alfalfa protein disulfide isomerase (PDISP; nucleotides 32-103 of SEQ ID NO: 57; Figure 22; Accession No. Z11499) was fused to the HA0 coding sequence of H1 from A/Brisbane/59/2007, and the resulting fragment was cloned into CPMV-HT as follows. The H1 coding sequence was amplified with primers SpPDI-H1B.c (SEQ ID NO: 28) and SacI-H1B.r (SEQ ID NO: 29) using construct 774 (SEQ ID NO: 61; Figure 26) as template. The resulting fragment consisted in the H1 coding sequence flanked, in 5', by the last nucleotides encoding PDISP (including a BglII restriction site) and, in 3', by a SacI restriction site. The fragment was digested with BglII and SacI and cloned into construct number 540 (SEQ ID NO: 59; Figure 24) previously digested with the same restriction enzymes. The coding sequence of intermediate cassette, named construct number 787 (SEQ ID NO: 67), is presented in Figure 32. Restriction sites ApaI (immediately upstream ATG) and StuI (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-SpPDI.c (SEQ ID NO: 30) and StuI-H1B.r (SEQ ID NO: 27) using construct number 787 (SEQ ID NO: 67; Figure 32) as template. Resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 62; Figure 27) digested with the same enzymes. Resulting cassette was named construct number 733 (SEQ ID NO: 68; Figure 33).

K- Assembly of H1 A/Brisbane/59/07 receptor-binding (RB) domain in H5 A/Indonesia/5/05 backbone in CPMV-HT expression cassette (construct number 734).

[00211] The coding sequence of chimeric HA consisting in RB domain from H1 A/Brisbane/59/07 in H5 A/Indonesia/5/05 backbone was cloned into CPMV-HT as follows. Restriction sites ApaI (immediately upstream of ATG) and StuI (immediately downstream of the stop codon) were added to the chimeric hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-H5 (A-Indo).1c (SEQ ID NO: 31) and H5 (A-Indo)-StuI.1707r (SEQ ID NO: 32) using construct number 690 (SEQ ID NO: 63; Figure 28) as

template. Resulting fragment was digested with *ApaI* and *StuI* restriction enzymes and cloned into construct number 828 (SEQ ID NO: 62; Figure 27) digested with the same enzymes. Resulting cassette was named construct number 734 (SEQ ID NO: 69; Figure 34).

**L- Assembly of SpPDI-H3 A/Brisbane/10/2007 in CPMV-*HT* expression cassette
(construct number 736).**

[00212] A sequence encoding alfalfa PDI signal peptide fused to HA0 from H3 A/Brisbane/10/2007 was cloned into CPMV-*HT* as follows. First, a synthetic fragment was synthesized comprising the complete hemagglutinin coding sequence (from ATG to stop) flanked in 3' by alfalfa plastocyanin gene sequence corresponding to the first 84 nucleotides (starting with a *DraIII* restriction site) upstream of the plastocyanin ATG. The synthetic fragment also comprised a *SacI* site immediately after the stop codon. Synthetic fragment was synthesized by Top Gene Technologies (Montreal, QC, Canada). The fragment synthesized is presented in SEQ ID NO: 70 (Figure 35) and was used as template for further PCR-based ligation.

[00213] Second, the signal peptide of alfalfa protein disulfide isomerase (PDISP) (nucleotides 32-103; Accession No Z11499; SEQ ID NO: 57; Figure 22) was linked to the HA0 coding sequence of H3 from A/Brisbane/10/2007 along with *ApaI* restriction site immediately upstream of ATG and *StuI* restriction site downstream of the stop codon as follows. PDISP was linked to the H3 coding sequence by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, PDISP signal peptide was amplified using primers *ApaI*-SpPDI.c (SEQ ID NO: 30) and H3B-SpPDI.r (SEQ ID NO: 33) with construct number 540 (SEQ ID NO: 59; Figure 24) as template. In parallel, another fragment containing a portion of the coding sequence of H3 A/Brisbane/10/2007 (from codon 17 to the stop codon) was amplified with primers SpPDI-H3B.c (SEQ ID NO: 34) and *StuI*-H3B.r (SEQ ID NO: 35) using previously synthesized fragment (SEQ ID NO. 70; Figure 35) as template. Amplification products were then mixed and used as template for a second round of amplification (assembling reaction) with primers *ApaI*-SpPDI.c (SEQ ID NO: 30) and *StuI*-H3B.r (SEQ ID NO: 35). The resulting fragment

was digested with *ApaI* and *StuI* restriction enzymes and cloned into construct number 828 (SEQ ID NO: 62; Figure 27) digested with the same enzymes. Resulting cassette was named construct number 736 (SEQ ID NO: 71; Figure 36).

M- Assembly of chimeric SpPDI-H3 A/Brisbane/10/2007 (ectodomain) + H5

5 **A/Indonesia/5/2005 (TmD + Cyto tail) in CPMV-*HT* expression cassette (construct number 737).**

[00214] A sequence encoding alfalfa PDI signal peptide fused to the ectodomain of H3 A/Brisbane/10/2007 and to the transmembrane and cytoplasmic domains of H5 A/Indonesia/5/2005 was cloned into CPMV-*HT* as follows. PDISP-H3 coding sequence was
 10 fused to the H5 transmembrane domain by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, a fragment comprising PDISP signal peptide and ectodomain from H3 Brisbane was generated by amplification (with *ApaI* restriction site upstream of the PDISP initial ATG) using primers *ApaI*-SpPDI.c (SEQ ID NO: 30) and TmD H5I-H3B.r (SEQ ID NO: 36) with construct
 15 number 736 (SEQ ID NO: 71; Figure 36) as template. In parallel, another fragment containing transmembrane and cytoplasmic domains of H5 Indonesia was amplified with primers H3B-TmD H5I.c (SEQ ID NO: 37) and H5 (A-Indo)-*StuI*.1707r (SEQ ID NO: 32) using construct number 660 (SEQ ID NO. 53; Figure 18) as template. Amplification products were then mixed and used as template for a second round of amplification (assembling reaction) with
 20 primers *ApaI*-SpPDI.c (SEQ ID NO: 30) and H5 (A-Indo)-*StuI*.1707r (SEQ ID NO: 32). The resulting fragment was digested with *ApaI* and *StuI* restriction enzymes and cloned into construct number 828 (SEQ ID NO: 62; Figure 27) digested with the same enzymes. Resulting cassette was named construct number 737 (SEQ ID NO: 72; Figure 37).

N- Assembly of SpPDI-HA B/Florida/4/2006 in CPMV-*HT* expression cassette (construct
 25 **number 739).**

[00215] A sequence encoding alfalfa PDI signal peptide fused to HA0 from HA B/Florida/4/2006 was cloned into CPMV-*HT* as follows. First, a synthetic fragment was

synthesized comprising the complete hemagglutinin coding sequence (from ATG to stop) flanked in 3' by alfalfa plastocyanin gene sequence corresponding to the first 84 nucleotides (starting with a DraIII restriction site) upstream of the plastocyanin ATG. The synthetic fragment also comprised a SacI restriction site immediately after the stop codon. The synthetic
 5 fragment was synthesized by Epoch Biolabs (Sugar Land, Texas, USA). The fragment synthesized is presented in SEQ ID NO: 73 (Figure 38) and was used as template for further PCR-based ligation.

[00216] Second, the signal peptide of alfalfa protein disulfide isomerase (PDISP) (nucleotides 32-103 of SEQ ID NO: 57; Figure 22; ; Accession No Z11499) was linked to the
 10 HA0 coding sequence of HA from B/Florida/4/2006 along with ApaI restriction site immediately upstream ATG and StuI restriction site downstream stop codon as follows. PDISP was linked to the HA coding sequence by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, PDISP signal peptide was amplified using primers ApaI-SpPDI.c (SEQ ID NO: 30) and HBF-SpPDI.r
 15 (SEQ ID NO: 38) with construct number 540 (SEQ ID NO: 59; Figure 24) as template. In parallel, another fragment containing a portion of the coding sequence of HA from B/Florida/4/2006 (from codon 16 to the stop codon) was amplified with primers SpPDI-HBF.c (SEQ ID NO: 39) and StuI-HBF.r (SEQ ID NO: 40) using previously synthesized fragment (SEQ ID NO. 73; Figure 38) as template. Amplification products were then mixed
 20 and used as template for a second round of amplification (assembling reaction) with primers ApaI-SpPDI.c (SEQ ID NO: 30) and StuI-HBF.r (SEQ ID NO: 40). The resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 62; Figure 27) digested with the same enzymes. Resulting cassette was named construct number 739 (SEQ ID NO: 74; Figure 39).

25 **O- Assembly of chimeric SpPDI-HA B/Florida/4/2006 (ectodomain) + H5 A/Indonesia/5/2005 (TmD + Cyto tail) in CPMV-HT expression cassette (construct number 745).**

[00217] A sequence encoding alfalfa PDI signal peptide fused to the ectodomain from HA B/Florida/4/2006 and to the transmembrane and cytoplasmic domains of H5 A/Indonesia/5/2005 was cloned into CPMV-*HT* as follows. PDISP-B/Florida/4/2006 ectodomain coding sequence was fused to the H5 transmembrane and cytoplasmic domains by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, a fragment comprising PDISP signal peptide fused to the ectodomain from HA B/Florida/4/2006 was generated by amplification using primers ApaI-SpPDI.c (SEQ ID NO: 30) and TmD H5I-B Flo.r (SEQ ID NO: 41) with construct number 739 (SEQ ID NO: 74; Figure 39) as template. In parallel, another fragment containing H5 Indonesia transmembrane and cytoplasmic domains was amplified with primers B Flo-TmD H5I.c (SEQ ID NO: 42) and H5 (A-Indo)-StuI.1707r (SEQ ID NO: 32) using construct number 660 (SEQ ID NO. 53; Figure 18) as template. Amplification products were then mixed and used as template for a second round of amplification (assembling reaction) with primers ApaI-SpPDI.c (SEQ ID NO: 30) and H5 (A-Indo)-StuI.1707r (SEQ ID NO: 32). The resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 62; Figure 27) digested with the same enzymes. Resulting cassette was named construct number 745 (SEQ ID NO: 75; Figure 40).

P-Assembly of chimeric SpPDI-HA B/Florida/4/2006+ H5 A/Indonesia/5/2005 (TmD + Cyto tail) in 2X35S-CPMV-*HT* expression cassette (construct number 747).

[00218] A sequence encoding alfalfa PDI signal peptide fused to HA0 from HA B/Florida/4/2006 and to the transmembrane and cytoplasmic domain of H5 A/Indonesia/5/2005 was cloned into 2X35S-CPMV-*HT* as follows. The promoter switch was performed using the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85 (1995)). A first fragment containing 2X35S promoter (SEQ ID NO: 88; Figure 50A) was amplified by PCR with primers **PacI-MCS-2X35S.c** (SEQ ID NO: 89) and CPMV 5'UTR-**2X35S.r** (SEQ ID NO: 90):

PacI-MCS-2X35S.c (SEQ ID NO:89)

AATTGTTAATTAAGTCGACAAGCTTGCATGCCTGCAGGTCAAC

CPMV 5'UTR-2X35S.r (SEQ ID NO:90)

TCAAAACCTATTAAGATTTTAATACCTCTCCAAATGAAATGAACTTCC

5

using a plasmid containing the 2X35S promoter as template. In parallel, a second PCR was performed using primers 2X35S-CPMV 5'UTR.c (SEQ ID NO: 91) and ApaI-M prot.r (SEQ ID NO: 92):

2X35S-CPMV 5'UTR.c (SEQ ID NO:91)

10

TTGGAGAGGTTATTAATAATCTTAATAGGTTTTGATAAAAGCGAACGTGGG

ApaI-M prot.r (SEQ ID NO:92)

15

TCTCCATGGGCCCGACAAATTTGGGCAGAATATACAGAAGCTTA

using construct 745 (SEQ ID NO 75; Figure 40) as template. The two fragments obtained were then mixed and used as template for a second round of PCR (assembling reaction) with PacI-MCS-2X35S.c (SEQ ID NO:89 and ApaI-M prot.r (SEQ ID NO:92) as primers.

20

Resulting fragment was the digested with PacI and ApaI and cloned into construct 745 (SEQ ID NO 75; Figure 40) digested with the same restriction enzymes. The sequence of the expression cassette, named construct 747 (SEQ ID NO:93), is presented in Figure 50B.

2. Assembly of chaperone expression cassettes

[00219] Two heat shock protein (Hsp) expression cassettes were assembled. In a first cassette, expression of the *Arabidopsis thaliana* (ecotype Columbia) cytosolic HSP70 (Athsp70-1 in Lin et al. (2001) Cell Stress and Chaperones 6: 201-208) is controlled by a chimeric promoter combining elements of the alfalfa Nitrite reductase (Nir) and alfalfa Plastocyanin promoters (Nir/Plasto). A second cassette comprising the coding region of the

25

alfalfa cytosolic HSP40 (MsJ1; Frugis et al. (1999) Plant Molecular Biology 40: 397-408) under the control of the chimeric Nir/Plasto promoter was also assembled.

[00220] An acceptor plasmid containing the alfalfa Nitrite reductase promoter (Nir), the GUS reporter gene and NOS terminator in plant binary vector was first assembled. Plasmid pNir3K51 (previously described in US Patent No. 6,420,548) was digested with HindIII and EcoRI. The resulting fragment was cloned into pCAMBIA2300 (Cambia, Canberra, Australia) digested by the same restriction enzyme to give pCAMBIA-Nir3K51.

[00221] Coding sequences for Hsp70 and Hsp40 were cloned separately in the acceptor plasmid pCAMBIANir3K51 by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26:77-85 (1995)).

[00222] For Hsp40, Msj1 coding sequence (SEQ ID NO: 76; Figure 41) was amplified by RT-PCR from alfalfa (ecotype Rangeland) leaf total RNA using primers Hsp40Luz.1c (SEQ ID NO: 43) and Hsp40Luz-SacI.1272r (SEQ ID NO: 44). A second amplification was performed with primers Plasto-443c (SEQ ID NO: 5) and Hsp40Luz-Plasto.r (SEQ ID NO: 45) with construct 660 (SEQ ID NO: 53; Figure 18) as template. PCR products were then mixed and used as template for a third amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO: 5) and Hsp40Luz-SacI.1272r (SEQ ID NO: 44). The resulting fragment was digested with HpaI (in the plastocyanin promoter) and cloned into pCAMBIANir3K51, previously digested with HpaI (in the Nir promoter) and SacI, and filed with T4 DNA polymerase to generate blunt ends. Clones obtained were screened for correct orientation and sequenced for sequence integrity. The resulting plasmid, named R850, is presented in Figure 42 (SEQ ID NO: 77). The coding region of the Athsp70-1 was amplified by RT-PCR from Arabidopsis leaf RNA using primers Hsp70Ara.1c (SEQ ID NO: 46) and Hsp70Ara-SacI.1956r (SEQ ID NO: 47). A second amplification was performed with primers Plato-443c (SEQ ID NO: 5) and Hsp70Ara-Plasto.r (SEQ ID NO: 48) with construct 660 (SEQ ID NO: 53; Figure 18) as template. PCR products were then mixed and used as template for a third amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO: 5) and Hsp70ARA-SacI.1956r (SEQ ID NO: 47). The resulting fragment was digested with HpaI (in

the plastocyanin promoter) and cloned into pCAMBIANir3K51 digested with HpaI (in the Nir promoter) and SacI and filled with T4 DNA polymerase to generate blunt ends. Clones obtained were screened for correct orientation and sequenced for sequence integrity. The resulting plasmid, named R860, is presented in Figure 43 (SEQ ID NO: 78).

5 [00223] A dual Hsp expression plasmid was assembled as follows. R860 (SEQ ID NO: 78; Figure 43) was digested with BsrBI (downstream the NOS terminator), treated with T4 DNA polymerase to generate a blunt end, and digested with SbfI (upstream the chimeric NIR/Plasto promoter). The resulting fragment (Chimeric Nir/Plasto promoter-HSP70 coding sequence-Nos terminator) was cloned into R850 (SEQ ID NO: 77; Figure 42) previously
10 digested with SbfI and SmaI (both located in the multiple cloning site upstream chimeric Nir/Plasto promoter). The resulting plasmid, named R870, is presented in Figure 44 (SEQ ID NO: 79).

3. Assembly of other expression cassettes

HcPro expression cassette

15 [00224] An HcPro construct (35HcPro) was prepared as described in Hamilton et al. (2002). All clones were sequenced to confirm the integrity of the constructs. The plasmids were used to transform *Agrobacterium tumefaciens* (AGL1; ATCC, Manassas, VA 20108, USA) by electroporation (Mattanovich et al., 1989). The integrity of all *A. tumefaciens* strains were confirmed by restriction mapping.

20 **P19 expression cassette**

[00225] The coding sequence of p19 protein of tomato bushy stunt virus (TBSV) was linked to the alfalfa plastocyanin expression cassette by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, a segment of the plastocyanin promoter was amplified using primers Plasto-443c (SEQ
25 ID NO: 5) and supP19-plasto.r (SEQ ID NO: 49) with construct 660 (SEQ ID NO: 53) as template. In parallel, another fragment containing the coding sequence of p19 was amplified

with primers supP19-1c (SEQ ID NO: 50) and SupP19-SacI.r (SEQ ID NO: 51) using construct 35S:p19 as described in Voinnet et al. (The Plant Journal 33: 949-956 (2003)) as template. Amplification products were then mixed and used as template for a second round of amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO: 5) and SupP19-SacI.r (SEQ ID NO: 51). The resulting fragment was digested with BamHI (in the plastocyanin promoter) and SacI (at the end of the p19 coding sequence) and cloned into construct number 660 (SEQ ID NO: 53; Figure 18), previously digested with the same restriction enzymes to give construct number R472. Plasmid R472 is presented in Figure 45.

Construct number 443

[00226] Construct number 443 corresponds to pCAMBIA2300 (empty vector).

Table 1. Oligonucleotide primers used for assembly of expression cassettes.

SEQ ID	Title	Oligonucleotide sequence (5'---3')
1	XmaI-pPlas.c	AGTTCCCCGGGCTGGTATATTTATATGTTGTC
2	SacI-ATG-pPlas.r	AATAGAGCTCCATTTTCTCTCAAGATGATTAATTAATTAATTAGTC
3	SacI-PlasTer.c	AATAGAGCTCGTTAAAATGCTTCTTCGTCTCCTATTTATAATATGG
4	EcoRI-PlasTer.r	TTACGAATTCTCCTTCCTAATTGGTGTACTATCATTTATCAAAGGGGA
5	Plasto-443c	GTATTAGTAATTAGAATTTGGTGTC
6	SpHA(Ind)-Plasto.r	GCAAGAAGAAGCACTATTTTCTCCATTTTCTCTCAAGATGATTA
7	Plasto-SpHA.c	TTAATCATCTTGAGAGAAAATGGAGAAAATAGTGCTTCTTCTTGC
8	HA(Ind)-Sac.r	ACTTTGAGCTCTTAAATGCAAATTCTGCATTGTAACGA
9	pBinPlus.2613c	AGGAAGGGAAGAAAGCGAAAGGAG
10	Mut-ATG115.r	GTGCCGAAGCACGATCTGACAACGTTGAAGATCGCTCACGCAAGAAA GACAAGAGA
11	Mut-ATG161.c	GTTGTCAGATCGTGCTTCGGCACCAGTACAACGTTTTCTTTCACTGAA GCGA
12	LC-C5-1.110r	TCTCCTGGAGTCACAGACAGGGTGG
13	E1 H1B-E1 H5Lr	TCATAGTCAGCGAAATGCCCTGGGTAACAGAGGTCATTGGTTGGATT GGCCT
14	E1 H5N-E1 H1B.c	ATGACCTCTGTTACCCAGGGCATTTCGCTGACTATGAGGAACTGAGG G
15	E2 H5L-RB H1B.r	CCAATTCACTTTTTCATAATTCCTGATCCAAAGCCTCTACTCAGTGCGA

SEQ ID	Title	Oligonucleotide sequence (5'--3')
16	RB H1B-E2 H5L.c	GGCTTTGGATCAGGAATTATGAAAAGTGAATTGGAATATGGTAACTG CAAC
17	E1 H1B-F'1 H5L.r	GGCTATTCCTTTTAATAGGCAGAGCTTCCCGTTGTGTGTCTTTCCAG
18	F'1 H5N-E1 H1B.c	AACGGGAAGCTCTGCCTATTAAAAGGAATAGCCCCACTACAATTGGG T
19	F'2 H5I-E2 H1B.r	GGAGTTTGACACTTGGTGTTCATTTATCCATTGGTGCATTTGAGTTG
20	E2 H1B-F'2 H5L.c	AATGCACCAATGGATAAATGCAACACCAAGTGTCAAACCTCCAATGGG G
21	E1 H5I-E1 H1NC.r	TCTTCATAGTCGTTGAAACTCCCTGGGTAAACATGTTCCATTCTCAGGA
22	E1 H1NC-E1 H5L.c	CTGAGAATGGAACATGTTACCCAGGGAGTTTCAACGACTATGAAGAA C
23	E2 H1NC-RB H5L.r	ATTTGAGGTGATGATTGCTGAGTCCCCTTTCTTGACAATTTGTATGC ATA
24	RB H5I-E2 H1NC.c	GTCAAGAAAGGGGACTCAGCAATCATCACCTCAAATGCACCAATGGA T
25	HA-SacI.r	TTAACTTAGAGCTCTTAGATGCATATTCTACACTGCAAAGACC
26	ApaI-H1B.c	TGTCGGGCCCCATGAAAGTAAACTACTGGTCCTGTTATGCACATT
27	StuI-H1B.r	AAATAGGCCTTTAGATGCATATTCTACACTGTAAAGACCCATTGGA
28	SpPDI-H1B.c	TTCTCAGATCTTCGCTGACACAATATGTATAGGCTACCATGCTAACA AC
29	SacI-H1B.r	CTTAGAGCTCTTAGATGCATATTCTACACTGTAAAGACCCATTGGAA
30	ApaI-SpPDI.c	TTGTCGGGCCCCATGGCGAAAAACGTTGCGATTTTCGGCTTATTGT
31	ApaI-H5 (A- Indo).1c	TGTCGGGCCCCATGGAGAAAATAGTGCTTCTTCTTGCAAT
32	H5 (A-Indo)- StuI.1707r	AAATAGGCCTTTAAATGCAAATTCTGCATTGTAACGA
33	H3B-SpPDI.r	TGTCATTTCCGGGAAGTTTTTGAGCGAAGATCTGAGAAGGAACCA
34	SpPDI-H3B.c	TCTCAGATCTTCGCTCAAAAACCTCCCGGAAATGACAACAGCACG
35	StuI-H3B.r	AAAATAGGCCTTCAAATGCAAATGTTGCACCTAATGTTGCCTTT
36	TmD H5I-H3B.r	ATTTGGTAAGTTCCTATTGACTTCAGCTCAACGCCCTTGATCTGG
37	H3B-TmD H5L.c	TGAGCTGAAGTCAATAGGAACTTACCAAATACTGTCAATTTATTCAA C
38	HBf-SpPDI.r	GTTATTCCAGTGCAGATTTCGATCAGCGAAGATCTGAGAAGGAACCAA CAC
39	SpPDI-HBf.c	CAGATCTTCGCTGATCGAATCTGCACTGGAATAACATCTTCAAACCTC ACC
40	StuI-HBf.r	AAAATAGGCCTTTATAGACAGATGGAGCATGAAACGTTGTCTCTGG
41	TmD H5I-B Flo.r	TGACAGTATTTGGTAGTTATCCAATCCATCATCATTTAAAGATGC
42	B Flo-TmD H5L.c	GGATTGGATAACTACCAAATACTGTCAATTTATTCAACAGTGGCGAG TTC
43	Hsp-40Luz.1c	ATGTTTGGGCGCGGACCAAC

SEQ ID	Title	Oligonucleotide sequence (5'---3')
44	Hsp40Luz-SacI.1272r	AGCTGAGCTCCTACTGTTGAGCGCATTGCAC
45	Hsp40Luz-Plasto.r	GTTGGTCCGCGCCCAAACATTTTCTCTCAAGATGAT
46	Hsp70Ara.1c	ATGTCGGGTAAAGGAGAAGGA
47	Hsp70Ara-SacI.1956r	AGCTGAGCTCTTAGTCGACCTCCTCGATCTTAG
48	Hsp70Ara-Plasto.r	TCCTTCTCCTTTACCCGACATTTTCTCTCAAGATGAT
49	supP19-plasto.r	CCTTGTATAGCTCGTTCCATTTTCTCTCAAGATG
50	supP19-1c	ATGGAACGAGCTATACAAGG
51	SupP19-SacI.r	AGTCGAGCTCTTACTCGCTTTCTTTTTCGAAG
89	PacI-MCS-2X35S.c	AATTGTTAATTAAGTCGACAAGCTTGCATGCCTGCAGGTCAAC
90	CPMV 5'UTR- 2X35S.r	TCAAAACCTATTAAGATTTTAATACCTCTCCAAATGAAATGAACTTCC
91	2X35S -CPMV 5'UTR.c	TTGGAGAGGT ATTAAAAATCTTAATAGGTTTTGATAAAAGCGAACGTGG
92	ApaI -M prot.r	TCTCCAT GGGCCC GACAAATTTGGGCAGAATATACAGAAGCTTA

Table2: Agrobacterium strains used for expression of influenza hemagglutinins with native or PDI signal peptides

Agro strain	HA expressed	Signal Peptide	Expression Cassette
AGL1/540	H1 (A/New Caledonia/20/99)	PDI	Plastocyanin
AGL1/774	H1 (A/Brisbane/59/2007)	native	Plastocyanin
AGL1/787	H1 (A/Brisbane/59/2007)	PDI	Plastocyanin
AGL1/732	H1 (A/Brisbane/59/2007)	native	35S/CPMV-HT
AGL1/736	H3 (A/Brisbane/10/2007)	PDI	35S/CPMV-HT
AGL1/660	H5 (A/Indonesia/5/2005)	native	Plastocyanin
AGL1/739	B (B/Florida/4/2006)	PDI	35S/CPMV-HT
AGL1/828	CPMV HT-LC C51	C51LC	35S/CPMV-HT
AGL1/690	H1/Bris RB + H5/Indo SDC	native	Plasto
AGL1/691	H1/Bri E1-RB-E2 + H5 SDC	native	Plasto
AGL1/696	H5/Indo RB+H1/NC SDC	PDI	Plasto
AGL1/733	H1/Bri	PDI	35S/CPMV-HT
AGL1/734	H1/Bri RB + H5/Indo SDC	native	35S/CPMV-HT
AGL1/737	H3/Bri Ectodomain+H5/Indo TDC	PDI	35S/CPMV-HT
AGL1/745	B/Flo ectodomain H5/Indo TDC	PDI	35S/CPMV-HT

AGL1/747	B/Flo ectodomain_H5/Indo TDC	PDI	2X35S/CPMV-HT
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4. Preparation of plant biomass, inoculum, agroinfiltration, and harvesting

[00227] *Nicotiana benthamiana* plants were grown from seeds in flats filled with a commercial peat moss substrate. The plants were allowed to grow in the greenhouse under a 16/8 photoperiod and a temperature regime of 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. Prior to transformation, apical and axillary buds were removed at various times as indicated below, either by pinching the buds from the plant, or by chemically treating the plant

[00228] *Agrobacteria* transfected with each construct were grown in a YEB medium supplemented with 10 mM 2-[N-morpholino]ethanesulfonic acid (MES), 20 µM acetosyringone, 50 µg/ml kanamycin and 25 µg/ml of carbenicillin pH5.6 until they reached an OD₆₀₀ between 0.6 and 1.6. *Agrobacterium* suspensions were centrifuged before use and resuspended in infiltration medium (10 mM MgCl₂ and 10 mM MES pH 5.6). Syringe-infiltration was performed as described by Liu and Lomonossoff (2002, *Journal of Virological Methods*, 105:343-348). For vacuum-infiltration, *A. tumefaciens* suspensions were centrifuged, resuspended in the infiltration medium 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* or *N. tabacum* 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. Following syringe or vacuum infiltration, plants were returned to the greenhouse for a 4-5 day incubation period until harvest. Unless otherwise specified, all infiltrations were performed as co-infiltration with AGL1/35S-HcPro in a 1:1 ratio, except for CPMV-HT cassette-bearing strains which were co-infiltrated with strain AGL1/R472 in a 1:1 ratio.

5. Leaf sampling and total protein extraction

[00229] Following incubation, the aerial part of plants was harvested, frozen at -80°C, 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, 0.15 M NaCl, 0.04% sodium metabisulfite and 1 mM phenylmethanesulfonyl fluoride. After

5 homogenization, the slurries were centrifuged at 20,000 g for 20 min at 4°C and these clarified crude extracts (supernatant) kept for analyses. 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.

6. Protein Analysis and Immunoblotting

10 [00230] Protein concentrations were determined by the BCA protein assay (Pierce Biochemicals, Rockport IL). Proteins were separated by SDS-PAGE under reducing conditions and stained with Coomassie Blue. Stained gels were scanned and densitometry analysis performed using ImageJ Software (NIH).

[00231] Proteins from elution fraction from SEC were precipitated with acetone
15 (Bollag et al., 1996), resuspended in 1/5 volume in equilibration/elution buffer and separated by SDS-PAGE under reducing conditions 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.

20 [00232] Immunoblotting was performed by incubation with a suitable antibody (Table 6), 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).
25 Horseradish peroxidase–enzyme conjugation of human IgG antibody was carried out by using the EZ-Link Plus® Activated Peroxidase conjugation kit (Pierce, Rockford, IL). Whole

inactivated virus (WIV), used as controls of detection for H1, H3 and B subtypes, were purchased from National Institute for Biological Standards and Control (NIBSC).

Table 3: Electrophoresis conditions, antibodies, and dilutions for immunoblotting of expressed proteins.

HA sub-type	Influenza strain	Electrophoresis condition	Primary antibody	Dilution	Secondary antibody	Dilution
H1	A/Brisbane/59/2007 (H1N1)	Reducing	FII 10-I50	4 µg/ml	Goat anti-mouse (JIR 115-035-146)	1:10 000
H1	A/New Caledonia/20/99 (H1N1)	Reducing	FII 10-I50	4 µg/ml	Goat anti-mouse (JIR 115-035-146)	1:10 000
H3	A/Brisbane/10/2007 (H3N2)	Non-Reducing	NIBSC 08/124	1:4000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H5	A/Indonesia/5/2005 (H5N1)	Reducing	ITC IT-003-005V	1:4000	Goat anti-rabbit (JIR 111-035-144)	1:10 000
B	B/Florida/4/2006	Non-Reducing	NIBSC 07/356	1:2000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000

FII: Fitzgerald Industries International, Concord, MA, USA;
 NIBSC: National Institute for Biological Standards and Control;
 JIR: Jackson ImmunoResearch, West Grove, PA, USA;
 ITC: Immune Technology Corporation, Woodside, NY, USA;

7. Clarification and concentration prior to SEC

[00233] To improve resolution and increase signal in elution fractions, extracts to be loaded on size exclusion chromatography, crude protein extracts were clarified and concentrated using the following method. Extracts were centrifuged at 70 000 g, 4°C for 20 min and the pellet was washed twice by resuspension in 1 volume (compared to the initial extract volume) of extraction buffer (50 mM Tris pH 8, 0.15 M NaCl) and centrifugation at

70 000 g, 4°C for 20 min. The resulting pellet was resuspended in 1/3 volume (compared to the initial extract volume) and proteins (including VLPs) were precipitated by the addition of 20% (w/v) PEG 3350 followed by incubation on ice for 1h. Precipitated proteins were recovered by centrifugation at 10 000 g, 4°C, 20 min, and resuspended in 1/15 volume (compared to the initial extract volume) of extraction buffer. After complete resuspension of proteins, a final centrifugation at 20 000 g, 4°C, 5 min was performed to pellet insolubles and the clear supernatant was recovered.

8. Size exclusion chromatography of protein extract

[00234] Size exclusion chromatography (SEC) columns of 32 ml Sephacryl™ S-500 high resolution beads (S-500 HR : GE Healthcare, Uppsala, Sweden, Cat. No. 17-0613-10) were packed and equilibrated with equilibration/elution buffer (50 mM Tris pH8, 150 mM NaCl). One and a half millilitre of crude protein extract was loaded onto the column followed by an elution step with 45 mL of equilibration/elution buffer. The elution was collected in fractions of 1.5 mL relative protein content of eluted fractions was monitored by mixing 10 µL of the fraction with 200 µL of diluted Bio-Rad protein dye reagent (Bio-Rad, Hercules, CA). The column was washed with 2 column volumes of 0.2N NaOH followed by 10 column volumes of 50 mM Tris pH8, 150 mM NaCl, 20% ethanol. Each separation was followed by a calibration of the column with Blue Dextran 2000 (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA). Elution profiles of Blue Dextran 2000 and host soluble proteins were compared between each separation to ensure uniformity of the elution profiles between the columns used.

Example 1: Domain swapping strategy for RB and/or Esterase domains on influenza subtype stems.

[00235] The RB subdomain of H5/Indo may be replaced by an RB subdomain of H1, H3 or B HA. The resulting chimeric HA provides an SDC H5/Indo to form VLPs and present the RB subdomain comprising immunogenic sites of H1, H3 or B. The H5/Indo RB subdomain may be inserted on an H1 stem (H1/NC) . Figures 15A and 15B illustrate the

amino acid sequences at the fusions of the indicated subdomains, and the amino acid sequences of the respective subdomains is provide in Figure 2 (constructs 690, 734, 696 and 691) and Tables 4 (constructs 900 and 745) and 5 (constructs 910, 920 and 930). Amino acid sequences illustrated in Figure 2, and Tables 4 and 5 do not include signal peptide sequences.

5 **Table 4 Subdomains and chimeric influenza HA. Chimeric influenza HA comprising heterologous RB subdomain.**

Const No	N-terminal Stem: -F'1+E1	RB head	C-terminal stem: E2+F'2-Stop
900 (SEQ ID NO: 105)	H5/Indo DQICIGYHANNSTEQ VDTIMEKNVTVTHA QDILEKTHNGKLCDL DGVKPLILRDCSVAG WLLGNPMCDEFINV PEWSYIVEKANPTND LCYP	H3/Brisbane YDVPDYASLRSLVASSGTLEFN NESFNWTGVTQNGTSSACIRRS NNSFFSRLNWLTHLKFKYPALN VTMPNNEKFDKLYIWGVHHPG TDNDQIFLYAQASGRITVSTKRS QQTVIPNIGSRPRVRNIPSRISY WTIVKPGDILLINSTGNLIAPRGY FKIRSGKSS	H5/Indo IMKSELEYGNCNTKCQTPMGAINSSMPFHNHPLTIGECPKY VKS NRLVLATGLRNSPQRESRRKKRGLFGAIAAGFIEGGWQG MVDGWYGYHHSNEQSGSYAADKESTQKAIDGVTNKVNSII DKMNTQFEAVGREFNLERRIENLNKKMEDGFLDVWTYNA ELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELGNG CFEFYHKCDNECMESIRNGTYNYPQYSEEARLKREEISGVKLE SIGTYQILSIYSTVASSLALAIMMAGLSLWMCNGLSLQCRICI
745 (SEQ ID NO: 106)	H5/Indo DQICIGYHANNSTEQ VDTIMEKNVTVTHA QDILEKTHNGKLCDL DGVKPLILRDCSVAG WLLGNPMCDEFINV PEWSYIVEKANPTND LCYP	B/Florida IMHDRTKIRQLPNLLRGYENIRL STQNVIDAEKAPGGPYRLGTSG SCPNATSKSGFFATMAWAVPK DNNKNATNPLTVEVPYICTEGE DQITVWGFHSDNKTQMKNLYG DSNPQKFTSSANGVTTHYVSQI GSFPDQTEDGGLPQSGRIVVDY MMQKPGKTGTIVYQRGVLLPQ KVWCASGRSK	H5/Indo IMKSELEYGNCNTKCQTPMGAINSSMPFHNHPLTIGECPKYV KSNRLVLATGLRNSPQRESRRKKRGLFGAIAAGFIEGGWQGMV DGWYGYHHSNEQSGSYAADKESTQKAIDGVTNKVNSIIDKM NTQFEAVGREFNLERRIENLNKKMEDGFLDVWTYNAELLV LMENERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFY HKCDNECMESIRNGTYNYPQYSEEARLKREEISGVKLESIGTY QILSIYSTVASSLALAIMMAGLSLWMCNGLSLQCRICI

[00236] Amino acids 1-92 of SEQ ID NO: 105 are an F'1+E1 domain of H5/Indo; amino acids 93-259 are the RB head domain of H3/Brisbane; amino acids 260-548 are the E2+F'2 domain of H5/Indo.

- 10 [00237] Amino acids 1-92 of SEQ ID NO: 106 are an F'1+E1 domain of H5/Indo; amino acids 93-276 are the RB head domain of B/Florida; amino acids 277-565 are the E2+F'2 domain of H5/Indo.

15 **Table 5 Subdomains and chimeric influenza HA. Chimeric influenza HA comprising heterologous RB subdomain.**

Construct No.	N-terminal Stem: F'1	E1-RB-E2 head	C-terminal stem: F'2- Stop
910 (SEQ ID NO: 107)	H5/Indo DQICIGYHANNSTE QVDTIMEKNVTVT HAQDILEKTHNGK LC	H3/Brisbane DSPHQILDGENCTLIDALLGDPQCDG FQNKKWDLFVERSKAYSNCYPYDV PDYASLRSLVASSGTLEFNNEFNFNT GVTQNGTSSACIRRSNNSFFSRLNWL THLKFYKYPALNVTMPNNEKFDKLYI WGVHHPGTDNDQIFLYAQASGRITV STKRSQQTVIPNIGSRPRVRNIPSRSI YWTIVKPGDILLINSTGNLIAPRGYFK IRSGKSSIMRSDAPIGK	H5/Indo CNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKS NRLV LATGLRNSPQRESRRKKRGLFGAAGFIEGGWQGMVD GWYGYHHSNEQSGSYAADKESTQKAIDGVTNKVNSII DKMNTQFEAVGREFNLERRIENLNKKMEDGFLDVW TYNAELLVLMENERTLDHFHDSNVKNLYDKVRLQLRDN AKELGNGCFEFYHKCDNECMESIRNGTYNYPQYSEEA RLKREEISGVKLESIGTYQILSIYSTVASSLALAIMMAGL SLWMC SNGSLQCRICI
920 (SEQ ID NO: 108)	H5/Indo DQICIGYHANNSTE QVDTIMEKNVTVT HAQDILEKTHNGK LC	B/Florida PDCLNCTDLDVALGRPMC VGTTPSA KASILHEVKPVTSGCFPIIMHDKIR QLPNLLRGYENIRLTQNVIDAEKAP GGPYRLGTSGSCP NATSKSGFFATM AWAVPKDNNKNATNPLTVEVPYICT EGEDQITVWGFHSDNKTQMKNLGY DSNPQKFTSSANGVTTHYVSQIGSFP DQTEDGGLPQSGRIVVDYMMQKPG KTGTIVYQRGVLLPQKVWCASGRSK VIKGSPLIGEAD	H5/Indo CQTPMGAINSSMPFHNIHPLTIGECPKYVKS NRLV LATGLRNSPQRESRRKKRGLFGAAGFIEGGWQGMVDGWYG YHHSNEQSGSYAADKESTQKAIDGVTNKVNSIIDKMN TQFEAVGREFNLERRIENLNKKMEDGFLDVWTYNAE LLVLMENERTLDHFHDSNVKNLYDKVRLQLRDN AKELGNGCFEFYHKCDNECMESIRNGTYNYPQYSEEARLKREE ISGVKLESIGTYQILSIYSTVASSLALAIMMAGLSLWMC SNGSLQCRICI
930 (SEQ ID NO: 109)	H1/NC DTICIGYHANNSTD TVDTVLEKNVTVT HSVNLLED SHNGK LC	H5/Indo DLDGVKPLILRDCSVAGWLLGNPMC DEFINVPWESYIVEKANPTNDLCYPG SFNDYEELKHLISRINHFEEKIIPKSS WSDHEASSGVSSACPYLGSPSFFRN VWVLIKKNSTYPTIKKSYNNTNQED LLVLWGIHHPNDAAEQTRLYQNPTT YISIGTSTLNQRLVPKIATRSKVNQGS GRMEFFWTILKPNDAINFESNGNFIA PEYAYKIVKKGDSAIMKSELEYGN	H1/NC CDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRS AKL RMVTGLRNIPSIQSRGLFGAAGFIEGGWGMVDGWYG YHHQNEQSGSYAADQKSTQNAINGITNKVNSVIEKMN TQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAE LLVLENERTLDHFHDSNVKNLYEKVKSQKKNNAKEIGN GCFEFYHKCNNECMESVKNGTIDYDPKYSEESKLNREK IDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWMC SNGSLQCRICI

[00238] Amino acids 1-42 of SEQ ID NO: 107 are an N terminal F'1 domain of H5/Indo; amino acids 43-228 are the E1-RB-E2 head domain of H3/Brisbane; amino acids 229-507 are the F'2 domain of H5/Indo.

[00239] Amino acids 1-42 of SEQ ID NO: 108 are an N terminal F'1 domain of H5/Indo; amino acids 43-281 are the E1-RB-E2 head domain of B/Florida; amino acids 282-556 are the F'2 domain of H5/Indo.

[00240] Amino acids 1-42 of SEQ ID NO: 109 are an N terminal F'1 domain of H1/NC; amino acids 43-273 are the E1-RB-E2 head domain of H5/Indo; amino acids 274-548 are the F'2 domain of H1/NC.

[00241] The fusion points for the various chimeras were selected so as to be as close to (but not necessarily directly at) the N and C termini of the various subdomains – without wishing to be bound by theory, these fusions were selected so as to maximize the stability of the chimeric HA. For example, structure and sequence conservation is observed at the N-terminus of the RB subdomain (Ha et al. 2002, EMBO J. 21:865-875; which is incorporated herein by reference). A less variable region in the primary sequence is found at the C-F/Y-P triad located at approximately 15 amino acids before, in the E1 subdomain. This cysteine is involved in disulfide bridge #3, which is conserved among HAs (see Figures 46 and 47). A junction at this Cys may provide for suitable, or superior, stability to the chimeric HA relative to the native sequence. The C-terminus end of the RB provides conserved features: for example, a conserved Ser residue at position -1 and the E2 subdomain starts with a beta-sheet observed in all HA on the alignment (Ha et al. 2002, EMBO J. 21:865-875; which is incorporated herein by reference). Therefore, the C-terminus of this RB may be fused to the initiating amino acid of this beta sheet structure of the E2 subdomain. Further, the disulfide bridge pattern is not changed, or is not substantively changed, for the chimeras comprising RB subdomains of H1/NC, H1Bri, H3/Bri, or B/Flo on an H5/Indo SDC, and for H5/Indo RB subdomain on H1 SDC (total of 6), but a disulfide bridge will be added (bridge #8) on the hybrid HA of B RB on H5 stem. This addition of disulfide bridge should not interfere with the folding of the HA (because it is located within the RB domain and the Cys are adjacent on the sequence), and may be produce a even more stable hybrid HA.

[00242] The E1-RB-E2 subdomains of a first influenza type were replaced by E1-RB-E2 subdomains of a second influenza type. Such an arrangement may present a greater number of amino acids of the second type at the surface of the H5-VLP. In this example, the HDC of H1, H3 or B was placed on an H5/Indo SDC, and an HDC of H5/Indo on an H1/NC SDC (Table 5).

[00243] The junction of the HDC was defined with a conserved cysteine residue (comprising disulfide bridge #6 of HA type A and #7 in HA type B). The junction of the HDC at the C-terminus of the E2 subdomain was defined with another conserved cysteine residue comprising disulfide bridge #6 (the second amino acid of the F'2 subdomain) of influenza

type H1 or H3 on an SDC of H5/Indo or for influenza type H5 on an SDC of H1. For the influenza B chimera, the junction was established the connection at the first Cysteine comprising of disulfide bridge #4 (located 4 amino acids away on the F'2 subdomain, and conserved among the HAs). The resulting chimeras do not exhibit any alteration in disulfide bridge patterns - the H1/H3/H5 hybrid HAs will contain 6 disulfide bridges and the B hybrid will have 7 of them.

Example 2: Replacement of receptor binding (RB) or receptor binding and esterase (E1-RB-E2) subdomains of H5 A/Indonesia/5/05 with that of H1 A/Brisbane/59/2007: Comparison of expression for chimeric and native forms.

[00244] To combine the high accumulation level of VLPs from H5 A/Indonesia/5/05 with the antigenicity characteristics of H1 A/Brisbane/59/2007, chimeric hemagglutinins were designed comprising domains from H1 A/Brisbane/59/2007 fused to an H5 A/Indonesia/5/05 stem domain cluster. Expression cassettes for the expression of the H5/H1 hemagglutinin fusions are represented in Figure 1 and amino acid sequences of the mature fusion proteins produced illustrated in Figure 2.

[00245] To compare the accumulation level of H5/H1 chimeric hemagglutinins with that of their native forms, *Nicotiana benthamiana* plants were infiltrated with AGL1/774, AGL1/691 and AGL1/690, and the leaves were harvested after a six-day incubation period. To determine the accumulation level of each HA form in the agroinfiltrated leaves, proteins were extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-HA monoclonal antibodies. A unique band of approximately 75 kDa (Figure 3), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin, was detected in extracts from leaves infiltrated with AGL1/690 but not in AGL1/774 or AGL1/691, indicating that the chimeric hemagglutinin comprising the receptor-binding region of H1 A/Brisbane/59/2007 fused to the H5 A/Indonesia/5/05 backbone accumulated to higher level than both the native form of H1 A/Brisbane/59/2007 (AGL1/774) and the chimeric hemagglutinin combining the esterase and receptor-binding regions of H1 A/Brisbane/59/2007 with the H5 A/Indonesia/5/05 backbone. The whole inactivated virus (WIV) (H1 A/Brisbane/59/2007)

used as a positive control was detected as multiple bands with a major band at approximately 80 kDa, corresponding to the molecular weight of the precursor HA0 of H1 A/Brisbane/59/2007. These results demonstrated that the replacement of the receptor-binding region from H5 A/Indonesia/5/05 with that of H1 A/Brisbane/59/2007 generated a chimeric hemagglutinin which presented the antigenic region of H1 and which accumulated at higher level than the native H1 A/Brisbane/59/2007 in plants. However, the chimeric hemagglutinin in which the esterase and receptor-binding regions from H5 A/Indonesia/5/05 were replaced by those of H1 A/Brisbane/59/2007 did not accumulate to a detectable level in the plants.

[00246] The fusion of the receptor-binding region from H1 A/Brisbane/59/2007 to the H5 A/Indonesia/5/05 backbone as a method of increasing accumulation of H1 antigen-presenting VLPs in plants was re-evaluated under the control of a strong CPMV-HT-based expression cassette. This fusion strategy was also compared to signal peptide replacement as mean of increasing accumulation level. Expression cassettes for the expression of the H5/H1 hemagglutinin fusions under CPMV-HT are represented in Figure 8 and amino acid sequence of the mature fusion protein produced is presented in Figure 2.

[00247] *Nicotiana benthamiana* plants were infiltrated with AGL1/732, AGL1/733 or AGL1/734, and the leaves were harvested after a six-day incubation period. To determine the accumulation level of each HA form in the agroinfiltrated leaves, protein were first extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-H1 (Brisbane) polyclonal antibodies. A unique band of approximately 75 kDa (Figure 6), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin, was detected in extracts from leaves infiltrated with AGL1/732, AGL1/733 and AGL1/734. However, although hemagglutinin was detected in all extracts analyzed, important differences in accumulation could be noticed. While H1 A/Brisbane/59/2007 expression was barely detectable under these conditions when using its natural signal peptide (732), replacement of signal peptide with that of PDI resulted in higher accumulation of mature H1 A/Brisbane/59/2007 (733), and the chimeric H5/H1 hemagglutinin (734) accumulated to the highest level. Taken together, these results show that the fusion of the receptor-binding domain from H1 on a H5 backbone leads to high accumulation of H1 antigens-presenting hemagglutinin and that the accumulation level

obtained for such fusion in plants is higher than that obtained with the native form with or without replacement of the signal peptide.

Example 3: Replacement of receptor binding (RB) subdomain of H1 A/New Caledonia/20/99 with that of H5 A/Indonesia/5/05. Comparison of expression for chimeric and native forms.

[00248] Use of an H1 backbone (from A/New Caledonia/20/99) for the presentation of H5 antigenic region was also evaluated. Expression cassettes for the expression of the H1/H5 hemagglutinin fusion are represented in Figure 1 and amino acid sequence of the mature fusion protein produced is presented in Figure 2.

[00249] To compare the accumulation level of H1/H5 chimeric hemagglutinin with that of its native form, *Nicotiana benthamiana* plants were infiltrated with AGL1/660 and AGL1/696, and the leaves were harvested after a six-day incubation period. To determine the accumulation level of each HA form in the agroinfiltrated leaves, proteins were extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-H5 (Indonesia) polyclonal antibodies. A unique band of approximately 75 kDa (Figure 7), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin, was detected in extracts from leaves infiltrated with AGL1/660 and AGL1/696, indicating that both the native H5 A/Indonesia/5/05 and the H1/H5 chimeric hemagglutinin accumulate at high level in plants.

Example 4: Replacement of the ectodomain of H5 A/Indonesia/5/05 with that of H3 or B. Comparison of expression for chimeric and native forms.

[00250] The fusion of the ectodomain from H3 A/Brisbane/10/2007 or B Florida/4/2006 to the transmembrane and cytoplasmic subdomains from H5 A/Indonesia/5/05 was evaluated as a strategy to present hemagglutinin antigenic regions from H3 and B strains while increasing their accumulation level in plants. Expression cassettes for the expression of the H5/H3 and H5/B hemagglutinin fusions are represented in Figure 10 and amino acids at the border of the fusions are presented in Figure 11.

[00251] Accumulation level of H5/B chimeric hemagglutinin (745) was compared with that of native HA B (739) in *Nicotiana benthamiana* plants. Plants were infiltrated with AGL1/739 and AGL1/745, and the leaves were harvested after a six-day incubation period. To determine the accumulation level of each HA form in the agroinfiltrated leaves, proteins were first extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-B (Florida) polyclonal antibodies. A unique band of approximately 75 kDa (Figure 14), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin, was detected in extracts from leaves of one plant infiltrated with AGL1/739 while the 3 plants infiltrated with AGL1/745 showed positive signal corresponding to hemagglutinin, indicating that the H5/B chimeric form of hemagglutinin accumulated more regularly at high level than the native form of B hemagglutinin.

[00252] Similarly, accumulation level of H5/H3 chimeric hemagglutinin (737) was compared with that of its native form (736) in *Nicotiana benthamiana* plants. Plants were infiltrated with AGL1/736 and AGL1/737, and the leaves were harvested after a six-day incubation period. To determine the accumulation level of each HA form in the agroinfiltrated leaves, proteins were extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-H3 (Brisbane) polyclonal antibodies. A unique band of approximately 75 kDa (Figure 15), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin, was detected in extracts from leaves infiltrated with AGL1/736 and AGL1/737. This result indicates that the fusion of transmembrane and cytoplasmic subdomains from H5 A/Indonesia/5/05 to the ectodomain of H3 A/Brisbane/10/2007 creates a chimeric hemagglutinin that accumulates to a similar level as the native H3 A/Brisbane/10/2007.

[00253] The production of VLPs from expression of the H5/B chimeric hemagglutinin (construct no. 745) was evaluated using size exclusion chromatography. Concentrated protein extracts from AGL1/745-infiltrated plants (1.5 mL) were fractionated by size exclusion chromatography (SEC) on Sephacryl™ S-500 HR columns (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA). As shown in Figure 16, Blue Dextran (2 MDa) elution peaked early in fraction 8. When proteins from 200 µL of each SEC elution fraction were concentrated (5-fold) by acetone-precipitation and analyzed by Western blotting using anti-B

(Florida) polyclonal antibodies (Figure 16), chimeric hemagglutinin was primarily found in fraction 7, indicating the incorporation of HA into high molecular weight structures. Without wishing to be bound by theory, this suggests that the chimeric HA protein had either assembled into a large superstructure or that it has attached to a high molecular weight structure. The results obtained indicate that the chimeric HA consisting in the ectodomain from HA B/Florida/4/2006 fused to the transmembrane and cytosolic subdomains from H5 A/Indonesia/5/05 assembles into high molecular weight particles and that the elution profile of these high molecular weight particles is indistinguishable to that of influenza VLPs.

Example 5: Co-expression of H5/B chimeric hemagglutinin (construct number 747; comprising B/Flo HDC and SDC fused with an H5/Indo TDC) with Hsp70 and Hsp40 in combination with signal peptide modification.

[00254] Expression of Hsp40 and Hsp70 in plants and co-expression with influenza hemagglutinins is described in co-pending application PCT/CA2009/000032. Cytosolic Hsp70 and Hsp40 (construct number R870) of plant origin may also be co-expressed with chimeric hemagglutinins, to increase their accumulation level in plants. The co-expression may be performed by agroinfiltration of *N. benthamiana* plants with a bacterial suspension containing a mixture (1:1:1 ratio) of AGL1 bearing the cassette for the expression of the desired chimeric HA with AGL1/R870 and AGL1/35SHcPro.

[00255] Accumulation level of H5/B chimeric hemagglutinin (B/Flo HDC and SDC fused with an H5/Indo TDC) was evaluated in co-expression with HSP40 and HSP70 in *Nicotiana benthamiana* plants. Plants were infiltrated with AGL1/747, AGL1/747+AGL1/443 (empty vector) or AGL1/747+AGL1/R870 (HSP40/HSP70), and the leaves were harvested after a six-day incubation period. To determine the accumulation level of H5/B chimeric HA in the agroinfiltrated leaves, proteins were first extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-B (Florida) polyclonal antibodies. A unique band of approximately 75 kDa (Figure 50), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin, was detected in extracts from leaves from 3 plants infiltrated with AGL1/747+AGL1/R870 while the 3 plants infiltrated with AGL1/747+ control vector (443)

showed no signal (under the exposure condition used) indicating that the H5/B chimeric form of hemagglutinin accumulated at high level when co-expressed with the HSP40 and HSP 70 chaperones.

[00256] All citations are herein incorporated by reference, as if each individual publication was specifically and individually indicated to be incorporated by reference herein and as though it were fully set forth herein. Citation of references herein is not to be construed nor considered as an admission that such references are prior art to the present invention.

[00257] In the description a number of terms are used extensively and definitions are provided to facilitate understanding of various aspects of the invention. Use of examples in the specification, including examples of terms, is for illustrative purposes only and is not intended to limit the scope and meaning of the embodiments of the invention herein. Numeric ranges are inclusive of the numbers defining the range. In the specification, the word “comprising” is used as an open-ended term, substantially equivalent to the phrase “including, but not limited to,” and the word “comprises” has a corresponding meaning.

[00258] One or more currently preferred embodiments of the invention have been described by way of example. The invention includes all embodiments, modifications and variations substantially as hereinbefore described and with reference to the examples and figures. 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. Examples of such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way.

WHAT IS CLAIMED IS:

1. A polypeptide comprising a chimeric influenza HA comprising a stem domain cluster (SDC), a head domain cluster (HDC) and a transmembrane domain cluster (TDC) wherein:

a) the SDC comprises an F'1, F'2 and F subdomain;

5 b) the HDC comprises an RB, E1 and E2 subdomain;

c) the TDC comprises a TmD and Ctail subdomain; and

wherein at least one subdomain is of a first influenza HA and the other subdomains are of one or more second influenza HA.

10 2. The polypeptide of claim 1 wherein the first and second influenza HA are independently selected from the group comprising H1, H3, H5 and B.

3. The polypeptide of claim 2 further comprising a signal peptide.

4. A nucleic acid encoding the polypeptide of claim 1.

5. A nucleic acid encoding the polypeptide of claim 3.

15 6. A method of producing chimeric influenza virus like particles (VLPs) in a plant comprising:

a) introducing the nucleic acid of claim 5 into the plant, or portion thereof, and

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

20 7. The method of claim 6, wherein in the step of introducing (step a), the nucleic acid is introduced in the plant in a transient manner.

8. The method of claim 6, wherein, in the step of introducing (step a), the nucleic acid is introduced in the plant so that it is stable.

9. The method of claim 6 further comprising a step of

c) harvesting the host and purifying the VLPs.

10. A virus like particle (VLP) comprising the polypeptide of claim 1.

11. The VLP of claim 10 further comprising plant-specific N-glycans, or modified N-glycans.

5 12. A composition comprising an effective dose of the VLP of claim 10 and a pharmaceutically acceptable carrier.

13. A method of inducing immunity to an influenza virus infection in a subject, comprising administering the virus like particle of claim 20.

10 14. The method of claim 13, wherein the virus like particle is administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

15. A plant, or portion thereof, comprising the polypeptide of claim 1.